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Inhibitors of indoleamine 2,3-dioxygenase 1 (IDO1) for cancer therapy

Petr Tomek

Abstract

Immune escape is a central hallmark of cancer. A tryptophan-catabolising enzyme indoleamine 2,3-dioxygenase-1 (IDO1) is a dominant immune escape mechanism in a broad range of human tumours and its expression is associated with a poor prognosis in cancer. Blockade of IDO1 by small-molecule inhibitors is a validated cancer therapy and two IDO1 inhibitors are showing promise in human clinical trials.

The work in this thesis contributed to an overall goal at the Auckland Cancer Society Research Centre to identify and investigate the mode of action of novel IDO1 inhibitors as potential anti-cancer agents. It entailed the establishment of a sensitive and automated IDO1 enzyme assay to screen compound libraries for potential drug development leads.

The new fluorescence IDO1 enzyme assay developed in this work is 30-fold more sensitive (limit of detection 153 nM $N$-formylkynurenine) than pre-existing assays. It is economical and features low interference from test compounds. The assay utilises a fluorescing tetrahydroquinolone adduct formed in a chemical reaction between $N$-formylkynurenine and cyclic amines involving transamidation and amine rearrangement, not previously described. This assay was automated and used to screen the National Cancer Institute Diversity Set III library (1,597 compounds) and to validate the thirty hits obtained from the screening of a commercial library of 40,000 molecules. This afforded eight IDO1 inhibitors from which pyrimidinone, indolonoxide, and isoxazole classes emerged as suitable drug development leads. All three showed excellent cell permeability and good potency (IC$_{50}$ 0.066 - 8 μM) in cell-based assays with negligible cell toxicity. Three of the top eight hits, including pyrimidinone, blocked IDO1 activity reversibly, identical to a well-studied IDO1 inhibitor, 4-phenyl-1H-imidazole. The other five hits, including isoxazole, elicited essentially irreversible IDO1 inactivation, an inhibitory mechanism not previously documented for IDO1 inhibitors. Testing the NCI library against serine-167 and cysteine-129 alanine replacement IDO1 mutants, established that serine-167 but not cysteine-129 in the IDO1 active site, is important for the binding of a broad range of inhibitors.

This project discovered three novel IDO1 inhibitors suitable for drug development. The isoxazole lead is currently being optimised by rational medicinal chemistry for development as a potential anti-cancer drug for the future.
Acknowledgements

The following lines are an expression of infinite thanks to many wonderful human beings without whose help this PhD journey would never have reached the successful end.

My most special thanks go to Associate Professor Lai-Ming Ching for inviting me to New Zealand to work with her on such an exciting multidisciplinary project. I would not have been writing these lines without her excellent supervision and guidance. I never regret for a single moment that I came to her lab. Thank you Lai-Ming for getting me where I am now, I have learnt so much from you.

Thanks to my co-supervisor, Dr Jack Flanagan, for the ton of encouragement, especially in the early days. His insights into science, constructive criticism on the results and helpful suggestions were instrumental in shaping the numerous research ideas of this work.

Associate Professor Brian Palmer helped me enormously in this project. I am grateful for his precious time, advices on chemistry and optimistic attitude. The structure of the novel fluorophore would not have been identified without him. I must not forget to thank Dr Jackie Kendall who also contributed to this project with her incredible knowledge about chemistry and NMR. Jackie was always so positive and happy to answer all my questions in such an elegant way. Thanks to Dr David Bridewell who was my guide in early days in the laboratory and taught me a lot about the enzyme purification and science in general.

Thanks to the whole Stromal Targeting Group, you are incredible. Particularly Sofian Tijono; I am thankful for your help on many occasions. You kept everything in the lab running so smoothly and always saved me when necessary. Thanks to Raymond Yung and Vahid Seyfoddin for their helpful discussions and perspectives on science. Thanks to Simon Fung for the fruitful chats about IDO1 which were instrumental in shaping a number of my ideas. Kimiora Henare, I thank you sincerely for your help with my thesis and some inspirational discussions about immunology. What an incredible time we all had outside of the lab and enjoying the barbecues organised by our generous hosts Lai-Ming and Brian. I will never forget this in my life, what an amazing “family”. I also have to thank all of you guys for constructive advices on my results during our weekly meetings. Such feedback was an immense help.

I would like to express my thanks to all the guys from Perkin-Elmer, particularly Alex Burnett and Shaun Stapleford for providing a valuable help about the JANUS robotic workstation used in my work. Special thanks go to Dr Michael Abdo who devoted his precious time to teach me the programming and scripting of the robotic workstation.
My thanks go to Professor Bruce Baguley from the Auckland Cancer Society Research Centre (ACSRC) for his encouragement and mental support during my PhD and playing the music with me and my friends on many occasions.

I should not forget to acknowledge my funding from University of Auckland, without which this PhD would not have been possible. The international doctoral scholarship has paid my doctoral fees and provided me with an adequate living allowance to survive in one of the most expensive cities in the world.

I would like to thank all the staff at the ACSRC for making the centre such a friendly environment to work in. I have always felt everyone is there to help me at all occasions. Special thanks to Vicki Scott, Mary Spellman and Yuli Quay for their wonderful administrative assistance and such a cheerful attitude. I would be lost in the administrative mess without you. You saved my day many times.

To all the students at the ACSRC who are my friends. Thanks to Shevan and Stacey who shared the love of music with me and cheered me up but also Cho and Mohammed, my fellow colleagues in the office for some incredible laughter.

To all my musician friends, Shyam, Ryan, Dickson, Michael, David, John and Trio Pohádka who I have been neglecting during the last few months of writing this thesis. What a shame. Yes, I have been missing having a good chat about music and playing with you guys so much.

To my most incredible Mum and Dad, thank you for your love and support during this stressful journey despite the 20,000 kilometres gap between us. Thanks for keeping calling me on Skype and constantly reminding me to finish that PhD so I can finally get a job. Thanks to my sisters Dana and Jarmila for their mental support and care. I am so lucky to have you all.

Finally, to my beloved partner Lisa who has endured with me in the worst of times. You had to sacrifice a lot so I can complete this PhD, but you persisted. Thank you for your patience, and thank you for your unconditional love which kept me going forward and kept me above the water. Thank you for taking care about me, giving me energy, and keeping me alive when the resources were running low. You are truly my angel.
CHAPTER 1
LITERATURE REVIEW

1.1 Introduction ............................................................................................................................................... 1
1.2 Escaping immune surveillance ............................................................................................................... 2
1.3 The kynurenine pathway (KP) ............................................................................................................... 5
1.4 The gatekeepers of the kynurenine pathway (IDO, TDO and IDO2) ............................................. 6
  1.4.1 IDO1 and TDO ............................................................................................................................... 6
  1.4.2 IDO2 ................................................................................................................................................. 12
1.5 IDO1-mediated immune suppression ................................................................................................. 13
1.6 The IDO1 immunomodulation mechanisms .................................................................................. 14
  1.6.1 Tryptophan depletion ................................................................................................................... 14
    1.6.1.1 TRP transporters ...................................................................................................................... 15
    1.6.1.2 General control non-derepressible 2 (GCN2) ...................................................................... 16
  1.6.2 Accumulation of tryptophan metabolites .............................................................................. 17
    1.6.2.1 Aryl hydrocarbon Receptor (AhR) ..................................................................................... 18
  1.6.3 IDO1 is a signalling molecule ................................................................................................... 22
1.7 Regulation of IDO1 expression and activity ................................................................................... 24
1.8 IDO1 is associated with a poor prognosis for cancer patients .................................................... 25
1.9 Validation of IDO1 as a therapeutic target for cancer therapy .................................................... 27
1.10 Small-molecule inhibitors of IDO1 ................................................................................................. 28
  1.10.1 Indoles ........................................................................................................................................ 28
  1.10.2 Quinones ..................................................................................................................................... 30
  1.10.3 Imidazoles .................................................................................................................................. 31
  1.10.4 Miscellaneous ............................................................................................................................ 31
1.11 Pharmacological IDO1 inhibitors in human clinical trials ......................................................... 32
  1.11.1 INCB024360 (Incyte Corp.) ........................................................................................................ 32
  1.11.2 NLG919 (NewLink Genetics) .................................................................................................... 33
  1.11.3 FLX-B (Flexus Biosciences Inc.) ............................................................................................... 33
CHAPTER 2

MATERIALS AND METHODS

2.1 Biology

2.1.1 Materials

2.1.2 Production and purification of recombinant human IDO1

2.1.3 Assays for determination of IDO1 inhibitory activity

2.1.3.1 Enzyme assays

2.1.3.2 Cell-based assay

2.1.4 PIP-THQ formation (Figure 3.3)

2.1.5 Isolation and purification of NFK from an enzymatic reaction

2.1.6 Enzyme kinetics, time-dependency and reversibility of rhIDO1 inhibition

2.1.7 Molecular biology methods

2.1.7.1 Primers

2.1.7.2 Restriction endonuclease digest and agarose gel electrophoresis

2.1.7.3 DNA sequencing

2.1.7.4 Denaturing protein acrylamide gel electrophoresis (SDS-PAGE)

2.1.7.5 Polymerase chain reaction (PCR)

2.1.7.6 Bacterial transformation and plasmid isolation

2.1.8 Production of mutant IDO1 enzymes

2.1.8.1 Serine-167 alanine replacement IDO1 mutant (S167A)

2.1.8.2 Cysteine-129 alanine replacement IDO1 mutant (C129A)

2.1.9 Production and purification of recombinant human tryptophan 2,3-dioxygenase (rhTDO)

2.1.9.1 Production of expression vectors

2.1.9.2 Small-scale rhTDO expression

2.1.9.3 Enzyme activity of Ni-NTA eluates

2.1.9.4 Large-scale purification of rhTDO from BL-21(DE3)/Construct 1
CHAPTER 3

NOVEL FLUORESCENCE ASSAY FOR DETERMINATION OF IDO1 ENZYMATIC ACTIVITY

3.1 Introduction .................................................................................................................. 68
3.2 Results .......................................................................................................................... 71
   3.2.1 A novel fluorescence assay for IDO1 ................................................................. 71
   3.2.2 Detection of a novel NFK-derived fluorophore ............................................... 72
   3.2.3 Optimization of PIP-THQ formation and assay conditions ....................... 74
   3.2.4 Adaptation of the fluorescence assay for use with JANUS automated workstation ........................................................................................................ 78
   3.2.5 Validation and comparison of the new assay with the absorbance assay ....... 80
3.3 Discussion ....................................................................................................................... 83

CHAPTER 4

STRUCTURE AND MECHANISM OF FLUOROPHORE PIP-THQ FORMATION

4.1 Introduction ................................................................................................................ 90
4.2 Experimentation and Discussion .............................................................................. 92
   4.2.1 Structure and properties of PIP-THQ fluorophore .......................................... 92
      4.2.1.1 NMR studies .............................................................................................. 92
      4.2.1.2 MS fragmentation studies to confirm position of the formyl group on PIP-THQ ..................................................................................................................................................................................... 93
CHAPTER 5

DISCOVERY AND CHARACTERISATION OF NOVEL IDO1 INHIBITORS FROM COMPOUND LIBRARIES

5.1 Introduction ............................................................................................................................................. 107
5.2 Experimentation and Discussion .................................................................................................... 109
   5.2.1 NCI library screening ................................................................................................................... 109
      5.2.1.1 Structural analyses ............................................................................................................... 112
      5.2.1.2 Validation of chemical structure and inhibitory activity of NCI hits 1-5 ..... 113
      5.2.1.3 Reactive compounds ............................................................................................................ 113
      5.2.1.4 Aggregators .............................................................................................................................. 114
      5.2.1.5 Cell-based assay ..................................................................................................................... 114
   5.2.2 Confirmation of hits from the WEHI library ...................................................................... 115
   5.2.3 Mechanism of IDO1 inhibition ................................................................................................. 117
      5.2.3.1 Reversibility and time-dependency .............................................................................. 119
   5.2.4 Structure-activity relationship (SAR) of the NCI hits 1-5 ............................................ 126
      5.2.4.1 Thioanilines (1) ...................................................................................................................... 126
      5.2.4.2 Phenantroimidazoles (2) ................................................................................................... 127
      5.2.4.3 Benzofurazans (BF) (3) ...................................................................................................... 127
      5.2.4.4 Pyrimidinones (4) ................................................................................................................. 128
      5.2.4.5 Indolonoxides (5) .................................................................................................................. 128
CHAPTER 6

IMPORTANCE OF SERINE-167 AND CYSTEINE-129 IN IDO1 ACTIVE SITE FOR BINDING OF IDO1 INHIBITORS

6.1 Introduction ............................................................................................................................................. 130
6.2 Results ........................................................................................................................................................ 132
  6.2.1 Characterisation of mutant enzymes .................................................................................... 132
  6.2.2 Screening of the NCI library for C129A or S167A sensitive IDO1 inhibitors ...... 134
  6.2.3 Structure-activity study of IDO1 inhibitors sensitive to the S167A mutation .... 137
6.3 Discussion ................................................................................................................................................. 139

CHAPTER 7

EXPRESSION AND PURIFICATION OF HUMAN TRYPTOPHAN 2,3-DI-OXYGENASE (TDO)

7.1 Introduction ............................................................................................................................................. 142
7.2 Results ........................................................................................................................................................ 143
7.3 Discussion ................................................................................................................................................. 146

CHAPTER 8

CONCLUDING DISCUSSION

8.1 Introduction ............................................................................................................................................. 148
8.2 Research Outcomes.................................................................................................................................. 148
  8.2.1 Development of an automated, sensitive assay for IDO1 .............................................. 148
  8.2.2 Identification of the structure and formation of the new fluorophore PIP-THQ ................................................................. 149
  8.2.3 Use of the PIP fluorescence assay for identification of IDO1 inhibitors ................. 149
  8.2.4 Importance of serine-167 and cysteine-129 for the binding of IDO1 inhibitors ................................................. 151
8.3 Future work .............................................................................................................................................. 151
  8.3.1 Resolving the mechanism of IDO1 inactivation by compound 8 ......................... 152
  8.3.2 Development of optimised analogues of compounds 4 and 5e ......................... 152
  8.3.3 TDO/IDO1 dual inhibitors ................................................................................................. 153
  8.3.4 Combination therapy of IDO1 inhibitors plus other cancer therapies ............ 154
8.4 Final summary ......................................................................................................................................... 155

APPENDIX .................................................................................................................................................. 156

Figure A. 1. JANUS script for IC50 automated protocol (part 1) .............................................. 156
Figure A. 2. JANUS script for IC50 automated protocol (part 2) .............................................. 157
Figure A. 3. HPLC chromatograms of the synthesised compounds..........................158
Figure A. 4. a) $^1$H NMR and b) $^{13}$C NMR spectrum of PIP-THQ...........................160
Figure A. 5. $^1$H-$^1$H Correlation spectroscopy NMR spectrum of PIP-THQ. .............161
Figure A. 6. $^1$H-$^1$H total correlation spectroscopy NMR spectrum of PIP-THQ. ..........162
Figure A. 7. $^1$H-$^{13}$C HMBC NMR spectrum of PIP-THQ. .......................................163
Figure A. 8. $^1$H-$^{13}$C Heteronuclear single-quantum correlation spectroscopy NMR spectrum of PIP-THQ. .................................................................164
Figure A. 9. $^{1}$H-$^{1}$H NOESY NMR spectrum of PIP-THQ. .........................................165
Figure A. 10. $^{1}$H-$^{15}$N Heteronuclear single-quantum correlation spectroscopy NMR spectrum of PIP-THQ. .................................................................166
Figure A. 11. HPLC chromatograms of 2 mM a) N-(4-nitrophenyl)formamide (MW166), b) methyl 2-formamidobenzoate (MW179) and c) l-tryptophan (l-TRP) acquired before (black line) and after (red line) incubation with 1 M piperidine (PIP) at 65°C for 20 min. ..............................................................................................................167
Figure A. 12. Mass fragmentation spectra of a) PYR-THQ, b) 3-MePIP-THQ and c) PIP-THQ in positive electrospray ionisation mode (QqQ). .................................................................168
Figure A. 13. $^1$H NMR spectrum of 3-MePIP-THQ. .......................................................169
Figure A. 14. $^{1}$H-$^1$H Correlation spectroscopy NMR spectrum of 3-MePIP-THQ. .........170
Figure A. 15. $^{1}$H-$^1$H NOESY NMR spectrum of 3-MePIP-THQ. ..................................171
Figure A. 16. Frequency distribution of total number of biological test results deposited in PubChem database amongst a) NCI library compounds and b) NCI hits. .......................172
Figure A. 17. Frequency distribution of percentual activity of a) NCI library compounds and b) NCI hits, deposited in PubChem database. .................................................................173
Figure A. 18. Inhibitory activity of a) known IDO1 inhibitors and b), c) IDO1 inhibitors identified in this study, after 25 min preincubation with rhIDO1 (600 nM, 1% DMSO, 21°C) and 100-fold dilution into the complete assay buffer, respectively. .........................174
Figure A. 19. Inhibitory activity of NCI and WEHI hits against recombinant hIDO1 or native hIDO1 expressed in Lewis Lung carcinoma (LLTC) cells. .................................................................175
Table A. 1. Chemical structures and properties of the thirty-five screening hits (compounds that inhibited rhIDO > 50% at 20 μM) obtained from the NCI Diversity Set III library. .........................................................................................................................................................176
Table A. 2. rhIDO1 inhibitory activity of benz(o)-imidazole/thiazole series. .................180
Table A. 3. Percent inhibition and apparent IC$_{50}$ of 18 NCI library compounds that showed significantly decreased inhibitory activity against S167A mutant IDO1. ..........181

LICENSE AGREEMENTS ..................................................................................................................183
REFERENCES ....................................................................................................................................193
List of Figures

Figure 1.1. Schematic overview of the kynurenine pathway................................................................. 4
Figure 1.2. Comparison of TDO and IDO1 protein structures............................................................... 9
Figure 1.3. Catalytic mechanism of IDO1.............................................................................................. 10
Figure 1.4. IDO1 effector pathways...................................................................................................... 19
Figure 1.5. Non-catalytic immunosuppression by IDO1 in a long-term immune tolerance........... 22
Figure 1.6. Structural classes of IDO1 inhibitors.................................................................................. 29
Figure 1.7. Overview of potential combinatorial strategies to complement IDO1 inhibitors....... 39
Figure 1.8. Overall strategy and the main objectives for the work in this thesis............................. 41
Figure 2.1. Expression vector F279-V5/hIDO1 for production of hIDO1 enzyme.............................. 48
Figure 2.2. Production of the rhTDO bacterial expression constructs using Gateway cloning........... 56
Figure 2.3. Synthesis of NFK............................................................................................................... 61
Figure 2.4. Synthesis of PIP-THQ........................................................................................................ 63
Figure 2.5. Synthesis of 3-MePIP-THQ............................................................................................... 64
Figure 2.6. Synthesis of PIP-KYN........................................................................................................ 65
Figure 2.7. Synthesis of KYN-CKA..................................................................................................... 66
Figure 2.8. Synthesis of NFK-CKA..................................................................................................... 67
Figure 3.1. Schematic pathway of microplate assays for measuring IDO1 activity.............................. 70
Figure 3.2. a) Calibration curve of KYN and signal of blank in assay buffer treated with 160 mM NaOH in 384-well plates (50 µL) incubated for 1 h at ambient temperature............................ 71
Figure 3.3. a) Excitation and b) emission fluorescence spectra obtained in enzyme assay mixture using 160 mM NaOH (solid line) or 200 mM PIP (dashed line).................................................. 72
Figure 3.4. a) HPLC chromatogram of PIP-THQ produced in the enzyme assay treated with 200 mM PIP showing two eluting substances at the indicated R ...................................................... 73
Figure 3.5. a) PIP-THQ formed with a length of incubation time at 55°C, 65°C, 80°C and 95°C determined using HPLC.................................................................................................................. 75
Figure 3.6. a) Calibration curve of NFK (0.3-18.7 µM) in assay buffer treated with 50, 100, 200, 500 and 1000 mM PIP...................................................................................................................... 76
Figure 3.7. Effect of various concentrations of a) Ascorbic acid, b) Catalase, c) Tween 20, d) Methylene blue and e) DMSO on fluorescence signal of 10 µM NFK in assay buffer (closed circles) or assay buffer alone (open circles) reacted with 200 mM PIP....................................................... 78
Figure 3.8. Initial User Query from the protocol for determining IC50 of IDO1 inhibitors.............. 79
Figure 3.9. a) Chemical structures of three published IDO1 inhibitors titrated for inhibition of IDO1 enzymatic activity measured using b) the PIP fluorescence assay and c) the TCA absorbance assay........................................................................................................................................... 81
Figure 3.10. IDO1 inhibitory activity of the compounds of the NCI Diversity Set III determined using fluorescence assay and plotted against that obtained using the a) absorbance assay and compared using b) Bland-Altman analysis. ............................................................... 82

Figure 3.11. Updated schematic pathway of microplate assays for measuring dioxygenase enzymes activity using (a,b,e) KYN formation, (c,d) NFK formation and d) TRP detection. .................................................................................... 87

Figure 4.1. Overview of major chemical transformations of KYN and NFK........................... 91

Figure 4.2. Mass fragmentation of PIP-THQ isotopes in triple quadrupole positive electrospray ionisation mode............................................................... ............................................................... ....................................................... 93

Figure 4.3. Fluorescence titration of 15 μM PIP-THQ measured in 0.1 M KCl at 25°C.............. 94

Figure 4.4. HPLC chromatogram of the reaction of a) NFK (2 mM) or b) KYN (2 mM) with PIP (1 M) incubated at 65°C for 20 min and immediately cooled on ice prior to analysis. ............ 95

Figure 4.5. HPLC chromatogram of reaction progress of 20 mM KYN incubated in 20 mM NaOH at 80°C ............................................................................................................................... 96

Figure 4.6. HPLC chromatograms (254 nm) of the reaction of NFK (800 μM) and PIP (1 M)..... 97

Figure 4.8. Decomposition of PIP-NFK into PIP-KYN after treatment with HCl.................... 99

Figure 4.9. Proposed pathway of NFK and KYN reactions with PIP ......................................... 100

Figure 4.10. Reaction progress of a) 2 mM NFK and 1 M PIP at 65°C and b) 5 mM KYN and 1 M PIP at 80°C ......................................................................................................................................................... 101

Figure 4.11. Formation of PIP-THQ in the reaction of a) variable NFK and 1 M PIP and b) 2 mM NFK and variable PIP........................................................................................................ 102

Figure 4.12. Schematic illustration of the effect of PIP methyl substituents on the formation of fluorophores.................................................................................................................. 105

Figure 5.1. IDO1 inhibitory activity of NCI library compounds (n = 1600) at 20 μM in 0.2% DMSO. ............................................................................................................................... 109

Figure 5.2. A number of NCI library hits that failed a) individual computational filters and b) the indicated number (0-6) of filters used in a)......................................................... 112

Figure 5.3. Confirmation of 28 hits identified from WEHI high-throughput screening............ 117

Figure 5.4. Kinetics of IDO1 inhibition by 4-PI and WEHI hits 6-8. ....................................... 118

Figure 5.5. Reversibility and time-dependence of IDO1 inhibitors ....................................... 121

Figure 6.1. The active site of human IDO1 (PDB: 2D0T). ......................................................... 132

Figure 6.2. Characterisation of mutant IDO1 enzymes .......................................................... 133

Figure 6.3. Screening of the NCI library compounds (at 20 μM) for inhibitory activity against a) wild-type and S167A mutant IDO1, and b) wild-type and C129A mutant IDO.............. 136

Figure 7.1. Schematic representation of two different rhTDO inserts subcloned into Novagen pET-62-DEST bacterial expression vector. ......................................................... 143
Figure 7.2. Expression and enzymatic activity of rhTDO isolated from bacterial strains
BL21(DE3) and BL21(AI) transformed with expression vectors rhTDO Construct 1 and
rhTDO Construct 2. ........................................................................................................... ................................ 145

List of tables

Table 1.1. The association of IDO1 or its surrogate markers with survival in cancer. .............. 26
Table 1.2. The ongoing human clinical trials of IDO1 inhibitors alone and in combination with
other cancer therapies ............................................................................................................. 34
Table 1.3. Antitumour efficacy of IDO1 inhibitors in combination with other cancer therapies.
.................................................................................................................................................. 36
Table 2.1. Sources of the compounds used for structure activity studies in sections 5.2.4 and
6.2.3 ........................................................................................................................................ 43
Table 2.2. A list of primers used in this work (Uppercase letters indicate bases complementary
to the amplified DNA template and vice versa) ........................................................................ 51
Table 2.3. PCR thermal cycling conditions .............................................................................. 52
Table 2.4. 1H and 13C resonances of PIP-THQ ........................................................................ 64
Table 3.1. Comparison of the microplate assays used for assaying activity of dioxygenase
enzymes (see also Fig. 3.11) .................................................................................................. 88
Table 4.1. Molecular weights (MW), retention times (Rt) and amount of amine-KYN, amine-NFK,
amine-THQ adducts formed in reaction of amines with NFK (red font), and the amine-KYN
adduct formed in reaction of amines with KYN (blue font) .................................................... 103
Table 5.1. Inhibitory activities and structural analysis of selected IDO1 inhibitors .................. 111
Table 5.2. Reversibility and time-dependency of tested IDO1 inhibitors (summary of the results
from Figure 5.5) ..................................................................................................................... 121
Table 5.3. IDO1 inhibitory activities of analogues of NCI hits 1-5 (highlighted in black
background) .......................................................................................................................... 122
Table 6.1. Absorbance maxima, enzyme activity and Soret’s ratio of wild-type and mutant IDO1
enzymes ...................................................................................................................................... 134
Table 6.2. Percent inhibition and apparent IC50 of three NCI library compounds that showed
increased inhibitory activity against C129A mutant IDO1 ......................................................... 136
Table 6.3. Structure-activity relationship of the three S167A sensitive compounds (2, 3d, 5)
from NCI library (in black background) against wtIDO1 and S167A ...................................... 138
# Glossary

<table>
<thead>
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<th>Abbreviation</th>
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<tr>
<td>1MT</td>
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<td>2-MePIP</td>
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**Chapter 3, sections 3.1, 3.2.2, 3.2.3, 3.2.5 and 3.3.**


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**CO-AUTHORS**

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<tr>
<td>Brian D. Palmer</td>
<td>edited the manuscript</td>
</tr>
<tr>
<td>Jack U. Flanagan</td>
<td>edited the manuscript, designed the experiments, analysed data</td>
</tr>
<tr>
<td>Sai-Parng Fung</td>
<td>edited the manuscript</td>
</tr>
<tr>
<td>David J. A. Bridewell</td>
<td>edited the manuscript</td>
</tr>
<tr>
<td>Joanne F. Jamie, Lai-Ming Ching</td>
<td>LMC analysed the data and designed the experiments. JFJ provided IDO1 expression construct and edited the manuscript</td>
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**Certification by Co-Authors**

The undersigned hereby certify that:
- the above statement correctly reflects the nature and extent of the PhD candidate’s contribution to this work, and the nature of the contribution of each of the co-authors; and
- in cases where the PhD candidate was the lead author of this work that the candidate wrote the text.

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**Last updated:** 25 March 2013
Co-Authorship Form

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Chapter 4, section 4.2 except for 4.2.3.


Nature of contribution by PhD candidate: designed and performed the experiments, analysed and interpreted the results, wrote the manuscript.

Extent of contribution by PhD candidate (%): 70

CO-AUTHORS

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Last updated: 25 March 2013
1.1 Introduction

Cancer is a leading cause of death in developed countries, and New Zealand leads the charts with 21,300 new cancer cases and 8,600 cancer-related deaths recorded in 2012 (Ferlay et al., 2013). Whilst improved understanding of cancer biology has provided many novel cancer therapies, there are none that a) are effective against all types of cancer, b) are curative, c) work in all patients, and d) show low toxicity. Commonly prescribed chemotherapies destroy rapidly dividing cancer cells as well as non-tumour proliferating cells, resulting in adverse events such as gastrointestinal toxicity, hair loss, reduced production of blood cells, or infertility. To avoid damage to healthy cells, researchers started to develop drugs that target mutant proteins expressed by cancer cells to stimulate their own growth. Although such drugs are initially effective for the majority of suitable patients, the tumour cells ultimately evolve to resist the drugs, resulting in the remission.

Paradoxically, while we are attempting to target and destroy the cancer cells with synthetic drugs, nature has already developed a superior anticancer mechanism of its own – the immune system. In the same way that it protects us from the invading pathogens on a daily basis; it can seek out and destroy the tumour cells with a deadly precision. Similarly to a memory generated of successfully defeated pathogens, the immune system remembers the cancer cells it encountered which limits their recurrence. In addition, the plasticity of the immune system allows it to adapt to a changing phenotype of tumour cells. However, cancers co-opt a variety of immune regulatory and suppressive mechanisms to “escape” the immunity, contributing to the emergence of malignant cancer. These mechanisms need to be relieved to allow our immunity to fight back.

Co-inhibitory molecules cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed cell death-1 (PD-1) (so called “immune checkpoints”) on T-lymphocytes are one of these suppressive mechanisms. Very recently, monoclonal antibody ipilimumab that blocks CTLA-4, and nivolumab that blocks PD-1, demonstrated spectacular tumour regressions in patients with various malignancies (Ott et al., 2013, Robert et al., 2013). With most mature data in metastatic melanoma, overall response rates pooled from multiple clinical trials range between 14-40% for ipilimumab and 38-45% for nivolumab (Berman et al., 2015). Importantly, the tumour remissions were long-lasting. Analysis of 4800 metastatic melanoma
patients treated by ipilimumab in pooled phase II/III trials showed that nearly 20% of them survived up to 10 years (Berman et al., 2015). For a disease where the 2-year survival rate is about 10% (Robert et al., 2011), this is an exceptional achievement. This demonstrates that a properly activated immune system can eradicate an aggressive disease such as metastatic melanoma, and thanks to the immunological memory, the antitumour responses can be sustained. These clinical results have forever changed the field of cancer therapy and sparked intense efforts to search for other immune-suppressive pathways that can complement the checkpoint inhibitors and increase the proportion of patients benefitting from the immune-modulation. Preclinical (Ngiow et al., 2011, Woo et al., 2012) and clinical (Robert et al., 2011, Wolchok et al., 2013) studies are already showing a significant benefit of combining immune checkpoint inhibitors with other cancer therapies.

One of the key immune escape mechanisms active in a broad range of human cancers with a substantial practical appeal for drug development is the tryptophan catabolising enzyme indoleamine 2,3-dioxygenase 1 (IDO1). The first part of this chapter will explore the tryptophan catabolism along the kynurenine pathway; the enzymes catalysing the initial step of this pathway with the focus on IDO1; the mechanisms of IDO1-mediated immune-suppression, and the association of IDO1 expression with a poor clinical prognosis of cancer patients. The second part will provide an overview of small-molecule IDO1 inhibitors, the ongoing human clinical trials, and combinatorial strategies that may complement IDO1 inhibitors in the clinic.

1.2 Escaping immune surveillance

The recent success of cancer immunotherapies confirmed that immune surveillance, a process in which the immune cells of the host seeks out and destroy cancer cells, exists. However, the concept of immune surveillance has been uncertain for a long time. In the 19th century, Rudolf Virchow observed that tumours commonly contain infiltrates of leukocytes (Katz et al., 2009). Paul Ehrlich later proposed that the immune system prevents a high occurrence of carcinomas in animals and that modulation of the immune system might be used to treat cancer (Dunn et al., 2002). William B. Coley put this theory to practice. In 1891, Coley inoculated terminal cancer patients with living bacteria that had a curative effect on many of them (Coley, 1891). This work has stimulated a large number of studies aimed to prove the existence of the immune surveillance against tumours, but a compelling conclusion about its existence could not be made for a long time.
Chapter 1- Escaping immune surveillance

Seminal studies by Robert Schreiber’s group in 2001 confirmed the essential role of the immune system in tumour suppression (Shankaran et al., 2001, Smyth et al., 2001). However, it became apparent that while the immune system can eliminate cancer cells, it inadvertently selects for cancer cells that can “escape” immune surveillance and form malignant cancer. This process was called “cancer immunoediting”, and involves three phases: Elimination, Equilibrium and Escape (Dunn et al., 2002, Schreiber et al., 2011).

Briefly, in the Elimination phase, the expression of tumour specific antigens and innate immunity ligands on tumour cells can be recognised by the innate and adaptive immune system leading to elimination or durable control of the growing cancer cells. This phase is clinically occult. If the tumour cells are not eradicated, the Equilibrium phase follows. Here, the immune system keeps the tumour in check but the constant thrust and parry between the tumour and immune cells slowly select for tumour cells able to escape immune recognition. Tumours may remain at this stage for the entire host’s life, not clinically evident. During the last phase, the Escape, the tumour cells capable of subverting the antitumour immunity start spreading and form a clinically relevant cancer. The immune escape phase is of a particular therapeutic interest because it is there that most human cancers are diagnosed.

Tumour cells utilise a large number of mechanisms to avoid antitumour immunity including down-regulation of strong tumour antigens or antigen presenting machinery (Drake et al., 2006). Moreover, most malignant cells express co-inhibitory ligands PD-L1 and PD-L2, which are capable of inducing cell death in activated T-cells (Singer et al., 2011). Soluble factors such as immunosuppressive cytokines transforming growth factor-beta (TGF-β) and interleukin (IL)-10 are also expressed by tumour cells. Non-tumour cells such as regulatory T-cells (Tregs) or myeloid-derived suppressor cells (MDSC) also strongly suppress antitumour immunity in the tumour microenvironment (Blankenstein et al., 2012, Turk, 2014, Vasievich & Huang, 2011).

However, one mechanism stands out as it affects numerous other immune escape mechanisms. It is the catabolism of tryptophan (TRP) along the kynurenine pathway (KP) (Fig. 1.1) mediated by indoleamine 2,3-dioxygenase 1 (IDO1) or tryptophan 2,3-dioxygenase (TDO) (Sono et al., 1996). Depletion of TRP and accumulation of toxic kynurenine pathway metabolites activate and differentiate Tregs, inhibit tumour-killing cells (T-cells and NK cells), and lead to the production of TGF-β and IL-10 (Mellor & Munn, 2004). IDO1 and TDO can be turned on in tumour cells, stromal cells, immune cells (mainly dendritic cells) and MDSC and therefore represent an attractive therapeutic target to reverse the tumour-induced immune suppression (Mellor & Munn, 2008).
Chapter 1- Escaping immune surveillance

Figure 1.1. Schematic overview of the kynurenine pathway. More than 95% of tryptophan enters the kynurenine pathway where it is converted by IDO1, TDO or IDO2 to N-formylkynurenine, which is subsequently hydrolysed into the first stable KP metabolite kynurenine by kynurenine formamidase (KF). Kynurenine has three possible fates. It is converted to kynurenic acid by kynurenine aminotransferase (KAT) or to 3-hydroxyanthranilic acid either through 3-hydroxykynurenine by kynurenine 3-monooxygenase (KMO) or via anthranilic acid. 3-hydroxyanthranilic acid can undergo either condensation reaction to cinnabarinic acid or enzymatic transformation by 3-hydroxyanthranilate 3,4-dioxygenase (HAO) to 2-amino-3-carboxymuconate-semialdehyde (ACMS), an unstable product that is converted enzymatically (by picolinic carboxylase (PC)) into picolinic acid or non-enzymatically into quinolinic acid; the latter being a precursor for the synthesis of nicotinamide adenine dinucleotide confined only to liver cells.
1.3 The kynurenine pathway (KP)

Tryptophan (TRP) is the least abundant essential amino acid which must be acquired in a diet. It is also the largest protein-coding amino acid (molecular weight = 204.23 g/mol) containing an indole functional group that confers its distinctive properties such as high hydrophobicity and aromaticity. TRP has many essential functions in mammals. Apart from biosynthesis of proteins, TRP serves as a precursor for tryptamine (the serotonin receptor agonist), serotonin itself (neurotransmitter), and melatonin (sleep hormone) (Claustrat et al., 2005, Hasegawa & Nakamura, 2010, Stone & Darlington, 2002). However, the majority (~95%) of ingested TRP is broken down in the KP (Stone & Darlington, 2002, Vecsei et al., 2013). The KP produces a number of bioactive metabolites and ultimately yields a co-enzyme nicotinamide adenine dinucleotide (NAD+) critical for many enzymatic processes such as dehydrogenation; although NAD+ is not essential and can be substituted by dietary intake of vitamin B3 (nicotinic acid) (Fatokun et al., 2013) (Fig. 1.1).

The decrease in TRP and increase in KP metabolites is profoundly immune-suppressive and plays a role in pregnancy (Munn et al., 1998) and tumoural immune escape (Uyttenhove et al., 2003). However, TRP depletion was shown to be important as a defence mechanism against certain microbes and intracellular parasites auxotrophic for TRP (Pfefferkorn, 1984, Schmidt & Schultze, 2014). The KP metabolites have a significant role also in brain function. Metabolites 3-hydroxyanthranilic acid (3-HAA), 3-hydroxykynurenine (3-HK) and quinolinic acid (QA) are neurotoxic whereas kynurenic acid (KA) and picolinic acid (PA) are neuroprotective (Maddison & Giorgini, 2015). QA is an excitotoxin that selectively activates N-methyl-D-aspartate receptors and is a potent generator of reactive oxygen species (ROS) in complexes with iron (Pláteník et al., 2001, Stone & Perkins, 1981). Similarly, 3-HK and 3-HAA produce harmful free radicals and 3-HK potentiates the neuronal toxicity of QA (Goldstein et al., 2000, Guidetti & Schwarcz, 1999, Vazquez et al., 2000). In contrast, KA is a scavenger of free radicals including superoxide anion, hydrogen peroxide or peroxynitrite (Lugo-Huirtón et al., 2011) generated by other KP metabolites and nitric oxide synthases and can antagonise binding of QA to the N-methyl-D-aspartate receptors (Maddison & Giorgini, 2015). The optimal balance of KP metabolites and KA is likely essential to limit the neuronal toxicity and proper function of the brain.

Ample evidence suggests that the imbalance in KP metabolites associates with serious neurodegenerative diseases including: Parkinson’s, Huntington’s and Alzheimer’s disease, and also dementia, schizophrenia and epilepsy (Vecsei et al., 2013). Moreover, diseases
characterised by the deregulated immune system are also associated with KP. For example, patients with Crohn’s disease, multiple sclerosis, rheumatoid arthritis or AIDS all showed high KYN levels and upregulated IDO1 compared to healthy controls (Gupta et al., 2012, Hartai et al., 2005, Huengsberg et al., 1998, Merlo et al., 2014). In addition, KP metabolites KYN and 3-HK covalently modifies human lens proteins contributing to the development of cataract (Taylor et al., 2002). Interestingly, stress might also be connected to KP. Rats exposed to stress by immobilisation or forced running expressed more tryptophan 2,3-dioxygenase (TDO) than non-stressed animals (Nomura, 1965).

The above evidence emphasises the critical role of KP in the pathogenesis of the most serious human diseases, but the prospect of KP modulation for the clinical application has been validated so far only in cancer.

1.4 The gatekeepers of the kynurenine pathway (IDO, TDO and IDO2)

1.4.1 IDO1 and TDO

The first and the rate-limiting step in the KP is the oxidation of TRP to N-formylkynurenine (NFK) which is then rapidly converted by the enzyme formamidase to kynurenine (KYN) (Mehler & Knox, 1950), a first stable metabolite in the KP (Fig. 1.1). Three enzymes can catalyse the conversion of TRP to NFK - IDO1, IDO2 and TDO. Let us first explore the two better understood of the three enzymes - IDO1 and TDO.

The discovery of IDO1 and TDO is tightly associated with the discovery of the KP. In 1853, a German chemist, Justus von Liebig, identified an unknown acid in the urine of the dogs and called it the kynurenic acid (KA) according to the source (Liebig, 1853). The idea that it is a metabolite of TRP, however, was not made until Ellinger noticed that the dogs fed with TRP excrete higher levels of KA in the urine (Ellinger, 1905). In 1925, Matsuoka reported that the urine of rabbits injected with TRP contains a yet unreported intermediate (Matsuoka & Yoshimatsu, 1925) – a missing link between TRP and KA – later determined to be KYN by Butenandt and colleagues (Butenandt et al., 1940). At that time, the enzymatic process responsible for TRP catabolism was unknown. In 1936, Kotake’s group showed that rat liver extracts metabolise TRP into KYN (Kotake, 1936) and the liver enzyme responsible for the production of KYN - tryptophan 2,3-dioxygenase (TDO) - was isolated for the first time in 1955 (Knox, 1955). Although TDO preferentially oxidises a levoratory TRP enantiomer (L-TRP), Kotake found that rabbits excrete KYN also when injected with d-TRP implying TDO is not the only enzyme capable of KYN production (Kotake & Ito, 1937). This
second enzyme, indoleamine 2,3-dioxygenase 1 (IDO1), was purified from a rabbit intestine in 1967 (Yamamoto & Hayashi, 1967).

Whilst both IDO1 and TDO catalyse the identical biochemical reaction, their physicochemical properties, structures, and biological roles are distinct. IDO1 is a monomer (molecular weight 45 kDa) (Takikawa et al., 1988) consisting of a small N-terminal and a large C-terminal domain that harbours the catalytic site (Sugimoto et al., 2006), whereas TDO is a tetramer (molecular weight 167 kDa) composed of subunits that are structurally similar to the large domain of IDO1 (Fig. 1.2) (Schutz & Feigelson, 1972, Thackray & Chapman, 2008). Catalytic sites of IDO1 and TDO contain prosthetic group haem b which is essential for catalysis (Fig. 1.2d). TDO and IDO1 monomers share only 10% amino acid sequence identity but the active site topology is comparable, indicating the binding of similar substrates (Fig. 1.2d) and catalytic mechanism (Fig. 1.3) (Chauhan et al., 2008). However, the evidence suggests otherwise: a) TDO preferably metabolises L-TRP, whereas IDO1 oxidises both TRP enantiomers (L and D) despite D-TRP having a ~10-fold lower affinity for IDO1; (Takikawa et al., 1988, Watanabe et al., 1980) b) IDO1 oxidises a broad range of substrates including tryptamine, serotonin or TRP analogues such as 5-hydroxy- or 5-methyl-TRP; (Pantouris et al., 2014, Takikawa et al., 1988) c) both IDO1 and TDO are catalytically more active in a reduced Fe^{2+} form, but TDO can oxidise L-TRP even in Fe^{3+} form suggesting L-TRP might play a role in activation of TDO (Batabyal & Yeh, 2007).

The reductive activation of IDO1 is necessary for its catalytic activity (Fig. 1.3). In isolated enzyme assays, the reduction equivalent is provided by the methylene blue/ascorbic acid (MB/AA) system; but the superoxide anion can also activate IDO1 (Hirata et al., 1977). The in vivo reducing cofactor of IDO1 is currently under hot debate. Early experiments showed that inhibition of a superoxide anion generating system (xanthine oxidase/inosine) attenuated activity of IDO1 expressed in rabbit enterocytes, suggesting the superoxide anion as the IDO1 activator (Taniguchi et al., 1977). The authors, however, used a strong reductant, methylene blue, which likely overpowered the potential of superoxide anion. Recently, two studies suggested that cytochrome b5 activates IDO1 in both yeast and mammalian cells (Brastianos et al., 2006, Maghzal et al., 2008). Specifically, the knockout of cytochrome b5 by siRNA in IDO1-transfected HEK293 cells significantly decreased KYN production compared to that of the controls, whereas superoxide dismutase (the scavenger of superoxide anions) did not affect KYN production in these cells. Therefore, superoxide anion is unlikely to be an activator of IDO1 in vivo, while cytochrome b5 is also not the only activator because IDO1 activity was observed even in cytochrome b5 knockout cells.
Figure 1.2. (See legend on page 9.)
Chapter 1- The gatekeepers of the kynurenine pathway (IDO, TDO and IDO2)

(See figure on page 8.)

**Figure 1.2.** Comparison of TDO and IDO1 protein structures.

**a)** The structure of *Drosophila melanogaster* TDO (PDB: 4HKA) formed from four identical monomers packed together as a dimer of two U-shaped dimers. One dimer is represented as a red and cyan surface model, and the other one as a yellow and magenta cylinder cartoon model for clarity. Each monomer harbours a single binding pocket containing haem b (green spheres). Whether all 4 active sites can catabolise tryptophan has not yet been elucidated. Note that the active site of each monomer contains N-terminal amino acid residues of the adjacent monomer.

**b)** The surface representation of human IDO1 (PDB: 2D0T) featuring an active site cavity accommodating haem b (green spheres). A ligand 4-phenyl-1H-imidazole and two buffer molecules of N-cyclohexyl-2-aminoethanesulfonic were removed from the active site.

**c)** Structural alignment of human IDO1 (orange) and a *Drosophila melanogaster* TDO monomer (cyan) demonstrating a marked structural overlap in the catalytic site domain (depicted as a magenta mesh). Both TDO and IDO1 each contains structurally different terminal segments.

**d)** Aligned binding pockets of human IDO1 (yellow-orange carbons) and *Drosophila melanogaster* TDO (cyan carbons) demonstrating a significant similarity of the active site topology and residues. Amino acid residues of TDO (in brackets) and IDO1 (without brackets) are labelled by a three-letter code and a protein sequence number. The purple dashed lines indicate hydrogen bonds. Atom colours: red (oxygen), blue (nitrogen), brown sphere (iron).

Images were generated using PyMOL, the Molecular Graphics System, Version 1.7.2, Schrödinger, LLC (www.pymol.org).
Chapter 1- The gatekeepers of the kynurenine pathway (IDO, TDO and iDO2)

Figure 1.3. Catalytic mechanism of IDO1. IDO1 requires activation by a single electron reduction of the haem iron from Fe$^{3+}$ to Fe$^{2+}$ to be able to oxidise its substrates. Whilst the catalytic mechanism of IDO1 is not yet fully understood, the first step is likely the abstraction of a proton from the indole nitrogen of tryptophan mediated by water molecules in the active site and the O$_2$-haem complex. This step produces a ternary complex tryptophan-O$_2$-haem inducing a breakage of the tryptophan indole ring and the insertion of two oxygen atoms via either the Criegee or dioxetane pathway to form a final product – N-formylkynurenine.

Abbreviations: MB/AA (methylene blue/ascorbic acid), H346 (histidine-346 – ligates the haem of IDO1).

Authors reported, however, that neither flavin nucleotides nor biopterin could activate IDO1 despite being reported to activate purified murine IDO1 (Maghzal et al., 2008). Cytochrome b5 and either NADH/cytochrome b5 reductase or NADPH/cytochrome P450 reductase systems were later confirmed as more efficient IDO1 activators than MB/AA in vitro (Kolawole et al., 2015, Pearson et al., 2010). In addition, the activation of IDO1 by cytochrome b5 system relieved the inhibition of IDO1 by l-TRP commonly observed in MB/AA assay (Pearson et al., 2010). Therefore, the kinetics of IDO1 in artificial MB/AA assay may not be representative of in vivo conditions. The role of cytochrome b5 in TDO activation is unclear.
Chapter 1 - The gatekeepers of the kynurenine pathway (IDO, TDO and iIDO2)

The biological roles of both IDO1 and TDO are strikingly different. IDO1 is induced mainly in immune cells by pro-inflammatory stimuli including interferons (IFN) of type I (IFN-α and IFN-β) and type II (IFN-γ), bacterial endotoxin lipopolysaccharide (LPS), CpG oligodeoxynucleotide (CpG) or prostaglandin E2 (PGE2) (Braun et al., 2005, Taylor & Feng, 1991, Trabanelli et al., 2015). IDO1 has an immune-regulatory role. IDO1 is not active in most tissues excluding placenta, uterus, lung, intestines, and lymphoid organs; (Théat et al., 2014) the tissues exposed to the non-self antigens where IDO1 is needed to dampen excessive immune reactions. In contrast, TDO is transcriptionally induced by glucocorticoids and mainly expressed in the liver where it regulates systemic L-TRP levels; (Feigelson & Greengard, 1962, Schimke et al., 1965) however, TDO was recently found also in placenta, pregnant uterus, brain, epididymis and testis (Lob et al., 2009). Consistent with its regulatory role of systemic TRP concentration, TDO has a low affinity for L-TRP ($K_m = 190 \mu M$) (Batabyal & Yeh, 2007) and degrades at low TRP concentrations (L-TRP is needed to stabilise the haem in the TDO active site) (Feigelson & Greengard, 1962, Schimke et al., 1965), which ensures that TDO does not deplete systemic TRP pool below physiological levels (around 60-80 $\mu M$) (Laich et al., 2002). TDO is also inhibited by nicotinic acid derivatives, the end products of KP (Cho-Chung & Pitot, 1967). Therefore, the absence of substrate and excess of the KP end products turns off TDO activity; a negative feedback loop not observed for IDO1. The regulation of systemic TRP levels by TDO is well demonstrated in TDO-knockout mice that show 10-fold higher systemic TRP concentration compared to wild-type animals; and have altered behaviour and neurogenesis thanks to the elevated levels of serotonin (Kanai et al., 2009). In contrast, IDO1 needs to deplete TRP levels to lower levels than TDO for immune-suppression activity, and its affinity for L-TRP is therefore stronger ($K_m = 21 \mu M$) (Batabyal & Yeh, 2007, Pantouris et al., 2014). In this way, both enzymes have specific physiological roles.

Of interest, both TDO and IDO enzymes are not exclusive to mammals and are expressed in various species ranging from bacteria to metazoans (Yuasa & Ball, 2015). However, the catalytic efficiency of TDOs has been more conserved in evolution than that of IDOs, which underlines the “housekeeping” biological role of TDO. In contrast, only the mammalian lineage features IDO1 gene with high catalytic efficiency (Yuasa & Ball, 2015). It is conceivable that IDO1 evolved in mammals specifically to regulate the increasingly complex immune system.
Chapter 1- The gatekeepers of the kynurenine pathway (IDO, TDO and IDO2)

1.4.2 IDO2

IDO2, identified in 2007 (Ball et al., 2007, Metz et al., 2007), is the least understood of all three dioxygenase enzymes. IDO2 is also a haem-enzyme of similar size as IDO1 (407 amino acids, a functional splice variant) (Meininger et al., 2011) located downstream of the IDO1 gene on chromosome 8p12 in humans (Metz et al., 2007). The chromosomal location suggests that IDO2 arose by duplication of the IDO1 gene in the evolution of mammals, as IDO2 is not present in prokaryotes, fungi or invertebrates (Yuasa & Ball, 2015). Despite this, IDO2 shows low similarity (63%) and identity (43%) to human IDO1 at the amino acid level, but the active site residues of IDO1 critical for enzyme activity are highly conserved in mammalian IDO2 (Metz et al., 2007). In contrast to IDO1, IDO2 does not seem to metabolise L-TRP or similar TRP analogues. The catalytic activity of IDO2 for L-TRP is extremely weak or undetectable in both the purified enzyme assays and the cell-based assays compared to that of IDO1 (Meininger et al., 2011, Pantouris et al., 2014, Qian et al., 2012). In one study, affinity of L-TRP for human IDO2 ($K_m = 6809 \mu M$) was 324-fold lower than that for IDO1 ($K_m = 21 \mu M$), and turnover number was 30-fold lower (3 s$^{-1}$ and 0.1 s$^{-1}$ for IDO1 and IDO2, respectively) using the MB/AA reducing system (Pantouris et al., 2014). In contrast, the affinity of L-TRP for recombinant murine IDO2 increased 23-fold when assayed using cytochrome b5 assay, a more physiologically relevant reducing agent than MB/AA (Austin et al., 2010). IDO2 might therefore be able to catalyse L-TRP at same catalytic efficiency as IDO1 under conditions that have not yet been identified.

The tissue expression of IDO2 is distinct to that of IDO1. IDO2 is expressed in mammalian liver, kidney, epididymis and brain, but also in the placenta; a major expression site of IDO1 (Kudo & Boyd, 2000, Metz et al., 2007). The induction of IDO2 expression also appears different to IDO1. Whilst IDO2 can be induced by IFN-γ or LPS in murine pre-dendritic cells and human tumour cells (Lôb et al., 2009, Metz et al., 2007), Trabanelli and colleagues showed that human dendritic cells (DCs) express IDO2 constitutively by Western blotting and KYN measurement. Furthermore, proinflammatory cytokines (IL-1β, tumour necrosis factor-α (TNF-α), IL-6) and PGE2 did not affect IDO2 expression while it upregulated IDO1 expression ~10-fold compared to unstimulated cells (Trabanelli et al., 2014). Although regulation of IDO2 appears to be distinct from IDO1, both proteins might interact. One study suggested that IDO1 affects splicing of the IDO2 transcript. In this work, peritoneal macrophages from IDO1-knockout mice produced a higher percentage (~60%) of alternatively spliced IDO2 mRNA (encoding catalytically inactive IDO2) than macrophages from wild-type mice (~20%). The splicing of IDO2 appears
complex. The literature reports six human IDO2 splice isoforms of which the four are catalytically inactive (Metz et al.,2007, Metz et al.,2014). In addition, the human IDO2 gene has two alternative N-terminal exons and the longer transcript (420 amino acids) does not metabolise TRP (Meininger et al.,2011). Single nucleotide polymorphisms encoding inactive IDO2 are abundant in the population (Metz et al.,2007). Up to 50% of individuals of European and Asian descent, and 25% of Africans may carry defective IDO2 alleles. In contrast, alleles encoding inactive IDO1 are rare in the population (~1%) (Arefayene et al.,2009, Lee et al.,2014a). The lack of genetic variability of the IDO1 gene within the population strongly suggests that IDO1 evolved primarily as a catalytic enzyme, unlike IDO2.

Taken together, this leads to a hypothesis that if IDO2 evolved to have some biological function in mammals, and is not simply an inactive copy of IDO1 gene, that function is likely non-catalytic. Evidence supports this hypothesis. In a mouse model of rheumatoid arthritis, IDO2-knockout but not IDO1-knockout mice showed less severe joint inflammation (Merlo et al.,2014). Others suggest that the IDO1 immune-suppression may be conditional on IDO2. In a study by Metz and colleagues, immune-suppression by Tregs isolated from wild-type mice but not IDO2-knockout mice could be abrogated by a mixture of PD-1/PD-L1/PD-L2 antibodies (Metz et al.,2014). As the expression of PD-L1 ligands is characteristic for IDO1-induced Tregs (Sharma et al.,2007), it suggests that IDO1 lost the ability to activate Tregs. On the other hand, Lee and co-workers proposed that IDO2 inhibits IDO1 catalytic activity by reducing the availability of haem cofactor in the cytosol (Lee et al.,2014b). When human IDO2 and IDO1 were co-expressed in HEK293 cells, the production of KYN by the cells decreased compared to IDO1-only transfected cells. This inhibition was relieved by a mutation of histidine-360 of IDO2 critical for the haem binding. This scenario is particularly conceivable since the haem co-factor is essential for IDO1 activity and succinylacetone (the inhibitor of haem biosynthesis) was shown to abrogate IDO1 activity in human macrophages (Thomas et al.,2001).

IDO2 might be instrumental for IDO1 immune-suppression, but its weak catalytic activity and high frequency of inactive alleles in the population makes the therapeutic targeting of IDO2 by small-molecule inhibitors an uncertain prospect.

1.5 IDO1-mediated immune suppression

The immunosuppressive function of IDO1 was first recognised in 1998 in a groundbreaking discovery by Munn and colleagues (Munn et al.,1998). This study offered an
Chapter 1- The IDO1 immunomodulation mechanisms

1.6 The IDO1 immunomodulation mechanisms

1.6.1 Tryptophan depletion

Active IDO1 in antigen presenting cells (APCs), stromal cells or tumour cells can efficiently deplete L-TRP in the microenvironment (Friberg et al.,2002, Munn et al.,1999, Munn & Mellor,2007). Activated T-cells are particularly sensitive to low levels of L-TRP which triggers their cell cycle arrest (in mid-G1 phase) and apoptosis (Frumento et al.,2001, Lee et al.,2002). It is surprising though that T-cell proliferation ceases at ~500 nM L-TRP in vitro. Depletion of L-TRP to such a low level is not consistent with the low affinity of common mammalian amino acid transporters for L-TRP (~20-30 μM) (Kaper et al.,2007, Seymour et al.,2006). Furthermore, how can the other cells in the tumour microenvironment survive at such low levels of TRP while T-cells are dying? Several studies have suggested that the effect of amino acid starvation is cell type dependent and regulated, at least in part, by kinase General control non-derepressible 2 (GCN2). Furthermore, both tumour cells and
DCs, but not T-cells, seem to express high-affinity TRP transporters that enable them to deplete TRP in the microenvironment and protect themselves from the TRP starvation.

1.6.1.1 TRP transporters

IDO1 is an intracellular enzyme (Kudo & Boyd, 2000, Kudo & Boyd, 2001) and requires TRP which cannot passively diffuse into cells. Therefore, the supply of TRP for IDO1 depends entirely on amino acid transporters. Transport of amino acids across the membrane of mammalian cells is chiefly regulated by system-L which is specific for large neutral amino acids. It is a heterodimer consisting of a one glycoprotein chain CD98 and a one catalytic chain such as LAT1 or LAT2. System-L acts as an antiporter, i.e., exchanges cytosolic amino acids for the extracellular amino acids and vice versa (Verrey, 2003). Using the fluorescence-based real-time measurement of intracellular TRP, Kaper and colleagues showed that KB oral cancer cells released intracellular TRP upon addition of exogenous KYN to the medium, indicating that system-L may be a KYN/TRP exchanger (Kaper et al., 2007). This would be quite relevant for circulation of TRP and KP metabolites in the tumour microenvironment.

Low-affinity system-L controls TRP degradation by IDO1 in villous placental extracts and may be the main amino acid transporter on T-cells (Kudo & Boyd, 2001). In contrast, human macrophages and mouse dendritic cells (DC2.4) were shown to express additional amino acid transporters specific for L-TRP (Bhutia et al., 2015, Seymour et al., 2006). These transporters show a 100-fold higher affinity for L-TRP (K_m 300-500 nM) than system-L explaining the ability of DCs to deplete extracellular TRP to such low levels. Interestingly, IFN-γ stimulated both TRP uptake and IDO1 expression in DC2.4 cells (Bhutia et al., 2015) indicating that the high-affinity TRP transporters are indispensable for IDO1 activity and likely co-evolved together; inducible by IFN-γ. Although cancer cells commonly express only system-L (Fuchs & Bode, 2005, Travers et al., 2004), a recent report showed that HeLa cells treated with IFN-γ expressed a high-affinity TRP transporter although with lower L-TRP specificity than the one found in APCs (Silk et al., 2011). It appears that even in cancer cells, expression of IDO1 and amino acid transporters might be coupled together. This presents a double trouble for T-cells, which apparently lack any high-affinity TRP transporters.

These data suggest a model where the APCs expressing IDO1 would efficiently draw TRP from the local microenvironment in exchange for the intracellular KYN, which will be forced into the T-cells in exchange for their TRP, thereby producing a continuous siphon of TRP towards IDO1-expressing cells (Kaper et al., 2007). It is important to realise, however, that this model assumes steady-state L-TRP concentration in the microenvironment. It is
uncertain whether L-TRP depletion plays a role in vivo where TRP concentration in plasma is ~70 μM (Masaki et al., 2015, Weinlich et al., 2007, Widner et al., 1997) and depleted L-TRP can be replenished from the surrounding tissues.

Nonetheless, considering the critical role of TRP transporters for IDO1 catalytic activity, their function is not adequately understood. The characterisation of these transporters, their regulation and expression in tumour cells and APCs need to be performed because they might be useful therapeutic targets to complement inhibition of IDO1.

1.6.1.2 General control non-derepressible 2 (GCN2)

GCN2 is a kinase activated by uncharged tRNAs during the amino acid starvation. Activated GCN2 phosphorylates serine-51 in the α-subunit of eukaryotic translation initiation factor 2 (eIF2α) leading to the shutdown of total protein biosynthesis (Castilho et al., 2014). However, phosphorylated eIF2α has a superior affinity for certain transcripts (Castilho et al., 2014). These include, for example, activated transcription factor 4 (ATF4) which initiates translation of genes such as amino acid transporters or amino acid synthetases needed to alleviate the amino acid deprivation (Ye et al., 2010). The response of GCN2 pathway to IDO1-mediated TRP starvation is not well understood but it is clear that the consequences of GCN2 activation are cell-type dependent.

In stimulated cytotoxic T-cells (CTL), activation of GCN2 pathway results in an anergy which is not observed in GCN2-knockout T-cells or when medium is supplemented with excess TRP (Munn et al., 2005). Likewise, the activation of suppressive Tregs by IDO1-expressing plasmacytoid DCs is contingent on an intact GCN2 pathway (Fallarino et al., 2006, Grohmann & Bronte, 2010, Sharma et al., 2007). The signalling pathways responsible for such disparate effects in CD4+ T-cells and CTL still remain to be identified.

Whilst the activated GCN2 pathway suppresses effector T-cells, it is critical for proliferation and survival of many spontaneous mammalian tumours and promotes angiogenesis in a number of cancer cell lines (Ye et al., 2010). The essential role of GCN2 for tumour proliferation is demonstrated in mouse models. Knockdown of GCN2 in head and neck cancer cell line, UM-SCC-22B, implanted in immune-deficient mice (SCID) was sufficient to inhibit formation of the tumour blood vessels and tumour growth (Wang et al., 2013). Similarly, fibrosarcoma cells (HT1080) with GCN2- or ATF4-knockdown completely stopped growing in nude mice whereas wild-type cells grew rapidly (Ye et al., 2010). The tumours obviously regressed even in immune-deficient hosts indicating the GCN2/eIF2α pathway has a direct cytostatic effect independent of the immune system.
Chapter 1- The IDO1 immunomodulation mechanisms

Although GCN2 may appear as a good therapeutic target, the pathway is critical for a number of vital processes such as regulation of cell cycle, metabolism of lipids (Guo & Cavener, 2007), gluconeogenesis and regulation of feeding behaviour (Hao et al., 2005, Sattlegger et al., 2011) which makes GCN2 targeting a challenge.

1.6.2 Accumulation of tryptophan metabolites

KYN and other downstream KP metabolites, with the exception of AA, are toxic to cells, and therefore augment the effect of TRP starvation (Bauer et al., 2005, Fallarino et al., 2002a, Frumento et al., 2001, Terness et al., 2002). KYN, 3-HK, 3-HAA, and PA all induce cell death in T-cells (CD4+ and CTL populations), NK-cells, but not B-cells suggesting that IDO1 preferentially suppresses cellular immunity (Th1 cells) (Frumento et al., 2002, Terness et al., 2002). However, the concentrations required to induce T-cell death in vitro are high (EC50 ~ 200 μM), inconsistent with their activity as single agents in vivo. On the other hand, mixtures of KP metabolites, e.g., KYN + 3-HAA (EC50 = each at 21 μM) enhanced the T-cell killing potency (Terness et al., 2002). It is therefore conceivable that KP metabolites act synergistically to destroy T-cells. The addition of exogenous TRP attenuated the toxicity of KYN and PA (Frumento et al., 2002) to activated T-cells, raising the idea that IDO1 immunosuppression might be contingent on simultaneous starvation of TRP and the accumulation of KP metabolites (Fallarino et al., 2006, Grohmann et al., 2003, Pallotta et al., 2011). One study suggested, however, that only TRP starvation in the absence of KP metabolites was sufficient to inhibit the growth of activated T-cells in vitro (Munn et al., 2004). Incompatible with these observations, restimulated alloreactive T cells grew even in the absence of TRP in vitro (Frumento et al., 2002). Such disparate results suggest that the microenvironment of the cells will play a significant role in IDO1-mediated immune suppression.

The mechanisms of toxicity of KP metabolites are not well known and focus mostly on 3-HAA and KYN. 3-HAA is a redox active molecule consistent with the finding that it depletes intracellular GSH which, in turn, triggers apoptosis in activated T-cells (Guidetti & Schwarcz, 1999, Hayashi et al., 2007, Lee et al., 2010). In DCs, 3-HAA downregulated T-cell co-activating ligands CD80 and CD86 leading to a reduced T-cell activation in BALB/c mice and in vitro (Soliman et al., 2013). KYN, on the other hand, was found to downregulate cell surface receptors NKG2D and NKp46 on NK cells which decreased their capacity to kill tumour cell lines (Chiesa et al., 2006). In vivo data support the immunosuppressive activity of KP metabolites. Chromium picolinate reduced T-cell proliferation, but not antibody production in lambs, indicating again that IDO1 immunosuppression primarily targets T and
NK cell compartments (Dallago et al., 2013). Consistent with these results, administration of 3-HAA and KYN to mice significantly enhanced survival of allogeneic murine transplants (Bauer et al., 2005, Lee et al., 2010).

In a similar vein to TRP starvation, the KP metabolites promote tumour growth. In one study, IDO1-knockout mice showed reduced growth of chemically induced colon cancer (Thaker et al., 2013). This was an unusual observation since IDO1 would be expected to suppress antitumour immunity thereby increase tumour growth. IDO1 promoted tumour proliferation independently of the immunosuppression by the production of KP metabolites QA and KYN which were subsequently found to activate the Wnt-signalling pathway in HCT-116 colon cancer cells (Thaker et al., 2013).

Although TRP metabolites can directly kill effector T-cells and promote tumour proliferation, some of them can bind to and activate aryl hydrocarbon receptor (AhR). Increasing evidence demonstrates that AhR is an essential signalling component in the IDO1 immunosuppressive programme that is responsible for the production of Tregs (Fig. 1.4) (Bessede et al., 2014, Pallotta et al., 2011, Quintana, 2014, Vogel et al., 2008).

1.6.2.1 Aryl hydrocarbon Receptor (AhR)

AhR is a ligand-activated transcription factor that resides in the cytosol in a complex with chaperoning proteins heat shock protein 90 (Hsp90) (Perdew, 1988) and heat shock protein 23 (Hsp23); a non-receptor tyrosine kinase Src (Src); (Enan & Matsumura, 1996) and an immunophilin hepatitis B virus X-associated protein 2 (XAP2) (Meyer et al., 1998). Amongst the number of its functions (Nguyen & Bradfield, 2007), AhR controls a differentiation between the two opposing populations of T-cells: Tregs, which are immunosuppressive; and Th17 cells, which causes autoimmunity (Fig. 1.4) (Quintana et al., 2008, Quintana et al., 2010, Veldhoen et al., 2008). AhR is mostly known as a receptor of the environmental toxin 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) which induces metabolic enzymes of cytochrome P450 class (Cyp1a1, Cyp1b1) (Kerkvliet, 2002). It was initially thought that AhR plays a detoxification role, but strong immunosuppressive effects of TCDD on mice indicated that AhR performs additional functions far beyond metabolism of toxic chemicals. Moreover, developmental defects of hypomorphic AhR mice (i.e., mice expressing low levels of AhR) could be eliminated by administration of TCDD, which strongly suggests that an endogenous AhR agonist must exist (Kerkvliet et al., 2002, Nguyen & Bradfield, 2007, Vorderstrasse et al., 2001).
Chapter 1 - The IDO1 immunomodulation mechanisms

Figure 1.4. IDO1 effector pathways. Enzymatically active IDO1 in antigen presenting cells, tumour cells or stromal cells depletes tryptophan that induces growth arrest or cell death in T-cells (T) but stimulates the growth of cancer cells by activating the GCN2 pathway in both cell types. Tryptophan metabolites produced by IDO1 synergises with tryptophan depletion and kills effector immune cells. Kynurenine (KYN) binds to AhR in tumour cells and promotes tumour aggressiveness. Some of the tryptophan metabolites bind to and activate AhR in T-cells. The activation of AhR by different metabolites controls the balance between the dark side (immunosuppressive regulatory T-cells (Treg)) and the light side (pro-inflammatory Th17 cells (Th17)) of the immunomodulatory force. This balance can affect the tumour progression (Opitz et al.,2011, Shin et al.,2013).
Chapter 1- The IDO1 immunomodulation mechanisms

A large number of endogenous ligands of AhR have been discovered so far including TRP metabolites (Denison & Nagy, 2003, Stejskalova et al., 2011). KYN was identified as an AhR activator already in 1998 (Denison & Nagy, 2003) but a direct binding to AhR was demonstrated only recently (Bessede et al., 2014). The activation of AhR in naïve T-cells by physiological concentrations of KYN (5-50 μM) triggers their differentiation into Tregs (Fig. 1.5) (Mezrich et al., 2010). The Tregs differentiation was also shown in vivo and appears to be contingent on IDO1. In C57BL/6 mice, IDO1 inhibitor (1MT) blocked proliferation of Tregs induced by an immunosuppressive AhR agonist TCDD (Vogel et al., 2008). Some studies further suggest that AhR controls even IDO1 expression. LPS-stimulated bone marrow-derived dendritic cells (BMDCs) deficient in AhR inhibited differentiation of naïve T-cells into Tregs in vitro but the addition of exogenous KYN to the co-culture restored production of Tregs (Nguyen et al., 2010). Similarly, induction of functional IDO1 by LPS or CpG was strictly dependent on AhR in BMDCs (Nguyen et al., 2010). The control of IDO1 expression by AhR is rational as it will increase the activation of AhR by IDO1 downstream metabolite KYN. Interestingly, the differentiation of Tregs by KYN synergises with immunosuppressive cytokine TGF-β, which upregulates AhR expression in the T-cells (Fig. 1.5) (Mezrich et al., 2010). The above evidence suggests that the differentiation of Tregs represents a dominant immunosuppression mechanism of IDO1. Furthermore, when these IDO1-induced Tregs recognise an MHC-II-restricted antigen on APCs in low TRP environment, they get activated into even more potent suppressors that utilise PD-1/PD-1L pathway to shut down T-cell proliferation (Sharma et al., 2007).

Some endogenous TRP metabolites other than KYN can bind to and activate AhR (Fig. 1.4). 2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE), likely a conjugate of TRP and cysteine (Song et al., 2002), elicited profound immunosuppressive effects comparable to KYN in mice and in vitro (Nugent et al., 2013, Quintana et al., 2010). In marked contrast, a KP metabolite cinnabarinic acid (CBA) that forms from 3-HAA (see Fig. 1.1), and a UV-photoproduct of TRP, 6-formylindolo[3,2-b]carbazole (FICZ), elicited opposite effects to that of KYN and ITE (Fig. 1.4). FICZ and CBA stimulated differentiation of proinflammatory Th17 cells and suppressed formation of Tregs (Lowe et al., 2014, Mezrich et al., 2010, Quintana et al., 2008). FICZ was also shown to be necessary for the cytolytic activity of NK cells against MHC-I-deficient lymphomas (RMA-S cells) in vivo that cannot be controlled by T-cells (Shin et al., 2013). Furthermore, the tumour-killing activity of AhR-deficient NK cells was severely compromised confirming the bipolar role of AhR (Shin et al., 2013).
Figure 1.5. (See legend on page 22.)
Figure 1.5. Non-catalytic immunosuppression by IDO1 in a long-term immune tolerance. In certain murine dendritic cells, IDO1 can act as a signalling molecule. IDO1 can be phosphorylated at its two putative immunoreceptor tyrosine-based inhibitory motifs (ITIMs) by tyrosine kinases Src or Fyn. IDO1 may trigger its phosphorylation by producing KYN, which binds to and activate AhR and Src tyrosine kinase. TGF-β signalling through phosphoinositide 3-kinase (PI(3)K) and SMAD upregulates Src-homology 2 domain-containing tyrosine phosphatases SHP1 and SHP2 that can dephosphorylate ITIMs on IDO1 and trigger the non-canonical NF-κB pathway (p52/Rel-B). This amplifies IDO1 itself and produces immunosuppressive TGF-β, which has two main roles. TGF-β differentiates naive T-cells into regulatory T-cells (Tregs) by upregulating FoxP3 transcription factor, and upregulates AhR in naive T-cells to enhance differentiation of Tregs by KYN. The potency of TGF-β in Treg cell differentiation appears higher than that of KYN but both molecules synergise together.

Different TRP metabolites can, therefore, produce opposite outcomes upon binding to AhR (Fig. 1.4). One group (KYN and ITE) drives AhR towards tolerance and the other (CBA and FICZ) drives AhR towards immunity. How is the balance between these two groups controlled? The formation of TRP conjugates FICZ and ITE is not enzymatically controlled but may increase when the IDO1 is inactive or inhibited and TRP levels elevate. Therefore, inhibition of IDO1 may be ineffective because it may lead to the production of immunosuppressive ITE. Concerning the KP metabolites, CBA is located downstream of KYN on the KP (Fig. 1.1), therefore the regulation of enzymatic activity of other KP enzymes might determine tolerance versus immunity in this case. Exactly how the activity of IDO1 or its inhibition affects the balance of TRP and KP metabolites in tumour microenvironment will need to be determined in future experiments.

The interaction of AhR with TRP metabolites stimulates a number of fascinating open questions. In particular, how a single receptor (AhR) can be activated to trigger such distinct pathways? Some studies suggest that binding of a ligand causes specific conformational changes in the structure of AhR so it can target different DNA sites (Nuti et al., 2014). For example, mutation of a single residue in the AhR binding site could divert otherwise agonistic ligand into an antagonist (Soshilov & Denison, 2014).

### 1.6.3 IDO1 is a signalling molecule

IDO1 contains two putative immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in its non-catalytic N-terminal domain, which are well conserved in mammalian IDO1 enzymes (Orabona et al., 2008). ITIMs play a significant role in immunological signalling. However, other than covering the catalytic site of the enzyme, the function of this domain in
IDO1 has long been unknown. In murine plasmacytoid DCs, TGF-β but not IFN-γ triggered phosphorylation of the two ITIMs on IDO1, which served as a scaffold for non-canonical NF-κB pathway signalling leading to the induction of IDO1 and TGF-β expression (Fig. 1.5) (Pallotta et al., 2011). This signalling function of IDO1 was independent of its catalytic activity. The study clearly demonstrated that the induction of IDO1 expression by IFN-γ fades out quickly, and TGF-β was necessary for the induction of a long-term IDO1 expression in plasmacytoid DCs. This leads to a sustained Treg cell generation through the combined effects of TRP starvation, AhR activation by KYN, and TGF-β production (Fig. 1.5) (Fallarino et al., 2006, Sharma et al., 2007, Sharma et al., 2009). A non-catalytic mechanism of IDO1 immune suppression was also observed in a mouse model of graft-versus-host disease (Rohlman et al., 2013), and in a long-term immune tolerance against bacterial endotoxin (LPS) in mice (Bessede et al., 2014). It is conceivable that the IDO1 signalling will be active also in tumours where TGF-β is a dominant late stage cytokine (Connolly et al., 2012). TGF-β appears to be the “alpha and omega” for the long-term IDO1 immunosuppression in vivo, but where does the initial TGF-β to initiate the IDO1 phosphorylation come from?

Cancer cells and a number of immune cells can produce TGF-β (Tu et al., 2014), but a recent study has suggested that the initial IDO1 phosphorylation may not require TGF-β. A knockout of AhR in LPS-stimulated murine DCs abrogated IDO1 phosphorylation, and the inhibitor (PP2) of AhR-associated tyrosine kinase Src attenuated both the IDO1 phosphorylation and TGF-β production (Bessede et al., 2014). Therefore, activated AhR can drive the phosphorylation of IDO1. It is tempting to speculate that IDO1 triggers its phosphorylation by producing AhR-agonist KYN (Bessede et al., 2014, Mezrich et al., 2010). Taken in sum, KP metabolites might play an important immunosuppressive role early during inflammation, but the non-catalytic function of IDO1 will dominate the long-term immune tolerance (Pallotta et al., 2011, Pallotta et al., 2014). Figure 1.5 summarises the IDO1-AhR-TGF-β signalling pathway.

The non-enzymatic immunosuppression by IDO1 is an intriguing finding but it may limit the clinical benefit of IDO1 inhibitors. However, this non-enzymatic function was identified in mouse models. Reproducing these results in human cells and determining whether it affects IDO1 inhibition by small-molecule inhibitors will be critical. Not compatible with this idea, crystal structures of human IDO1 (PDB: 2D0T and 4PK5) show that the tyrosine residues in question are buried inside the enzyme, inaccessible to the solvent (Pallotta et al., 2014). That makes the phosphorylation of ITIMs difficult to rationalise. Some conformational change in IDO1, induced either by post-translational modification or binding
by a substrate/ligand, is therefore needed to expose these residues. Alternatively, the Src kinase itself might induce the conformational change of IDO1 prior to phosphorylation.

The phosphorylation of IDO1 has an additional and contrasting function. During inflammation, cross-linking of the surface molecule CD28 on T-cells to CD80/86 on murine DCs was shown to induce IL-6-dependent upregulation of suppressor of cytokine signalling 3 (SOCS3), which bound phosphorylated IDO1 and activated its proteasomal degradation (Orabona et al., 2008). This proteasomal degradation of IDO1 is likely an important mechanism to reverse IDO1 induced tolerance in DCs (Orabona et al., 2004). Interestingly, activation of AhR by yet another KP metabolite KA produces IL-6 in breast cancer cells but only when stimulated by IL-1β (DiNatale et al., 2010). This suggests that KA, a downstream metabolite of IDO1, might enhance the proteasomal degradation of IDO1 in the inflammatory milieu. The differential consequences of the AhR activation by three distinct KP metabolites (KA, KYN, CBA) markedly underlines the complexity of interactions of IDO1 metabolites with AhR.

1.7 Regulation of IDO1 expression and activity

Since TRP catabolism is toxic to cells, IDO1 activity needs to be tightly regulated. The absence of IDO1 mRNA in most tissues (Theáte et al., 2014) suggests that the control of IDO1 expression represents a major mechanism of IDO1 regulation. Interferons of type I (α,β) and II (γ) can induce expression of IDO1 in a number of cell types but IFN-γ induces IDO1 expression more effectively than IFN-α (Taylor & Feng, 1991). For example, many cancer cells were shown to upregulate IDO1 in response to IFN-γ but not to IFN-α or IFN-β (Ozaki et al., 1988, Werner-Felmayer et al., 1989).

A number of other inflammatory molecules can induce IDO1 such as TNF-α, LPS, or PGE2 under certain conditions (Braun et al., 2005, Nguyen et al., 2010, Trabanelli et al., 2014, Trabanelli et al., 2015). However, neither LPS nor TNF-α alone induces IDO1 expression in human DCs and the IDO1 expression was found to be contingent on the additional signal from either PGE2 or IFN-γ (Braun et al., 2005, Trabanelli et al., 2015). Similarly, IFN-γ alone cannot induce maximal IDO1 expression in human IDO1-positive mature DCs and additional co-stimulation from CTLA-4 on activated T-cells is required (Munn et al., 2004). This multistep IDO1 activation in DCs involving T-cell interactions likely ensures that the tolerance induced by IDO1 takes place only during cellular immunity to dampen the excessive immune reaction. It is also evident that IDO1 can be expressed devoid of catalytic activity (Mellor & Munn, 2004). Mature human DCs produced high levels of IDO1 protein by flow cytometry but showed high KYN production only after CD80/86 cross-linking and IFN-
Chapter 1- IDO1 is associated with a poor prognosis for cancer patients

γ stimulation (Munn et al.,2004). Likewise, comparable amounts of IDO1 observed by Western Blot were expressed by two subsets of murine splenic DCs, but only one subset produced KYN (Fallarino et al.,2002b). Therefore, some post-translational mechanisms that regulate IDO1 catalytic activity have to exist.

Haem b and reductive cofactors such as cytochrome b5 or NADH are essential for IDO1 activity but given that regulation of the biosynthesis of both molecules will affect other haem-dependent proteins, it does not appear to be a specific IDO1 regulatory mechanism. In contrast, phosphorylation of IDO1 on tyrosine residues of ITIMs is specific and can likely have additional functions to those already discussed in section 1.6.3. IDO1 can also be acetylated at the N-terminal alanine-2 residue (Fujigaki et al.,2007). Although this modification is known to increase the stability of certain mammalian enzymes, the role it plays for IDO1 is uncertain (Fujigaki et al.,2012). Nitric oxide plays a significant regulatory role for IDO1. The binding of nitric oxide to the IDO1 active site inhibits enzyme activity and accelerates its proteasomal degradation (Hucke et al.,2004). Moreover, nitric oxide reacts with superoxide anions to form peroxynitrite which inactivates IDO1 by nitrilation of the three tyrosine residues (Tyr15, Tyr315 and Tyr353) (Fujigaki et al.,2006, Fujigaki et al.,2007). Similarly, hydrogen peroxide irreversibly inactivates IDO1 by oxidation of IDO1 cysteine residues (Poljak et al.,2006). The latter two findings suggest that the oxidative stress in the cell may regulate IDO1 activity. Consistent with this idea, catalytic activity of IDO1 increased the oxidative stress in human macrophages; moreover, antioxidants such as 2-mercaptoethanol decreased the expression of IDO1 mRNA (Thomas et al.,2001). Paradoxically, it indicates that the oxidative stress that can be harmful to IDO1 may upregulate IDO1 expression. This idea is supported by the finding that inhibition of the glutamate/cystine transporters, which are necessary for uptake of cystine and synthesis of glutathione, stimulated IDO1 expression and catalytic activity in human LPS-treated DCs in IFN-γ independent manner (D’Angelo et al.,2012, Mattox et al.,2012). The signalling pathways regulating IDO1 activity in response to the oxidative stress are yet to be identified.

1.8 IDO1 is associated with a poor prognosis for cancer patients

Using immunohistochemistry on nearly 1,000 human cancer samples, two extensive studies (Table 1.1, columns Ref1 (Théate et al.,2014) and Ref2 (Uyttenhove et al.,2003)) found that IDO1 is expressed in a broad range of malignancies. Given that the female reproductive tract, digestive tract, and lung endothelial cells basally express IDO1 (Théate et al.,2014), it is not surprising to see a high frequency of IDO1 expression in the cancers of these tissues. The lowest expression of IDO1 was found in glioblastomas, breast, pancreatic and prostate carcinomas. However, the results for prostate and pancreatic carcinomas, and
Chapter 1 - IDO1 is associated with a poor prognosis for cancer patients

glioblastomas are not consistent between the two studies. Théate and colleagues (Table 1.1 - column Ref1) used a highly specific IDO1 antibody generated in-house (4.16 H1) which gives confidence in their results.

Table 1.1. The association of IDO1 or its surrogate markers with survival in cancer.

<table>
<thead>
<tr>
<th>Cancer type</th>
<th>IDO1+/total&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Global Survival</th>
<th>Associated markers</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>endometrial/</td>
<td>45/48 5/5</td>
<td>Reduced</td>
<td>(↑IDO1, ↑invasion)</td>
<td>(Ino et al., 2006)</td>
</tr>
<tr>
<td>uterine</td>
<td></td>
<td>Reduced</td>
<td>(↑IDO1, ↓CTL, ↓NK cell)</td>
<td>(Ino et al., 2008)</td>
</tr>
<tr>
<td>cervical</td>
<td>48/58 10/10</td>
<td>Reduced</td>
<td>(↑IDO1)</td>
<td>(Inaba et al., 2010)</td>
</tr>
<tr>
<td>kidney</td>
<td>48/60 5/10</td>
<td>Reduced</td>
<td>(↑IDO1)</td>
<td>(Riesenberg et al., 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prolonged</td>
<td>(↑IDO1)</td>
<td>(Yuan et al., 2012)</td>
</tr>
<tr>
<td>lung (non-small)</td>
<td>45/57 9/11</td>
<td>Not evaluated</td>
<td>(↑KYN/TRP)</td>
<td>(Suzuki et al., 2010)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reduced</td>
<td>(↑IDO1)</td>
<td>(Astigiano et al., 2005)</td>
</tr>
<tr>
<td>colorectal</td>
<td>46/59 10/10</td>
<td>Not evaluated</td>
<td>(↑KYN/TRP)</td>
<td>(Huang et al., 2002)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reduced</td>
<td>(↑IDO1, ↓CD3+)</td>
<td>(Brandacher et al., 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reduced</td>
<td>(↑IDO1)</td>
<td>(Ferdinande et al., 2012)</td>
</tr>
<tr>
<td>stomach</td>
<td>45/59 9/10</td>
<td>No correlation</td>
<td>(↑IDO1)</td>
<td>(Kim et al., 2014)</td>
</tr>
<tr>
<td>ovarian</td>
<td>35/53 8/10</td>
<td>Reduced</td>
<td>(↑IDO1)</td>
<td>(Okamoto et al., 2005)</td>
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<td></td>
<td></td>
<td>Reduced</td>
<td>(↑IDO1)</td>
<td>(Takao et al., 2007)</td>
</tr>
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<td></td>
<td></td>
<td>Reduced</td>
<td>(↑IDO1, ↓CTL)</td>
<td>(Inaba et al., 2009)</td>
</tr>
<tr>
<td>bladder</td>
<td>36/58 8/10</td>
<td>Not available</td>
<td></td>
<td></td>
</tr>
<tr>
<td>melanoma</td>
<td>32/60 11/25</td>
<td>Reduced</td>
<td>(↑KYN/TRP)</td>
<td>(Weinlich et al., 2007)</td>
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<td></td>
<td></td>
<td>Reduced</td>
<td>(↑IDO1, ↑FoxP3)</td>
<td>(Brody et al., 2009)</td>
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<tr>
<td>head and neck</td>
<td>27/59 7/11</td>
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<td></td>
<td></td>
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<tr>
<td>esophageal</td>
<td>25/60 7/10</td>
<td>Reduced</td>
<td>(↑IDO1, ↓CTL)</td>
<td>(Zhang et al., 2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reduced</td>
<td>(↑IDO1, ↓Bin1)</td>
<td>(Jia et al., 2015)</td>
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<tr>
<td>prostate</td>
<td>25/60 11/11</td>
<td>Not evaluated</td>
<td>(↑IDO1, ↑KYN/TRP)</td>
<td>(Feder-Mengus et al., 2008)</td>
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<tr>
<td>pancreatic</td>
<td>22/58 10/10</td>
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<td>(Witkiewicz et al., 2008)</td>
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<tr>
<td></td>
<td></td>
<td>Reduced</td>
<td>(↑IDO1, ↑FoxP3, ↓CTL)</td>
<td>(Kobayashi et al., 2010)</td>
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<tr>
<td>breast</td>
<td>21/57 3/10</td>
<td>Reduced</td>
<td>(↑IDO1, ↑COX-2, ↑mets)</td>
<td>(Chen et al., 2014)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prolonged</td>
<td>(↑IDO1)</td>
<td>(Soliman et al., 2013)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Not evaluated</td>
<td>(↑IDO1, ↑FoxP3, ↑mets)</td>
<td>(Mansfield et al., 2009)</td>
</tr>
<tr>
<td>glioblastoma</td>
<td>5/60 9/10</td>
<td>Reduced</td>
<td>(↑IDO1 in 72/75)</td>
<td>(Mitsuka et al., 2013)</td>
</tr>
<tr>
<td>thyroid</td>
<td>N/A 2/10</td>
<td>Not evaluated</td>
<td>(↑IDO1)</td>
<td>(Sonia Moretti et al., 2014)</td>
</tr>
<tr>
<td>T-cell/myeloid</td>
<td>N/A 4/18</td>
<td>Reduced</td>
<td>(↑KYN/TRP)</td>
<td>(Masaki et al., 2015)</td>
</tr>
<tr>
<td>leukemia/lymphoma</td>
<td></td>
<td>Reduced</td>
<td>(↑KYN)</td>
<td>(Mabuchi et al., 2015)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reduced</td>
<td>(↑IDO1)</td>
<td>(Fukuno et al., 2014)</td>
</tr>
<tr>
<td>liver</td>
<td>N/A 2/5</td>
<td>Prolonged</td>
<td>(↑IDO1)</td>
<td>(Ishio et al., 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reduced</td>
<td>(↑IDO1)</td>
<td>(Pan et al., 2008)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Number of IDO1-positive (IDO<sup>+</sup>) out of the total examined cancer samples by immunohistochemistry. Ref1 ((Théate et al., 2014)), Ref2 ((Uyttenhove et al., 2003)).

Abbreviations: mets (metastasis). Red font indicates conflicting results.
Expression of IDO1 or presence of its surrogate markers such as staining for FoxP3 (a marker of Tregs) or the elevated KYN/TRP ratio in the plasma of cancer patients strongly correlates with a poor prognosis in essentially all common cancers (Table 1.1). However, conflicting survival results were reported for breast, liver and kidney carcinoma patients. The interpretation of immunohistochemistry staining and RT-PCR in these experiments has to be tempered for two main reasons. Firstly, certain commercial IDO1 antibodies may not be specific to IDO1 (Théate et al., 2014), and secondly, the presence of IDO1 mRNA or IDO1 protein in the cell may not equate to catalytically active IDO1 (Fallarino et al., 2002b, Munn et al., 2004). This might be the explanation for some of the conflicting results observed. The measurement of KYN/TRP ratio is the most reliable indicator of functional IDO1; again is not without its pitfalls, KYN can be converted into other downstream KP metabolites in an unpredictable manner. In summary, however, it appears that IDO1 expression significantly contributes to the cancer progression in humans.

1.9 Validation of IDO1 as a therapeutic target for cancer therapy

As IDO1 is expressed in a broad range of human tumours and its expression correlates with a poor prognosis, IDO1 inhibition might provide a promising cancer treatment. The IDO1 inhibitor 1MT was shown to reverse immune-suppression during pregnancy in mice, suggesting that it might reverse tumour-mediated immune suppression as well. In one of the first experiments to test this hypothesis, 1MT significantly suppressed the growth of IDO1-transfected mastocytoma cells (P815B) in mice (Uyttenhove et al., 2003). This result was reproduced in several other mouse models of cancer including melanoma and orthotopic glioblastoma (Wainwright et al., 2014, Zheng et al., 2006). The antitumour effect of 1MT was immune-mediated and contingent on the host’s IDO1 (Hou et al., 2007, Uyttenhove et al., 2003). While these studies provided a proof-of-concept for therapeutic inhibition of IDO1, complete tumour regressions were not achieved. This may not be surprising given that the tumour associated antigens are less immunogenic than the allogeneic paternal antigens of a foetus. It could also be partly due to the low potency of 1MT. In support of this idea, silencing of IDO1 in melanoma cells (B16F10) prior to their inoculation into the mice or injection of IDO1 siRNA into the tumours of the mice was significantly more effective than treatment by 1MT alone (Zheng et al., 2006). In another study, all IDO1-knockout mice bearing IDO1-silenced glioblastoma cells in the brain survived for at least 5 months, whereas mice with non-silenced tumour cells died in less than 30 days (Wainwright et al., 2014). This showed that the total loss of IDO1 in the body can have a curative effect under certain
conditions. The potency of early IDO1 inhibitors may therefore not be sufficient to fully inactivate IDO1.

Interestingly, the IDO1 inhibitors 5-bromobassinin and vitamin K3 (menadione) showed slightly improved efficacy over 1MT in a mouse melanoma model (B16F10), but the effect was weaker in a breast cancer mouse model (mouse mammary tumour virus- Neu, MMTV-Neu) at identical treatment regimens (Banerjee et al.,2007, Kumar et al.,2008b). This suggested that reversal of immune suppression by IDO1 inhibitors might not be sufficient in a more “natural” and weakly immunogenic model like MMTV-Neu.

1.10 Small-molecule inhibitors of IDO1

IDO1 is an excellent immune-modulatory target because it is amenable to therapeutic inhibition by small-molecule inhibitors, unlike most “immune checkpoints” that have to be targeted by monoclonal antibodies. This brings several advantages. Firstly, compared to monoclonal antibodies or cell-based therapies, the costs associated with production and formulation of small molecules are considerably lower. Secondly, the pharmacodynamics of IDO1 inhibitors can be easily determined by quantification of the KYN/TRP ratio in blood serum of laboratory animals or human patients. Thirdly, mice genetically deficient in IDO1 are healthy and show no spontaneous autoimmunity (Mellor et al.,2003) suggesting pharmacological inhibition of IDO1 is reasonably safe. In contrast, mice genetically deficient in CTLA-4 die of an excessive autoimmunity and destruction of the major organs (Khattri et al.,1999). Lastly, IDO1 is not activated in most healthy tissues but is abundant in a broad range of tumours emphasising IDO1 as an ideal target for a therapeutic blockade. The first discovered IDO1 inhibitor was paradoxically the substrate itself. At high concentrations, L-TRP but not D-TRP inhibits IDO1 activity (Yamamoto & Hayaishi,1967). This observation provided a clue that analogues of TRP might inhibit IDO1.

1.10.1 Indoles

A large number of IDO1 inhibitors based on the indole nucleus have been identified to date (see examples in Fig. 1.6, top row) but they show a weak potency (IC\textsubscript{50} 10 – 100 μM) in cell-based and cell-free enzyme assays. Some indoles were found to suppress tumour growth \textit{in vivo} including 1MT, which is used in most biological IDO1 studies. The D-enantiomer of 1MT (D-1MT) was the first IDO1 inhibitor to enter human clinical trials but current evidence suggests that D-1MT (aka Indoximod or NLG-8189) is not a pharmacological IDO1 inhibitor.
### INDOLES

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ki (μM)</th>
<th>EC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-methyltryptophan (1MT)</td>
<td>34.6</td>
<td>266.9 (COS-1)</td>
</tr>
<tr>
<td>5-Bromo-brassinin (5-BB)</td>
<td>24.5</td>
<td>24.0 (COS-1)</td>
</tr>
<tr>
<td>Methylthiohydantoin-tryptophan (MTH)</td>
<td>11.4</td>
<td>12.85 (COS-1)</td>
</tr>
<tr>
<td>Indol-2-yl ethanone</td>
<td>13% at 20 μM (P815B)*</td>
<td></td>
</tr>
</tbody>
</table>

*(Muller et al., 2005a) (Banerjee et al., 2007) (Muller et al., 2005a) (Dolusić et al., 2011)*

### QUINONES

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (μM)</th>
<th>EC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-lapachone</td>
<td>0.44</td>
<td>1 (HeLa)</td>
</tr>
<tr>
<td>Menadione</td>
<td>1.1</td>
<td>28.9 (T-Rex)</td>
</tr>
<tr>
<td>Annulin B</td>
<td>0.12</td>
<td></td>
</tr>
</tbody>
</table>

*(Flick et al., 2013) (Kumar et al., 2008b) (Pereira et al., 2006)*

### IMIDAZOLES

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (μM)</th>
<th>EC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-phenylimidazole (4PI)</td>
<td>48</td>
<td>0.08 (P815B)</td>
</tr>
<tr>
<td>Triazole</td>
<td>0.33</td>
<td>&lt; 100 nM (HeLa)</td>
</tr>
<tr>
<td>Anilinotriazole</td>
<td>28 nM</td>
<td>75 nM (T-Rex)</td>
</tr>
</tbody>
</table>

*(Kumar et al., 2008a) (Röhrig et al., 2012) (Boyall et al., 2014) (Mautino et al., 2013)*

### MISCELLANEOUS

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (μM)</th>
<th>EC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyamidine 5L</td>
<td>71.8 nM</td>
<td>7.1 nM (HeLa)</td>
</tr>
<tr>
<td>Aminonitrile</td>
<td>&lt; 200 nM (HeLa)</td>
<td></td>
</tr>
<tr>
<td>Imidazothiazole</td>
<td>77 nM</td>
<td></td>
</tr>
<tr>
<td>N,N-disubstituted urea</td>
<td>0.61 nM (HEK293)</td>
<td></td>
</tr>
</tbody>
</table>

*(Yue et al., 2009) (Banerjee et al., 2014) (Tojo et al., 2014) (Markwalder et al., 2015)*

**Figure 1.6.** Structural classes of IDO1 inhibitors. Human IDO1 inhibitory activities in cell-free and cell-based assays are shown in the upper and lower row of the shaded area, respectively. Cell lines used for cell-based assays are indicated in parentheses next to the respective value. Ki denotes inhibitory constant in a cell-free enzyme assay. Clinical candidates are indicated in red. *Cells were transfected with murine IDO1.
D-1MT is a significantly weaker IDO1 inhibitor than L-1MT in both cell-free and cell-based enzyme assays (Löb et al., 2009, Pantouris et al., 2014, Qian et al., 2012). Despite this limitation, D-1MT showed similar or better \textit{in vivo} efficacy to L-1MT in a number of mouse models of cancer including the already mentioned MMTV-Neu or orthotopic glioblastoma (Hou et al., 2007, Wainwright et al., 2014). This suggested that the pharmacological target of D-1MT might not be IDO1. D-1MT inhibited IDO2 more potently than L-1MT in T-Rex cells transfected with IDO2 (Metz et al., 2007) but L-1MT was the stronger inhibitor in other studies (Qian et al., 2012, Yuasa et al., 2010). 1MT (a racemic mixture) does not inhibit TDO (Muller et al., 2005a) indicating that D-1MT may not be a strong inhibitor of any dioxygenase in the KP. A recent study proposed that D-1MT interferes with the mammalian Target Of Rapamycin (mTOR) signalling pathway (Metz et al., 2012). Similar to GCN2, mTOR is a sensor of amino acid deficiency but the signalling pathway of mTOR differs to GCN2. Metz and co-workers showed that HeLa cells expressing IDO1 deplete TRP in the medium that activates cell autophagy in an mTOR-dependent manner. The natural substrate L-TRP or IDO1 inhibitor D-1MT reversed the autophagy in these cells (Metz et al., 2012). Therefore, D-1MT acted as an L-TRP mimic providing an amino acid sufficiency signal to mTOR. This study not only offered a rational explanation for the D-1MT clinical activity but suggested that D-1MT might synergise with IDO1 inhibitors to reverse immunosuppression. D-1MT might thus require reclassification as a TRP mimic rather than an IDO1 inhibitor.

\textbf{1.10.2 Quinones}

Extracts of marine invertebrates provided IDO1 inhibitors such as annulins and exiguamine A based on a quinone core (Fig. 1.6), which inspired a number of synthetic analogues. Although quinones showed much higher potency than indoles, they are associated with poor cell permeability and toxicity in cell-based enzyme assays (Carvalho et al., 2014, Flick et al., 2013, Kumar et al., 2008b). Current evidence even suggests that quinones may not be the pharmacological IDO1 inhibitors. Some quinones exhibit uncompetitive inhibition kinetics not compatible with their binding to the vacant active site of IDO1 as predicted by computer docking (Carr et al., 2008, Flick et al., 2013). Also, a recent study demonstrated that menadione (2-methyl-1,4-naphthoquinone) inhibits IDO1 activity only in the conventional MB/AA assay and not in a more physiologically relevant cytochrome b5/NADPH/cytochrome P450 reductase assay (Pearson et al., 2010). This strongly suggests
that quinones inhibit the reductive activation of IDO1 by MB/AA and may not interact with the IDO1 active site.

1.10.3 Imidazoles

In 1989, Sono and Cady discovered 4-phenylimidazole (4PI), a non-competitive IDO1 inhibitor of modest potency that preferentially binds to the inactive Fe$^{3+}$ form of IDO1, thereby competing with the reductive activation of IDO1 (Sono & Cady, 1989). The binding of 4PI to the enzyme active site was confirmed in the first crystal structure of IDO1 in 2006 (Sugimoto et al., 2006). This crystal structure enabled a rational drug design of optimised 4PI analogues including triazoles and ultimately yielded the clinical candidate NLG919 (NewLink Genetics) (Fig. 1.6). Vertex Pharmaceuticals Inc. recently claimed anilinotriazoles likely derived from the triazole nucleus. Their patent lists close to 50 analogues that show sub-$\mu$M potency in cell-based enzyme assays but in vivo data were not published (Fig. 1.6).

1.10.4 Miscellaneous

In 2009, a high-throughput screening of Incyte Corporation’s corporate collection identified a potent hydroxyamidine, which was a milestone in IDO1 drug discovery (Yue et al., 2009). This compound exhibited competitive inhibition kinetics and was shown to interact with the IDO1 active site by spectroscopic and molecular docking studies. It did not cross-react with either TDO or IDO2. A diverse range of analogues with improved potencies were synthesised including hydroxyamidine 5L and ultimately led to the development of a clinical candidate INCB024360 (Fig. 1.6). The success of the hydroxyamidine series likely attracted the attention of Curadev Pharma Pvt. Ltd., which recently claimed aminonitriles in which the hydroxyamidine moiety is replaced by nitrile. Their patent lists over 100 compounds and some of them showed potency < 200 nM in cell-based IDO1 assays (Fig. 1.6).

Amg-1, a reversible competitive inhibitor discovered by the biotech company Amgen Inc. in 2011 (Meininger et al., 2011), likely inspired the rational drug design of a novel series of potent imidazothiazoles (Fig. 1.6). This was another milestone in IDO1 drug discovery. Two novel IDO1 crystal structures complexed with Amg-1 and imidazothiazole 13b were generated and demonstrated the remarkable flexibility of the IDO1 active site (Tojo et al., 2014). The imidazothiazole analogues containing a urea moiety (Fig. 1.6) showed enhanced IDO1 inhibitory activity due to the increased number of contacts with the IDO1 active site. The urea moiety appears to be important for some of the most potent IDO1 inhibitors. Bristol-Myers Squibb recently claimed N,N-disubstituted ureas as IDO1 inhibitors.
Chapter 1- Pharmacological IDO1 inhibitors in human clinical trials

Their patents show close to 1000 analogues eliciting potencies reaching pM levels in cell-based assays (Markwalder et al., 2015). Their work demonstrates a trend in IDO1 drug development towards larger molecules, increasing the number of interactions with IDO1 active site residues and bringing increased metabolic stability.

The list of IDO1 inhibitors discussed here is not exhaustive and keen readers are referred to detailed reviews covering the academic literature and patents up to the year 2012 (Dolušić & Frédérick, 2013, Muller et al., 2005b, Pucchio et al., 2010). Considering the number of advances in the field over the last three years, an updated review of IDO1 inhibitors would be timely. Two IDO1 inhibitors are currently in human clinical trials, INCB024360 and NLG919 (Table 1.2). A third IDO1 inhibitor, FLX-B is expected to begin human trials soon.

1.11 Pharmacological IDO1 inhibitors in human clinical trials

1.11.1 INCB024360 (Incyte Corp.)

INCB024360 was the first potent and orally bioavailable IDO1 inhibitor to enter human clinical trials in 2012. Oral administration of INCB024360 decreased KYN levels by 20-40% for at least 8 hours in both mice and dogs. Similar decreases in KYN levels were also observed in plasma, tumours and lymph nodes of tumour-bearing mice (Koblish et al., 2010). INCB024360 modestly retarded tumour growth of colon (CT26) and pancreatic (PAN02) cancer cell lines in mice. The antitumour effect of INCB024360 was not seen in athymic and IDO1-deficient mice suggesting that the target of this drug in vivo is IDO1 (Koblish et al., 2010). An important mechanistic observation was that INCB024360 attenuated differentiation of suppressive Tregs by IDO1-expressing DCs, and increased proliferation of T-cells and CTLs and expression of a T-cell co-stimulatory ligand CD86 on DCs. This likely contributed to the antitumour effect in vivo (Liu et al., 2010).

In the first human clinical study in 52 patients, INCB024360 was well tolerated up to 700 mg twice daily but the systemic reduction of KYN was not durable at lower than 100 mg per dose indicating a short half-life in humans (Beatty et al., 2013). Unexpectedly, in a phase 1/2 trial that enrolled 12 immunotherapy-naïve patients, 300 mg twice daily dose of INCB024360 elicited a significant increase in liver enzymes in 5 out of 7 patients and doses had to be adjusted down to levels which previously showed inferior IDO1 inhibitory activity (Gibney et al., 2014). Still, one patient showed a complete response and five patients an objective response. Overall, INCB024360 seems to be a good IDO1 inhibitor in humans but the toxicity limits the use of more effective doses. Several clinical trials of INCB024360 are...
currently enrolling patients and the drug will be tested at a relatively low dose (25 mg twice daily) (Table 1.2).

### 1.11.2 NLG919 (NewLink Genetics)

NLG919 is an orally bioavailable IDO1 inhibitor disclosed in 2013 (Mautino et al., 2013). It has more favourable drug-like properties and toxicity profile compared to INCB024360. NLG919 is a poor substrate for cytochrome P450 enzymes indicating better metabolic stability than Incyte’s clinical candidate. Interestingly, NLG919 shows some cross-reactivity with TDO but the impact of this in clinical trials is unclear at the moment (Mautino et al., 2013). The metabolic half-life in mouse and dog is moderate, around 1 h and 3 h, respectively. The antitumour activity of NLG919 was demonstrated in mouse models of melanoma and pancreatic cancer and the efficacy was comparable to INCB024360. Tumour remissions were not achieved. NLG919 is currently being evaluated in a human clinical trial for the safety and toxicity at doses 50-800 mg twice daily (Table 1.2).

### 1.11.3 FLX-B (Flexus Biosciences Inc.)

The existence of a next generation IDO1 inhibitor called FLX-B was disclosed at the AACR meeting in Philadelphia in April 2015 (Juan et al., 2015). This inhibitor is scheduled to enter human clinical trials in 2015. It shows superior properties to the previous two clinical candidates. The potency in cell-based assays is around 2 nM and the predicted half-life of FLX-B is ~20 h determined by intrinsic hepatocyte clearance. One capsule of 17 mg is expected to inhibit IDO1 in patients by at least 90% throughout the whole day. Such low doses are expected to minimise toxicities. In a mouse model of colon cancer (CT26 cancer cells), the antitumour efficacy of FLX-B was comparable to that of INCB024360 using a similar dosing regimen (Juan et al., 2015, Koblish et al., 2010). This is surprising as FLX-B has superior drug-like properties and metabolic stability compared to INCB024360.

Overall, current preclinical data suggest that the inhibition of IDO1 alone will not induce profound tumour regressions, regardless of the inhibitor potency and metabolic stability. The most likely explanation is the presence of other immunosuppressive mechanisms or the absence of ongoing antitumour immunity (Mellman et al., 2011). However, small molecule IDO1 inhibitors may still have a very useful role to play in modern immunotherapies. Evidence suggests that IDO1 inhibitors can unleash the true potential of cancer therapies that are limited by the active tumour immunosuppression, such as cancer vaccines or adoptive T-cell therapies (Maus et al., 2014, Vasaturo et al., 2015). The last section of this chapter will detail the current status of these combinations.
### Table 1.2. The ongoing human clinical trials of IDO1 inhibitors alone and in combination with other cancer therapies.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Combination</th>
<th>Combination type</th>
<th>Phase</th>
<th>Cancer type</th>
<th>Estimated Enrolment</th>
<th>Start Date</th>
<th>Completion Date</th>
<th>ClinicalTrials.gov ID</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NLG919</td>
<td>none</td>
<td></td>
<td>I</td>
<td>Solid Tumours</td>
<td>36</td>
<td>04/2014</td>
<td>04/2015</td>
<td>NCT02048709</td>
<td>(Khleif et al., 2014)</td>
</tr>
<tr>
<td>INCB024360</td>
<td>none</td>
<td>pilot study</td>
<td></td>
<td>Gynaecological cancers</td>
<td>12</td>
<td>12/2013</td>
<td>08/2015</td>
<td>NCT02042430</td>
<td></td>
</tr>
<tr>
<td>INCB024360</td>
<td>Ipilimumab (Bristol-Myers Squibb)</td>
<td>αCTLA-4 mAb</td>
<td>I/II</td>
<td>Melanoma</td>
<td>136</td>
<td>03/2012</td>
<td>10/2014</td>
<td>NCT01604889</td>
<td>(Gibney et al., 2014)</td>
</tr>
<tr>
<td>INCB024360</td>
<td>Pembrolizumab (Merck &amp; Co.)</td>
<td>αPD-1 mAb</td>
<td>I/II</td>
<td>Solid Tumours</td>
<td>120</td>
<td>06/2014</td>
<td>05/2017</td>
<td>NCT02178722</td>
<td></td>
</tr>
<tr>
<td>INCB024360</td>
<td>MEDI4736 (AstraZeneca)</td>
<td>αPD-L1 mAb</td>
<td>I/II</td>
<td>Solid Tumours</td>
<td>157</td>
<td>12/2014</td>
<td>03/2017</td>
<td>NCT02318277</td>
<td></td>
</tr>
<tr>
<td>INCB024360</td>
<td>MPDL3280A (Genentech)</td>
<td>αPD-L1 mAb</td>
<td>I</td>
<td>Lung cancer (non-small cell)</td>
<td>80</td>
<td>11/2014</td>
<td>01/2017</td>
<td>NCT02298153</td>
<td></td>
</tr>
<tr>
<td>INCB024360</td>
<td>MELITAC 12.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Cancer vaccine</td>
<td>II</td>
<td>Melanoma</td>
<td>12</td>
<td>09/2013</td>
<td>04/2016</td>
<td>NCT01961115</td>
<td></td>
</tr>
<tr>
<td>INCB024360</td>
<td>CDX-1401 and adjuvant poly-ICLC&lt;sup&gt;b&lt;/sup&gt; (Celldex Therapeutics)</td>
<td>Cancer vaccine</td>
<td>I/IIb</td>
<td>Gynaecological cancers</td>
<td>98</td>
<td>08/2014</td>
<td>02/2018</td>
<td>NCT02166905</td>
<td></td>
</tr>
<tr>
<td>INCB024360</td>
<td>NK cells + IL-2, fludarabine/cyclophosphamide</td>
<td>Immuno/</td>
<td>I</td>
<td>Gynaecological cancers</td>
<td>20</td>
<td>04/2014</td>
<td>08/2017</td>
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<tr>
<td>INCB024360</td>
<td>Tamoxifen</td>
<td>Chemo-therapy</td>
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<td>Gynaecological cancers</td>
<td>83</td>
<td>08/2012</td>
<td>02/2015</td>
<td>NCT01685255</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> MELITAC 12.1 - a multipeptide vaccine consisting of a 12 MHC I restricted melanoma peptides and a MHC II restricted tetanus toxoid helper peptide  
<sup>b</sup> CDX-1401 - fusion monoclonal antibody of dendritic endocytic receptor DEC-205 and tumour antigen NY-ESO-1; poly-ICLC, stabilized polyriboinosinic/polyribocytidylic acid, Toll-like receptor 3 ligand. Start and completion dates are shown in a month/year format. 

**Abbreviations:** mAb (monoclonal antibody)
1.12 Combination therapies to complement IDO1 inhibition

1.12.1 Checkpoint inhibitors, cancer vaccines, chemo- and radio-therapies

Immune checkpoint inhibitors have demonstrated exceptional antitumour efficacy in advanced melanoma, although not all patients benefit from this therapy to the same extent (Berman et al., 2015). The variability in responsiveness of patients may be due to immunosuppressive microenvironment. This provides a rationale for combining immune checkpoint inhibitors and IDO1 inhibitors. The combination of IDO1 and checkpoint inhibitors is also supported mechanistically. CTLA-4 induces IDO1 expression in DCs (Grohmann et al., 2002, Munn et al., 2004) and PD-1 is the effector mechanism of IDO1-induced Tregs (Fallarino et al., 2006, Sharma et al., 2007). Using a melanoma (B16F10) mouse model, the antitumour efficacy of anti-PD-1 and anti-CTLA-4 monoclonal antibodies markedly improved in IDO1-deficient mice (Holmgaard et al., 2013), confirming the hypothesis. Table 1.3 summarises the beneficial effects of these combinations in various preclinical cancer models. Notably, an IDO1 inhibitor was necessary to provide significant survival benefit in a poorly immunogenic B16-BL6 (van Elsas et al., 1999) melanoma model where other therapies alone had failed (Table 1.3).

Even more striking results were seen in combinations with chemo- and radio-therapies. Radiation or cyclophosphamide (CPM) treatments were effective only in combination with IDO1 inhibitors. However, not all the chemotherapies will produce synergy with IDO1 inhibitors. Alkylating agents (CPM and temozolomide) appear to be more effective than the antimetabolite methotrexate. This suggests that methotrexate may not induce immunogenic cell death that stimulates antitumour immunity (Apetoh et al., 2007, Muller et al., 2005a). Triplets of radio/chemotherapy and IDO1 inhibitors did not show any additional benefit to doublet combinations. The bottom section of the Table 1.3 (Various) demonstrates the ability of IDO1 inhibitors to realise the potential of cancer vaccines which cannot overcome the active immunosuppression on their own. Taken together, IDO1 inhibitors potently enhance the antitumour efficacy of a broad range of cancer therapies which are weakly effective alone. IDO1 inhibitors will likely be necessary to treat poorly immunogenic tumours. The reversal of tumour-induced immune suppression by IDO1 inhibitors may be essential for effective modern cancer treatments. Some of these combinations will be investigated in human clinical trials of IDO1 inhibitors but it will take at least two years before final data will be available (Table 1.2).
<table>
<thead>
<tr>
<th>Agent</th>
<th>Antitumour efficacya</th>
<th>Tumour type</th>
<th>Mouse strain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IDO1i Tx</td>
<td>IDO1i +Tx</td>
<td>OS/GD</td>
<td></td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GL261b</td>
<td>C57BL/6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B16-F10</td>
<td>C57BL/6</td>
</tr>
<tr>
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<td>B16-8L6</td>
<td>C57BL/6</td>
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<td></td>
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<td>B16-8L6</td>
<td>C57BL/6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B16-BL6</td>
<td>C57BL/6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4T1 BALB/c</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>B16-SIY</td>
<td>C57BL/6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B16-SIY</td>
<td>C57BL/6</td>
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<td></td>
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<td></td>
<td>B16-F10</td>
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<td>GL261b</td>
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<td>B16-SIY</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>GL261b</td>
<td>C57BL/6</td>
</tr>
</tbody>
</table>

**Table 1.3.** Antitumour efficacy of IDO1 inhibitors in combination with other cancer therapies.

- Antitumour efficacy was assessed according to the overall survival (OS) or growth delay (GD) as indicated. b Intracranial inoculations. 1MT indicates racemic mixture of enantiomers. Abbreviations: IDO1i (IDO1 inhibitor), Tx (other cancer therapy), GM-CSFvac (cancer cell vaccine secreting granulocyte/macrophage colony stimulating factor) (Gupta & Emens, 2010), TMZ (temozolomide), CPM (cyclophosphamid), RT (radiotherapy), gp100 (melanoma peptide vaccine), αPD-mix (cocktail of PD-1/PD-L1/PD-L2 monoclonal antibodies).
1.12.2 Blockade of the high-affinity TRP transporters

In addition to checkpoint blockade, standard chemo/radio therapies and cancer vaccines, there are other potential combinations that may synergise with IDO1 inhibitors. As discussed in section 1.6.1.1, the high-affinity TRP transporters on DCs and tumour cells represent a promising therapeutical target to limit IDO1 enzymatic activity. The first identified inhibitor of the high-affinity TRP transporters on DCs is L-1MT (Bhutia et al., 2015). It shows a moderate affinity of ~5 μM in a cell-based assay, whereas its isomer D-1MT is barely active (Bhutia et al., 2015). This further stresses the functional dichotomy between the two 1MT enantiomers. Besides small-molecules, monoclonal antibodies could be used to block these TRP transporters.

1.12.3 Inhibition of IDO1 expression

1.12.3.1 Signal transduction inhibitors

Imatinib mesylate (Gleevec) is one of the most successful tyrosine kinase inhibitors for treatment of chronic myelogenous leukemia and gastrointestinal stromal tumours. Surprisingly, a recent study suggested that IDO1 mediates the therapeutic effect of Gleevec (Balachandran et al., 2011). In a mouse model of gastrointestinal stromal tumours, Gleevec downregulated the IDO1 expression through blockade of the oncogenic KIT signalling. This resulted in an increased apoptosis of Tregs and enhanced activity of cytotoxic T-cells, the surrogate markers of IDO1 inhibition. 1MT recapitulates the effects of Gleevec but does not synergise with it (Balachandran et al., 2011). A more potent IDO1 inhibitor might deliver better results. This discovery also suggests the possibility that the spectacular therapeutic effects of some targeted molecular therapies may be acting through the blockade of IDO1 expression.

1.12.3.2 Cyclooxygenase-2 (COX-2) inhibitors

An exciting combinatorial avenue is the inhibition of COX-2, the enzyme catalysing the rate-limiting step in the synthesis of prostaglandins, particularly prostaglandin E2 (PGE2) which was shown to induce expression of IDO1 in tumour cells and DCs (Braun et al., 2005, Trabanelli et al., 2014). COX-2 and IDO1 co-expression is associated with the worst prognosis in breast cancer (Chen et al., 2014). In a transgenic mouse model of breast cancer, administration of the selective COX-2 inhibitor celecoxib significantly reduced tumour-associated IDO1 expression (Basu et al., 2006). This likely resulted in an excellent antitumour
efficacy of celecoxib in combination with a breast cancer vaccine. Similar results were observed in a mouse model of pancreatic ductal adenocarcinoma treated with Mucin-1 cancer vaccine and celecoxib (Mukherjee et al., 2009). The treatment not only blocked the formation of metastases but was associated with reduced serum KYN levels, and a decrease in the amount of Tregs and MDSC in the tumour microenvironment. This suggests that the therapeutical effects of COX-2 inhibitors may be mediated through IDO1 expression.

1.12.3.3 JAK/STAT1, NF-κB pathway inhibitors

A number of phytochemicals including curcumin and resveratrol inhibit expression of functional IDO1 via blockade of the Janus Kinase (JAK)/Signal transducer and activator of transcription 1 (STAT1) signalling pathways (Arumuggam et al., 2015). In a mouse injected with thymoma EG7 cells, resveratrol retarded tumour growth by blockade of IDO1 expression (Noh et al., 2013). Similarly, the food additive ethyl pyruvate and its analogues were shown to reduce IDO1 expression in human monocytes cell line (U937) by blockade of the canonical NF-κB signalling pathway (Muller et al., 2010). Ethyl pyruvate markedly stimulated immune-mediated rejection of melanoma cells (B16F10) in mice. These compounds may elicit a synergy in combination with IDO1 inhibitors.

1.12.4 TGF-β/TGF-β-receptor inhibitors

TGF-β is an immunosuppressive cytokine and a major driver of late stage tumour progression (Connolly et al., 2012). Perhaps not coincidentally, TGF-β is an inducer and an effector in long-term immune tolerance mediated by the non-catalytic activity of IDO1, at least in mice (see section 1.6.3). Disrupting TGF-β signalling could provide synergy with IDO1 inhibition. The pan-TGF-β neutralising antibody Fresolimumab (GC-1008) and the type I TGF-β receptor kinase inhibitor LY2157299 showed disappointing results in human clinical trials (Connolly et al., 2012, Maier et al., 2015, Rodon et al., 2015) as single agents but evidence suggests that the combination of LY2157299 with other therapies might work. In a transgenic mouse melanoma model, LY2157299 and anti-CTLA-4 monoclonal antibody were ineffective as monotherapy but stimulated deep tumour regressions in combination (Holtzhausen et al., 2014). Since IDO1 inhibitors potentiate the antitumour efficacy of anti-CTLA-4 monoclonal antibodies, the combination with Fresolimumab or LY2157299 may be a promising prospect.
Figure 1.7. Overview of potential combinatorial strategies to complement IDO1 inhibitors. Abbreviations: COX-2 (Cyclooxygenase-2), CAR-T (Chimeric antigen receptor T-cell adoptive therapy), TGF-β (Transforming growth factor β).
1.13 Thesis objectives and research outline

The future of IDO1 inhibitors is bright. Although their antitumour efficacy as single agents is modest, preclinical results have demonstrated that IDO1 inhibitors can dramatically enhance the antitumour efficacy of a broad range of anticancer therapies. This emphasises a dominant role of IDO1 in the tumour immune escape and the need for blockade of IDO1 activity for effective cancer treatment. The Auckland Cancer Society Research Centre has initiated a drug discovery programme for the development of novel IDO1 inhibitors.

The overall objective of the work in this thesis is to identify and characterise novel IDO1 inhibitors from compound libraries as potential leads for the drug development. To achieve this objective, the following strategy is proposed (see Fig. 1.8):

A) I will firstly aim to develop a novel bioassay for detection of IDO1 enzymatic activity that could be miniaturised and adapted for use in a robotic workstation. This would enable a high-throughput testing of a large number of compounds for IDO1 inhibitory activity.

B) My second objective will be to screen compound libraries against wild-type IDO1, and IDO1 harbouring mutations of the active site residues. The screening against wild-type IDO1 is designed to identify novel IDO1 inhibitors, whereas the screening against the mutant IDO1 has potential to shed light on the importance of the active site residues for the binding to IDO1 inhibitors. I believe this knowledge could sharpen the rational drug design of IDO1 inhibitors.

C) The work in the third objective will focus on identifying IDO1 inhibitors suitable for drug development. I will use computational structural filters and the “wet” experimental methods to remove aggregating, chemically-reactive or cytotoxic IDO1 inhibitors unsuitable for drug development. The tractability of these IDO1 inhibitors for rational medicinal chemistry will be further assessed in a small structure-activity study of commercially available analogues. Furthermore, I plan to investigate the inhibitory mechanism of selected IDO1 inhibitors which is not well understood the IDO1 literature.
Figure 1.8. Overall strategy and the main objectives for the work in this thesis.
2.1 Biology

2.1.1 Materials

δ-Aminolevulinic acid (ALA), 1,4-naphthoquinone (NQ), 4-phenyl-1H-imidazole (4PI), ascorbic acid, boric acid, bromophenol blue, catalase (bovine, Cat. No. C40), chloramphenicol, deoxyribonuclease I (from bovine pancreas, Cat. No. D4527), ethylenediaminetetraacetic acid (EDTA), reduced glutathione (GSH), Ficoll-400, hemin (porcine, Cat. No. 51280), imidazole, L-kynurenine (L-KYN), L-tryptophan (L-TRP), lysozyme (from chicken egg white, Cat. No. L6876), methylthiazolyldiphenyl-tetrazolium bromide (MTT), p-dimethylaminobenzaldehyde (p-DMAB), piperidine (PIP), phenylmethysulfonyl fluoride (PMSF), puromycin dihydrochloride, ribonuclease A (from bovine pancreas, Cat. No. R5125), sodium dodecyl sulphate (SDS), Trizma® base (Tris), Tris-hydrochloride (Tris-HCl), Tween 20 and yeast extract were purchased from Sigma–Aldrich (St Louis, MO, USA). Agarose (UltraPure), One Shot chemically competent strains of *E. coli* BL21(DE3), BL21(AI) and TOP10, carbenicillin (disodium salt), Luria Broth (LB) agar, LB medium, Platinum® Pfx DNA polymerase (Pfx Pol) and ethidium bromide solution (10 mg/mL) were from Invitrogen Life Technologies (Carlsbad, CA, USA). Bacterial strain *E. coli* SG13009 (pREP4) was from Qiagen (Hilden, Germany). α-MEM medium was from GIBCO (Grand Island, NY, USA). Bacteriological agar was from Scharlau (Spain). EDTA-free protease cocktail inhibitor tablets were from Roche Diagnostics (Mannheim, Germany); dimethylsulfoxide (DMSO) was from ECP Ltd (Auckland, New Zealand) and methylene blue was from Chem-Impex International Inc (Wood Dale, IL, USA). Magnesium sulphate heptahydrate (MgSO$_4$) was a product of Ajax Chemicals (Auburn, Australia), calcium chloride (CaCl$_2$) was from BDH Chemicals Ltd (Poole, England), and magnesium chloride hexahydrate (MgCl$_2$) was purchased from Riedel-de Haën (Seelze, Germany). Tryptone was from Applichem (Darmstadt, Germany); isopropyl-β-D-thiogalactopyranoside (IPTG) and kanamycin sulphate were from EMD Chemicals (San Diego, CA, USA). All organic solvents were products of Merck (Darmstadt, Germany), and trichloroacetic acid (TCA) was from Avantor Performance Materials Inc. (Phillipsburg, NJ, USA). The NCI Diversity Set III (http://dtp.nci.nih.gov/branches/dseb/div2_explanation.html) was obtained from The National
Cancer Institute (NCI; Bethesda, MD, USA). Microplates were purchased from Greiner Bio-One (Frickenhausen, Germany). Stratagene QuikChange Lightning Site-directed mutagenesis kit was a product of Agilent Technologies (Santa Clara, CA, USA). Restriction enzymes and buffers were from New England Biolabs Inc. (Herts, UK). Human tryptophan 2,3-dioxygenase fully sequenced cDNA clone was obtained from Source Bioscience (Product name: IRAUp969G0942D; Nottingham, UK).

Analogues of the NCI hits for the structure activity studies were sourced from Chembridge (San Diego, CA, USA), Maybridge (Cambridge, UK), NCI, Auckland Cancer Society Research Centre (ACSRC) chemical repository, Ambinter (Orléans, France), Sigma-Aldrich, Life Chemicals (Kiev, Ukraine), Asinex (Moscow, Russia) and ChemDiv (San Diego, CA, USA) (Table 2.1). NLG919 (Cat. No. S7111) was obtained from Selleck Chemicals (Houston, TX, USA) and was a mixture of stereoisomers by \(^1\)H-NMR. 2-(1H-imidazol-4-yl)phenol (2-OH-4PI) (Kumar et al., 2008a) and 4-amino-N-(3-chloro-4-fluorophenyl)-N‘-hydroxy-1,2,5-oxadiazole-3-carboximidamide (HA) (Yue et al., 2009) were synthesized at ACSRC according to the published procedures.

### Table 2.1. Sources of the compounds used for structure activity studies in sections 5.2.4 and 6.2.3.

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2.1.2 Production and purification of recombinant human IDO1

The procedure of Austin and co-workers was used (Austin et al., 2004) with minor modifications. All centrifugation steps were carried out at 5000 g, for 20 min at 4°C, unless stated otherwise. Briefly, *E. coli* EC538 transformed with both pREP4 and pQE9-hIDO1 was grown on LB agar supplemented with carbenicillin (100 μg/mL) and kanamycin (50 μg/mL). A single colony was inoculated into LB medium (100 mL) and grown overnight at 37°C with shaking, and 5 mL of this starter culture was used to inoculate 500 mL LB medium and grown in 2 L culture flasks at 30°C with shaking. At an OD$_{600}$ of 0.6, ALA and IPTG were added to a final concentration of 500 μM and 10 μM, respectively, and cultures were allowed to grow for a further 8 h in the dark with shaking. Cells were harvested by centrifugation and the pellets resuspended in ice-cold PBS without Ca$^{2+}$ and Mg$^{2+}$ (pH 7.4; Sigma) containing PMSF (1 mM) and EDTA (1 mM). Cell pellets were collected by centrifugation and stored at -20°C until lysis. Prior to lysis, pellets were resuspended in an ice-cold solution A (25 mM Tris-HCl (pH 7.4) containing 150 mM sodium chloride (NaCl), 10 mM imidazole, 10 mM MgCl$_2$, 1 mM PMSF), centrifuged and lysed using solution A containing EDTA-free protease cocktail inhibitor tablets (1 tablet per 15 mL), 300 nM hemin, 100 μg/mL lysozyme, 10 μg/mL RNase A and 12 μg/mL DNase I. The mixture was gently agitated on a rocker at room temperature for 30 min, and then centrifuged. Supernatant was applied to a 5 mL HisTrap-FF column (GE Healthcare Life-Sciences AB, Uppsala, Sweden), rinsed with 25 mL Milli-Q water (MQW), and equilibrated with 25 mL solution A and eluted using a stepwise gradient (25 ml each) of imidazole (10 mM, 30 mM, 60 mM, 300 mM) in 25 mM Tris-HCl (pH 7.4) containing 500 mM NaCl and 1 mM PMSF. The red fraction containing rhIDO1 typically eluted with 300 mM imidazole and was collected and desalted on a PD-10 column (GE Healthcare) according to manufacturer’s instructions using Tris-HCl buffer (25 mM, pH 7.4) as an eluent. The fractions containing rhIDO1 protein were pooled and concentrated by centrifugation at 2500 g, 4°C using a spin concentrator (Vivaspin 20, MWCO 30 kDa, GE Healthcare) until the volume reached ~500 μL. The sample was washed with a further 500 μL of 25 mM Tris-HCl and concentrated to a final volume of <250 μL. Holoenzyme concentration (rhIDO1) was determined using the extinction coefficient at 405 nm ($\varepsilon = 172 \text{ mM}^{-1}\text{cm}^{-1}$) (Chauhan et al., 2008). Protein purity, estimated using SDS-PAGE, was 95%, and the amount of haem incorporation of the protein (expressed as $A_{280}:A_{405}$ of the
sample) ranged typically between 1:1.3-1.8. A purified rhIDO1 was then mixed with 80% glycerol in 25 mM Tris-HCl (1:1) and stored at -20°C.

2.1.3 Assays for determination of IDO1 inhibitory activity

2.1.3.1 Enzyme assays

Reaction mixes were set up in transparent polystyrene and black polypropylene 384-well microplates (Greiner Bio-One GmbH, Frickenhausen, Germany) for absorbance and fluorescence assays, respectively, and consisted of assay medium (30 μL) containing 50 mM phosphate buffer (pH 6.5), 10 mM ascorbic acid (freshly prepared, neutralised with equimolar amount of sodium hydroxide (NaOH)), 10 μM methylene blue, 100 μg/mL catalase (freshly prepared), 80 μM L-TRP, 0.01% (v/v) Tween 20 mixed with a concentration of rhIDO1 (15 μL) that depletes < 30% of the initial substrate concentration (typically 6 nM). The plates were covered with sealing film and incubated at 37°C for 25 min.

TCA absorbance assay: The absorbance assay was performed as described by Takikawa and co-workers (Takikawa et al.,1988) with modifications. The enzymatic reaction was terminated by 40% TCA (10 μL) and heated at 65°C for 15 min. p-DMAB (20 μL, 37.5 mg/mL in glacial acetic acid) was added to the reaction, which was shaken at 1200 rpm for 3 min in a plate shaker (Grant-Bio PHMP-4, Grant Instruments, Shepreth, Cambridgeshire, UK) and absorbance was read at 480 nm after 10 min incubation in the dark at ambient temperature on a SpectraMax M2 (Molecular Devices, Sunnyvale, CA, USA).

PIP fluorescence assay: For the PIP fluorescence assay, the enzymatic reaction was terminated by adding PIP from stock solutions prepared in MQW, to give the indicated final concentration over the range 200 mM-1000 mM during the method development and 200 mM for all the subsequent experiments. The plates were then covered with a sealing film and heated at 60°C for 30 min or 65°C for 20 min in a heated plate shaker (Grant-Bio PHMP-4). After 0.5-4 h, depending on the required sensitivity, the fluorescence signal of each well was read using an EnSpire 2300 Multimode Plate Reader (Perkin-Elmer, Singapore) at λex 400 nm and λem 500 nm (25°C, 3 mm measurement height, 100 flashes; if not stated otherwise).

The reaction setup was automated and performed using JANUS Automated Workstation (Perkin-Elmer). Test compounds were dissolved in 100% DMSO and aliquoted into small-volume polypropylene 384-well plates (Cat. No. 784201, Greiner Bio-One) from which they were transferred into the enzyme assay medium, followed by the addition of rhIDO1. Final DMSO concentration in the assay did not exceed 1% (v/v). IC50
determinations were carried out in duplicates of eight different concentrations together with background controls for each concentration to correct for the possible autofluorescence of test compounds. If the fluorescence signal of the background control exceeded the signal of the uninhibited enzyme reaction, the IC$_{50}$ of the concerned compound was determined in the absorbance assay. rhIDO1 inhibition (\%) was determined according to the Equation (Eq.) 1, and IC$_{50}$ was calculated by fitting the rhIDO1 inhibition (\%) values obtained in Eq. 1 to Eq. 2 where $y = 50$. Statistical analyses were conducted in Prism v6 (Graphpad Software, Inc., La Jolla, CA, USA). The limit of detection (LoD) was calculated using the equation $LoD = 3.3\sigma/S$, where $\sigma$ and $S$ are standard deviation of y-intercept of the regression line and the slope of the calibration curve, respectively. The $Z'$ factor value was calculated using Eq. 3, developed by Zhang and colleagues where $c^+$ and $c^-$ stand for positive control (enzyme in assay medium) and negative control (assay medium), respectively, and $\mu$ and $\sigma$ denote mean and standard deviation, respectively (Zhang et al., 1999). The normalised signal of test compounds was calculated according to Eq. 4.

\[
\frac{(|\text{uninhibited enzyme assay signal}| - |\text{inhibited enzyme signal}|)}{(|\text{uninhibited enzyme assay signal}| - |\text{assay medium signal}|)} \times 100 \quad \text{Eq. 1}
\]

\[
y = a + \frac{100 - a}{1 + \left(\frac{IC_{50}}{x}\right)^{Hill\text{slope}}} \quad \text{Eq. 2}
\]

\[
Z' = 1 - \frac{3\sigma_{c^+} + 3\sigma_{c^-}}{|\mu_{c^+} - \mu_{c^-}|} \quad \text{Eq. 3}
\]

\[
\frac{(|\text{test compound signal}| - |\text{assay medium signal}|)}{(|\text{uninhibited enzyme assay signal}| - |\text{assay medium signal}|)} \quad \text{Eq. 4}
\]

**PIP fluorescence assay notes:**

The solutions of PIP in MQW progressively develop a yellow tint which correlates with a decrease in the assay fluorescence signal. Therefore, PIP solution should be prepared fresh when a yellow tint or a decrease in the fluorescence signal is observed.

The fluorescence signal generated by the enzyme assay performed in the polypropylene plates (Greiner Bio-One, Cat. No. 781209) was significantly higher than that in the polystyrene plates (Greiner Bio-One, Cat. No. 781076) probably due to inactivation of rhIDO1 on the polystyrene surface.
2.1.3.2 Cell-based assay

Murine wild-type Lewis Lung carcinoma cells adapted to tissue culture (LLTC) stably transfected with the expression vector F279-V5/hIDO1 (Fig. 2.1) (a hybrid of the Gateway region of plasmid pcDNA6.2/V5-DEST (Invitrogen) and the backbone of plasmid pIRES-P (Hobbs et al., 1998)) harbouring Gateway-cloned human IDO1 under the control of CMV promoter, was used for the cell-based assays (LLTC-hIDO1) (Fig. 2.1). The rhIDO1 is expressed from this construct on a single mRNA transcript together with a puromycin resistance gene ensuring that puromycin resistant cells also produce hIDO1. The expression of the hIDO1 enzyme and KYN production by the LLTC-hIDO1 were validated previously (Fung et al., 2013). LLTC-hIDO1 were cultured in α-MEM medium containing 49 μM L-TRP, supplemented with 10% fetal calf serum, penicillin (100 U/mL) and streptomycin (100 μg/mL) and puromycin (2.5 μM). To determine the IC50 of the test compounds against hIDO1 expressed in LLTC-hIDO1, 2x10^4 cells were seeded in a sterile 96-well plate (100 μL/well) in a complete growth medium lacking puromycin (solution M1), outer wells of the plate were filled with 250 μL MQW to slow down the evaporation, and incubated (37°C, 5% CO2) for 30 min. Then, the triplicates of 6-9 different concentrations of test compounds in solution M1 were added to the cells (100 μL, final concentration 0.5% DMSO (v/v)). LLTC-hIDO1 treated with 0.5% DMSO (6 wells) served as negative controls. After 24 h of incubation (37°C, 5% CO2), which results in < 50% depletion of L-TRP, culture supernatants (120 μL) of each well were transferred into a fresh 96-well plate, mixed with TCA (40 μL, final concentration 10% (w/v)), heated at 60°C for 20 min, and spun at 2500 g for 10 min. Supernatants were mixed with p-DMAB (20 mg/mL in glacial acetic acid), and after 10 min incubation in the dark, the absorbance was read at 490 nm on a SpectraMax M2 (Molecular Devices). The concentration of L-KYN in the supernatants was determined from a calibration curve of L-KYN standard. IC50 values were calculated using Eq. 1 and Eq. 2 shown in section 2.1.3.1.

The viability of LLTC-hIDO1 cells were determined using the MTT colourimetric assay (Mosmann, 1983). When the cell culture supernatants were removed for the L-KYN measurement, filter-sterilised (0.2 μm) MTT solution (1 mg/mL in 1x PBS, 80 μL) was added to the wells and incubated (37°C, 5% CO2) until crystal formation was observed (typically in ~1 h). Then, plates were spun at 2000 g for 10 min, supernatant was decanted, the formazan crystals were dissolved in DMSO (100 μL), and the absorbance of the wells was measured at
560 nm (formazan absorption peak) and 690 nm (background MTT absorbance). Data were normalised by subtracting \( A_{690\text{ nm}} \) from \( A_{560\text{ nm}} \).

**Figure 2.1.** Expression vector F279-V5/hIDO1 for production of hIDO1 enzyme. A bicistronic mRNA transcript contains both the hIDO1 gene and the puromycin resistance gene (PuroR) under control of a strong cytomegalovirus (CMV) promoter ensuring high-level production of both hIDO1 and puromycin N-acetyl transferase (PAC). The internal ribosome entry site (IRES) allows translation of the PuroR gene inside the mRNA sequence. **Abbreviations:** attB1 and attB2 (Gateway recombination sites), ori (origin of replication), AmpR (encodes \( \beta \)-lactamase conferring resistance to ampicillin, carbenicillin and similar antibiotics). SV40 poly(A) (simian virus 40 polyadenylation signal for transcription termination).

### 2.1.4 PIP-THQ formation (Figure 3.3)

An enzyme assay was prepared with 250 \( \mu \text{M} \) L-TRP and 28 nM rhIDO1. The reaction was incubated at 37°C and aliquots (taken at 0, 15, 30, 45 and 60 min) were immediately mixed with PIP (final concentration 200 mM) and heated at 65°C for 20 min. The reaction was evaporated to dryness, resuspended in a volume of MQW equal to the volume of the initial aliquot, and analysed using HPLC. TRP and PIP-THQ present in the samples were quantified by integrating the respective peak areas in the chromatogram.
2.1.5 Isolation and purification of NFK from an enzymatic reaction

A reaction mixture (250 mL) containing 50 mM phosphate buffer, 5 mM ascorbic acid, 10 μM methylene blue, 100 μg/mL catalase, 1 mM L-TRP and ~ 90 nM rhIDO1 was incubated until the substrate was depleted. All evaporation steps were performed under high vacuum at 32°C, and centrifugation was carried out at 3200 g and 10°C. Solid-phase extraction (SPE) was performed using a Visiprep SPE Vacuum Manifold (Sigma-Aldrich) connected to a bench-top vacuum pump. The reaction mixture was evaporated to near dryness, resuspended in MQW (6 mL) and centrifuged for 10 min. The supernatant was mixed with ice-cold ethanol (1:3.5 ratio), agitated, re-centrifuged for 20 min, and the supernatant was evaporated to near dryness and resuspended in 6 mL MQW. In order to remove methylene blue, the sample was loaded onto a Strata-X SPE cartridge (100 mg, 6 mL, 33 μm; Phenomenex), which was firstly pre-conditioned (5 mL acetonitrile (MeCN)) and subsequently equilibrated (5 mL MQW), and eluted firstly with 10 mL MQW and subsequently with 3 mL 10% MeCN. Both elution fractions were collected and pooled. The resultant dark orange eluate was evaporated to near dryness and resuspended in 6 mL MQW. This solution was loaded onto a Strata C18-E cartridge (5 g, 20 mL, 55 μm, 70 Å; Phenomenex), which was firstly pre-conditioned (50 mL MeCN) and subsequently equilibrated (50 mL MQW), eluted with MQW and 5 mL fractions of eluate were continuously collected and analysed for the presence of NFK (using liquid chromatography/UV/mass spectrometry). When NFK was detected in the eluting fraction for the first time, 10% MeCN was applied to the column and 5 mL fractions were collected again until NFK was no longer detectable in the eluting fractions. The fractions containing significant amount of NFK were pooled, evaporated and freeze-dried. The identity and purity of the isolated NFK was assessed by LC-MS, absorption spectroscopy, and formation of KYN after exposure to a strong acid. A purified NFK solution (5 mM) was kept in 0.1 M phosphate buffer (pH 7.0) in the freezer.

2.1.6 Enzyme kinetics, time-dependency and reversibility of rhIDO1 inhibition

Enzyme kinetics were determined by measuring the initial rate of NFK formation at 37°C. The enzyme assay was performed as described previously (section 2.1.3.1) except that the length of the enzyme assay was 4 min to avoid substrate depletion at low initial concentrations of TRP. The TRP concentrations used were 5, 10, 20, 40, 80, 160 and 320
μM. The enzyme reaction was initiated by addition of rhIDO1 to the wells of the 384-well plate containing the inhibitors diluted in the assay medium (final concentration of DMSO was 0.4% (v/v)), and terminated by addition of PIP after 4 min. The initial rate of NFK formation was expressed as the amount of NFK (determined from the calibration curve of NFK standard) formed in 4 min. The reciprocal initial rates of NFK formation were plotted against the reciprocal TRP concentrations to determine the type of inhibition (Lineweaver-Burk plot) and the inhibition constant (K_i) was determined by fitting the data to a nonlinear regression equation describing the observed mode of inhibition in GraphPad Prism v6. As TRP inhibits rhIDO1 at concentrations greater than 80 μM, only the TRP concentrations below 80 μM were plotted.

For reversibility measurements, rhIDO1 (~600 nM, holoenzyme) was incubated with inhibitors (diluted from DMSO stock solutions, final DMSO concentration 1% (v/v)) at 21°C in solution A (potassium phosphate buffer (50 mM, pH 6.5), ascorbic acid (10 mM, neutralised by an equimolar amount of NaOH) and methylene blue (10 μM)) for 25 min or 90 min. Subsequently, this preincubated solution was 100-fold diluted into solution A containing catalase (200 μg/mL) and TRP (80 μM) and immediately mixed. At desired time points, aliquots (45 μL) of the enzyme reaction were mixed with piperidine (10 μL, 1.1 M in MQW) in a 384-well black plate (Greiner, Cat. No. 781209) and after the last aliquot, plates were processed as outlined in section 2.1.3.1 – PIP fluorescence assay. The amount of NFK produced by rhIDO1 at each time point was quantified using the calibration curve of authentic NFK standard. The assay was conducted at 21°C.

Time dependence of inhibition was carried out according to the procedure for the standard enzyme assay in section 2.1.3.1 but Tween 20 was absent and the assay was performed at 21°C instead of 37°C. Aliquots of the enzyme reaction were processed as described in the previous paragraph.

2.1.7 Molecular biology methods

2.1.7.1 Primers

Custom DNA oligonucleotides (primers) were synthesised by Integrated DNA Technologies Pte. Ltd. (Singapore). The primers for site-directed mutagenesis were purified on acrylamide gel, whereas sequencing primers were only desalted. Sequencing and mutagenic primers were designed using Primer3Plus (http://www.primer3plus.com/cgi-bin/dev/primer3plus.cgi) and PrimerX websites (http://www.bioinformatics.org/primertools/cgi-bin/DNA_1.cgi), respectively.
Table 2.2. A list of primers used in this work (Uppercase letters indicate bases complementary to the amplified DNA template and *vice versa*).

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Primer</th>
<th>Sequence (5’ → 3’)</th>
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<tr>
<td>pQE-30/S167A-rhIDO1</td>
<td>P1</td>
<td>TATGAGAGGATCGCATCACC</td>
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<td>P2</td>
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<td></td>
<td>P3</td>
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<td>inserts</td>
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<tr>
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<tr>
<td></td>
<td>P12</td>
<td>AGAGATTTTTTGCAGGCGGC</td>
</tr>
</tbody>
</table>

2.1.7.2 Restriction endonuclease digest and agarose gel electrophoresis

The purity and identity of the plasmids were determined by agarose gel electrophoresis and restriction endonuclease digest. A plasmid (400 ng) was incubated with endonucleases (10 units) in an appropriate restriction buffer for 60-80 min at 37°C, chilled on ice, mixed 6:1 with a gel loading buffer (6x) (40 mM Tris.HCl, 90 mM EDTA pH 8, 15% Ficoll-400, 0.1% SDS (w/v), 0.04% (w/v) bromophenol blue), loaded together with undigested control plasmid and a 1 kb Plus DNA ladder (Invitrogen) into the agarose gel (0.7 – 2% (w/v) cast in a TBE buffer (90 mM Tris base, 90 mM boric acid, 2.5 mM EDTA). Gels were run in a TBE buffer typically at 5 V per 1 cm of the electrode distance for 60 min, stained in 0.75 μg/mL ethidium bromide for 15-30 min, and imaged using Gel-Doc.

2.1.7.3 DNA sequencing

Plasmids were sequenced by Genetic Analysis Service (University of Otago, New Zealand; [http://gas.otago.ac.nz/services/sequencing.html](http://gas.otago.ac.nz/services/sequencing.html)) using BigDye® Terminator Version 3.1 Ready Reaction Cycle Sequencing Kit on a 3730xl DNA Analyzer (Applied Biosystems). Two forward and a two reverse primers were typically supplied for sequencing of ~ 1500 bp plasmid sections containing IDO/TDO gene, and successful sequencing results
were multi-aligned in a DNA editor ApE (A Plasmid Editor, v2.0.47, programmed by M. Wayne Davis, http://biologylabs.utah.edu/jorgensen/wayned/ape/) to generate a consensus DNA sequence. Prior to sending the primers and plasmids for sequencing, the primers were tested in a PCR reaction for their ability to amplify desired gene segments from the plasmids.

2.1.7.4 Denaturing protein acrylamide gel electrophoresis (SDS-PAGE)

Protein samples were mixed in a ratio 3:1 with a NuPAGE® LDS Sample Buffer (4X) (Invitrogen), heated at 70°C for 10 min, loaded into a precast Mini-PROTEAN® Stain-Free™ gradient (4-15%) acrylamide gel (Bio-Rad Laboratories Inc, CA, USA) and run using a standard Laemmli buffer (Laemmli,1970) (25 mM Tris.HCl, 192 mM glycine, 0.1% SDS, pH 8.3) in a Mini-PROTEAN® Tetra Cell at 200 V for 30 min at ambient temperature. Precision Plus Protein™ Unstained Standards (Bio-Rad, #161-0363) was used as a marker. Proteins on the gel were visualised by a UV-induced (2.5 min induction time) fluorescence labelling of TRP residues by the trihalo-compounds in the TGX Stain-Free™ gel using a ChemiDoc MP System (Bio-Rad), and photographed.

2.1.7.5 Polymerase chain reaction (PCR)

PCR (50 μL total volume) was carried out in a 96-well block thermal cycler (Mastercycler epGradient S, Eppendorf, Hamburg, Germany). Reactions were assembled on ice and contained: Pfx Pol amplification buffer (1x), Pfx Pol (1 unit), deoxynucleotide triphosphate mix (dATP, dCTP, dGTP, dTTP, each at 300 μM), MgSO₄ (1 mM), primers (300 nM), DNA template (variable between 10–100 ng) and sterile MQW. Thermal cycling conditions for each experiment are indicated in Table 2.3. Note: the QuikChange Lightning site-directed mutagenesis kit uses its own proprietary reagents distinct to those listed in this paragraph.

Table 2.3. PCR thermal cycling conditions.

<table>
<thead>
<tr>
<th>Step</th>
<th>C129A mutagenesis (section 2.1.8.2)</th>
<th>rhTDO inserts (section 2.1.9)</th>
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</thead>
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<tr>
<td></td>
<td>18xB</td>
<td>25xB</td>
</tr>
<tr>
<td>A</td>
<td>T (°C) t (min)</td>
<td>T (°C) t (min)</td>
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<tr>
<td></td>
<td>95  2</td>
<td>95  3</td>
</tr>
<tr>
<td>B (cycle)</td>
<td>95  0.3</td>
<td>95  0.25</td>
</tr>
<tr>
<td></td>
<td>58  0.25</td>
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<tr>
<td></td>
<td>68  2.5</td>
<td>68  2</td>
</tr>
<tr>
<td>C</td>
<td>68  5</td>
<td>68  5</td>
</tr>
<tr>
<td>D</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>
2.1.7.6 Bacterial transformation and plasmid isolation

Competent bacterial strain (typically 20-100 $\mu$L) was incubated in a 14 mL polystyrene round-bottom tube (BD Falcon, #352057) on ice for 20 min, heat-shocked in a water bath (42°C, 30 s), cooled on ice (5 min), and a whole transformation reaction or its part was diluted (5- to 10-fold) into S.O.C. medium (Invitrogen) and incubated for 1 h (37°C, 150 rpm). Bacteria carrying a plasmid of interest were selected on an LB agar supplemented with appropriate antibiotic(s) (either carbenicillin (100 $\mu$g/mL), kanamycin (50 $\mu$g/mL) or chloramphenicol (34 $\mu$g/mL)), and incubated overnight at 37°C. Next day, if the transformed plasmid was not meant for protein expression, a single colony was inoculated in an LB medium with an appropriate antibiotic, grown until the OD$_{600}$ ~ 1.5 (37°C, 250 rpm), and the plasmid was isolated using QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer’s instructions.

2.1.8 Production of mutant IDO1 enzymes

2.1.8.1 Serine-167 alanine replacement IDO1 mutant (S167A)

Firstly, *E. coli* SG13009(pREP4) were made competent to accept plasmid DNA. A bacterial starter culture grown overnight on a rotary shaker (37°C, 225 rpm) in a kanamycin-supplemented (50 $\mu$g/mL) LB medium was inoculated into an identical medium (100 mL) and grown until the OD$_{600}$ = 1.22. The preparation of competent bacteria was performed with ice-cold solutions, labware and centrifuges set at 4°C. A pellet of a centrifuged (3,200 g, 16 min) bacterial culture was carefully resuspended in a sterile CaCl$_2$ solution (100 mM in MQW, 30 mL), spun again (3,200 g, 10 min) and resuspended in a sterile CaCl$_2$/glycerol solution (85 mM/15% (v/v) in MQW, 2 mL). Subsequently, a plasmid pQE-30/S167A-rhIDO1 (courtesy of Prof. Emma Lloyd Raven, University of Leicester, UK) was sequenced using primers P1-P4 (Table 2.2) to ensure no random mutations had occurred in the rhIDO1 gene, and (5 ng) was added to ice-cold competent SG13009(pREP4) (100 $\mu$L), transformed as described in section 2.1.7.6 and selected with carbenicillin and kanamycin. S167A-rhIDO1 mutant enzyme was grown, induced, purified and stored exactly as for wild-type rhIDO1 in section 2.1.2 The concentration of S167A holoenzyme was calculated from the extinction coefficient at 404 nm ($\varepsilon = 144$ mM$^{-1}$cm$^{-1}$) (Chauhan et al.,2008).
2.1.8.2 Cysteine-129 alanine replacement IDO1 mutant (C129A)

Cysteine-129 of the rhIDO1 gene in an expression vector pQE-9/rhIDO1 (kindly provided by Assoc. Prof. Joanne Jamie, Macquarie University, Australia), was mutated to alanine using a Stratagene Quikchange Lightning Site-directed mutagenesis kit according to the manufacturer’s instructions. Briefly, pQE-9/rhIDO1 plasmid was amplified using the mutagenic primers C129A-F and C129A-R (Table 2.2, see also PCR conditions in Table 2.3), parental (hemi)-methylated plasmid was digested by endonuclease Dpn I, the reaction was transformed into XL10-Gold competent bacteria and the plasmid harbouring the C129A mutant rhIDO1 was isolated according to the procedure in section 2.1.7.6. Bacteria were selected on carbenicillin. Restriction digests (using combination of endonucleases PvuI and PvuII, and HincII and NsiI alone) and sequencing (primers P5-P8, Table 2.2) confirmed the identity of pQE-9/C129A-rhIDO1 with the desired C129A mutation. The isolated plasmid was transformed, expressed and purified identically to wild-type IDO1 (section 2.1.2) with the exception that bacterial cells were lysed using a French press (single pass at 20,000 psi; One Shot model, Constant Systems Ltd., Northants, UK). The extinction coefficient of rabbit intestinal IDO1 at 406 nm (ε = 140 mM⁻¹cm⁻¹) (Shimizu et al., 1978) was used to calculate the concentration of C129A holoenzyme.

2.1.9 Production and purification of recombinant human tryptophan 2,3-dioxygenase (rhTDO)

2.1.9.1 Production of expression vectors (see Figure 2.2).

Human tryptophan 2,3-dioxygenase (hTDO) cDNA clone (IMAGE ID: 4071714, GenBank: BC005355.1) was amplified (see PCR conditions in Table 2.3) using two primer sets, TDO1-N/TDO2-C and TDO1-N/TDO3-C (Table 2.2) flanking the open reading frame of hTDO sequence (Met₁ - Asp₄₀₆). The primers contained Gateway-recombination sites attB1 (TDO1-N) and attB2 (TDO2-C, TDO3-C), bacterial ribosome binding site (TDO1-N), and C-terminal hexahistidyl tag (TDO2-C). This PCR reaction produced two Gateway-compatible inserts (Insert 1 and Insert 2) that were recombined (5 μL of the PCR reaction) into pDONR221 plasmid (70 ng; Invitrogen) using BP Clonase II enzyme mix (Invitrogen) according to the manufacturer’s instructions (10 μL total reaction volume). Each reaction was transformed (1 μL) into TOP10 E. coli (50 μL) and bacterial clones harbouring the recombined entry vectors (pENTR221/rhTDO) were selected on kanamycin. The plasmids
were extracted as described in section 2.1.7.6, and validated in a restriction digest (using combination of NsiI and HindIII endonucleases, and PvuI alone) and by sequencing (primers P9-P12).

Subsequently, each of the entry vectors (pENTR221/rhTDO) were recombined into bacterial expression vector pET-62-DEST (Novagen; containing C-terminal hexahistidyl tag and Strep-Tag II) using LR Clonase II enzyme mix (Invitrogen) according to the manufacturer’s instructions, and the reactions were transformed into TOP10 E. coli. Bacterial clones harbouring pET-62-DEST with recombined rhTDO gene were selected on carbenicillin, plasmids were extracted as described in section 2.1.7.6, and validated in a restriction digest (using combination of HindIII and HincII endonucleases, and PvuI alone).

This generated two recombinant rhTDO expression vectors (rhTDO Construct 1 and rhTDO Construct 2). rhTDO from Construct 1 was expressed with a C-terminal hexa-histidyl tag (derived from the primer TDO2-C) immediately after the last amino acid of hTDO sequence (Asp406), whereas rhTDO from Construct 2 was expressed with both a C-terminal hexa-histidyl tag and a Strep-Tag II derived from the expression vector pET-62-DEST. Both constructs were subsequently transformed into competent bacterial strains BL21(AI) and BL21(DE3) according to the procedure outlined in section 2.1.7.6 and selected on carbenicillin.

2.1.9.2 Small-scale rhTDO expression

To determine which construct and bacterial strain expressed rhTDO more efficiently, a small-scale protein expression was performed. A single colony of BL21(AI) and BL21(DE3) carrying either rhTDO Construct 1 or rhTDO Construct 2 was inoculated into LB medium (10 mL) containing carbenicillin (100 μg/mL) and grown until the OD<sub>600</sub>=1.5 (37°C, 250 rpm) in conical tubes (BD Falcon, 50 mL) capped with perforated lids. Subsequently, the cultures were 20-fold diluted in to a fresh carbenicillin-supplemented (100 μg/mL) LB medium (5 mL) and grown until OD<sub>600</sub>=0.9 (37°C, 250 rpm). ALA and IPTG were then added at a final concentration of 500 μM to the cultures of both BL-21(DE3) and BL-21(AI), while L-arabinose (final concentration 0.2% (w/v)) was additionally added to BL-21(AI) cultures, and bacterial cultures were grown in the dark with shaking (21°C, 170 rpm, 21 h). Next, the cultures were centrifuged (3200 g, 4°C, 20 min) and the pellets were put in a -80°C freezer. To lyse the bacteria, 400 μL of ice-cold solution L (Tris-HCl (40 mM, pH 7.6), KCl (100 mM), hemin (2 μM), PMSF (1 mM), MgCl₂ (10 mM) and CaCl₂ (1 mM)) containing DNase I (20 μg/mL), lysozyme (200 μg/mL) and EDTA-free protease inhibitor
Figure 2.2. Production of the rhTDO bacterial expression constructs using Gateway cloning. a) The PCR insert containing hTDO gene flanked by the Gateway recombination sites attB1 and attB2 was recombined into a donor vector pDONR221 (at attP1 and attP2 sites, BP recombination) to generate the entry vector pENTR221/rhTDO. b) The rhTDO gene in the entry clone was subsequently recombined into a bacterial expression vector pET-62-DEST (LR recombination) which produced c) the rhTDO Construct 1 to be transformed into a bacterial strain for protein production. When the plasmids were isolated using Miniprep, their identity was confirmed by restriction digest using indicated endonucleases. pENTR/rhTDO was sequenced to ensure no spurious mutation had occurred during the PCR. Restriction endonucleases PvuI, NsiI, HindIII and HincII, and sequencing primers P9-P12 are indicated. Abbreviations: ccdB (encodes a DNA gyrase inhibitor lethal to bacteria), CmR (a chloramphenicol resistance gene), KanR (a kanamycin resistance gene), lacI (encodes repressor protein that blocks transcription of a gene under control of lactose (lac) operator), 6xHis (hexa-histidyl tag for metal affinity protein purification), Strep-Tag II (a tag for protein purification that binds Strep-Tactin).
cocktail (1 tablet / 15 mL) were added to the bacterial pellets, blank (empty tube, negative control) and purified rhIDO1 (400 pmol, positive control). The lysis solutions were incubated for 30 min on ice, sonicated by three rounds of 8 pulses (pulse duration 1 s, low intensity) separated by 1 minute intervals, and centrifuged (3200 g, 4°C, 20 min).

Subsequently, about one-half of the Ni-NTA resin from the His-Trap FF column (Qiagen) was extracted, resuspended in MQW (500 μL), and aliquots (30 μL) of this Ni-NTA slurry were washed two times with MQW (1 mL) in 1.5 mL Eppendorf tubes, and one time with solution L (1 mL) supplemented with imidazole (10 mM) and NaCl (200 mM). After each wash step, the Ni-NTA resin slurry was centrifuged (10000 g, 4 min) to facilitate removal of the supernatants. The lysates prepared in the previous paragraph were supplemented with imidazole and NaCl at a final concentration of 10 mM and 200 mM, respectively, mixed with the washed Ni-NTA resin, incubated at 13°C for 80 min on a lab-roller and centrifuged (10000 g, 4 min). The pellets (Ni-NTA resin bound with enzymes) were resuspended in MQW (60 μL) and 25 μL of this solution was run on an SDS-PAGE (see section 2.1.7.4)

2.1.9.3 Enzyme activity of Ni-NTA eluates

All centrifugation steps in this section were performed at 13000 g, 4°C and 5 min. The enzyme-bound Ni-NTA resin suspensions from the previous section were centrifuged, the pellets were washed in a solution L1 (1 mL) (50 mM Tris.HCl, 300 mM NaCl, 4% (v/v) glycerol) supplemented with 15 mM imidazole, centrifuged again, and the supernatant was discarded. The three latter steps were repeated once more. Subsequently, enzymes were extracted from the Ni-NTA resin by mixing (5 min) in the solution L1 containing 250 mM imidazole (50 μL), spun, and the supernatants and the purified rhIDO1 (1 μM in a solution L1 containing 250 mM imidazole) were diluted 10- and 100-fold into the potassium phosphate buffer (50 mM, pH 6.5) and kept on ice prior to the enzyme activity assay.

A modified PIP fluorescence assay was used to determine the enzyme activity. The assay medium (36 μL) consisted of Tris.HCl (pH 6.5, 50 mM), ascorbic acid (10 mM), methylene blue (10 μM), catalase (100 μg/mL) and L-TRP (160 μM). The concentration of L-TRP was two-fold higher than that used for rhIDO1 assay since the affinity of L-TRP for rhTDO (Km = 222 μM) is about ~ 30-fold lower than that for rhIDO1 (Km = 7 μM). The eluted enzymes (4 μL) prepared in the previous paragraph were added to the assay medium, incubated at 37°C for 30 min, and the reaction was terminated by addition of PIP (20 μL, 600 mM in MQW) and processed as for the standard PIP fluorescence assay. The enzyme activity
was expressed as the fluorescence signal of the enzyme sample divided by the fluorescence signal of the blank. The measurements were carried out in duplicate.

### 2.1.9.4 Large-scale purification of rhTDO from BL-21(DE3)/Construct 1

The bacterial strain *E. coli* BL-21 (DE3) was freshly transformed with the rhTDO expression vector Construct 1 according to section 2.1.7.6 and selected on carbenicillin. One colony was inoculated into LB medium (10 mL) supplemented with carbenicillin (100 μg/mL) and was grown (37°C, 275 rpm) until OD\textsubscript{600} = 1.8. Then, this starter culture was inoculated into a carbenicillin-supplemented (100 μg/mL) LB medium (400 mL), and when the culture reached OD\textsubscript{600} = 0.7, IPTG and ALA were added to the final concentration of 500 μM, and the culture was allowed to grow for a further 22 h (21°C, 250 rpm) in the dark. Bacterial cells were harvested by centrifugation and the pellets were resuspended in an ice-cold PBS without Ca\textsuperscript{2+} and Mg\textsuperscript{2+} (pH 7.4; Sigma), supernatant was discarded and the pellets were stored at -20°C. Prior to lysis, the bacterial pellets were resuspended in an ice-cold solution B (potassium phosphate buffer (50 mM, pH 7.7), KCl (300 mM), L-TRP (5 mM), PMSF (1 mM)) containing hemin (10 μM), EDTA-free protease inhibitor cocktail (1 tablet/15 mL) and imidazole (10 mM), lysed using a French press (2 passes at 20,000 psi) and centrifuged (20,000 g, 6°C, 30 min). The supernatant was purified on a HisTrap-FF column using an identical procedure to that for rhIDO1 purification with the exception that imidazole concentrations were dissolved in solution B, and the PD-10 column was eluted with potassium phosphate buffer (50 mM, pH 7.7). The purified rhTDO eluate (5 mL) from the PD-10 column was loaded into the spin concentrator and centrifuged (3200 g, 7°C). After 35 min, the volume in the concentrator decreased to 1 mL, and an equal volume of potassium phosphate buffer (50 mM, pH 7.7) was added. After an additional 50 min of concentration, the volume decreased to ~0.65 mL and rhTDO precipitated on the membrane of the concentrator.

### 2.1.10 Structural filters

The analyses using AlarmNMR, Blake, PAINS and Glaxo filters were performed via the SMARTS filter applet ([http://pasilla.health.unm.edu/tomcat/biocomp/smartsfilter](http://pasilla.health.unm.edu/tomcat/biocomp/smartsfilter)) kindly provided by Dr. Jeremy Yang (University of New Mexico, Albuquerque, NM, USA). This application assigned each compound either “pass” or “fail” status for any of the selected structural filters and the output was downloaded as an SD file.
Chapter 2 - Materials and Methods

For PubChem BioAssays filter, each compound in the NCI library was assigned with the number of “active” and total screening test results deposited in a PubChem BioAssay database (https://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi). Data were extracted from the database using a PubChem PUG (Power User Gateway) REST HTTP-based procedure in a Mozilla Firefox (v24.0.2) plugin HttpRequester (v1.0.5). Firstly, generic substance identifiers (SID) of each compound in the NCI library were converted to their unique compound identifiers (CID). Then, the results of biological screenings for each compound were obtained using request: http://pubchem.ncbi.nlm.nih.gov/rest/pug/compound/cid/assaysummary/CSV. The percent activity of each compound was expressed as a “number of test results in which compound was active” divided by “total number of test results for the compound” multiplied by 100. The cutoff for classifying a compound as failing the BioAssays filter was set at 2.53%, the average of the percent activity of all the NCI library compounds (see Fig. A.16).

The NCI library compounds (n = 1600) and the NCI hits (n = 35) have 526 ± 251 and 496 ± 317 (mean ± SD) screening results deposited in the PubChem database, respectively, and > 90% of the compounds in both groups had > 100 entries (see Appendix Figures A.16 and A.17).

2.2 Chemistry

2.2.1 Materials

DL-kynurenine (DL-KYN, > 95%, cat. no. 69791) obtained from AK Scientific (Union City, CA, USA) was only 62% pure by HPLC compared to crystalline L-kynurenine (cat. no. K8625) from Sigma-Aldrich, which was used with no further purification. 13C-labelled formic acid (99%, cat. no. 14C-428) was purchased from Cambridge Isotope Laboratories (Tewksbury, MA, USA). 2-methylpiperidine (2-MePIP), 3-methylpiperidine (3-MePIP), 4-methylpiperidine (4-MePIP), azepane (AZP), diethylamine (DEA), cis-2,6-dimethylpiperidine (DMP), dipropylamine (DPA), N-methylpiperidine (NMePIP), N-methylpyrrolidine (NMePYR), piperidine (PIP), pyrrolidine (PYR), trifluoroacetic acid (TFA), 2,2,6,6-tetramethylpiperidine (TMP) were obtained from Sigma-Aldrich. All other chemicals and solvents were purchased from either Merck or Sigma-Aldrich.

2.2.2 General analytical methods

NMR spectra were obtained on a Bruker Avance 400 spectrometer at 400 MHz for 1H and 100 MHz for 13C spectra and were referenced to tetramethylsilane. High resolution mass
spectra (HRMS) were acquired on a Bruker micrOTOF-Q mass spectrometer operating under electrospray ionisation conditions.

Mass spectrometry experiments were performed on Agilent 1200 HPLC/6460 triple quadrupole MS (QqQ) equipped with an Agilent JetStream electrospray ionisation interface (drying gas temperature 250°C, flow 10 L/min, nebulizer pressure 40 psi, capillary voltage + 2.75 kV and - 3.5 kV, sheath gas temperature 250°C, sheath gas flow 6 L/min, collision cell accelerator voltage 7 V, fragmentor voltage 50 V). MS2 scans were performed over the 50 – 1000 m/z range. Samples were injected into the system without a column in a mobile phase consisting of 4.5 mM ammonium formate pH 3.5 and 0.1% (v/v) formic acid in MeCN (6:4) at flow rate 0.3 mL/min. Data were analysed in Agilent MassHunter software.

LC-MS analyses were carried out on Agilent 1100 HPLC/6150 single quadrupole electrospray mass spectrometer. Ionisation conditions were: drying gas temperature 270°C, gas flow 10 L/min, nebulizer pressure gauge 35 psi, capillary voltage + 3 kV and - 3 kV, fragmentor voltage 70 V. Spectra were acquired over 107 – 1000 m/z range. Analytical separations were carried out on a Luna C\(_18\) column (5 µm, 100 Å, 150 x 2 mm; Phenomenex, Torrance, CA, USA) eluted using 80% MeCN (A) and formic acid buffered MQW (pH ~ 2.7) (B) using two different binary gradients:

- **G1** (Chapter 3 analyses): 0 min (6% A), 5 min (25% A), 15 – 16 min (49% A), 18 min (6% A) at flow rate 0.4 mL/min and 40°C.

- **G2** (Chapter 4 analyses): 0 min (5% A), 11 min (58% A), 14 – 17 min (96% A), 18-22 min (5% A) at flow rate 0.4 mL/min and 40°C.

UV/VIS chromatograms were acquired using diode-array detection at multiple wavelengths simultaneously ranging from 240 to 480 nm, and fluorescence signals of amine-THQ fluorophores were acquired at emission and excitation wavelengths of 500 nm and 400 nm, respectively, using an in-line fluorescence detector. Data were analysed on Agilent Chemstation software.

The purity of synthesised compounds was determined by HPLC analysis (detection at 254 nm) (see Appendix Fig. A.3) as a peak area of compound of interest divided by the peak area of all analysed peaks in the chromatogram. Peaks were selected manually in Agilent Chemstation software.

Fluorescence titration of PIP-THQ (Fig. 4.3) was performed on an EnSpire 2300 Multimode plate reader (Perkin-Elmer) in a black polypropylene 384-well plate.
Chapter 2 - Materials and Methods

(Cat. No. 781209, Greiner Bio-One). Fluorescence emission spectra of the fluorophores amine-THQ (products of the chemical reaction between an amine and NFK, see Fig. 2.4 for an example) were acquired at 400 nm excitation wavelength during HPLC analysis. Extinction coefficients were determined in a potassium phosphate buffer (0.1 M in MQW, pH 7) at 25°C on an Agilent 8453 UV/VIS spectrophotometer (Hewlett-Packard).

2.2.3 Small-scale reactions of amino acids and amines

Amino acids (2 mM) were incubated with amines (1 M) in 100-200 µL MQW in 1.5 mL Eppendorf tubes heated in Thermomixer Comfort (Eppendorf) at 65°C for 20 min, then immediately cooled on ice and analysed on LC-MS (single quadrupole). Peak areas (mV.s) of corresponding adducts were quantified from 254 nm (amine-KYN and amine-NFK) and 400 nm (amine-THQ) chromatograms. Amine-amino acid adducts were identified in chromatograms by their a) predicted molecular ions in positive and negative mass spectra and b) virtually identical absorption spectra to those of a parent amino acids KYN and NFK. Amine-THQ fluorophores were further validated from the fluorescence chromatogram. Since amine-NFK and amine-KYN were incompletely separated, the double peak in chromatograms was split at the trough as depicted on Figure 4.4a (dashed line) to allow quantification. Aliquots (10-20 µL) for each time point during the reaction progress experiments (Fig. 4.10) were taken from the same tube and immediately chilled on ice prior to HPLC analysis.

2.2.4 Compound synthesis and characterisation

The isolation procedures described below are a result of optimisations. The yields are calculated based on 62% purity of commercial DL-KYN.

2.2.4.1 2-amino-4-(2-formamidophenyl)-4-oxobutanoic acid (NFK)

![Figure 2.3. Synthesis of NFK.](image)

Figure 2.3. Synthesis of NFK.
Acetic anhydride (0.24 mL, 2.54 mmol) was added to formic acid (0.48 mL, 12.7 mmol) and the solution was warmed at 50-55°C for 15 min, then cooled to room temperature. A solution of DL-KYN (0.50 g, 2.40 mmol) in formic acid (14 mL) was added and the mixture was stirred at room temperature for 2 h. Ether was added to precipitate out the product, which was washed further with ether and dried *in vacuo* to give NFK as a hygroscopic tan powder (0.38 g, ~99%). Purity was 96%. $^1$H NMR $\delta$ (400 MHz, D$_2$O, 298 K) (rotamers about the formanilide group evident) 8.90 (s, 0.35H, CHO, rotamer A), 8.40 (s, 0.65H, CHO, rotamer B), 8.20 (d, $J$=8.2 Hz, 0.65H, H-6', rotamer B), 8.09 (d, $J$=8.2 Hz, 0.35H, H-6', rotamer B), 8.05 (d, $J$=8.0 Hz, 0.65H, H-3', rotamer B), 7.71 (dd, $J$=8.2, 7.7 Hz, 1H, H-5', rotamers A and B), 7.60 (d, $J$=8.0 Hz, 0.35H, H-3', rotamer A), 7.41 (dd, $J$=8.2, 7.7 Hz, 1H, H-4', rotamers A and B), 4.20 (t, $J$=4.9 Hz, 1H, H-2), 3.80 (d, $J$=4.9 Hz, 2H, H-3). MS m/z 237.0 (100%, [M+H]$^+$). HRMS m/z calcd for C$_{11}$H$_{13}$N$_2$O$_4$ 237.0870, found 237.0864 [M+H]$^+$. λ$_{\text{max}}$(H$_2$O)/nm 261 and 322 ($\varepsilon$ /M$^{-1}$ cm$^{-1}$ 6289 and 1936), lit. (Dalgliesh,1952), nm 260 and 321 ($\varepsilon$ /M$^{-1}$ cm$^{-1}$ 10980 and 3750). Other authors reported λ$_{\text{max}}$(H$_2$O)/nm 321 ($\varepsilon$ /M$^{-1}$ cm$^{-1}$ 3152) from commercially available product (Dick et al.,2001). NFK isolated from enzymatically catalysed oxidative cleavage of TRP showed λ$_{\text{max}}$(H$_2$O)/nm 323 ($\varepsilon$ /M$^{-1}$ cm$^{-1}$ 3066).

### 2.2.4.2 $^{[13]}$C-formyl labelled NFK

This material was prepared from dl-kynurenine as described above for NFK, except that the acetic anhydride was added to $^{[13]}$C-HCOOH in the first step. The kynurenine was added to the resulting solution, dissolved in normal $^{[12]}$C formic acid. The $^1$H NMR spectrum of the product was the same as that described above for NFK, except that there were two additional doublets present from the rotamers of the $^{13}$C-labelled formyl group, due to $^1$H-$^{13}$C coupling, at $\delta$ 8.86 (d, $J_{\text{H-C}}$=234.3 Hz, CHO, rotamer A) and 8.40 (d, $J_{\text{H-C}}$=203.1 Hz, CHO, rotamer B). $^1$H NMR analysis indicated the sample to consist of an approximately 3:2 mixture of $^{13}$C:$^{12}$C-labelled NFK. MS m/z 237.0 (64.88%, $^{[12]}$C M+H)$^+$), 238.0 (100%, $^{[13]}$C M+H)$^+$).
2.2.4.3 2-(1-formylpiperidin-2-yl)-4-oxo-1,2,3,4-tetrahydroquinoline-2-carboxylic acid (PIP-THQ)

![Chemical Structure]

**Figure 2.4.** Synthesis of PIP-THQ.

NFK (1.84 g, 7.8 mmol) was incubated with PIP (45.5 mL, 460 mmol) in 368 mL MQW at 60°C for 20 min. The reaction was cooled on ice, evaporated to dryness and subsequently redissolved in 0.04% (v/v) trifluoroacetic acid (TFA) in MQW (15 mL). This solution was loaded onto a Strata C18-E cartridge (5 g/20 mL, Phenomenex) preconditioned with 70 mL MeCN and equilibrated with 70 mL of 0.04% (v/v) TFA in MQW. The column was eluted with mixtures of MeCN/0.04% TFA on a vacuum manifold and fractions (10 mL) were analysed on HPLC. PIP-THQ fractions were pooled, evaporated, resuspended in NaOH (143 mM, 3.5 mL) and evaporated again. The sample was resuspended in MQW (0.5 mL), loaded onto a C18 cartridge (1 g/6 mL, Varian) and eluted successively with MQW, 50% MeCN in MQW and 100% MeCN. MeCN eluates were pooled, evaporated and dried at high vacuum to yield PIP-THQ as a yellow powder after freeze-drying (17.6 mg, 0.96%). Purity was 94%. The product was a 1:1 mixture of diastereomers. 

\[ ^1H \text{NMR} \delta (400 \text{ MHz}, D_2O, 298 K) 8.06 (s. 0.5H, CHO), 8.02 (s, 0.5H, CHO), 7.50-7.46 (m, 2H, H-5,7), 6.92 (dd, J=8.2, 0.8 Hz, 1H, H-8), 6.80-6.74 (m, 1H, H-6), 4.45 (br d, J=6.5 Hz, 0.5H, H-2'), 4.01 (dd, J=14.6, 4.4 Hz, 0.5H, H-6'), 3.80 (dd, J=6.8, 3.2 Hz, 0.5H, H-2'), 3.50 (dd, J=13.1, 4.6 Hz, 0.5H, H-6'), 3.40 (m, 0.5H, H-6'), 2.71 (m, 0.5H, H-6'), 2.52 (2*d, J=15.1 Hz, 1H, H-3), 2.49 (2*d, J=15.1 Hz, 1H, H-3), 1.59-1.20 (m, 6H, H-3', 4' 5').

\[ ^13C \text{-NMR spectrum-see Table 2.4. MS m/z 303.1 (90.94%, [M+H]^+), 112.1 (100%, N-formyl-2,3,4,5-tetrahydropyridin-1-ium), 627.1 (40.22%, [2M+Na]^+). HRMS m/z calcd for C_{16}H_{18}N_{2}NaO_{4} 325.1159, found 325.1162 [M+Na]^+. \lambda_{\text{max}}(H_2O)/nm 400 (\varepsilon /M^{-1} \text{ cm}^{-1} 2136). \]
**Table 2.4.** $^1$H and $^{13}$C resonances of PIP-THQ.

<table>
<thead>
<tr>
<th>Atom Number</th>
<th>$^1$H Resonance Position$^1$</th>
<th>$^{13}$C Resonance Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>-</td>
<td>71.52 (71.02)$^2$</td>
</tr>
<tr>
<td>3</td>
<td>2.52 (2.51)$^2$</td>
<td>42.32 (42.29)</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>206.92 (206.57)</td>
</tr>
<tr>
<td>5</td>
<td>7.49 (7.49)</td>
<td>123.55 (123.55)</td>
</tr>
<tr>
<td>6</td>
<td>6.75 (6.75)</td>
<td>118.15 (118.08)</td>
</tr>
<tr>
<td>7</td>
<td>7.50 (7.50)</td>
<td>138.34 (138.15)</td>
</tr>
<tr>
<td>8</td>
<td>6.92 (6.92)</td>
<td>112.06 (112.04)</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>176.15 (175.87)</td>
</tr>
<tr>
<td>2'</td>
<td>4.45 (3.80)$^3$</td>
<td>53.21 (59.92)</td>
</tr>
<tr>
<td>3'</td>
<td>1.69-1.13 (1.69-1.13)</td>
<td>24.93 (24.55)</td>
</tr>
<tr>
<td>4'</td>
<td>1.88-1.75</td>
<td>19.30 (19.03)</td>
</tr>
<tr>
<td>5'</td>
<td>1.69-1.13 (1.69-1.13)</td>
<td>23.44 (23.00)</td>
</tr>
<tr>
<td>6'</td>
<td>3.40, 3.50 (4.01, 2.71)$^3$</td>
<td>44.09 (37.06)</td>
</tr>
<tr>
<td>7'</td>
<td>8.06 (8.02)</td>
<td>164.79 (164.68)</td>
</tr>
</tbody>
</table>

$^1$Chemical shifts in ppm ($\delta$) downfield from tetramethylsilane using D$_2$O as solvent. $^2$Shifts in parentheses are the corresponding values for the other isomer. $^3$Bracketed proton and carbon resonances correlate, as do non-bracketed.

### 2.2.4.4 [$^{13}$C-formyl] labelled PIP-THQ

[$^{13}$C-formyl]-NFK (2.56 mg, 0.011 mmol) was incubated with PIP (0.3 mL, 3.04 mmol) in MQW (3 mL) at 65°C for 20 min. The reaction was cooled on ice, acidified to ~ pH 1 with HCl and extracted twice with ethyl acetate (EtOAc). The EtOAc extract was washed twice with saturated NaCl solution containing 0.3% (v/v) HCl, evaporated and resuspended in methanol (MeOH) prior to MS analysis.

### 2.2.4.5 2-(1-formyl-5-methylpiperidin-2-yl)-4-oxo-1,2,3,4-tetrahydroquinoline-2-carboxylic acid (3-MePIP-THQ)

![Figure 2.5. Synthesis of 3-MePIP-THQ.](image)

NFK (80.24 mg, 7.8 mmol) was incubated with 3-methylpiperidine (3-MePIP; 2.0 mL, 17.04 mmol) in MQW (17 mL) at 65°C for 30 min. The reaction was evaporated and
subsequently redissolved in 15 mL MQW, acidified to ~ pH 1 with HCl and extracted with EtOAc. The dried extract was dissolved in 3 mL MeOH:MeCN (5:1) and diluted with 30 mL MQW. The extract was fractionated on a Strata C18-E cartridge (5 g/20 mL, Phenomenex) using mixtures of MeCN/MQW containing 0.04% (v/v) TFA. Fractions containing 3-MePIP-THQ were pooled, evaporated and dissolved in MQW, adjusted to ~ pH 8 by NaOH and mixed with ammonium bicarbonate buffer (0.02 M final concentration, pH 8). 3-MePIP-THQ was subsequently purified on a Strata C18-E cartridge (2 g/12 mL, Phenomenex) and eluted by mixtures of MeCN/MQW containing ammonium bicarbonate (0.02 M, pH 8). Eluted 3-MePIP-THQ was acidified to ~ pH 1 and extracted with EtOAc, washed with saturated NaCl solution and dried over sodium sulphate. Subsequent evaporation and drying at high vacuum afforded 3-MePIP-THQ as a yellow powder (1.47 mg, 1.83%). Purity was 94%. The product was a mixture of diastereomers. $^1$H NMR $\delta$ (400 MHz, CDCl$_3$, 298 K) 8.21 (s, 0.5H, CHO), 8.03 (s, 0.5H, CHO), 7.64 (dd, J=8.4, 8.2 Hz, 1H, ArH), 7.53 (ddd, J=8.2, 8.2, 1.2 Hz, 0.5H, ArH), 7.48 (ddd, J=8.2, 8.2, 1.2 Hz, 0.5H, ArH), 7.02 (d, J=8.2 Hz, 0.5H, ArH), 6.96-6.84 (m, 1.5H, ArH), 6.48 (br s, 0.5H, NH), 6.24 (br s, 0.5H, NH), 4.88 (br d, J=4.1 Hz, 0.5H, CHN), 4.24 (dd, J=13.4, 3.4 Hz, 0.5H, CHHN), 3.97 (d, J=6.6 Hz, 0.5H, CHN), 3.23 (dd, J=13.8, 4.1 Hz, 0.5H, CHHN), 2.97 (d, J=16.3Hz, 0.5H, CHHCO), 2.87 (dd, J=11.3, 11.3Hz, 0.5H, CHHN), 2.69 (d, J=16.0Hz, 0.5H, CHHCO), 2.61 (dd, J=12.3, 12.3 Hz, 0.5H, CHHN), 2.48 (d, J=16.3 Hz, 0.5H, CHHCO), 2.23 (d, J=16.0 Hz, 0.5H, CHHCO), 1.77-1.28 (m, CH$_2$), 0.94 (d, J=6.4 Hz, 1.5H, CH$_3$), 0.90 (d, J=6.0 Hz, 1.5H, CH$_3$). MS $m/z$ 317.1 (98.18%, [M+H]$^+$), 126.1 (100%, N-formyl-3-methyl-2,3,4,5-tetrahydropyridin-1-ium), 339.0 (60.60%, [M+Na]$^+$), 655.2 (32.11%, [2M+Na]$^+$). $\lambda_{\text{max}}$(H$_2$O)/nm 402 (ε/M$^{-1}$cm$^{-1}$2431).

2.2.4.6 4-(2-aminophenyl)-4-oxo-2-(piperidin-1-yl)butanoic acid (PIP-KYN)

Figure 2.6. Synthesis of PIP-KYN.

KYN (16.64 mg, 0.08 mmol) was incubated with PIP (0.395 mL, 4 mmol) in MQW (4 mL) at 80°C for 30 min. The reaction was evaporated, redissolved in MQW and acidified to pH ~ 4 with TFA. Purification was carried out on a C18-E cartridge (2 g/12 mL,
Phenomenex) using MeCN/MQW mixtures containing 0.016% (v/v) TFA. Freeze-drying of purified PIP-KYN yielded a brown powder (4.7 mg, 45%). Purity was 95%. $^1$H NMR $\delta$ (400 MHz, D$_2$O, 303 K) 7.99 (dd, J=8.1, 1.3 Hz, 1H, H-6’), 7.57 (ddd, J=7.2, 7.1, 1.2 Hz, 1H, H-4’), 7.25 (ddd, J=8.1, 7.2, 1.2 Hz, 1H, H-5’), 7.19 (dd, J=7.1, 1.2 Hz, H-3’), 4.35 (dd, J=7.0, 4.4 Hz, 1H, H-2), 3.85 (dd, J=18.2, 7.0 Hz, 1H, H-3), 3.72 (dd, J=18.2, 4.4 Hz, 1H, H-3), 3.51 (br d, J=11.7 Hz, 1H, CH/NN), 3.40 (br d, J=11.7 Hz, 1H, CH/NN), 3.13-2.95 (m, 2H, CH$_2$N), 1.93-1.68 (m, 5H, CH$_2$), 1.47-1.34 (m, 1H, CH). MS $m/z$ 277.2 (100%, [M+H]$^+$). HRMS $m/z$ calcd for C$_{15}$H$_{21}$N$_2$O$_3$ 277.1547, found 277.1538 [M+H]$^+$.

$\lambda_{max}$(H$_2$O)/nm 259 and 364 (ε/M$^{-1}$ cm$^{-1}$ 7213 and 4010).

2.2.4.7 4-(2-aminophenyl)-4-oxo-2-butenoic acid (KYN-CKA)

![Figure 2.7. Synthesis of KYN-CKA.](image)

KYN (58.24 mg, 0.28 mmol) was incubated with NaOH (7 mg, 0.175 mmol) in MQW (8.75 mL, pH ~ 12) at 80°C for 9 min, immediately cooled on ice and brought to pH ~ 2 using HCl. This solution was extracted three times with EtOAc, and the organic layer was pooled and washed four times with saturated NaCl solution. Subsequent evaporation and drying at high vacuum afforded KYN-CKA as a bright orange powder (9 mg, 24%). Purity was 96%. $^1$H NMR $\delta$ (400 MHz, DMSO-d$_6$, 298 K) 7.74 (dd, J=8.2, 1.5 Hz, 1H, H-6’), 7.53 (d, J=15.7 Hz, 1H, H-3), 7.32 (dd, J=7.1, 7.2, 1.5 Hz, 1H, H-4’), 6.78 (dd, J=8.7, 0.9 Hz, 1H, H-3’), 6.70 (ddd, J=8.2, 7.1, 0.9 Hz, 1H, H-5’), 6.62 (d, J=15.7 Hz, 1H, H-2). MS $m/z$ 192.0 (100%, [M+H]$^+$), 213.9 (85.37%, [M+Na]$^+$), 189.9 (100%, [M-H]). HRMS $m/z$ calcd for C$_{10}$H$_9$NNaO$_3$ 214.0475, found 214.0466 [M+Na]$^+$. $\lambda_{max}$(H$_2$O)/nm 392 (ε/M$^{-1}$ cm$^{-1}$ 4205). $\lambda_{max}$(EtOH)/nm 411 (ε/M$^{-1}$ cm$^{-1}$ 4493).
2.2.4.8 4-(2-formamidophenyl)-4-oxo-2-butenoic acid (NFK-CKA)

NFK (65.7 mg, 0.316 mmol) was heated with PIP (1.78 mL, 18 mmol) in 81% (v/v) 2-propanol in MQW (20 mL) at 52°C for 55 min. Subsequently, the reaction was cooled on ice and rapidly neutralised to ~ pH 7 by HCl and evaporated to dryness. The residue was dissolved in ammonium bicarbonate (0.05 M, pH 7), loaded onto a Strata C18-E cartridge (5 g/20 mL) and eluted using MeCN/MQW mixtures containing ammonium bicarbonate (0.03 M, pH 7). Fractions containing NFK-CKA adduct were put into a freezer (-20°C). Next day, isolated NFK-CKA was left at room temperature for > 3 hours, acidified with HCl to pH 2 and extracted with 2 volumes of EtOAc. The EtOAc extract was washed with saturated NaCl solution and evaporated. Dry material was resuspended in MeOH, sonicated, and centrifuged for 5 min at 14000 g. The supernatant, which contained impurities and a small amount of NFK-CKA was removed and the pellet was dried at high vacuum to afford NFK-CKA as a pale yellow powder (12.55 mg, 19.1%). Compound was unstable in MS but appeared pure from NMR and HPLC. Purity was 98%. 1H NMR δ (400 MHz, DMSO-d6, 298 K) 13.20 (br, 1H, COOH), 10.82 (s, 1H, NHCHO), 8.38 (s, 1H, CHO), 8.14 (d, J=7.8 Hz, 1H, H-6'), 7.87 (d, J=7.4 Hz, 1H, H-3'), 7.64 (dd, J=7.4, 7.3 Hz, 1H, H-4'), 7.62 (d, J=15.6 Hz, 1H, H-3), 7.29 (dd, J=7.8, 7.3Hz, 1H, H-5'), 6.56 (d, J=15.6 Hz, 1H, H-2). HRMS m/z calcd for C$_{11}$H$_9$NNaO$_4$ 242.0424, found 242.0425 [M+Na$^+$]. $\lambda_{\text{max}}$(H$_2$O)/nm 285 and 350 (ε /M$^{-1}$ cm$^{-1}$ 1584). $\lambda_{\text{max}}$(THF)/nm 285 and 350 (ε /M$^{-1}$ cm$^{-1}$ 4355 and 2596).
CHAPTER 3

NOVEL FLUORESCENCE ASSAY FOR DETERMINATION OF IDO1 ENZYMATIC ACTIVITY

This chapter is a modified version of the original manuscript entitled “Formation of an N-formylkynurenine-derived fluorophore and its use for measuring indoleamine 2,3-dioxygenase 1 (IDO1) activity” which was accepted for publication in journal “Analytical chemistry and biochemistry” (2014 Impact Factor 3.44) in December 2012 (Tomek et al., 2013). The manuscript was authored by Petr Tomek, Brian D Palmer, Jack U Flanagan, Sai-Parng S Fung, David J A Bridewell, Joanne F Jamie and Lai-Ming Ching. The final publication is available at Springer via http://dx.doi.org/10.1007/s00216-012-6650-y.

I have conceived, designed and performed the experiments, analysed and interpreted the data and wrote the manuscript. JUF, BDP and LMC edited the manuscript, contributed to the design and analysis of the experiments. SPF and JFJ edited the manuscript.

There are many differences in the chapter presented in this thesis and the publication. Mainly, figures and figure legends were modified and new figures were added. The first paragraph of the introduction, sections 3.2.1 and 3.2.4 were added and discussion was modified. The methods were moved to chapter 2. Fluorophore NFK-PIP was renamed to piperidine-tetrahydroquinoline (PIP-THQ; see Fig. 2.4) because the original name no longer matched its recently determined structure. Characterisation of the reaction mechanism and chemical structure of PIP-THQ is the subject of Chapter 4.

3.1 Introduction

The first objective of my PhD project was to screen the National Cancer Institute (NCI) Diversity Set III library comprising of 1597 compounds for inhibition of IDO1 activity that could be used as leads in the drug development programme for cancer therapy. To efficiently test such a large number of compounds, there are at least two essential requirements; a) an accurate, high-density (at least 384-well) microplate assay for measuring IDO1 enzymatic activity, and b) an automated workstation adapted to perform reliably the enzymatic assay. The JANUS robotic workstation (Perkin-Elmer) was available at Auckland Cancer Society Research Centre (ACSRC), but a sensitive, economic, simple assay showing low interference from test compounds was not available in 2011 when the project started.
Chapter 3- Introduction

The majority of the assays for determining IDO1 activity (Cook et al.,1980, Matin et al.,2006, Takikawa et al.,1988, Vignau et al.,2004) rely on the enzymatic conversion of TRP to NFK, its subsequent hydrolysis to KYN and the quantification of various end products (Fig. 3.1). The absorbance assay is based on quantification of a Schiff base obtained in a reaction between KYN and Ehrlich’s reagent, p-(dimethylamino)benzaldehyde (p-DMAB) (Fig. 3.1a) (Takikawa et al.,1988). The reaction between p-DMAB and KYN is not specific however, and test compounds can cross react with p-DMAB generating false negatives. Moreover, the assay requires many steps and its low sensitivity limits the miniaturisation. A radiometric assay, utilising radioactive D-[ring-2-14C]-tryptophan, offers better sensitivity than the absorbance assay but is cumbersome to perform, requires handling of radioactive material and is not microplate compatible (Cook et al.,1980). HPLC methods have been developed and are best suited for measuring KYN in biological matrices as they avoid interference of drugs and other metabolites present in the samples, but they are not highly amenable for use in high throughput screening (Vignau et al.,2004). A fluorescence assay is measuring KYN, generated following the treatment of the enzyme reaction with sodium hydroxide (NaOH) (Fig. 3.1b), has a simple experimental protocol but low sensitivity (LoD = 5.6 µM) (Matin et al.,2006). The Bridge-It® tryptophan fluorescence assay (Mediomics, LLC, St. Louis, MO, USA) used by Meininger and colleagues for detection of IDO1 activity (Meininger et al.,2011), measures the amount of remaining substrate (TRP) after the IDO1 reaction as opposed to the more common measurement of formed product (KYN). This assay is based on the association of the two fluorophore-labelled DNA half-sites in the tryptophan repressor protein bound by TRP (Fig. 3.1d). This commercial assay is complex but highly sensitive (~ 0.1 µM TRP) and simple to execute. However, the kit has a short shelf-life and a high cost (1,000 NZD per 384 measurements). The work in this chapter describes the development of a fluorescence assay that measures IDO1 activity from the generation of a novel fluorophore called PIP-THQ derived from NFK (Fig. 3.1c). This assay is economical, offers high sensitivity together with a simple experimental procedure that can be miniaturized for use in high throughput screens (HTS) - a staple of present day drug discovery campaigns (Pope et al.,1999).
Figure 3.1. Schematic pathway of microplate assays for measuring IDO1 activity using (a,b) KYN formation, (c) PIP-THQ formation and (d) TRP detection. Abbreviations: \( \lambda_{\text{ex}} \) (excitation wavelength), \( \lambda_{\text{em}} \) (emission wavelength); TrpR (tryptophan repressor protein), PIP (piperidine), \( p \)-DMAB (para-(dimethylamino)benzaldehyde), TCA (trichloroacetic acid), PIP-THQ (piperidine-tetrahydroquinoline).
3.2 Results

3.2.1 A novel fluorescence assay for IDO1

The previously published fluorescence assay (Matin et al., 2006) is reported to have a low sensitivity, and this is confirmed in the calibration curve of KYN shown in Figure 3.2a using this detection method (LoD = 5.4 µM KYN). Moreover, the coefficient of the fit ($r^2 = 0.8268$) was poor with a high signal variability and a high signal to background ratio (S/B), 1.9 at 25 µM KYN (Fig. 3.2a). Also, peaks other than KYN (360 nm) were observed in the excitation spectrum of the NaOH-treated enzyme reaction incubated at 21°C for > 30 min (data not shown), indicating that KYN is unstable at high pH consistent with previous reports (Taylor et al., 2002, Tsentalovich et al., 2006).

![Figure 3.2. a) Calibration curve of KYN and signal of blank in assay buffer treated with 160 mM NaOH in 384-well plates (50 µL) incubated for 1 h at ambient temperature. Fluorescence was measured at excitation and emission wavelength 370 and 490 nm, respectively. b) rhIDO1 inhibitory activity of NaOH and piperidine determined using TCA absorbance assay. Abbreviations: RFU, relative fluorescence units.](image_url)

In an attempt to improve the assay, a weaker base was used to substitute for NaOH. Since aliphatic amines are either too volatile or poorly soluble in water, cyclic secondary amine piperidine (PIP) was selected because of its complete aqueous solubility and a high boiling point (106°C at 1 atm), lowering its evaporation rate.

Firstly, the concentration of PIP that would effectively inhibit the enzyme reaction was determined. PIP was titrated into complete assay buffer and after addition of rhIDO1, the reaction was incubated for 60 min at 37°C, and the enzyme activity was determined using the TCA absorbance assay. NaOH was used as a control. The enzyme activity was completely
inhibited at PIP concentrations >50 mM (Fig. 3.2b). NaOH showed an identical concentration-response relationship to that of PIP, indicating that the inhibition of rhIDO1 results from an increase in pH. PIP at 200 mM was selected as a robust concentration for subsequent experiments.

3.2.2 Detection of a novel NFK-derived fluorophore

The excitation and emission fluorescence spectra of the rhIDO1 enzyme reaction treated with 200 mM PIP or 160 mM NaOH were measured. When 200 mM PIP was used, a radical difference in the fluorescence spectrum compared to that obtained using 160 mM NaOH was observed. The latter displayed an excitation spectrum with a broad, low-intensity peak with the maximum at 335 nm (Fig. 3.3a). When 200 mM PIP was used, a sharply defined peak at 400 nm was detected, with 25x higher fluorescence intensity at the maximum compared to that of 160 mM NaOH (Fig. 3.3a). The fluorescence emission spectrum of the NaOH-treated enzyme assay medium showed two poorly defined peaks with low intensity at 440 nm and 490 nm, suggesting that both KYN and NFK were present (Fig. 3.3b). In comparison, the emission spectrum of the 200 mM PIP-treated assay medium displayed an intense and well-defined peak at 500 nm (Fig. 3.3b). This was surprising as it strongly indicated that the fluorophore emitting the fluorescence at 500 nm is not KYN.

Figure 3.3. a) Excitation and b) emission fluorescence spectra obtained in enzyme assay mixture using 160 mM NaOH (solid line) or 200 mM PIP (dashed line). Excitation spectrum a) was measured at emission wavelength 480 nm. Emission spectrum b) was measured at excitation wavelength 360 nm. c) The amount of PIP-THQ (circles) and TRP (squares) in an enzymatic assay determined by HPLC. PIP-THQ was detected using in-line fluorescence detector (excitation and emission wavelengths 400 and 500 nm, respectively). TRP was detected using diode-array detector at 280 nm.
Chapter 3- Results

When 200 mM PIP was reacted with 80 μM TRP or 80 μM KYN in assay medium and the fluorescence spectrum measured, neither of the treatments produced the peaks at 400 nm and 500 nm (data not shown), suggesting that the unknown fluorophore was most likely formed from the reaction of PIP with the end product of the enzyme reaction, NFK. This fluorophore was named PIP-THQ. The amount of PIP-THQ formed with time was shown to be inversely correlated with the amount of TRP remaining in the enzymatic reaction (Fig. 3.3c), confirming that the formation of PIP-THQ is related to the IDO1 enzymatic activity.

In an attempt to characterise this new fluorophore, the reaction products present in the enzyme assay medium after treatment with 200 mM PIP and incubation for 20 min at 65°C, were analysed by LC-MS. Two entities with retention times (Rt) of 11.5 min and 13.1 min, respectively, were seen in the chromatogram in a ratio of 1 to 3 (Rt 11.5 to Rt 13.1) (calculated using integrated areas of the respective peaks) (Fig. 3.4a). The positive ionisation mass spectrum of the compound with Rt 13.1 min showed an intense peak at m/z 303.2 (Fig. 3.4b) whilst the mass spectrum of the compound with Rt 11.5 min showed the most abundant ion at m/z 291.0 (Fig. 3.4c).

**Figure 3.4.** a) HPLC chromatogram of PIP-THQ produced in the enzyme assay treated with 200 mM PIP showing two eluting substances at the indicated Rt. The chromatogram was acquired using fluorescence detector set at excitation and emission wavelengths 400 and 500 nm, respectively. b) The positive electrospray ionisation mass spectrum of the compound eluting at Rt 13.1 min and c) the compound with Rt 11.5 min. Relative ion intensity at 100% was 2.4x10^6 in b) and 5.7x10^5 in c). Proposed molecular ions are indicated below m/z values.
Both ions were identified as a protonated molecular ion \([M+H]^+\) based on the presence of sodium dimer adducts \([2M + Na]^+\) in both spectra, typical for ionisation of acids in the gas phase. This suggested that the molecular weight of compound \(R_t 11.5\) and \(R_t 13.1\) is 290 and 302 Da, respectively, and that both compounds contain the carboxylic acid group. Fluorescence spectra of both compounds were identical and showed excitation and emission maxima at 400 nm and 486 nm, respectively (data not shown). An NFK standard was needed to confirm that the fluorophore is formed directly from the reaction of NFK with PIP. Since NFK is not available commercially; it was isolated from the rhIDO1 enzymatic reaction as described in section 2.1.5. Briefly, EtOH precipitation to remove salts was followed by purification on two different reverse-phase matrices, and the purified NFK was freeze-dried. The isolated NFK showed molecular adduct ions \([M+H]^+\) at \(m/z\) 237.0, \([2M+H]^+\) at \(m/z\) 473.2 and \([2M+Na]^+\) at \(m/z\) 495.0 in single quadrupole MS, consistent with the expected molecular weight of 236 Da and the presence of a carboxylic acid group. The absorption maximum of NFK was 323 nm \((ε_{323} = 3066 \text{ M}^{-1} \text{ cm}^{-1})\). HPLC analysis of 1 mM NFK hydrolysate \((65°C, 25 \text{ min})\) in 0.2 M trichloroacetic acid showed ~96% peak area compared to that of 1 mM crystalline L-Kynurenine (Sigma-Aldrich, Cat. No. K8625) processed at identical conditions. Moreover, when 20 \(µM\) L-tryptophan (Sigma-Aldrich, Cat. No. T0254) was incubated with a concentration of IDO1 that can convert all the TRP into NFK, the fluorescence signal \((λ_{ex} 400 \text{ nm}, λ_{em} 500 \text{ nm})\) of this enzymatic reaction was ~93% of 20 \(µM\) NFK solution in an identical enzyme assay buffer. This validated the identity of NFK. Since NFK is prone to hydrolysis, the solutions were kept in 100 mM phosphate buffer (pH 7) at -20°C.

LC-MS analysis of the reaction mix of isolated NFK \((500 \mu\text{M})\) treated with PIP \((1 \text{ M})\) at 65°C for 20 min showed products identical to those found in the enzyme assay mix shown in Figure 3.4, strongly indicating that the fluorophore is derived from the reaction of NFK with PIP.

### 3.2.3 Optimization of PIP-THQ formation and assay conditions

Since PIP-THQ formation is correlated with rhIDO1 enzymatic activity (Fig. 3.3c), it could form the basis of a new, more sensitive fluorescence assay for IDO1 than the earlier KYN-based fluorescence assay. The optimal conditions for PIP-THQ formation and detection were therefore determined. The rate of PIP-THQ formation at different temperatures was measured after reacting NFK \((500 \mu\text{M})\) with PIP \((1 \text{ M})\) in the assay medium, and quantifying the amount of PIP-THQ formed by integrating the area of the PIP-THQ peak in the HPLC chromatogram. The rate of PIP-THQ formation increased with increasing incubation
温度，但高于80°C，快速形成的速率会被快速降解所抵消（图3.5a）。在65°C时，PIP-THQ的形成稳步增加，达到最大值后20 min之前水平下降。在55°C时，PIP-THQ的形成速率是65°C的2.75倍，需要60 min才能达到在65°C时20 min内达到的相同浓度的PIP-THQ。20 min的孵育时间在65°C下被选作是使用在分析中最为合适的。

孵育后在常温下读取荧光的后孵育期的长度影响是被进一步研究的（图3.5b）。背景荧光保持不变，但S/B比值随着后孵育期的长度增加而增加，这并不是由于PIP-THQ增加形成的。孵育4 h后在常温孵育的样本的S/B比值是孵育后0.5 h（图3.5b）的2.5倍。在延长分析程序的情况下，可以增加分析的灵敏度通过增加后孵育期的时间。选择了1 h的后孵育期作为后续实验的最优平衡点，既保证了灵敏度，又缩短了分析时间。

图3.5. a) PIP-THQ形成随时间的变化，以55°C, 65°C, 80°C和95°C的孵育温度，采用HPLC测定。b) 在65°C孵育20 min后，在0.5 h (黑色条形)，2 h (白色条形)和4 h (斜纹条形)后，分别在常温读取的S/B比值。c) NFK (12.5 µM) 和 PIP (200 mM) 在65°C孵育20 min后，在室温、冰箱或冰箱温度下保存24 h后的荧光强度。值在c)中是相对于室温孵化后30 min测量的。PIP-THQ的量从400 nm的色谱图中量化。

- 75 -
Chapter 3- Results

The stability of the assay signal over 24 h was determined (Fig. 3.5c). NFK (12.5 µM) was mixed with PIP (200 mM) in assay buffer in three separate 384-well plates, incubated at 65°C for 20 min and the fluorescence was measured after 30 min incubation at room temperature (21°C). Subsequently, the plates were incubated at three different temperatures (benchtop 21°C, fridge 5.5°C and freezer -18.9°C) for 23.5 h prior to the second readout. The fluorescence signal decreased by 38% in the plate incubated on the bench, but increased 52% and 38% in plates kept in the fridge and the freezer, respectively (Fig. 3.5c). Plates that needed to be stored overnight before readout, were placed in the fridge for maximum signal the next day.

The linearity of the response and sensitivity of the fluorescence signal was assessed using five different PIP concentrations (50 mM, 100 mM, 200 mM, 500 mM and 1000 mM) across NFK concentrations between 0.3 – 18.7 µM. Perfect linear fits were obtained with all PIP concentrations across the NFK concentration range used (Fig. 3.6a), but signal intensity decreased with PIP concentrations above 200 mM and below 100 mM.

Figure 3.6. a) Calibration curve of NFK (0.3-18.7 µM) in assay buffer treated with 50, 100, 200, 500 and 1000 mM PIP. r^2 value of the linear fit of each titration curve is indicated. b) Relative fluorescence of 18.75 µM NFK replotted from a), and relative amount of PIP-THQ formed in a reaction of 61 µM NFK (produced by incubation of 28 nM rhIDO1 in complete assay mix with 250 µM TRP for 60 min at 37°C) and various PIP concentrations in 20 min at 65°C. pH of the enzyme reaction after addition of different PIP concentrations are indicated next to the data points. Data in b) were normalised to the highest and lowest value in each dataset. PIP-THQ was quantified from fluorescence chromatogram (excitation and emission wavelength 400 nm and 500 nm, respectively).
Chapter 3- Results

To determine whether the signal intensity correlates with the amount of PIP-THQ formed, 61 µM NFK (determined by HPLC compared to NFK standard) produced in an IDO1 assay, was reacted with the same concentrations of PIP as in Fig. 3.6a, and analysed by HPLC to quantify PIP-THQ. Figure 3.6b shows that the amount of PIP-THQ formed and the fluorescence signal increased only with PIP concentrations between 50 - 100 mM. At higher than 100 mM PIP, the fluorescence signal decreased even though the amount of PIP-THQ formed had increased. This inverse correlation suggests that the fluorescence of PIP-THQ might be pH-sensitive. A concentration of 200 mM PIP was used for subsequent experiments to maximise the sensitivity of the assay whilst accommodating variability in preparation of assay buffer or PIP solutions. The lower limit of detection value determined with 200 mM PIP and 1 h post-incubation time was 153 nM NFK, approximately 30-fold lower than that reported in the previously-described fluorescence assay for IDO1 activity (Matin et al., 2006). Post-incubation times between 2-4 h increased the sensitivity to LoDs < 100 nM NFK with 200 mM PIP (data not shown). The effect of other essential components in the assay mixture on the fluorescence signal of the complete assay medium (background), and the reaction of 10 µM NFK and 200 mM PIP in the complete assay medium was also examined. Titration of each component was performed in assay buffer containing all the other remaining assay components at concentrations described in methods section 2.1.3.1, but excluding rhIDO1. TRP was excluded when rhIDO1 was titrated. Ascorbic acid, a reducing agent necessary to keep IDO1 in its active ferrous (Fe2+) form (Takikawa et al., 1988, Yamamoto & Hayaishi, 1967), was shown to dramatically reduce the fluorescence signal of the PIP-THQ formed, and to increase the background at concentrations greater than 50 mM (Fig. 3.7a). Ascorbic acid is best used at concentrations between 1 - 50 mM. Catalase, which is required as a scavenger of inhibitory hydrogen peroxide formed from auto-oxidation of ferrous-oxy (Fe2+-O2) haem or methylene blue/ascorbic acid (Hirata & Hayaishi, 1975, Hirata et al., 1977, Poljak et al., 2006), is best kept at concentrations below 400 µg/mL (1.6 µM) (Fig. 3.7b). Tween 20, added to minimize aggregate formation by test compounds (Ryan et al., 2003), increased the background signal, and its concentration should not exceed 1% (v/v) (Fig. 3.7c). Methylene blue, a reducing co-factor transferring electron from ascorbic acid to haem (Hirata & Hayaishi, 1975), had no effect on the background or PIP-THQ fluorescence signal at concentrations between 0.1 µM – 100 µM (Fig. 3.7d). DMSO, used for dissolving the test compounds, had no effect on fluorescence signal when used between 0.1% – 10% (v/v) (Fig. 3.7e).
3.7e). TRP (0.5 μM – 1000 μM) and rhIDO1 (3.5 nM – 430 nM) also had no effects on the background or PIP-THQ fluorescence signal (data not shown).

![Graph showing effects of various concentrations of a) Ascorbic acid, b) Catalase, c) Tween 20, d) Methylene blue and e) DMSO on fluorescence signal of 10 μM NFK in assay buffer (closed circles) or assay buffer alone (open circles) reacted with 200 mM PIP. Each titration was performed in complete assay buffer without rhIDO1.]

**Figure 3.7.** Effect of various concentrations of a) Ascorbic acid, b) Catalase, c) Tween 20, d) Methylene blue and e) DMSO on fluorescence signal of 10 μM NFK in assay buffer (closed circles) or assay buffer alone (open circles) reacted with 200 mM PIP. Each titration was performed in complete assay buffer without rhIDO1.

### 3.2.4 Adaptation of the fluorescence assay for use with JANUS automated workstation

Manual testing of a large number of compounds can be problematic due to a number of reasons including precision, reliability and risk of repetitive use syndrome for the investigator. Therefore, an automated protocol was developed taking advantage of the JANUS automated workstation available at ACSRC.

This required a) calibration of all labware (plates, troughs, pipette racks, etc.) dimensions in the JANUS software, b) determination of optimal pipetting parameters (aspiration and dispensing speeds, maximum number of repeated dispensions per given volume, optimum waste volume for aspiration), and c) programming of the actual liquid handling protocol for the assay. Firstly, liquid handling programmes were created where the
parameters (e.g. volumes, source and destination wells and the number of samples) were defined and changed manually for every experiment. Adjusting a large number of variables manually can be a source of random errors in liquid handling, and to eliminate this problem, a set of scripts written in C# programming language, was created that re-calculated the majority of the variables in the protocol automatically according to the number of compounds entered into an Initial User Query appearing at the start of the protocol (Fig. 3.8) (see the scripts in Appendix Figures A.1 and A.2). However, compound-specific variables; including the position in the plate or dilution could not be calculated with a script. Instead, these variables were linked to the Microsoft Excel spreadsheet containing the database of the compounds and their properties (e.g., plate coordinates and stock concentrations) which were loaded into the JANUS before each protocol. Also, spreadsheets were designed to automatically generate experimental protocols (buffer preparation and corresponding volumes to be mixed) and to convert the raw data acquired from the fluorescence reader into an array that could be directly pasted into the graphing application (e.g. GraphPad Prism).

Using these approaches, programmes for two major protocols were successfully developed: A) determination of IC$_{50}$ of the IDO1 inhibitors and B) testing compound libraries at a single concentration for IDO1 inhibitory activity. Using Protocol A, a batch of 40 inhibitors can be processed in duplicates of 8 different concentrations together with background controls for each concentration, in ~ 100 min. Protocol B can process 640 compounds in 2 h.

![Initial User Query](image)

**Figure 3.8.** Initial User Query from the protocol for determining IC$_{50}$ of IDO1 inhibitors. The script is programmed in a way requiring only entering the number of compounds to test and pressing Start button (ticks select instructions to be executed in the protocol).
Chapter 3- Results

3.2.5 Validation and comparison of the new assay with the absorbance assay.

To assess whether the new fluorescence assay performed using the JANUS automated workstation has sufficient dynamic range and reproducibility to be applied in an HTS setting to reliably identify inhibitors of IDO1, the Z’ factor was determined according to the Eq. 3 described in the methodology section 2.1.3.1 using a set of 320 positive and 16 negative controls in two 384-well plates. Ideally, the Z’ value should lie between 0.5 – 1, while Z’ close to 0 indicates poor reliability. The Z’ value obtained for the two plates of controls was 0.88 ± 0.003 (two plates) demonstrating that the assay is most suitable for this format (Zhang et al.,1999).

To evaluate the performance of the new fluorescence assay, the IC50 values of three known IDO1 inhibitors, namely 1,4-napthoquinone (NQ), 4-amino-N-(3-chloro-4-fluorophenyl)-N’-hydroxy-1,2,5-oxadiazole-3-carboximidamide (HA), and 4-phenyl-1H-imidazole (4PI) (Fig. 3.9a), were determined using the new fluorescence assay (Fig. 3.9b) and compared to that obtained using the standard absorbance assay (Fig. 3.9c). The slopes of the titration curves of the three compounds in the two assays were similar (-21.32 vs -19.64, -0.9 vs -0.85, 0.013 vs 0.018 (fluorescence assay vs absorbance assay), respectively for HA, NQ and 4PI). The IC50 values from the two assays were also comparable (0.051±0.003 vs 0.043±0.003, 0.48±0.04 vs 0.44±0.04 and 37.8±1.14 vs 35.6±3.53 μM (fluorescence assay vs absorbance assay) respectively for HA, NQ and 4PI, and were consistent with the published values for the three compounds (Kumar et al.,2008a, Kumar et al.,2008b, Yue et al.,2009). The standard deviations of the IC50 values obtained from at least two independent experiments were also comparable between the two assays and the result of a representative experiment is shown in Figs. 3.9b and 3.9c.

The IDO1 inhibitory activity was then determined for the 1597 compounds of the NCI Diversity Set III, together with the three standards, 4PI, HA and NQ (Fig. 3.9), each at 20 μM using the two assays. Thirty-five ‘hits’ were identified (compounds that inhibited IDO1 activity >50% at 20 μM; including known IDO1 inhibitors HA and NQ) of which 25 were identified in both assays (Fig. 3.10a – upper right quadrant) and 10 appeared as ‘hits’ only in the fluorescence assay (Fig. 3.10a – upper left quadrant). Considering the small difference in the inhibitory values of these 10 compounds between the two assay formats (12.4 ± 5.9%) and close proximity to the 50% threshold, they were probably not identified in the absorbance assay due to experimental variability.
Chapter 3- Results

Figure 3.9. a) Chemical structures of three published IDO1 inhibitors titrated for inhibition of IDO1 enzymatic activity measured using b) the PIP fluorescence assay and c) the TCA absorbance assay. Data were fitted by nonlinear regression analysis using built-in GraphPad Prism 6 equation One Site-Total: $y = a \left( \frac{x}{k+x} \right) + slope \cdot x + b$. Indicated IC$_{50}$ values (μM) were interpolated from the curves fitted to the data at $y$ equal to 50.

Furthermore, the majority of these compounds were found to absorb light at the absorbance assay readout at 480 nm; another contributing factor to the observed differences in inhibition values obtained in the two assays. The fluorescence assay gave positive compounds a marginally higher inhibitory activity value than those obtained in the absorbance assay (Fig. 3.10a), although the values determined in the two assays correlated reasonably well ($r^2 = 0.86$). Bland-Altman analysis (Bland & Altman, 1986), comparing the difference between the results determined by the two methods for each sample (y-axis) plotted against their corresponding averages (x-axis) of the identical dataset as in Figure 3.10a, confirmed that the two methods are in excellent agreement (mean difference = 2.0064, with 95% limits of agreement ranging from -8.8664 to 12.8801) (Fig. 3.10b).
Figure 3.10. IDO1 inhibitory activity of the compounds of the NCI Diversity Set III determined using fluorescence assay and plotted against that obtained using the a) absorbance assay and compared using b) Bland-Altman analysis. Bold and dotted lines in b) denotes mean difference and 95% upper and lower limit of agreement, respectively. c) Compounds in the library that interfered with the assay readout (> 0.25) are denoted by the dashed line in the fluorescence assay (y-axis) and the absorbance assay (x-axis). Note: compounds that interfered with both assays (upper right quadrant) were not detected.

A further experiment was carried out to determine the types of compounds that may interfere with the readout in each of the assays. Each compound in the NCI Diversity Set III was incubated in the standard assay medium without rhIDO1, and processed for fluorescence detection and absorbance detection. The amount of interference of the test compounds for each assay was calculated according to the Equation 4 given in the Materials and methods section 2.1.3.1, using the uninhibited enzyme signal value. Compounds with a signal ≥ 0.25 of the enzyme-catalysed reaction were considered as compounds that interfered with that
assay. Ninety-three of the compounds in the NCI diversity set III interfered with the absorbance read-out, compared to only 18 compounds with the fluorescence read-out (Fig. 3.10c). Aldehydes and ketones have previously been shown to interfere with the absorbance read-out (Alegre et al., 2005), and our data showed that aromatic amines may interfere as well. On the other hand, compounds that gave interfering fluorescence signals were mainly those with conjugated systems and fused cycles. Since no compounds were observed to interfere with both assays (Fig. 3.10c), it is possible to obtain a correct determination of the IDO1 inhibitory activity for all compounds by using the fluorescence and the absorbance assays together.

3.3 Discussion

The work in this chapter describes a novel fluorescence assay for assaying IDO1 enzymatic activity that utilises the formation of a previously unreported fluorophore formed from the reaction of NFK with PIP. The fluorescence intensity of this fluorophore, referred to as PIP-THQ, is significantly stronger than that of KYN, conferring improved S/B ratios and greater sensitivity compared with the current assays that measure KYN production (Matin et al., 2006). The low sensitivity of the KYN-based fluorescence assay could be caused by the instability of KYN at high pH and its poor fluorescence quantum yield (\(\Phi = 5.8 \times 10^{-4}\) at pH 7.5) (Fukunaga et al., 1982) which, for example is 1600-fold lower than that of fluorescein (\(\Phi = 9.3 \times 10^{-1}\) in 0.1 M aqueous NaOH, pH ~ 13) (Magde et al., 2002). The new PIP fluorescence assay is simple to execute, requiring only a single step addition of PIP to the reaction mix, and heating at a recommended temperature of 65°C for 20 min for maximal PIP-THQ formation. The assay can also accommodate minor changes in reagent concentrations, temperature and post-incubation times to balance sensitivity and length of assay times.

In this work, pure NFK was isolated from the IDO1-catalysed oxidation of TRP. NFK showed an extinction coefficient (\(\varepsilon_{323} = 3066 \text{ M}^{-1} \text{ cm}^{-1}\)), which differs from the value determined by Dalgliesh and colleagues in 1952 (\(\varepsilon_{321} = 3750 \text{ M}^{-1} \text{ cm}^{-1}\)) (Dalgliesh, 1952), but agrees with those determined by Walrant and coworkers (\(\varepsilon_{318} = 3180 \text{ M}^{-1} \text{ cm}^{-1}\)) (Walrant & Santus, 1974) and by Ren and coworkers (\(\varepsilon = 3152 \text{ M}^{-1} \text{ cm}^{-1}\)) (Ren et al., 1996) who sourced NFK from commercial suppliers. Extinction coefficients of NFK synthesised in recent studies were not reported (Kurnasov et al., 2003, Savige, 1971, Simat et al., 1994). A large number of studies that have used the extinction coefficient of NFK (\(\varepsilon_{321} = 3750 \text{ M}^{-1} \text{ cm}^{-1}\)) reported by Dalgliesh et al may have underestimated the spectrophotometric quantifications of NFK by ~ 20% (Basran et al., 2008, Batabyal & Yeh, 2007, Batabyal & Yeh, 2009, Dreaden et al., 2011,

The optimal incubation temperature for maximum formation of the fluorophore PIP-THQ is 65°C for 20 min. PIP-THQ can form however at 55°C using a longer incubation (55 min) to obtain a similar amount of PIP-THQ to that acquired in a reaction heated at 65°C for 20 min. Assuming the time required to form a fixed amount of PIP-THQ increases 2.75-fold with each 10°C decrease in temperature, at 37°C the assay would need to be incubated with PIP for about 5.5 h. Therefore, the assay could be used without a heating step at 65°C at the cost of extended incubation at 37°C.

One of the advantages of the assay is the possibility of increasing the sensitivity by extending the post-incubation time at ambient temperature (Fig. 3.5b). It is possible that a decrease in temperature when the plate moves from 65°C to a cooler ambient environment of 21°C increases the fluorescence signal. However, the temperature in such a small volume (55 µL x 320 wells) is unlikely to be decreasing over a 1-2 h period. A more likely explanation is that the fluorescence of PIP-THQ increases over time as PIP evaporates, decreasing the pH of the solution. This is consistent with the fluorescence signal increasing 2.5-fold in 3.5 h post-incubation at 21°C (Fig. 3.5c), whereas it increased by only 1.5-fold in 23.5 h at 5.5°C; and even less at -18.9°C. The process responsible for increasing the fluorescence signal post-incubation slows down by a decrease in temperature, supporting the idea of PIP evaporation.

Concentrations of PIP above and below 100 mM decreased the fluorescence signal of PIP-THQ (Fig. 3.6a). The decrease of fluorescence signal at PIP concentrations below 100 mM was caused by the low fluorophore formation. However, it was surprising to observe that the fluorescence signal of PIP-THQ decreased at PIP concentrations above 100 mM despite the increased amount of formed PIP-THQ as quantitated using HPLC (Fig. 3.6b). It indicates that the fluorescence of PIP-THQ is pH-dependent which is typical for most polar fluorophores. Moreover, the data from our studies to characterise the structure of the novel fluorophore, suggests that PIP-THQ exists in a dynamic equilibrium between two states. LC-MS analyses of the reaction products from the fluorescence assay, or from the reaction of purified NFK with PIP, showed the presence of two entities (Fig. 3.4a) that contribute to the PIP-THQ fluorescence band detected at 500 nm. That PIP-THQ exists in more than one inter-converting forms has made both the data from mass spectrometry and NMR analyses extremely challenging to interpret in the efforts presented in the following chapter to identify the structure of PIP-THQ.
Ascorbic acid/methylene blue is used to maintain IDO1 in its catalytically active Fe$^{2+}$ form (Yamamoto & Hayaishi, 1967) and to limit auto-oxidation to the inactive Fe$^{3+}$ form (Hirata et al., 1977). The same reducing system was used for both the absorbance and the fluorescence assay. Ascorbic acid concentrations above 50 mM however, were found to decrease dramatically the PIP-THQ signal in the fluorescence assay (Fig. 3.7a). It is not clear if the decreased signal is due to altered rates of PIP-THQ formation or its fluorescence properties. Ascorbic acid is a known quencher of harmful fluorescence of proteins and TRP in the eye (Ringvold, 1995, Ringvold, 1996), and it is possible that ascorbic acid at concentrations above 10 mM may also quench the fluorescence signal of PIP-THQ. However, ascorbic acid at concentrations < 10 mM decreased the assay fluorescence signal (Fig. 3.7a) indicating that increasing concentrations of ascorbic acid up to 10 mM will enhance PIP-THQ formation, whilst concentrations above 10 mM will quench its fluorescence.

The assay has been adapted successfully for use with the JANUS automated robotic workstation to test a large number of compounds for IDO1 inhibitory activity. This has enabled the ACSRC to test large numbers of compounds for inhibition of IDO1 in their drug development programme. In addition to the obvious benefits of high reliability and speed of the automated assay, running costs are significantly reduced as the workstation requires disposable tips only for pipetting of test compound solutions. Whilst the throughput of 640 compounds per 2 h at a single concentration is not as high as expected, the limitation is caused by the 8-channel dispensing head of the current JANUS setup, and could be replaced by a 96-channel dispensing head that would increase the liquid handling speed by 12-fold.

An acceptable correlation was obtained between the two sets of data generated when the entire NCI library was screened for inhibition of IDO1 activity using the new fluorescence PIP assay and the absorbance TCA assay ($r^2 = 0.86$) (Fig. 3.10a). However, this correlation is likely to have been affected by compounds that produce false-positive, high signals at assay readouts, and the actual correlation of IDO1 inhibitory activities between the two methods could be higher. The majority of the compounds that produced widely differing inhibition values in the two assays had either a high fluorescence at detection wavelengths of the new fluorescence assay, or a high absorbance at the readout of the TCA absorbance assay.

The conditions for generation of PIP-THQ have been optimised for assaying IDO1 enzymatic activity in vitro in cell-free systems only. It is not known if such fluorophores are formed in cells or in vivo and whether the assay could be adapted for measuring dioxygenase activity under physiological conditions. Currently, determinations of dioxygenase activity in patients rely on HPLC and LC-MS (Huang et al., 2002, Laich et al., 2002, Suzuki et al., 2010, Vignau et al., 2004) measurements of tryptophan to kynurenine ratios in their serum samples.
In cells, NFK is rapidly hydrolysed to KYN by kynurenine formamidase ($V_{\text{MAX}}$ of mouse kynurenine formamidase = 53 mM KYN.min$^{-1}$) (Pabarcus & Casida, 2005). The relatively low affinity of mouse kynurenine formamidase for NFK ($K_m = 180 \, \mu\text{M}$) (Pabarcus & Casida, 2005) however suggests that total depletion of the NFK pool would take a long time. NFK turnover may differ between cell types, and stress conditions, and determination of dioxygenase activity based on the quantification of NFK in cell based assays may not be reliable.

Since the publication of this fluorescence assay (Tomek et al., 2013), two other dioxygenase assays have appeared in print (Table 3.1; Fig. 3.11e,f) (Klockow & Glass, 2012, Seegers et al., 2014). One of the methods is based on a reaction of KYN and a coumarine aldehyde called Sensor 1 that forms a Schiff base strongly fluorescing at acidic pH (in 50 mM H$_3$PO$_4$, pH 1) (Fig. 3.11e) (Klockow & Glass, 2012). The excitation wavelength of this Schiff base (555 nm) is the highest of all the fluorescence assays for determination of IDO1 enzymatic activity (Table 3.1), promising high specificity and low interference from test compounds. However, Sensor 1 reacts with aniline (Klockow & Glass, 2012), suggesting a non-specific reactivity similar to that of the TCA absorbance assay (Alegre et al., 2005, Takikawa et al., 1988). This method has not yet been used for determining IDO1/TDO activity, but may provide an alternative for the absorbance TCA assay (Fig. 3.11a).

The second method, NFK GreenScreen™ is developed by a private company NTRC (Oss, The Netherlands) (Fig. 3.11f) (Seegers et al., 2014). This assay detects NFK similarly to our PIP assay, but utilises a proprietary reagent NFK Green™ which forms an undisclosed fluorophore in reaction with NFK. This fluorophore has identical excitation and emission wavelengths as PIP-THQ (see Fig. 3.11c,f or Table 3.1). NFK GreenScreen™ assay requires a long incubation time of 240 min at 37°C, and has a lower sensitivity (~ 1 µM) than the PIP fluorescence assay, and the cost of $2,000 NZD for 1000 measurements is 3000-fold more expensive (see also Table 3.1). Seegers at al. successfully used NFK Green™ in a cell-based assay (A-172 glioblastoma line), suggesting the availability of NFK in cells. However, the ability to detect NFK is cell-line dependent. Out of 44 cell lines tested for production of NFK after overnight incubation with L-TRP, only 2 lines, the glioblastoma A-172 and the colorectal carcinoma SW48 produced detectable NFK without IFN-γ stimulation, and 3 colon cancer lines, including SW48, in the presence of IFN-γ. Given the evidence of dioxygenase expression in a broad range of cancers, a likely explanation for the 40 out of 44 negative cell lines is the rapid depletion of cellular NFK as discussed previously (Pabarcus & Casida, 2005).
Figure 3.11. Updated schematic pathway of microplate assays for measuring dioxygenase enzymes activity using (a,b,e) KYN formation, (c,d) NFK formation and (d) TRP detection. The assays that were published after December 2012 (e,f) are indicated by a dashed rectangle. Abbreviations: $\lambda_{ex}$ (excitation wavelength), $\lambda_{em}$ (emission wavelength), TrpR (tryptophan repressor protein).
### Table 3.1. \(\text{Comparison of the microplate assays used for assaying activity of dioxygenase enzymes (see also Fig. 3.11).}\)

<table>
<thead>
<tr>
<th>Assay</th>
<th>(a) TCA</th>
<th>(b) NaOH</th>
<th>(c) H(_3)PO(_4)</th>
<th>(d) PIP</th>
<th>(e) NFK Green Screen™</th>
<th>(f) Bridge-It(^*) TRP assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Readout</td>
<td>Abs</td>
<td>FI</td>
<td>FI</td>
<td>FI</td>
<td>FL</td>
<td>FL</td>
</tr>
<tr>
<td>Metabolite</td>
<td>KYN</td>
<td>KYN</td>
<td>KYN</td>
<td>NFK</td>
<td>NFK</td>
<td>TRP</td>
</tr>
<tr>
<td>Measured analyte</td>
<td>KYN-p-DMAB</td>
<td>KYN</td>
<td>KYN-coumarine</td>
<td>PIP-THQ</td>
<td>proprietary</td>
<td>Oyster-645(^*), fluorescein labelled DNA</td>
</tr>
<tr>
<td>Incubation</td>
<td>65°C 20 min</td>
<td>65°C 20 min</td>
<td>N/A</td>
<td>65°C 20 min</td>
<td>37°C 240 min</td>
<td>25°C 30 min</td>
</tr>
<tr>
<td>LoD (µM)</td>
<td>5.6 µM</td>
<td>6.7 µM</td>
<td>N/A</td>
<td>0.15 µM</td>
<td>1 µM</td>
<td>0.1 µM</td>
</tr>
<tr>
<td>Detection wavelengths (nm)</td>
<td>(\lambda_{\text{ex}} 480)</td>
<td>(\lambda_{\text{em}} 360)</td>
<td>(\lambda_{\text{ex}} 555)</td>
<td>(\lambda_{\text{em}} 400)</td>
<td>(\lambda_{\text{ex}} 400)</td>
<td>(\lambda_{\text{em}} 665)</td>
</tr>
<tr>
<td>Single step</td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Cost per 1000 measurements (NZD)(^a)</td>
<td>1.03</td>
<td>0.05</td>
<td>N/A</td>
<td>0.66</td>
<td>1923</td>
<td>2683</td>
</tr>
</tbody>
</table>

\(^a\) Calculation of “Cost per 1000 measurements” assumed that 1000 measurements equal 50 mL of the assay mixture (50 µL/1 well per measurement). Final concentrations of reagents used for calculations were: TCA (5%, w/v), p-DMAB (10 mg/mL), NaOH (160 mM), PIP (200 mM). Prices of reagents were collected from Sigma-Aldrich website (New Zealand region) on 3/3/2015 using following product numbers: TCA (T4885-500G (NZD 141.5)), p-DMAB (109762-500G (NZD 329.0)), NaOH (S5881-500G (NZD 76.2)), PIP (104094-500 mL (NZD 157.5)). Price of NFK GreenScreen™ is based on product number NTRC-GSCell-1K obtained from manufacturer website (NTRC, Oss, The Netherlands). Price of Bridge-It\(^*\) TRP fluorescence assay is based on product number 1-1-1002B obtained from manufacturers website (Mediomics, LLC, St.Louis, MO, USA). Abbreviations: Abs, absorbance; FI, fluorescence intensity; Y, yes; N, no; N/A, not available, NZD (New Zealand Dollar). LoD (Limit of Detection).

In summary, the formation of PIP-THQ has provided the basis for a novel fluorescence assay that has a number of advantages over current assays for IDO1 enzymatic activity (Fig. 3.11, Table 3.1). It is economical, offers low-nM sensitivity, and has a low interference rate from test compounds and a single step experimental protocol (Table 3.1). The assay was used to screen the NCI Diversity Set III compound library, and identified 35 compounds that gave greater than 50% inhibition of IDO1 enzyme activity at 20 µM. With its ease of set-up and miniaturization, this new fluorescence assay would be the method of choice for use in HTS of compound libraries in drug discovery programmes for novel IDO1 inhibitors for development.
into potential therapies for cancer and other diseases. Our fluorescence assay could also be applied to determine activity of related dioxygenase enzymes TDO and IDO2 since both produce NFk, which can be converted to fluorophore PIP-THQ. The PIP assay can be used both in industry (Julie Rainard, Sarah Maxfield; RedX Oncology; personal communication) as well as in academic research (Cheung, 2013). Cheung demonstrated a novel application for utilising formation of PIP-THQ fluorophore to detect TRP oxidation in a $\gamma$-irradiated peptide WLBU2 (Cheung, 2013).
CHAPTER 4

STRUCTURE AND MECHANISM OF FLUOROPHORE PIP-THQ FORMATION

The work in chapter 3 showed that NFK, the product of IDO1-catalysed oxidation of TRP, reacts with PIP to form a strong fluorophore PIP-THQ that can be used to measure IDO1 activity. The isolation and characterization of the chemical structure of PIP-THQ is presented in the work in this chapter, together with experimental evidence for a previously unreported chemical transformation pathway leading to its formation.

This chapter is a modified version of a manuscript entitled “Formation of fluorophores from the kynurenine pathway metabolite N-formylkynurenine and cyclic amines involves transamidation and carbon-carbon bond formation at the 2-position of the amine” that has been published in a respectable journal Biochimica et Biophysica Acta (BBA) - General Subjects (2014 Impact Factor 4.38) (Tomek et al., 2015). The final publication is available at Elsevier B.V. via http://dx.doi.org/10.1016/j.bbagen.2015.04.007. The manuscript was authored by Petr Tomek, Brian D. Palmer, Jackie D. Kendall, Jack U. Flanagan and Lai-Ming Ching. I conceived the study, designed and performed the experiments, analysed and interpreted all the results except the NMR studies that were carried out in collaboration with Assoc. Prof. Brian D. Palmer and Dr Jackie D. Kendall from ACSRC. BDP synthesised NFK and [13C]-NFK, analysed NMR experiments and wrote the experimental details for the synthesis of NFK and the interpretation of NMR spectra. JK and LMC critically edited the manuscript.

The results of the spectral and analytical characterisations of all synthesised compounds can be found in section 2.2.4 (Materials and Methods) and Appendix (pages 158 - 171).

4.1 Introduction

The formation of fluorophores from NFK has not been reported in the literature, and the only reported transformation of NFK is photooxidation into N-formylantranilic acid and 4-hydroxyquinoline,(Pirie, 1971) and the hydrolysis into KYN (Fig. 4.1) (Chen & Guillemin, 2009, Mehler & Knox, 1950). KYN can spontaneously deaminate at physiological pH to form a reactive α,β-carboxyketalkene (KYN-CKA) (Taylor et al., 2002), which can either form adducts with biological nucleophiles (Garner et al., 2000, Parker et al., 2007), undergo reduction or cyclise to form kynurenic acid or kynurenine yellow
Chapter 4- Introduction

(Fig. 4.1) (Kopylova et al., 2009, Tsentalovich et al., 2006, Yu et al., 2008). Since PIP is a both nucleophilic and basic molecule, it is likely that NFK may undergo similar reactions to those described for KYN (Fig. 4.1).

Figure 4.1. Overview of major chemical transformations of KYN and NFK. MW stands for molecular weight in Daltons.
4.2 Experimentation and Discussion

4.2.1 Structure and properties of PIP-THQ fluorophore

4.2.1.1 NMR studies

NFK was synthesised by formylation of KYN using a modification (addition of a heating step) of a published method (see section 2.2.4.1) (Dalgliesh, 1952). NFK was then reacted with an excess of PIP at 60°C under aqueous conditions, and the fluorophore was purified using reversed phase preparative chromatography (see section 2.2.4.3). The product displayed a molecular ion adduct [M+H]^+ at m/z 303.1 (Fig. 4.2a - asterisk) on low-resolution triple quadrupole mass spectrometry, and a sodium adduct [M+Na]^+ at 325.1159 amu on high-resolution time-of-flight mass spectrometry, respectively. The data is consistent with a molecular formula of C_{16}H_{18}N_{2}O_{4} and a molecular weight of 302 Da. The majority of the resonances in the ^1H and ^13C NMR spectra is doubled up, consistent with a 1:1 mixture of isomers in the sample (Appendix Fig. A.4a,b). The four aromatic resonances of the starting NFK are still present, and resonances characteristic of a PIP group are seen at \( \delta \) 4.4-2.7 ppm (CH-N) and \( \delta \) 1.8-1.3 ppm (CH2CH2). Singlets at \( \delta \) 8.06 and 8.02 ppm are consistent with the presence of a formyl group. Particularly diagnostic are resonances resulting from two overlapping AB spin systems centred at around 2.5 ppm, each with a large coupling constant of approximately 15 Hz, indicating the presence of an isolated CH2 group, most likely in a ring system. Two-dimensional NMR studies (Figs. A.5 – A.10) were used to identify coupled proton systems and to correlate proton and carbon resonances (Table 2.4). From these, it is evident that there are only three protons adjacent to the nitrogen atom of the PIP, with two of these displaying geminal coupling to each other. The remaining proton did not display geminal coupling and appear as a much narrower resonance. This indicated that PIP has reacted at one of the carbon atoms adjacent to the nitrogen of the PIP with the loss of one of its hydrogen atoms. A nitrogen NMR spectrum was also obtained and indicated the presence of two nitrogen atoms in the PIP-THQ (\( \delta \) 88 and 134 ppm) (Fig. A.10). Considering all the data, the structure of PIP-THQ shown in Fig. 4.2 is proposed. The product is a mixture of two racemic diastereomers at the 2 and 2’ positions (Table 2.4). The placement of the formyl group on the PIP nitrogen and not the nitrogen of the quinolone ring is based on the Nuclear overhauser effect spectroscopy (NOESY) spectrum, which suggested it is close to the PIP ring protons, although the correlation peaks are not strong (Fig. A.9).
4.2.1.2 MS fragmentation studies to confirm position of the formyl group on PIP-THQ

To confirm the position of the formyl group in the structure of PIP-THQ, mass fragmentation studies of the compound were conducted (Fig. 4.2). The fragmentation spectrum of $[^{12}\text{C}\text{-formyl}]$-PIP-THQ ($m/z$ 303.1) shows a prominent ion at $m/z$ 112.2, likely corresponding to the $N$-formyl-2,3,4,5-tetrahydropyridin-1-ium fragment (Fig. 4.2a). Further fragmentation of this ion provided $m/z$ 84.1, indicating a loss of the formyl group ($\Delta m/z$ 28), and $m/z$ 56.1, 42.1 and 28.1 ($\Delta m/z$ 14) typical for PIP fragmentation (Fig. 4.2b) (Daasch,1965). To confirm that $m/z$ 112.2 is indeed an $N$-formyl-2,3,4,5-tetrahydropyridin-1-ium ion, NFK was synthesised using $^{13}\text{C}$-labelled formic acid and it was reacted with PIP. This yielded a mixture of $[^{13}\text{C}\text{-formyl}]$-PIP-THQ and $[^{12}\text{C}\text{-formyl}]$-PIP-THQ in a ratio of 1.73:1 according to MS. Fragmentation of $[^{13}\text{C}\text{-formyl}]$-PIP-THQ is expected to yield M+1 ion ($m/z$ 113), and indeed the fragmentation spectrum of $[^{13}\text{C}\text{-formyl}]$-PIP-THQ ($m/z$ 304.3) shows a dominant ion at $m/z$ 113.1 (Fig. 4.2c), consistent with the formyl group in PIP-THQ being attached to PIP. Other ions produced were the same as those observed in the fragmentation spectrum of $[^{12}\text{C}\text{-formyl}]$-PIP-THQ, consistent with their formation following the loss of the labelled formyl group (Fig. 4.2b,d).

![Figure 4.2](image)

Figure 4.2. Mass fragmentation of PIP-THQ isotopes in triple quadrupole positive electrospray ionisation mode. Panel shows fragmentation spectra of ion a) $m/z$ 304.3 ($[^{13}\text{C}\text{-formyl}]$ PIP-THQ), b) $m/z$ 113.1, c) $m/z$ 303.1 ($[^{12}\text{C}\text{-formyl}]$ PIP-THQ) and d) $m/z$ 112.1. Voltage applied to collision cell was 10 V (a,c) and 20 V (b,d), respectively. The asterisk marks the ion being fragmented.
4.2.1.3 Fluorescence titration and absorption spectrum of PIP-THQ

Changes in pH between 3 and 11.5 did not affect fluorescence intensity of 15 μM PIP-THQ, but highly acidic (< 1) or basic (> 13) pH destroyed the signal (Fig. 4.3). A decrease in fluorescence of PIP-THQ is likely caused either by ionisation of the quinolone nitrogen or carboxylic acid groups of PIP-THQ (pKa 1.78 and 12.56, respectively), or decomposition at pH extremes. The absorption spectrum of PIP-THQ shows distinct bands at 400 nm and 235 nm, and a shoulder at 260 nm (see Fig. 4.4a - inset), which differs to those of previously reported KYN-related compounds.

![Fluorescence titration of 15 μM PIP-THQ measured in 0.1 M KCl at 25°C. pH was adjusted with KOH and HCl. Excitation and emission wavelengths were 400 nm and 500 nm, respectively. Data were fitted with bell shaped curve in GraphPad Prism v6 which afforded to calculate values at inflection points (pKa1 and pKa2).](image)

**Figure 4.3.** Fluorescence titration of 15 μM PIP-THQ measured in 0.1 M KCl at 25°C. pH was adjusted with KOH and HCl. Excitation and emission wavelengths were 400 nm and 500 nm, respectively. Data were fitted with bell shaped curve in GraphPad Prism v6 which afforded to calculate values at inflection points (pKa1 and pKa2).

4.2.2 Reaction pathway leading to the formation of PIP-THQ

Considering that PIP-THQ is produced in a moderately heated aqueous reaction without obvious catalysts, the N-formylation and 2-substitution of PIP to form PIP-THQ raises an intriguing question as to the reaction mechanism involved. The UV trace from the LC-MS chromatogram of the reaction products of NFK (2 mM) and PIP (1 M) at pH 12.56 shows a large number of compounds of which some were identified (Fig. 4.4a). The most intense peaks correspond to the PIP-THQ fluorophore eluting at R_r 9.6 min. Molecular ions [M+H]^+ at m/z 305.2 and m/z 277.4 coeluting at R_r 5.7 and 5.9 min (peak area ratio 1:3.4) observed in the spectrum were tentatively assigned to PIP-NFK and PIP-KYN, respectively, whilst KYN eluted at R_r 2.8 min. KYN is a substantial product of the PIP and NFK reaction and could potentially be the intermediate in fluorophore formation. However, only m/z 277.4 and KYN were observed in the chromatogram from the reaction of KYN and PIP (Fig. 4.4b). The product in the m/z 277.4 peak was isolated and confirmed to be PIP-KYN.
(see section 2.2.4.6). The results suggest that the formyl group is necessary for fluorophore formation, and KYN or its adduct PIP-KYN appear not to be intermediates in this process.

The unsaturated amino acid KYN-CKA has been shown to form adducts with nucleophilic protein residues and glutathione (Garner et al., 2000, Yu et al., 2008), which led to the investigation of the reaction of KYN-CKA or NFK-CKA (both at 2 mM) with 1 M PIP heated at 65°C for 20 min. KYN-CKA or NFK-CKA were not present in any of the chromatograms and had to be synthesised de novo.

Figure 4.4. HPLC chromatogram of the reaction of a) NFK (2 mM) or b) KYN (2 mM) with PIP (1 M) incubated at 65°C for 20 min and immediately cooled on ice prior to analysis. The detection wavelength was 254 nm. Compound name and retention time (R_t) is indicated. The absorption spectrum of PIP-THQ is shown in the inset. Dashed line denotes the separation of PIP-KYN and PIP-NFK peaks for quantification purposes.

4.2.3 Isolation of KYN-CKA and NFK-CKA

Procedure for preparation of KYN-CKA is available, but it is quite complex requiring incubation of KYN at 70°C and pH 8.3 for 22 h, followed by chromatographic purification to provide a 30% yield (Kopylova et al., 2007). To simplify the method, KYN was incubated at higher pH to increase the reaction rate. The only major compounds detectable in a reaction of
equimolar amounts of KYN and NaOH (pH ~ 12) after 10 min incubation at 80°C were KYN and KYN-CKA. Longer incubations showed presence of impurities despite a higher yield of KYN-CKA (Fig. 4.5). Extraction of the reaction incubated for < 10 min with EtOAc in acidic aqueous solution provided very pure KYN-CKA in a comparable yield (24%) to the previous method in 30 min without any chromatographic purification. It should be noted that impurities at Rt 1.7 min and 2.4 min (Fig. 4.5) are soluble in EtOAc, and incubation for less than 10 min is necessary to avoid chromatographic separation (see this procedure in section 2.2.4.7).

![HPLC chromatogram](image.png)

**Figure 4.5.** HPLC chromatogram of reaction progress of 20 mM KYN incubated in 20 mM NaOH at 80°C. The aliquots of the reaction were chilled on ice at the designated time and immediately analysed. Chromatograms were recorded at 254 nm. The retention time of KYN, impurities, and KYN-CKA were 0.8 min, 1.7 and 2.4 min, and 3.5 min, respectively. The chromatograms at 10 min and 25 min were shifted diagonally for clarity.

Preparation of NFK-CKA has not previously been published and could not be prepared by incubation in diluted NaOH as KYN-CKA or by direct formylation of KYN-CKA due to large amounts of impurities and the low yields. NFK-CKA was successfully isolated, however, from a failed attempt to purify PIP-NFK as described in the following paragraph.

PIP-NFK and PIP-KYN co-elutes tightly together at Rt 5.9 and 6.1 min, respectively (Figure 4.6a), and various attempts (change of pH, temperature, gradient optimisation) to resolve the two compounds on a reverse phase were unsuccessful. It was noted, however, that incubation of NFK and PIP in > 80% 2-propanol suppressed formation of PIP-KYN, KYN, PIP-THQ and other products (Fig. 4.6b) typically observed in the aqueous reactions (Fig. 4.6a). Taking advantage of this phenomenon, NFK and PIP were reacted in 81% (v/v) 2-propanol for 55 min at 52°C (optimal conditions for maximal yield) and immediately neutralised by HCl to produce PIP-NFK in ~ 70% purity. Unexpectedly, fractionation of this neutralised reaction on a reverse-phase column yielded NFK-CKA as a major product.
Chapter 4- Experimentation and Discussion

(Fig. 4.7a,b). A large amount of NFK-CKA in fraction No 7 converted back to PIP-NFK after overnight storage in a freezer (Fig. 4.7c) but the ratio of products observed on the previous day was re-established after 3 h incubation at room temperature (21°C) (Fig. 4.7d). This indicated that the formation of PIP-NFK is in an unstable equilibrium with NFK-CKA. The failed attempt to obtain PIP-NFK led to a development of a procedure for isolation of NFK-CKA. Acidic extraction of the mixture in Fig. 4.7d by EtOAc and a wash in MeOH to remove the impurity in Fig. 4.7e provided 98% pure NFK-CKA (Fig. 4.7f).

Figure 4.6. HPLC chromatograms (254 nm) of the reaction of NFK (800 μM) and PIP (1 M) in a) MQW and b) 84% (v/v) 2-propanol incubated for 10 min at 65°C, immediately chiled on ice and analysed. c) HPLC chromatogram (254 nm) of the NFK-CKA dissolved in reacted with PIP (1 M) in 90% (v/v) DMSO at 21°C for 5 min. The compounds eluted faster in c) because the chromatogram was acquired at a different composition of mobile phases.

4.2.4 Reaction of KYN-CKA and NFK-CKA with PIP

The reaction of KYN-CKA and PIP showed only one dominant product, PIP-KYN, establishing that KYN-related analogues are not involved in fluorophore formation, and the hydrolysis of NFK into KYN on reaction with PIP is only a side reaction. On the other hand, the reaction of NFK-CKA (2 mM) and PIP (1 M) dissolved in MQW gave a comparable chromatogram as in Fig. 4.4a, consistent with deamination of NFK being a first step in fluorophore formation. When an aliquot of NFK-CKA dissolved in DMSO was mixed with PIP (final concentration 1 M) at 21°C, the product corresponding to PIP-NFK (MW 304 Da, absorption spectrum comparable to NFK) was formed.
rapidly at ~ 80% purity (Fig. 4.6c). This strongly indicated that the formation of PIP-NFK adduct is the second step in the fluorophore formation from PIP (Fig. 4.9). PIP-NFK could not be isolated for structural studies because it decomposed back to NFK-CKA during purification. However, it was found that PIP-NFK completely converts to PIP-KYN when heated at 60°C for 10 min at pH 1 (Fig. 4.8), lending support to the proposed structure of PIP-NFK. As expected, weakly acidic pH 4 was not sufficient to hydrolyse the formyl group of PIP-NFK but induced PIP-NFK degradation (Fig. 4.8).

**Figure 4.7.** Reversible conversion of PIP-NFK to NFK-CKA during purification. a) Fractionation of a neutralised reaction of PIP and NFK on a C18 reverse-phase column in an ammonium bicarbonate-buffered mobile phase (pH 7, MeCN/MQW). Note this reaction afforded ~70% pure PIP-NFK before purification with only a small contamination of NFK-CKA. Peak areas were quantified from 325 nm chromatograms. HPLC chromatogram (254 nm) of fraction 7 b) immediately after purification, c) after 19 h incubation at -18°C, d) after additional 3 h incubation at 21°C, e) extracted with acidified EtOAc (pH 2) and f) pellet of EtOAc extract after MeOH wash.
Figure 4.8. Decomposition of PIP-NFK into PIP-KYN after treatment with HCl. HPLC chromatogram at 254 nm of a) the reaction of NFK and PIP previously neutralised by HCl, evaporated and resuspended in MQW, b) the same reaction acidified to pH 4 and 21°C for 120 min and c) the reaction b) acidified to pH 1 and incubated for another 10 min at 60°C. The figure is a cut from the complete chromatogram. Compounds corresponding to the peaks are indicated by arrows. Please refer to Fig. 4.9 for their chemical structures.

No reaction products were observed when TRP was heated with PIP at 65°C for 20 min (Fig. A.11c), indicating that the γ-keto group on the amino acid chain of KYN and NFK is critical for the addition of the amine. Moreover, formanilides lacking the amino acid side chain, do not undergo any reaction other than hydrolysis when incubated with PIP (Fig. A.11a,b). This demonstrates that the interaction of the amino acid side chain with the formanilide group is essential for fluorophore formation, and neither of the groups alone produces any relevant intermediate. The specificity of the reaction of NFK and PIP is remarkable. In the 2 years of testing a wide range of compounds using the PIP fluorescence assay, no other molecule has been identified that could form fluorophores with PIP. Although compounds showed high fluorescence in the assay mixture when incubated with PIP, they also fluoresced in diluted NaOH solution, but not in potassium phosphate buffer (pH 6.5). This demonstrated that these compounds were not converted into a fluorophore in the reaction with PIP (data not shown).

Greater than 80% 2-propanol or DMSO in the reaction of NFK and PIP or NFK-CKA and PIP suppressed the formation of PIP-THQ and PIP-KYN but not the PIP-NFK intermediate (Fig. 4.6b,c). Considering that the conversion of PIP-NFK to PIP-KYN is a hydrolysis reaction, the speed of which is dependent on water availability; there is a possibility that the formation of PIP-THQ fluorophore may also involve hydrolysis. The possibility of the involvement of organic solvents affecting the pH being involved in the conversion to PIP-THQ cannot be excluded, however.
Figure 4.9. Proposed pathway of NFK and KYN reactions with PIP. Note that PIP-KYN does not undergo any further modification when incubated with PIP. The percent yield of a purified solid material is indicated in parentheses next to the compound name.
4.2.5 Rate of formation and consumption of PIP-NFK, PIP-KYN and PIP-THQ

The formation of PIP-NFK is very fast compared to that of PIP-KYN (Fig. 4.10). When NFK (2 mM) was incubated with PIP (1 M) in MQW at 65°C, the maximal amount of PIP-NFK was formed after 3 min and then decreased exponentially (Fig. 4.10a). In contrast, a 30 min incubation of KYN (5 mM) and PIP (1 M) at 80°C is necessary to reach maximal formation of PIP-KYN (Fig. 4.10b). Rates of PIP-KYN formation ($k_{obs} = 8.2 \times 10^{-2} \text{min}^{-1}$) and PIP-NFK consumption ($k_{obs} = 4.5 \times 10^{-2} \text{min}^{-1}$) are comparable. As expected, KYN decomposes at a 10-fold slower rate ($k_{obs} = 2.9 \times 10^{-2} \text{min}^{-1}$) than NFK ($k_{obs} = 3.4 \times 10^{-1} \text{min}^{-1}$) when incubated with PIP, consistent with the higher reactivity of NFK to KYN. While PIP-NFK decreases at a nearly linear rate, PIP-THQ forms exponentially ($k_{obs} = 1.3 \times 10^{-1} \text{min}^{-1}$) and reaches a plateau before PIP-NFK is depleted. This suggests two things. Firstly, PIP-THQ is decomposing as the reaction progresses. Secondly, PIP-THQ is not formed directly from PIP-NFK. The missing step is likely a rearrangement and transamidation (see Fig. 4.9).

Figure 4.10. Reaction progress of a) 2 mM NFK and 1 M PIP at 65°C and b) 5 mM KYN and 1 M PIP at 80°C. Each time point aliquot was analysed by HPLC and peak areas of corresponding compounds were quantified from 400 nm (PIP-THQ), 360 nm (PIP-KYN and KYN), and 325 nm (PIP-NFK and NFK) chromatograms. Data points were fitted with one-phase exponential curves. Data point of PIP-KYN at 40 min in panel b) was not included in the fit.

4.2.6 Effect of PIP and NFK concentration on the formation of fluorophore PIP-THQ

The effect of varying concentrations of PIP on the fluorophore formation was investigated. When a fixed concentration of PIP (1 M) was incubated with different concentrations of NFK (0.5 – 9 mM), PIP-THQ formation was initially nonlinear in relationship to NFK concentration and reached a plateau at 9 mM NFK (Fig. 4.11a).
the concentration of NFK was kept constant (2 mM), and reacted with varying concentrations of PIP (0.4, 1 and 3 M) (Fig. 4.11b), PIP-THQ formation at both 3 M and 0.4 M PIP was approximately half of that formed at 1 M PIP. The large molar excess (~ 500-fold) of PIP to NFK is necessary for efficient fluorophore formation; however, the concentration of PIP higher than 1 M appear to decrease PIP-THQ formation, possibly due to decomposition induced by a high pH.

In contrast, the reaction of KYN and PIP does not require a high molar excess of PIP. When KYN (20 mM) was heated at 80°C for 10 min with either a 50-fold (1 M) or 10-fold (0.2 M) excess of PIP, the yield of PIP-KYN by HPLC was comparable (data not shown).

**Figure 4.11.** Formation of PIP-THQ in the reaction of a) variable NFK and 1 M PIP and b) 2 mM NFK and variable PIP. Reactions were initiated by addition of PIP into an aqueous solution of NFK, incubated at 65°C for 20 min, chilled on ice and analysed on HPLC. Peak area corresponding to PIP-THQ was quantified from 400 nm chromatogram.

### 4.2.7 Formation of fluorophores from amines other than PIP

#### 4.2.7.1 Cyclic amines

Fluorophore formation from NFK and 5- or 7-membered cyclic amines was compared to that of the 6-membered ring PIP. Fluorophore formation with 5-membered ring pyrrolidine (PYR), or with 7-membered ring azepane (AZP), were decreased 12% and 10% respectively, compared to PIP (Table 4.1). The mass and Rₜ of the fluorophores AZP-THQ (MW 316, Rₜ 10.7 min) and PYR-THQ (MW 288, Rₜ 8 min) were as expected in relation to the mass of the reacted amine ring (Table 4.1). The fragmentation pattern of PYR-THQ resembled that of PIP-THQ indicating that the amine ring was similarly formylated and connected (Fig. A.12a). This showed that the fluorophore formation is not significantly affected by the size of the amine ring.
Table 4.1. Molecular weights (MW), retention times (Rt) and amount of amine-KYN, amine-NFK, amine-THQ adducts formed in reaction of amines with NFK (red font), and the amine-KYN adduct formed in reaction of amines with KYN (blue font). (See footnotes on page 104.)

<table>
<thead>
<tr>
<th>amine</th>
<th>amine-KYN</th>
<th>amine-NFK</th>
<th>amine-THQ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MW (Da)</td>
<td>Rt (min)</td>
<td>peak area (%)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NFK rxn&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>PIP</td>
<td>276</td>
<td>5.9</td>
<td>100*</td>
</tr>
<tr>
<td>NMePIP</td>
<td>276</td>
<td>5.9</td>
<td>16</td>
</tr>
<tr>
<td>2-MePIP</td>
<td>290</td>
<td>6.3</td>
<td>0</td>
</tr>
<tr>
<td>3-MePIP</td>
<td>290</td>
<td>7.1</td>
<td>94</td>
</tr>
<tr>
<td>4-MePIP</td>
<td>290</td>
<td>7.1</td>
<td>114</td>
</tr>
<tr>
<td>DMP&lt;sup&gt;e&lt;/sup&gt;</td>
<td>290</td>
<td>7.1</td>
<td>71</td>
</tr>
<tr>
<td>TMP (40% 2-propanol)</td>
<td>N/O</td>
<td>0</td>
<td>N/T</td>
</tr>
<tr>
<td>PYR</td>
<td>262</td>
<td>4.9</td>
<td>190*</td>
</tr>
<tr>
<td>NMePYR</td>
<td>N/O</td>
<td>0</td>
<td>N/T</td>
</tr>
<tr>
<td>AZP</td>
<td>N/O</td>
<td>0</td>
<td>N/T</td>
</tr>
<tr>
<td>DEA</td>
<td>236</td>
<td>4.1</td>
<td>0</td>
</tr>
<tr>
<td>DPA (40% 2-propanol)</td>
<td>250</td>
<td>5.2</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 4.1 footnotes (See table on page 103.)

N/O = not observed; N/T = not tested. Molecular weights were determined by presence of [M+H]+ and [M-H]- ions in mass spectra of respective compounds at given retention time (Rt). Reactions of TMP and DPA were performed in 40% 2-propanol due to their poor water solubility.

a PYR-KYN and PYR-NFK adducts coeluted in a single peak, and the value represents their combined peak areas
b peak areas are expressed relative to PIP reactions (100%) in each respective column. The percent value in parentheses denotes the yield of purified solid relative to PIP-THQ.
c these two columns show peak areas of amine-KYN adducts observed in reactions of NFK (NFK rxn) and KYN (KYN rxn) with amines
d The fluorescence emission maximum at 400 nm excitation acquired during HPLC analysis
e The products in the reaction of DMP with amino acids are likely formed from impurities in the DMP reagent
* asterisk denotes that the compound was purified and isolated

4.2.7.2 Monomethyl-substituted PIP and acyclic amines

Fluorophore formation from the reaction of NFK with isomeric methyl substituted PIPs was investigated (Table 4.1). When the methyl-substituent was located on the nitrogen of PIP (NMePIP), the mass and Rt of the formed fluorophore (NMePIP-THQ) was identical to that of PIP-THQ (MW 302, Rt 9.5 min), but 2.5-fold lower yields were obtained. A similar pattern was also observed comparing fluorophore formation between PYR and NMePYR. This indicated that the N-methyl group of the tertiary amine had to undergo demethylation. Much lower yields of fluorophore were obtained using 2-methylpiperidine (2-MePIP), cis-2,6-dimethylpiperidine (DMP) and 2,2,6,6-tetramethylpiperidine (TMP) (4%, 3% and 0%, respectively, compared to PIP). Furthermore, the mass of the fluorophore DMP-THQ (MW 316) formed from DMP was identical to fluorophores formed from monomethylated amines (3-MePIP-THQ and 4-MePIP-THQ) (Table 4.1). DMP forms both amine-KYN and amine-NFK adducts with slight decreases in efficiency compared to PIP (by 29% and 36% respectively). This suggested that the commercial DMP used in the study (Sigma-Aldrich, 98%) might have been contaminated with isomers of monomethyl PIP. Indeed, comparing 13C NMR spectra of DMP, 2-Me, 3-Me and 4-Me PIP, showed that DMP contains about 1% of 3-MePIP but other impurities were also present. In section 4.2.6, it was shown that formation of PIP-KYN adducts does not require a high molar excess of PIP, in contrast to the formation of fluorophores. This strongly indicates that the adducts observed in the reaction of DMP and NFK are 3-MePIP adducts and that DMP itself is only marginally reactive. It also
Chapter 4- Experimentation and Discussion

corroborates the requirement for a high molar excess of PIP (and most likely also other amines) to NFK for the fluorophore formation.

Formation of the amine-THQ fluorophore from 3-MePIP and 4-MePIP was increased 1.84-fold and 1.38-fold respectively compared to PIP. Acyclic amines diethylamine (DEA) and dipropylamine (DPA) formed THQ products in much lower quantities than with PIP. The effect of methyl substituents of PIP on fluorophore formation is schematically shown in Figure 4.12.

The fluorescence emission maximum of PIP-THQ and the other amine-THQ fluorophores are comparable (Table 4.1), indicating that the structure of amine has a negligible effect on electronic properties of the fluorophores. 3-MePIP-THQ was purified and mass fragmentation (Fig. A.12b) and NMR studies (Figs. A.13 – A.15) confirmed it was indeed an analogue of PIP-THQ, differing only in the attached amine. Moreover, comparable extinction coefficients of 3-MePIP-THQ ($\varepsilon_{402} = 2431$ M$^{-1}$cm$^{-1}$) and PIP-THQ ($\varepsilon_{400} = 2136$ M$^{-1}$cm$^{-1}$) strongly indicates that the yields in Table 4.1 obtained from peak areas of 400 nm chromatograms are genuine, and not a result of different extinction coefficients.

Figure 4.12. Schematic illustration of the effect of PIP methyl substituents on the formation of fluorophores.
Chapter 4- Experimentation and Discussion

4.2.8 Reactions of amines with KYN

In a final aspect of our investigations, reactions of amines with KYN, an NFK analogue without the formamide group, were investigated. Chromatograms of the reaction of KYN with amines showed only unreacted KYN and amine-KYN adduct(s) (Table 4.1). NMePIP-KYN and DMP-KYN adducts also showed a loss of the methyl substituent, similar to their respective fluorophores NMePIP-THQ and DMP-THQ formed from NFK (Table 4.1). 4-MePIP did not significantly increase the yield of amine-KYN adduct relative to PIP. This contrasts with the increased yields of amine-NFK and amine-THQ that were obtained in the reaction of NFK with 4-MePIP and 3-MePIP compared to PIP. Thus, a 4-methyl substituent on the PIP skeleton is favoured during fluorophore formation but has a negligible effect on amine-KYN adduct formation.

Acyclic secondary amines DEA and DPA formed DEA-KYN and DPA-KYN adducts with 86% and 39% efficiency, respectively, compared to PIP-KYN. This again contrasts with NFK reactions where the acyclic amines did not form any adducts apart from small amounts of fluorophore amine-THQ. Moreover, acyclic amines DPA and DEA, produced two different KYN adducts; the more abundant adduct having apparently lost one of the alkyl chains, whilst the lower yielding adduct did not. In contrast, reactions of cyclic amines produced only a single amine-KYN adduct with the exception of 2-MePIP that also formed two products but with identical mass (MW 290, Table 4.1). The two 2-MePIP-KYN adducts are probably diastereomers.

4.2.9 Summary of reactions of amines with NFK

1) Cyclic amines form fluorophores more efficiently than acyclic amines of comparable size and basicity. This suggests that the rearrangement and transamidation step requires a spatial arrangement, not in favour of acyclic structures.

2) Only cyclic amines without substitutions at both carbons adjacent to nitrogen (2- and 6-position on PIP ring) appear to be able to form fluorophores efficiently. One position next to nitrogen atom is required for bond formation during the rearrangement step, but why both positions next to nitrogen need to be unsubstituted is not yet clear. As suggested in point 1), a methyl group adjacent to nitrogen might sterically hinder the interaction of the formyl group with the amine nitrogen.

3) Fluorophore formation is enhanced with 3- and 4-methyl substituents on PIP.
In chapter three, the development of a novel sensitive fluorescence assay for detection of rhIDO1 enzymatic activity and its adaptation for use with a robotic workstation has been described. The fluorescence PIP assay was subsequently used to screen NCI Diversity Set III (1,597 compounds) for their rhIDO1 inhibitory activity. This chapter focuses on analysis and characterisation of the hits obtained from the screening of the NCI library and 40,000 compounds from The Walter and Eliza Hall Institute of Medical Research (WEHI; Victoria, Australia) library.

5.1 Introduction

There is much interest in the identification of IDO1 inhibitors due to their ability to reverse tumour-mediated immune suppression and to potentiate the efficacy of other cancer therapies including anti-cancer vaccines, antibody blockades to immune-checkpoint proteins CTLA-4 and PD-1 (Spranger et al., 2014, Wainwright et al., 2014), and chemo- and radio-therapies (Hou et al., 2007, Li et al., 2014). A number of small-molecule IDO1 inhibitors has been identified to date from natural products, and from high-throughput screenings of compound libraries (reviewed e.g. in Dolušić & Frédérick, 2013 and Pucchio et al., 2010). Some of the most potent IDO1 inhibitors include hydroxyamidines (Liu et al., 2010), triazoles (Boyall et al., 2014, Röhrig et al., 2012) or imidazothiazoles (Tojo et al., 2014), which exhibit rhIDO1 inhibitory activities between 10-100 nM. Two IDO1 inhibitors are currently in human clinical trials; hydroxyamine INCB024360 (Liu et al., 2010) developed by Incyte Corp., and imidazole NLG919 (Mautino et al., 2013) produced by NewLink Genetics.

Although their most promising hits were not disclosed, a number of high-throughput screens of compound libraries have been carried out recently to identify novel IDO1 inhibitors. The ChemBridge library of 50,000 compounds was screened in a yeast-based assay and provided 101 hits (0.2% hit rate). Of those, 76% were confirmed in mammalian cells and 84% displayed safe toxicity profiles (Cerejo et al., 2012). The Amgen library of 245,000 compounds was screened using a fluorescence assay and 3439 hits (1.4%) were identified. The most potent compound disclosed, Amg-1, exhibited an IC_{50} = 3 μM against the isolated enzyme (Meininger et al., 2011). A proprietary library of 87,000 compounds
Chapter 5 - Introduction

(Specs, Delft, The Netherlands) was screened using a fluorescence assay and identified 174 hits (0.2%) (Seegers et al., 2014). Cheng and colleagues screened a library compiled of Chembridge Co., ChemDiv Inc. and Enamine Ltd. compounds and found a class of potent sulfonylhydrazides exhibiting IC_{50} < 100 \text{nM} against the isolated enzyme (Cheng et al., 2014). Prestwick Chemical Library (1,200 FDA-approved drugs) and Maybridge Hitfinder Collection (14,000 chemicals) were screened using an absorbance assay and provided 36 hits (3%) and 186 hits (1.3%), respectively. The majority of these hits inhibited IDO1 by a nonspecific mechanism and only 4 out of 60 of the tested compounds were active in cell-based assays (Röhrig et al., 2014).

Both the experimental and virtual screening hit lists are known to include compounds that are unsuitable for drug development (Baell & Holloway, 2010, McGovern et al., 2002). These undruggable hits inhibit target enzymes by the formation of aggregates, nonspecific chemical modifications or protein oxidation (Dahlin & Walters, 2014, McGovern et al., 2002, Rishton, 1997, Röhrig et al., 2014). Early triaging of hits unsuitable for development into a therapeutic drug would reduce the time and effort that would be wasted (Baell & Holloway, 2010, Dahlin & Walters, 2014).

IDO1 inhibitors that inactivate enzyme by chemical modification have been described. Röhrig and coworkers identified compounds that were chemically reactive to IDO1 in the analysis of their IDO1 screening hits (Röhrig et al., 2014). Terentis and colleagues showed that ebselen inhibits IDO1 by covalent modification of cysteine residues (Terentis et al., 2009), and phenylhydrazine could inactivate IDO1 by chemical modification of the haem (Fung et al., 2013) similar to that shown with catalase (Ortiz de Montellano & Kerr, 1983). All in all, IDO1 appears to be particularly vulnerable to inactivation by chemical modification by reactive compounds.

Aggregators were identified amongst many approved drugs (Seidler et al., 2003), lead candidates (mainly kinases) (McGovern & Shoichet, 2003) and screening hits (McGovern et al., 2002), but there are no reports on aggregating IDO1 inhibitors. The aggregators inhibit enzymes by forming large oligomers (~10^4 monomers) similar to micelles of surfactant that can inhibit a broad range of unrelated enzymes (McGovern et al., 2002). Aggregating inhibitors have several hallmarks. Their potency decreases in the presence of increasing enzyme concentration and non-ionic detergents; (McGovern et al., 2002, Ryan et al., 2003) and their concentration-response curves show steep slopes > 1 (Shoichet, 2006). These properties can be used to experimentally identify aggregators, given that in silico prediction of aggregation is unreliable (Röhrig et al., 2014, Seidler et al., 2003).
This chapter presents studies focused on analysis and characterisation of the hits for IDO1 inhibition obtained from the screening of the NCI and WEHI libraries. Thirty-five initial hits from the NCI library were analysed by structural filters and 5 shortlisted compounds were tested for their structural integrity, aggregation, chemical reactivity and ability to inhibit IDO1 in cells. The mechanism of inhibition of selected IDO1 inhibitors was investigated, and a small structure-activity relationship of validated NCI hits was performed to assess their suitability for drug development programme.

5.2 Experimentation and Discussion

5.2.1 NCI library screening

The screening of the NCI library performed in Chapter 3 identified 35 compounds (2.2% hit rate) which inhibited rhIDO1 activity > 50% at 20 μM (Fig. 5.1). The positive controls that were included, HA and NQ both inhibited rhIDO1 enzymatic activity > 90%, whilst 4PI inhibited rhIDO1 activity 45% at 20 μM, in agreement with its IC$_{50}$ value of 35.6 μM determined in Chapter 3 (see Fig. 3.9).

![Figure 5.1](image_url)  
**Figure 5.1.** IDO1 inhibitory activity of NCI library compounds (n = 1600) at 20 μM in 0.2% DMSO. This screening also included three known IDO1 inhibitors HA, 4PI and NQ (see their structures in Fig. 3.9a). Inhibitory activity was normalised to IDO1 treated with 0.2% (v/v) DMSO. Compounds were classified into three categories based on their % IDO1 inhibition values: inactives/interferences (< 20%), weak inhibitors (20-50%), and hits (> 50%). The number of compounds (n) in each category and a fraction of the whole NCI library (%) is indicated below the category labels.
Fifty-three compounds were classified as weak inhibitors and 1512 as inactive (Fig. 5.1). The inhibitory activity of 18 compounds that interfered with the fluorescence assay readout was re-determined using the TCA absorbance assay. All 18 compounds showed < 50% inhibition of rhIDO1 and were not classified as hits. Thirteen of the 35 hits (37%) are para-quinones and include anticancer natural products mitomycin C (NSC26980), porfiromycin (NSC56410), streptonigrin (NSC45383 and NSC26980) and streptonigrin methyl ester (NSC45384). Other hits are antibiotics streptovaricin C (NSC19990) and a nibomycin derivative (NSC275428), two arsenic chemotherapeutic agents (NSC12646 and NSC13785), two nitroquinolinols (NSC57103 and NSC92207), benzothiazole (NSC503425), two diazenyl compounds (NSC134199, NSC338106), thiadiazine (NSC163639), thiourea (NSC201634), as well as the 5 compounds listed in Table 5.1 that were selected for follow-up studies. Please refer to Appendix Table A.1 for a complete list of the hits and their structural analysis.

Quinones (Brastianos et al.,2006, Carr et al.,2008, Pereira et al.,2006), benzothiazoles (Röhrig et al.,2010), quinolines (Röhrig et al.,2010) and thioureas (Matsuno et al.,2010, Serra et al.,2014) have previously been reported as IDO1 inhibitors. As discussed in the literature, quinones are most likely not pharmacological inhibitors of IDO1 but rather interfere with the reductive activation of IDO1 by MB/AA which is used in most IDO1 assays (Pearson et al.,2010). In addition, quinones react with biological macromolecules (Bittner,2006, Chang et al.,1992), deplete intracellular GSH, and generate oxidative stress (Chang et al.,1992). This all suggests that quinones are unlikely candidates for successful drug development (Baell & Holloway,2010).

Two earlier NCI Diversity Set libraries were screened for inhibition of IDO1 activity and provided 16 IDO1 inhibitors (Pantouris & Mowat,2014, Vottero et al.,2006). Of these, para-quinone mitomycin C is also identified as a hit in our study. However, NSC201863 (benzamide) identified in a yeast-based assay (EC$_{50} = 2$ μM) (Vottero et al.,2006) was inactive (8% inhibition) in our PIP fluorescence assay. Of note, the 14 other IDO1 inhibitors present in the earlier libraries were not included in the NCI Diversity Set III library used in the current study.
Table 5.1. Inhibitory activities and structural analysis of selected IDO1 inhibitors.

<table>
<thead>
<tr>
<th>ID</th>
<th>Structure</th>
<th>Primary screen</th>
<th>Confirmation (re-sourced compounds)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>enzyme assay</td>
<td>IC$_{50}$</td>
<td>enzyme assay</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GSH + Tw20</td>
<td></td>
</tr>
<tr>
<td>NSCa 89349 (1)</td>
<td>3.2 Alarm NMR</td>
<td>2.4 ± 0.8</td>
<td>3.4 ± 0.1</td>
</tr>
<tr>
<td>NSCa 332670 (2)</td>
<td>4.2 Alarm NMR, 40/617 Blake</td>
<td>7.8 ± 0.2</td>
<td>3.3 ± 0.1</td>
</tr>
<tr>
<td>NSCa 228150 (3)</td>
<td>5.1 Alarm NMR, 41/267 Blake</td>
<td>7.6 ± 2.9</td>
<td>6.6 ± 2.5</td>
</tr>
<tr>
<td>NSCa 44556 (4)</td>
<td>6.1 Alarm NMR</td>
<td>4.3 ± 0.2</td>
<td>6.5 ± 0.2</td>
</tr>
<tr>
<td>NSCa 330796 (5)</td>
<td>3.2 Alarm NMR, Blake, PAINS</td>
<td>9.3 ± 2.9</td>
<td>4.1 ± 0.8</td>
</tr>
<tr>
<td>WEHI 1 (6)</td>
<td>1.5 Alarm NMR, Blake</td>
<td>N/A</td>
<td>3.2 ± 1.7</td>
</tr>
<tr>
<td>WEHI 2 (7)</td>
<td>3.1 Alarm NMR, Blake</td>
<td>N/A</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>WEHI 3 (8)</td>
<td>5.0 Blake</td>
<td>N/A</td>
<td>1.8 ± 0.0</td>
</tr>
<tr>
<td>control 4PI</td>
<td>- no alert</td>
<td>17/44</td>
<td>31.0 ± 8.1</td>
</tr>
</tbody>
</table>

*identifier of National Cancer Institute compounds. IC$_{50}$ values are in µM. Alerts shows the structural filters (Alarm NMR, Blake, Glaxo, PAINS) the compound has failed. BioAssays displays a number of active / total biological test results deposited in PubChem database. Enzyme assay was performed at standard conditions with or without GSH (5 mM reduced glutathione) or Tw20 (0.01% (v/v) Tween-20). Data are shown as a mean ± SD of at least 2 independent experiments. LC$_{50}$ (concentration of the compound that decreases viability of the cells by 50%).
5.2.1.1 Structural analyses

To eliminate inhibitors that are unsuitable for drug development, four publicly available structural filters were applied to the list of hits. AlarmNMR (Huth et al., 2004) and Blake (Blake, 2005) filters are designed to detect reactive functional groups. In addition, the Blake filter also scans for mutagenic or carcinogenic compounds. The Glaxo filter (Hann et al., 1999) aims to detect unsuitable leads, natural products and reactive chemicals. PAINS is the most recent filter to be included in filtering out unsuitable compounds for drug development (Baell & Holloway, 2010). In addition, the hits were filtered by molecular weight > 500 Da (MW>500) and by their biological activities listed in a PubChem BioAssay database (BioAssays filter) (https://pubchem.ncbi.nlm.nih.gov/assay), a repository of close to 2 million molecules and their screening results against a wide range of enzymes (Wang et al., 2014). Please see materials and methods section 2.1.10 for details of these computational analyses.

The majority of the hits (30 out of 35) failed at least 3 filters and only 5 hits failed 1 or 2 filters. None of the hits passed all the filters (Fig. 5.2b). The structural filters AlarmNMR and Blake, detecting reactive molecules, and the BioAssays filter flagged on average 29 out of 35 the hits. PAINS, Glaxo and MW>500 failed a lower proportion of the compounds (ranging from 6 – 14) (Fig. 5.2a). The results from the use of the filters indicate a large number of the hits may be chemically-reactive or non-specific inhibitors that are active against a wide range of enzymes.

![Figure 5.2](image_url)

**Figure 5.2.** A number of NCI library hits that failed (a) individual computational filters and (b) the indicated number (0-6) of filters used in a).
Chapter 5- Experimentation and Discussion

AlarmNMR and Blake collectively flagged all 35 hits making a selection according to these two filters not practical. On the other hand, Glaxo, MW>500 and BioAssays filters identified most of the quinones and unsuitable natural products; and could be useful filters to apply for selection of suitable inhibitors. However, the BioAssays filter based on a real experimental data is perhaps the most valuable and most relevant to use.

The hits that were prioritised for further study were selected based on their novelty, successful pass of the Bioassays filter, chemical accessibility, absence of the quinone moiety, and a pass of the Glaxo, MW>500, PAINS, Blake and AlarmNMR filter in a descending order of priority. This selection process yielded 5 compounds (1-5) listed in Table 5.1.

Interestingly, hydroxyamidine HA from the clinical candidate INCB024360 chemical class (Liu et al., 2010) failed 3 out of 4 structural filters. Imidazoles 4PI and the clinical candidate NLG919 did not fail any of the structural filters, although 4PI was active in 17 out of 44 biological test results deposited on PubChem.

5.2.1.2 Validation of chemical structure and inhibitory activity of NCI hits 1-5

Re-testing of the compounds 1-5 from the original NCI-plated solutions gave IC_{50} 3.2 – 8 μM, which is consistent with the inhibitory activity being > 50% at 20 μM in the initial screening. Powders of compounds 1-5 were obtained from NCI and were freshly prepared and assayed. The IC_{50} values obtained for the freshly prepared solutions were consistent with the previous determinations (Table 5.1). The chemical structures of compounds 1-5 were all confirmed using MS and 1H-NMR analyses (data not shown). Inhibitors 1-5 were next investigated for non-specific mechanisms of IDO1 inhibition that might potentially reduce their suitability as leads for drug development.

5.2.1.3 Reactive compounds

All 5 hits were flagged by either the AlarmNMR or Blake filter, or both, suggesting that they might inhibit IDO1 by chemical modification. The strong nucleophilic trapping agent, reduced glutathione (GSH), was used (Foti et al., 2011, Röhrig et al., 2014) to detect potential electrophiles. Compounds 1-5 were pre-incubated with 5 mM GSH (~ 10^6 molar excess of rhIDO1) which has previously been shown to attenuate the activity of chemically reactive IDO1 inhibitors (Röhrig et al., 2014, Whitehouse & Ghosh, 1968). The GSH solution was prepared fresh for each experiment and 1H-NMR was used to confirm its reduced form. GSH had no effect on the activity of rhIDO1 alone, or the inhibitory activity of the negative control.
Chapter 5- Experimentation and Discussion

4PI (Table 5.1). The potency of compounds 1-5 in the presence of GSH and Tween-20 did not decrease but increased 8.5, 6.5 and 2.8-fold for 1, 4 and 5, respectively (Table 5.1).

Kumar and colleagues found that glutathione conjugates of menadione (2-methyl-1,4-naphthoquinone) exhibited 1.3-fold (quinone form) and 3.2-fold (hydroquinone form) higher rhIDO1 inhibitory activity than the free menadione (Kumar et al..2008b). Also, the N-oxide functional group was reported to react with the glutathiol radical (GS*) (Polovyanenko et al.,2008). These reports suggest that the compounds 1, 4 and 5 might form more potent inhibitors upon reaction with GSH. It is conceivable that glutathione in these conjugates will interact with the amino acid residues at the entrance of the IDO1 active site, whilst the inhibitors themselves will provide the interaction with haem. Glutathione conjugation could therefore provide a useful approach for increasing the selectivity and potency of IDO1 inhibitors. In summary, inhibitors 1-5 have been found not to chemically modify rhIDO1 in a GSH-sensitive reaction. However, GSH is known to react poorly with strong electrophiles (Ketterer et al.,1983), and an analogue of compound 3, 7-nitro-4-(phenylthio)benzofurazan has been shown to be a strong electrophile (Patridge et al.,2012). Further work is needed to establish if GSH-conjugates are indeed formed with the hits in this study.

5.2.1.4 Aggregators

Hits 1-5 were titrated and assayed for inhibition of rhIDO1 activity with or without 0.01% (v/v) detergent Tween 20. The titration curves of 1-5 did not display Hill slopes > 1 with or without Tween 20, and their potency did not significantly decrease in the absence of the detergent (Table 5.1). The potency of compounds 2 and 5 was increased when assayed with the detergent, which is likely to due to their enhanced solubilisation mediated by the detergent. The IC$_{50}$ of the negative control 4PI was largely unaffected by the detergent (Table 5.1). These results indicated that compounds 1-5 do not inhibit rhIDO1 by aggregation in an isolated enzyme assay.

5.2.1.5 Cell-based assay

Since IDO1 is an intracellular enzyme (Kudo & Boyd,2000), the IDO1 inhibitory activity of 1-5 was assessed in using an engineered Lewis Lung carcinoma cell line (LLTC) that was constitutively expressing human IDO1 (LLTC-hIDO1). This experiment tests not only the IDO1 inhibitory activity of the compound but also its cellular permeability and potential toxicity to the cells.
Chapter 5- Experimentation and Discussion

Compounds 1, 4 and 5 showed good cell penetration and inhibited hIDO1 activity in cells with a similar potency as that measured against the isolated recombinant enzyme (Table 5.1). In contrast, the potency of inhibitors 2 and 3 decreased 20- and 3-fold, respectively, in the cell-based assay. Moreover, the titration curve of 2 showed a steep slope (Hill = 3.8) that suggested inhibition by non-specific mechanisms in the cell-based assay that was not observed in the isolated enzyme assay (Fig. A.19). One possible explanation is that the cell growth medium promotes the aggregation of 2 and the decreased concentration of the active monomer results in a concomitant reduction in inhibitory activity. In addition, as suggested by McGovern and colleagues, excess protein in the growth medium could neutralise the activity of some of the aggregates, resulting in a decrease in potency (McGovern et al., 2002). In a similar vein, the excess molar concentration of catalase in the enzyme assay might absorb formed aggregates and protect rhIDO1 from their damage (McGovern et al., 2002).

Compounds 1-5 did not reach LC50 even at the highest tested concentration, although the viability curves showed a decreasing trend at the highest inhibitor concentrations (Fig. A.19). This was most apparent with compound 3 which decreased cell viability of cells by 34% at 125 μM (6.4-fold of its IC50 in the cell-based assay). Cellular toxicity of an analogue of 3, 7-nitro-4-(phenylthio)benzofurazan was attributed to a production of reactive oxygen species (Patridge et al., 2012). Hits 1 and 4 were the least toxic to the LLTC cells (Table 5.1; Fig. A.19).

5.2.2 Confirmation of hits from the WEHI library

The ACSRC contracted the screening of 40,000 structurally-diverse, drug-like compounds from the WEHI library as part of our efforts to identify novel IDO1 inhibitors suitable for development into therapeutic agents. This HTS identified 191 hits (0.5 % hit rate). Twenty-eight compounds were selected and retested for IDO1 inhibitory activity using freshly-prepared solutions in both the PIP fluorescence assay and the LLTC-hIDO1 cell-based assay (Fig. 5.3). The correlation between the IC50 measured using the PIP fluorescence assay with those determined by WEHI - using the suboptimal fluorescence-based quantification of NFK - was low (Pearson correlation coefficient = 0.47, P = 0.012) (Fig. 5.3a), with 71% of the compounds showing a higher potency in the WEHI assay than in the PIP assay. Bland-Altman method of comparison showed high 5.32-fold mean difference (bias) between the results obtained by the two methods (95% limits of agreement were between -7.9 to 18.6), suggestive of systematic differences between the results produced by the two methods.
Figure 5.3. (See legend on page 117.)
Chapter 5- Experimentation and Discussion

Figure 5.3. Confirmation of 28 hits identified from WEHI high-throughput screening. Comparison of the WEHI enzyme assay results with the ACSRC a) enzyme assay and b) cell-based assay. c) Bland-Altman analysis of a) with indicated bias (dashed line) and 95% limits of agreement (dotted lines). Open circles in c) indicate that IC$_{50}$ in the cell-based assay was not reached and could not be extrapolated from the concentration-response curve because the inhibition at a maximal tested concentration (50 μM) was < 30%. Abbreviations: r (Pearson correlation coefficient), P (statistical significance).

Furthermore, the IC$_{50}$ of 4PI determined in the WEHI assay was 259 μM and differs to the IC$_{50}$ between 35-40 μM determined in our assay (see Fig. 3.9) and by others (Kumar et al., 2008a). Since the IC$_{50}$ for the 4PI measured using the PIP assay is more in line to those in the literature, we suggest that our assay provides a more accurate and reliable determination of IDO1 enzymatic activity.

The IC$_{50}$ of the 28 WEHI hits were subsequently determined in a cell-based assay and compared with results from the PIP fluorescence enzyme assay. It was found that 61% of the hits had good cell permeability and four inhibitors showed 12-, 16-, 27- and 33-fold higher potency in the cell-based assay compared with the isolated enzyme assay. One of these, WEHI-3 (8), is also one of the most potent inhibitors. WEHI-3 (8), together with WEHI-1 (6) and WEHI-2 (7) were taken forward for our drug development programme (Table 5.1, Fig. 5.3b). These three hits all showed good cell penetration and did not decrease cell viability at concentrations that inhibited the enzyme (see Fig A.19). Compounds 6-8 had no records in the PubChem database, but were detected by chemical reactivity filters AlarmNMR and Blake suggesting they may chemically modify IDO1.

The basis for the dramatic 27-fold higher potency of WEHI-3 (8) in the cell-based assay compared to the isolated enzyme is not clear, but such observations are not unique or unprecedented. Three other structurally distinct WEHI hits also showed between 12 to 33-fold higher potency in the cell-based assay (Fig. 5.3b). Furthermore, 4-phenyl-1,2,3-triazole in study by Röhrig and colleagues exhibited 10-fold higher potency against human IDO1 in cells (Röhrig et al., 2012). Further studies are necessary, but one possible explanation for the increased potency in cells is that these compounds are actively transported and sequestered in cells.

5.2.3 Mechanism of IDO1 inhibition

To understand how each of the three hits interact with IDO1, competition with l-TRP for the enzyme active site was investigated by measuring initial velocity of NFK formation. In the first experiment, WEHI hits (6-8) and the control 4PI was investigated (Fig. 5.4a).
Chapter 5 - Experimentation and Discussion

Figure 5.4. Kinetics of IDO1 inhibition by 4-PI and WEHI hits 6-8. a) Lineweaver-Burk double reciprocal plot of 4PI incubated with IDO1 at 37°C for 4 min in a standard assay mixture containing 5 – 40 μM L-TRP. Ki was determined by nonlinear regression fitted to non-competitive enzyme inhibition equation in GraphPad Prism v6. b) The effect of enzyme assay length on the inhibitory activity of 6-8 and 4PI. The assay was carried out at 37°C with 80 μM L-TRP. The inhibitory values at 25 min were extrapolated from concentration-response curves of 6-8 and 4PI shown in Appendix A.19 at concentrations indicated in parentheses. Statistical significance was calculated using Sidak’s multiple comparisons test in GraphPad Prism v6. ns = not significant, **** = significant.

The Lineweaver-Burk plot of 4PI shows a family of lines converging on the x-axis, demonstrating that 4PI does not affect the affinity of L-TRP for IDO1, but lowers the reaction velocity. This indicates that 4PI is a non-competitive inhibitor with L-TRP, and is consistent with previous reports for this compound (Sono & Cady, 1989). The Ki of 4PI in this study, 26.9 μM, is higher than the Ki = 8 μM determined by Sono and Cady. However, their experiments were carried out at different conditions, at pH 7 and with D-TRP as the substrate (Sono & Cady, 1989). Unexpected, the potency of compounds 6-8 was extremely weak in this experiment and did not allow reliable interpretation (see next paragraph and Fig. 5.4b). Considering that the enzyme assay was performed using a 4 min incubation in this experiment instead of the 25 min incubation used normally, the time-dependency of the inhibitory activity of 6-8 was investigated. The inhibitory activity of hits 6-8 and 4PI obtained in a 4 and a 25 min incubation time were compared at identical inhibitor and substrate concentrations (Fig. 5.4). The inhibitory activity of 4PI was unaffected by the
length of incubation time, but compounds 6, 7 and 8 showed 6.2, 8.8 and 2.4-fold increases respectively, in inhibitory activity with 25 min incubation compared to 4 min incubation (Figure 5.4b).

Based on the above observations, hits 6-8 appear to be slow-binding inhibitors. Slow binding IDO1 inhibitors have not yet been reported. Considering that hits 6-8 are chemically distinct, and yet all showed a time-dependent increase in inhibitory activity, slow-binding might be a common mechanism of action for IDO1 inhibitors. The slow-binding of cyclooxygenase inhibitors has been shown to result in essentially irreversible but non-covalent binding to the enzyme (Copeland et al.,1994, Kulmacz & Lands,1985). The results with cyclooxygenase inhibitors prompted the investigation of both time-dependency and reversibility of the inhibition by all shortlisted compounds (Table 5.1). These studies are an essential part of inhibitor characterisation that provides important mechanistic insights into its mode of inhibition (Copeland,2005, Silverman,1995).

5.2.3.1 Reversibility and time-dependency

Firstly, reversibility (dissociation) of the inhibitor-enzyme complex was examined. rhIDO1 was pre-incubated in a buffer containing the reduction cofactors, MB/AA and a saturating concentration of inhibitor (rhIDO1 inhibition > 75%) for 25 min. This reaction mixture was then diluted 100-fold with an assay buffer containing saturating concentration of the substrate (TRP). The enzyme activity was monitored for 60 min following dilution (Fig. A.18). In this situation, the decrease in inhibitory activity should correlate with the dissociation rate of the IDO1-inhibitor complex. A reversible inhibitor would show a rapid loss of activity to < 25% enzyme inhibition after dilution, whilst the inhibitory activity of an irreversible enzyme inactivator remains above 75%. The inhibitory activity of a slow-reversible inhibitor decreases slowly over time. Except NLG919, which reached equilibrium in ~15 minutes (see Fig. A.18), the inhibitory activity after dilution of all the other inhibitors tested, remained constant during the entire 60 min. Therefore, a subsequent experiment was carried out with a longer pre-incubation time of 90 min compared to 25 min (please refer to the experimental outline on top of Fig. 5.5).

After diluting 100-fold, 4PI, NLG919, and hits 1, 2 and 4 exhibited less than 30% inhibition. This indicated that they are reversible inhibitors with high dissociation rates (Fig. 5.5a). Dissociation of NLG919 from rhIDO1 was significantly slower than that of the other reversible inhibitors, due perhaps to its higher potency (IC50 60.4 nM determined in our PIP enzyme assay). Strikingly, 4PI and hit 1 increased enzyme activity by 50% and 64%, respectively, compared to the enzyme without inhibitor (Fig. 5.5a). This suggests that 4PI and
hit 1 can stabilise rhIDO1 which slowly degrades during the experiment. This could be due to a preferential binding of 4PI and hit 1 to Fe³⁺-IDO1 (previously shown for 4PI) (Sono & Cady, 1989), thereby decreasing a reductive IDO1 activation and concomitant formation of hydrogen peroxide (Hirata et al., 1977, Sono, 1989, Sutton et al., 1976) that can irreversibly inactivate IDO1 by oxidation of cysteine residues (Poljak et al., 2006).

**Figure 5.5.** (See legend on page 121.)
(See figure on page 120.)

**Figure 5.5.** Reversibility and time-dependence of IDO1 inhibitors. 

- **a)** Inhibitory activity of IDO1 inhibitors after preincubation (25 or 90 min) with rhIDO1 (600 nM, 1% DMSO, 21°C) and 100-fold dilution into assay buffer, respectively. Enzyme assay was carried out for 15 min at 21°C. Concentration of compounds preincubated with rhIDO1 (before 100-fold dilution into assay) is indicated in parentheses in front of the compound name. Time-dependence of rhIDO1 inhibition of **b)** known IDO1 inhibitors and **c), d)** inhibitors identified in this study. In experiments **b) – d)**, rhIDO1 (6 nM) was added to the compounds (concentration in parentheses) or 1% DMSO control in complete assay buffer at time = 0 min, and aliquots of enzyme assay performed at 21°C were taken every 3 min and mixed with PIP (final concentration 200 mM) to stop the reaction. Inhibitory activities were normalised to rhIDO1 preincubated with 1% DMSO and processed identically as an inhibitor-treated enzyme. Data are presented as a mean ± SD of at least two independent measurements carried out in technical duplicates. Note: The activity of uninhibited (DMSO-treated) rhIDO1 determined at 90 min incubation was one-half of the activity measured at 25 min indicating IDO1 was degrading relatively fast at the experimental conditions. **Abbreviations:** MB/AA (methylene blue/ascorbic acid), IC$_{75}$ (concentration of inhibitor inducing 75% maximal inhibition of rhIDO1)

Interestingly, **4PI** and hit **1** are the only reversible inhibitors that protected rhIDO1 from spontaneous degradation, and binding of **NLG919** may not be competing with reductive activation of the enzyme like **4PI** and hit **1**. In contrast to these reversible inhibitors, benzofurazan **3** and its analogues **3a** and **3g** (see structures in Table 5.3), **HA**, **5a** (a more soluble analogue of **5**, see structure in Table 5.3) and the WEHI hits **6, 7** and **8** all show > 75% inhibitory activity after either 25 min or 90 min preincubation with rhIDO1 (Fig. 5.5a) indicating they are irreversible enzyme inactivators.

The time-dependence of rhIDO1 inhibition was next examined (Fig. 5.5b,c,d). The enzyme was added to the complete assay mix containing inhibitors or DMSO (uninhibited control) at time zero and the formation of NFK was followed over 60 min. Compounds **4PI, 1, 2, 3, 3a, 3g** and **5a** all exhibited rapid onset of inhibition. Compounds **HA, NLG919, 4** and WEHI hits **6, 7, 8** all showed significant time-dependent inhibition. In the case of **HA** and WEHI hits **6, 7, 8**, it resulted in essentially irreversible inactivation of the enzyme. Inhibition of rhIDO1 by hit **4** and **NLG919** could be reversed by dilution (Fig. 5.5a). The observations from these experiments are summarised in Table 5.2 below.

**Table 5.2.** Reversibility and time-dependency of tested IDO1 inhibitors (summary of the results from Figure 5.5)

<table>
<thead>
<tr>
<th>Inhibition</th>
<th>4PI</th>
<th>HA</th>
<th>NLG919</th>
<th>1, 2</th>
<th>3, 3a, 3g</th>
<th>4</th>
<th>5a</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time-dependent</td>
<td>x</td>
<td>✓</td>
<td>✓</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Reversible</td>
<td>✓</td>
<td>x</td>
<td>✓</td>
<td>✓</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>
## Table 5.3. IDO1 inhibitory activities of analogues of NCI hits 1-5 (highlighted in black background).

<table>
<thead>
<tr>
<th>Structure</th>
<th>ID</th>
<th>X₁</th>
<th>X₂</th>
<th>R₁</th>
<th>R₂</th>
<th>IC₅₀ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Structure 1" /></td>
<td>1</td>
<td>CH₂</td>
<td>-</td>
<td>2-Cl</td>
<td>4'-Cl</td>
<td>3.4 ± 0.1</td>
</tr>
<tr>
<td><img src="image2.png" alt="Structure 1a" /></td>
<td>1a</td>
<td>CH₂</td>
<td>-</td>
<td>4-NO₂</td>
<td>4'-Cl</td>
<td>4 ± 0.7</td>
</tr>
<tr>
<td><img src="image3.png" alt="Structure 1b" /></td>
<td>1b</td>
<td>CH₂</td>
<td>-</td>
<td>2-NO₂</td>
<td>4'-Cl</td>
<td>63.3 ± 20.9</td>
</tr>
<tr>
<td><img src="image4.png" alt="Structure 1c" /></td>
<td>1c</td>
<td>C=O</td>
<td>-</td>
<td>4-Cl</td>
<td>2'-Cl</td>
<td>103 ± 32</td>
</tr>
<tr>
<td><img src="image5.png" alt="Structure 1d" /></td>
<td>1d</td>
<td>CH₂</td>
<td>-</td>
<td>4-Cl</td>
<td>4'-Cl</td>
<td>533 ± 38*</td>
</tr>
<tr>
<td><img src="image6.png" alt="Structure 1e" /></td>
<td>1e</td>
<td>CH₂</td>
<td>-</td>
<td>4-CH₃</td>
<td>4'-Cl</td>
<td>-1 ± 2% (250 μM)*</td>
</tr>
<tr>
<td><img src="image7.png" alt="Structure 2" /></td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>2-OH-phenyl</td>
<td>-</td>
<td>3.3 ± 0.1</td>
</tr>
<tr>
<td><img src="image8.png" alt="Structure 2a" /></td>
<td>2a</td>
<td>-</td>
<td>-</td>
<td>3-OH-phenyl</td>
<td>-</td>
<td>112 ± 18</td>
</tr>
<tr>
<td><img src="image9.png" alt="Structure 2b" /></td>
<td>2b</td>
<td>-</td>
<td>-</td>
<td>2-F-phenyl</td>
<td>-</td>
<td>231 ± 196</td>
</tr>
<tr>
<td><img src="image10.png" alt="Structure 2c" /></td>
<td>2c</td>
<td>-</td>
<td>-</td>
<td>2-CH₃-phenyl</td>
<td>-</td>
<td>512 ± 149*</td>
</tr>
<tr>
<td><img src="image11.png" alt="Structure 2d" /></td>
<td>2d</td>
<td>-</td>
<td>-</td>
<td>4-OH-phenyl</td>
<td>-</td>
<td>522 ± 228*</td>
</tr>
<tr>
<td><img src="image12.png" alt="Structure 2e" /></td>
<td>2e</td>
<td>-</td>
<td>-</td>
<td>H</td>
<td>-</td>
<td>8 ± 3% (250 μM)*</td>
</tr>
<tr>
<td><img src="image13.png" alt="Structure 3a" /></td>
<td>3’a</td>
<td>S</td>
<td>-</td>
<td>NO₂</td>
<td>-</td>
<td>320 ± 18</td>
</tr>
<tr>
<td><img src="image14.png" alt="Structure 3b" /></td>
<td>3’b</td>
<td>S</td>
<td>-</td>
<td>H</td>
<td>-</td>
<td>-8 ± 4%*</td>
</tr>
<tr>
<td><img src="image15.png" alt="Structure 3c" /></td>
<td>3’c</td>
<td>O</td>
<td>-</td>
<td>H</td>
<td>-</td>
<td>-9 ± 5%*</td>
</tr>
<tr>
<td><img src="image16.png" alt="Structure 3" /></td>
<td>3</td>
<td>S=O</td>
<td>CH</td>
<td>NO₂</td>
<td>4-CH₃</td>
<td>6.6 ± 2.5</td>
</tr>
<tr>
<td><img src="image17.png" alt="Structure 3a" /></td>
<td>3a</td>
<td>S</td>
<td>CH</td>
<td>NO₂</td>
<td>4-F</td>
<td>8.8 ± 0.4</td>
</tr>
<tr>
<td><img src="image18.png" alt="Structure 3b" /></td>
<td>3b</td>
<td>S</td>
<td>CH</td>
<td>NO₂</td>
<td>5-Cl</td>
<td>12.4 ± 0.4</td>
</tr>
<tr>
<td><img src="image19.png" alt="Structure 3c" /></td>
<td>3c</td>
<td>S</td>
<td>CH</td>
<td>NO₂</td>
<td>4-CH₃</td>
<td>18.7 ± 0.6</td>
</tr>
<tr>
<td><img src="image20.png" alt="Structure 3d" /></td>
<td>3d</td>
<td>S</td>
<td>N'O'</td>
<td>NO₂</td>
<td>-</td>
<td>24.1 ± 1.0</td>
</tr>
<tr>
<td><img src="image21.png" alt="Structure 3e" /></td>
<td>3e</td>
<td>S</td>
<td>CH</td>
<td>NO₂</td>
<td>4,6-di-Cl</td>
<td>24.4 ± 0.9</td>
</tr>
<tr>
<td><img src="image22.png" alt="Structure 3f" /></td>
<td>3f</td>
<td>NH</td>
<td>CH</td>
<td>NO₂</td>
<td>4-Br</td>
<td>67.7 ± 3.6</td>
</tr>
<tr>
<td><img src="image23.png" alt="Structure 3g" /></td>
<td>3g</td>
<td>NH</td>
<td>CH</td>
<td>NO₂</td>
<td>-</td>
<td>76 ± 10</td>
</tr>
<tr>
<td><img src="image24.png" alt="Structure 3h" /></td>
<td>3h</td>
<td>NH</td>
<td>CH</td>
<td>NO₂</td>
<td>4-OCH₃</td>
<td>96 ± 6.8</td>
</tr>
<tr>
<td><img src="image25.png" alt="Structure 3i" /></td>
<td>3i</td>
<td>NH</td>
<td>CH</td>
<td>NO₂</td>
<td>4-CH₃</td>
<td>96.9 ± 11.7</td>
</tr>
<tr>
<td><img src="image26.png" alt="Structure 3j" /></td>
<td>3j</td>
<td>NH</td>
<td>CH</td>
<td>H</td>
<td>4-CH₃</td>
<td>22 ± 0%*</td>
</tr>
<tr>
<td><img src="image27.png" alt="Structure 3k" /></td>
<td>3k</td>
<td>N</td>
<td>-</td>
<td>5-F, 6-R₅, 7-COOH</td>
<td>-</td>
<td>13.5 ± 4.2</td>
</tr>
<tr>
<td><img src="image28.png" alt="Structure 3l" /></td>
<td>3l</td>
<td>N'O'</td>
<td>-</td>
<td>5-COOH, 6-R₄</td>
<td>-</td>
<td>22.7 ± 2.3</td>
</tr>
<tr>
<td><img src="image29.png" alt="Structure 3m" /></td>
<td>3m</td>
<td>N</td>
<td>-</td>
<td>5-COOH, 6-R₄, 7-Br</td>
<td>-</td>
<td>40.2</td>
</tr>
<tr>
<td><img src="image30.png" alt="Structure 3n" /></td>
<td>3n</td>
<td>N</td>
<td>-</td>
<td>5-COOH, 6-R₄</td>
<td>-</td>
<td>41.3 ± 2.4</td>
</tr>
<tr>
<td><img src="image31.png" alt="Structure 3o" /></td>
<td>3o</td>
<td>N</td>
<td>-</td>
<td>4-F, 6-COOH, 7-R₃</td>
<td>-</td>
<td>43.4 ± 2.8</td>
</tr>
<tr>
<td><img src="image32.png" alt="Structure 3p" /></td>
<td>3p</td>
<td>N</td>
<td>-</td>
<td>4-F, 6-COOCH₃, 7-R₃</td>
<td>-</td>
<td>3 ± 3%*</td>
</tr>
<tr>
<td><img src="image33.png" alt="Structure 4a" /></td>
<td>4a</td>
<td>NH</td>
<td>CH</td>
<td>-</td>
<td>-</td>
<td>6.5 ± 0.2</td>
</tr>
<tr>
<td><img src="image34.png" alt="Structure 4b" /></td>
<td>4b</td>
<td>NH</td>
<td>CH</td>
<td>6-CH₃</td>
<td>-</td>
<td>571 ± 7*</td>
</tr>
<tr>
<td><img src="image35.png" alt="Structure 4c" /></td>
<td>4c</td>
<td>CH</td>
<td>NH</td>
<td>1-CH₃</td>
<td>-</td>
<td>-3 ± 2% (250 μM)*</td>
</tr>
<tr>
<td><img src="image36.png" alt="Structure 4d" /></td>
<td>4d</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>15 ± 1%*</td>
</tr>
</tbody>
</table>
Table 5.3. continued.

<table>
<thead>
<tr>
<th>Structure</th>
<th>ID</th>
<th>X₁</th>
<th>X₂</th>
<th>R₁</th>
<th>R₂</th>
<th>enzyme assay</th>
<th>cell-enzyme assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IC₅₀ (μM)</td>
<td>IC₅₀ (μM) LC₅₀ (μM)</td>
</tr>
<tr>
<td>5a</td>
<td>N’O’</td>
<td>C=O</td>
<td>-</td>
<td>2’-CH₃</td>
<td></td>
<td>0.23 ± 0.03</td>
<td>3.5 &gt; 25</td>
</tr>
<tr>
<td>5b</td>
<td>N’O’</td>
<td>C=O</td>
<td>-</td>
<td>-</td>
<td></td>
<td>0.43 ± 0.11</td>
<td>1.6 &gt; 125</td>
</tr>
<tr>
<td>5c</td>
<td>N’O’</td>
<td>C=O</td>
<td>-</td>
<td>4’-OCH₃</td>
<td></td>
<td>0.60 ± 0.04</td>
<td>2.7 &gt; 50</td>
</tr>
<tr>
<td>5d</td>
<td>N-</td>
<td>C-CN</td>
<td>-</td>
<td>4’-Cl</td>
<td></td>
<td>2.5 ± 0.4</td>
<td>19.1 &gt; 100</td>
</tr>
<tr>
<td>5</td>
<td>N’O’</td>
<td>C=O</td>
<td>-</td>
<td>5,6-(OCH₃)₂</td>
<td>-</td>
<td>4.1 ± 0.8</td>
<td>5.2 &gt; 125</td>
</tr>
<tr>
<td>5e</td>
<td>N</td>
<td>C=O</td>
<td>-</td>
<td>-</td>
<td></td>
<td>8.9 ± 0.6</td>
<td>3.8 &gt; 125</td>
</tr>
<tr>
<td>5f</td>
<td>N</td>
<td>C=NH</td>
<td>-</td>
<td>-</td>
<td></td>
<td>23.9 ± 0.79</td>
<td>-</td>
</tr>
<tr>
<td>5g</td>
<td>C=O</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td>266 ± 111ᵇ</td>
<td>-</td>
</tr>
<tr>
<td>5h</td>
<td>NH</td>
<td>C=O</td>
<td>-</td>
<td>-</td>
<td></td>
<td>510 ± 95.0ᶜ</td>
<td>-</td>
</tr>
<tr>
<td>5i</td>
<td>NH</td>
<td>CH</td>
<td>-</td>
<td>4’-OCH₃</td>
<td></td>
<td>16 ± 3% (200 μM)*</td>
<td>-</td>
</tr>
<tr>
<td>5j</td>
<td>C-NH</td>
<td>C=O</td>
<td>-</td>
<td>-</td>
<td></td>
<td>14 ± 3% (100 μM)*</td>
<td>-</td>
</tr>
<tr>
<td>5k</td>
<td>C-NH₂</td>
<td>C=O</td>
<td>-</td>
<td>-</td>
<td></td>
<td>14 ± 1% (100 μM)*</td>
<td>-</td>
</tr>
<tr>
<td>5l</td>
<td>C-NH₂</td>
<td>C=O</td>
<td>-</td>
<td>4’-OCH₃</td>
<td></td>
<td>13 ± 3% (50 μM)*</td>
<td>-</td>
</tr>
<tr>
<td>5m</td>
<td>CH₂</td>
<td>C=O</td>
<td>-</td>
<td>-</td>
<td></td>
<td>21 ± 2% (200 μM)*</td>
<td>-</td>
</tr>
<tr>
<td>5n</td>
<td>CH₂</td>
<td>C=O</td>
<td>-</td>
<td>5-OCH₃</td>
<td>4’-OCH₃</td>
<td>29 ± 1% (200 μM)*</td>
<td>-</td>
</tr>
<tr>
<td>5o</td>
<td>C-ethyl</td>
<td>C=O</td>
<td>-</td>
<td>-</td>
<td></td>
<td>20 ± 1% (150 μM)*</td>
<td>-</td>
</tr>
<tr>
<td>5p</td>
<td>C=O</td>
<td>C=O</td>
<td>-</td>
<td>-</td>
<td></td>
<td>20 ± 4%*</td>
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</tr>
</tbody>
</table>

Data are presented as a mean ± SD of at least two independent experiments. Each compound was assayed at 8 concentrations in technical duplicates for enzyme assay and at 6 concentrations in technical triplicates in cell-enzyme assay (Lewis lung carcinoma cells constitutively expressing hIDO1). When IC₅₀ value could not be reached but the inhibition of hIDO1 at the highest assayed concentration was > 30%, IC₅₀ was calculated as for other compounds using fitted equation $y = a + \frac{100-a}{1+\left(\frac{IC}_{50}\right)^{Hill\text{ slope}}}$ where $y = 50$.

Superscript letters indicate maximal assayed concentrations:ᵃ400 μM,ᵇ100 μM,ᶜ250 μM.

*Percent rhIDO1 inhibition at the concentration indicated in parentheses.

*Inhibitory activity of rhIDO1 was determined using absorbance TCA assay due to high autofluorescence of the compound in the fluorescence assay.
The inhibitors can be classified into four categories (A-D) based on the two possible outcomes from both the reversibility and time-dependency experiments (see Table 5.2):

A) Rapid binding, reversible inhibitors (4PI, 1 and 2). Reversible inhibitors showing fast on-rates that do not form covalent adducts with rhIDO1.

B) Slow binding, reversible inhibitors (clinical candidate NLG919 and 4). These inhibitors behaved similarly to A) but appear to bind enzyme much slower. The possible source of slow-binding will be discussed in D).

C) Rapid binding inactivators (benzofurazans (BF) 3, 3a, 3g and 5a). Such inhibitors appear to completely inactivate rhIDO1 in less than 3 min (Fig. 5.5), and rapid chemical modification of IDO1 is proposed, although GSH did not decrease the inhibitory activity of either 3 or 5. GSH has been shown in a study by Whitehouse and colleagues, to attenuate the activity of 4-nitro-7-thiosubstituted benzofurazans (NT-BF), analogues of hit 3 (Whitehouse & Ghosh,1968). The higher pH used by Whitehouse and coworkers (pH 7.4) compared to this study (pH 6.5) could have increased the proportion of a more reactive thiolate anion GS⁻ (pKa of GSH = 8.8) (Winterbourn & Metodiewa,1999). While covalent modification of IDO1 by 3 and 5 is possible, there is also a convincing body of evidence that NT-BFs (3) and indolonoxides (5) are potent generators of reactive oxygen species. Consumption of dioxygen by 25 μM NT-BF was shown to be close to 100 μM per min (37°C, pH 7.4) generating amounts of hydrogen peroxide and superoxide radicals (O₂⁻) that would be sufficient to inactivate rhIDO1 in < 3 min. It is surprising, however, that catalase (a scavenger of hydrogen peroxide present in the enzyme assay) (Hirata et al.,1977) could not protect rhIDO1 from hydrogen peroxide inactivation. Catalase could have been overwhelmed by a rapid burst of hydrogen peroxide or became inactivated itself by O₂⁻ (Pigeolet et al.,1990) since hydrogen peroxide has minimal effect on its activity (Kirkman & Gaetani,1984). Poljak and colleagues also showed that 5 mM DTT but not 5 mM GSH could completely protect rhIDO1 from hydrogen peroxide inactivation, probably due to lower reduction potential of DTT (-330 mV, pH 7) (Cleland,1964) compared to GSH (-262 mV, pH 7) (Millis et al.,1993). This might explain the inability of GSH present in both the enzyme assay (5 mM) and the cell-based assay (GSH concentration in cells is expected to be up to 10 mM) (Chakravarthi et al.,2006) to protect IDO1 from hydrogen peroxide inactivation by 3 and 5 (see structures in Table 5.3). Furthermore, the maximal inactivation of rhIDO1 by 3g and 5a (see structures in Table 5.3) was reached faster when the inhibitors were directly added to the enzyme assay without...
preincubation (Fig. 5.5c,d) compared to the assay with preincubation (Fig. 5.5a). This is consistent with the observation of Freewan and colleagues that ascorbic acid alone (preincubation conditions in Fig. 5.5), but not a mixture of ascorbic acid and TRP (assay conditions) is more effective in protecting rhIDO1 from hydrogen peroxide damage (Freewan et al., 2013). This evidence suggests that 3 and 5a may inhibit rhIDO1 by the generation of hydrogen peroxide rather than binding to the active site of the enzyme (Freewan et al., 2013, Poljak et al., 2006).

D) Slow binding inactivators (HA, WEHI hits 6, 7 and 8). The rhIDO1 inhibition by the most abundant group of rhIDO1 inhibitors in the study increased slowly over time and appeared essentially irreversible. Irreversibility could have arisen from multiple sources:

1) Covalent modification of apoprotein. Ebselen has been found to inactivate rhIDO1 by modification of cysteine residues. However, in contrast to our results, the inactivation by ebselen is rapid (< 5 min), potent (IC<sub>50</sub> 200 nM), and could by reversed by GSH (1 mM, pH 7) (Terentis et al., 2009).

2) Destruction or covalent modification of the haem cofactor. This has been shown with cytochrome P450 enzymes (Ernest et al., 2005, Foti et al., 2011, Roberts et al., 1998) and is a likely scenario since 6 and 7 can chelate the iron atom (Baell & Holloway, 2010, Richardson et al., 1995, Singh et al., 2013). Inhibitors 6 and 7 might cross-react with other haem enzymes including liver cytochrome P450s, which is in line with the poor pharmacokinetics of hit 7 in liver microsomes (data not shown).

3) Inhibitors with very low dissociation rates similar to e.g. dihydrofolate reductase inhibitor methotrexate (Williams et al., 1979), some kinase inhibitors (Chaikuad et al., 2014, Wood et al., 2004) or inhibitors of haem enzymes cyclooxygenase (COX)-1 and COX-2 (Copeland et al., 1994, Kulmacz & Lands, 1985). Interestingly, the slow-binding of COX inhibitors was attributed to flexibility of the COX active site (Kulmacz & Lands, 1985, Luong et al., 1996). Tojo and coworkers recently demonstrated using X-ray crystallography that IDO1 inhibitors could induce significant conformational change to the IDO1 active site (Tojo et al., 2014) and the slow binding of IDO1 inhibitors in the groups C) and D) could relate to the flexibility of the IDO1 active site as well. Expulsion of water molecules from the IDO1 active site may also cause slow-binding (Silverman, 1995). Further experiments are necessary to identify the inhibition mechanism of HA and WEHI hits 6-8. This aspect is important as the drugs that act by inactivating specific enzymes are now desirable due to their irreversible
engagement with the target enzyme (Copeland, 2005). Drugs with this type of interaction can provide a sustained therapeutic effect even when the free inhibitor is eliminated from the body. A prime example is finasteride, a mechanism-based inactivator of steroid 5α-reductase which exhibits a half-life of 6-8 h in humans but the therapeutic effect persists for 7 days (Vermeulen et al., 1991). Irreversible IDO1 inactivators, even those with poor pharmacokinetics, may still be able to provide prolonged IDO1 inhibition in vivo. The results here also suggest that the increased potency of hit 8 in the cell-based assay (see Table 5.1, Fig. 5.3b) might be caused by its essentially irreversible inactivation of IDO1.

5.2.4 Structure-activity relationship (SAR) of the NCI hits 1-5

In this section, commercially available analogues of hits 1-5 were purchased and tested in an effort to assess the SAR of each hit to define their suitability as leads for the IDO1 drug development programme (Table 5.3).

5.2.4.1 Thioanilines (1)

The substitution of CH₂ group at X₁ position by carbonyl resulted in a 30-fold decrease of activity (1c) (Table 5.3). The position of the chlorine substituent on the aniline ring appears to play a significant role in potency for this scaffold. 2-Cl analogue (1) showed 157-fold higher potency than 4-Cl compound (1d). Strikingly, substitution of the 4-Cl group of 1d by 4-NO₂ restored the potency back to the level of 1 but methyl group at the 4-position induced loss of the inhibitory activity. The SAR of these analogues appears quite confusing and it is likely that the active inhibitors are degradation products of 1-1e. Since the thioaminal bond between the two rings is unstable, the respective thiols and amines could form. However, the ¹H-NMR analysis demonstrated higher than 90% purity of 1. Degradation products might therefore be active at extremely low concentrations or form on storage or during the enzyme assay.

Hit 1 is a reversible, non-aggregating IDO1 inhibitor with good cell permeability and low cell toxicity. It has a favourable record in PubChem BioAssay (active in 4 out of 616 test results), and this hit could be a good candidate for further IDO1 drug development upon substitution of the linker between the sulphur and nitrogen rings.
5.2.4.2 Phenantroimidazoles (2)

The position of the hydroxyl substituent on the phenyl ring was found to dramatically affect potency. Substitution at the 2- position was preferred over 3- (2a) or the 4-position (2d) (Table 5.3). Replacement of the 2-hydroxy group with fluorine (2b) or a methyl (2c) group, results in a > 100-fold decrease in inhibitory activity, indicating the importance of the hydroxyl group in mediating a specific interaction with IDO1. Compounds with reduced number of fused cycles to increase solubility were investigated to see if they retained inhibitory activity. When phenanthrene of 2 was substituted by benzene, inhibitory activity was completely lost, and changing the substituents on the phenyl ring did not recover activity (Table A. 2).

Hit 2 is a reversible inhibitor that appeared as an aggregator in the cell-based assay but not in the enzyme assay. PubChem Bioassay indicates promiscuity of 2 (active in 40 out of 617 test results). Interestingly, the inhibitory activity of 2 was significantly reduced against the alanine replacement IDO1 mutant of serine-167, which indicates a specific interaction with the IDO1 active site (discussed in chapter 6). However, in summary, 2 is not a preferred lead for IDO1 drug development.

5.2.4.3 Benzofurazans (BF) (3)

The 4-nitro group and 7-phenylthio/amino substituents were both critical for the low μM inhibitory activities in this series (Table 5.3). Moreover, sulphur is preferred over nitrogen at position X₁ in the series 4a-j. The properties of the substituents and their positions on the phenyl ring (R₂) did not affect the inhibitory activity. These observations are in line with previous studies of 4-nitro benzofurazan analogues on different enzymes (Ghosh & Whitehouse, 1968, Korolev et al., 2013, Ricci et al., 2005).

4-nitro BFs are reactive electrophiles that form adducts with thiols, amines and even water at physiological conditions (Crampton et al., 2003, Patridge et al., 2012) explaining their toxicity in cell-based assays. The 4-nitro group appears to be responsible for high reactivity. Several analogues lacking 4-nitro group (3k-o) showed rhIDO1 inhibitory activities comparable to the most potent analogues in the 4-nitro series (3,3a-e). However, substitution of 4-nitro group (3k) by carboxy group did not abrogate toxicity to the LLTC cells compared to 3 (data not shown). Interestingly, substitution of the carboxyl group in this series by its methyl ester abrogated the IDO1 inhibitory activity (see 3o vs 3p in Table 5.3), similar to the loss of the 4-nitro group (see 3i vs 3j in Table 5.3). This indicated that the carboxy group plays a similarly critical role as the 4-nitro group for inhibiting IDO1.
Hit 3 appears to be a non-specific inhibitor (active in 41 out of 267 test results on PubChem Bioassay, flagged by 2 structural filters) that was reported to inhibit protein and nucleic acid synthesis (Ghosh & Whitehouse, 1968, Ghosh et al., 1972), and a range of enzymes including glutathione S-transferases (Ricci et al., 2005), HIV-1 integrase (Korolev et al., 2013) and stimulate the activity of the epidermal growth factor receptor (Sakanyan et al., 2014). Hit 3 is not a preferred IDO1 drug lead. However, possible development of this class might involve substitution of benzoxadiazole ring by phthalazine ring which was successfully used in the optimisation of benzothiadiazoles as cholecystokinin-2 antagonists (Allison et al., 2006).

5.2.4.4 Pyrimidinones (4)

Hit 4 appears as the best candidate amongst the 5 NCI inhibitors for IDO1 drug development. It is a slow-binding reversible inhibitor that has good cell permeability and is not toxic to the LLTC cells at concentrations that inhibit the enzyme. PubChem BioAssay also confirms the low promiscuity of 4 (activity in only 1 out of 615 test results). SAR studies of this group were limited to the commercial availability of only four analogues, however. The addition of a single 6-CH₃ group on the pyrimidinone ring of 4 (4a) resulted in a nearly 90-fold decrease in IDO1 inhibitory activity (Table 5.3), and additional modifications to the parent molecule 4, including the substitution of pyrimidinone ring by triazole (4b) exhibited weak or no IDO1 inhibitory activity.

5.2.4.5 Indolonoxides (5)

Removing the dimethoxy group increased the potency 10-fold from 4.1 μM (5) to 0.43 μM (5b). The addition of substituents (R₂) on the phenyl ring of 5b did not significantly influence the activity (5a, 5c) in the enzyme assay (Table 5.3). However, the potency of compounds 5a-c in the cell-based assay is much lower, and their IC₅₀ values are comparable to that of the original hit 5 (IC₅₀ ranged from 1.6 to 5.2 μM). This indicates a non-specific mechanism of inhibition that may not involve actual binding to the IDO1 active site. N-oxide and carbonyl groups appear critical for the potency of this series. Replacement of the N-oxide group by nitrogen reduced the potency 20-fold from 0.43 μM (5b) to 8.9 μM (5e). More importantly, 5e shows no cellular toxicity up to 125 μM (data not shown) compared to other N-oxide analogues tested that reduced cell viability > 25% at concentrations that inhibited hIDO1 activity (Table 5.3, see LC₅₀ values). Other substituents of N-oxide (X₁) including acetate (5g), acetamide (5j), amine (5k) or carbonyl (5p) decreased potency further, except
5d, which has the N-oxide group and carbonyl group substituted by N-hydroxy and nitrile groups, respectively. The IC₅₀ of 5d was comparable to that of the original hit 5 in the enzyme assay (2.5 μM) but significantly higher (19.1 μM) in the cell-based assay (Table 5.3). The slope of the IC₅₀ curve of 5d in the cell-based assay was > 2 indicating aggregating inhibitory mechanisms. Toxicity of 5d is comparable to that of 5 and other N-oxide analogues. Hit 5 is flagged by 3 structural filters and is likely a redox active non-specific inhibitor. 5b has been reported to inhibit the respiratory chain (Green et al., 1974, Sweetman et al., 1971) and analogues of 5 are known inhibitors of parasitic, bacterial and fungal growth (Ibrahim et al., 2012, Pantaleo et al., 2012).

Analogue 5e lacking the N-oxide group is essentially non-toxic to LLTC cells, exhibits comparable potency to parent hit 5, and has very good cell penetration (Table 5.3). Therefore, it would be a good lead for IDO1 drug development programme. 5e failed only one out of the five structural filters (AlarmNMR) compared to the parent compound 5 which failed three of the filters.

In summary, the most recommended NCI hits for IDO1 drug development programme are indolone 5e and pyrimidinone 4. Thioaniline 1 could also be considered, and benzoxadiazole 3 might be optimised.
CHAPTER 6

IMPORTANCE OF SERINE-167 AND CYSTEINE-129 IN IDO1 ACTIVE SITE FOR BINDING OF IDO1 INHIBITORS

6.1 Introduction

Crystal structures of wild-type human IDO1 in complex with two buffer molecules of N-cyclohexyl-2-aminoethanesulfonic acid and 4PI (Protein data bank (PDB) entry: 2D0T) or cyanide (PDB entry: 2D0U) were determined by Sugimoto and colleagues in 2006 (Sugimoto et al., 2006). Human IDO1 consists of one small N-terminal and one large C-terminal domain harbouring catalytic active site with prosthetic group haem b. The active site is remarkably hydrophobic, in order to perhaps facilitate binding of nonpolar substrates including TRP and other indoles (Fig. 6.1) (Kudo & Boyd, 2000, Takikawa et al., 1988). The entrance of the IDO1 active site contains phenylalanine-226 (F226), phenylalanine-227 (F227), arginine-231 (R231) and serine-235 (S235) which form pocket B, whilst phenylalanine-163 (F163), leucine-234 (L234), alanine-264 (A264), serine-263 (S263), glycine-261 (G261), glycine-262 (G262), together with phenylalanine-164 (F164), valine-130 (V130), serine-167 (S167) and cysteine-129 (C129) form pocket A (Fig. 6.1b). Histidine-346 (H346) ligates haem and its mutation results in an inactive enzyme (Littlejohn et al., 2003). Similarly, mutation of residues in pocket B (F226, F227, R231) and S263 abrogates enzyme activity (Sugimoto et al., 2006). In contrast, mutation of residues in pocket A (F163, C129, S167) has a negligible effect on enzyme activity, indicating they are not essential for catalysis (Sugimoto et al., 2006). However, dissociation constants of L-TRP for alanine replacement IDO1 mutants of F163 (1.08 and 0.87 mM, Fe$^{3+}$ and Fe$^{2+}$ form, respectively) and S167 (1.37 and 1.01 mM, Fe$^{3+}$ and Fe$^{2+}$ form, respectively) are 3-fold higher than that for wild-type IDO1 (0.32 and 0.53 mM, Fe$^{3+}$ and Fe$^{2+}$ form, respectively) (Sugimoto et al., 2006). This indicates that the structure of the active site might be slightly altered in the F163A and S167A IDO1 mutants.

Hydrogen bonds to the ligands inside the active site of pocket A, can potentially be provided by the carboxy group of the 7-propionate of haem, the carbonyl of G262, the amide of A264, the hydroxyl of S167 and the thiol of C129 (the latter four are depicted with green carbons in Figure 6.1b). Larger ligands can potentially hydrogen bond with R231 and S235 in the entrance to the active site (Fig. 6.1b). The hydroxyl of S263 is an unlikely hydrogen
bonding site for inhibitors, as it is already engaged in a hydrogen bond to the carboxyl of the 7-propionate of haem (Fig. 6.1b).

Figure 6.1. (See legend on page 132.)
Kumar and colleagues showed that the addition of a hydroxyl or a thiol group on 2- and 6-position, or 3- and 4- position of 4PI phenyl ring, respectively, increased IDO1 inhibitory activity of 4PI 6-10-fold (Kumar et al., 2008a). This was rationalised by hydrogen bonding to S167 and C129 using computer-aided molecular docking. Similarly, Röhrig and colleagues used a 2-hydroxy substitution on the phenyl ring of 4-phenyl-1,2,3-triazole to increase the molecule’s potency (Röhrig et al., 2012). Hydrogen bonding to hydroxyl of S167 was predicted as a key interaction for optimised IDO1 inhibitors (Lancellotti et al., 2011, Röhrig et al., 2010) and several groups have utilised this concept in a structure-based design of IDO1 inhibitors (Dolušić et al., 2011, Röhrig et al., 2010, Smith et al., 2012). However, none of the computer-simulated predictions has been verified experimentally, and this has led to the experimental investigations into the importance of S167 and C129 in the IDO1 active site for binding of inhibitors.

In the work in this chapter, the IDO1 alanine-replacement mutants of S167 (S167A) and C129 (C129A) were produced. The inhibitory activity of the NCI library was tested against each mutant IDO1 compared to the wild-type IDO1. Analogues of three inhibitors that showed a significant reduction of activity against S167A IDO1 were examined in a small SAR study to identify their potential hydrogen bonding groups.

### 6.2 Results

#### 6.2.1 Characterisation of mutant enzymes

Expression constructs for mutant enzymes were produced using Stratagene QuikChange Lightning site-directed mutagenesis kit (Expression construct for S167A was a...
kind gift from Prof Emma Lloyd Raven, University of Leicester, England), and was purified exactly as wild-type IDO1 (wtIDO1). S167A, C129A and wtIDO1 all showed identical bands on SDS-PAGE suggesting a molecular weight ~ 40 kDa for the rhIDO1 monomer (Fig. 6.2). A faint band at ~80 kDa likely corresponds to the rhIDO1 dimer. Enzyme preparations were ~ 95% pure. All three enzymes showed a Soret band at 405 nm indicating that the haem was oxidised (Fe\(^{3+}\) form). wtIDO1 and S167A displayed absorption maximum in visible spectrum consistent with literature values (Table 6.1) (Chauhan et al., 2008). The absorption maxima of C129A were comparable to those of S167A with the exception of the peak at 566 nm which was red-shifted in S167A to 572 nm (Fig. 6.2, Table 6.1). Soret ratio (A\(_{405}\):A\(_{280}\)) of all three enzymes ranged from 1.23 to 1.63, indicating that the haem was incorporated in ~ 60-75% of the enzyme molecules based on the Soret ratio of 2.17 for native human IDO1 (Takikawa et al., 1988). Enzyme activities (mol NFK/mol holoenzyme per min) of wtIDO1 and C129A were comparable (146.3±0.2 and 121.5±5.3, respectively) but the activity of S167A was 3.6-fold lower (40.5 ± 3.0) than that of wtIDO1 (Table 6.1).

**Figure 6.2.** Characterisation of mutant IDO1 enzymes. a) UV and visible absorption spectra of enzymes in potassium phosphate buffer (100 mM, pH 7) at 21°C. The plots in region of 460-700 nm were multiplied by a factor of 8 and shifted along the y-axis for clarity. Plots were normalised to the absorption at 405 nm. b) Acrylamide gradient gel (4-15%) showing purified rhIDO1 enzymes. Molecular weights (kDa) of marker proteins are indicated on the left side of the gel. Proteins were ~ 95% pure based on gel densitometry analysis performed in ImageJ software and considering rhIDO1 degradation product not as an impurity.
Table 6.1. Absorbance maxima, enzyme activity and Soret’s ratio of wild-type and mutant IDO1 enzymes.

<table>
<thead>
<tr>
<th>property</th>
<th>rhIDO1</th>
<th>WT</th>
<th>S167A</th>
<th>C129A</th>
</tr>
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<td>absorbance maxima (nm)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>280, 405, 500, 635</td>
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<td>280, 405, 500,</td>
<td>280, 405, 502,</td>
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<tr>
<td>activity</td>
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<td>expressed as mol of NFK / mol of holoenzyme per min. Activity was determined in a standard assay performed at the following conditions: 37°C, 25 min, pH 6.5, 80 μM TRP. Each enzyme was diluted to produce ~10 μM NFK.</td>
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6.2.2 Screening of the NCI library for C129A or S167A sensitive IDO1 inhibitors

Screening of NCI library compounds at 20 μM for inhibition of S167A and C129A IDO1 was performed on different days. Therefore, to control for potential inter-experimental variations, wtIDO1 was screened simultaneously with each mutant. Results from the screens are presented in Figure 6.3. Two criteria were applied in the selection of inhibitors that were considered significantly affected by the mutation. Firstly, the % inhibition values were converted to their “apparent IC₅₀” using Equation 5 below obtained by rearranging the sigmoidal equation for the concentration-response relationship (Equation 6; assuming Hillslope = 1 and concentration (x) = 20 μM).

\[
\text{apparent } IC_{50} = \frac{2000}{\% \text{ inhibition at } 20 \mu M} - 20 \quad \text{(Eq. 5)}
\]

\[
y = \frac{100}{1 + \left(\frac{IC_{50}}{\chi}\right)^{\text{Hillslope}}} \quad \text{(Eq. 6)}
\]

Differences in “apparent IC₅₀” between wtIDO1 and mutant enzymes ≥ 10-fold were considered significant as prescribed in similar studies (Ennion et al., 2000, Evans, 2010). This threshold is graphically shown in Figure 6.3 by two inverse exponential curves. This ensured that identical % inhibition differences are weighted correctly at different parts of the graph, i.e. difference between 40% and 60% inhibition will be negligible, whereas the difference between 100% and 80% will be significant. Secondly, only compounds showing ≥ 25% inhibition of wtIDO1 were considered (dotted lines in Fig. 6.3).
Figure 6.3. (See legend on page 136.)
(See figure on page 135.)

**Figure 6.3.** Screening of the NCI library compounds (at 20 μM) for inhibitory activity against a) wild-type and S167A mutant IDO1, and b) wild-type and C129A mutant IDO. $r^2$ of the linear regression fit is indicated at the top of the y-axis. Highlighted areas show regions of significant difference between % inhibitory activity of wild-type and mutant enzymes according to the procedure described in the text (see Eq. 5 and Eq. 6 in section 6.2.2). The ideal correlation is indicated by a thick dashed line. Dotted lines denote 25% enzyme inhibition.

According to the above criteria, the S167A mutation significantly reduced inhibitory activity of 18 out of 69 (26%) compounds that inhibited wtIDO1 $\geq$ 25% at 20 μM, and no inhibitors showed greater activity against S167A compared to the wtIDO1 (Fig. 6.3). The three hits obtained from the initial screening of the NCI library (hits 2, 3, 5) against wtIDO1 showed decreased activity against S167A (see the full list of 18 compounds in Appendix Table A.3). In contrast, the C129A mutation did not reduce the inhibitory activity of the 65 compounds that inhibited wtIDO1 $\geq$ 25%; and increased the activity of 3 compounds shown in Table 6.2. Interestingly, only benzothiazole (NSC503425) out of the total of 10 thiol (SH)-containing compounds in the NCI library inhibited wtIDO1.

It is clear that IDO1 inhibitors are considerably more sensitive to S167A than C129A mutation ($r^2 = 0.55$ and 0.97 for S167A and C129A, respectively). Overall, the studies indicate that serine-167 but not cysteine-129 in the IDO1 active site is important for interaction with a wide range of inhibitors.

<table>
<thead>
<tr>
<th>Structure</th>
<th>NSC #</th>
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<tr>
<td></td>
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<td>percent inhibition</td>
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<td></td>
<td></td>
<td>at 20 μM</td>
<td></td>
<td>IC$_{50}$ (μM)</td>
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<tr>
<td></td>
<td></td>
<td>wild-type IDO1</td>
<td></td>
<td>C129A mutant IDO1</td>
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<td>wild-type IDO1</td>
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<td></td>
<td></td>
<td></td>
<td>C129A mutant IDO1</td>
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<td></td>
<td>Fold change</td>
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<td>44.9</td>
</tr>
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<td>70.8</td>
<td>115.4</td>
<td>8.2</td>
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<tr>
<td><img src="structure3.png" alt="Structure" /></td>
<td>155698</td>
<td>4.5</td>
<td>38.8</td>
<td>421.6</td>
<td>31.6</td>
</tr>
</tbody>
</table>

Apparent IC$_{50}$ was calculated using formula: $\text{apparent IC}_{50} = \frac{\text{IC}_{50}^{2000} \times \text{percent inhibition at 20 μM}}{1000}$ as described in the text. Fold change was calculated by dividing apparent IC$_{50}$ of wild-type IDO1 by that of C129A mutant IDO1. Percent inhibition values $\leq 0$ were converted to 1000 μM.
6.2.3 Structure-activity study of IDO1 inhibitors sensitive to the S167A mutation

To determine whether the decreased activity of IDO1 inhibitors sensitive to S167A mutation results from the importance of hydrogen bonding to hydroxyl of S167, a small structure-activity study of 3 compounds and their analogues was performed. 4PI and its analogue 2-(1H-imidazol-4-yl)phenol (2-OH-4PI) that had been predicted by Kumar and colleagues to hydrogen bond to S167, were included as the negative and positive control, respectively.

2-OH-4PI is 34-fold less potent against S167A (IC\textsubscript{50} = 41.1 μM) than wtIDO1 (1.2 μM), whereas the potency of 4PI is unaffected by the S167A mutation (IC\textsubscript{50} 37.7 μM against wtIDO1 vs. IC\textsubscript{50} 56.9 μM against S167A, respectively) (Table 6.3). Inhibitor 2 is 7.8-fold less potent to S167A (IC\textsubscript{50} = 25.5 μM) compared to wtIDO1 (IC\textsubscript{50} = 3.3 μM). Moreover, substitution of its 2-hydroxy group by 2-methyl (2c) or 2-fluoro (2b) eliminated sensitivity to S167A mutation (Table 6.3). Similarly, hydroxy substituents at 3- (2a) or 4- (2d) positions do not show a significant decrease of potency against S167A. Compound 3d shows a 4.5-fold decrease in potency against S167A compared to wtIDO1. Other substituents on the phenyl ring (R\textsubscript{1}), including fluorine, chlorine or methyl groups (3a-e) show an even lower fold change ranging from 0.6 to 1.9 (Table 6.3). In the case of benzofurazan 3l which inhibits wtIDO1 at 22.7 μM but lost activity against S167A (2% inhibition at 400 μM), the N-oxide group appears to be hydrogen bonding to S167, as its analogue, 3n which lacks the N-oxide group, is not affected by the S167A mutation (IC\textsubscript{50} = 41.3 μM vs. 42.3 μM for wtIDO1 and S167A, respectively). Unlike 2-hydroxyl of hit 2, the N-oxide of compound 3l did not result in a higher potency (Table 6.3).

The 50.3-fold decrease in potency of indolonoxide 5 to S167A (IC\textsubscript{50} = 4.1 μM against wtIDO1 vs. 206 μM against S167A) is particularly striking (Table 6.3). Removal of the 5,6-dimethoxy group in compound 5b resulted in only a 4.1-fold difference in potency between wtIDO1 and S167A. However, compound 5b is 10-fold more potent than 5 against wtIDO1 (IC\textsubscript{50} = 0.43 μM for 5b). This differs from compounds 2-OH-4PI and 2 where the putative hydrogen bonding to S167 is associated with increased potency. The potency of the WEHI hits 6-8 (see structures in Table 5.1) was not affected by the S167A mutation (< 2.7-fold difference between IC\textsubscript{50} against wtIDO1 and S167A; data not shown).

In summary, these studies suggest that the inhibitory activity of IDO1 inhibitors 2, 3d, 3l and 5 is mediated by hydrogen bonding to hydroxyl of S167 in the IDO1 active site.
Table 6.3. Structure-activity relationship of the three S167A sensitive compounds (2, 3d, 5) from NCI library (in black background) against wtIDO1 and S167A. 4PI and 2-OH-4PI served as negative and positive controls, respectively.

<table>
<thead>
<tr>
<th>Structure</th>
<th>No</th>
<th>X₁</th>
<th>X₂</th>
<th>R₁</th>
<th>R₂</th>
<th>IC₅₀ (μM)</th>
<th>fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>wild-type</td>
<td>S167A mutant</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IDO1</td>
<td>IDO1</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>2-OH</td>
<td>-</td>
<td>-</td>
<td>3.3 ± 0.1</td>
<td>25.5 ± 0.55</td>
</tr>
<tr>
<td>2d</td>
<td>-</td>
<td>-</td>
<td>4-OH</td>
<td>-</td>
<td>-</td>
<td>522 ± 228</td>
<td>14 ± 8% *</td>
</tr>
<tr>
<td>2a</td>
<td>-</td>
<td>-</td>
<td>3-OH</td>
<td>-</td>
<td>-</td>
<td>112 ± 17.9</td>
<td>173 ± 41</td>
</tr>
<tr>
<td>2c</td>
<td>-</td>
<td>-</td>
<td>2-CH₃</td>
<td>-</td>
<td>-</td>
<td>512 ± 149</td>
<td>464 ± 229</td>
</tr>
<tr>
<td>2b</td>
<td>-</td>
<td>-</td>
<td>2-F</td>
<td>-</td>
<td>-</td>
<td>231 ± 196</td>
<td>108 ± 53</td>
</tr>
<tr>
<td>3d</td>
<td>-</td>
<td>-</td>
<td>6-N’O’</td>
<td>-</td>
<td>-</td>
<td>24.1 ± 1.0</td>
<td>108 ± 43.0</td>
</tr>
<tr>
<td>3a</td>
<td>-</td>
<td>-</td>
<td>4-F</td>
<td>-</td>
<td>-</td>
<td>8.80 ± 0.4</td>
<td>17.1 ± 14.8</td>
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<tr>
<td>3b</td>
<td>-</td>
<td>-</td>
<td>5-Cl</td>
<td>-</td>
<td>-</td>
<td>12.4 ± 0.4</td>
<td>17.7 ± 15.3</td>
</tr>
<tr>
<td>3c</td>
<td>-</td>
<td>-</td>
<td>4-CH₃</td>
<td>-</td>
<td>-</td>
<td>18.7 ± 0.6</td>
<td>10.8 ± 8.60</td>
</tr>
<tr>
<td>3e</td>
<td>-</td>
<td>-</td>
<td>4,6-Cl₂</td>
<td>-</td>
<td>-</td>
<td>24.4 ± 0.9</td>
<td>15.1 ± 14.3</td>
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<tr>
<td>3l</td>
<td>N’O’</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>22.7 ± 2.3</td>
<td>2 ± 6% *</td>
</tr>
<tr>
<td>3n</td>
<td>N</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>41.3 ± 2.4</td>
<td>42.3 ± 2.3</td>
</tr>
<tr>
<td>5</td>
<td>N’O’</td>
<td>C=O</td>
<td>5,6-</td>
<td>-</td>
<td>-</td>
<td>4.1 ± 0.8</td>
<td>206 ± 19.1</td>
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<tr>
<td></td>
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<td>(OCH₃)₂</td>
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</tr>
<tr>
<td>5a</td>
<td>N’O’</td>
<td>C=O</td>
<td>2'-CH₃</td>
<td>-</td>
<td>-</td>
<td>0.23 ± 0.03</td>
<td>1.70 ± 0.22</td>
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<tr>
<td>5b</td>
<td>N’O’</td>
<td>C=O</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.43 ± 0.11</td>
<td>1.77 ± 0.38</td>
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<tr>
<td>5f</td>
<td>N</td>
<td>C=NH</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>23.9 ± 0.80</td>
<td>21.4 ± 0.51</td>
</tr>
<tr>
<td>5e</td>
<td>N</td>
<td>C=O</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>8.9 ± 0.6</td>
<td>6.34 ± 2.90</td>
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<tr>
<td>5h</td>
<td>NH</td>
<td>C-CH=NOH</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>510 ± 95.0 b</td>
<td>366 ± 134 b</td>
</tr>
<tr>
<td>4PI</td>
<td>-</td>
<td>-</td>
<td>2-OH</td>
<td>-</td>
<td>-</td>
<td>37.7 ± 0.20</td>
<td>56.9 ± 7.5</td>
</tr>
<tr>
<td>2-OH-4PI</td>
<td>-</td>
<td>-</td>
<td>2-OH</td>
<td>-</td>
<td>-</td>
<td>1.2 ± 0.4</td>
<td>41.1 ± 2.0</td>
</tr>
</tbody>
</table>

Data are presented as a mean ± SD of at least two independent experiments performed in technical duplicates. When IC₅₀ was not reached but the inhibition of IDO1 at the highest assayed concentration was > 30%, IC₅₀ was calculated as for other compounds using fitted equation $y = a + \frac{100-a}{1 + \left(\frac{IC₅₀}{100-a}\right)^{1/\text{Hill slope}}}$ where $y = 50$. Superscript letters indicate maximal assayed concentrations: a400 μM, b250 μM. Fold change was calculated by dividing IC₅₀ of S167A by that of wtIDO1. *% IDO1 inhibition at the concentration indicated in parentheses. Values marked by an asterisk (*) were considered to be > 1000 μM to allow calculation of the fold change.
6.3 Discussion

In this chapter, the two alanine replacement mutants of S167 and C129 were successfully created. The properties of the mutant IDO1 enzymes produced in this study are consistent with those previously reported, although the enzymatic activity of the S167A mutant compared to the wtIDO1 is different to the studies of other groups (Austin et al., 2013, Chauhan et al., 2008, Sugimoto et al., 2006). The activity of wtIDO1 and S167A with L-TRP measured here is 146.3 and 40.5 mol NFK/mol enzyme per min, respectively; showing that the S167A mutation decreases activity by 3.6-fold compared to wtIDO1. In the studies of Chauhan and coworkers (Chauhan et al., 2008) and Sugimoto and colleagues (Sugimoto et al., 2006) the activity of the S167A mutant and wtIDO1 were comparable. The other reports used a higher pH 8 than pH 6.5 used for our studies, and may explain the contrasting results to ours. It is somewhat surprising, however, that those two groups obtained similar activity values when their measurements were carried out at different temperatures, and there was a 6-fold difference in the affinity of L-TRP for Fe3+ S167A reported by the two groups (Kd 1.37 mM for Chauhan et al. cf 8 mM for Sugimoto et al.) (Chauhan et al., 2008, Sugimoto et al., 2006).

The NCI library was tested concomitantly against wtIDO1 and a mutant enzyme. Activity against C129A compared to wtIDO1 was tested nearly 1 year after the screening of the library against S167A compared to the wtIDO1. The number of compounds that inhibited wtIDO1 > 25% in the two experiments is comparable (54 vs. 59, see Fig. 6.3), indicating that there was no significant degradation of the library compounds on storage at -20°C during that period. The alanine mutation of S167 affected the inhibitory activity of a range of compounds to a much greater extent than C129. S167 confers potency to IDO1 inhibitors and alanine mutation of S167 did not increase the activity of any of the inhibitors. There were three non-thiol compounds that inhibited C129A more than they inhibited wtIDO1. The increased potency of these three compounds (Table 6.2) may be due to higher hydrophobicity or an altered structure of the roof of pocket A (see Fig. 6.1b).

The findings in this section are consistent with the computer docking predictions for S167 but not those for C129 (Kumar et al., 2008a). There are two possible explanations for the nonessential role of C129. Firstly, cysteine-129 might be buried too deep in pocket A to allow interaction with the ligands. Secondly, compounds containing thiol groups can easily be oxidised to form dimers (R-S-S-R) (Poole, 2015) which may affect their inhibitory activity and hydrogen bonding capacity compared to their respective monomers (R-SH). Consistent
with dimerization as a cause of reduced IDO1 inhibitory activity, the IC\textsubscript{50} of 4-(4-thiophenyl)-1\textit{H}-imidazole decreased from 5.1 μM to 15.9 μM after 2 weeks of storage in the fridge at 50 mM in DMSO. The thiol of C129 appears difficult to reach for most inhibitors and may not be relevant for use in structure-based drug design of IDO1 inhibitors.

Structure-activity studies were carried out to identify potential hydrogen bonding substituents of \textit{2-OH-4PI} and the three NCI inhibitors that showed sensitivity to the S167A mutation (see Table 6.3). The potency of \textit{2-OH-4PI} decreased 34.3-fold to S167A compared to wtIDO1, but the potency of \textit{4PI} was unaffected by S167A mutation. The results indicated that the 2-hydroxyl group on the phenyl ring of \textit{2-OH-4PI} hydrogen bonds to hydroxyl of S167 consistent with the predicted computer docking by Kumar and coworkers (Kumar et al., 2008a). Moreover, the active site of S167A does not appear to be structurally different to that of wtIDO1 as the potency of \textit{4PI} to both is similar, confirming the usefulness of the S167A mutant for studying the binding of IDO1 inhibitors.

Phenantroimidazole \textit{2} exhibited only a 7.8-fold decrease in potency against S167A vs. wtIDO1, in comparison to the 34.3-fold decrease obtained with \textit{2-OH-4PI} (Table 6.3). However, the substitution of the 2-hydroxy group of \textit{2} by non-polar groups eliminates the sensitivity to S167A mutation, consistent with an interaction between the hydroxyl of \textit{2} with the hydroxyl of S167. Moreover, only hydroxy substituent at the 2-position appears to hydrogen bond with S167. Hydroxyl groups on other positions of the phenyl ring of \textit{2} did not show sensitivity to S167A (Table 6.3).

The \textit{N}-oxide appears to be the hydrogen bonding group of benzofurazans \textit{3d} and \textit{3l} (Table 6.3). However, the difference in potency of \textit{3d} between wtIDO1 and S167A is low (4.5-fold), and there were no analogues available in the study that could establish the significance of the \textit{N}-oxide group on \textit{3d} for hydrogen bonding. In the pair of benzofurazans \textit{3l} and \textit{3n}, potency to S167A was decreased (> 44-fold) only when the \textit{N}-oxide group was present. The \textit{N}-oxide group had no effect on the inhibitory activity to wtIDO1, however. This suggests that the \textit{N}-oxide group may be affecting the orientation of \textit{3l} and \textit{3n} in the IDO1 active site without changing the potency. The substituent that binds to S167 is unclear as \textit{3l} and \textit{3n} contain multiple hydrogen bonding groups. However, it has been shown that binding of a charged substituent on the ligand to the uncharged enzyme residue results in a larger difference in free binding energy compared to that of an uncharged substituent (Fersht et al., 1985). While K\textsubscript{i} values were not determined to calculate the free binding energy, the difference in potency of compound \textit{3l} against wtIDO1 and S167A is one of the highest in this study, supporting the notion of the \textit{N}-oxide binding to the hydroxyl of S167.
Chapter 6- Discussion

Compound 5 shows the greatest (50.3-fold) decrease in potency against S167A compared to wtIDO1 (Table 6.3). Removal of the 5,6-dimethoxy substituent of 5 abrogates sensitivity to S167 and increases the potency against wtIDO1. This indicates that the hydrogen bonding of one of the methoxy groups of 5 to S167 impedes the more favourable orientation where the phenyl ring of 5a and 5b sits in the pocket A or at the entrance to the IDO1 active site. The observed specific binding of inhibitor 5 to the IDO1 active site as demonstrated in this study with the mutants, is not compatible with the previous suggestion that its analogue 5a non-specifically inactivates IDO1 by irreversible oxidation (see Fig. 5.5 and section 5.2.3.1). A conciliatory possibility is that wtIDO1 might be more susceptible to oxidative inactivation than S167A.

In conclusion, S167 but not C129 can be reliably used for the structure-based design of IDO1 inhibitors.
In a final aspect, we aimed to develop an assay to measure recombinant human TDO (rhTDO) activity as part of an overall goal to identify dual IDO1/TDO inhibitors. This aim was not completed, however, due to time constraints for completion and submission of the thesis.

7.1 Introduction

IDO1 is a well-established target for immunomodulation in cancer, but recently, TDO is emerging as an equally important target (Opitz et al., 2011, Pilotte et al., 2012). Expression of TDO in tumour cells accelerates their growth in mice, and the prototypic TDO inhibitor LM10, has been shown to slow this tumour growth. Similar to that by IDO1 inhibitors, inhibition of tumour growth by LM10 is seen only in mice with functional lymphocytes (Pilotte et al., 2012) indicating that TDO suppresses antitumour immunity. In a broad range of 104 human tumour cell lines, 20 expressed only TDO, 17 only IDO1, and 16 both TDO and IDO1. This suggests that dual IDO1/TDO inhibitors would be able to target a significantly higher proportion of tumours than inhibitors specific to IDO1 alone (Pilotte et al., 2012). The number of studies on dual IDO1/TDO inhibitors is currently limited, despite the keen interest. This is due in part to the instability of mammalian TDOs in the purified form (Batabyal & Yeh, 2009, Meng et al., 2014, Ren et al., 1996, Schimke, 1970, Schutz & Feigelson, 1972). Human TDO (hTDO) can be partly stabilised by truncation of both N- (Δ17 residues) and C-termini (Δ18 residues) (Batabyal & Yeh, 2009). These residues are not present in bacterial TDOs and may not be essential for the activity of the human enzyme as well (Batabyal & Yeh, 2009, Forouhar et al., 2007). It is not clear if truncated hTDO can fully substitute for the wild-type hTDO, but truncated hTDO shows comparable kinetics (K_m and k_cat) to wild-type enzyme in respect to l-TRP (Meng et al., 2014), although d-TRP appears a better substrate for truncated hTDO. Truncated hTDO shows activity with concentrations of d-TRP as low as 1 mM (Batabyal & Yeh, 2007), but wild-type hTDO has very little activity with d-TRP at 50 mM (Basran et al., 2008). In addition, wild-type hTDO expressed in HeLa cells produces negligible amounts of d-KYN after 3 days of incubation with d-TRP (Schmidt et al., 2009). This suggests that the binding of d-TRP to the truncated hTDO is affected by the truncation and the binding of inhibitors could be affected as well. A striking example is seen with HA, a
potent IDO1 inhibitor from which the clinical candidate INCB024360 was derived (Liu et al., 2010, Yue et al., 2009). Whilst HA shows similar potencies against truncated rhTDO (IC₅₀ 41 nM) and wild-type rhIDO1 (IC₅₀ 16 nM) in enzyme assays (Seegers et al., 2014), it inhibits wild-type hTDO (IC₅₀ 70 μM) 1170-fold less potently than hIDO1 (IC₅₀ 0.06 μM) in cell-based assays (Röhrig et al., 2012). Yue and colleagues also reported a decreased potency of HA against hTDO in cell-based assay (IC₅₀ > 10 μM) (Yue et al., 2009). HA inhibits truncated hTDO more efficiently than wild-type TDO suggesting that the active site of truncated hTDO has been altered by a missing N-terminal sequence. The crystal structure of wild-type hTDO without the active site only has been determined (Meng et al., 2014). The structure of wild-type Drosophila melanogaster TDO, which has 60% sequence identity and high structural similarity to hTDO (root mean square deviation 1.4 Å), shows that the N-terminal residues of neighbouring TDO monomers indeed participate in formation of the neighbouring active site (see Fig. 1.2) (Huang et al., 2013).

7.2 Results

Whilst truncated hTDO has better solubility, the concerns that this truncation may alter the active site prompted us to express full-length hTDO carrying 6xHis tag at the C-terminus. Two constructs for bacterial expression were tested (Fig. 7.1). Both contain an identical N-terminus but the C-terminus of Construct 1 had a 6xHis tag and a stop codon immediately after the last amino acid (Asp₄₀₆) of the wild-type hTDO sequence. Construct 2 utilised the C-terminal tags of the expression vector (6xHis and Strep-Tag II) making it 19 amino acids longer than Construct 1 (Fig. 7.1).

**Figure 7.1.** Schematic representation of two different rhTDO inserts subcloned into Novagen pET-62-DEST bacterial expression vector. Abbreviations: attB1 and attB2 (Gateway cloning recombination sites), RBS (ribosome binding site), 6xHis (hexa-histidyl tag), Stop (stop codon).
Chapter 7- Results

The expression of these constructs was each examined in the two *E. coli* bacterial strains, BL21(DE3) and BL21(AI). The expression and purification protocol was based in part on the procedure of Dolusic and colleagues (Dolusić et al., 2011). The bacterial lysates were induced to express rhTDO, lysed, concentrated on Ni-NTA resin and then subjected to SDS-PAGE and enzyme activity assays.

Expression of rhTDO from Construct 1 is significantly higher than that from Construct 2 (Fig. 7.2a). The BL21 (DE3) strain produced slightly more rhTDO than BL21 (AI) but the expression conditions have yet to be optimised. The molecular weight of rhTDO expressed from Construct 1 was ~ 40 kDa, similar to rhIDO1, but not consistent with the expected 48.7 kDa calculated from the gene sequence of the Construct 1 (Fig. 7.2a; see also Fig. 7.1).

The enzymes used for SDS-PAGE were extracted from Ni-NTA resin as described in section 2.1.9.1 of Materials and Methods, and assayed using the PIP fluorescence assay. The amount of activity of the extracted enzymes correlated well with the intensity of the bands observed on the SDS-PAGE (Fig. 7.2b). rhTDO expressed from Construct 1 exhibited nearly 10-fold higher activity than Construct 2 (based on the activity of the lysates at 100x dilution), and a 10-fold dilution of enzyme samples produced the expected 10-fold decrease in enzyme activity. The activity of purified rhIDO1, spiked into the lysis buffer at 1 μM and processed identically as the rhTDO lysates, was ~ 26-fold lower compared to the enzyme activity of a fresh rhIDO1 at 1 μM (Fig. 7.2b). This indicated the purification procedure caused a significant degradation of the enzymes, although their relative activity was still determinable.

Since rhTDO expression from Construct 1 was superior to Construct 2, a scale-up preparation of rhTDO was attempted using Construct 1 in bacterial strain BL21(DE3) (please see methods section 2.1.9.3 for details). The expression and purification procedure was similar to that of rhIDO1 but with the inclusion of 5 mM L-TRP to stabilise the enzyme. Cells were harvested and lysed using a French Press, followed by purification on a Ni-NTA column. This preparation produced a dark red fraction, presumed to contain active haem-containing rhTDO. The red fraction was loaded onto a size exclusion column to remove small-molecules (TRP, imidazole, salts) and was then concentrated on a spin concentrator. After 30 min of concentration, the original red solution turned colourless and the membrane of the concentrator turned red. The rhTDO had obviously precipitated on the membrane, making it nearly impossible to recover the enzyme.
Figure 7.2. Expression and enzymatic activity of rhTDO isolated from bacterial strains BL21(DE3) and BL21(AI) transformed with expression vectors rhTDO Construct 1 and rhTDO Construct 2. a) Acrylamide gradient gel (4-15%) showing lysates of rhTDO expressing bacteria concentrated on Ni-NTA resin. Lane rhIDO1 spiked (1 μM) is a purified rhIDO1 that was added into the lysis buffer and processed identically as the rhTDO samples. b) Enzyme activity of samples in a) extracted from Ni-NTA resin and assayed by PIP fluorescence assay. The enzyme activity is expressed as a fluorescence signal of the enzyme sample divided by a fluorescence signal of the blank lysis buffer. Note that spiked rhIDO1 (1 μM) sample was concentrated by a factor of ~ 6 during extraction from Ni-NTA agarose.
7.3 Discussion

Our data shows that the length of the C-terminal sequence of rhTDO, i.e. amino acids replacing stop codon of wild-type hTDO, considerably affects the expression of the enzyme in bacterial cultures. Extension of C-terminus from 6 to 25 amino acids decreased protein expression and lowered enzyme activity 10-fold. The likely reason is the formation of rhTDO inclusion bodies in bacterial cells; however, their presence was not experimentally investigated. It is also possible that the specific DNA sequence at the C-terminus of Construct 2 causes errors during transcription or translation, or the transcript could simply have low stability. Both the rhTDO expressed from Construct 1 and previously purified rhIDO1 migrated at molecular weight ~ 40 kDa which is 20% lower than the expected value calculated from their respective gene sequence (rhIDO1 - MW 47 kDa, rhTDO Construct 1 – 48.7 kDa). It is very unlikely that the proteins are 7 kDa shorter, as that would result in a complete loss of enzyme activity. This anomalous migration has previously been observed with dioxygenase enzymes. Recombinant human IDO2 and native IDO1 isolated from human placenta or rabbit intestine are all expected to have molecular weights > 40 kDa, but have been shown to migrate to ~ 40 kDa (Meininger et al., 2011, Shimizu et al., 1978, Takikawa et al., 1988) consistent with the observations in this thesis. Likewise, recombinant full-length rat liver TDO migrated unexpectedly slow on 9% linear acrylamide gel (Ren et al., 1996). On the other hand, in the studies by Littlejohn and colleagues and by Austin and coworkers (Austin et al., 2004, Littlejohn et al., 2000), rhIDO1 migrated at the expected ~ 47 kDa position on 12% linear gels using identical running conditions to the ones used in the current work. The inconsistencies could stem from using different protein standards (not specified in recombinant human IDO1 studies) or from the gradient gels used in the work presented in this thesis. To confirm the identity of the enzymes, mass spectrometry or amino acid sequencing will need to be used.

The purification procedure of full-length recombinant human TDO was successful up to the point when the eluate from Ni-NTA column was concentrated in a spin concentrator, where it aggregated on the membrane. This suggests that TRP is critical for stabilisation of human TDO and its removal is deleterious as previously shown by various groups (Batabyal & Yeh, 2009, Meng et al., 2014, Ren et al., 1996, Schutz & Feigelson, 1972). However, Basran and colleagues was able to purify full-length hTDO using similar conditions to ours without using L-TRP and they concentrated rhTDO using a regenerated cellulose membrane concentrator (Microcon YM-30, MWCO 30 kDa) (Basran et al., 2008). A
Chapter 7 - Discussion

concentrator with a polyethersulfone membrane (Vivaspin 20, MWCO 30 kDa) was used in the current work, which may explain the aggregation of rhTDO. Surprisingly, the report of Basran and colleagues is the only description of successful purification of full-length rhTDO (Basran et al., 2008). Other groups have used full-length rhTDO without purification, i.e. only desalted bacterial lysate (Dolušić et al., 2011, Pilotte et al., 2012) or hTDO transfected cell lines. Substitution of the membrane type or avoiding concentration step altogether might be viable options to try in future studies to solve the aggregation problem. hTDO expression vectors for transfection of mammalian cells have been constructed and sequenced in readiness for preparing a cell-based assay to measure hTDO activity. However, due to time constraints for completing this thesis, transfection of cells has yet to be carried out.
8.1 Introduction

IDO1 is a potent immune-suppressive enzyme co-opted by a broad range of cancers in order to escape immune-mediated destruction and promote malignant cancer growth. This has led to the recent intense interest in the identification and development of IDO1 inhibitors to reverse the tumour-mediated immune suppression. The work in this thesis has contributed significantly to the overall programme at the Auckland Cancer Society Research Centre to discover and develop novel IDO1 inhibitors as potential anti-cancer agents. In this final chapter, the research outcomes and future directions are presented.

8.2 Research Outcomes

8.2.1 Development of an automated, sensitive assay for IDO1

The overall aim in Chapter 3 was to develop an automated and sensitive IDO1 enzyme assay for use in screening of the compound libraries, for the identification and evaluation of potential leads for the drug development programme. Whilst a number of assays were in existence, they all had limitations. The radiometric and the HPLC assay are not suitable for a high-throughput screening format. The low sensitivity of absorbance TCA assay and previous NaOH fluorescence assay limits miniaturisation; moreover, high interference from test compounds in the TCA absorbance assay increases the number of false positives. Although the Bridge-It® TRP fluorescence assay and recently introduced NFK GreenScreen™ fluorescence assay offer a good sensitivity, the price is high. The new fluorescence enzyme assay developed in the initial part of this work was able to incorporate many of the more desirable properties of the other assays into the single procedure. It is economical, making use of a widely available and inexpensive reagent, piperidine, added in a single step, easy to execute protocol. The superior sensitivity of the assay allowed for its miniaturisation and use in high-density microplates. The assay was used in the work in this thesis in a 384-well format, but it should be possible with a minor technical modification to the robotic workstation setup to allow testing of compounds in the 1536-well format in volumes as small as 5 μL. Of note, the low interference from test compounds may potentially allow discovery of wider spectrum of chemical classes of IDO1/TDO inhibitors.
One possible drawback to the PIP assay is that it requires NFK as a standard. NFK is no longer commercially available, as it was withdrawn from the market due to stability issues. We prepared NFK easily from commercially available KYN, and surprisingly, we did not observe significant degradation of NFK either in the powder form or in solution (pH 7 buffer) over 1 year.

8.2.2 Identification of the structure and formation of the new fluorophore PIP-THQ

In the development of the new fluorescence assay, a novel class of fluorophores formed in a previously unreported reaction between cyclic amines and NFK was discovered and this work is presented in Chapter 4.

The proposed structure of the novel fluorophore PIP-THQ was derived from a comprehensive set of 1D and 2D NMR, UV/VIS and mass spectrometry studies. The purity of PIP-THQ observed in NMR experiments and the striking agreement of results obtained from the different types of spectroscopic analyses gives confidence in the proposed structure of PIP-THQ. The precise molecular mechanism of PIP-THQ formation, however, remains a fascinating open question. Specifically, and considering that the reaction occurs in the absence of catalysts and harsh reaction conditions; the reactivity of the carbon on the 2-position of the piperidine ring is hard to explain. Although transamidations can proceed catalyst-free at ambient temperature, the production of 2-substituted piperidines without catalysts has not previously been reported.

Importantly, in determining if substituted piperidines could also form fluorophores, it emerged that using 3-MePIP increased the fluorophore yield relative to PIP; and replacing PIP with 3-MePIP could therefore provide an IDO1 enzyme assay with even greater sensitivity than the current one used in this project. This avenue has not yet been pursued, however.

8.2.3 Use of the PIP fluorescence assay for identification of IDO1 inhibitors

We used the automated PIP fluorescence assay in the work in chapter 5 to screen the NCI library (1,597 compounds) for novel IDO1 inhibitors; and to validate the hits acquired from both the NCI library and those previously obtained by the ACSRC from the screening of 40,000 compounds from the WEHI library. Validated hits were then characterised for their suitability as chemical leads for drug development.
The NCI library provided an unusually high hit rate (2.2%), due to the high content (~37%) of quinones, a chemical class that is known to contain IDO1 inhibitory activity. That our assay accurately identified known IDO1 inhibitors in the library was encouraging, and gave us confidence in the screen, which identified several other chemical classes with activity but had not previously been reported in the literature as IDO1 inhibitors. All compounds showing strong IDO1-inhibitory activity in the screens were rigorously put through a number of different filters that would detect those considered unsuitable for drug development. Two compounds emerged from the screening of the NCI library; indolonozone 5e and pyrimidinone 4, as being excellent candidates for optimisation by rational medicinal chemistry for IDO1 inhibitory activity in a drug development programme. Both compounds failed only 1 computational structural filter. Both shows good cell penetration to reach the cytoplasmic IDO1, but negligible toxicity to the cells. They were reversible non-aggregating inhibitors and did not covalently modify IDO1. Whilst 4 and 5e are ~50-fold less potent than the optimised IDO1 inhibitors HA or NLG919, their potency is comparable to that of the original parent hit from which HA was derived. Also of relevance, both 4 and 5e have not previously been reported to be IDO1 inhibitors. Currently, however, due to the limited number of analogues available for purchase, the SAR around these two compounds has not been extensively characterised.

From the initial list of 30 hits obtained from the screening of the WEHI library, three chemical classes were selected for further evaluation. Two classes were subsequently de-prioritised following initial SAR studies (A/ Prof B. Palmer, personal communications), and subsequent work focussed on WEHI hit 3 (8). This compound has not previously been claimed as an IDO1 inhibitor, providing the clear freedom to operate. Studies in chapter 4 showed that compound 8 has excellent cell permeability and was 27-fold more potent in a cell-based assay compared to the enzyme assay. Toxicity to cells was not observed at concentrations that provided 100% inhibition of IDO1 activity. The potency of the non-optimized hit 8 in the cell-based assay is comparable to that of the optimised clinical candidate NLG919. Studies in this section also showed inhibitor 8 to be a slow irreversible inactivator of IDO1. An analogue of the Incyte clinical candidate INCB024360 (HA) was shown in this study to have a similar mode of inhibition; suggesting that slow irreversible inactivation of the IDO1 enzyme might be a mechanism of action common to potent IDO1 inhibitors.
8.2.4 Importance of serine-167 and cysteine-129 for the binding of IDO1 inhibitors

Studies in chapter 6, examined the importance of serine-167 and cysteine-129 in the IDO1 active site for interaction with ligands. A role for both these residues for binding to ligands have been implicated in computational studies. However, experimental evidence of their interaction with inhibitors is not available. In the studies of chapter 6, we obtained alanine replacement mutants of serine-167 (S167A) and cysteine-129 (C129A) in the IDO1 active site and screened the entire NCI library for inhibitory activity against each mutant IDO1 compared to that against the wild-type enzyme.

The results clearly showed the involvement of serine-167 but not that of cysteine-129 in the interaction with a wide range of IDO1 inhibitors. However, it should be noted that the S167A IDO1 mutant showed a lower catalytic activity than the wild-type IDO1, which may have biased the results. Structure-activity studies of the three IDO1 inhibitors with sensitivity to the S167A mutation suggested that the binding to serine-167 is a result of hydrogen bonding of polar substituents on the IDO1 inhibitors. Additional experimental evidence would be needed to validate the interaction of the inhibitors with serine-167 of IDO1. Although molecular docking of the serine-167 sensitive inhibitors to the IDO1 active site may generate useful hypotheses about the inhibitor binding, it would not provide any real evidence for the binding to serine-167. The molecular docking approach is further hampered by the unavailability of the S167A IDO1 crystal structure. On the other hand, X-ray crystallography of the S167A IDO1 mutant bound by inhibitors would be the preferred experimental platform for investigating the interaction of the serine-167 sensitive inhibitors with the IDO1 active site.

Overall, the data from these studies highlight the importance of the interaction with the serine-167, which will sharpen the rational design of IDO1 inhibitors.

8.3 Future work

The work achieved for this thesis has contributed significantly to the programme at the ACSRC for the development of IDO1 inhibitors for cancer therapy. At the same time, the studies have raised a number of intriguing research questions that should be addressed in further investigations. These follow-on studies are discussed in this final section of the concluding discussion.


8.3.1 Resolving the mechanism of IDO1 inactivation by compound 8

Compound 8 and HA were shown to irreversibly inactivate IDO1 enzymatic activity. Irreversible inactivation has not previously been described for IDO1 inhibitors, and yet, would be one of the more desirable modes of inhibition to have in a therapeutic agent due to the potential longevity of the enzyme blockade. It would be worthwhile to identify the precise mechanism as to how compound 8 inactivates the IDO1 enzyme. These studies will entail mass spectrometry of the inhibitor-treated IDO1 and haem, followed by UV/VIS spectroscopy, as well kinetic analyses. Other potent IDO1 inhibitors including N,N-disubstituted ureas, imidazothiazoles, or aminonitriles should also be investigated to elucidate if they also inhibit IDO1 enzymatic activity by a similar mechanism.

Another property of compound 8 that should be investigated is the basis for its improved activity against the IDO1 expressed in cells compared to the purified enzyme. A possible explanation is that the compound is actively transported into, and accumulated inside the cell. Determining the concentration of 8 over time inside the cell compared to that in the culture medium, together with identification of potential transporter proteins in the cellular membrane would be informative. Since many transporters are ion-dependent, the variation of extracellular ion concentrations or competition studies with known substrates of high-affinity transporters could be valuable. Of interest, 3-aminoisoxazolopyridines, which are structurally related to 8, have been patented as analgesics, and act as agonists of the KCNQ2/3 K⁺ gated channels (Kühnert et al., 2010). An alternative explanation proposes that cells can metabolise compound 8 to a more potent inhibitory molecule. LC-MS could be applied to cellular homogenates to identify the metabolites of 8.

8.3.2 Development of optimised analogues of compounds 4 and 5e

Compounds 4 and 5e identified in the screening of the NCI library emerged from the studies in chapter 5 as being the most suitable for use as chemical leads for development as IDO1 inhibitors. Analogues of the novel hit compound should be optimised by rational drug design for improved potency, ligand-binding efficiency, metabolic stability, oral bioavailability and water solubility. Increasing the size of the molecules might be a successful strategy to enhance the potency and selectivity for IDO1, as a larger molecule may be able to interact with more residues in the IDO1 active site and also limit the binding to other haem-containing proteins such as cytochrome P450 that may cause off-target toxicities and poor pharmacokinetics. The interaction with the serine-167 in the IDO1 active should be designed
into IDO1 inhibitors, as we have shown in the work in chapter 6 to be important for the activity of a wide range of IDO1 inhibitors. Pursuant to the studies in chapter 5, we suggest that conjugation to glutathione might also provide a novel approach to increasing the potency and selectivity of IDO1 inhibitors.

### 8.3.3 TDO/IDO1 dual inhibitors

There is intense interest from both academia and industry to develop dual TDO/IDO1 inhibitors due to the attractive prospect of the dual inhibitors being able to target a broader range of clinical cancers than each of the mono-specific inhibitor alone. Studies on dual TDO/IDO1 inhibition are limited, and it is not clear whether dual inhibition may be associated with side effects not encountered with IDO1 inhibition alone, due to the differential tissue distributions and homeostatic roles of the two dioxygenases (Batabyal & Yeh, 2007, Thackray & Chapman, 2008). TDO maintains the homeostasis of TRP and TDO-knockout mice have ~10-fold higher TRP concentrations in the blood and elevated serotonin levels compared to the wild-type counterparts (Kanai et al., 2009). The elevated levels of TRP and serotonin affect the anxiety-related behaviour of mice, but may not pose a major risk for using TDO inhibitors in humans.

Most of the dual IDO1/TDO inhibitors that have been identified from screening of compound libraries have been quinones and natural products, and would not be the most suitable leads for drug development due to their redox activity and potential toxicity to cells (Pantouris & Mowat, 2014). In the work in chapter 7, we made an effort to establish a TDO enzymatic assay for use to screen the NCI library for TDO inhibitors and dual TDO/IDO1 inhibitors. We were unable to complete the work for this aim in the time frame of this thesis, due to the difficulties posed by the instability and precipitation of the full-length recombinant human TDO during the purification procedure. In further work, to circumvent this problem, we would try omitting the concentration step all together and use non-purified TDO in a bacterial lysate. A disadvantage of using non-purified preparations of the enzyme is that the presence of various proteins and other bacterial components in the lysate could interfere with the inhibitory activity of test compounds and their binding to TDO. We could also try using spin concentrators with a different type of membrane to the ones used in the work in chapter 7. Basran and colleagues have successfully concentrated full-length human TDO using spin concentrators with cellulose-based membranes (Basran et al., 2008). Alternatively, at the cost of a lower throughput, we can screen for TDO inhibitors using a cell-based assay instead of an isolated enzyme assay. Constructs for engineering tumour lines to constitutively express
human TDO have been prepared, but have yet to be used for transfections. Once these engineered TDO-expressing lines have been established, we propose, firstly, to test our identified hits with IDO1 inhibitory activity for inhibition of human TDO in the cell-based assay to separate out those with dual activity from those with IDO1 or TDO inhibitory activity only.

8.3.4 Combination therapy of IDO1 inhibitors plus other cancer therapies

Whilst IDO1 inhibitors have shown modest antitumour activity as monotherapy, there is an increasing evidence of synergistic antitumour effects when IDO1 inhibitors are given in combination with other treatment modalities such as radiotherapy and chemotherapy (Hou et al., 2007, Li et al., 2014, Muller et al., 2005a, Wainwright et al., 2014) (see section 1.12). Moreover, tumour mediated immune suppression represents a major stumbling block to anticancer vaccines and adoptive T cell immunotherapies. Current approaches to overcoming the tolerogenic environment of patients before receiving immunotherapies include total body lymphodepletion or multiple-dosing with cyclophosphamide (Barrett et al., 2014, Maus et al., 2014). The use of IDO1 inhibitors might be a better tolerated and less invasive approach to restoring the functioning immune system to patients with cancer. The most exciting use of IDO1 inhibitors is in combination with immune checkpoint blockades; anti-CTLA-4 antibodies and anti-PD-1 antibodies. An increasing number of preclinical studies have established the ability of these combinations to induce complete long-term regression of tumours (Holmgaard et al., 2013, Spranger et al., 2014, Wainwright et al., 2014).

As different chemical classes of IDO1 inhibitors may exhibit different synergistic potential with various other therapies, it would be important to evaluate which therapies combine best with the class of IDO1 inhibitors that the ACSRC wish to develop. At the time of writing, WEHI hit 3 (8) is the most advanced in terms of analogue development, pharmacology and in vivo antitumour activity testing. Pilot studies have shown that daily dosing of compound 8 causes a modest growth delay against a murine lung carcinoma model and a murine glioma model (LM Ching, personal communications). When compound 8 is given in combination with the immune checkpoint blockades; complete long-term regressions of the tumours can be obtained in the majority of the treated mice.
8.4 Final summary

In summary, in this project:

- a novel, robust and sensitive fluorescence assay for detecting compounds with inhibitory activity against the rhIDO1 enzyme was developed;
- the chemical structure of the novel fluorophore and the reaction mechanism of its formation was characterised;
- the assay was miniaturised and used to efficiently and reproducibly assay compound libraries for novel IDO1 inhibitors;
- from the original list of hits identified from screening of the NCI library and the WEHI library, two NCI compounds (4 and 5e) and one WEHI compound (8) emerged as being suitable for use as leads for drug development programme;
- compound 8 inhibited IDO1 by an irreversible mechanism of inactivation of the enzyme that has not previously been reported for IDO1 inhibitors;
- the importance of serine-167 in the IDO1 active site in binding of a range of IDO1 inhibitors was established.

These findings have contributed to the selection of compound 8 as a chemical lead for the IDO1 inhibitor drug development programme at the ACSRC.
int Uf_Procedure_1( // 0=Normal; 3=Abort; 12=Stop Procedure char* pPCX ) // Address of procedure context information {
    int nRet = 0;                       // Load return value into nRet
    MP2_PROC_CONTEXT_DEF* pPC = pPCX;   // Cast pPCX into
    local procedure context ptr

    int Ctrl = 300;
    int CtrlDiv2 = 160;

    if ( Rt_CmpdNumber <= 10) // 10 or less compounds
    {
        Rt_Ctrl_BufDMSO_Nbr = 7;
        Rt_Ctrl_BufDMSO_Start = ((Rt_CmpdNumber) * 7) + 1;
        Rt_TopBuf_Nbr = Rt_CmpdNumber + 1;
    }

    if ( (Rt_CmpdNumber+1) <= 12) // 12 or less compounds with
    control
    {
        Rt_MovePlate1 = 1;
    }

    if ( (Rt_CmpdNumber+2) > 21) && ( (Rt_CmpdNumber+2) <= 24 ) // 21-24 compounds
    {
        Rt_MovePlate1 = 1;
        Rt_MovePlate2 = 1;
    }

    return nRet;
} // End of Uf_Procedure_1()
Figure A. 2. JANUS script for IC\textsubscript{50} automated protocol (part 2)

```c
// Appendix

int Uf_Procedure_1( // 0=Normal; 3=Abort; 12=Stop Procedure
    char*  pPCX )  // Address of procedure context information
{
    int nRet = 0;  // Load return value into nRet
    MP2_PROC_CONTEXT_DEF* pPC = pPCX;  // Cast pPCX into
    local procedure context ptr

    int controls = 32;
    int halfcontrol = controls / 2;

    // 1) number of cmpds to process multiplied by 8 dilutions
    Rt_nNbrOfSamples_S1 = (Rt_CmpdNumber * 8);

    // if (Rt_CmpdNumber <= 10)
    if (Rt_CmpdNumber <= 10)
    {
        Rt_bUseThis_S2 = 1;
        Rt_IDONbr1 = ( (Rt_CmpdNumber * (halfcontrol)) + (halfcontrol * 1) );
        Rt_VehNbr = ( (Rt_CmpdNumber * (halfcontrol)) + (halfcontrol * 1) );
        Rt_IDO1 = 1;
        Rt_PIP1 = 1;
    }

    if ((Rt_CmpdNumber > 10) && (Rt_CmpdNumber <= 20))
    {
        Rt_bUseThis_S2 = 1;
        Rt_IDONbr1 = ( (10 * (halfcontrol)) + (halfcontrol * 1) );
        Rt_IDONbr2 = ( ((Rt_CmpdNumber - 10) * (halfcontrol)) + (halfcontrol * 1) );
        Rt_VehNbr = ( (Rt_CmpdNumber * (halfcontrol)) + (halfcontrol * 2) );
        Rt_IDO1 = 1;
        Rt_IDO2 = 1;
        Rt_PIP1 = 1;
        Rt_PIP2 = 1;
    }

    if ((Rt_CmpdNumber > 20) && (Rt_CmpdNumber <= 30))
    {
        Rt_bUseThis_S2 = 1;
        Rt_bUseThis_S7 = 1;
        Rt_IDONbr1 = ( (10 * (halfcontrol)) + (halfcontrol * 1) );
        Rt_IDONbr2 = ( (10 * (halfcontrol)) + (halfcontrol * 1) );
        Rt_IDONbr3 = ( (10 * (halfcontrol)) + (halfcontrol * 1) );
        Rt_IDONbr4 = ( ((Rt_CmpdNumber - 30) * (halfcontrol)) + (halfcontrol * 1) );
        Rt_VehNbr = ( (Rt_CmpdNumber * (halfcontrol)) + (halfcontrol * 4) );
        Rt_IDO1 = 1;
        Rt_IDO2 = 1;
        Rt_IDO3 = 1;
        Rt_IDO4 = 1;
        Rt_PIP1 = 1;
        Rt_PIP2 = 1;
        Rt_PIP3 = 1;
        Rt_PIP4 = 1;
    }
    //
    // if (Rt_CmpdNumber <= 10)
    if (Rt_CmpdNumber <= 10)
    {
        Rt_bCtrlBuf_Nbr = 8;
        Rt_bCtrlBuf_Start = ((Rt_CmpdNumber * 8) + 1);
        Rt_RFU2_2 = 0;
        Rt_RFU1Drop = 1;
    }

    if ((Rt_CmpdNumber > 10) && (Rt_CmpdNumber <= 20))
    {
        Rt_bCtrlBuf_Nbr = 8;
        Rt_bCtrlBuf_Start = ((Rt_CmpdNumber * 8) + 1);
        Rt_RFU2_2 = 1;
        Rt_RFU2Drop = 1;
    }

    if ((Rt_CmpdNumber > 20) && (Rt_CmpdNumber <= 30))
    {
        Rt_bCtrlBuf_Nbr = 8;
        Rt_bCtrlBuf_Start = ((Rt_CmpdNumber * 8) + 1);
        Rt_RFU2_2 = 1;
        Rt_RFU3_4 = 1;
        Rt_RFU4_4 = 0;
        Rt_RFU3Drop = 1;
    }

    if ((Rt_CmpdNumber > 30) && (Rt_CmpdNumber <= 40))
    {
        Rt_bUseThis_S2 = 1;
        Rt_bUseThis_S7 = 1;
        Rt_IDONbr1 = ( (10 * (halfcontrol)) + (halfcontrol * 1) );
        Rt_IDONbr2 = ( (10 * (halfcontrol)) + (halfcontrol * 1) );
        Rt_IDONbr3 = ( (10 * (halfcontrol)) + (halfcontrol * 1) );
        Rt_IDONbr4 = ( ((Rt_CmpdNumber - 30) * (halfcontrol)) + (halfcontrol * 1) );
        Rt_VehNbr = ( (Rt_CmpdNumber * (halfcontrol)) + (halfcontrol * 4) );
        Rt_IDO1 = 1;
        Rt_IDO2 = 1;
        Rt_IDO3 = 1;
        Rt_IDO4 = 1;
        Rt_PIP1 = 1;
        Rt_PIP2 = 1;
        Rt_PIP3 = 1;
        Rt_PIP4 = 1;
    }

    return nRet;
    // End of Uf_Procedure_1()
```
Appendix

Figure A. 3. HPLC chromatograms of the synthesised compounds. $R_t$ and Area% in tables indicate retention time of individual peaks in the chromatogram and their percent peak areas (relative to sum of all peaks), respectively. Compound name is indicated in the upper left corner of each chromatogram. The yellow background marks the data for each analysed compound.
Appendix

Chromatographic analyses for Figure A. 3
Instrument: Agilent 1100 HPLC. Column: Luna C\textsubscript{18} (5 µm, 100 Å, 150 x 2 mm; Phenomenex, Torrance, CA, USA). Mobile phases: A (25 mM formic acid, ~ pH 2.7), B (80% MeCN v/v), C (35 mM NH\textsubscript{4}HCO\textsubscript{3}, pH 8).

- analysis of NFK, PIP-KYN, NFK-CKA, KYN-CKA: Binary gradient A/B (13 min): 0 min (5% B), 5 min (62.5% B), 5.5 min (90% B), 7.5 min (90% B), 8.5 min (5% B), 13 min (5% B). Flow rate 0.45 mL/min, injection 5 µL, 40°C

- analysis of PIP-THQ: Binary gradient A/B (23 min): Binary gradient A/B (23 min): 0 min (6% B), 5 min (25% B), 20 min (60% B), 22 min (60% B), 23 min (6% B). Flow rate 0.45 mL/min, injection 5 µL, 40°C

- analysis of 3-MePIP-THQ: Binary gradient C/B (13 min): 0 min (5% B), 5 min (62.5% B), 5.5 min (80% B), 7.5 min (80% B), 8.5 min (5% B), 13 min (5% B). Flow rate 0.45 mL/min, injection 5 µL, 30°C
Figure A. 4. a) $^1$H NMR and b) $^{13}$C NMR spectrum of PIP-THQ.
Appendix

Figure A.5. $^1$H-$^1$H Correlation spectroscopy NMR spectrum of PIP-THQ.
Appendix

Figure A. 6. $^1$H-$^1$H total correlation spectroscopy NMR spectrum of PIP-THQ.
Appendix

Figure A. 7. $^1\text{H}-^{13}\text{C}$ HMBC NMR spectrum of PIP-THQ.
Figure A. 8. $^1$H-$^{13}$C Heteronuclear single-quantum correlation spectroscopy NMR spectrum of PIP-THQ.
Appendix

Figure A. 9. $^1$H-$^1$H NOESY NMR spectrum of PIP-THQ.
Appendix

Figure A. 10. $^1$H-$^{15}$N Heteronuclear single-quantum correlation spectroscopy NMR spectrum of PIP-THQ.
Figure A. 11. HPLC chromatograms of 2 mM a) N-(4-nitrophenyl)formamide (MW166), b) methyl 2-formamidobenzoate (MW179) and c) L-tryptophan (L-TRP) acquired before (black line) and after (red line) incubation with 1 M piperidine (PIP) at 65°C for 20 min. Detection wavelength was 240 nm (a,b) and 254 nm (c). Analyses were carried out as described in Materials and methods section 2.2.2 but the gradient for separation in a) and b) was: 0-5 min (2.5 – 10% A), 5 – 22 min (10 – 100% A), 22 -26 min (100% A), 26 – 27 min (100 – 2.5% A), 27 – 30 min (2.5% A). Sample of MW179 was found to be partially hydrolysed into MW151 before addition of PIP. Note that hydrolysis was the only observed reaction of MW166 and MW179. TRP did not react with PIP.
Figure A. 12. Mass fragmentation spectra of a) PYR-THQ, b) 3-MePIP-THQ and c) PIP-THQ in positive electrospray ionisation mode (QqQ). PIP-THQ and 3-MePIP-THQ were injected as pure compounds, PYR-THQ was injected as a crude evaporated reaction of NFK and PYR redissolved in H₂O. Collision cell voltage was 10 V. Blue diamond marks the ion being fragmented.
Figure A. 13. $^1$H NMR spectrum of 3-MePIP-THQ.
Figure A.14. $^1$H-$^1$H Correlation spectroscopy NMR spectrum of 3-MePIP-THQ.
Appendix

Figure A. 15. $^1$H-$^1$H NOESY NMR spectrum of 3-MePIP-THQ.
Figure A. 16. Frequency distribution of total number of biological test results deposited in PubChem database amongst a) NCI library compounds and b) NCI hits. Column statistics was calculated in Prism v 6.03 (GraphPad, La Jolla, CA, USA). Note that close to 50% of NCI library compounds (n = 742) displayed between 600-700 biological test results in PubChem database. Only 3.75% (n = 60) of NCI library compounds showed less than 100 test results. Only 3 out of 35 hits (NSC 344494, hydroxyamidine HA and 5) displayed less than 100 test results.

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</tr>
<tr>
<td>Median</td>
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<td>75% Percentile</td>
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<table>
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<tr>
<td>Actual confidence level</td>
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<tr>
<td>Lower confidence limit</td>
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Figure A. 17. Frequency distribution of percentual activity of a) NCI library compounds and b) NCI hits, deposited in PubChem database. Percentual activity for each compound was calculated as „number of active outcomes indexed in PubChem“ divided by „number of total biological test results deposited in PubChem“ multiplied by 100. Note that the majority (n = 1212, 75.8%) of NCI library compounds was active in less than 2.53% of total biological test results on PubChem. Only 7 out of total 35 hits (80%) identified in this study displayed < 2.53% active outcomes out of total corresponding test results on PubChem (thus appears to be non-promiscuous inhibitors according to PubChem). These 7 hits included hydroxyamidine HA with no PubChem results and NSC 344494 with only 1 entry.
Figure A. 18. Inhibitory activity of a) known IDO1 inhibitors and b), c) IDO1 inhibitors identified in this study, after 25 min preincubation with rhIDO1 (600 nM, 1% DMSO, 21°C) and 100-fold dilution into the complete assay buffer, respectively. Concentrations of compounds preincubated with rhIDO1 (before 100-fold dilution) are denoted in parentheses. For chemical structures of inhibitors (in **bold** typeface) please refer to Table 5.1 in the main text. Inhibitory activities were normalised to rhIDO1 preincubated with 1% DMSO and processed identically as inhibitor treated reactions. Assay was performed at 21°C. Data are representative experiments. Note: The activity of uninhibited (DMSO-treated) rhIDO1 determined at 90 min incubation was one half of the activity measured at 25 min indicating IDO1 was degrading relatively fast at the experimental conditions.
**Figure A. 19.** Inhibitory activity of NCI and WEHI hits against recombinant hIDO1 or native hIDO1 expressed in Lewis Lung carcinoma (LLTC) cells. LLTC cells were incubated with compounds (0.5% DMSO) for 24 hours at 37°C and L-kynurenine content and viability were determined from the same well. Activity and viability data are normalised against cells treated with 0.5% DMSO. Data are presented as a mean ± SD of technical triplicates.
Table A. 1. Chemical structures and properties of the thirty-five screening hits (compounds that inhibited rhIDO > 50% at 20 μM) obtained from the NCI Diversity Set III library.

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<tr>
<th></th>
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<th># failed filters</th>
<th>PubChem biological test results a</th>
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<th>active</th>
<th>% active</th>
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*a PubChem Biological test results: total (total number of test results deposited on PubChem), active (number of times the compound was reported as active), % active (active / total*100).

*b # failed filters (Number of filters that was failed by a compound (out of Glaxo, Blake, AlarmNMR, PAINS, PubChem BioAssays > 2.53%)).

c MW (moleculer weight in Daltons).

NSC (NCI compound idetifier)
### Table A.2. rhIDO1 inhibitory activity of benz(o)-imidazole/thiazole series.

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<thead>
<tr>
<th>ID</th>
<th>X₁</th>
<th>X₂</th>
<th>R₁</th>
<th>R₂</th>
<th>IC₅₀ (μM)</th>
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<td>2'</td>
<td>N</td>
<td>S</td>
<td>-</td>
<td>2'−OH</td>
<td>-2 ± 3% *</td>
</tr>
<tr>
<td>2'a</td>
<td>N</td>
<td>NH</td>
<td>-</td>
<td>2'−OH</td>
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<td>2'b</td>
<td>N</td>
<td>NH</td>
<td>5−CH₃</td>
<td>2'−OH</td>
<td>6 ± 5%</td>
</tr>
<tr>
<td>2'c</td>
<td>N−CH₃</td>
<td>N−CH₃</td>
<td>-</td>
<td>2'−OH</td>
<td>6 ± 3%</td>
</tr>
<tr>
<td>2'd</td>
<td>N</td>
<td>NH</td>
<td>-</td>
<td>-</td>
<td>-6 ± 2%</td>
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<tr>
<td>2'e</td>
<td>N</td>
<td>NH</td>
<td>-</td>
<td>2'−CH₃</td>
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<tr>
<td>2'f</td>
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<td>2'g</td>
<td>N</td>
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<td>2'h</td>
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<td>2'j</td>
<td>O</td>
<td>N</td>
<td>-</td>
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<td>-5 ± 1%</td>
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</table>

IC₅₀ was not reached in these experiments, therefore data show a mean ± SD of rhIDO1 inhibition at highest assayed concentration (400 μM) from at least two independent experiments.

*Inhibitory activity of IDO1 was determined using absorbance TCA assay due to high autofluorescence of the compound in the fluorescence assay.
### Table A. 3. Percent inhibition and apparent IC$_{50}$ of 18 NCI library compounds that showed significantly decreased inhibitory activity against S167A mutant IDO1.

<table>
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<tr>
<th>Structure</th>
<th>NSC #</th>
<th>percent inhibition at 20 μM</th>
<th>apparent IC$_{50}$ (μM)</th>
<th>Fold change</th>
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<td></td>
<td></td>
<td>wild-type IDO1</td>
<td>S167A mutant IDO1</td>
<td>wild-type IDO1</td>
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Apparent IC₅₀ was calculated using formula $IC_{50} = \frac{2000}{y} - 20$ where $y$ equals percent inhibition at 20 μM (see section 6.2.2, Equations 5 and 6) for details. Fold change was calculated by dividing apparent IC₅₀ of wild-type IDO1 by that of S167A mutant IDO1. Percent inhibition values ≤ 0 were converted to 1000 μM.
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