Inhibition of Herpes Simplex Virus 1 by Novel Heparan Sulfate Mimetics

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By
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Abstract

The identification and development of novel antiviral drugs is one of the most important areas of current medical research. Such therapeutics may serve to control treatment resistant diseases as well as having the potential to contain viral epidemics that occur in the future. Compounds which are able to inhibit herpes simplex virus type 1 (HSV-1) merit thorough investigation as this is a human pathogen that is globally distributed, highly prevalent and capable of producing severe morbidity.

The HSV-1 infection cycle begins with the attachment of a virion to a suitable host-cell, this initial attachment is facilitated by binding between viral attachment proteins (VAPs) on the surface of the virion and a class of host polysaccharides known as heparan sulfate glycosaminoglycans (HS GAGs). Of the compounds currently being investigated as potential HSV therapeutics, many are functional mimetics of HS GAGs. These compounds (by enlarge) act by binding to HSV-1 VAPs in the place of HS GAGs and thereby block the virions ability to attach to host-cells. These compounds are of special interest to virologists as HS GAGs serve as attachment receptors for numerous viruses, including significant human pathogens such as hepatitis B virus (HBV), human immunodeficiency virus (HIV) and server acute respiratory syndrome coronavirus-2 (SARS Cov-2).

This study, completed as part of a MSc through the University of Auckland, examines four synthetic HS GAG mimetics developed by the Ferrier Research Institute (FRI) and assesses their ability to inhibit HSV-1 infection. Viral inhibition was measured via plaque inhibition assay, qPCR and yield reduction assays. The first two of these assays indicated that the

compounds exerted varying levels of inhibition with the two compounds having tetrameric molecules showing promisingly high levels of HSV-1 inhibition corresponding with IC_{50} values less than 1μM. The yield reduction assay, while indicating some level of HSV-1 inhibition, failed to replicate the potent HSV-1 inhibition seen in the preceding assays.

The mode of action of the most efficacious of the four compounds was interrogated via experiments which measured the level of virion neutralisation (aka "virucidal effect"), attachment inhibition and entry inhibition produced by this compound. These assays revealed that the anti-HSV-1 effect of the compound could be entirely explained by its capacity to inhibit viral attachment. The compound in question also displayed an ability to inhibit virion entry to the host-cell cytoplasm, however there was no apparent correlation between the concentration of the compound and the degree of entry inhibition. Because of this it was inferred that the entry inhibition results were due to a down-stream effect of the binding between compound molecules and the HSV-1 VAPs (rather than being the direct result of interaction between the compound and the HSV-1 entry mechanism).

The observed disparity between the yield reduction results and other measures of compound efficacy were most unexpected. This anomaly may be partially due to the observed ability of HSV-1 infections from different MOIs to produce convergent yields at 24 hours post infection (24hpi). Additional possibilities are discussed in the final chapter of this thesis.

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Glossary

Term	Definition (for the purposes of this text)
3-O sulfated HS	The sub-group of all heparan sulfate glycosaminoglycan species that are defined by the constituent saccharide moieties having a sulfate group bound to the oxygen atom at the number three position of the sugar ring.
Acyclovir	The drug of first preference in the treatment of most HSV related conditions.
Aetiological agent	The agent responsible for producing an illness or infection.
Adsorption	The name given to the stage of viral infection which consists of free virions binding to the surface of a new host-cell via interaction between viral attachment proteins and attachment receptors located on the cell surface.
Adsorption period	The period of time at the at the beginning of an experimental infection during which cultured cells are exposed to growth media containing infectious virions. This media and any un-adsorbed virions therein are removed via aspiration at the conclusion of the adsorption period.
Affinity	The term used to define the strength of a non-covalent binding relationship between cognate bio-molecules, e.g. between a viral attachment protein and its receptor. High affinity interactions are defined by strong binding forces, the opposite is true for low affinity interactions.
Analogue	Used as a contraction of the term “functional analogue”: A chemical/molecule which is able to replicate some or all of the biological roles of a specific host molecule. E.g. The administered compound “*y*” triggers the same biological responses as host molecule “*x”* hence “*y”* is said to be an analogue of “*x”*.
Anti-herpetic	The property of inhibiting the replication of herpes viruses. For the purposes of this paper the term is always used in reference to the inhibition of HSV-1 and/or HSV-2.

ART	Antiretroviral therapy: a general term for modern drug regimens which supress HIV replication.
ASFV	African swine fever virus
Attachment	Synonymous with adsorption (see above).
Attachment receptor	A molecule or class of molecules expressed on the surface of host-cells that a given species/strain of virus binds to in order to initiate infection of a newly encountered host-cell.
Avidity	As per affinity except "avidity" is only applied to situations in which the binding interaction is multivalent. The sum of force vectors produced by multiple atomic-level binding interactions is the avidity of the gross binding interaction. Additionally, multiple low affinity interactions may create a high avidity interaction between two molecules.
Bioavailability	A measure of the relationship between the amount of compound administered to a live subject and the concentration of said compound in the target tissue. Bioavailability is always specific to the administration route e.g. the oral bioavailability of a given compound is rarely the same as the bioavailability produced by administering the compound intravenously or subcutaneously.
Biological replicate	An experimental replicate carried out in such a way that the biological entities in the experiment are totally independent. For the purposes of the current paper the cells used in separate biological replicates were both seeded and treated on different days from those in other biological replicates.
cDNA	Complementary DNA: DNA that forms a complimentary strand with the RNA of interest.
CDV	Canine distemper virus
Charge density	The net spatial-density of potential electrostatic bonding sites for a given molecule. Where two molecules have the same number of available dipoles the smaller molecule is said to have the higher charge density.
Cognate	Possessing complementary molecular motifs, i.e. when appropriately orientated cognate molecules readily form a "lock-and-key" type binding relationship.
Control relative	When conducting an experiment, the response variable is said to be recorded in "control relative" units if the values are expressed as a percentage of the response observed in the control cohort from the same biological replicate.
Ct	Cycle threshold value: The number of discrete qPCR amplification cycles that were required before the instrument was able to detect a fluorescence signal coming from the amplified DNA. The Ct is inversely

	proportional to the concentration of the targeted cDNA sequence in original sample.
Cytotoxicity	A property of chemicals which produce cell death.
DAA	Direct acting antiviral: Antiviral compounds which directly interfere with viral replication, these stand in contrast to compounds such as interferon therapies which manipulate host biology in a manner that hinders viral replication.
Daltons (Da)	The unit of molecular weight (abbreviated as Da).
Daughter virions	Those virions directly produced by the infection of a single host-cell by a specific virion (the "parent virion").
Degree of sulfation	With reference to a polysaccharide- the proportion of all potential sulfate incorporation sites that carry a sulfate group. The unit of sulfation is the average number of sulfate groups per monosaccharide moiety (sulfates per saccharide or sulfates per sugar).
Dendrimeric compound	A compound composed of dendrimer molecules (see next entry).
Dendrimer	A radially symmetrical molecule composed of identical branching arms arranged like the spokes of a wheel. The biologically active functional groups are located at the outer edge of the molecule (the end of the arms farthest from the centre of the molecule).
Dendritic effect	The phenomenon whereby dendrimeric compounds produce a biological effect which exceeds the level of response produced by a dose of the equivalent monomeric compound containing an identical concentration of the functional moiety (i.e. the effect is greater than a simple additive effect due to an increased concentration of active sites).
DENV	Dengue virus
DEV	Duck enteritis virus
dNTPs	Deoxy-nucleoside triphosphates (the monomeric units which DNA-polymerases incorporate into the growing daughter strand during DNA replication).
DTT	1,4-dithiothreitol
EBV	Ebola virus
ECM	Extra cellular matrix
Electrostatic (interactions or bonding)	Attractive forces which exist between complementary areas of positive and negative charge. Herein the term is most often used to describe the forces that create binding affinity between biological molecules with complementary charges.
Entry	A step in the viral replication cycle during which a ell bound virion transits into the intracellular space either by endocytosis, fusion between the viral envelope and the cell plasma membrane or direct penetration of the plasma membrane.

Entry receptor	A host-cell molecule which when bound to an infecting virion triggers some vital process (or processes) leading to virion entry.
FMDV	Foot and mouth disease virus
Fomite	An inanimate object which may transmit infectious particles from one individual to another and thereby disseminate the associated disease.
GAGs	Glycosaminoglycans: A family of carbohydrates that are highly expressed in animal tissue (for further explanation see section 1.8 in the text).
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase: A multifunctional protein found in the cytoplasm of all eukaryote cells. It is primarily directed at the regulation of metabolic energy production (glycolytic production of ATP and NADPH/ NADH)
Ganglia	Ganglia (singular = ganglion) are globular masses of neural tissue that contain the cells bodies of neurons.
gB, gC, gD, gH gL	HSV glycoproteins B, C, D, H and L respectively.
ICP47	Infected cell protein 47: An HSV gene that is expressed at high levels very early in the HSV replication cycle.
Glycolipid	Chemical compounds in which the molecules feature a carbohydrate domain bound to a lipid molecule.
Glycosaminoglycans	A family of carbohydrates highly expressed in animal tissues (see section 1.8 in the text).
gp150	Glycoprotein 150 of murid herpesvirus
Haemocytometer	An instrument used to measure the concentration of eukaryote cells in a sample of fluid dispersed cells. Cells are enumerated by adding 10uL of the sample to a well in the haemocytometer and counting the cells in the indicated portion of the well via a light microscope.
HCV	Hepatitis C virus
Herpes genitalis	A HSV infection (either HSV-1 or HSV-2) associated with the genital region.
HSVE	Herpes simplex viral encephalitis
HIV/AIDS	Human immune virus/ acquired immunodeficiency syndrome
hpi	Hours post infection
HPV	Human papilloma virus
HS	Heparan sulfate (see section 1.8 and 1.9 in the text)
HS GAGs	Heparan sulfate glycosaminoglycan (see section 1.8 in the text)
HSPGs	Heparan sulfate proteoglycans (see section 1.8 and 1.9 in the text)
Hydrophilic	The property of chemical compounds, molecules, or molecular domains that identifies them as having a high degree of water solubility.
Hydrophobic	Of chemical compounds, molecules, or molecular domains that have very low solubility in water (typically these are highly lipid soluble).

IC_{50}	Inhibitory concentration 50%: The concentration of a given compound that inhibits the process of interest (e.g. viral replication) by 50% of the level produced under control conditions ("control conditions" typically means a zero concentration of the compound in question).
Immediate early gene	The cohort of viral genes that are very first to be expressed upon initiation of infection.
Immunoprivileged	The term is used with reference to specific animal tissues. The cells of immunopriveleged tissue are subject to lower level of immune surveillance and are typically protected from T-cell directed cell death.
In vitro	A Latin term which literally translates to "in glass", used to refer to experimental procedures which do not involve any living animals and are carried out under laboratory conditions.
In vivo	A Latin term which literally translates to "in life", used to describe experimental procedures conducted on live organisms (cultured cells are not considered to be organisms hence cell culture is *in vitro*).
Intra-replicate mean	Used to refer to mean-values generated using data from one biological replicate only (i.e. by averaging the technical replicates within a single biological replicate)
JEV	Japanese encephalitis virus
JCV	John Cunningham virus
Kilodaltons (KDa)	A unit of molecular mass equal to 1000 Daltons.
Log	logarithm base 10
Lysate	The emulsion of cell constituents and growth media produced by artificially lysing (destroying the structure of the cell membranes) a sample of cells
Mannose	A monosaccharide with the formula $C_6H_{12}O_6$.
Mimetic	A chemical compound which is capable of replicating the biological role or another chemical. If chemical "*y*" reproduces the biological role of "*x*" then *y* is an *x* mimetic.
M-MLV reverse transcriptase	Moloney murine leukemia virus reverse transcriptase: A commercially available reverse transcriptase produced by Thermo Fisher Scientific.
MOI	Multiplicity of infection: the average number of virions per cell in a controlled infection.
MW	Molecular weight
Monomeric-compound	A compound in which the molecular structure is defined by comparison to the structure of a different compound with larger molecules (a dendrimer or polymer). Molecules of the dendrimer/polymer will be composed of multiple repeating subunits with each subunit being identical (or near identical) to entire molecules of the monomeric-compound.

MTT	A water soluble compound, yellow in colour with the proper name 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium. Healthy cells convert MTT to a blue/purple formazan in a manner that requires the conversion of NADPH to $NADP^+$.
Mucosa	Also called "mucus membranes", mucus secreting layers of endothelial tissues with relatively high levels of molecular absorbance.
MuHV	Murid herpes virus
Multivalent	Molecular interactions defined by multiple binding forces simultaneously acting within the frame of reference. The frame of reference is defined by the context in which the term is used e.g. multivalent binding during viral infection refers to multiple identical binding forces acting on the surface of a single virion, whereas multivalent ligand binding refers to the multiple binding forces acting on a single ligand molecule.
MV	Measles virus
MVE	Murray Valley encephalitis virus
NADP/NADPH	Nicotinamide adenine dinucleotide phosphate (NADPH is the reduced form of the same, the "H" indicating the proton that has been incorporated into the $NADP^+$ molecule). The conversion of NADPH to $NADP^+$ provides the chemical reduction in a wide variety of cellular processes. A consistent supply of cellular NADPH is dependent on normal metabolic energy production within the cell.
Nano drop	A piece of equipment that measures the concentration certain biomolecules (e.g. DNA) in a solution by shinning a laser through a 1-2uL sample of the solution and measuring the resulting photo absorbance over wavelengths that are absorbed by the solute of interest.
NDV	Newcastle Disease virus
Oroloabial	Pertaining to the area surrounding the mouth (i.e. the lips and adjacent tissue).
p-value	A statistical device used to describe the probability that the observed data would be produced under "x" conditions (e.g. the null hypothesis is correct).
Paracellular adsorption	A form of molecular adsorption occurring in the gut by which dissolved molecules travel between adjoining cells via gaps in the cell-cell junctions. In this manner solutes can be absorbed without entering the cytoplasm of the gut endothelial cells.
Passage (in the phrase "to passage cells")	The process by which cells being cultured *in vivo* are maintained for extended periods without exceeding the desired number of cells per vessel. Typically passaging

	consists of removing the cells from the culture vessel, discarded the majority and returning a small subset of the cells to the culture vessel to continue growing.
PFU/ mL	Plaque forming units per millilitre: The unit used to describe the concentration of a viral inoculum. The term "plaque forming units" is used instead of "virions" as it cannot be assumed that all of the virions in a sample are capable of forming a plaque under the test conditions.
Pharmacokinetic	A broad term used to indicate manner in which the intra-host concentration of an administered compound varies both between tissues and over time. Indicated drug properties include but are not limited to: the speed of adsorption, the speed of elimination, the tissue specific concentrations of the compound and the intracellular concentrations of the compound (across the total period of exposure).
PI-88	An anti-tumour drug composed of a mixture of polysaccharides 4-8 sugars in length (aka Murapfrost).
Plaque-forming period	Used to describe the latter phase of a viral infection carried out *in vitro*. During the plaque-forming period infected cells are maintained without further manipulation (usually after the viral inoculum has been removed) under conditions which prevent the free diffusion of progeny virions but permit cell-to-cell spread of virions. In this manner infection spreads in a localised fashion allowing visible viral plaques to form.
Polyanionic	Pertaining to a compound having multiple sites of negative charge spread on each molecule.
PowerUp SYBR	A commercially available preparation of qPCR reagents sold by Thermo Fisher Scientific. It contains the necessary substrates, enzyme, dye and buffer to run a qPCR assay.
Progeny	The total population of virions produced during a specified infection interval.
Proteoglycan	Proteins with polysaccharide groups bound to their exterior.
PV	Polio virus
Quasi-species	A subset of the viral progeny in an infected host (or cell culture) all of which are descended from a single virion and exhibit certain unique mutations as a result of this common descent. Many viruses are known to produce multiple distinct quasi-species within a single infected host.
Random hexamer primers	A solution random hexamer primers contains fragments of DNA (or RNA) six bases in length. The total diversity of random six base sequences should be represented in the solution so as to guarantee that all of

	the sampled DNA/RNA fragments are equally amplified.
Saccharide	A sugar or carbohydrate
SARS-CoV 2	Severe acute respiratory syndrome corona virus-2, the aetiological agent of Covid-19 infections.
AU SBS	Auckland University School of Biological Science
Sequelae	Secondary illnesses or disease symptoms occurring as a downstream effect of the original condition.
Sofosbuvir	A nucleoside analogue pro-drug used in the treatment of HCV.
Sonicator	A piece of equipment which creates a uniform dispersion of molecules/particles within a solution/suspension. The effect is achieved via the application of high frequency sonic vibrations.
Spinoculate	The process of initiating a viral infection *in vitro* by using the assistance of centrifugation to bring the virus and the cells into tight contact.
Stochastic	An adjective applied to research data which is seemingly random in distribution. Stochastic patterns are more likely to be witnessed when dealing with small data sets, the central limit theorem predicts that the total data set will tend towards a normal distribution as the total number of samples is increased.
Technical replicate(s)	Repeated iterations of the same treatment conditions carried out within a single biological replicate. Technical replicates are always averaged and treated as single data point for all downstream analysis.
Test-compound	The collective name applied to all four compounds examined herein. The term is also used as a generic identifier for the compound or compounds tested in various cited papers.
Transcellular	Intestinal adsorption whereby the absorbed compound is able to pass through the cellular membrane of gut endothelial cells and is thereby delivered into capillaries that feed the hepatic portal vein.
Tropism	The subset of cells/ tissues within a host in which a specific virus is capable of inducing a productive infection (i.e. cells in which a virus can successfully produce progeny)
Trypsinise	Verb- To treat an *in vitro* cell layer with trypsin resulting in the exfoliation of the cells and their subsequent dispersal into a suspension of individual cells (i.e. no cell is bound to any other cell).
Uptake	With reference to viral replication “uptake” is synonymous with “entry”.
Uptake receptor	With reference to viral replication “uptake receptor” is synonymous with “entry receptor”.
Valency	The number of inter-molecular (or inter-entity) attractive forces that are acting simultaneously in a

	single binding event (i.e. between two entities only). See “multivalent” for further explanation.
VAPs	Viral attachment proteins
Viremic	A viral infection in which there is a medically relevant concentration of virions present in the host’s bloodstream.
Virion	An individual virus-particle. Properly the term “virus” is used as a collective noun for a viral species or in reference to a virus infected cell and does not refer to the infectious particles/virions.
Virucidal	The property of permanently inactivating free virions.
WNV	West Nile virus
YFV	Yellow fever virus

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Introduction

1.1 The Relative Difficulty of Controlling Viral Diseases

Viral diseases are among the most pernicious of current global health concerns, they spread rapidly, can produce severe morbidity and, at a population level, are very difficult to control. The relative impotence of existing virus control strategies is illustrated by comparing the meagre outcomes achieved in the control of viral diseases to the substantial progress that has been made in controlling non-viral infections. Between 1990 and 2013, the total mortality attributable to infectious disease decreased markedly, during the same period however the absolute mortality due to viral hepatitis and human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) increased by 63% and 344% respectively (Murray *et al* 2014; Stanaway *et al* 2016). These two infections (HIV and viral hepatitis) were the only two definitively viral diseases that featured in the 15 most common causes of death globally in the year 2013 ((Stanaway et al 2016). While the number of annual deaths due to HIV is now steadily decreasing (having peaked in 2005) the number of infected individuals continues to grow, this is due to the irresolvable (i.e. life-long) nature of HIV infection, the incurable nature of HIV is in itself a pertinent example of the difficulties associated with treating certain viral infections (Murray *et al* 2014).

Attempts to reduce the total burden of viral diseases have been further hampered by the rapid emergence of drug-resistant phenotypes in numerous notable viruses. A meta-analysis of studies monitoring drug-resistant HIV in China found that after receiving anti-retroviral therapy (ART) for 12 months over ten percent of patients harboured a strain of the virus that was resistant to at least one of the drugs they were receiving (Liu *et al* 2014). The frequency of patients harbouring resistant infections increased to 80% after patients had been receiving

treatment for 72 months (Liu *et al* 2014).

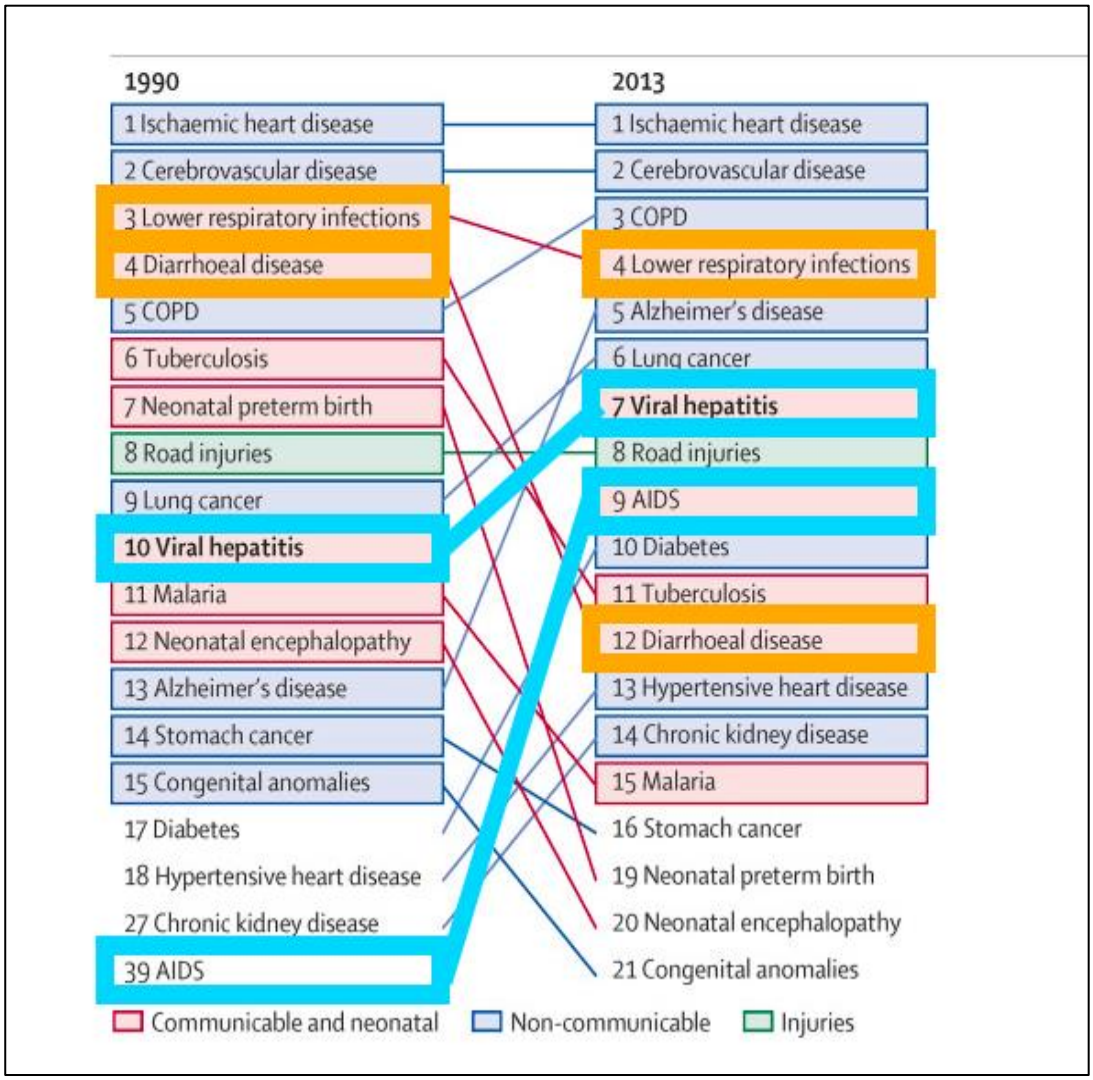


Figure 1.1: Causes of death ranked by global prevalence for the years 1990 (left) and 2013(right). Unambiguously viral causes are depicted in cyan boxes. Causes of death that are sometimes associated with viral infections are depicted in orange boxes. Of the causes of death that are depicted, all of those which are transmissible decreased in relative prevalence between 1990 and 2013 with the exception of the two unambiguously viral diseases (HIV/AIDS and viral hepatitis).

Figure adapted from Stanaway *et al* 2016, reproduced with the authors' permission.

1.2 Novel Anti-Viral Drugs and the Management of Existing Diseases

Novel antiviral compounds are likely to play a valuable role in the fight against viral diseases. The successful development of new antiviral drugs could potentially provide therapies where none currently exist and could facilitate the replacement of those drugs which have been rendered obsolete through the ubiquity of resistant virus.

The magnitude of what can be achieved with novel antivirals is exemplified by the case of sofosbuvir (and sofosbuvir based drug regimens) in the treatment of hepatitis C virus (HCV). Sofosbuvir is a direct-acting antiviral (DAA) which first became available in 2014 and has since shown unprecedented efficacy in the treatment of HCV infections (Keating & Vaidya 2014). In the case of the United States, drug therapies based around sofosbuvir have been projected to reduce the prevalence of viremic HCV to less than 24% of the pre-sofosbuvir level by the year 2030 (Chatwal *et al* 2016). Additionally, sofosbuvir has been shown to have remarkably low susceptibility to viral resistance; the most commonly identified resistance genotype incurs a substantial fitness cost for the virus meaning that when sofosbuvir is removed the resistant strain is rapidly displaced by non-resistant variants and sofosbuvir based therapy can be resumed after a suitable withholding period (Svarovskaia et al 2014; Walker *et al* 2017). Theoretically there is no reason to doubt that the level of success achieved by sofosbuvir could be reproduced for any or all of the viral diseases that are current health concerns.

1.3 Antiviral Drug Development as an Epidemic Preparedness Strategy

Novel anti-viral drugs may also prove to be a useful in combating the viral epidemics which will inevitably occur in the future. Recent examples of viral epidemics and the associated harm they can cause include the 2013-2016 outbreak of Ebola in West Africa, the 2015 spread of

Zika virus in South America and the SARS-CoV2 pandemic which began in December 2019 (Fauver *et al* 2020; Gatherer & Kohl 2016; Richardson and Fallah 2019).

An extensive catalogue of functionally diverse and (ideally) broad acting antivirals is one of several fundamental elements in optimal epidemic preparedness (Beckerman & Einav 2015; Milne, Halder & Kelso 2013; Osterhaus and Mackenzie 2020; Webby & Webster 2003). Limitations in terms of available funding, the ability to predict which pathogens pose an imminent threat and the impossibility of performing efficacy trials for virus variants that are yet to evolve mean that it will often be impossible to develop effective vaccines until an epidemic infection is already beginning to spread (Plotkin 2017). Even when the process of vaccine development is maximally expedited it still takes between six months and a year to have vaccines which are ready for rollout (six months is the time line for seasonal influenza vaccine design and production and 12 months was the approximate duration of vaccine development for the various SARS-CoV 2 vaccines)(Funk, Laferriere & Ardakani 2020; Gerdil 2003). Conversely, broad spectrum antiviral drugs can be produced and stockpiled in anticipation of future outbreaks, making them a vital component in the immediate response to an emergent epidemic (Webby and Webster 2003). No single intervention stands to improve population level outcomes as significantly as an anti-viral drug possessed of both prophylactic and a therapeutic activity (a number of existing antiviral drugs possess this dual capacity for treatment and prevention) (Asano et al 1993; Monto, Gunn, Bandyk & King; Gani et al 2005; Gupta et al 2020; McCaw & McVernon 2007; Milne, Halder & Kelso 2013; Romanowski, Yates, & Gordon 2001).

1.4 Theoretical Considerations in Antiviral Drug Design

Administration of a compound that triggers comprehensive disruption of any viral process is likely to result in reduced virus production and improved clinical outcomes, as such novel antiviral drugs can be directed at impeding any stage in the replication cycle (or any combination of stages) (De Clerq & Herdewijn 2010; De Palma, Vilegen, De Clercq & Neytsl 2008; Leyssen De Clercq & Neyts 2008). One of the first lines of assessment that a candidate antiviral must pass is *in vitro* assessment of its capacity to inhibit replication in the targeted virus or viruses (Saxena et al 2010). Apart from antiviral efficacy, candidate compounds must also show appropriate bioavailability, low toxicity, good intra-host stability, an absence of off target effects and must be suitably cheap to produce (Bowman & Zon 2010; Guengerich 2010; Kakkar, Traverso, Forokhzad, Weissleder & Langer 2017; Saxena et al 2010)

The investigations carried out in chapter three pertain to several novel compounds that are potentially capable of inhibiting the adsorption and entry of a multitude of noteworthy viral pathogens, most prominent among these is herpes simplex virus type 1 (HSV-1). By inhibiting one or both of these essential replication processes (adsorption and entry) it is hoped that these compounds might be able to treat or prevent HSV and other functionally similar infections.

In order to present at thorough explanation of these compounds, their putative mode of action and potential benefits it is necessary to first give a brief description of the processes involved in viral attachment and entry, this is the topic of the following section.

1.5 Viral Attachment and Entry Receptors

The attachment of a virion to the surface of a host-cell is the first stage in the viral replication cycle (Miller 2011). Attachment (often called adsorption) takes place via binding between viral

attachment proteins (VAPs) and cognate molecules on the host-cell surface (Haywood 1994). In the majority of the studied cases VAP-receptor binding by itself is not sufficient to bring about infection, rather the attached virion must pass through a least one subsequent molecular interaction before it is able to transit into the cytoplasm (a process called “viral entry” or “uptake”). Viral entry takes place either by membrane fusion (between the cell membrane and the viral envelope) or via endocytosis (a normal host-cell process that many virus exploit or direct in order to access the cytosol) (Haywood 1994; Boulant, Stanifer & Lozach 2015).

In most instances the infection process is reliant on numerous host factors and multiple viral proteins e.g. HSV requires at least four viral glycoproteins (gB, gD, gH and gL (additionally gC often serves as a VAP though it is dispensable)) and two host-cell receptors,(an attachment receptor and an entry receptor) in order to successfully initiate a new infection (MacLeod & Minson 2010).

The host factors which VAPs bind to are termed “attachment receptors” and (for the purpose of the current text) those receptors which a virus must bind in order to trigger entry are termed “entry receptors” or “uptake receptors”. The term “entry receptor” is sometimes used to identify the class of receptors that includes both attachment and entry receptors collectively, however for clarity’s sake the present text always denotes the two receptor types separately.

Attachment and entry receptors show great diversity both in terms of their chemical structure and the functions that they perform in host biology (Boulant, Stanifer & Lozach 2015; Haywood 1994). It is worth noting that the name “receptor” does not indicate that these molecules preform a receptor function in the biological processes of the host, rather the term is solely intended to indicate the manner in which they are utilised by the virus in question.

The only feature that can reasonably be attributed to all attachment receptors is that they are expressed at the cell surface or in the extracellular space/ extracellular matrix (ECM) (Baranowski, Ruiz-Jarabo & Domingo 2001; Marsh & Helenius 2006).

Some viruses are thought to rely on a single type of receptor to mediate both attachment and the subsequent passage to the cell cytoplasm (e.g. polio virus), however the majority of viruses for which the attachment process has been studied utilise discrete receptors for attachment and entry (Hogle 2002; Marsh & Helenius 2006). In addition to this, many viruses are capable of targeting multiple different receptors to trigger adsorption, uptake or (in some cases) both processes (Marsh & Helenius 2006). Affinity for multiple receptor types can confer wider tropism or it can reflect the necessity of multiple sequential interactions in the cell-entry process (Marsh & Helenius 2006; Tee, Zainol, Sam & Chan 2021).

Affinity for multiple receptor types (as a means to produce broader tropism) can be achieved by at least two non-exclusive viral strategies (i.e. a single virus particle is capable of exploiting both strategies) (Haywood 1994; Marsh & Helenius 2006). The first strategy is to express two or more VAPs or viral entry proteins with each protein type binding a different class of host-cell receptor. This strategy is displayed by Epstein Barr virus (EBV) and African swine fever virus (ASFV) both of which rely on different viral protein-receptor pairings to initiate infection in different cell types (Gomez-Puertas et al 1998; Tugizov, Berline & Palefsky 2003). The second strategy describes the situation whereby a single viral protein features multiple receptor-binding epitopes, with each binding region targeting a different type of receptor molecule. Canine distemper virus (CDV), EBV and measles virus (MV) are all examples of viruses which possess one or more attachment or entry proteins having affinity for multiple receptor types (discrete binding epitopes at different regions of the same protein)(Chen &

Longnecker 2019; Colf, Juo & Garcia 2007; Langedijk et al 2011; Matsuura, Krischner, Longnecker & Jardetzky 2010; Zhang et al 2013). The fact that these two strategies can be simultaneously exhibited by a single virus is illustrated by the case of EBV. Epstein-Barr virus is capable of attaching to host cells via viral glycoprotein gp350, protein BMRF-2 or the gH/gL glycoprotein dimer; each of these proteins is capable of attaching to receptor types that cannot be bound by the other two proteins (Chen & Longnecker 2019; Nemerow, Mold, Schwend, Tollefson & Cooper 1987; Tugizov, Berline & Palefsky 2003). Additionally, the gH/gL complex is capable of binding to at least three different types of EBV attachment/entry receptors, this function is due to the complex containing numerous receptor binding regions (the specific function due to some of these binding motifs remains unclear) (Chen & Longnecker 2019; Matsuura, Krischner, Longnecker & Jardetzky 2010, Connolly, Jackson, Jardetzky & Longnecker 2011).

1.6 The Importance of Regulating Receptor Binding

In order for a virus to replicate efficiently it is essential that the virus possess means of regulating the strength and duration of the binding between extracellular virions and host-cell receptors. Binding that is too transient, too tenacious or prevents lateral movement of the virion incurs a substantial fitness cost (Delguste et al 2018; Marsh & Helenius 2006). Though the specific nature of the interactions between viruses and their attachment receptors are multifarious there are two frequently occurring (though non-universal) principles that can be used to understand the selective pressures that shape the evolution of virion-receptor interactions.

Firstly, a virus needs to regulate the interaction between viral proteins and their receptors so that binding is terminated in a time appropriate manner (an evolutionary requirement that will

henceforth be referred to as the necessity of“ regulating binding duration”). Binding duration regulation is necessary to facilitate the efficient release of daughter virions that have become bound to the surface of the parent-cell. Newly released virus particles are often trapped at the surface of the parent cell as a results of their attachment to the same type/types of receptors that mediated the attachment and entry of the parent virion(s) (Baum & Paulson 2001; de Groot 2006; Desforges, Desjardins, Zhang & Talbot 2013; Hadigal et al 2015; Trybala et al 2021; Wagner, Matrosovich & Klenk 2002). The regulation of binding duration is also a necessary condition for one of the two mechanism by which virions move laterally across the surface of host-cells in the interval between attachment and entry (“viral surfacing”), this phenomenon is discussed in more detail below.

One of the most widespread mechanisms by which viruses satisfy need for regulation of binding duration is by using targeted enzymes to progressively degrade the virion-receptor linkages. Enzymatic cleavage can be achieved either by the expression of viral enzymes or by the manipulation of host-cell enzymes. The most often cited example of the former scenario is the influenza envelope protein neuraminidase. Neuraminidase acts by enzymatically degrading host-cell receptors (salicylic acid) thereby ensuring that daughter virions can migrate away from the parent cell (Baum & Paulson 1991; Gong, Xu & Zhang 2007; Wagner, Matrosovich & Klenk 2002).

Herpes simplex virus-1 on the other hand uses a host enzyme called heparanase to regulate the duration of receptor binding. Heparanase exists to regulate the membrane concentration and progressive turnover of a host-cell molecule called heparan sulfate (the HSV-1 attachment receptor). By manipulating the host cell to secrete an increased concentration of heparanase in

synchronicity with progeny maturation, HSV-1 is able to accelerates the rate of heparan sulfate degradation and thereby the rate of virion egress (Hadigal et al 2015).

Another mechanism by which certain viruses discharge the need to regulate binding duration is by controlling the number of simultaneous binding interactions taking place between each individual virion and the host-cell (i.e. the valency). Assuming that firstly, VAP-receptor interactions are of such low affinity that multiple interactions are necessary to keep a virion tethered to the cell surface, and secondly, that these VAP-receptor bounds are prone to spontaneous dissociation after a relatively short interval, then it follows that rate of virion release will be inversely proportional to the average receptor valency. Unduly high valency not only decrease the rate at which new virions are released from the parent-cell but can also slow or even completely halt the lateral movements that certain viruses display immediately after attachment (this is true for viruses which use viral surfacing but not for those that rely on viral surfing, see section 4.7 for more information on viral surfing/surfacing). This is the situation described in the paper by Delguste *et al* (2018) wherein the authors showed that one of the murid herpesvirus-4 (MuHV-4) envelope glycoproteins (gp150) regulates the valency of interactions between MuHV-4 VAPs and their receptors in such a manner that each cell-bound virion consistently displays a valency of either 2 or 3 VAP- receptor linkages. Optimal receptor valence ensures that an incoming virion is likely to be bound to a cell long enough to ensure that entry takes place, while also creating a suitably high probability that each daughter virion will at some point experience a total loss of receptor binding (due to the random timing of receptor disengagement and the stochastic forces that emerge at low valency) and thus be able to migrate away from the parent cell (Delguste et al 2018).

The second of the evolutionary imperatives discussed in this section can be summarised as follows: the efficient replication of a virus relies on said virus possessing a means by which to ensure that an attached virion will interact with all of the necessary host-cell structures. Following adsorption, the majority of viruses need to interact with an entry receptor, or in some cases multiple entry associated receptors and/or cell-structures (e.g. endocytotic pits, regions of high endocytotic/pinocytotic uptake) (Boulant, Stanifer & Lozach 2015). A virus particle which binds to a random attachment receptor in an immobile manner is unlikely to encounter the secondary receptors that are necessary for penetration of the cell membrane. To overcome this restriction, the vast majority of viruses display strategies which increase the likelihood that an attached virion will encounter the required receptors and other necessary host factors (the host of mechanisms that serve this evolutionary requirement will henceforth be referred to as means of "promoting post-attachment proximity to essential structures") (Boulant, Stanifer & Lozach 2015).

Broadly speaking, the means by which viruses promote post-attachment proximity to essential structures fall into two categories: Those that allow the rapid lateral movement of membrane tethered virions and those that trigger rearrangement of the host-cell plasma membrane (thereby bringing the required receptors into the vicinity of the attached virion) (Boulant, Stanifer & Lozach 2015). As alluded to on the previous page the valency regulating function of gp150 (from MuHV-4) allows attached virions to move laterally across the cell surface and thereby come into contact with distant entry receptors (Delguste et al 2018). By promoting the regular disassociation of VAP-receptor bonds and the formation of new VAP-receptor bonds, gp150 allows MuHV-4 virions to roll across the surface of the host-cell, with receptors being released at the trailing edge and engaged at the leading edge of the virion (Delguste et al 2018). This process is known as "viral surfacing", and it allows virions to roll across the cell surface

via sequential binding and release of receptors (Sheerer, Jin & Mothes 2010). Viral surfacing should not be confused with “viral surfing”, a process under which a virion remains anchored to the same receptor (or collection of receptors) and transits across the cell surface by means of cell-directed rearrangement of the underlying cytoskeleton (Oh, Akhtar, Desai & Shukla 2010; Sherer, Jin & Mothes 2010; Boulant, Stanifer & Lozach 2015;). Viral surfing and surfacing are discussed in more detail in Chapter 4 (4.7).

The second category of mechanisms which promote proximity to essential structures are those whereby the attached virion triggers the activation of cell-signalling pathways leading to the rearrangement of the cytoskeleton in such a manner that the necessary cell structures are presented to the bound virion without the virion being relocated, this is sometimes called the “land and stick” method of virion uptake (Boulant, Stanifer & Lozach 2015). This usually manifests as pit formation (as a precursor to virion endocytosis).

1.7 Heparan Sulfate Proteoglycans

Of all the host-cell molecules used as attachment receptors one of the most widely exploited classes is a family of carbohydrates known as heparan sulfates. Heparan sulfate proteoglycans (HSPGs) are a highly diverse class of biomolecules consisting of a central protein with one or more heparan sulfate (HS) polysaccharide molecules bound to their surface (properly called HS glycosamminoglycans) (Sarrazin, Lamannal and Esko 2011). The proteinaceous cores of HSPGs fall into several distinct categories: membrane spaning proteins (having both intra and extra-cellular domains), membrane-associated-extracellular proteins (being anchored to the cellular membrane via phospholipid moieties), lysosomal secretions and components of the ECM (Bishop, Schuksz and Esko 2007).

HSPGs carry between one and five HS side chains, they have been found on the surface of every class of mammalian cells and are so widely distributed in the ECM that proteins with HS binding sites are a near ubiquitous component of these matrices (Bishop, Schuksz and Esko 2007; Sarrazin, Lamannal and Esko 2011). Notable roles of HSPGs include acting as receptors/co-receptors for growth factors, presentation of chemokines, endocytosis of ligands, intracellular trafficking, providing structural integrity to ECM, and cell adhesion, (both cell-cell and cell-ECM) (Bishop, Schuksz and Esko 2007).

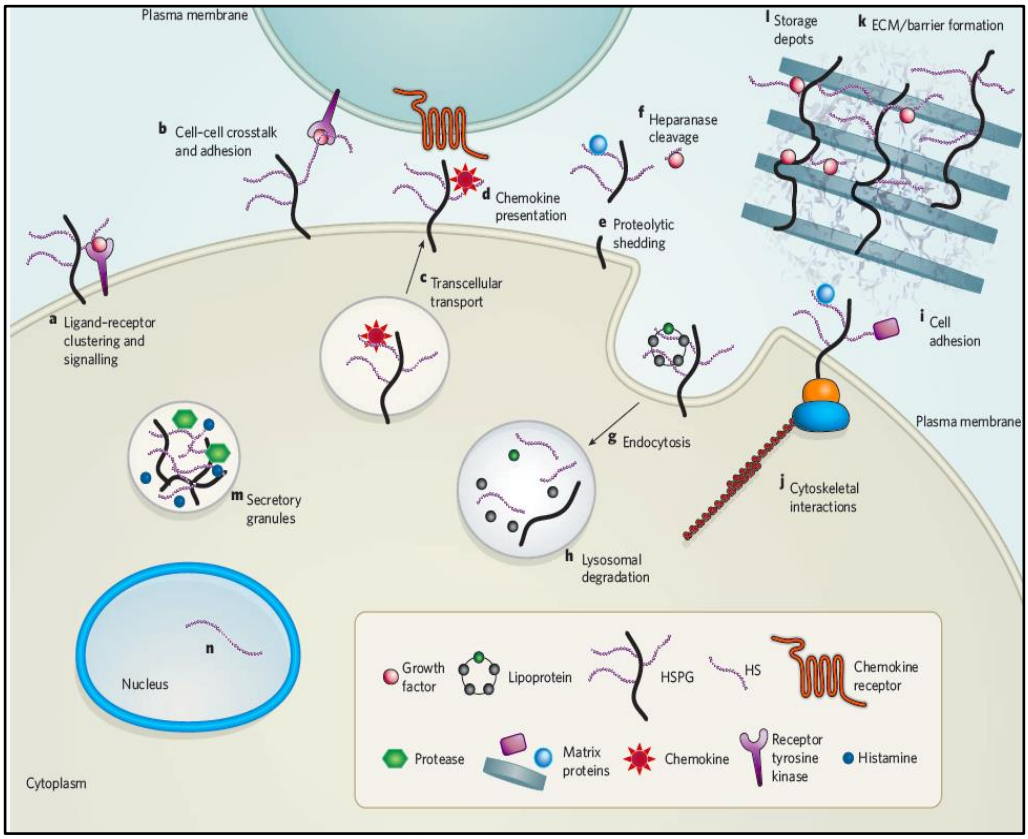


Figure 1.2: Generalised representation of the roles that heparin sulfate performs in animal tissues.

Reproduced with permission from Bishop, Schuksz and Esko 2007.

1.8 Heparan Sulfate Glycosaminoglycans

The HS side-chains of HSPGs belong to the class of polysaccharides called glycosaminoglycans (GAGs). These are composed of repeating disaccharide subunits forming long unbranched molecules, GAGs possess the highest negative charge density of any biological compound giving them unparalleled water holding capacity and a high affinity for electrostatic interactions (Gandhi and Mancera 2008). High concentrations of GAGs are present in mucosal secretions and confer the viscosity and water adsorbing behaviour that define these fluids (Gandhi and Mancera 2008).

Heparan sulfate GAGs are a specific sub-group of GAGs in which the disaccharide motifs consist of a uronic acid (occurring as either L-iduronic acid or D-glucuronic acid) and N-acetyl D-glucosamine (Rabenstein 2002). Despite the apparent simplicity of their basic structure HS GAGs are exceedingly heterogeneous, this diversity is the result of varied chain lengths (40-300 saccharide units) and, more importantly, highly varied patterns of sulfation (Rabenstein 2002, Habuchi, Habuchi and Kimata 2004; Bishop, Schuksz and Esko 2007).

Sulfation involves the replacement of one or more of the component atoms in a saccharide subunit with a sulfate group (SO_4^{-2}) (Rabenstien 2002). The uronic acid subunits of HS are capable of incorporating a single sulfate group, whereas D-glucosamine monomers are capable of accepting up to three sulfate groups, including one in place of the N-linked amine (this alters the applied naming convention giving D-N-sulfo-glucosamine) (Sarrazin, Lamanna and Esko 2011; Rabenstein 2002). When different patterns of sulfation are taken into account there are 24 possible disaccharide subunits from which a given HS molecule may be composed, though only 19 of these have been obtained from living tissue (Rabenstein 2002).

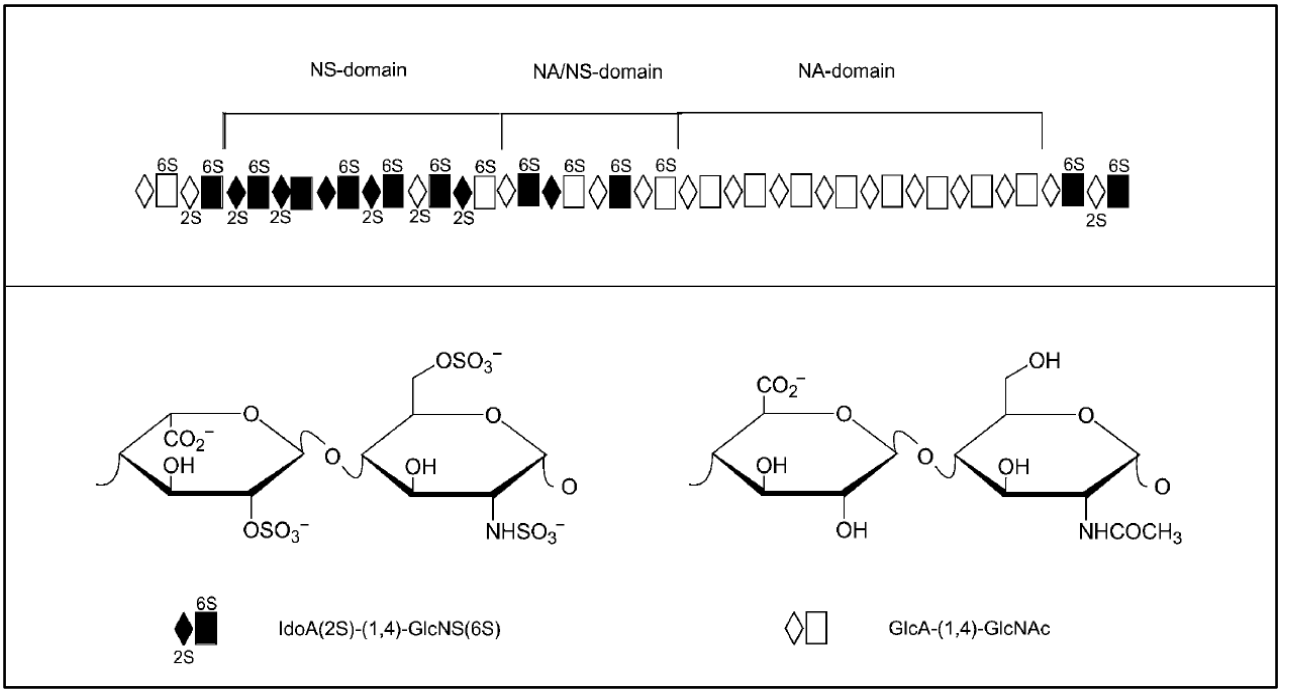


Figure 1.3, Bottom Panel: The diagram on the left is represents a disaccharide subunit consisting of a 2-O-sulfated-$_L$-iduronic acid (IdoA(2S)) and a 6-O-sulfated, N-sulfated glucosamine (GlcNS(6S)). This is one of the most highly represented subunits within the sulfated regions of HS GAG chains. The diagram on the right represents a non-sulfated disaccharide consisting of a D-glucuronic acid (GlcA) and a N-acetylglucosamine (GlcNAc), a primary component of non-sulfated domains in HS GAGs.

Upper Panel: A diagram depicting the manner in which HS GAG chains are divided into sulfated and non-sulfated domains with interspersing regions of partial sulfation ("NA/NS-domain).Solid Black shapes represent sulfated disaccharide subunits; white filled shapes represent non-sulfated subunits (see key at bottom of figure).

"NS" =N-sulfated
NA", = N-acetylated

Reproduced with permission from Rabenstein 2002.

Heparan sulfate differs from other GAGs in that sulfated and non-sulfated monomers tend to occupy discrete regions of the saccharide backbone (Figure 1.3), zones of sulfation result in an extremely high negative charge density at the corresponding region of the saccharide molecule (Rabenstein 2002). With regards to any specific HSPG it is believed that many of its biochemical interactions (i.e. its role in host physiology) are variously determined by the specific saccharide moieties in the GAG chain, the conformational flexibility of the HS GAGs, the type/degree of sulfation present on the sugar moieties and the pattern of sulfated and non-sulfated domains on the GAG chain (Habuchi, Habuci & Kimata 2004; Schuksz and Esko

2007; Ori Wilkonson & Fernig 2008, Jastrebova, Vanwildemeersch, Lindahl & Spillmann, 2010).

1.9 Virus Utilisation of Heparan Sulfate

Though the biological relevance of the interaction is in many cases poorly understood, the published evidence indicates that over 50 different viruses from 11 different families are capable of exploiting host-cell HS as an attachment receptor (Cagno, Tseeligka, Jones & Tapparel 2019). Despite the sheer diversity of viruses that exploit HS, it appears that the majority of these do so only under certain circumstances (Zhu, Li & Liang 2011; Cagno, Tseeligka, jones and Tapparel 2019). Interestingly, all but one of these viruses (herpes simplex virus-1 (HSV-1)) require at least one additional receptor type to act as an entry receptor and all but one (human papilloma virus (HPV)) have been shown to possess affinity for attachment receptors other than HS GAGs (Zhu, Li & Liang 2011; Cagno, Tseeligka, jones and Tapparel 2019). These finding raise doubts as to whether some, or possibly any, of these viruses are inescapably dependent on host-cell HS GAGs (Zhu, Li & Liang 2011; Cagno, Tseeligka, jones and Tapparel 2019)

While it is clear that, for certain viruses, moderate to high levels of HS binding affinity correlate with optimal virulence, other viruses have been shown to display the inverse correlation. Enzymatic digestion of cell surface HS and blocking of HS GAG availability have been shown to prevent infection with HSV and HPV-16 indicating that HS-VAP binding is a virulence associated trait for these viruses (Giroglou, Florin, Schafer, Streek & Sapp 2001; Tiwari, Liu, Valyi-Nagy& Shukla 2011). On the other hand animal studies with Sindbis virus, foot and mouth disease virus (FMDV), yellow fever virus (YFV) and Japanese encephalitis virus (JEV) have shown that strains with increased HS affinity show reduced virulence *in vivo*, ostensibly

as a result of virus particles being sequestered in HS rich tissues leading to reduced intra-host spread, shortened duration of viremia and lower viral titre in the tropism tissues (JByrnes and Griffin 2000; Cagno, Tseligka, Jones & Tapparel 2019; Lee & Lobigs 2002; Lee & Lobigs 2008; Sa-Carvalho et al 1997; Zhu, Li & Liang 2011). It appears that for these virus high affinity for HS GAGs is a maladaptive trait, likely produced by extended periods of *in vitro* replication; this hypothesis is in accordance with the widely cited relationship between extended lab cultivation and attenuation of viral virulence in animal hosts (Badgett, Auer, Carmichale, Parrish & Bull 2002; Cagno, Tseligka, Jones & Tapparel 2019; Dunster, Gibson, Stephenson, Minor & Barrett 1990; Liu *et al* 2016; Spatz 2010; Zhu, Li & Liang 2011).

In addition to the above cases where HS it utilisation is exclusively confined to lab cultivated virus, viruses which do not adsorb via HS have been found to evolve HS mediated adsorption over the course of infection (Badgett, Auer, Carmichale, Parrish & Bull 2002). Initial infection with John Cunningham virus (JCV) is understood to occur via the transmission of virions that lack the capacity to bind HS GAGs, it has been shown intra-host evolution over the course of infection results in the emergence of viral quasi-species which chiefly rely on HS mediated attachment (Geoghegan et al 2017; Kondo et al 2014; McIlroy, Halary & Bressellette-Bodin 2019). This is thought to be reflective of the manner in which the operative selection pressure shifts during infection. Initially virions are selected for their capacity to infect and reinfect the primary tropism tissue (epithelium of the urethra) however latter in the infection the virus is selected for escape from host immunity, in JEV a single mutation in the VP1 protein leads to erasure of the binding site targeted by host antibodies and confers VAP affinity for HS, thereby allowing the virus to infect the immunoprivileged tissue of the central nervous system (Geoghegan et al 2017; Kondo et al 2014; McIlroy, Halary & Bressollette-Bodin 2019).

While the extent to which certain viruses rely on HS GAGS as a receptor remains a contentious issue, practical investigations into the anti-viral efficacy of HS analogues have in some cases (e.g. treatment of HSV-1) outpaced the theoretical understanding. Some of the studies which examined such HS mimetics will be addressed in brief in the following section.

1.10 Heparan Sulfate Analogues as Inhibitors of Herpes Simplex Virus

By far the greatest body of work concerning the antiviral application of HS mimetics focuses on the treatment of HSV. The attention given to HSV is not merely due to the fact that it is among the most unambiguously HS dependent viruses, but also because HSV-1 can utilise 3-O-sulfated-HS as a post-attachment entry receptor (This is thought to occur primarily in the tissues which lack the two other classes of HSV-1 entry receptor) meaning that HS analogues are theoretically capable of hindering both the adsorption and the uptake of HSV particles (O'Donnell & Shukla 2008; Shukla *et al* 1999; Tiwari et al 2007). Additionally, the primary route of infection for HSV (through dermal abrasions, and oral/genital mucosa) means that topical prophylactics may be an effective means of controlling the spread of HSV (de Witte *et al* 2011; Antoine, Park and Shukla 2013; Balzarini *et al* 2013; Yadavalli *et al* 2020). It is a frequently occurring problem in the search for new medicinal compounds that drug candidates which perform well *in vitro* cannot be developed into a marketable form due to poor solubility, low oral bioavailability or insufficient dissemination in the targeted tissue (Goldberg & Gomez-Orellana 2003; Aungst 2017; Kakkar, Traverso, Farokhzad, Weissleder & Langer 2017). Topical administration of locally acting compounds by enlarge negates these concerns meaning the anti-herpetic compounds can be developed into a marketable form even if they lack the correct pharmaco-kinetic profile for internal administration. The potential to be marketed as an externally administered biocide also applies to compounds targeted at HPV and

HIV, both of which are known to use HS GAGs as attachment receptors (Zhu, Li & Liangl 2011).

Of the HS mimetics which show anti-HSV activity *in vitro* the majority are polysaccharides, an exceptionally diverse array of polysaccharides have been tested for their anti-herpetic activity, including both synthetic compounds and those isolated from biological sources. In general the antiviral activity of these saccharides is due to (or is inferred to be due to) the compound adhering to the HSV VAPS in a manner that blocks the virions ability to attach to host-cells. In HSV the VAPs are the envelope glycoproteins identified as HSV glycoprotein B and glycoprotein C (gB and gC), some HS mimetics are also thought to act post-adsorption by blocking the entry of HSV-1 by blocking the activity of glycoprotein D (gD) (HSV-1 gD can bind 3-O sulfated HS as an entry receptor) (O'Donnell & Shukla 2008; Shukla *et al* 1999; Tiwari et al 2007). Select saccharide compounds have however been found to exert their inhibitory effects via various other mechanisms, either in addition to, or in the apparent absence of VAP binding, see Figure 1.4 for a brief overview of the most noteworthy HS mimetics tested against HSV their relative efficacy and putative mode of action.

Relevant Paper (Lead Author and Year)	Tested Compound's Name and/or Source Organism	HSV-1 IC_{50}	Compounds' Mode of HSV-1 Inhibition	MW (kDa) MW reference paper In brackets
Hayashi et al 1996	Calcium spirulan (from seaweed *Spirulina platensis*)	0.85	One of the early process in the replication cycle, probably adsorption and/or entry.	74.6 (Lee et al 1998)
Hayashi et al 1996	Dextran sulfate	0.92	early stage, probably adsorption or entry	8
Carlucci et al 1997	A partially cyclised carrageenan from the red seaweed *Girgartina skottsbergii*	0.7	Attachment (Inferred)	198
Carlucci et al 1997	A lambda carrageenan extracted from the seaweed *Girgartina skottsbergii*	0.6	Attachment (Inferred) Antiviral effect appears to be of a virucidal nature (i.e. cannot be abrogated following the treatment of cell-free virions).	83
Xu, Lee, Lee, White & Blay 1999	An anionic polysaccharide isolated from Chinese herb *Prunella vulgaris*	10	Apparently the primary mode of action is adsorption blocking. There is some disagreement in the results as to whether it can act in an intracellular manner.	3.5
Nyberg et al 2004	Commercially available anti-tumour drug PI88 (a mixture of saccharides 2 -6 sugar moieties in length) derived from sulfated mannose.	6	Marginal inhibition of adsorption, significant inhibition of cell to cell spread (syncytia formation).	2.4 - average
Chi-Ming Chiu, Zhu & Ooi et al 2004	A non-specific extract of hydrophilic compounds derived from P*runella vulgaris* (a Chinese herb).	20.6	The authors did not comment on the mode of action.	Not published
Zhang et al 2007	An acidic lignin-carbohydrate complex extracted *Prunella vulgaris* (compound is speculated to be similar to that studied by Xu et al(1999))	18	Acts by blocking attachment and entry. Antiviral effect appears to be of a virucidal nature (i.e. cannot be abrogated by washing the virus prior to adsorption).	8.5

Copeland et al 2008	A synthetic compound produced by treating heparan derived octasaccharides with 3-O-sulfotransferase	47.7	Both attachment and entry.	2.39
Karmarkar et al 2010	An artificially sulfated fucoidan extracted from the seaweed *Padina tertrastromatica*	0.74	Blocks absorption.	~20
Sinha, Astani, Gosh, Schnitzler & Ray 2010	Artificially sulfated fucoidan extracted from *Sargarssum tenerrimum*	0.5	Blocks adsorption and or entry.	25 - 35
sinha et al 2010	Artificially sulfated form of alginic acid (sulfated sodium alginate) extracted from *Sargarssum tenerrimum*	6	Blocks adsorption and or entry.	26 - 31
Ekblad et al 2010	Anti-Tumour drug PI88 modified by the incorporation of a lipophilic functional group	2.1	Compound identifies as "14" is both virucidal (envelope disruption), adsorption inhibiting, and capable of binding to cell surface prior to virus addition. Also hindered HSV cell to cell spread.	~1.7
Luganini et al 2011	A peptide derived dendrimer called "SB105_A10" (a branched peptide core presenting four peptide functional groups)	1.26 - 1.9	prevents adsorption by blocking HS (not VAP)	4.6829 (Luganini et al 2010)
Lopes et al 2013	An artificially sulfated polysaccharide extracted from the seeds of *Caesalpina ferrea* (a species of Brazilian tree)	405	Blocks adsorption, possibly blocks gene expression and or cell to cell spread	not published
Mader et al 2016	Calcium spirulan (extracted from the micro algae *Spirulina platensis)*	0.04 - 0.07	Blocks attachment only	74.6
Lopes et al 2017	Artificially sulfated polysaccharide (SU1F1) extracted from the seaweed *Enteromorpha compressa*	28.25	Appears to inhibit viral gene transcription and genome replication	34

With regard to polysaccharides with anti-HSV activity, in increased level of viral inhibition tends to be correlated with an increased degree of sulfation (sulfation is measured as the average number of sulfate groups per sugar moity) (Chen and Huang 2018). This phenomenon is particular evident in cases where artificially sulfated compounds have been tested alongside their parent compound (the parent compound being either non-sulfated or having alow sulfate content). The difference in the resulting IC_{50} values (i.e. the concentration of the test compound at which the titre of viral progeny is 50% of that in the mock-treated control group) clearly illustrate the role that sulfation plays in determining the anti-hepretic properties of GAG mimetics. Karmarker Pujol, Damonte, Gosh & Ray (2009)and Sinha *et al* (2010) both tested the anti-herpetic effects of polysaccharides extracted from marine seaweeds, these compounds

gave IC_{50} values of 50 and 1.4µg/mL in the respective studies. Artificially sulfated forms of the same compounds produced values of 0.74 and 0.54µg/mL, this equates to a 98.5% and a 35.7% reduction in their respective IC_{50} values (Karmarker Pujol, Damonte, Gosh & Ray 2009; Sinha *et al* 2010).

The influence of sulfation on a compounds anti-herpetic properties is ostensibly due to the relationship between GAG sulfation and high affinity interaction of said GAG with HSV-1 gB and gC molecules. Heparan sulfate GAGs are very highly sulfated and as such, highly sulfated polysaccharides mimic the electrostatic binding properties of HS GAGs more closely than their low sulfate counterparts. As an aside, it can be observed in Figure 1.4 that there is little or no correlation between sulfation and the IC_{50} values displayed by compounds which do not act via binding to the HSV VAPs (gC and gB).

Increasing molecular weight of GAG mimetics is another property that tends to correlate with increasing inhibition of HSV, that is to say where highly similar compounds have been tested side by side it tends to be the compounds with the higher molecular weight that show the greater level of HSV inhibition (Chen and Huang 2018). The effect of increasing molecular weight is evinced by the trend observable in Figure 1.4, the compounds are listed in order of increasing molecular weight hence the relationship between molecular weight and IC_{50} values can be observed by reading down the column.

It is worth noting that while compounds having a molecular mass of 20 kilo Daltons (kDa) or more (refer to Figures 1.4 and 1.5) tend to be the most efficacious inhibitors of adsorption and entry *in vitro,* such heavy compounds are unlikely to find clinical utility as a result of the pharmaco-kinetic draw backs of large hydrophilic molecules. Compounds exceeding 500 DA

in mass typically show poor oral bioavailability as a result of degradation by digestive enzymes and low adsorption in the gastrointestinal tract, (Donovan, Flynn & Amidon 1990; Goldberg & Gomez-Orellana 2003; Mitragotri, Burke & Langer 2014). This is because these compounds are very poorly adsorbed via the paracellular route (in the ileum) , in combination with the fact that hydrophilic molecules are almost totally unable to be absorbed by the transcellular route. As such hydrophilic molecules exceeding 2 kDa (the vast majority of viral adsorption/entry inhibitors) show highly restricted oral bioavailability (Donovan, Flynn & Amidon 1990; Goldberg & Gomez-Orellana 2003; Mitragotri, Burke & Langer 2014). While compounds which lack oral bioavailability may still be suitable for parenteral administration this largely restricts a drug to in patient scenarios, thereby imposing a considerable reduction in terms of practicality and profitability.

In addition to hindering oral adsorption, it seems that high molecular weight also restricts the capacity of adsorbed compound molecules to interact with virus particles . This was pointedly illustrated by Nyberg et al's (2004) research into the anti-herpetic properties of PI-88 (an artificially sulfated anti-tumour drug hydrolytically extracted from the yeast *Pichia holstii*), PI-88 produces only modest HSV-1 inhibition *in vitro*, having an IC_{50} of 6 µg/ml as compared to the 1 µg/mL produced by treatment with medicinal heparin (Nyberg et al 2004). Despite this, PI-88 outperformed heparin *in vivo*, apparently as a result of its low molecular weight (2.4 kDa as compared to 15 kDa for the tested heparin species) and the ostensible impact that molecular diameter has in deterring a compounds capacity to inhibit the direct cell-to-cell spread of HSV (Nyberg et al 2004). Furthermore Lee, Pavy, Young, Freeman and Lobigs (2006) have shown that while several heavy polysaccharides are considerably more effective *in vitro*, PI-88 is by far the most well tolerated and efficacious of the compounds they investigated when used to

treat live subjects challenged with dengue virus (DENV), JEV or Murray Valley encephalitis virus (MVE). The authors tentatively attributed this discrepancy to the problematic properties of the high molecular weight (MW) compounds, i.e. shorter plasma half-life and greater propensity for off target binding (Lee, Pavy, Young, Freeman & Lobigs 2006).

1.11 Herpes Simplex Type-1

Seeing as the experimental section of this paper is concerned with assessing several novel HS mimetics in terms of their ability to inhibit HSV-1 it is pertinent to first examine the public health concerns associated with herpes simplex viruses. This is done so as to provide context regarding the biological attributes of HSV-1 and to give an understanding of the public health concerns that novel anti-HSV drugs migh seek to address.

Herpes simplex type one is the etiological agent responsible for an incurable infection that is defined by lifelong latency and intermittently recurring lesions in the region of the mouth or genitals (Bradley, Markowitz, Gibson & McQuillan; Looker et al 2013; Wheeler 1988). Looker et al (2015) estimated that 67% of all individuals globally (in the 0 – 49 age cohort, data collected in 2012) were infected with HSV-1, prevalence was found to vary between geographic regions with no area showing lower than 40% community infection. As the rate of infection was found to be higher in older cohorts it is quite possible that the total prevalence of HSV-1 is even higher than the 67% found in the studied age group (0-49 years old) (Looker et al 2015). It is likely that part of the reason for the extremely high prevalence of HSV-1 is that it can be spread by individuals who do not realise that they are contagious (Bernstein et al 2013; Bradley, Markowitz, Gibson & McQuillan 2014; Sacks et al 2004; Wheeler 1988). Infectious HSV virions are known to be shed by persons who are not manifesting visible lesions, additionally, viral shedding has been found to precede the initial

manifestations of herpes, meaning that HSV can be spread by those who have never presented any visible symptoms (Bernstein et al 2013; Bradley, Markowitz, Gibson & McQuillan 2014; Finger-Jardim et al 2017; Sacks et al 2004).

Cold sores are HSV-1 associated lesions occurring in the vicinity of the mouth and are properly termed orolabial herpetic lesions (Looker et al 2015; Wheeler 1988). Herpetic lesion occurring in the genital region (herpes genitalis) are most frequently caused by HSV-2, however HSV-1 is also capable of producing genital herpes (Wheeler 1988). The proportion of herpes genitalis cases that are due to HSV-1 (as opposed to HSV-2) has been shown to be steadily increasing in a number of western countries, this has tentatively been explained as the result of increased rates of oral-genital transmission (i.e. via oral sex) and decreasing prevalence of orolabial HSV-1 among adolescents (a phenomenon which might reduce the proportion of the adolescent population whom are producing HSV-1 antibodies at sexual debut) (Lafferty, Downey, Celum & Wald 2000; Nilsen & Myrmel 2009; Roberts, Pfister & Spear 2003; Tran, Druce, Catton, Kelly& Birch 2004; Vyse et al 2000 Xu et al 2006). In many western countries genital associated HSV-1 is on track to surpass HSV-2 as the leading cause of herpes genitalis cases, in certain locations HSV-1 already accounts for a substantial majority of new genital herpes infections (Nilsen & Myrmel 2009; Roberts, Pfister & Spear 2003; Tran, Druce, Catton, Kelly& Birch 2004).

Initial infection with HSV (genital of orolabial) occurs when virion laden secretions are transferred from one individual to another. Herpes simplex virions quickly lose viability once the infectious secretion has evaporated (saliva was shown to dry after 35 minutes under experimental conditions), as such direct transfer from one individual to another is by far the most likely means of transmission (Bardell 1990; Bardell 1995). That being said, low level

virion viability has been detected on cotton wool three days after inoculation with HSV particles, accordingly fomite transmission is a conceivable if unlikely mode of transmission (Larson & Bryson 1985). Most often the site of initial infection is the oral or urogenital mucosa, cells of the epidermis also offer a propitious site for initial infection (Goel, Docherty, Fu, Zimmerman & Rosenthal 2002; Petti & Lodi 2019).

It is believed that successful invasion of a new host requires that virions come into contact with an area of damaged epithelium/epidermis, this damage need only be very minor in nature, in the case of epidermal invasion it is actually necessary that surface abrasion be extremely superficial in order to facilitate a long lived latent infection (Goel, Docherty, Fu, Zimmerman & Rosenthal 2002; Peti & Lodi 2019). Once cells of the epidermis/epithelial mucosa are infected the resulting progeny spread from the site of primary infection and invade the free nerve endings of nearby sensory neurons (Petti & Lodi 2019). Virus particles travel through the neural axon until they reach the cell nucleus, located in the nerve ganglion (Petti & Lodi 2019). Latent infection is established in the immunoprivileged cells of nerve ganglia; for oroloabial infections latent HSV resides in the gasserian and trigeminal ganglia, in cases of herpes genitalis latency is typically maintained in the sacral ganglia (Petti & Lodi 2019; Wassoner-Fountain & Grossman 2004). Secondary outbreaks are the result of reactivations in this viral population, upon reactivation daughter virions disseminate down the neural axons and are released via exocytosis at the free nerve endings of sensory neurons (Petti & Lodi 2019). Daughter virions released from the nerve endings reinfect epithelium and/or epidermis, resulting in either asymptomatic shedding (the most common type of HSV reoccurrence) or the manifestation of herpetic lesions (Koyuncu, Hogue & Enquist 2013; Petti & Lodi 2019)

The classical model of HSV spread within a host is based on what has been observed in the murine model of infection, this is believed to be a sound analogy for human infections, however it is worth noting that humans are the only natural host for HSV-1(Goel, Docherty, Fu, Zimmerman & Rosenthal 2002). As such there is a degree of speculation involved in the classically accepted model of HSV transmission as the majority of data comes from mice, the initial processes in naturally occurring infections (i.e. human infections) are notably absent from the literature.

For the purposes of the present investigation the relevant molecular processes in HSV-1 infection are as follows. Each new cellular infection is initiated when a free virion attaches to the surface of a host-cell via binding between the VAPs gB and gC and host HS GAGs. Glycoprotein C is the primary VAP in HSV-1 attachment with gB serving to facilitate adsorption in the absence of sufficiently high valency of gC-HS interactions (Delguste et al 2019). Once the virion is adsorbed, it moves across the cell surface via the sequential binding and release of HS-GAGs by gB. This interaction is controlled by the "mucin-like-region" of gC which serves to regulate gB-HS duration of binding and overall valancey (Delguste et al 2019). Lateral movement continues until the gD encounters and binds one of its cognate receptors, nectin-1, nectin-2, herpes virus entry mediator (HVEM) or 3-O-sulfated HS (Cocchi, Menotti, Dubreuil, Lopez & Campadelli-Fiume 2000; Geraghty, Krummenacher, Cohen, Eisenberg & Spear 1998; O'Donnell and Shukla 2008). This triggers a conformation al shift in gD and triggers the envelope glycoproteins gB, gH and gL to associate with gD in a manner that produces an active fusion complex, this in turn leads to fusion between the viral envelop and host-cell membrane (Karasneh & Sukla 2011) Following membrane fusion the viral nucleocapsid is transported to the nucleus where genome replication and gene transcription occur (Hochberg &Becker1968). The

nucleocapsids of progeny virions are been assembled in the nucleus after which they are \transported via the golgi apparatus wherein they receive the surrounding membrane which will become the HSV viral envelope (Carter & Saunders 2007). The golgi vesicles then migrate to cellular membrane and release the mature virions via fussion between the vesicular membrane and the cell membrane.

1.12 Serious Complications Associated with Herpes Simplex Type-1

For most patients HSV-1 infection does not result in serious harm, though it is often a source of embarrassment and discomfort, this is especially true for herpes genitalis which despite its relatively high prevalence still carries a significant social stigma (Bickford, Barton & Mandalia 2007).

In addition to these commonplace concerns, HSV-1 occasionally produces life threatening sequelae, most notably in neonates and the immunocompromised. Mahant et al (2019) found that 4.5 out of every 10,000 infants in the Cincinnati area (United States) became infected with HSV during or soon after birth (the authors did not differentiate between HSV-1 and HSV-2, however HSV-1 is believed to account for the majority of neonatal infections), resulting in 6% mortality (Finger-Jardim et al 2017; Mahant et al 2019). Additionally, the authors found a statistically significant increase in the rate of neonatal HSV infection over the period 2009-2015, this was attributed to the increasing rates of genital infections with HSV-1 and the corresponding increase in perinatal exposure to the herpes simplex type (HSV-1) which shows higher infectivity in infants (Finger-Jardim et al 2017; Mahant et al 2019).

Jorgensen, Dalrd, Ostergaard, Norgaard & Morgensen (2017) monitoring of Herpes simplex virus encephalitis (HSVE) in Danish hospitals gave an estimate of 4.64 cases of HSVE per

million persons per year. Of these cases 72.8% were due to HSV-1 (this number is believed to be higher in neonatal HSVE) with mortality of 8.3% at 30 days follow up and mortality of 18.6% at 12 months post infection (Jorgensen et al 2017). Data collected from other nations indicate that these figures conform to the global trend (Hjalmarsson, Blomqvist & Skoldenberg 2007, Sheybani, Aribikhan & Nader 2013, Jouan et al 2015; Modi et al 2017).

Other HSV-1 associated ailments include infections of the eye, skin (eczema hereticum) and persistent oral infection (Farooq, Shah & Shukla 2010; Valerio & Lin 2019; Wheeler 1988;). Ocular HSV-1 can lead to significant corneal damage and even blindness; HSV-1 is believed to be the leading cause of infectious blindness in developed nations (Farooq et al 2010; Valerio & Lin 2019).

The immunocompromised are particularly at risk from HSV-1 related illness and death; Tan, McArthur, Venkatesan & Nath (2012) found that immunocompromised patients with HSVE were six times more likely to die than their immunocompetent counterparts. Luzzati et al's (2019) study of antibiotic-resistant-pneumonia in immunocompromised patients found that HSV-1 related pneumonia resulted in 27.3% mortality. By way of comparison, mortality in the remainder of the study group was 20.6% making HSV-1 significantly more deadly than other forms of treatment resistant pneumonia they studied (Luzzati et al 2019).

Herpes simplex-1 is also notable for the high rate at which drug resistant viral variants emerge in immunocompromised sufferers. Under normal conditions treatment with acyclovir (the recommended first line antiviral for HSV-1) rarely results in viral escape, however acyclovir resistant HSV-1 strains are alarmingly common among immunocompromised patients (Wassoner-Fountain &Grossman). A study of acyclovir users in the Netherlands

found that only 0.27% of the immunocompetent subjects were harbouring a resistant strain of HSV-1, this figure was 7% for the immunocompromised participants in the same study (Stranska et al 2005).

The creation of a novel treatment for HSV-1 would serve to remedy a pressing public health concern, this is because HSV-1 produces debilitating symptoms in vulnerable persons, regularly acquires resistance to the preferred treatment and is capable of manifesting multi-drug resistant phenotypes (these can only be treated by drugs which have unfavourable toxicity profiles) (Wilson, Fakioglu & Herold 2009; Jiang, Feng, Lin & Guo 2016). Additionally, non-drug strategies for managing the spread and symptoms of HSV-1 are unlikely to be effective, behaviour and hygiene based initiatives are hindered by the fact that HSV-1 is nearly ubiquitous and can be transmitted by asymptomatic individuals (Bernstein et al 2013; Bradley, Markowitz, Gibson & McQuillan 2014; Finger-Jardim et al 2017; Looker et al 2015; Sacks et al 2004). Numerous attempts at developing an HSV-1 vaccine are yet to produce any clinically approved treatment, furthermore there is considerable doubt as to the plausibility of producing an immune response which would be capable of preventing HSV-1 shedding by chronically infected individuals (Galasso, Whitely & Merigan 1997; Johnstone, Koelle & Wald 2014; Rajcani, Banati, Szenthe & Szathmary 2018; Stanberry et al 2002)

1.13 Heparan Sulfate Analogues as Inhibitors of Other Viruses

Excluding HSV, experimental HS mimetics (and similar putative attachment/ uptake inhibitors) are most often investigated for their potential as inhibitors of HIV and human papillomavirus (HPV). As is the case with HSV, these viruses are especially propitious subjects for treatment with glycomimetics, this is because their primary modes of transmission are amenable to inhibition by topical prophylactics (i.e. vaginally administered

gels). This means that there is a clearly defined utility for efficacious compounds that are unsuitable for internal administration.

A number of polyanionic compounds (compounds having high negative charge density) have shown considerable *in vitro* inhibition of HIV-1, with the most promising of these having IC_{50} values in the range of 0.1- 5 µg/mL (see Figure 1.5). The few clinical trials that have been conducted with such compounds indicated that they are only marginally effective or in some instances could actually enhance HIV infectivity *in vivo*. Continuous infusion with intravenous dextran sulfate was found to significantly increase the plasma titre of HIV p24 antigen in HIV infected patients (Flexner et al 1991). Stage III trials with vaginally administered gels containing carrageenan and naphthalene sulfonate both showed no discernible protection against the sexual transmission of HIV-1, while a trial with a similar gel containing cellulose sulfate indicated that this product might actually increase the rate of HIV-1 transmission (though the validity of this finding is subject to debate) (Bolognesi 2007; Van Damme et al 2008; Pirrone, Wigdahl & Krebs 2011, Pirrone, Passic, Wigdahl & Krebs 2012). Subsequent re-examination of the *in vitro* properties of cellulose sulfate confirmed that at low concentrations (~0.1 µg/mL) the compound produced a marked enhancement of HIV-1 replication with viral yields being up to nine times higher than those in the control group (Tao, Richards & Harner 2008).

Polyanionic compounds tested for *in vitro* inhibition of HPV showed substantial variation between compounds with the same putative mode of action (binding to HPV VAPs and/or uptake related proteins) with IC_{50} values ranging from 0.1 - >100 µg/mL (see figure 1.5). In addition to this HPV subtypes tend to differ in their susceptibility to specific GAG mimetics, in many cases there was greater than eight-fold difference in the IC_{50} values evinced by

different HPV subtypes treated with the same compound (Buck et al 2006; Christensen et al 2001; Gao, Liu, Wang, Zhang & Zhao 2018; Lembo et al 2008; Rodriguez et al 2014).

Various other viruses have been used as test pathogens in assessing the clinical potential of HS mimetics, the above mentioned study by Lee, Pavy, Young, Freeman & Lobigs (2006) is a pertinent example. The authors showed that PI-88 is capable of inhibiting DENV and JEV, ostensibly by binding to their respective VAPs in place of HS (Lee, Pavy, Young, Freeman & Lobigs 2006). Many of the studies conducted along these lines targeted viruses where the WT virus is not known to exploit host HSPGs, but rather adsorbs to host-cells via non-HS GAG species. Because HS and other types of GAG are somewhat analogous in terms of their chemistry and biological functions it is conceivable that compounds intended as HS mimetics will be able to inhibit viruses that don't typically utilise host HS and vice versa (Gandhi & Mancera 2008). Accordingly, the various studies in which GAG mimetics inhibited viruses that do not exhibit HS mediated attachment may still have some relevance in informing the predicted function of the compounds examined in this thesis.

Garcia-Vaillalon and Gil-Fernandez (1991) demonstrated the inhibition of African swine fever virus (ASFV) by pentosane polysulfate, fucoidan and carrageenan (both lambda and kappa isoforms), these are all naturally occurring polysaccharides (Chen & Huang 2018; Damonte, Matulewicz & Cerezo 2004; Sinha, Astani, Gosh, Schnitzler & Ray 2010). Song *et al* (2013a) and Song *et al* (2013b) demonstrated that artificial sulfation of a polysaccharide extracted from *Chuanmingshen violaceum* (a medicinal herb) produced a compound having moderate antiviral activity against duck enteritis virus (DEV) and Newcastle Disease Virus (NDV). Of special relevance to human disease is the demonstration by Lopes et al (2013) that a sulfated extract

from *Caesalpina ferrea* (a Brazilian tree species) inhibits poliovirus *in vitro* with an IC_{50} of 1.73µg/ml, a value that places it well within the realm of clinical relevance.

1.14 Polysaccharides Showing Unusual Inhibitory Mechanisms

For the most part investigations into the antiviral capacity of sulfated polysaccharides (and similar polyanionic compounds) are premised on the notion that these compounds likely bind to VAPs or entry-proteins in the place of HS or a host receptor possessed of a similar electrostatic profile (e.g. the poliovirus receptor family- see Lopes *et al* 2013). Rather paradoxically some of the most effective antiviral polysaccharides have been shown to exert their inhibitory effect by a means other than the disruption of viral adsorption or uptake. Lopes *et al* (2017) found that a sulfated seaweed extract inhibited HSV-2 with a IC_{50} of 2.2µg/mL. While some of this activity was due to inhibition of viral adsorption the authors found that the greater part of the compounds inhibitory effect was due to an unknown interaction occurring late in the replication cycle (Lopes et al 2017). Similarly, Garcia-Vaillalon and Gil-Fernandez (1991), Gao *et al* (2018) and Lopes *et al* (2013) all found evidence that they considered to indicate some degree of post-entry inhibition due to the polysaccharides they tested.

There have also been several noteworthy instances of sulfated polysaccharides showing virucidal activity, this means that rather than creating a transient retardation of a virus-particles replicative capacity these compounds interact with cell-free virions in a manner that renders them permanently neutralised. The tenacity of this neutralising activity indicates that the compounds are acting in a manner that is unlike that shown by heparin, HS and the majority of the mimetic compounds which resemble them. Under normal circumstances binding between HS and cognate VAPs does not appear to induce any permanent modification of viral proteins

and tends to be terminated after a relative short duration (Delguste et al 2018; Delguste et al 2019; Peerboom et al 2017) Plant extracts studied by Huheihel , Ishanu, Tal & Arad (2002), Carlucci, Scolaro, Noseda, Cerezo & Damonte(2004), Lopes *et al* (2013) and Ghosh, Pujol, Damonte, Sinha & Ray (2009) all showed evidence of having permanently altered the external aspects of the tested viruses resulting in a loss of function (membrane disruption affecting the viral envelope or permanent binding between compounds and viral proteins are putative mechanisms for this loss of function). All four papers found that their respective compounds had a virucidal impact on HSV, additionally, Lopes *et al* (2013) found that a highly sulfated form of the plant extract they studied had a moderate virucidal effect on polio virus (PV).

While it is anticipated that any anti-herpetic activity displayed by the test compounds will be the result of interference with adsorption or entry, other modes inhibition should not be ruled out a priori. Additionally, it is worth examining whether the test compounds are possessed of any virucidal effect.

1.15 Anomalous Upregulation of Viral Replication by Known Inhibitory Compounds

Finally, it is worth mentioning that there have been instances in which polyanionic compounds reproducibly inhibiting viral replication *in vitro* have produced the opposite effect under specific test conditions. The studies discussed in section 1.13 in which cellulose sulfate showed unexpected upregulation of HIV infectivity are noteworthy illustrations of this phenomenon.

Other examples of polyanionic compounds producing anomalous upregulation include studies by Lee, Pavy, Young, Freeman & Lobigs (2006), Ekbald et al (2010) and Bartolini *et al* (2003), these studies found that treatment with specific concentrations of proven inhibitory substances lead to an increase in viral production, these studies examined West Nile virus (WNV), HSV-1 and HIV respectively. This upregulation was observed even though treatment with higher concentrations of the same compound resulted in manifest inhibition of these viruses (in the case of WNV the upregulation was only observed when cells were pre-treated with PI-88 then washed) (Bartolini *et al* 2003; Ekbald et al 2010; Lee, Pavy, Young, Freeman & Lobigs 2006).

The mechanism behind this phenomenon is unknown, one possibility is that the sometimes observed relationship between reduced *in vivo* fitness (see section 1.9) and elevated HS binding might produce an analogous relationship *in vitro (*Byrnes & Griffin 2000; Lee & Lobigs 2002). This is to say that if the studied viral strain was expressing VAPs which bound to HS with deleteriously high avidity or valance then it follows that a limited degree of binding inhibition (resulting from the presence of the polyanionic test compound) could optimise viral attachment dynamics and thereby enhance replication (this theory is discussed in more detail in Chapter 4).

Another possibility is that the interaction between viral surface proteins and certain GAG sequences may produce a conformational change in said proteins that helps facilitate down-stream processes in the infection cycle. Cerqueira et al (2013) discovered that HPV-16 interacts with free heparin in a manner that promotes infectivity, ostensibly by producing a necessary conformational change in pentamers of the L1 capsid protein. This type of interaction exerts a bi-directional influence on viral replication, viral attachment is hindered by the VAP binding

properties of the test-compound, however some post-attachment process (likely uptake/membrane fusion) is upregulated by conformational changes produced by interaction between the virus and the test-compound. It stands to reason the two opposing forces may not respond equally to dose alterations, meaning that at certain concentrations the test-compound's adsorption hindering properties may override its uptake enhancing effects, whereas at different concentrations (presumably lower concentrations) the effects of increased viral uptake may override the reduction in efficiency of adsorption .

Where increased infectivity has been observed for vaginally administered gels in live subjects (either human clinical trials or mouse based studies) several possible mechanisms have been postulated. Mesquita et al (2009) showed that cellulose sulfate is capable of disrupting the barrier function of human epithelium and Tan et al (2013) highlighted an interaction between various polyanionic compounds (including cellulose sulfate) and human semen which resulted in the formation of microscopic structures (amyloid fibrils) which enhanced viral infectivity *in vivo.* These findings may explain the putative elevation in the rate of HIV transmission observed in human subjects using vaginally administered cellulose sulfate gels (Van Damme et al 2008). Additionally, the effects of generic ingredients (excipients) present in some anti-viral gels (Moench, Mumper, Hoen, Sun & Cone 2010) and the effect of rapid compound dilution in the vaginal environment (Cone et al 2006) are generalisable principles that may lead to microbicides producing increased infectivity regardless of what the active ingredient is.

1.16 The Dendritic Effect

The present study focuses on a group of novel compounds developed by the Ferrier Research Institute (FRI) (a chemical innovation group associated with Victoria University in Wellington, New Zealand) and seeks to evaluate their efficacy as inhibitors of HSV-1 replication *in vitro.*

The compounds examined in the experimental section of this thesis consist of three dendritic compounds (compounds with dendrimer molecules)(henceforth referred to as the "test compounds") and a compound included as an outgroup comparison (henceforth the "monomeric compound").

It is anticipated that the test-compounds will exhibit an impressive capacity to mimic the biological functions of HS by virtue of a pharmacological principle known as the "dendritic effect". Dendrimers are radially symmetrical molecules featuring multiple identical active sites (functional groups with some sort of specific binding affinity) at the outer perimeter of the molecule, with repeating molecular chains linking these to the molecular midpoint (Boas & Heegaard 2003; Caminade, Ouali, Laurent, Turrin & Majoral 2015). The dendritic effect is revealed when comparing two bioactive compounds where one of the compounds is dendrimeric in structure and the other is a monomer featuring a single functional group, identical to those present in the dendrimeric compound. Where the dendritic effect is involved the magnitude of the biological response produced by the dendrimeric compound will exceed that produced by the monomer by a greater magnitude than can be explained as a mere additive effect (Boas & Heegaard 2003; Caminade, Ouali, Laurent, Turrin & Majoral 2015). An additive effect is that whereby multivalent compounds (such as dendrimers) show a dose wise response that is n times greater than the response evinced by the corresponding monovalent compound (where n is the valency of each molecule of the multivalent compound) (Boas & Heegaard 2003). By way of example Page and Roy (1997) found that tetra and octavalent dendrimers featuring mannopyranoside functional groups showed target (pea lectin) binding activity that was 28 times greater that shown by the monomeric mannopyranoside on a per-functional-group basis (net binding activity was 113 and 220 times higher for the tetramer and octamer respectively).

Biological processes which are amenable to the dendritic effect and the associated amplification of drug efficacy tend to be those which rely on multivalent interactions (i.e. monovalent interactions are insufficient to produce the expected downstream effects) (Boas & Heegaard 2003). The interaction between HSV-1 and host-cell HS GAGs during viral attachment requires an appropriate level of multivalence in order to function properly, deleteriously low valency is likely to lead to virion disengagement before successful infection can occur (Delguste et al 2019). The importance of multivalent binding in HSV-1 adsorption means that HSV-1 adsorption is likely to be susceptible to the dendritic effect and ipso facto there is reason to anticipate that the test-compounds will prove to be highly efficient inhibitors this process.

1.17 The Study Compounds

In addition to their dendritic structure there are several other reasons for hypothesising that the test-compounds will display a high level of HSV-1 inhibition. Firstly, the putative binding moieties of all three test-compounds (and that of the monomeric compound as well) are very highly sulfated being maltose moieties which have been modified by the maximal incorporation of sulfate groups(Zubkova et al 2018). By being based on maltose these molecules closely resemble the disaccharide subunits of HS GAG chains, the primary difference being that the oversulfated maltose carries more sulfate moieties than any of the HS GAGs found in nature (Rabenstein 2001). As mentioned in 1.10, the anti-herpetic properties of HS mimetics tends to increase in direct proportion to their degree of sulfation. It follows that the maximal sulfation at the active domains of the test compounds is a highly promising trait.

Secondly, two of the three test compounds have previously been studied by Zubkova et al (2018) and have been shown to be well tolerated by live hosts and to be capable of replicating at least one of the biological function of HSPGs. It should be noted that this study did not examine the ability of these compounds to alter the course of viral infection so the relevance of the findings to the present study remains to be seen (Zubkova et al 2018)

Additionally, the test-compounds have an advantage over all but a few of the currently studied HS mimetics/polyanionic anti-virals in that their molecular weight puts them at the lower end of the range in terms of the molecular masses of previously studied compounds. The test-compounds are between 3.9 and 5.5 kDa in size, meaning that they are closer in mass to the biologically propitious PI-88 (2.4kDa average molecular weight) than they are to the multitude of biologically derived saccharides which have been studied as HS mimetics (Nyberg et al 2004). The potential advantage of relatively low molecular weight has been illustrated by studies on PI-88 which is notably more bioavailable and apparently has higher *in vivo* efficacy than is typically observed for HS analogues with higher molecular weights (e.g. polystyrene sulfonate, heparin) (Ekblad et al 2010; Lee *et al* 2006, Nyberg et al 2004). The antiviral effects of PI- *in vitro* are somewhat underwhelming, yet in live subjects it outperforms the majority of heavier GAG mimetics (Ekblad et al 2010; Lee *et al* 2006, Nyberg et al 2004).

The test compounds (depicted in figure 1.6) consist of two tetramers and a trimer. The tetrameric compounds are structurally identical bar the length of the carbon backbones that links each maltose moity to the centre of the dendrimer, accordingly these are identified as the long-armed and short-armed tetramers (the long-armed tetramer is identified as the “PEG tetramer” in Zubkova et al (2018)). The trimeric compound is henceforth identified as the

"glycolipid trimer" on the basis that the over-sulfated maltose moieties are connected to the central domain of the dendrimer by glycolipid chains.

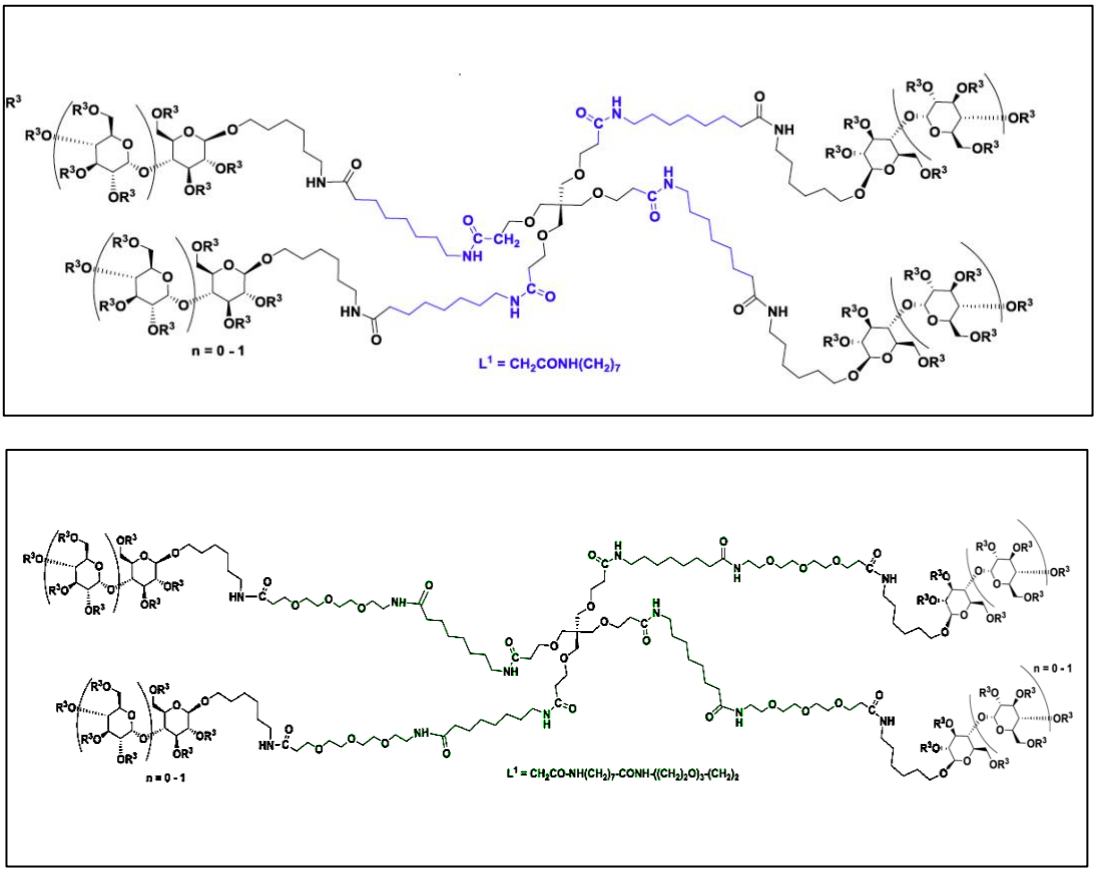


Figure 1.6: Molecular diagrams of the two tetrameric compounds (PET tetramer top, short-armed tetramer bottom). Note that the compounds are chemically identical apart from the composition of tetramer arms. "R^3O" and "OR^3" both indicate a sulfate group. All functional moieties are disaccharides hence the brackets surrounding the outer glucose moiety can be ignored.
Figure adapted from Zubkova *et al* 2018.

Due to the fact that there is a pending patent application pertaining to the glycolipid trimer it cannot be depicted in the present paper (for fear of jeopardising the intellectual property of the compound's creators). Suffice to say that the only differences that exist between the chemical structures for the tetrameric compounds and the glycolipid trimer are the eponymous number of dendrimer arms (four and three respectively) and the chemical structure of the "spoke-like" section of each dendrimer arm (i.e. region between the centre of the molecule and the functional

moieties). All compounds feature the same over-sulfated maltose moiety as the terminal group on each dendrimer arm.

The out-group compound is structurally identical to a single monomeric arm of the glycolipid trimer. As such any difference between the biological effects of the glycolipid trimer and the out-group monomer will presumably serve to illustrate the extent to which the dendrimer effect determines the anti-herpetic efficacy of the test-compounds. All four compounds are collectively referred to as the study-compounds or the studied compounds.

1.18 Aims of this Thesis

The aims of the study described in this thesis are as follows:

1) Identify the cytotoxicity of the study-compounds
2) Assess the inhibitory effect, if any, that each of the study-compounds has on the replication of HSV-1 *in vitro* and identify the IC_{50} values pertaining to the same.
3) Elucidate the extent to which any HSV-1 inhibition is likely to be attributable to the dendrimer-effect by comparing the IC_{50} of the glycolipid-trimer and the monomeric compound.
4) As much as is possible elucidate the mechanism of any observed HSV-1 inhibition produced by the study- compounds (e.g. blocking of adsorption, blocking of viral entry, post entry inhibition).

Chapter 2: Materials and Methods

2.1 List of Materials and Reagents

In-Text Nomenclature	Specific Description and Source
Acidic Glycine	Glycine (100mM), sodium chloride (150mM) dissolved in type I water, adjusted to pH 3 by the addition of $HCl_{(aq)}$.
Cell Culture Media (sometimes called growth media)	Dulbecco's modified Eagle's medium supplemented with 5% foetal bovine serum, 1 µg/mL penicillin and 1 µg/mL streptomycin
Crystal Violet	Crystal violet stain supplied by Ajax Finechem
Culture Flasks	BD Falcon brand tissue culture flasks (750mL) supplied by BD Biosciences
Culture Plate (10cm)	Cellstar brand culture plates supplied by Greiner Bio-One
Culture Pate (multi well)	BD Falcon branded 12, 24 and 48-well culture plates supplied by BD Biosciences
dNTPs (Deoxy-Nucleotide Triphosphate Solution)	dNTPs (equal concentrations of adenine, guanine, cytosine and thymine incorporating dNTPs) supplied by Bioline
DMEM (Dulbecco's modified Eagle's medium)	DMEM containing phenol red indicator, supplied by Gibco
DTT (1,4-dithiothreitol)	1,4-dithiothreitol supplied by Invitrogen
FBS (Fetal Bovine Serum)	Sterile filtered FBS supplied by Moregate Biotech
HSV-1	Kos strain HSV-1, supplied,, propagated and titred by Dr Carol Wang (University of Auckland School of Biological Science)

Infection Media	Identical to cell culture media with the omission of the antibiotics (penicillin/streptomycin)
IgG (Immunoglobulin G)	Pooled human IgG supplied by Sigma
Microtube	1.5mL microtube supplied by Eppendorf
MTT solution	5mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-(thiazole blue) (supplied by Sigma) dissolved in PBS
MTT solvent	4mM Hydrochloric acid and 0.1% NP-40 detergent dissolved in Propan-3-ol
NP-40	Np-40 detergent supplied by BDH Chemicals Ltd
PBS (Phosphate Buffered Saline)	A solution of 2g/L KCl, 2g/L KH_2PO_4, 80g/L NaCl and 11.5g/L Na_2HPO_4 dissolved in type I water and adjusted to pH 7.4 via dropwise addition of $HCl_{(aq)}$
Primers for qPCR	Supplied by Integrated DNA Technologies. See figure 2.1 for specific primer sequences.
Random Hexamer Primers	Hexamer primers for reverse transcription of total sample RNA, supplied by Bioline
Reverse transcriptase	M-MLV reverse transcriptase enzyme for conversion of RNA to cDNA, supplied by Invitrogen

SYBR Green	Applied Biosystems branded PowerUp SYBR Green master mix for qPCR. Mix contains: fluorescent dye (SYBR green) dNTPs, DNA polymerase, Uracil DNA glycosylase, appropriate buffers and a passive reference dye (ROX). Supplied by Thermo Fisher
TRIzol	Ambion branded TRIzol reagent- supplied by Life Technologies (Thermo Fisher)
Trypan Blue	Supplied by Invitrogen
Trypsin/EDTA	0.25% solution of Trypsin and Ethylenediaminetetraacetic acid (EDTA) supplied by Gibco
Type I water (all water used unless otherwise stated)	Distilled water further purified via an Arium Pro type I water unit (reverse osmosis and UV treatment)
Ultra-Pure Water	Ultra-pure water (RNAse free) supplied by Invitrogen
Vero Cells (immortalised African green monkey kidney cells)	Internal stock belonging to the University of Auckland School of Biological Science (Original source unknown, imported prior to year 2000).

2.2 List of Equipment Used

In-Text Nomenclature	Brand and Model
Camera phone	Huawei p10 Leica front facing camera
Haemocytometer	Hausser Scientific bright line haemocytometer
Heat block	VWR heat block
Incubator	A Sanyo incubator was used for cell culture, a Panasonic for any protocol involving live virus.
Inverted microscope	Leicia DM IL
Light box	Manufactured by University of Auckland Technical Staff
Microtube centrifuge	Eppendorf centrifuge 5424R
Minus 80 Freezer	Phcbi TwinGuard ULT (ultra low temperature freezer)-MDF DU702VX
Nano drop	Implen N60
Pipette	Eppendorf manual pipettes, various volumes between 1000 and 10μL
Plate reader	Spectra Max ID3
qPCR machiine	Thermo Fisher

Serological pipette	Eppendorf Easpet 3 (various sizes)
Shaker	IKA KS 260
Thermocycler	Gene Amp PCR System 9700

2.3 Cell Culture Practices

2.3.1 Culture Conditions and Cell Maintenance

Vero cells (Immortalised African green monkey kidney cells) were used to carry out all experiments; these were cultured in Dulbecco's modified Eagle's media (DMEM) supplemented with 5% foetal bovine serum (FBS), 1µg/mL of Streptomycin and 1 µg/mL of Penicillin. It is standard practice to rear mammalian cells in growth media supplemented with 10% FBS, however the past experience of the laboratory staff proved that media containing 5% FBS rendered indistinguishable cell viability and growth rates while also reducing the fiscal cost associated with cell culture maintenance.

Cells were maintained in an incubator at 37 degrees Celsius and an atmosphere of 5% carbon dioxide, these were the incubation conditions used for all procedures unless otherwise stated..

2.3.2 Passaging Cells

Cells were passaged when they were between 80 and 95% confluent with the interval between passages being between 60 and 96 hours depending on cell growth rates. At each passage 1/5 of the cell population was retained with the remainder being either discarded or used to seed plates for upcoming experiments.

The protocol for passaging consisted of aspirating the culture media, washing the cell monolayer in phosphate buffered saline (PBS) and applying 0.25% trypsin/EDTA at a rate of 18μL per square centimetre. The cells were returned to the incubator for three minutes to allow the trypsin to enzymatically degrade cell-cell attachment after which a serological pipette (Eppendorf) was used to disperse the monolayer via repeated ejection and re-aspiration of fresh culture media. The concentration of the resultant cell suspension was enumerated by staining a 20μL aliquot of the cells with trypan blue and counting on a hemocytometer under an inverted light microscope (Lecia). After 1/5 of the original cell population had been returned to the culture flask/dish (with the addition of fresh media) the remaining cells were diluted as necessary to seed culture plates at the correct volume and cell density for experiments being conducted the following day. Any cells that were not required for seeding experimental plates were destroyed by exposure to aqueous hydrogen peroxide in accordance with the laboratory protocol.

2.3.3 Seeding Cells onto New Culture Plates

An aliquot of singly suspended cells was obtained as per the passaging procedure described in the preceding paragraph. After the concentration of the cell suspension had been found (via enumeration on a manual haemocytometer), a calculator was used to find the volume of the suspension that need be added to each new plate (or each well of a multiwall plate) I order to give the desired cell-confluence in 24 hours' time. This volume of cell suspension was added

to each plate/well immediately followed by fresh culture media to bring the total volume up to the working volume of the plate/well in question. The cell suspension in each well/plate was aspirated and ejected three to five times in order to create an even suspension of single cells, each culture vessel was then placed on the work surface and slid along the surface in an oscillating motion, five times left to right followed by five times being slid along the perpendicular axis of motion (towards and away from the researcher) and finally five more times left to right. The culture vessels where then labelled and placed in the incubator.

2.4 Testing Compound Cytotoxicity.

2.4.1 MTT Conversion Assay

Cell viability assays were conducted via MTT conversion assays; this test is so named because it relies on a yellow MTT solution being reduced to a purple formazan precipitate in a manner that requires the participation of mitochondrial enzymes (Kumar, Nagarajan & Uchil2018). Accordingly the colour of the cell lysate at the end point gives a relative indication of the metabolic activity which occurred during the assay and indirectly indicates the level of cell viability.

Vero cells were seeded on a 48 well plate with 50,000 cells and 200μL of media being added to each well. The following day the plates were inspected to assess that the cells were between 70 and 80% confluent, the lower than normal seeding density was applied as a precaution against cell layer overgrowth during the subsequent assay period. The culture media was aspirated from the wells and replaced with 200μL of fresh media containing one of the test-compounds or the monomeric compound at a concentration of either 320, 160, 40, 20, 10 or

5μM. Every time an MTT assay was conducted each of the four experimental compounds was applied to triplicate wells at each of the above stated concentrations .

Each 48-well plate also featured four control wells and two blank wells. Control wells were treated as per the test wells with the exception that the media added to them contained no candidate compound. Blank wells contained only 200μL of the culture media without the presence of any cells and were included to measure the 590nm absorbance of the combined growth media MTT reagent and MTT solvent .

After 48 hours' incubation in the presence of the test-compounds 20μL of 5mg/mL MTT solution was added to each well (with the exception of the blank wells) and the plate was returned to the incubator for a further 3 hours. The three-hour incubation period allowed the cells to metabolise the MTT thereby producing the associated colour change. At the conclusion of the three hour period the purple formazan present in each well was solubilised by adding 300μL of MTT solvent and repeatedly aspirating/discharging the liquid in the well so as to disrupt the cell layer (thereby hastening cell lysis and dissolution of the intracellular formazan). Plates were then incubated at room temperature, shielded from light, for 15 minutes. Gentle agitation was provided by a plate rocker so as to confer even dissolution and diffusion of the purple formazan.

The relative extent of the colour change in each well was measured by reading absorbance on a plate reader set to detect photo-absorbance in the region of 590nm spectral wavelength. The level of absorbance shown by the blank wells was used to gauge the proportion of absorbance that was displayed by a blank well and the growth media/MTT reagent/solvent therein (i.e. being unrelated to enzymatic reduction of MTT by the cells). This value was subtracted from

each individual reading and the resultant values were expressed as a percentage of the mean absorbance shown by control wells on the same plate.

Each iteration of the MTT assay featured two technical replicates for all of the tested concentrations and four blank wells.

2.4.2 Trypan Blue Exclusion Assay

Cells were seeded on 24-well plates at 90,000 cells per well in 500μL of media, this was done to produce 70% confluence at 24 hours follow up. Culture media containing various concentrations of the study compounds (or without any added compound in the case of control wells) was added as per the method described in 2.3.1. The culture plates were returned to the incubator for 48 hours at the conclusion of which the cell layer was washed with PBS and trypsinised for 3 minutes at 37 degrees Celsius. The cells were then dislodged via repeated pipetting to create a single cell suspension and transferred to labelled micro tubes containing 1000μL of growth media. Aliquots of 20μL were removed from each micro tube, stained with trypan blue (1:1) and enumerated via haemocytometer (viewed on an inverted light microscope).

Separate counts were kept for cells that were ostensibly viable or dead within each sample, the distinction between viable and non-viable cells was made on the basis of the observed staining pattern. Cells with a visibly blue/stained cytoplasm were considered to be dead where as those showing a colourless cytoplasm were deemed to be viable. The number of dead cells was divided by the total number of cells counted (the sum of both counts) to give an estimate of the proportion of dead cells in each treatment well. Each replicate of this live/dead assay featured duplicate wells for each specific treatment condition (compound and the concentration thereof).

Cytotoxicity was inferred on the basis of reproducible differences between the mean cell viability observed in a treatment group and the control group.

2.5 Infection of Cultured Cells with Herpes Simplex Virus

All experimental infections were carried out with HSV-1 virus belonging to the F- strain of the KOS variant, virus was taken from the laboratories existing stock of HSV-1 aliquots. Stock aliquots were prepared and titred by Dr Carol Wang of Auckland Universities' School of Biological Science (AU SBS).

The HSV-1 stock aliquots contained 3.17 x 10^8 plaque forming units per mL (PFU/mL) suspended in a 1:1 mixture of culture media and skim milk (skim milk serves to enhance virion preservation and protect against the destructive impact of repeated freeze-thaw cycles as documented by Lepine, Artzet & Ling (1960)) and were stored in a minus 80 freezer.

Prior to infection viral stock was defrosted on ice, sonicated for 1 minute (in a water bath sonicator containing ice water) and agitated on a vortex in order to create an even dispersion of free, individual virions. The per-well number of cells in the to-be-infected culture plate were enumerated by trypsinising one of the wells, staining the cells with trypan blue and counting an aliquot on a haemocytometer (under an inverted light microscope). Virus stock was then diluted with infection media so as to produce the required multiplicity of infection (MOI). The volume of the viral inoculum placed onto the cell monolayer was equal to between half and one third of the recommended working volume for the culture well in question.

The specific inoculation process consisted of removing growth media from the culture vessel containing the cells that were going to be infected and adding the virus containing infection

media (hence forth called “the inoculum”) to the cell monolayer. The inoculum was left on the cells for two hours with the culture vessel being gently agitated every 15 minutes to ensure that all cells were equally exposed to free virions and to prevent drying of the cell layer. At the conclusion of the two-hour period (called “the adsorption period” henceforth) the inoculum was aspirated and replaced with a standard volume of culture media.

2.6 Plaque Inhibition Assay

Plaque inhibition assays are so-named as the measured response variable is a count of the viral plaques formed on a cell layer in response to an inoculum of fixed concentration and an independent variable that may or may not inhibit the infectivity of the virus (and thus reduce the number of plaques formed). If the count of plaques is the same for all treatments it can be inferred that the virus’s ability to infect the cells has not been inhibited. Plaque inhibition assays were conducted to assess the extent to which continuous exposure to the each of the studied compounds (at concentrations of 1.25, 2.5, 5, 10, 20 and 40 μM, (controls= 0μM)) inhibited the replication of HSV-1 in vitro.

Twelve well culture plates were seeded with 2.3 x 10^5 Vero cells per well and inspected the following day so as to ensure that the cell layer was at least 90% confluent, this was necessary to give plaques that stood in high contrast to the surrounding cell layer. Viral inocula were prepared such that each was 300μL in volume, contained an estimated 90 PFUs (based on serial dilution from the stock concentration) and one of the of the study compounds at one of the test concentrations. These inocula were used to infect each well as per the method described in 2.5 with the notable exception that the culture media added to the wells at the end of the adsorption period contained the same concentration of study-compound as applied during the adsorption period in addition to 3μg/ mL of pooled human immunoglobulin G (IgG). Each set of treatment

conditions (compound and the concentration thereof) was applied to duplicate wells each time the assay was repeated (i.e. duplicate technical replicates were carried out for each biological replicate).

The addition of IgG to the culture media is a technique that negates the need for a traditional methylcellulose overlay. The HSV binding action of human IgG prevents HSV virions from infecting naïve cells via free diffusion of virions (syncytial spread can still occur between adjacent cells) ensuring that the number of plaques formed on the cell-layer closely corresponds to the number of cells to which virions attached during the adsorption period (see Grosche et al (2019) for further details on this method).

Following adsorption period, the plates were returned to the incubator for 48 hours to allow plaques to form (henceforth called the "plaque forming period"). At the conclusion of the plaque forming period the media was aspirated, the cell layer was washed once in PBS and then immersed in a solution of 20% methanol and 1% crystal violet (made up with type I water). The cell layer was left in the crystal violet/methanol stain for 15 minutes at room temperature to allow thorough staining, the stain was then poured off and the cells were washed with type I water. Plates were inverted for 10 minutes to allow the fixed layer of cells to dry and the plaques were manually counted with the aid of alight box. At least one replicate of each plaque assay was photographed by a camera phone for record keeping purposes.

2.7 Titration of Viral Suspensions by Plaque Assay

Any time there was a sample containing an unknown concentration of virus particles (as was the case with total progeny assays- see 2. 8) the sample was titrated as follows. A ten-fold dilution series (concentrations ranging from 1×10^{-1} to 1×10^{-8}) was created and then aliquots of the three or four most propitious sample concentrations ("test inoculum concentrations" henceforth) were used to inoculate triplicate wells of 12-well plate containing near-confluent Vero cells. Infection of the Vero cells and staining of the resultant plaques were carried out as per the method described in 2.6 with the exception that there was no compound added to either the inoculum or the culture media.

A mean plaque count was taken from each set of triplicate wells belonging to each of the tested inoculum concentrations where the number of plaques formed was suitable for counting. Wells containing too many plaques (more than 70) were omitted from counting due to the risk that crowding of the cell monolayer might lead to overlapping plaques being counted as one, thereby leading to undercounting of the total plaque number. Wells containing fewer than 6 plaques were also not recorded in the results as these were considered likely to show unacceptably high error as a result of the amplified stochastic effects that influence small samples (Borda & Frost 1968). Mean plaque numbers were then multiplied by the inverse of the inoculum size (expressed in mL) and divided by the dilution factor to give an estimate of the number of PFUs per millilitre. Where the results obtained from a single experimental sample showed showed greater than 25% divergence the titration processes was repeated with the inclusion of additional dilution increments (additional dilutions were inserted between those that gave the disparate estimates of viral titre).

2.8 Quantitative Polymerase Chain Reaction for Abundance of Viral mRNA

2.8.1 Collecting Total RNA from HSV Infected Vero cells

Vero cells were seeded on 12-well plates and 24 hours later they were infect with HSV-1 in the presence of various concentrations of the studied compounds. Cell seeding and infection were carried out in the same manner as described for plaque inhibition assays (see 2.5) with the exception that the cells were infected at a MOI of 1 (as opposed to with a total inoculum of ~90 PFU) and the media that was added after the adsorption period did not contain IgG. The number of virions required to give an MOI of 1 was calculated by trypsinising one of the wells on the 12-well plate, staining the cells with trypan blue and counting the cells on a haemocytometer so as to generate an estimate of the number of cells per-well.

At four hours post infection (HPI) the culture media was aspirated, 300μL of TRIzol was added to each well and the cell layer was completely exfoliated via repeated aspiration/discharging o the TRIzol with a manual pipette. The resultant suspension was placed in to a micro tube labelled with the corresponding treatment conditions and placed in a minus eighty-degree freezer.

2.8.2 Purification of the Extracted RNA

The following day the suspension of cell lysate and TRIzol was removed from the freezer, thawed at room temperature, mixed with 60μL of chloroform and centrifuged. Centrifugation was carried out in a micro tube centrifuge under a force equivalent to 12,000 times the force exerted by gravity (12,000 x rfg) for a period of 15 minutes. The sample was then mixed with isopropanol and subject to further centrifugation at 12,000 x rfg in order to precipitate the RNA. The RNA pellet was washed in 70% ethanol, dried at room temperature and resuspended in ultra-pure water.

2.8.3 Conversion to cDNA

The concentration of RNA in each sample was quantified with a nano-drop photo spectrometer and the concentration of each sample was standardised by the addition of ultra-pure water to give a final concentration of 200ng/μL. Each sample was combined with 10mM of dNTPs, random hexamer primers (these serve to ensure all RNA fragments are primed), DTT and M-MLV reverse transcriptase and converted into cDNA according to the manufacturers protocol provided with the reverse transcriptase. The duration and temperature of the process intervals applied in the thermocycler are shown in figure 2.1. At the conclusion of the theromocycler run all samples were retained overnight at four degrees Celsius.

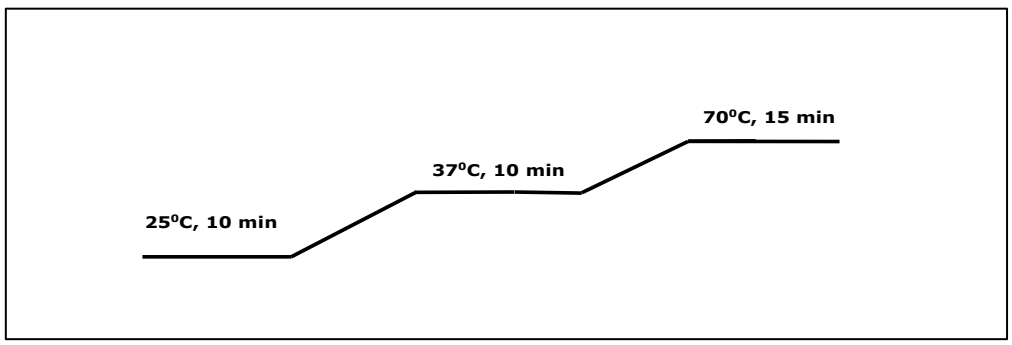
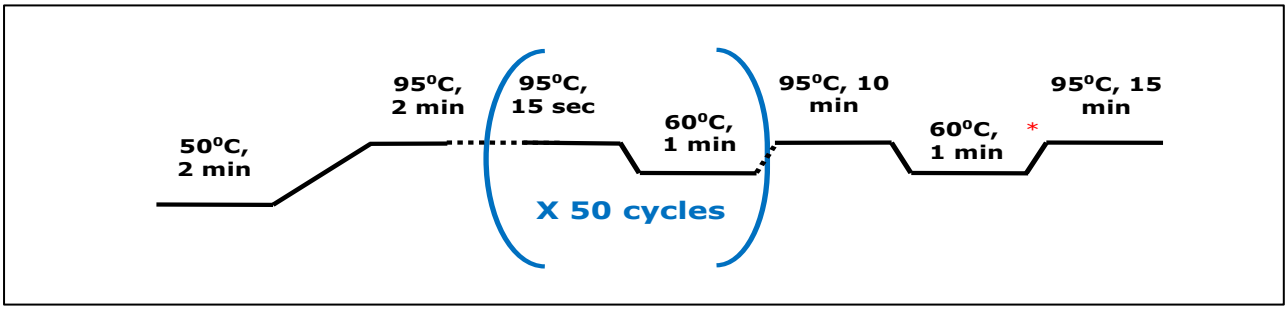


Figure 2.1: Thermocycler temperature intervals and duration applied during reverse-transcriptase conversion of sample RNA to cDNA

2.8.4 qPCR Protocol

The following day samples were added to a 384-well qPCR plate, each well contained 5μL PowerUp SYBR green master mix, 300nM of both forward and reverse PCR primers (either GAPDH or ICP-47) and 20ng of sample cDNA. Each of the individual cDNA samples was applied to four wells with duplicate wells measuring expression of GAPDH (host cell protein) and ICP47 (HSV-1 protein) respectively, for the HSV-1 gene ICP47. The primer sequences that were used to amplify GAPDH and ICP47 cDNA, and the profile of the qPCR thermocycling are shown in Figure 2.2.

A)



B)

Primer	Sequence
GAPDH forward	5’ GTCTCCTCTGACTTCAACAG 3’
GAPDH reverse	5’ ACCACCCTGTTGCTGTAGCC 3’
ICP47 forward	5’ ATGGCGGACACCTTCCTGGA 3’
ICP47 reverse	5’ TTAATGGCCGCCGTCCGCCA 3’

Figure 2.2:

A) Temperature time of each successive stage in the qPCR protocol

NB- All ramp rates were 1.6^{0}C/second with the exception of that indicated by “*”

*- Ramp rate= 0.15^{0}C/second

B) Forward and Reverse primer sequences used to amply host GAPDH cDNA (upper) and HSV-1 ICP47 cDNA(lower).

2.8.5 qPCR Data Analysis

The level of transcription for the host protein GAPDH (as measured by the number of qPCR cycles required to produce detectable fluorescence (i.e. the cycle threshold/ Ct)) was used as a means to standardise the level of ICP47 expression to the concentration of host cDNA in each well (GAPDH transcription was assumed to be approximately constant across all of the tested cells regardless of treatment). For each set of treatment conditions, the average Ct value from GAPDH wells was compared to the average Ct value obtained from ICP47 wells by what is known as the delta-delta-Ct method (Zhang, Ruschhaupt & Biczok 2010).

Briefly put the delta-delta-Ct method (as applied in the present case) uses the difference between the expression levels of an endogenous "house keeping gene" and the gene of interest as observed in the control group (this is called delta Ct) to the same metric as observed in the various treatment groups and finds the difference between these ("delta-delta-Ct). The delta-delta Ct value for each treatment is then raised to the second power so as to give values that represent the number of fold by which the housekeeping gene relative level of protein expression has changed in each treatment group, thereby giving directly comparable data points (Zhang, Ruschhaupt & Biczok 2010).

2.9 Total Progeny Assay

Vero cells were seeded in a 48-well plate at a concentration of 7 $x10^4$ cells per well, the following day these cells were inoculated with HSV-1 KOS at an MOI of 0.01, 0.1, or 1 (as determined by the requirements of the experiment in question). Cells were exposed to one of the study compounds during both the adsorption period and the post adsorption replication interval, in some subsequent experiments compound exposure was altered so that cells were only exposed to test compounds during the adsorption period alone or only during the post-

adsorption period. All experiments in which total progeny was measured featured control wells which were not exposed to study-compound at any time. All infections were carried out as per the description in section 2.5 with the exception that study compounds were applied at the stated time points.

Following infection, the cells were returned to the incubator until such time as they were to be harvested, this was determined by the requirements of the experiment in question (the times of cell harvesting correspond to the time points listed on the x-axis of progeny graphs in the results section (Chapter 4)). At the time of harvesting wells being sampled for supernatant only had the culture media aspirated and placed into appropriately labelled micro-tubes. Wells for which cell lysate was being collected had the cell layer exfoliated by using a 200μL pipette to repeatedly scrape and aspirate the cell layer, the supernatant and the suspended cells were then transferred to a labelled micro-tube. Lysate samples were subjected to three freeze/thaw cycles at minus 80 degrees to ensure complete lysis of the sample. The concentration of virions in each sample was estimated by titrating as per the method in section 2.7.

2.10 Controlled Synchronisation of Viral Adsorption and Entry

In order to disambiguate the stage of the viral infection cycle that the test compounds may have been affecting it was necessary to create conditions whereby viral adsorption could take place without proceeding to cellular uptake until a controlled time point. By pre-chilling 95% confluent Vero cells at 4 degrees Celsius and infecting these with aliquots of similarly chilled virus it was possible to allow adsorption while inhibiting cellular-entry. This is because virus adsorption has been found to readily occur at low temperatures whereas viral uptake is a temperature dependant process, cell entry comes to a near-total halt when the temperature is lowered to 4 degrees.

During the two hour adsorption interval cells were maintained at four degrees with gentle rocking every 15 minutes. At the conclusion of the adsorption interval the cell layer was washed three times with cold PBS so as to remove any test compound and any non-adhered virions that may have been present. Growth media that had been pre-warmed to 37 degrees Celsius was then added to the cells either with or without the addition of a test compound.

When the effect that the compounds have on adsorption was being tested cells were inoculated in the presence of one of the study-compounds, maintained 4 degrees for 2 hours and, at the conclusion of the adsorption period, washed and covered in warm media that was devoid of any study-compound (but did contain pooled human IgG to facilitate plaque formation (see section 2.6).

The inverse protocol was applied when interaction between test-compound and cellular entry was being investigated: chilled cells were inoculated with chilled HSV-1 in the presence of blank media, at the end of the adsorption period the cells received pre-warmed media containing one of the study-compounds. The re-warmed cells were then left in the presence of the test compound for two hours at 37 degrees Celsius. This was done to allow cellular uptake of the virus in as much as was possible in the presence of the specific test-compound. At the conclusion of this uptake window the cells were washed once with PBS so as to remove free virions and any compound residue, exposed to acidic glycine for two minutes (to destroy any extra-cellular virions), washed with fresh media (so as to eliminate the cytotoxic effects of the acidic glycine) and overlaid with media containing pooled human IgG. The use of acidic glycine for the inactivation of extra-cellular virions is in accordance with the practice described by MacLean (1998) (a modified version of the practice was applied by Karger & Mettenleiter

(1993)). Staining with crystal violet and subsequent plaque enumeration were carried at the conclusion of the 48 hour incubation period.

Chapter 3: Results

3.1 Cytotoxicity of the Test Compounds

3.3.1 MTT Conversion Assay to Test for Cytotoxicity

Results obtained from the MTT assays indicated that Vero cells experience no loss of metabolic activity when exposed to any of the study-compounds over a 48 hour period. By extension this

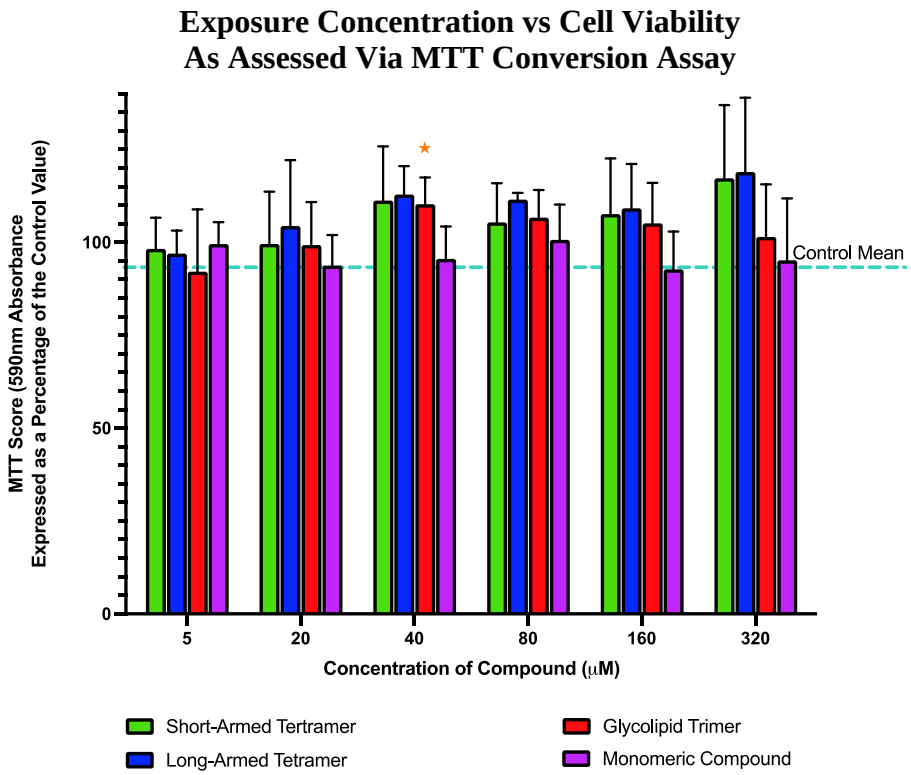


Figure 3.1: Control relative level of MTT conversion by Vero cells as measured after 48 hours in the presence of the indicated test compound. The level of MTT conversion was measured by reading the 590nm optical absorbance of combined cell lysate and supernatant after three hour incubation in the presence of aqueous MTT. All bars represent the mean obtained from across five biological replicates. Error bars represent the standard deviation evinced across all five biological replicates.

The cyan coloured horizontal line indicates 100%- i.e. MTT conversion shows no difference from the control mean. The orange star indicates the only set of treatment conditions for which a one sample T-test (two tailed) produced a p-value lower than 0.05 (indicating that the data from this sample exceeds the 95% confidence threshold for declaring a difference between the mean MTT conversion produced by this treatment and the control (at the population level)).

indicates that the compounds do not have any cytotoxic activity at concentrations up to 320μM (Figure 3.1).

Rather surprisingly there was an apparent increase in the level of MTT conversion when the two tetrameric compounds were applied at the highest of the exposure concentrations (320μM). Cells exposed to 320μM of the short and long-armed tetramers produced mean MTT scores which exceeded the control value by 17 and 19% respectively.

One sample T-tests (two -tailed) were conducted to determine the likelihood that each of treatment responses might be produced under a scenario whereby the population mean for MTT conversion was identical to that in the control population (identical MTT conversion is expressed as "100%" in the present data set) (Figure 3.2). Of the twenty-four treatment groups, six produced t-tests wherein the p-value was less than 0.1 (Figure 3.2) indicating that there was a greater than 90% probability that the treatment conditions corelated with an altered level of MTT conversion. Of these six treatments, five showed mean MTT conversion levels that exceeded the level produced in the control group. Accordingly. if the observed alteration in MTT conversion is the result of compound exposure (as opposed to being the result of experimental noise or systematic error) it follows that the compound has apparently increased the rate of MTT conversion, an effect that bears no apparent correlation with compound cytotoxicity.

Compound	Concentration (μM)					
	5	20	40	80	160	320
Glycolipid Trimer	92.1 (70.1 -114.2)	99.3 (80.1 – 118.4)	110.3 (100.7 -119.8)	106.6 (94.8 - 118.5)	105.1 (85.1 - 125.2)	101.5 (81.4 - 121.5)
Short-Armed Tetramer	98.3 (83.9 - 112.6)	99.6 (85.2 – 114.1)	112.6 111.2 (92.1 - 130.4)	105.4 (89.7 - 121.1)	107.6 (88.0 - 127.3)	117.3 (95.4 - 139.2)
Long-Armed Tetramer	97.0 (84.8 – 109.2)	104.4 (81.8 – 127.0)	112.9 (103.4 - 122.4)	111.5 (109.3 - 113.7)	109.2 (92.9 - 125.5)	118.9 (91.4 - 146.5)
Monomeric Compound	99.5 (91.4 – 107.7)	93.7 (86.3 – 101.2)	93.2 (84.1 - 102.3)	100.7 (93.1 – 108.3)	92.8 (78.2 – 107.3)	95.2 (71.1 - 119.3)

P-Values from One Sample T-tests (two-tailed) for the Likelihood that the Same Results Would be Produced if the Mean Level of MTT Conversion was Identical in Both the Treatment and Control Populations.						
Compound	Concentration (μM)					
	5	20	40	80	160	320
Glycolipid Trimer	0.35	0.87	**0.02**	**0.08**	0.26	0.79
Short-Armed Tetramer	0.63	0.94	0.12	0.27	0.22	**0.06**
Long-Armed Tetramer	0.34	0.57	0.10	**0.07**	0.12	**0.07**
Monomeric Compound	0.86	**0.07**	0.22	0.87	0.11	0.48

Figure 3.2: Upper table- mean MTT conversion and corresponding 95% confidence intervals (95% C.I.) for the true mean of the total population of cells within the treatment group. All numbers are expressed as a percentage of the MTT conversion in the control group (100% equates to zero difference between the treatment and the control),.

Lower table- The p-values obtained by performing one sample T-tests (two-tailed) on the MTT conversion levels observed over three replicates of the MTT conversion assay. These values represent the confidence with which one can infer that there is no difference between mean MTT conversion in the treatment group and the mean of the control group (at the population level). Values less than 0.1 appear in bold and underlined, the results from these groups carry a greater than 0.9 likelihood of indicating a difference between the two means.

The present results cannot be taken to indicate a cytotoxic effect on the part of the test-compounds at any concentration below 320μM. The two treatment which showed an average reduction in the level of MTT conversion and p-values that were less than 0.2 were the groups exposed to 20 and 160μM of the monomeric compound. These results do not to appear to indicate any level of cytotoxicity on the part of the monomeric compound as both the highest and the lowest of the tested concentrations of the monomeric compound gave sample means that were close to 100% (95% and 99.5%) and t-test derived p-values of 0.475 and 0.856 respectively, strongly indicating that this compound is not cytotoxic across the range of tested concentrations.

The unexpected increase in the level of MTT conversion evinced by the tetrameric compounds appears to be due to some property of the compounds themselves, rather than an experimental artefact. This is based on the fact that all of the tested compounds appear to produce a dose-wise alteration in the level of MTT conversion that closely mirrors the their dose-wise inhibition of HSV plaque formation. In the plaque inhibition assay (3.3) the tetrameric compounds produced the highest level of HSV-1 inhibition and both compounds consistently produced very similar results. In the same assay the glycolipid trimer displayed inhibition of HSV plaque formation at around half the level of that displayed by an identical concentration of either tetrameric compound. The monomeric compound produced no HSV inhibition at any of the tested concentrations. All of these observations reflect the relative MTT score elevation shown by each of the tested compounds. It stands to reason that both of these data sets (MTT score and HSV inhibition) may be indicative of the various test-compounds' ability to replicate the biological functions of HS GAGs. This theory is expanded upon in chapter 4.

It should be noted that while the results for cells treated with 40 and 80μM of the long-armed tetramer conform to the observable trends these results carry lower statistical weight than the rest of the above data. This is because these concentrations were only able to be tested two and three times respectively as a result of the research group running out of this compound at a time when it was not possible to obtain more.

3.1.2 Trypan Blue Exclusion Assay to Measure Compound Cytotoxicity

Due to the unexpected nature of the above results (an apparent increase in the level of MTT conversion upon exposure to 320μM concentration of both the tetrameric compounds) it was deemed necessary to perform an additional assay to confirm that the MTT assay was correct in predicting that the compounds did not have any cytotoxic activity at the tested concentrations. The secondary assay for cytotoxicity was a trypan blue exclusion assay performed in accordance with the protocol described in section 2.4.2.

Because access to the study compounds was extremely limited the trypan blue assay was only carried out on a single compound (the short-armed tetramer) on the basis that it displayed the highest levels of HSV inhibition a significantly larger quantity of this particular compound was supplied to the research group.

The trypan blue exclusion assay indicated that there was no difference in the ratio of viable to nonviable cells in the control group and the treatment groups after 48 hours of exposure to the short-armed tetramer (figure 3.3).

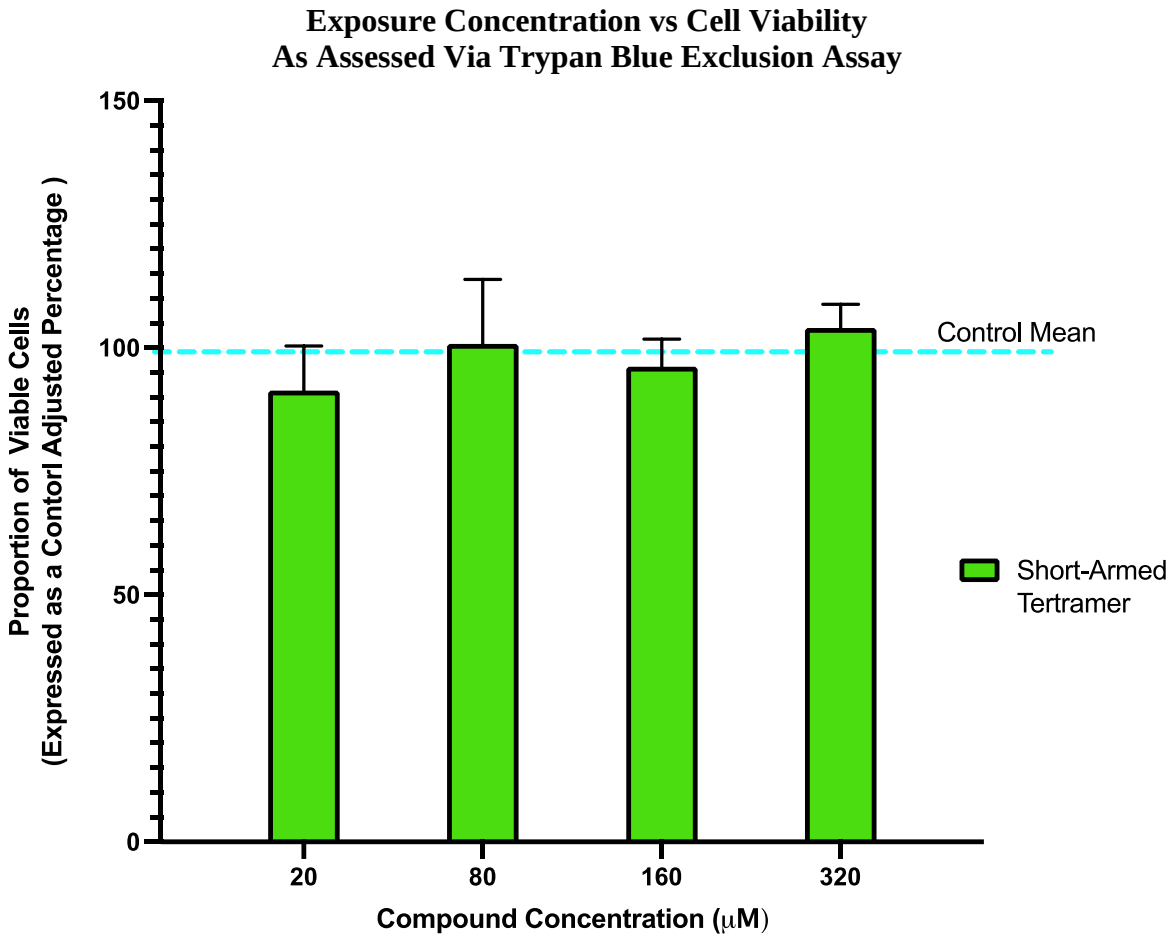


Figure 3.3 Bar graph representing the proportion of the total cell population that remained viable after 48-hour exposure to the short-armed tetramer at concentrations between 20 and 320μM. Cell viability was assessed via trypan blue exclusion assay. All values are the control adjusted percentage of viable cells, i.e. "100%" indicates that cell viability was identical in the treatment group and the control group, 50% indicates that viability in the treatment was half of that in the control group. The cyan coloured dashed line indicates identical viability to the control group. All bars represent mean values obtained from at least three biological replicates (20, 160 and 320μM treatments were replicated four times, the fourth replicate of the 80μM treatment showed less than 50% the control viability and was deemed to be an outlier). Error bars represent the standard deviation between replicates.

	Concentration (μM)			
	20	80	160	320
Mean Percentage of Viable Cells (Standardised against Control)	89.9	100.8	96.2	104.6
Standard Deviation	10.4	13.0	5.5	5.7
P-Value	0.15	0.92	0.27	0.19

Figure 3.4: Mean levels of Cell viability observed in the trypan blue exclusion assay (values expressed as a percentage of the proportional cell viability observed in the control group from the same biological replicate). The P-values were obtained via one sample, two-tailed T-tests.

One sample t-tests (two-tailed) were conducted to establish the probability that the observed results could be reconciled with a circumstance in which there was zero difference between the population means for the control and the various treatment conditions. Three of Four t-tests gave p-values greater than or equal to 0.2 (see figure 3.4) indicating a relatively low level of confidence that the control and treatment means are identical at the population level. This is almost certainly a reflection of the frustratingly low statistical power which applies to these results, a flow on effect of the limited sampling and high level of inter-replicate variation in total viability (this variation affected both treatments and controls). It is doubtful that these P-values indicate any biologically relevant effect occurring because of the study-compounds, this is indicated by the fact that the mean cell-viability was close to 100% of the control at all concentrations. Additionally there was no dose-wise trend in the discrepancy between the

treatment means and the control values with the highest of the tested concentrations (320uM) showing a slightly elevated proportion of viable cells.

The combined results of the MTT and trypan blue exclusion assays give a clear indication that the test compounds are not cytotoxic at concentrations up to and including 320μM. If compound concentration is to be described in terms of weight per unit volume (a metric which is favoured by some authors in the field of drug development) the three dendrimer test-compounds can be said to produce nil cytotoxicity in Vero cells at concentrations in excess of 1000μg/mL (specific values are given in figure 3.1.3). The monomeric compound was tested at a maximum of 369.8 μg/mL and showed no cytotoxicity at this concentration

Compound	**Maximum Concentration Tested (μg/mL)***
Long-Armed Tetramer	1772.9
Short-Armed Tetramer	1592.1
Glycolipid Trimer	1240.5
Maltose Monomer	369.8

Figure 3.5 The highest assayed concentration for each of the test compounds expressed in μg/mL. All concentrations are equivalent to 320μM.

*None of the concentrations tested exhibited any degree of cytotoxicity

3.2 Time Point Corresponding to Maximum Yield in a Single Step Infection

Before carrying out further experiments it was necessary to first establish the growth dynamics of HSV-1 (KOS variant, F strain) under control conditions. A single-step infection assay was carried out at various MOIs so as to give a clear indication as to the MOI that would be most suitable for infecting cells in subsequent experiments. Additionally, the results of a single-step infection assay can provide the additional benefit of revealing the time point (the time post-infection) at which maximum viral yield is achieved.

The single-step infection assay was conducted by infecting Vero Cells at MOIs of 1, 0.1 and 0.01 according to the protocol described in chapter 2. Cells were grown in 48-well culture plates and infected when they were between 80 and 90% confluent. Duplicate wells from each of the three MOI groups were harvested at 4 hourly intervals over a 36 hour period. At each time point one of the harvested wells had only the supernatant collected (so as to measure cell-free virions at the corresponding time point) and the other had both the cell layer and supernatant collected (as per method in 2.9)(so as to measure the total number of viable virions). The collected samples were stored at -80 C to allow titration at a later date. The growth curves produced by titrating the samples are shown below (Figure 3.6).

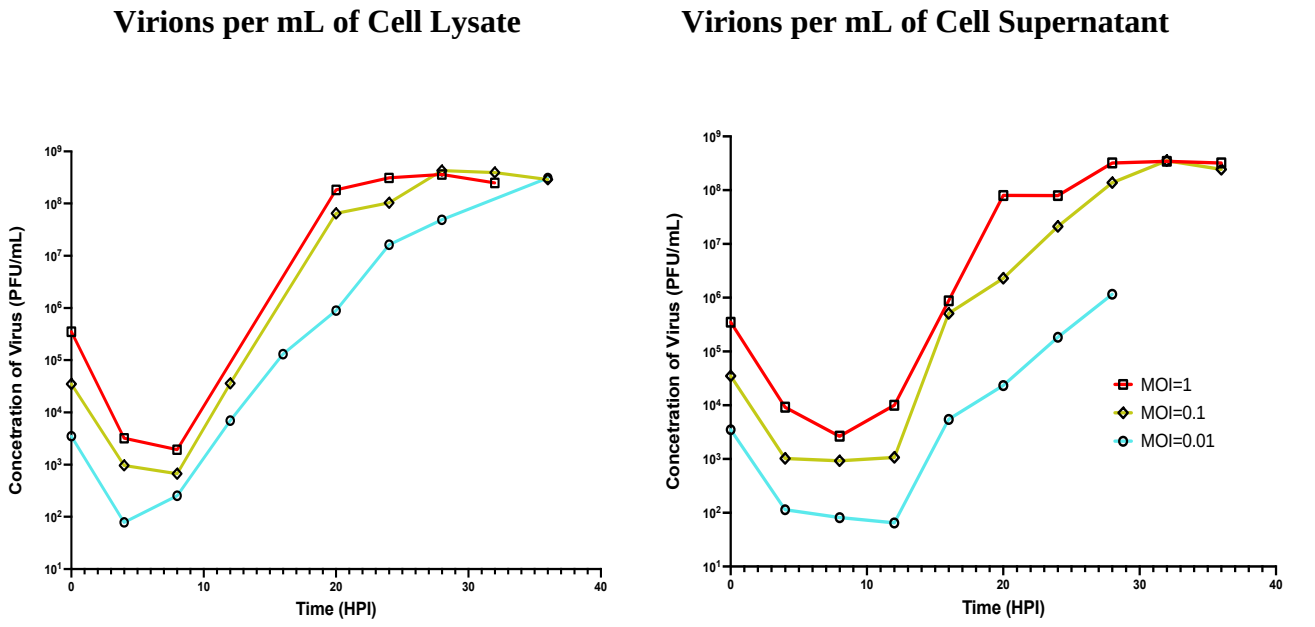


Figure 3.6: Line graphs of the progeny produced by infection of Vero cells with HSV-1 (KOS) at multiplicities of infection of 0.01, 0.1 and 1 virions per cell (line colour indicates multiplicity of infection, see key). Numbers are expressed as the number of plaque forming units per mL of cell lysate (left hand graph) or cell supernatant (right hand graph). Numbers are the product of two experimental replicates and for this reason error bars are not depicted. Inconsistencies in the sampling intervals depicted (e.g. between 8 and 20 hpi) reflect occasions when sampling was not possible due to laboratory protocols regarding after-hours access.

All three MOIs gave rise to the same approximate yield (4 x10^8 PFU/mL), however the time taken to reach this upper limit varied in direct proportion to the applied MOI. Cells infected at an MOI of 1 achieved maximum viral yield at between 20 and 24 hours post infection (hpi), whereas the MOIs or 0.1 and 0.01 didn't produce peak progeny concentration until 28 and 36 hpi respectively.

Comparison of the viral titre present in lysate and supernatant samples revealed that the two metrics were nearly identical from 0 to 16 hpi. After 16 hours the concentration of PFUs in the lysate samples increased far more rapidly than those in the supernatant samples. This was

presumably the result of intracellular virions maturing at a rate that exceeded the rate of virion release.

The less rapid growth rate evinced by the supernatant samples meant that supernatant taken from cells infected at an MOI of 0.01 did not reach the same maximum viral yield as was seen in the lysate samples. In spite of this there is no reason to suspect that the two figures would not have converged had the sampling period been extended, the gradient of the line for MOI= 0.01 in figure 3.6 indicates that this would have been the case.

Based on the results obtained in these experiments it was decided to conduct the subsequent total progeny assay (section 3.6) with an MOI of 0.1 and end point sampling being conducted at 24 hpi. The MOI of 0.1 was selected on the basis that it would provide a conveniently rapid rate of virus production while also reducing the likelihood that an overabundance of virus particles in the applied inoculum might overwhelm the test compound.

Working on the assumption that the test compounds bind to the exterior of HSV-1 particles (the intended mode of action) it follows that an overabundance of virions in the inoculum might lead to the entire dose of test-compound being bound to cell-free virus particles while some virions retain unaltered infectivity . This scenario is theoretically possible regardless of how effective the test-compound may be and would require the compounds to be applied at unreasonably high rate so as to gain a clear indication of their efficacy.

The lower MOI of 0.01 was deemed too low due to the impractically long time it took to produce maximum viral yield. Both time and the supply of test-compounds were constraining factors in the research programme, for this reason the MOI of 0.1 was selected.

3.3 Plaque Inhibition Assay

3.3.1 Plaque Inhibition Assay

In order to gain an indication of the anti-herpetic activity that each of the test-compounds possessed a plaque inhibition assay was conducted as per the method described in 2.6. In this assay the viral inoculum and the host-cells were exposed to study-compound during both the adsorption period and the subsequent plaque formation period (the 48 hour incubation following adsorption). In this way both pre-entry and post-entry inhibitory mechanisms were able to be assessed in a single assay. Additionally the effect of any synergistic interaction between pre and post-entry inhibition mechanisms could be captured by this method.

The assay was carried out by inoculating Vero cell monolayers with a suspension of HSV-1 made up from a previously titrated viral stock, virus was diluted with infection media so as to produce a suspension containing close to 533 PFU/mL, the necessary value to produce 80 plaques per well in the control group.

Two control wells were included on each 12-well plate, the first step in data interpretation was to express the raw plaque counts as a percentages of the plaque number present in the control group from the same biological replicate. This was done so as to correct for any inconsistency in the number of PFUs in the inoculum, (the intra-replicate variation in control plaque counts was observed to be somewhat lower than the inter replicate variation). Test compounds were applied at concentrations of 1.25, 2.5, 10, 20 and 40μM with identical compound concentrations being applied during both the adsorption and plaque-formation periods.

Additional concentrations of 0.156, 0.313 and 0.625 μM were applied as part of an expanded examination of the short-armed tetramer. These additional concentrations were included due

to the fact that the short-armed tetramer was found to be active at very low concentrations additionally the abundant supply of this particular compound allowed for more extensive testing than could be performed with the other compounds. The additional exposure concentrations of the short-armed tetramer were applied to two biological replicates. The monomeric compound was tested at an additional exposure level of 80μM, this was done so as to verify that the zero inhibition observed for this compound held true for elevated exposure levels.

The average number of plaques per well and the corresponding percentage inhibition are shown below (Figure 3.7 and 3.8**)**. All values were obtained by conducting three biological replicates of this assay except where otherwise indicated (Stars above bars in figure 3.7 and 3.8 indicaye

treatments for which only two biological replicates were conducted), with each set of treatment conditions being applied in duplicate (two technical replicates).

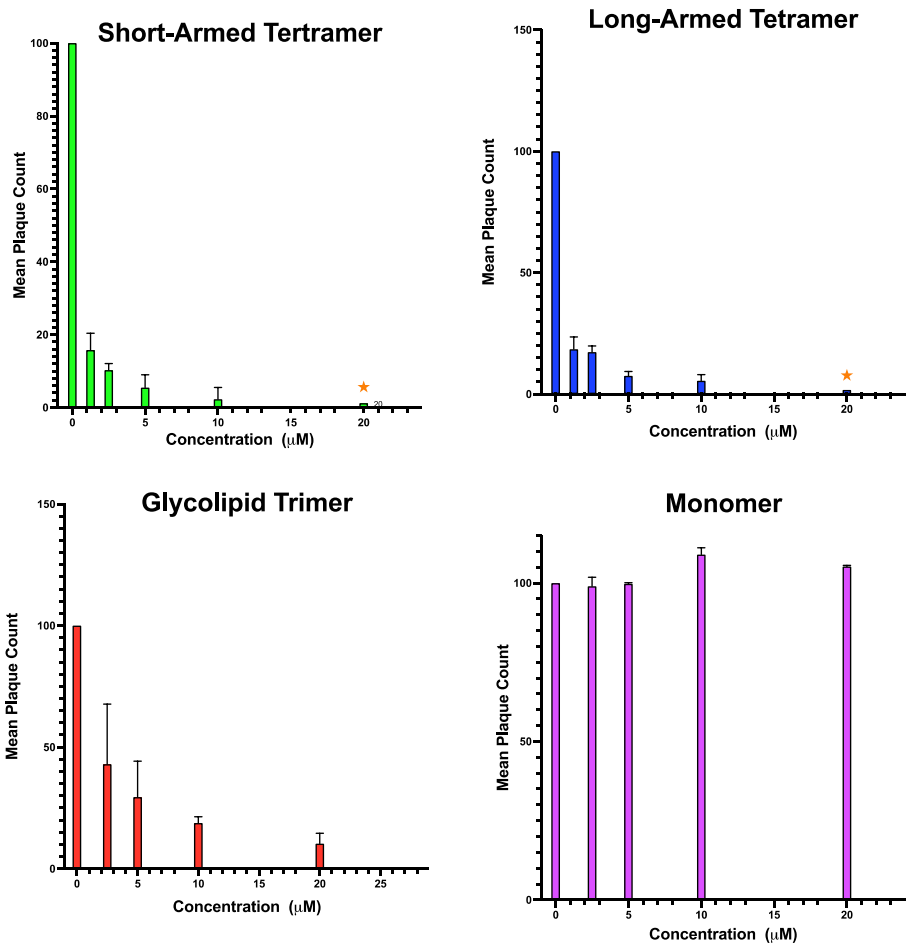


Figure 3.7: Bar graphs of the mean number of plaque per well in the plaque inhibition assays.

Each set of axes corresponds to one of the test compounds, cells were exposed to between 1.25 and 20 µM of test-compound during viral inoculation and the subsequent incubation period (48 hours).

All bars represent the mean plaque number from across three biological replicates unless otherwise indicated (diamonds above bars indicate treatment for which only two replicates were conducted), error bars represent the standard deviation between biological replicates.

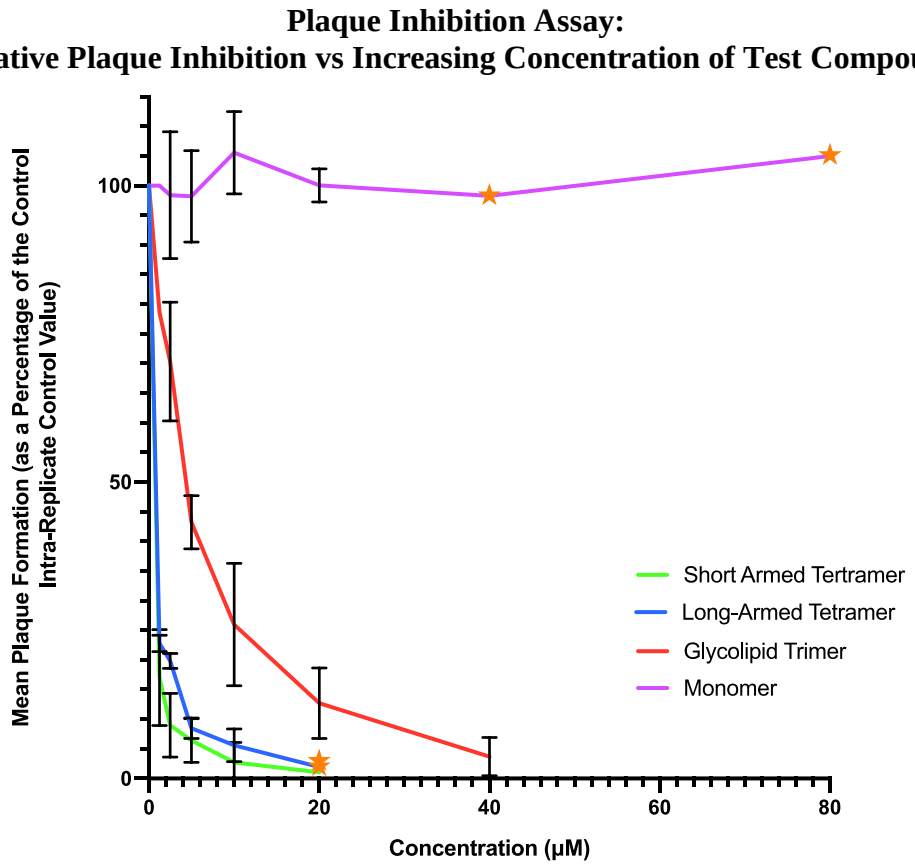


Figure 3.8: Line graph of the relative level of plaque formation observed in plaque inhibition assays conducted in the presence of increasing concentrations of each of the four test compounds. All numbers expressed are control adjusted based on the mean number of plaques observed in control wells from the same experimental replicate. Error bars represent the inter-replicate standard deviation. All data points are means values obtained from three experimental replicates with the exception of points displaying a yellow star and an absence of error bars. These are indicative of means obtained from two experimental replicates (an unfortunate consequence of lost data).

NB- Figures 3.8 and 3.7 display the same data set with 3.7 showing the values produced by taking means of the raw plaque numbers and 3.8 displaying the means obtained after the same values were standardized by converting them to a percentage of the intra-replicate control value.

The results from the plaque inhibition assays clearly indicated that of the four test compounds the tetrameric compounds were by far the most efficient inhibitors of HSV-1. The dose-wise levels of inhibition they produced were near identical (see figures 3.7 and 3.8)). This was somewhat unsurprising given that these compounds have highly similar molecular structures, the length of the dendrimer arms (i.e. the number of carbon atoms in these chains) being the only difference between the two compounds.

The monomeric compound showed no appreciable effect on HSV-1 plaque formation at any of the tested concentrations. The glycolipid trimer showed a modest inhibitory effect, consistently producing fewer plaques than the control wells, however also producing between 2 and 3 times more plaques than were formed in the presence of the tetrameric compounds.

3.3.2 Non-Linear Regression to Find Fifty Percent Inhibitory Concentration

The data from the plaque inhibition assay expressed as percentages of the number of plaques formed in the control group (all numbers were standardised to the control belonging to the same biological replicate as there was some inter-replicate variation in the concentration of the viral inoculum) was plotted against the base 10 logarithm (log) of the compound concentration. Prism GraphPad was used to fit non-linear regressions using the "find absolute IC_{50}" function with the "Top" and Bottom" inputs being set as fixed parameters with values 100 and 0 respectively (because the x variable is the number of plaques expressed as a percentage of the control, these parameters are 100 and 0 by definition). Because zero can't be expressed as a logarithm control concentrations were approximated as log -5 (0.00001 uM). The fitted regression curves are shown alongside the data for "percentage control-plaques" in figure 3.9). The IC_{50} values interpolated from the regression curves are listed below (Figure 3.10). Neither fitted regression curve nor IC_{50} could be generated for the monomeric compound as the data

strongly indicates that this compound has zero anti-herpetic effect at the tested concentrations.

This was reflected by the fact that the best fit values gave a slope gradient of zero.

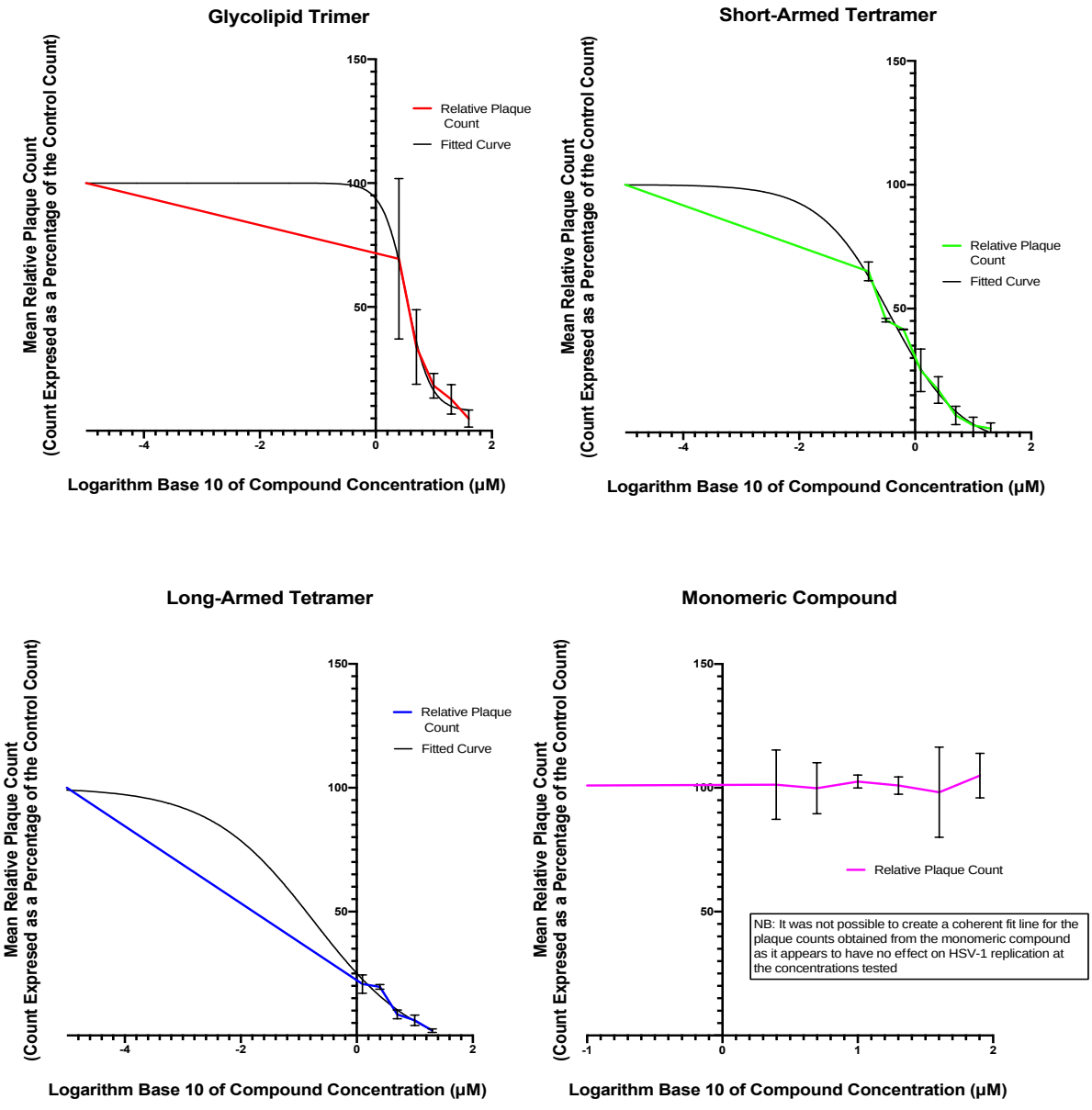


Figure 3.9: Line graphs of the percentage plaque formation graphed against the Log (base 10) of the compound concentration. Each set of axes also features the line of best fit generated by a non-linear regression performed via the "absolute IC_{50}" function of the Prisim GraphPad software. Data points are identical to those in Figure 3.8 with the exception that the x values (compound concentration) have been Log transformed

	IC_{50} (μM)	95% Confidence Interval	R^2
Glycolipid Trimer	3.7	2.7 - 4.9	0.88
Short-Armed Tetramer	0.32	0.26 - 0.38	0.98
Long-Armed Tetramer	0.13	0.06 - 0.33	0.99
Monomeric Compound	NA	NA	NA

Figure 3.10: Parameters derived from the non-linear regression performed on a plot of control-relative plaque inhibition against logarithim base 10 of compound concentration.

3.4 Quantitative Polymerase-Chain-Reaction of Viral RNA

The two tetrameric compounds were selected for analysis via qPCR. This was done in order to confirm that the plaque inhibition assay accurately reflected the antiherpetic properties of these compounds. The glycolipid trimer and the monomeric compound were omitted from this assay due to the need to conserve reagents and the fact that these compounds respectively displayed a limited and an undetectable inhibitory effect.

Because RNA samples were collected at 4 hours post infection (hpi) this assay also served to elucidate whether the observed inhibitory effect was primarily due to the interruption of events occurring early in the replication cycle or if the targeted inhibition of latter event was also a pertinent factor.

The HSV-1 gene for infected cell protein 47 (ICP47) was selected for qPCR quantification. Because ICP47 is an immediate early gene, it was inferred that reduction in ICP47 expression (at 4 hpi) could be taken as a reliable indicator that total viral gene expression had been reduced to a similar extent. It is highly unlikely that any other HSV-1 gene transcript would be expressed at a level that is substantially higher than ICP47 expression, at 4hpi.

If the pertinent aspects of the test compounds' activity took place prior to the expression of viral proteins, then it was anticipated that the qPCR results would show a near identical dose response to that seen in the plaque inhibition assay. Conversely, total failure to recapitulate the results from the plaque inhibition assay would likely indicate that the tetrameric-compounds operated by inhibiting a viral process (or processes) occurring at some point after viral uptake. A partial resemblance between the two assays might indicate that the tetrameric-compounds possess multiple modes of action.

Side by side comparison of line graphs representing the results of the plaque inhibition assay and the qPCR results (figure 3.11) show that the dose-wise responses are near identical, thereby confirming the accuracy of the results from the plaque inhibition (3.3). The similarity between the two data sets also indicates that the two tetrameric compounds act on one or more of the early processes in the HSV-1 replication cycle. What differences can be discerned between the two data sets are likely attributable to the higher sensitivity of qPCR process, especially when measuring samples containing very low concentrations of virus/viral RNA.

It should be noted that that the qPCR results are the product of a single experiment and as such do not have attendant standard deviation values or error bars. The lack of repetition in the qPCR assay was due to the limited availability of PCR reagents.

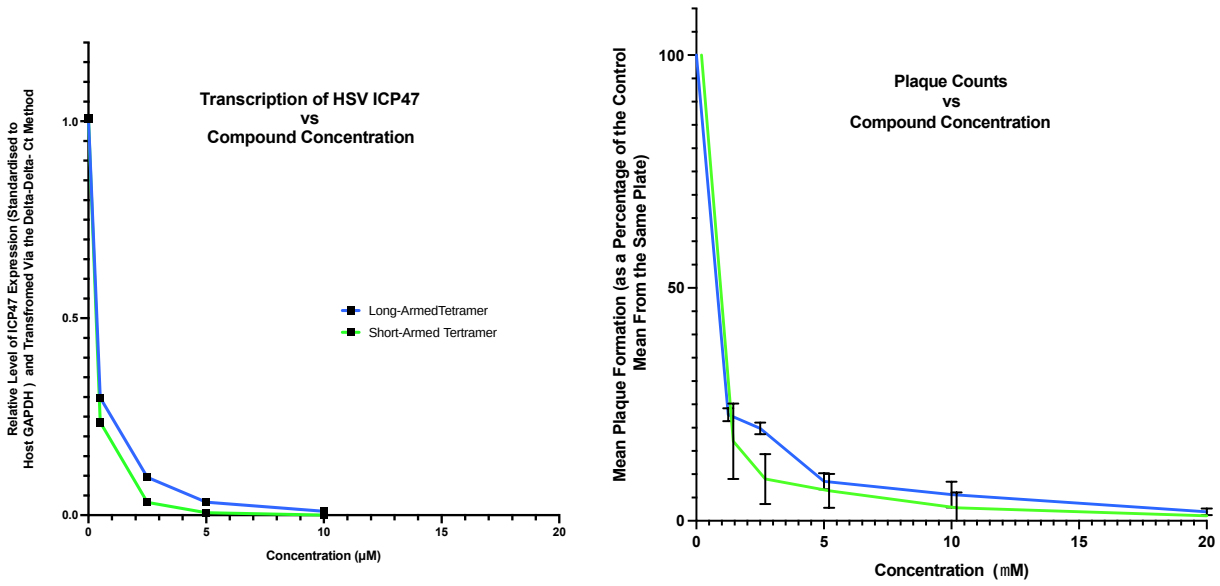


Figure 3.11 Side by side comparison of line graphs showing the qPCR (left) and the plaque inhibition results (right) for the two tetrameric test compounds.

As was the case for the plaque inhibition assay, the results from qPCR were plotted against the log of compound concentration and analysed via the non-linear regression function in Prisim GraphPad. This was done to compare the IC_{50} estimates produced by the two data sets. When the whole data set was analysed it was found that the long-armed tetramer produced an IC_{50} that was lower than that for the short-armed tetramer (0.16µM vs 0.20µM). This was unexpected as the qPCR assay had indicated that short-armed tetramer produced a measurably greater level of HSV-1 inhibition at all of the tested exposure concentrations (see figures 3.9 and 3.10) and as such should have the lower IC_{50} value. Because the regression analysis that was used to find the IC_{50} values required that compound concentration be log transformed the horizontal (x-axis) distance between data points at the 0.5µM exposure level and control values exceeded the distance between the lowest and highest of the tested concentrations. For this reason it was theorised that the control values may exerting an

unrepresentative effect on the line-of-best-fit. When the regression analysis was performed again without the inclusion of the control values not only did the long-armed tetramer give the higher IC_{50} value, as was originally anticipated, but the long-armed tetramer also displayed improved fit with increased R^2 (0.999 vs 1) and a decreased sum of squares (0.00009 vs 0) (see figure 3.12). Under the control-excluding regression the short-armed tetramer also showed a perfect fit (R^2= 1, sum-of-squares=0) and as such this was chosen as the more accurate reflection of the IC_{50} values attributable to these compounds.

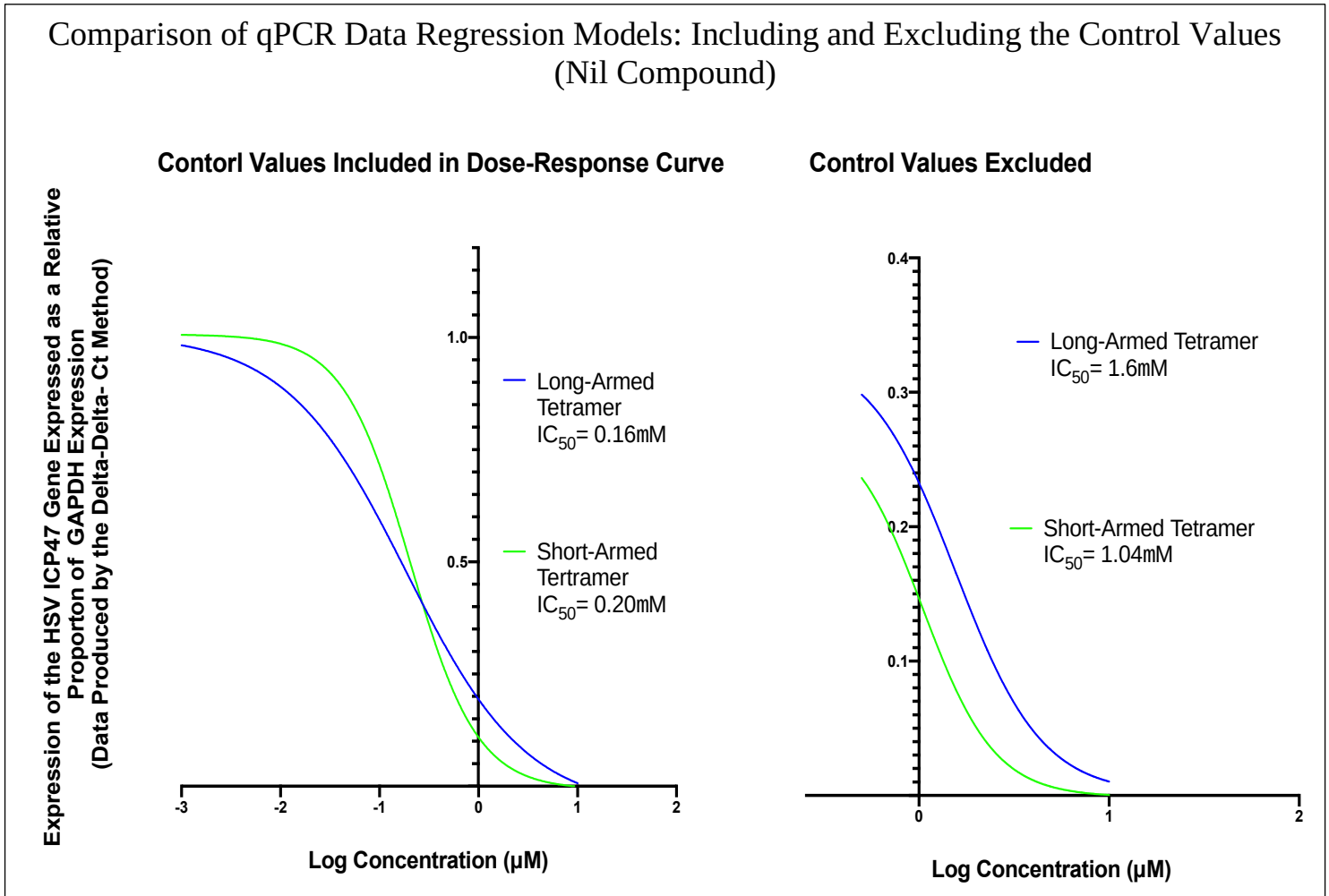


Figure 3.12: Comparison of the line-of-best-fit generated by regression analysis which included all of the qPCR data points (left axes) and a regression analysis that was identical except for the exclusion of the control values. The depicted curves are the line-of-best-fit generated by a regression analysis performed using the "find absolute IC_{50}, X is log (concentration)" function in Prism GraphPad. The exclusion of the control values provided a fitted curve that had a more perfect fit and better conformed to the expected hierarchy of anti-herpetic potency (all observed data indicated that the short-armed tetramer outperformed the long-armed tetramer).

3.5 Investigations into Short-Armed Tetramer Mode of Action

3.5.1 Virucidal Assay

A virucidal assay was carried out to assess whether the short-armed tetramer was capable of inactivating HSV-1 virions in a long-lasting manner. The extensively sulfated maltose saccharide is the intended functional domain of the test compounds in that the theoretical mode of action is one whereby this saccharide acts as ligand for the biological target molecule (the VAPs of HSV-1). Because all of the test compounds feature the same saccharide moiety it is likely that the all three test-compounds will evince similar levels of virucidal activity.

The concentrated HSV-1 stock (3.17×10^8 PFU/mL) was added in a 1:1 ratio to preparations of the short-armed tetramer dissolved in ultra-pure water. The suspension of highly concentrated virions/ test-compound was incubated at 37 $^{\circ}$C for one hour. During the incubation period the virus/test compound solutions all contained 1.585×10^8 PFU/mL and test-compound at concentrations of either 4, 0.4 and 0.04 mM. At the conclusion of the incubation period the virus/test compound was diluted by a factor of 1×10^6 and each preparation was used to infect three wells of a 12 well plate (method described in 2.5). The extensive dilution carried out between the compound exposure period and the inoculation of the Vero cells ensured that each 300µL inoculum contained approximately 50 virions and a maximum test-compound concentration of 0.004µM (substantially lower than the IC_{50} observed in the plaque inhibition and qPCR assays. Control wells were inoculated with viral stock that had been incubated in the presence of PBS instead of test-compound.

There was no discernible difference between the mean number of plaques produced by wells inoculated with the control preparation and those infected with virus that had been exposed to

the short-armed tetramer. Accordingly, it can be inferred that this compound (and in all likelihood all four compounds) do not exert any level of virucidal activity on HSV-1.

3.5.2 Adsorption Inhibition Assay

The test compounds were theorized (both on the basis of their chemical structure and previously published *in vivo* findings (Zubcova et al 2018)) to be capable of binding to HS receptor molecules and therefore stood a reasonable probability of binding to the HS receptors that serve as the VAPS of HSV-1. If these inferences were accurate it was expected that the test-compounds would inhibit HSV-1 adsorption, As such the adsorption assay served to confirm whether the observed levels of plaque inhibition (section 3.3) were primarily due to the theorised mode of action or some as yet undiscovered mechanism.

Six-well plates of 95% confluent Vero cells were chilled at 4 ˚C in order to prevent cellular uptake of virions while still allowing adsorption (this is in keeping with the method described by Karger & Mettenleiter (1993) and published in adapted format by MacLean (1998)). Cells were inoculated with pre-chilled preparations containing both the short-armed tetramer (at concentrations ranging from 0.78 to 160μM) and HSV-1 virions at a concentration that would give close to 80 PFU per-well. Cells were exposed to the virus/test-compound for 2 hours at 4˚C (agitation provide every 15 minutes), before being washed three times with chilled PBS (so as to remove non-adsorbed virions), and then overlayed with warm culture media (37 ˚C) containing 2 μg/mL of pooled human IgG. Human IgG was added to the media so as to ensure the formation of distinct plaques. Control wells were inoculated in the same fashion as the treatment wells with the exception that there was no test-compound present in the inoculum. Because the PBS wash step removed both the test-compound and non-attached virions from the cell layer (and was conducted prior to the re-warming of the cells) it follows that any

difference between the number of plaques formed in control and treatment wells was likely due to the inhibition of virion adsorption.

As can be seen in figure 3.13 the dose wise effect of the short-armed tetramer was almost identical to that observed in the plaque inhibition assay (3.3). Additionally the fitted curve generated via regression analysis in Prism GraphPad (using the non-linear regression function and the specific setting "absolute IC_{50}, X is log (concentration)") indicated that the short armed tetramer has an IC of 0.09µM (See Figure 3.14). This is lower than the IC_{50} found by analysing either the plaque inhibition data (section 3.3) or the qPCR data (section 3.4) and the (IC_{50}= 1.04 µM and IC_{50} = 0.32µM respectively).

The lack of agreement between the IC_{50} values obtained via the three different assays (sections 3.3, 3.4 and 3.5.2) indicates an undesirably high level of error and, in the case of qPCR, an undesirable lack of biological replication (i.e. insufficient number of biological replicates). That said the fact that three biological replicates of the adsorption inhibition assay gave the lowest IC_{50} value (for the short-armed tetramer) and also displayed a lower level of intra-treatment standard deviation than was seen in the plaque inhibition assay (mean of concentration specific standard deviations was 4.2% of the control mean in the plaque inhibition assay and 3.3% in the adsorption inhibition assay) indicates that these results provide sufficient evidence with which to conclude that adsorption inhibition is the primary mode of action for the short-armed tetramer.

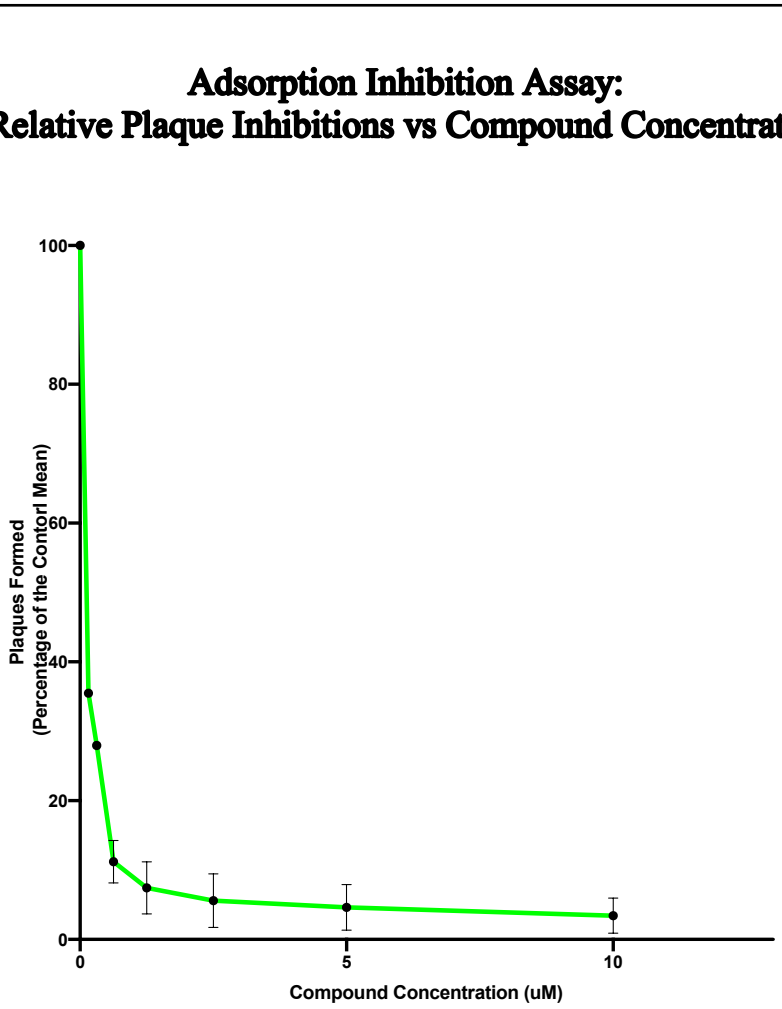


Figure 3.13 Line graph of the results from the adsorption inhibition assay conducted with the short-armed tetramer compound. Points represent the mean number of plaques formed expressed as a percentage of the plaque number observed in control wells from the same experimental replicate. Points with error bars are mean values obtained from three independent experimental replicates, error bars represent the standard deviation of the mean. Points without error bars (at x= 0.156 and x= 0.313) represent plaque numbers taken from a single biological replicate (three technical replicates).

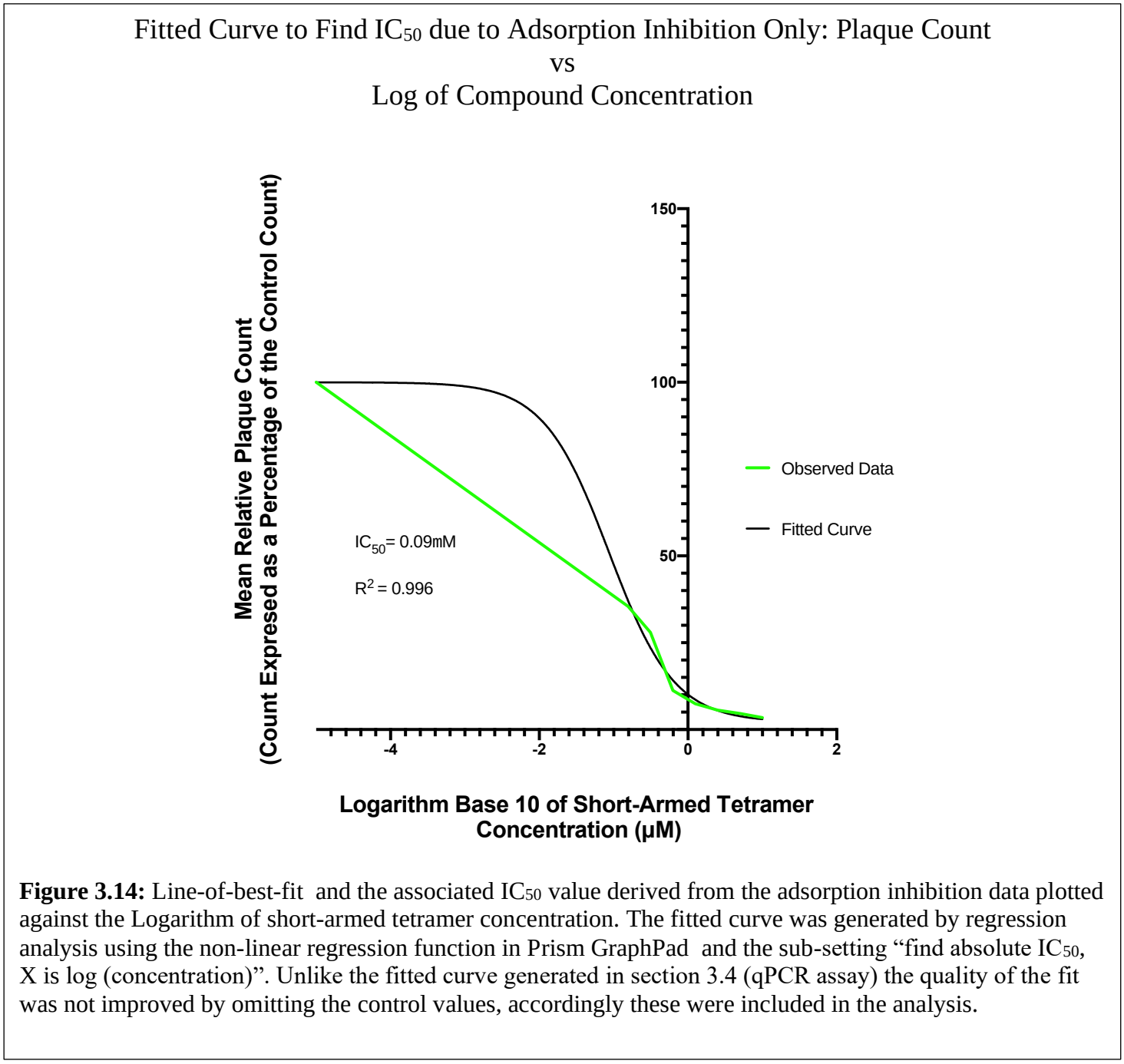


Figure 3.14: Line-of-best-fit and the associated IC_{50} value derived from the adsorption inhibition data plotted against the Logarithm of short-armed tetramer concentration. The fitted curve was generated by regression analysis using the non-linear regression function in Prism GraphPad and the sub-setting "find absolute IC_{50}, X is log (concentration)". Unlike the fitted curve generated in section 3.4 (qPCR assay) the quality of the fit was not improved by omitting the control values, accordingly these were included in the analysis.

The results of the adsorption inhibition assay indicated that the HSV-1 inhibition displayed by the short armed tetramer (and in all likelihood the other two test-compounds) is entirely the result of its capacity to block HSV-1 adsorption. While it is conceivable that the test compounds may possess additional modes of action, to invoke these as having a role in the plaque inhibition and qPCR results (sections 3.3. and 3.4) would be an over-determination of the observed dose-response. This is because adsorption is the very first step in the replication cycle, if the magnitude of adsorption blocking can entirely explain the observed level of viral

inhibition it is anti-parsimonious to invoke other mechanisms as having an causal role in producing the observed inhibitory effect.

3.5.3 Entry Inhibition Assay

As with the previous assay, 95% confluent Vero cells were chilled at 4 degrees C prior to infection, once the cells were evenly chilled each culture well was inoculated with 900μL of chilled culture media containing approximately 120 pfu of HSV-1 (KOS). The viral inoculum was allowed to adsorb for 2.5 hours at 4 degrees with gentle agitation performed every 15 minutes to ensure even distribution of nascent plaques. The adsorption interval took place before test-compound was added to any of the treatment groups so as to allow maximal virion attachment with minimal virion entry (viral entry does not take place at 4 degrees)(Shogan et al 2006). At the conclusion of the adsorption period non-attached virus particles were removed by washing the cells three times with chilled PBS, the cells were then covered with pre-warmed (37 C) culture media containing the short-armed tetramer at one of the selected exposure levels (0.625 1.25, 2.5, 5, 10, 80 or 160μM), control wells were covered with media that did not contain any test-compound. The cells were incubated at 37 degrees for 2 hours to allow the attached virions to enter the host-cell (in as much as was possible in the presence of the test compound). At the conclusion of the entry period the cells were washed once with PBS then once with acidic glycine (pH3) so as to neutralise any virions that had not been internalised, this is as per the method used in Shogan et al 2006) and finally overlayed with culture media containing 2μg/mL of pooled human IgG. Cells were then incubated at 37 degrees for 48 hours to allow plaque formation, after which cells were fixed with methanol, and stained with crystal violet. The plaques were counted and the number recorded with the difference between the control wells and the treatment wells being taken as a measure of the extent to

which the applied concentration of the short-armed tetramer inhibited the entry of attached HSV-1 virions.

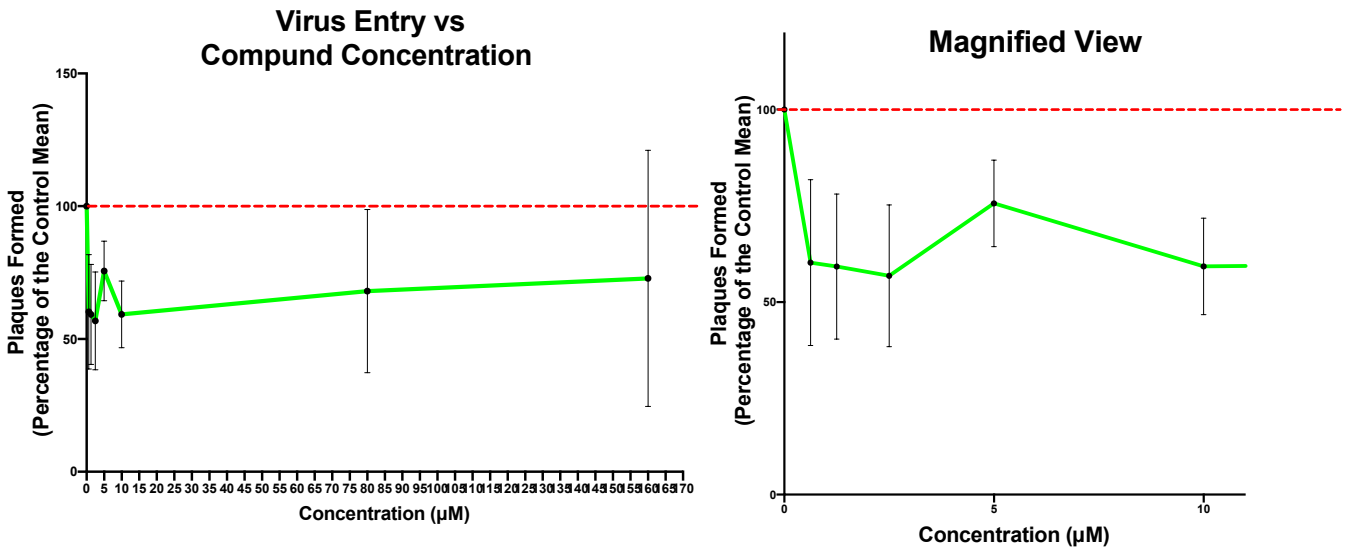


Figure 3.15 Line graph showing the relationship between the concentration of the short-armed tetramer and the rate of plaque formation in an entry inhibition assay for HSV-1. The left hand set of axes represents the full data set, the right hand figure is an isolated section of the former which has been expanded to better illustrate the effect produced by low concentrations of the test compound. The dashed red line indicates 100% plaque formation as standardised to the control wells (i.e. zero inhibition). All results were recorded as the number of plaques present in the treatment well expressed as a percentage of the mean number of plaques present in the intra-replicate control wells. All points represent the mean of plaque percentages obtained from four independent replicates (with the exception of the 2.5, 5 and 80µM treatments which were performed three times and the 160µM treatment which was performed twice- as a tentative confirmation of the observed patterns persistence at high concentrations). Error bars represent the standard deviation of the corresponding mean. The treatment of outliers is described in the text below.

The initial results of the entry-inhibition assay revealed a unacceptably high level of inter-replicate variation for the 5, 80 and 160M treatment groups. It was found that this was primarily due to the presence of one particularly egregious outlier in each of the hyper-variant treatment groups. Outliers were identified as those values which showed a greater than 50% disparity from the treatment mean. Under this definition five outlying values were identified, One in the 2.5µM treatment, one in the 5µM group and two in both the 80 and 160µM treatment

groups. It was only necessary to remove one of the outliers from the 80µM treatment as omission of the most disparate outlier reduced the distance between the second outlier and the new treatment mean. Because the 160µM treatment only consisted of two data points (these were an ad-hoc inclusion performed in the last two rounds or experimentation in an attempt to confirm the limits of the apparent data trend) both of these were equally disparate from their common mean and as such were retained in the total data set, though they were omitted from subsequent two-sample t-tests due to the high level of uncertainly associated with data from the 160µM group. Overall three outliers were omitted from subsequent analysis, one from the 2.5µM, one from the 5 µM and one from the 80µM treatment groups. The improved coherence of the apparent data trend can be seen by comparing the line graph produced by the full data set and the line graph produced by the data set with the three outliers removed (lower part of Figure 3.16).

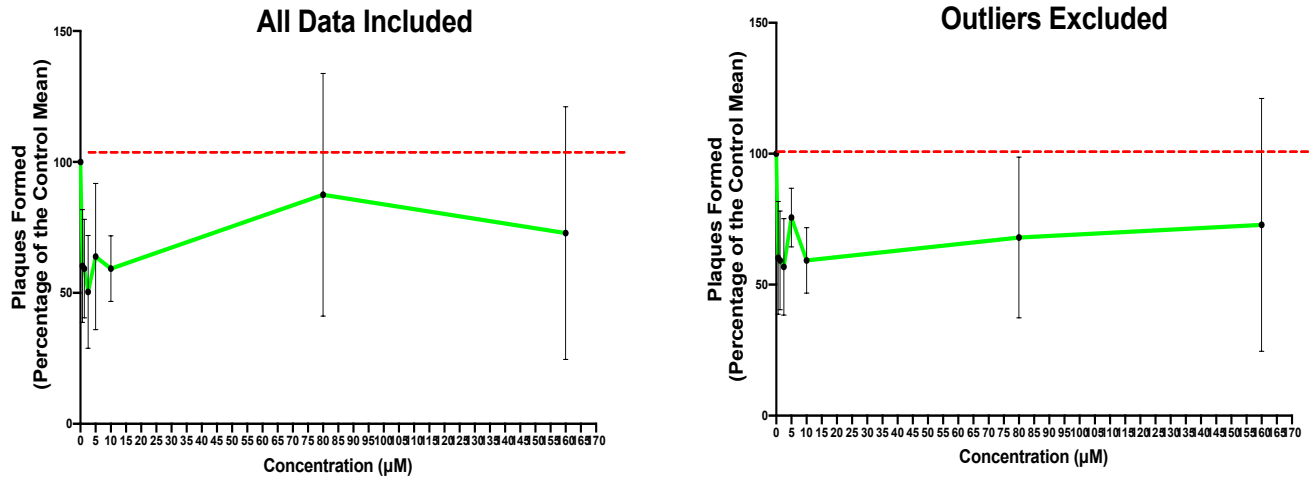


Figure 3.16: Comparison of the spread of raw data from the entry inhibition assay (left-hand set of axes) and the same data following the removal of three outlying values (values which showed greater than 50% disparity from the treatment mean) (right-hand set of axes). One outlier was removed from the 2.5, 5 and 80 µM treatment groups respectively. Data represents the mean number of plaques formed standardised to the number of plaques formed in intra-replicate control wells. Error bars represent the standard error of treatment means. Prior to removal of outliers all values are the mean of four biological replicates with the exception of the 80µM and 160µM treatments (three and two replicates respectively).

The results of the entry inhibition assay (figure 3.15) seemed to indicate that the short-armed tetramer produces slight to moderate inhibition of HSV-1 entry. Viral entry (as assessed by plaque formation) was reduced to 67% of the control level under exposure to 0.625 μM of the short armed tetramer. Somewhat paradoxically, a 128-fold increase in the concentration of the test-compound (to 80 μM) resulted in negligible shift in the level of viral entry/entry-inhibition.

Compound Concentration (μM)

Compound Concentration (μM)	**0.625**	**1.25**	**2.5**	**5**	**10**	**80**
0.625		0.98	0.80	**0.22**	0.93	0.73
1.25	0.98		0.85	**0.15**	0.9998	0.69
2.5	0.80	0.85		**0.14**	0.84	0.61
5	**0.22**	**0.15**	**0.14**		**0.10**	0.72
10	0.93	0.9998	0.84	**0.10**		0.67
80	0.73	0.69	0.61	0.72	0.67	

Figure 3.17: Table representing the various p-values obtained by performing two-sample T-tests (two-tailed) to establish the likelihood that the compared sets of sample data were obtained from populations with disparate mean values for the number of plaques formed under entry-inhibition assay conditions. The only treatment groups pairings which showed a p-value lower than 0.6 (indicating a greater than 0.4 likelihood that the two populations have identical means-on the basis of what can be inferred from the available data) were 5μM x 0.625μM, 5μM x 1.25μM, 5μM x 2.5μM and 5 μM x 10 μM. These pairing are indicated in bold/underlined text. No pairs of treatment responses produced a p-value lower than 0.1.

The statistical validity of the apparent insensitivity of HSV-1 to altered concentrations of the short-armed tetramer was tested by performing two-sample t-tests that compared the difference between the mean level of entry inhibition produced by all possible pairing of treatment groups used in the entry inhibition assay (Figure 3.17). Only four of the treatment pairings showed p-values less than 0.6 in magnitude, and all of the pairings showing $p<0.6$ included the 5µM treatment group. If the mean level of entry inhibition observed in the 5µM group is taken to be the result of unidentified experimental error (a parsimonious inference given the shape of the line in figure 3.16) the these t-tests give a reasonable indication that the level of entry inhibition produced by exposure to the short-armed tetramer is invariant at concentrations between 0.625 and 80uM. It would be highly surprising to observe such high p-values in a group-wise comparison where there was a biologically relevant difference between the mean response of magnitude in the different groups.

It should be noted that prior to the removal of outlying data points, all of the treatment groups displayed alarmingly high inter-replicate variation. This inter-replicate variation means that any population level shift in the level of entry inhibition occurring between the exposure concentrations 0.625 and 80µM is too small to be reliable inferred from the present data set and should be a priority for future investigations. The level of entry inhibition produced by exposing cells to 160µM of the short-armed tetramer was tested in only two biological replicates that were conducted during the final week of ,the ad-hoc inclusion of the 160µM treatment group was intended to give abroad indication as to whether or not the the apparent lack of correlation between compound exposure and the level of entry inhibition continued to

be displayed at extremely high concentrations of the short-armed tetramer, this is apparently the case.

The fact that exposure to 160µM of the test compound gave indication entry inhibition above that which was observed in the 0.625µM made it appear highly unlikely that the observed reduction in plaque numbers (36-50% at all concentrations between 0.156 and 80µM) was the result of direct interaction between the test compound and the viral entry processes. A dose wise response like that seen in 3.3, 3.4 and 3.5.2 should have been observed if this were the case. A possible mechanism by which the test-compound may have produced these anomalous findings is discussed in chapter 4.

3.6 Yield Reduction Assay

The first replicate of the total yield reduction assay (also referred to as the "total progeny assay") was carried out using MOIs of both 1 and 0.1, this was done so to ascertain what affect the MOI might have on the inhibitory effect of the compounds. Host-cell lysate was collected at 24 hpi and titrated to determine the total viral yield at this time point (this is in accordance with the protocol established in section 3.2). Cells and virus were exposed to the test compounds at concentrations of 0.156, 0.3125, 0.625. 1.25, 2.5 and 20 µM. The cells were exposed to the compounds during both the adsorption period and the subsequent incubation/replication period (incubation period was 22 hours at 37 degrees C). The monomeric compound was excluded from the yield reduction assay as its lack of anti-herpetic activity was well demonstrated by the plaque-inhibition assay. While further demonstration of this point may well have been desirable, scarcity of the monomeric compound and the time consuming nature of the yield reduction assay proved prohibitive.

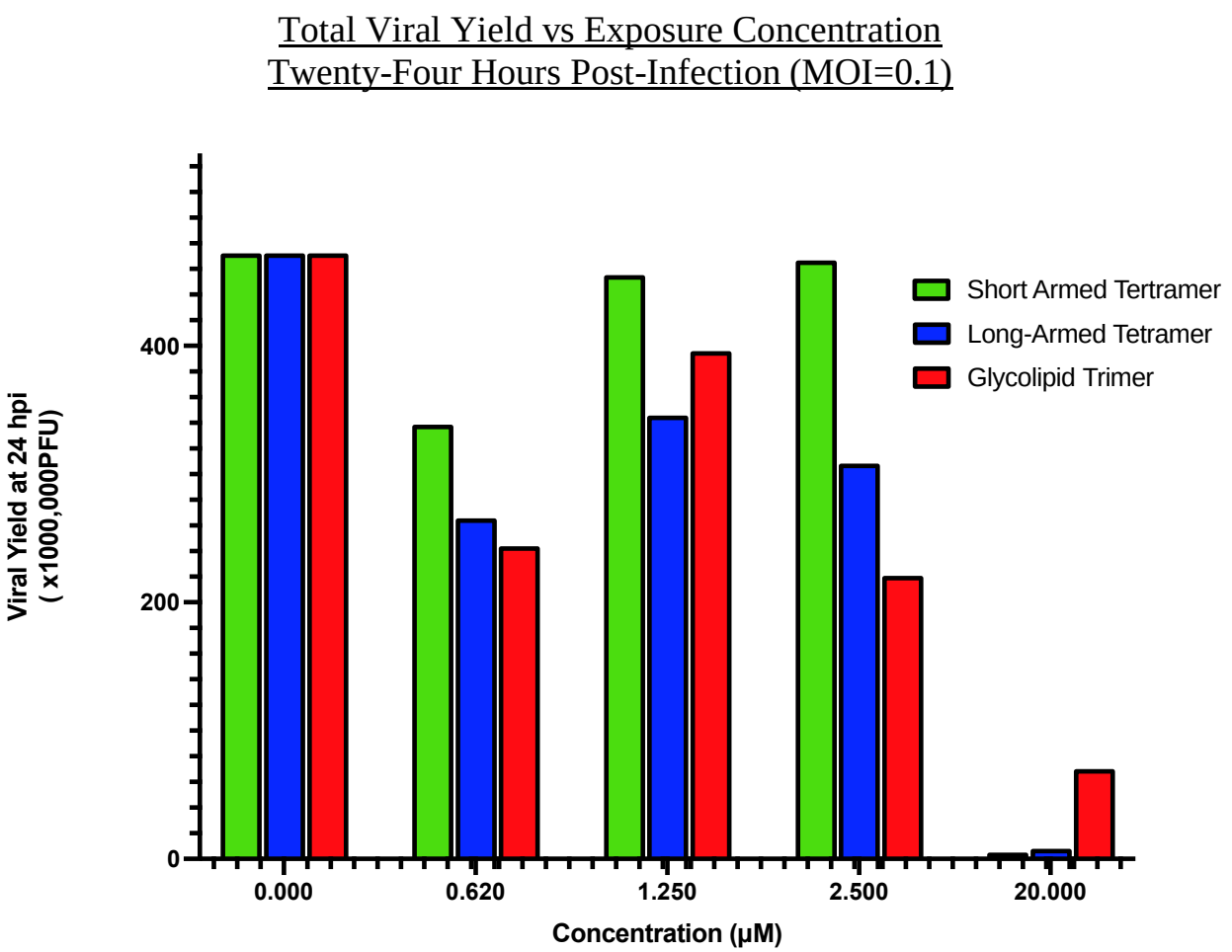


Figure 3.18: Bar graph illustrating the relationship between exposure to varying concentrations of the test compounds and the total viral yield produced by a 24 hour HSV-1 infection initiated at an MOI of 0.1. All points are the product of a viral yields obtained from a single experimental replicate (an undesirable consequence of time pressure).

The yield reduction assay carried out with a MOI of 1 did not show any tangible correlation between exposure to any of test-compounds at any of the applied concentrations . The assay conducted with an MOI of 0.1 showed some degree of response, however the dose-wise levels of viral inhibition were highly disparate from those observed in the plaque inhibition and qPCR assays. The only treatment concentration which produced a clear inhibitory effect was 20µM, this was true for all three of the (figure 3.18). In the plaque inhibition assay (section 3.3)

exposure to 2.5μM of the either tetrameric compound resulted in between 80 and 86% reduction in the number of viral plaques formed yet in the first round of yield reduction assay the same treatment failed to produce any manifest reduction in viral yield (though the considerable experimental noise present may have been partially responsible for this). Given the substantial reduction in plaque formation produced by 2.5μM the same treatment's inability to reduce the viral yield is a phenomenon that demands explanation.

It was hypothesised that the discrepancy between these two data sets might be due to the differing incubation conditions present in the two assays. Because there was no human IgG added to the yield reduction-assay the doses of test-compound needed to exert their anti-herpetic properties over a considerably longer time period than those added to the plaque inhibition assay (the total 24 hour incubation period vs the 2 hour adsorption period alone). If the test compounds happen to be chemically unstable (under cell culture conditions) it is plausible that lower doses of test-compound may have their inhibitory effect abrogated as the assay progresses. A dose of test-compound that is highly efficacious over the two hour adsorption window may be affected by molecular decay durring the incubation period resulting in a reduced concentration of said compound and possibly an elevated rate of viral replication. This potential phenomenon would not affect the results of the plaque inhibition assay as the overlay media therein contains pooled human IgG meaning that once the overlay media is added a total loss of compound efficacy would have no bearing on the number of plaques formed (though they might conceivably attain a larger size) as any extracellular virions would be neutralised by the IgG present in the media.

As such it is not entirely surprising that the plaque inhibition assay and the total progeny assay gave highly incongruous results. Additionally, the two assays are directed at measuring

different response variables which in turn assess quite different aspects of viral replication efficacy. Plaque inhibition assays are primarily sensitive to the proportion of virions in the initial inoculum that are able to establish a productive infection, whereas yield reduction assays give an aggregate assessment of the total number of virions produced over the total incubation period. If the viral population is dynamic in growth patterns (i.e. if temporary inhibition can be abrogated by enhanced replication at a latter point and vice versa) then it is plausible that quite considerable treatment effects may not be detectable via yield reduction assay.

In order to circumvent the possibility that compound stability was a confounding factor the assay protocol was adjusted to incorporate removal and replacement of the culture media (and the test compound therein) every 3.5 hours (210 minutes). Additionally, the range of tested concentrations was extended to include 40 and 160 μM treatments in the hope that dose wise inhibition might be observed at these higher concentrations. As with the first round of yield reduction assays MOIs of both 1 and 0.1 were tested. Due to insufficient supply of the other two compounds this assay examined the tetrameric compounds only. The results from this protocol are displayed in figure 3.19.

When the yield reduction assay was carried out with 3.5-hourly replacement of the test-compound it was found that concentrations of 2.5μM and above produced considerable inhibition of the total viral yield. Rather surprisingly it was found however that there was no dose-wise reduction in the total-viral yield at treatments exceeding 10uM of test-compound. Total viral yield for cells cultured in the presence of the short-armed tetramer was approximately the same in the 10, 40 and 160μM treatment groups. The applied MOI (1 or 0.1) correlated with an apparent 6-10 fold difference in total yield, but the considerable inter-

replicate variation and consequently overlapping confidence intervals mean that this assertion should be regarded as requiring further investigation.

Total Viral Yield vs Exposure Concentration
Following Three-and-a-Half Hourly Replacement of Media and Test-Compound

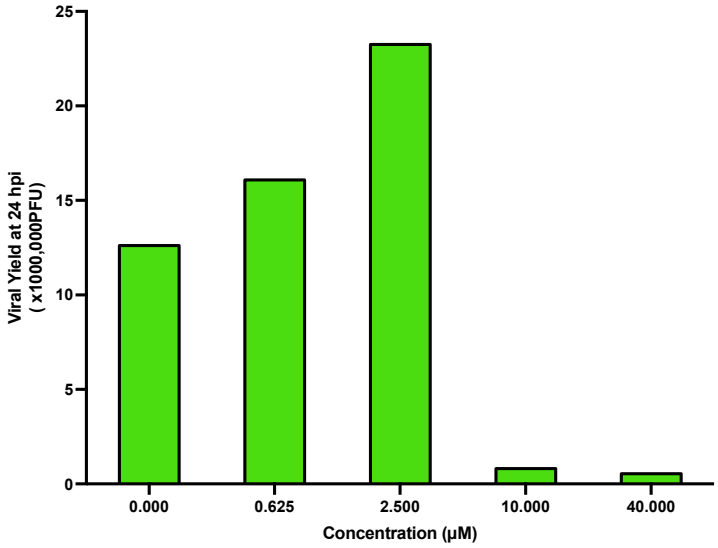


Figure 3.19 Bar graph illustrating the relationship between exposure to varying concentrations of the short-armed tetramer and the total viral yield at 26.5 hpi. Infections were conducted with an MOI of 0.1 and media (and the compound therein) was replaced every 3.5 hours to correct for the effects of possible compound decay. Points represent viral yield at 26.5 hpi, all points are the product of a viral yields obtained from a single experimental replicate (an undesirable consequence of time pressure).

These results go some way to redressing the apparent contradiction between the first set of yield reduction results and those from the preceding assays. These results confirm two of the research groups initial expectations in that even modest doses of the tetrameric compounds (<10µM) were shown to be capable of significantly reducing the total yield of HSV-1 and a ten-fold difference in the applied MOI (MOI=1 vs MOI=0.1) resulted in a roughly comparable difference in the total yield at 24hpi. That said these results leave much to be desired, the response pattern shown by the short-armed tetramer seems to defy logic,

primarily because the mean viral titre of the control differs substantial from the control mean in the long-armed treatment group, but is also lower than the viral titre in the 0.625 and 2.5 μM treatment groups. Insufficient replication of the experiment is the most likely explanation for these unexpected data points. Additional hypotheses will be discussed in the next chapter.

Chapter 4: Discussion

4.1 Apparent Utility of the Test Compounds

The results from the plaque inhibition, qPCR, and adsorption inhibition assays give a very clear indication that the two tetrameric compounds are highly promising as candidates for future antiviral development studies. If the results obtained from these assays are accurate then the IC_{50}s of the two tetrameric-compounds may be as low as 0.2µM (approximately 1µg/mL), this indicates a higher level of anti-herpetic activity than is evinced by numerous other adsorption inhibiting compounds including promising therapeutic candidates such as heparin, dextran sulfate, PI-88 and the peptide derived dendrimer studied by Luganini et al (2011)(Carlucci et al 1997; Ekblad et al 2010; Karmarker, Pujol, Damonte, Gosh & Ray 2009; Nyberg et al 2004). While the apparent IC_{50} of the tetrameric compounds is higher than those of the polyanionic compounds studied by Hayashi, Hayashi & Kojima (1996), Carlucci et al (1997), Sinha et al (2010) and Mader et al (2016), the fact that the tetrameric compounds have significantly lower molecular weights than the aforementioned is reason to suspect that they may be possessed of substantially better oral bio-availability, this in turn is likely to confer the tetrameric compounds with greater utility in a healthcare setting (Goldberg & Orellana 2003).

While the results in Chapter three indicate that the glycolipid trimer is considerably less efficacious than the tetramers, its unique molecular structure may confer advantageous pharmacokinetic properties that cannot be ascertained by straightforward *in vitro,* assays such as those herein. The glycolipid trimer's'IC_{50} (approximately 3.7µM (14.3µg/mL)) while being at the upper limit of what is considered useful for internal administration, is still low enough to confer reasonable efficacy as a topical prophylactic, as such this compound may warrant

further investigation, particularly if the tetrameric compounds are found to possess unfavourable pharmacokinetic properties.

The monomeric-compound was not found to inhibit HSV-1 at any of the tested concentrations, however it may have future utility as a negative control in studies of antiviral dendrimers. The failure of the monomer also served as proof that the inhibitory effect of the other three compounds is almost entirely due to the so-called dendrimer effect (also known as the multivalency effect).

Despite the fact that all four compounds possess the exact same functional group (over sulfated maltose moieties) the fact that the monomeric compound exhibited nil anti-herpetic effect strongly indicates that the efficacy of the three test-compounds was due to their valency, and by extension was the result of the dendrimer effect. It follows from this that it may be possible to develop structurally similar compounds which show even higher levels of HSV-1 inhibition, this could be achieved by increasing their valency or adding additional "generations" to the structure of the dendrimer core (With reference to dendrimers "generation" describes the number of steps used to incorporate successive layers of bifurcation onto the arms of the molecule- i.e. for generation n the number of functional groups at the periphery of the molecule=(the number of functional groups on dendrimer n-1)x2)) (Boas & Heegaard 2004) .

4.2 Implications of the MTT Assay Results

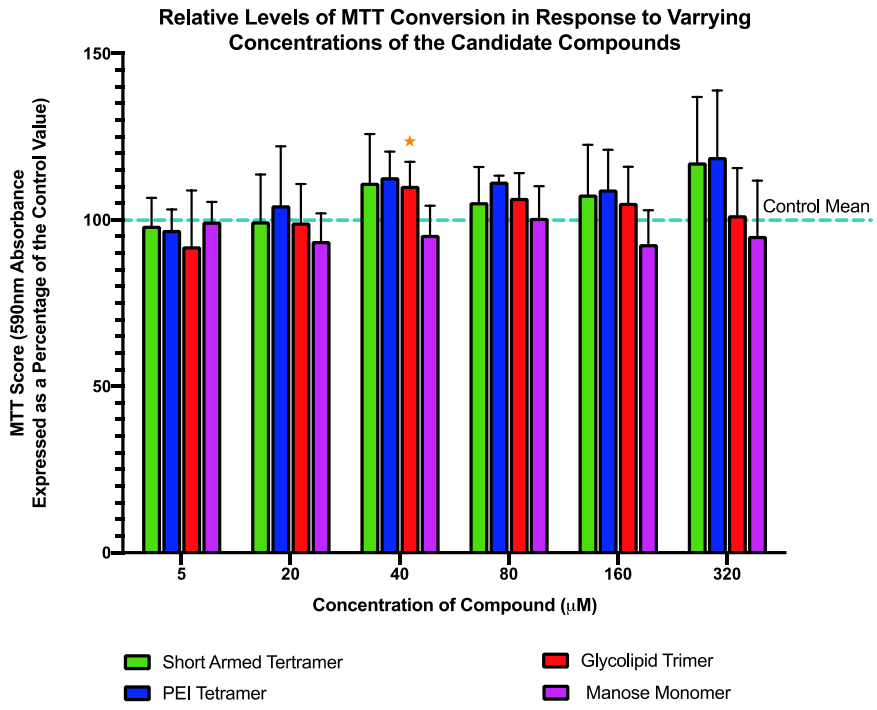


Figure 4.1 (Reproduction of Figure 3.1): Bar graph showing MTT conversion by Vero cells (expressed as a percentage of the intra-replicate control value) after 48 hours in the presence of each of the test compounds. The level of MTT conversion was measured by reading the optical absorbance (at the 590nm wavelength) of combined cell lysate and supernatant after three hour incubation in the presence of aqueous MTT. All bars represent the mean level of MTT conversion obtained from across five biological replicates. Error bars represent the standard deviation evinced across all five biological replicates. The cyan coloured horizontal line indicates 100%- i.e. MTT conversion shows no difference from the control mean. The orange star indicates the only set of treatment conditions for which a one sample T-test (two tailed) produced a p-value lower than 0.05 (indicating that the data from this sample exceeds the 95% confidence threshold for declaring a difference between the mean MTT conversion produced by this treatment and the control (at the population level)).

The first conclusion that can be drawn from the MTT assay is that none of the tested compounds produces measurable cytotoxicity in Vero cells at concentrations below 320μM. This follows

from the fact that none of the tested concentrations produced a statistically significant reduction in the level of MTT conversion (see section 3.1).

A somewhat harder to explain aspect of these results is the apparent increase in the level of MTT conversion evinced by Vero cells incubated in the presence of either 160μM or 320μM of either tetrameric compound. While one sample T-test comparing these observation to the control mean failed to demonstrate statistical significance at the preselected level of $p \leq 0.05$, the 320μM exposure concentrations came very close to achieving this level of significance (short-armed tetramer 320μM treatment $p = 0.06$; long-armed tetramer 320μM treatment $p = 0.07$). Additionally the ostensible pattern displayed in Figure 4.1 (a reproduction of Figure 3.1) gives a fairly clear indication that increasing concentration of the tetrameric compounds corelates with increased MTT conversion. Conversely, exposure to increasing concentrations of the monomeric compound appeared to correlate with slightly reduced levels of MTT conversion.

The divergent patterns displayed by exposure to high concentrations of the monomeric and tetrameric compounds tend to suggest that the MTT conversion enhancing phenomenon is due to the tetrameric compounds capacity to alter biological processes that rely on HS (or similar GAGs). This inference is further supported by the fact that the glycolipid trimer appears to show some elevation in the level of MTT conversion, though at a lesser magnitude than is produced by the tetrameric compounds. The relative level of MTT conversion upregulation evinced by each of the four compounds mirror the respective levels of HSV-1 inhibition produced by these compounds. If the impressive level of HSV-1 inhibition produced by the tetrameric compounds and the somewhat modest inhibition produced by the glycolipid trimer are both determined by these compounds ability to accurately replicate the VAP binding

properties of HS GAGs, it stands to reason that these compounds may also produce increased MTT conversion as a direct result of this same capacity (i.e. the efficiency with which they hijack biochemical roles that are normally performed by HS GAGs).

The most obvious function of HS that might be able to produce the observed results (without necessarily having a genuine impact on MTT conversion) is its function in the structure of host ECM. All known mammalian tissues express HS binding proteins as part of the network of connections that bind cells to the ECM(Bishop, Schuksz and Esko 2007). There is good reason to suspect that the measured increase in 590nm absorbance was the result of the reduced cell-cell adhesion in the Vero cell monolayer. Heparin and other HS like GAGs have been shown to interfere with cellular adhesion to the ECM thereby increasing the ease of cell exfoliation *in vitro* (Gao & Brigstock 2004; Rocha et al 2001). As such the possibility that high concentrations of the test- compounds degraded the integrity of the ECM/cell monolayer is not without precedent.

This theory also fits with the casual observations made by the research group, whom noted that cells having been exposed to high concentrations of the tetrameric compounds were far more easily exfoliated than those in the other treatment groups. Additionally, the research team noted that during the final stage of MTT assays (immediately prior to the addition of the MTT solvent), those wells containing high levels of the tetramers showed a faint purple colour in the supernatant, whereas control wells showed purple colour in cell layer only (the supernatant being faintly amber/yellow in colour. Furthermore, it was noted that upon addition of the MTT solvent (MTT solvent acts as both a lysis buffer and a formazan solvent) an additional degree of formazan associated colour change (yellow → blue) took place almost instantly whereas

other treatments did not display the full extent of the colour change colour until after the 30min incubation period.

The above observations could potentially be explained by a scenario whereby the integrity of the cell monolayer has been significantly degraded thereby facilitating the easy dispersal of cells to give a single cell suspension. Theoretically, if an MTT assay was performed on two identical cell samples with the only difference being that one was exfoliated in such a way as to give a suspension of single cells while the other was suspended as clusters of between two and ten cells, the latter will have a much smaller surface of contact between cells and the surrounding fluid, with cells at the centre of larger clusters being almost entirely shielded from the actions of the MTT solvent. The additional contact area present in a single cell suspension is likely to result in a more rapid rate of cell-lysis and a higher rate of formazan dissolution. As such, if these two cell suspension are possessed of identical formazan content, the sample in which cells are singularly dispersed would be expected to show greater intensity of blue colouration, and hence a higher level of 590nm absorbance. In this way it is conceivable that ECM disruption caused by the test-compounds could result in increased MTT scores without necessarily having a direct impact on the rate of cellular MTT conversion.

The apparent (though statistically non-significant) reduction in the level of MTT conversion produced by the monomeric compound is partially in keeping with the supposition that alterations in the rate of MTT conversion are due to the HS mimicking properties of the compound in question, (i.e. the monomeric compound produced nil HSV-1 inhibition and can therefore be inferred to possess very limited capacity for mimicking biological functions of HS GAGs. The fact that the treatment with very high doses of the monomeric compound appears to produce a slight decrease in the level of MTT conversion (as opposed to having no effect at

all) might be attributable to a non-specific disruption of biological processes on the part of the compound. It is conceivable, though entirely speculative, that the over sulfated maltose moiety of the monomeric compound may form transitory and non-specific electrostatic interactions with various cell membrane proteins. It is also conceivable that the glycolipid domain could associate with the hydrophobic interior of the phospho-lipid bilayer. By either of these mechanisms the monomeric compound might produce a minor inhibition of transmembrane transport processes, thereby decreasing the rate of background cellular processes (e.g. NADP/NADPH homeostasis). If this were the case it might not only explain this apparent reduction in MTT conversion, it could also offer an explanation for the elevated standard deviation evinced by the highest exposure concentrations of all four compounds. The reasoning behind this inference is that high concentrations of the test-compounds might produce two opposing effects in the exposed cells: the rate of transmembrane transport being hindered by high concentrations of the test-compound, while the level of 590nm optical absorbance is simultaneously increased by the ECM degrading effect of the compounds. The magnitude of the error stemming from the latter of these two forces is presumably subject to considerable variation as a result of minor inconsistencies in the level of mechanical force applied to disrupting the cell layer, the thickness of the cell layer, and the length of exposure to the MTT solvent.

4.3 Implications of the Trypan Blue Exclusion Assay

The results of the trypan blue exclusion assay give sufficient evidence with which to accept the hypothesis that the short-armed tetramer is not possessed of any cytotoxicity in Vero cells at concentrations up to and including 320µM. By extension this lends support to the ideas

discussed in the preceding section regarding the apparently anomalous MTT conversion results being due to factors other than altered cell viability.

With regards to the long-armed tetramer, it is highly likely that this compound effects cell viability in a manner that is very similar to that shown by the short-armed tetramer. This is inferred on the basis of the near identical chemical structures and anti-herpetic effects evinced by these two compounds. The more substantial divergence between the short-armed tetramer and the remaining two test-compounds reduce the certainty with which one can infer the effects that these might have on cell-viability based on the results obtained with the former. Nonetheless the results of the MTT conversion assay give suitable indication that these compounds are unlikely to have produced misleading plaque inhibition results on the basis of compound cytotoxicity.

4.4 Implications of the Time to Maximum Yield in a Single Step Infection

The results of the time to maximum yield assay are fairly self-explanatory; the maximum rate of titre increase took place between 12 and 20 hpi, this was true for both the tested MOIs (MOI= 1 and MOI=0). It is reasonable to infer that this is the window of time during which virions are released from cells that were infected during the initial adsorption period, this cohort of infected cells will henceforth be referred to as “first generation parent cells”. Virions released after 20 hpi are presumably the result of secondary infections stemming from daughter virions released from the primarily infected cells invading previously uninfected cells (i.e. the progeny of second and (possibly) third generation parent cells).

The average yield produced by the cells inoculated at a MOI of 0.01 are somewhat more ambiguous; following the production and release of the first progeny virions (at

approximately 8 and 12hpi respectively) the titre of progeny virions increased at a near constant rate for the remainder of the observation period. It seems likely that this is due to relative abundance of naïve cells in this group. The fact that newly released virions are likely to encounter an uninfected cell immediately after release from the parent cell means that the first generation of daughter virions likely initiated a totally asynchronous cascade of subsequent generations with a high (though presumably gradually decreasing) number of secondary infections being generated every hour from 12hpi until the end of observation period (38 hpi).

In terms of analysing subsequent results it is pertinent to note that all three MOIs (1, 0.1 and 0.01) appeared to converge upon a single total yield figure, the only difference being the amount of time it took to achieve this number.

As with certain other assays conducted as part of the present investigation, the time to maximum yield assay would have benefited from repetition, both as a means to obtain greater statistical power/ clarity of results, and as a means to prove the reproducibility of certain unexpected findings.

4.5 Combined Implications of the Plaque Inhibition and qPCR Assays

Both the plaque inhibition and qPCR assays demonstrate quite clearly that the two tertrameric compounds are the most efficacious of the test-compounds and they show similar relative HSV-1 inhibition across all of the tested concentrations. Additionally the results from the plaque inhibition assay give a clear indication that the tetrameric compounds are considerably more effective anti-herpetic agents than the glycolipid trimer and that all three dendrimers

are more effective than the monomeric compound which produced close to nil anti-herpetic effect in this assay.

The fact that the two assays provided quite different estimates of the IC_{50} value for both tetrameric compounds is not particularly surprising given that both sets of IC_{50} estimates were outside the range of concentrations that were tested for the long-armed tetramer in the plaque inhibition assay (In the case of the short-armed tetramer a wider range of exposure concentrations were tested due to the additional supply of this compound). Additionally the sensitivity of the plaque inhibition assays is known to be considerably lower than what is achievable by qPCR.

The experimental variable that likely bears the greatest responsibility for the discrepancy between these two data sets is the MOI at which the two assays were infected. The total number of virions in the plaque inhibition assay was between 80 and 100 virions per well. Given that this assay was conducted in confluent 12-well plates this means that the cells were infected at an approximate MOI of 0.0002, thereby creating a virus-to-compound ratio that was 5000 times lower than the virus-to-compound ratio produced when testing the same concentration of the test-compound under the qPCR protocol (the qPCR assay was carried out with an MOI of 1). Given that absorption inhibition is the putative mode of action for the short-armed tetramer the ratio of virus to compound is expected to play an important role in determining the IC_{50} *in vitro.* The initial attachment of HSV-1 virions relies on multi-valent VAP-receptor interactions and as such each virion expresses substantially more VAPs than the minimum number capable of producing stable attachment. When attempting to block adsorption *in vitro* it is necessary to block a substantial proportion of the total VAP population for if the number of blocked-VAPs-per-virion is too low then the infection is likely to proceed in a totally unimpaired manner due to the binding compound failing to

overcome the level redundancy inherent in the average number of VAPs-per-virion. The exact proportion of a virions' VAPs that must be blocked by inhibitory compound is unknown and presumably depends on the properties of the viral species (and probably even the specific virion) in question, however for the sake of illustration it seems reasonable to assume that an average binding rate of 1% of total VAPS per virion would be insufficient whereas 90% would likely confer a high level of inhibition (at the population level). Given that there was a 5000 fold difference between the virion-to-compound ratios applied in the qPCR and plaque inhibition assays it stands to reason that in the qPCR assay the average level of per-virion VAP binding would be 5000 times lower than would be produced by the same treatment concentration in the plaque inhibition assay. This means that there is a non-trivial difference between the infection conditions present in the two assays and as such the two sets of results are not directly comparable.

With these considerations in mind it is hard to identify the IC_{50} estimates provided by one assay as being inherently superior to those provided by the other. The nature of the qPCR assay lends itself to more accurate measurements however the statistical power of the qPCR results are stymied by the fact that the assay was only performed once. As such it is not possible to gauge what the inter-replicate variability for this assay might have been. Conversely the extremely low MOI applied in the plaque inhibition assay means that this assay may somewhat exaggerate the efficacy of the compounds. The fact that IC_{50} estimates produced by the two assays are considerably closer than the disparate magnitude of their MOIs might lead one to expect should be taken as an indication that the interaction between virus and VAP binding inhibitory substances is more complicated and possibly much more dynamic than the simple VAP-to-compound scenario described in the preceding paragraph.

As such there is reason to believe that vastly different assay procedures might give IC_{50} estimates that are close to those obtained in this paper

4.6 Shot-Armed Tetramer Mode of Action

The results from the virucidal assay make it abundantly clear that the HSV-1 inhibition observed in the plaque inhibition and qPCR assays was in no way due to virucidal activity, on the part of the short-armed tetramer. Given that all four compounds have the same functional moity it stands to reason that none of the tested compounds are likely to exert virucidal activity under normal testing conditions.

The adsorption inhibition assay indicates that the vast majority of the anti-herpetic activity observed in the plaque inhibition assay is due to adsorption blocking on the part of the short-armed tetramer. These results actually conferred and IC_{50} estimate that was lower than the ones produced in the less specific (with regards t mode-of-acton) qPCR and plaque inhibition assays. As such any additional modes of action that may exist are likely superfluous in explaining the results from these two general assays.

The results from the entry inhibition assay are somewhat more ambiguous. The confusion around these results is two pronged; firstly the dose-wise level of adsorption inhibition seems to fully account for the inhibitory action of the short-armed tetramer, the IC_{50} obtained in the adsorption inhibition assay (0.09μM) was lower than the IC_{50} estimate produced by any of the other assays conducted. It would typically be expected that an adsorption inhibiting effect acting in unison with an entry inhibiting effect would result in an increased level of total inhibition, however the results from the plaque inhibition and qPCR assays indicate that when both effects occur side by side, the total anti-herpetic effect is either unchanged or

possibly even slightly reduced. Secondly, the entry inhibition results are perplexing because the level of inhibition appears to be almost totally invariant across the entire range of treatment concentrations (0.625 – 160 μM), with a 256 fold increase in the concentration of the compound failing to produce any clear indication of increased entry inhibition.

Given these two rather paradoxical findings there is insufficient evidence to claim that inhibition of viral entry is a pertinent mechanism in the anti-herpetic activity that the short-armed tetramer displays under normal test-conditions (i.e. assays where the timing of adsorption and entry are not manipulated). To gain a proper understanding of the short-armed tetramers role in entry inhibition it will be necessary to conduct further experiments (see section 4.10). Factors that may have played a role in producing these unusual results are discussed in the following section.

4.7 Possible Explanations for the Results of the Entry Inhibition Assay

It is tempting to infer that the short-armed tetramer inhibits the entry of HSV-1 by blocking the HSV-1 entry receptor, such a capacity would be an unsurprising given the compound's structural resemblance to the saccharide subunits of 3-O-sulfated HS (a recognised HSV-1 entry receptor) (Shukla et al 1999; Tiwari et al 2007). However, this possibility seems unlikely given that an interaction between the short-armed tetramer and HSV-1 entry receptor (gD) would be expected to show a dose-wise inhibition pattern under entry-inhibition assay, the direct opposite of the pattern which was observed in this study (Karasneh & Sukla 2011). In the present study it was found that a very low dose of the short-armed tetramer (0.625μM) reduced viral entry by 33% however every subsequent increase in concentration, up to 80μM (a 128 fold increase in exposure concentration), failed to produce any additional inhibition that could clearly be separated from the experimental noise. Majmudar et al (2019) tested a

compound that inhibits the entry of HSV-1 and demonstrated a clear correlation between incremental increases in the exposure concentration and a comparable reduction in the number of viral plaques formed under entry- inhibition conditions, thus confirming the expected relationship between the dose of entry inhibiting compounds and the level of HSV-1 inhibition. This relationship was not observed.

Instead of appealing to direct inhibition of the HSV entry process, the observed phenomenon might be better explained by a hypothesis the that the test-compound disrupts the diffusive lateral movements that HSV-1 virions make in the milliseconds immediately after attaching to a host-cell.

Scientific understanding of the post-attachment movements displayed by virus particles has recently been revolutionised by the use of single-particle tracking, high-frame-rate single-virion imaging and atomic force microscopy (Huang et al 2017; Peerboom et al 2017; Delguste et al 2019). The number of viruses for which diffusive lateral motions have been thoroughly described remains relatively small, as such it would be premature to ascribe ubiquity to these motions, nonetheless there is sufficient evidence make reliable predictions about the mechanism by which HSV-1 virions travel across the cell surface in the interval between attachment and entry.

The type of virion translocation that is pertinent to the current hypothesis is one whereby particles move across the host-cell surface in a passive manner (i.e. the motion does not expend

metabolic energy), and is characterised by a rolling type motion (Sheerer, Jin & Molthes 2010; Peerboom et al 2017)

This type of diffusive movement has come to be termed “viral surfacing” and is produced by the successive association and disassociation of VAPs and their attachment-receptors (Sheerer, Jin & Molthes 2010). Successful viral surfacing relies upon VAP-receptor interactions having a relative short duration and the virus possessing a mechanism to regulate the total valency of such interactions (Boulant, Stanifer & Lozach 2015; Delguste et al 2018; Delguste et al 2019; Peerboom et al 2017). The combination of these two features allows virions to engage new receptors in the direction of travel while simultaneously releasing bound receptors at their trailing edge, valency regulation ensures that the virion does not bind so many receptors as to become immobilised (Boulant, Stanifer &Lozach 2015; Delguste et al 2018; Delguste et al 2019; Peerboom et al 2017).

Surfacing should not be confused with “viral-surfing”, another type of lateral movement that certain virus particles undergo in the interval between adsorption and internalisation (Sheerer, Jin & Molthes 2010). In surfing the virion is tenaciously bound to its attachment receptors, the translocation of both the virion and the adjoining attachment receptors are produced by rearrangement of the underlying cell cytoskeleton (Lehmann et al 2005; Spear & Wu 2014).

The short-armed tetramer ostensibly inhibits HSV-1 adsorption, the most parsimonious explanation of the mechanism by which this occurs is that the compound binds to one or both of the HSV VAPs (glycoproteins B and C (gB and gC)), if this putative mode of action is correct then it is also highly likely that the compound is capable of disrupting viral surfacing. Because the findings of Deguste et al (2019) and Peerboom et al (20017) strongly indicate that

HSV-1 surfacing is dependent on the unimpaired and appropriately regulated (with regard to total valency) interaction between cell surface GAGs and HSV-1 VAPs then it follows that binding between the test-compound and gC and/or gB might hinder or even totally block post-attachment surfacing. Because surfacing is thought to be an indispensable processes (for those viruses which display it) it logically follows that reduced viral surfacing might lead to reduced viral entry due to attached virions having a reduced capacity to interact with entry receptors (Burkhardt & Greber 2009; Boulant, Stanifer & Lozach 2015).

The research group formed the following hypothesis to explain why disruption of normal viral-surfacing (by the short-armed tetramer) may have led to entry-inhibition that was invariant across an extremely wide range of exposure concentrations (in line with the findings in Chapter Three) . Viral surfacing is only necessary for those virions that fail to attach to the host-cell in the immediate vicinity of an entry receptor. A certain percentage of virions are likely to adsorb to the host-cell surface at a location that allows immediate binding between an entry-receptor molecule and a viral entry protein. Virions that adsorb in this manner would be able to enter the host cell without any lateral movement, hence they would be expected to infect the host cell even in the presence of a surfacing inhibiting compound. Additionally, it would be expected that such virions would be largely immune to the effects of entry-inhibiting compounds as assessed by the commonly applied entry-inhibition protocol (i.e. the same protocol described in Chapter Three).

If this theory accurately reflects the process which produced the concentration impervious results of the entry-inhibition assay then it stands to reason that somewhere between 60 and 70% of the experimental inoculum came into contact with an entry receptor in immediate unison with adsorption. This could explain why the level of inhibition never exceeded 40%, as

this would be the average proportion of the administered virus particles that were required lateral movement (via viral surfacing) in order to successfully interact with an entry-receptor making these the only virions which could be stymied by the surfacing inhibiting properites of the short-armed tetramer.

4.8 Possible Reasons for the Incoherence of the Yield Reduction Results

The results observed in yield reduction assay present a stark contrast to those produced by the plaque inhibition, qPCR and adsorption inhibition assays. The latter three assays indicated that the tetrameric were moderately to highly antiherpetic when administered at between 0.625 and 2.5μM whereas the yield reduction assay indicated that the same treatment had no discernible effect on HSV-1 replication, such a substantial divergence between different assays measuring the same essential property (HSV-1 replication) is most unexpected. The magnitude of this discrepancy is most stark when considering the divergence between the results of the qPCR and the yield reduction assays; the former indicated that 2.5μM of the short-armed tetramer was sufficient to produce greater than 90% inhibition of HSV-1 infectivity as assessed at 4hpi, while the total yield assay indicated that after 24 hours there was no measurable effect on hsv-1 replication produced by identical treatment conditions. Additionally, it should be noted that the data obtained in the yield inhibition assay shows a very incongruous and apparently random distribution of data points in the low concentration range. Such results speak to some degree of experimental error having produced unacceptable levels of noise. Both aspects of these results (divergent levels of inhibition and ostensibly random distribution of data points) require at least some degree of explanation lest the whole outcome be deemed the product of egregious operator error.

With regard to the unexpectedly poor inhibition that the test-compounds displayed in the yield reduction assay (this will henceforth be referred to as the "inconsistency of inhibition") the phenomenon cannot be adequately explained by appeal to a single cause alone. Instead it appears that the inconsistency of inhibition was most likely due to the interaction of several factors which synergistically abrogated the anti-viral effect of the test-compounds in a manner that could not have been achieved by any of the factors individually.

Firstly, it is pertinent to point out that the inconsistency of inhibition cannot be explained merely by appeal to the elevated MOI applied in the yield reduction assay. This theory can be rejected on the basis that the highest MOI applied in the yield reduction assay was also used in the qPCR analysis. In the qPCR analysis infection with an MOI of 1 in the presence of 2.5 µM of the short-armed tetramer produced a 91.4% reduction in the level of ICP47 gene expression, ostensibly indicating a comparable reduction in HSV-1 infectivity. In the total yield assay the same treatment conditions produced a 1.2% reduction in the number of PFUs/mL of cell lysate.

Additionally, the use of plaque inhibition experiments (defined by exceptionally low MOIs) followed by yield reduction assays featuring very high MOIs is a paradigm that has been applied to many previously published HSV inhibitors without producing the level of inconsistency observed in the present study (Yoosook et al 1999; Tolo et al 2010; Jaheel Alkaby, Falah & Hasan 2020). A particularly pertinent example of the expected relationship between MOI and inhibitory effect is found in Luganini et al (2011). This work focuses on compounds which, like the compounds examined herein, are dendrimers with the capacity to block the attachment of HSV-1 virions. The tested compounds were found to inhibit HSV-1 adsorption with approximately the same level of efficacy that shown by the study's' positive

control , heparin), making them impressively efficacious *in vitro(* Luganini et al 2011). It was shown that a 2 µM concentration of the most effective test compounds was sufficient to reduce HSV-1 infectivity by approximately 89% in both yield reduction and a plaque inhibition assays (MOIs of 0.1 and 0.0004 respectively), these two assays featured near identical MOIs to those applied in the corresponding experiments described in Chapter Three of this work (Luganini et al 2011). Accordingly it is reasonable to conclude that MOI is highly unlikely to have been a causative factor in the observed inconsistency of inhibition.

A factor that may have been responsible for the test-compounds' inconsistent inhibitory effect is low stability/ short half-life (under the conditions present in cell culture). This is speculation as the study laboratory lacked the means to directly measure the half-life of the test-compounds, there is however a published precedent for such a phenomenon. In reviewing the clinical suitability of sulfated polysaccharides derived from macro-algae (a class of compounds which are frequently studied as HS mimetics) Damonte, Matulewicz and Cerezo (2004) found that the class wide half-life for such compounds was between 1.5 and 2 hours *in vivo.* This theory is corroborated by the fact that the second iteration of the yield reduction assay (in which growth media/ test-compound was aspirated and replaced every 3.5 hours) showed clear inhibition produced by 2.5 µM of the tetrameric compounds indicating that refreshing the compound somewhat alleviates the inconsistency of inhibition.

Taken on its own, short compound half-life does not have sufficient explanatory power to account for the observed inconsistency of inhibition, this is because the level of inhibition indicated by the qPCR and plaque- inhibition assays would logically be expected to produce a reduction in HSV-1 replication of such magnitude that the viral titre would remain lower than that in the control group even many hours after the compound concentration had been reduced

to negligible levels. There are however two additional phenomena which, if they synergise with the short half-life, form a plausible explanation for the inconsistency of inhibition.

It seems implausible that a 90% reduction in size of the initial inoculum (the level of inhibition expected under exposure to 2.5 µM of the short armed tetramer) could be abrogated by secondary infections and syncytial spread over a 24 hour period. Nonetheless this exact phenomenon is demonstrated by the HSV-1 growth curve produced under control conditions (section 3.2). Even though the initial inoculum is 90% smaller for cells infected at an MOI of 0.1 as compared to those infected at an MOI of 1, these groups show overlapping confidence intervals for the viral titre at 24 hpi (from cell lysate) and at 28 hpi the two groups show near identical values for PFU/mL (MOI=1at 28hpi: 3.6×10^8 PFU/mL, MOI=0.1at 28hpi: 4.3×10^8 PFU/mL).

This phenomenon might explain the rather precipitous appearance of an inhibitory effect at exposure to 20 µM of the short-armed tetramer, a 90% difference in the infectivity of the inoculum (the level of inhibition produced by 2.5 µM of short armed tetramer) is within the range of what uninhibited secondary infection and syncytial spread can erase over the course of 24 hours. Greater disparity than this takes longer to erase as is manifest by the difference in the concentration of progeny produced by MOIs of 1 and 0.01 in section 3.2.

There is another factor that may have had a hand in the observed inconsistency of inhibition, this phenomenon is briefly detailed in the introduction subsection “Anomalous Upregulation of Viral Replication by Known Inhibitory Compounds” (section 1.15). Enhancement of HSV-1 infectivity at very low concentrations of the test-compounds would have allowed abnormally accelerated viral replication once the administered test-compound had broken down to a

sufficient extent. In theory this would allow accelerated viral replication occurring in the latter half of the assay to rapidly abrogate the reduction in viral titre produced by inhibition in the earlier part of the yield-reduction assay.

The research group theorised that instances of anomalous up regulation by inhibitory compounds (termed "anomalous upregulation" henceforth) where the applied compound was an attachment inhibitor might be explained by appealing to the role of VAPs in viral surfacing and the need to balance the valency of VAP-receptor interactions in order to optimise infectivity.

4.9 A Possible Explanation for Anomalous Upregulation

For the viruses that attach to a host-cell and then use viral surfacing to search for a secondary receptor(s), maintaining the a number of VAP-receptor bonds that is close to the optimal number is crucial for ensuring successful infection occurs. The importance of maintaining the appropriate attachment valence has been demonstrated by a number of studies showing that increased receptor-VAP avidity (stemming from *in vitro* evolution/attenuation) leads to more rapid clearance of virus and decreased virulence *in vivo (*Kobayashi et al 2018; Lee & Lobigs 2002; Lee et al 2006; Lee & Lobigs 2008). In these studies the loss of virulence appears to stem from virus being sequestered in HS rich tissues that that do not represent the viruses tropism. Of more relevance to the present hypothesis are the findings discussed in Delguste et al 2018, Delguste et al 2019, De Vreis et al 2020 and Trybala et al 2021; these papers reveal that deleteriously VAP-receptor avidity leads to attached virions becoming immobilised on the cell surface prior to entry/uptake. Additionally these papers theorise (or in the case of Delguste et al 2019 demonstrate) that in order for attached virions to enter the host-cell they require a means of limiting the total valency of VAP-receptor interactions so as to preserve

virion motility and by extension, infectivity. In theory it also stands to reason that valency that is too low is likely result in spontaneous disassociation of virions during the surfacing process meaning that the affected virion fails to initiate a productive infection. This phenomenon appears to be absent from existing studies as the current experimental techniques are predisposed to either return no data for abortive attachments or to identify such transient interactions as a failure to attach.

These findings may relate to the anomalous upregulation phenomenon in that they show that an incremental reduction in the level of VAP-receptor avidity can actually increase virus virulence in that they reduce likelihood that any given virion might become immobilised prior to engaging with the necessary uptake receptor(s).

If one is culturing virus in which the expressed availability of VAPs exceeds the optimal level for the cell culture in question (as determined by the density of attachment receptors on the surface of the cell layer in question) then it follows that a low level of VAP blocking might enhance the fitness of the virus under the conditions in question. Obviously this enhancement of infectivity will be reversed if the concentration of the VAP blocking compound is elevated to a level that prevents stable attachment, with the level of inhibition increasing with each subsequent increase in the concentration of the VAP blocking compound.

4.10 Potential Future Experiments

The results produced in this work leave many areas of uncertainty, the following is a brief description of experiments that should be conducted in order to resolve the present ambiguities surrounding the clinical utility of the test-compounds and anti-viral GAG mimetics in general.

Firstly it is desirable that the three dendrimeric compounds should be subject to all of the relevant assays so as to make up for instances where one or more of these was omitted due to scarcity of supply. By way of example, it is desirable that both the trypan blue assay and the qPCR assay should be repeated with the inclusion of the long-armed tetramer, the glycolipid trimer and the monomeric compound. Additionally, experiments that were performed with only one or two experimental repeats should be carried out with at least three experimental repeats so as to give results with greater statistical power. As it stands, inadequate repetition and failure to thoroughly interrogate all of the compounds represent substantial departures from the standard of research that was desired.

In terms of gaining insight regarding the biochemical factors that produced the results observed in this project, the yield reduction assay is of special interest. By repeating this experiment with a number of differing protocols, defined by a variety of different compound replacement intervals it should be possible to get a better indication of the extent to which compound breakdown was a causative factor in the inconsistency of inhibition (see section 4.8). It is also desirable that this experiment should be carried out with a high number of experimental replicates (i.e.>5) in order to disentangle the pertinent biological phenomena from the considerable noise present in the results that this assay produced.

In addition to the above method for indirectly inferring compound half-life, it is also desirable that the stability and pharmacokinetics of these compounds should be established via direct observation. Results obtained by administration of the compounds to live subects are especially necessary for gauging the potential clinical utility of these compounds. Liquid chromatography is one potential method for establishing both the *in vitro* and *in vivo* half-life

of the test-compounds, although it is by no means simple this procedure is favoured by many academics due to its suitability for small scale laboratories and its impressive accuracy (Hagan 1994; Li et al 2003; Loregian et al 2007).

An area of ambiguity that affects several of the above findings is the extent to which alterations in the applied MOI can influence the subsequent results regarding with regard to antic-herpetic potency. If one assumes that test-compound operate by binding to viral proteins then theoretically it should be possible to entirely abrogate their effects by applying a MOI that is high enough to sequester all compound molecules while a large cohort of the applied virus experiences only marginal inhibition of infectivity. This data could be easily obtained by repeating the qPCR and yield inhibition assays under protocols that apply a wide range of MOIs (e.g. MOI= 1, 2, 4, 8, 16 and 32). The findings obtained by such an approach might be pertinent for one of two reasons; firstly by understanding the approximate compound to virion ratio at which the anti-herpetic effect is entirely lost one will gain a better understanding of the potential clinical utility of these drugs. Secondly, failure to totally abrogate the inhibitory effects of the test-compounds, even at very high MOIs, would indicate that these compounds act by a mechanism or mechanisms beyond a simple binding interaction between the compound and viral proteins. Such a finding would be grounds to re-examine the majority of the above results and might even raise questions about the mode of action attributed to other glycomimetic antivirals.

Another experiment that should be conducted in future in order to better interpret the results presented herein relates to the single-step infection protocol presented in section 3.2. One of the tentative conclusions derived from this assay is that all three of the applied MOIs (0.01, 0.1 and 1) eventually converge on the same total viral yield with the MOI determining the

time taken to reach this number. This result could be corroborated by testing additional MOIs (i.e. MOI= 0.5, 0.25, 0.05 and 0.025) and continuing to sample these for at least 48 hours post-infection. The corroboration of the apparent convergence of total viral yield is desirable as this phenomenon was invoked as a partial explanation of the unexpected results in the yield reduction assay (see section 4.8). If the final yields obtained from disparate MOIs fail to show convergence then it will be necessary to revisit the unexpected results obtained in the yield reduction assay.

Of all the matters requiring further investigation, perhaps the most fascinating and conceptually important is the role of viral surfacing (and the disruption thereof) in producing the above findings. The ability of the test-compounds to interfere with viral surfacing was invoked as a potential explanation for the unexpected results observed in the viral entry assay (section 4.7), it was also invoked as a potential mechanism for the "anomalous upregulation" phenomenon described in 1.15. Anomalous upregulation may have contributed to the results of the yield-reduction assay by producing enhanced viral replication over a specific range of compound concentrations (see 4.8 and 4.9). One relatively simple (though indirect) way to test the influence of viral surfacing would be to carry out the entry-assay protocol across cell populations with artificially modified expression of known HSV-1 entry recetoprs. The logic behind this protocol is that increased concentration of entry receptors should reduce the importance of post-attachment surfacing whereas reduction in the concentration of entry-receptors would make surfacing necessary for the vast majority of attached virions.

Another method to interrogate the same relationship (the importance of viral surfacing in determining the maximum level of inhibition that can be achieved in an entry inhibition assay) is to repeat the methods utilised in Peerboom et al (2017) and then observe the extent

that virion movements are altered by various concentration of the test-compounds (starting from extremely low concentrations so as to establish a dose wise relationship if possible). If incremental increases in the concentration of the test-compound initially result in increased speed of virion diffusion/surfacing, and subsequent increases result in surfacing that is quickly terminated, these observations are in keeping with the research groups hypothesis regarding the relationship between the test-compounds and viral surfacing.

The hypothesis regarding the potential nature of anomalous upregulation (see sections 1.15 and 4.9) could be tested by selecting a cell line that expresses HS GAGs at very high levels and artificially supressing the expression or availability HSV-1 entry receptors (by siRNA or targeted antibodies respectively) to such a level that HSV-1 infection can still occur but at a very limited level. If the above hypothesis about the mechanism of anomalous upregulation is correct it should be produce comparable increases in HSV-1 infection by treatment with either a VAP blocking compound or exogenous hepranase as both of these treatments should reduce the avidity of virion-HS binding, with the correct dose of either treatment confering the optimal level of VAP-HS valency.

One of the curious results that was produced by the experiments in this research programme was the observation that high levels of compound exposure appeared to produce MTT scores that exceeded those produced in the control group. The research group theorised that this may be the result of the test-compounds disrupting cell-cell adhesion and thereby enhancing the cell lysing step of the MTT assay (see section 4.2). It is desirable that this hypothesis be examined in future, this could be achieved by repeating the MTT assay under a protocol that is altered so as to ensure that all cell samples are entirely dispersed and lysed prior to the 20min incubation period (this is the period over which the formazan associated colour change

occurs). Sonication is likely to be an effective means of ensuring effective dispersion and lysis of the entire cell population though it is necessary that this should be verified via microscopy before proceeding to apply this protocol to all of the various treatment groups. If there is still an ostensible increase in MTT conversion associated with exposure to high concentrations of the test-compounds then this phenomenon will require further investigation, compound induced alterations in cell biology should be considered as likely candidates under this scenario.

4.11 Prospects for the Clinical Utility of Attachment Inhibitors in General

If the results obtained in the present study are representative of the antiherpetic activity that the test compounds will produce *in vitro* then it stands to reason that the two tetrameric compounds have much potential as therapeutic agents in the treatment and prevention of HSV-1 infections. Under the same understanding the glycolipid trimer should be considered to possess modest clinical potential whereas the monomeric compound has none what so ever.

This level of predicted utility relies on two assumptions: firstly that the compounds show similar performance in live subjects to that which was evinced in this study, and secondly that the medical and scientific communities do not totally discount the antiviral utility of attachment inhibiting compounds. The first assumption has been shown to be unjustified for a number of previously examined attachment inhibiting compounds, the fact that a compound produces very high levels of virus inhibition *in vitro* provides no grantee that biologically significant effect will be achievable in an animal host. A noteworthy example of this phenomenon (i*n vitro* findings having little correlation with *in vivo* results) is the case of dextran sulfate which is a potent inhibitor of HIV in cell culture, yet in live subjects the

compound is useless as a treatment due to having a short plasma half-life and its propensity to increase the titre of HIV in the patient (Flexner et al 1991; Ito et al 1987). Lee et al (2006) also gives a good illustration of this phenomenon affecting the relative *in vivo* efficacy of glycomimetic attachment inhibitors (in this case being used to treat DEN and encephalitic flaviviruses).

With regards to the second of the two assumptions there is reason to think that both expert opinion and empirical data are somewhat opposed to future development of glycomimetic compounds as viral attachment inhibitors. This is the stance that is advocated in Pirrone, Wigdahl and Krebs'(2011) appropriately titled review- "The Rise and Fall of Polyanionic Inhibitors of the Human Immunodeficiency Virus Type 1", this paper examines the manner HIV targeting attachment inhibitors have largely fallen out of favour due to the repeated failure of apparently promising drug candidates and the fact that certain vaginally applied biocides appear to increase the rate of HIV infection rather than reducing it. Nonetheless the public health necessities discussed in the introduction of this paper require that the search for new antiviral compounds continue. It is primarily in response to this need that glycomimetic compounds continue to be investigated as potential treatments for viral infections. With this in mind there is moderate cause for optimism, the development of glycomimetic drugs may be beset with obstacles but alternative strategies (such as dendrimer compounds) may yet yield successes.

4.12 Concluding Remarks

With the exception of the monomeric compound, the compounds examined in this document are clearly capable of inhibiting the replication of HSV-1 *in vitro.* The mechanism by which these compounds inhibit viral replication is ostensibly by binding to the VAPs of cell-free

virions and thereby reducing the rate of successful viral attachments. The exact mechanism by which these compounds acts requires further investigation, particularly with regard to the impact that these have on viral surfacing and post-attachment entry to the host cell.

The role of the dendrimer effect in producing the observed results can be inferred from the fact that the monomeric compound showed nil anti-herpetic activity, while the tetrameric compounds displayed very high levels of HSV-1 inhibition and the trimeric compound (the glycolipid trimer) showed an intermediate level of inhibition. With this In mind it stands to reason that the basic design of these compounds could be improved upon by adding additional generations to the same basic dendrimer structure, this is in line with a commonly observed property of dendrimer drugs: altering the number of generations (i.e. branching points per molecule) alters the magnitude of the drug affect (though the correlation between increasing generation number and drug effectiveness is not always positive) (Caminade et al 2015).

Additionally, it may be possible to use the techniques deployed in creating the study dendrimers (Tyler et al 2015) to create a more targeted drug in a manner similar to the approach used by by Connell et al (2012). By conjugating molecules that targeted two different binding sites on the HIV VAP (gp120) (binding sites corresponding to the initial attachment receptor and the necessary co-receptor) Connell et al (2012) where able to produce a compound with markedly higher anti-HIV activity than was evinced by either of the parent compounds (i.e. the non-conjugated forms of the two rgp120 binding molecules) acting in isolation. If the above study compounds were altered to incorporate a second species of active domain (e.g. a nectin analogue targeting HSV-1 gD) then it is conceivable that the resulting compound would have unprecedented anti-herpetic activity.

There is much to that remains to be understood about the clinical suitability of the compounds studied in this paper, that said there is good reason to think that these compounds present a promising avenue for further research and compound development. The research team hope that the findings presented herein will facilitate further research into the anti-viral properties of these and similar compounds.

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