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Screening Tools for the Identification of Alphavirus Inhibitors

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ACADEMIC DISSERTATION

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ABSTRACT

Alphaviruses (genus *Alphaviridae* in the family *Togaviridae*) are positive-stranded RNA viruses with a nucleocapsid of eicosahedral symmetry and a lipid envelope carrying glycoprotein spikes. The viral genome is a single 11.5 - 11.8 kb RNA strand that has a 5' cap structure and 3' poly-A tail. The replication cycle in vertebrate cells involves entry into the host cells via receptor-mediated endocytosis, translation of the RNA to form the replicase proteins and successive transcription of RNAs with both polarities, production of structural proteins and packaging of the material into newly formed virions. Even though the genus includes emerging pathogens such as the Ross-River virus and Chikungunya virus, no vaccine or chemotherapy is currently available to prevent or suppress alphavirus infections in humans.

Studies on alphavirus inhibitors have been scarce and mainly involved the description of broad-spectrum antiviral agents targeting host cell enzymes. Many of these agents demonstrate a narrow therapeutic window or have immunosuppressive activities, which limit their clinical use as antiviral agents. Thus far, the only clinical study on antiviral chemotherapy for alphavirus infections was conducted in 2006 using chloroquine and failed to show any benefits when compared to placebo-treated individuals.

The general challenge in bioactivity screening is the need for robust, reproducible, cost-effective and biologically accurate assays for the screening of small organic molecules against the target of interest. Antiviral screening can be conducted via two basic strategies, either by phenotypic screens using general endpoints to measure virus replication or by screening for ligands against isolated target proteins validated for their relevance in virus replication. The limitation caused by the use of pathogenic viruses in screening can be overcome by the use of surrogate viruses or by the creation of replicon-containing cell lines. Replicon cell lines are generated by transfecting cells with RNA constructs encoding the viral replicase proteins, but lacking the genes for structural proteins. The constructs are typically inserted with selection markers and reporter genes and for viruses that induce a cytopathic effect, attenuating mutations may be required to achieve a stable cell line.

In the current study, two antiviral screening assays were developed and used for the identification of alphavirus inhibitors. The SFV-*Rluc* marker virus carrying a *Renilla* luciferase insertion between nonstructural proteins (nsP) 3 and 4 was demonstrated to be genetically stable and similar to wild-type virus in terms of infectivity in BHK21 cell culture infections. This virus allowed for the development of a robust luminometric assay that resulted in a Z' value of 0.52, and approximately 10% deviation in the normalised mid-signal. Furthermore, a replicon approach was used in the development of an antiviral assay against CHIKV. A BHK21-based cell line persistently expressing CHIKV replicase proteins with adaptive mutations in nsP2 was adopted for antiviral screening applying the fluorescent readout of enhanced green fluorescent protein (EGFP) marker under the subgenomic promoter of the replicon and activity of the *Renilla* luciferase produced as a fusion protein with nsP3. Z' value of 0.79 and 0.74 were achieved for the fluorescent and luminescent readouts, respectively, and the normalised mid-signal showed approximately 5% deviation. Both assays were optimised for screening environment in 96-well format and validated with previously known alphavirus inhibitors. In addition to the new antiviral assays, methods for cell viability evaluation were compared and validated in automated environment to provide counter-screening methods to be combined with the antiviral assays..

The SFV-*Rluc* assay was used as the primary assay for the antiviral screening of 29 nucleoside analogues, 51 semisynthetic betulin-derived compounds, 124 natural compounds and 234 synthetic drug molecules. The identified hits were counter-screened in mammalian cell viability assays. The confirmed hits were further characterised in secondary assays using CPE reduction, measurement of SFV yield and CHIKV replicon assays. 3'-amino-3'-deoxyadenosine, 3,28-*O*-diacetylbetulin and coumarin 30 were identified as the most potent SFV inhibitors with IC_{50} values of 16.2 µM, 9.1 µM and 0.4 µM, respectively. The 5,7-dihydroxyflavonoids apigenin, chrysin, naringenin and silybin were found to suppress expression of the CHIKV replicon marker gene at micromolar concentrations. Among the pharmaceutical compounds, the core structure of 10*H*-phenothiazine was found in 6 of the 12 confirmed hit compounds and was considered a suitable building block for future antiviral studies. In conclusion, the newly developed assays were successfully used as a panel of antiviral and cell viability assays to identify alphavirus inhibitors with diverse chemical structures.

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following publications referred to in the text by the Roman numerals **I** - **IV**. Some unpublished results are also presented.

I Pohjala, L., Tammela, P., Samanta, S.K., Yli-Kauhaluoma, J. & Vuorela, P. 2007, "Assessing the data quality in predictive toxicology using a panel of cell lines and cytotoxicity assays", *Analytical Biochemistry*, vol. 362, no. 2, pp. 221-228.

II Pohjala, L., Barai, V., Azhayev, A., Lapinjoki, S. & Ahola, T. 2008, "A luciferase-based screening method for inhibitors of alphavirus replication applied to nucleoside analogues", *Antiviral Research*, vol. 78, no. 3, pp. 215-222.

III Pohjala, L., Alakurtti, S., Ahola, T., Yli-Kauhaluoma, J. & Tammela, P. 2009, "Betulin-derived compounds as inhibitors of alphavirus replication", *Journal of Natural Products*, vol. 72, pp. 1917-1926.

IV Pohjala, L., Vargus, M., Ott, A., Merits, A., Ahola, T. & Tammela, P. 2010, Identification of alphavirus inhibitors using virus-based assay and Chikungunya replicon. Manuscript.

ABBREVIATIONS

aa	amino acid
ATP	adenosine triphosphate
BSA	bovine serum albumin
CAT	chloramphenicol acetyltransferase
$CCID_{50}$	cell culture 50% infective dose
CHK	Chikungunya virus
CMC	carboxymethyl cellulose
CNS	central nervous system
CPE	cytopathic effect
CPV	cytopathic vacuole
CTP	cytosine triphosphate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EGFP	enhanced green fluorescent protein
FBS	foetal bovine serum
FRET	fluorescence resonance energy transfer
GFP	green fluorescent protein
HBSS	Hank's balanced salt solution
HCS	high-content screening
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HTS	high-throughput screening
I	interaction index
IC_{50}	50% inhibitory concentration
IFN	interferon
IL	interleukin
IMPDH	inositol monophosphate dehydrogenase
kb	kilobase
LD_{50}	50% lethal dose
LDH	lactate dehydrogenase
MEM	minimum essential medium
MOI	multiplicity of infection
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NC	natural compound
nsP	nonstructural protein
ONNV	O'nyong-nyong virus
PBS	phosphate-buffered saline
PC	pharmaceutical compound
PFU	plaque-forming unit
QSAR	quantitative structure-activity relationship
RC	replication complex
RdRp	RNA-dependent RNA polymerase
RFU	relative fluorescent unit
RLU	relative luminescent unit
Rluc	*Renilla* luciferase
RNA	ribonucleic acid

RRV	Ross-River virus
S/B	signal-to-background ratio
S/N	signal-to-noise ratio
SAH	S-adenosylhomocysteine
SAR	structure-activity relationship
SARS CoV	severe acute respiratory syndrome coronavirus
SDS	sodium dodecyl sulphate
SFV	Semliki Forest virus
SINV	Sindbis virus
siRNA	small interfering ribonucleic acid
SPA	scintillation proximity assay
TCA	trichloroacetic acid
TNF	tumour necrosis factor
TRF	time-resolved fluorescence
VEEV	Venezuelan equine encephalitis virus
WNV	West Nile virus
WST-1	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt
wt	wild type
Z’	screening window coefficient

1. INTRODUCTION

Several viral diseases remain major health problem worldwides, decreasing the quality of life and causing deaths in both developing countries and developed Western societies (Strauss and Strauss, 2007). The central challenge in the development of antiviral drug therapies is due to the nature of viruses as intracellular pathogens, which exploit host cell macromolecules and organelles to replicate their DNA or RNA genomes and have only a limited number of specific structures of their own. Although some successful antiviral agents target cellular proteins, the goal of antiviral therapy is typically inhibition of the processes specific and essential for virus replication to minimise the adverse effects on host cells. The dawn of such an approach occurred serendipitously with the synthesis of an acyclic guanosine analogue, acyclovir in 1973 (De Clercq, 2008). Acyclovir triphosphate, the active agent formed from acyclovir, is a DNA polymerase inhibitor whose inhibitory activity requires intracellular phosphorylation of the drug. DNA viruses, such as herpes simplex virus encode a deoxythymidine kinase, which has a significantly higher affinity for acyclovir than the host cell kinases. This feature leads to production of the active form mainly in infected cells, which allows for specific targeting of the antiviral effect.

In 2002, a review listed a total of 20 antiviral drugs approved for clinical use (De Clercq, 2002). By 2009, the number increased to approximately 50 licensed compounds, about half of which are indicated against human immunodeficiency virus (HIV) (De Clercq, 2010). Therapeutic strategies currently in clinical use include viral DNA and RNA polymerase inhibition, targeting of retrovirus reverse transcriptase by nucleosides and nonnucleoside-structured compounds, inhibition of viral proteases, and targeting the host cell enzymes crucial for virus replication (e.g. blockage of *de novo* guanosine synthesis by inhibiting inositol monophosphate dehydrogenase IMPDH). Besides HIV, antiviral drug development has mainly focused on influenza viruses and DNA viruses such as herpes simplex virus types I and II, while positive-stranded RNA viruses have received little attention in the past (Leyssen et al., 2008). However, the picture has recently changed due to the rise of chronic hepatitis C virus (HCV) infection as the main cause of liver failure worldwide and the emergence of severe acute respiratory syndrome coronavirus (SARS CoV).

Single-stranded RNA viruses also include the arthropod-borne viral species (arboviruses) that circulate between vertebrates and invertebrate (arthropods, most commonly mosquitoes) (Weaver,

2005). Arboviruses are distributed in different taxonomical families (*Togaviridae*, *Flaviviridae*, *Bunyaviridae* and *Rabdoviridae)*, and the group includes historically important pathogens such as the dengue virus, yellow fever virus and Japanese equine encephalitis virus. Except for the dengue virus, which has fully adapted for the mosquito-human-mosquito transmission cycle, most arboviruses spread as zoonotic infections and typically use birds and rodents as amplification and reservoir hosts. Recent arboviral epidemics has returned the arboviruses to general consideration and also revealed some unexpected features related to these viruses. The changing epidemiological patterns of arboviruses were probably most dramatically illustrated by spread of the West Nile virus (WNV) into the Western hemisphere in 1999. WNV caused several cases of fatal encephalitis in the New York area, and the virus has currently established itself in Northern America causing small epidemics in humans and domestic and wild animals (Nash et al., 2001). Since then several other arboviruses have been discussed as re-emerging or resurging threats due to similar changes in epidemiology (Gobler, 2002). From 2005 to 2007, an outbreak of the Chikungunya virus (CHIKV, alphavirus within the family *Togaviridae*) took place, estimated to affect more than 6 million people worldwide and involved an unexpectedly high prevalence of serious neurological disorders strongly, indiciatng that such concerns have not been overestimated (Pialoux et al., 2007). Notably, the relatively sporadic prevalence and benign clinical picture of most arboviral diseases in the past has contributed to the lack of vaccines and antiviral chemotherapy against many of these pathogens.

2. REVIEW OF THE LITERATURE

2.1. Bioactivity screening

Bioactivity screening refers to a spectrum of methodologies used to identify previously undescribed interactions between small organic molecules and biological macromolecules, most often proteins. The technologies currently in use for bioactivity screening have mainly been developed for the needs of the pharmaceutical industry as bioactivity screening currently forms the basis for lead discovery process (Drews, 2000). In this context, the term high-throughput screening (HTS) is often used to refer to bioactivity screening campaigns in which more than 10,000 assay points are run daily. Academic research organisations, on the other hand, typically apply more focused, medium-throughput screening strategies to use bioactivity screening as a tool to benefit the needs of basic science (Inglese et al., 2007). Chemical probes identified in bioactivity screening campaigns can improve our understanding of the specific biological processes that they target, help to dissect the relationships of related enzyme or receptor classes and give rise to structure-activity relationship (SAR) models.

2.1.1. Terminology and basic principles

Individual screening organisations may have developed their own procedures and implementation paths for their HTS campaigns. The procedures and terms presented here represent a generally accepted strategy of bioactivity screening, which is also used in the HTS facilities of the National Institute of Health in the United States (Bronson et al., 2001; NIH, 2009).

The initial steps of an HTS screening campaign include HTS implementation and validation of the selected bioassay. Implementation of an assay in the HTS environment usually requires modification of the assay protocol and optimisation to minimise assay costs and provide the best possible reproducibility (inter-plate and inter-day variation). The optimised assay is further validated by conducting a pilot screen and assaying suitable standard compounds.

The actual screening for novel ligands of interest is initiated by conducting the primary screen with the validated assay, typically using a single concentration and a very limited number of replicate samples of each compound. Compounds scoring above the threshold values set during the HTS assay implementation phase are considered primary screening actives and are subjected to confirmatory assays. The confirmatory assay involves cherry picking of the active compounds from the chemical library and retesting them using the primary screening assay. The confirmed active compounds exhibiting dose-dependent activity are nominated as screening hits to be further analysed. Depending on the nature of the chemical library used, chemical validation of the hits may be required at some stage of the process. The chemical identity of the hit compound is confirmed by reordering the sample from the vendor, obtaining the pure dry compound and/or resynthesis of the compound.

Hit compounds typically undergo secondary assay protocols in which their activity on the target of interest is tested in a different assay from the primary screen to confirm the order or hit compound potency. Furthermore, profiling assays can be conducted in which the hit compounds are characterised, e.g. for their selectivity by counter-screening for affinity to related targets such as receptor/enzyme subclasses or assaying for related microbial species, and pathogen vs. host cell selectivity.

2.1.2. Assay formats and detection modalities

Concerning the assay design, two main categories can be distinguished: i. screening for ligands in assays based on isolated target molecules and ii. phenotypic assay formats to identify small organic molecules that shift the assay system in the direction of the desired endpoint (Bronson et al., 2001). The previous emphasis on screening against individual, isolated targets stems from the current view of a validated target molecule, most often an enzyme or receptor protein to serve as a starting point for lead discovery in the drug development process. A variety of assay platforms as well as focused chemical libraries have been published and are commercially available for the most intensely studied target classes such as kinases, proteases and G-protein coupled receptors.

Despite the earlier focus on isolated targets, most of the recent advances in assay development have been seen in phenotypic assays (Johnston and Johnston, 2002). Functional cell-based assays yield

multiparametric readouts for the characterisation of complete signalling cascades in their native environments, and the introduction of high content screening (HCS) tools has rendered the monitoring of dynamic cellular events such as protein-protein interactions and changes in subcellular localisation accessible to large-scale screens (Giuliano et al., 2003). Yet phenotypic assays can sometimes be based on general endpoints such as cell viability (see section 2.3.); the most widely used approach to building phenotypic screening assays is the use of reporter gene systems. Reporter genes (the most commonly used of which are listed in Table 1) are markers whose expression level in the system is easily detectable by either the protein's inherent properties (fluorescence) or by the addition of a suitable substrate (Beonstein et al., 1996; New et al., 2003). A variety of applications of these systems have been developed such as the use of green fluorescent protein (GFP) or its derivatives as a portion of a fusion protein for localisation studies (Zhang *et al.*, 2002). . Alternatively, by placing a reporter gene sequence in the genomic region of interest the changes in transcriptional activity resulting from a given signalling cascade can be readily monitored via a generally applicable method. Still another application of reporter genes is the detection of protein-protein interaction by splitting the reporter gene into two and fusing each of the halves with a partner in an interaction pair. The proximity of the two partners recovers the catalytic site of the reporter and any changes in the interaction can be read by the reporter signal (Cubitt et al, 1995; Giepmans *et al.*, 2006).

Generally speaking, fluorescence is the most widely used detection mode in bioactivity screening campaigns due to the wide variety of fluorophores and fluorescent protein variants (Eggeling *et al.*, 2003; Jager et al., 2003; Thomsen et al, 2007). The energy emitted as light by fluorophores can also be transmitted to a second fluorophore as a function of distance, which allows for the use of assay

Table 1. Most commonly used reporter genes and their properties.

Name	**EC**	**Origin**	**Detection mode**
β-galactosidase	3.2.1.23	Various bacterial species	colorimetric
Chloramphenicol aetyltransferase	2.3.1.28	Various bacterial species	radiometric/fluorometric
Adcquorin		*Aequorea victoria*	luminometric
GFP		*Aequorea victoria*	fluorometric
Luciferase	1.13.12.7	*Photinus pyralis* firefly)	luminometric
Renilla luciferase	1.13.12.5	*Renilla reniformis* (sea pansy)	luminometric
Alkaline phosphatase	3.1.3.1	Various	colorimetric
β-lactamase	3.5.2.6	Various bacterial species	colorimetric

formats detecting the proximity of two fluorophores (fluorescence resonance energy transfer (FRET)). In addition to the measurement of fluorescence intensity, the readout may utilise changes in fluorescence lifetime and speed of fluorophore rotation (fluorescence lifetime and fluorescence polarisation, respectively). However, the sensitivity of fluorescent assays may be compromised by various experimental factors, such as fluorophore bleaching, light scattering and reagent autofluorescence (Gribbon et al., 2003). On the other hand, luminescence assays do not require excitation light but generate the emitted light from the energy released from chemical or enzymatic reactions. Thus, they are free from the interference caused by excitation light (Fan and Wood, 2007; Roda et al., 2003). Luminescent assays have wide dynamic ranges and are able to linearly detect signals over several orders of magnitude due to the negligible background and intense light emission capacity. Bioluminescent luciferase enzymes are of particular use in reporter gene systems, and the light produced by these enzymes can also be combined with fluorescence in the phenomenon known as bioluminescence resonance energy transfer (BRET), which is performed in a similar manner to FRET (Roda *et al.*, 2004).

Classical spectrophotometric and radiometric detection modes can also be used in bioactivity screening, but with some limitations (Bronson et al., 2001). Intensity of the absorbed light is directly proportional to its pathlength (diameter of the assay liquid column), which makes the miniaturisation of spectrophotometric assay formats difficult due to the diminished signal window. The use of radioactive isotopes is limited in the screening environment by the production of large amounts of radioactive waste, economical reasons and the need for a filtration step to separate the bound radioligand from the free fraction, which may be challenging to automate and may lead to high signal deviation in microplate formats. The latter problem can be overcome by a specific scintillation proximity assay (SPA) platform where the liquid scintillate cocktail is replaced by the scintillating matter coated in the solid surface of assay plates or plastic or glass beads (Udenfriend et al., 1987). The SPA beads or the wells also contain an affinity tag for assay components and the light signal can be read as a response to the proximity of the radioligand and the scintillating matter.

More recently, label-free detection systems have been developed for screening applications as this approach offers the opportunity to monitor the native biological process without the disturbance of probes (Cooper, 2006). Furthermore, label-free approaches typically allow kinetic rather than endpoint measurements and thus allow for more informative characterisation of the biological

event. Surface plasmon resonance (SPR) technologies are becoming a validated approach for binding studies characterising both the association and dissociation rates of binding events, and a variety of other assay formats using SPR are emerging (Maynard et al., 2009; Szabo et al., 1995). With regard to cell-based assay formats, impedance-based measurements of cell monolayers grown on the surface of detection electrodes have been demonstrated to be a robust and sensitive means for various applications involving cellular signalling and cell viability (Panke et al., 2008).

2.1.3. Data quality and assay artefacts

Assessment of assay performance. When large numbers of samples are run in bioactivity assays with a limited number of replicates the role of assay robustness and reproducibility have been underlined to generate reliable data. Different detection modes yield signal windows with varying dynamic ranges and signal and background deviations, which can sometimes make assay comparison and optimisation a difficult task. Assay quality parameters, such as signal-to-background (S/B), signal-to-noise (S/N) and the assay quality coefficient Z', are used as tools to control the signal window in assay optimisation and HTS implementation (Zhanf et al., 1999). The formulas for the three parameters are as follows:

$$S/B = \frac{\mu_{signal}}{\mu_{background}} \qquad (>5)$$

$$S/N = \frac{\mu_{signal} - \mu_{background}}{\sqrt{(\sigma_{signal}^2 - \sigma_{background}^2)}} \qquad (>10)$$

$$Z' = 1 - \frac{3\sigma_{signal} + 3\sigma_{background}}{\mu_{signal} - \mu_{background}} \qquad (>0.5)$$

μ_{signal} = signal mean
$\mu_{background}$ = background mean
σ_{signal} = signal standard deviation
$\sigma_{background}$ = background standard deviation.

The typical threshold values for each of the parameters indicating reasonable signal robustness are also given in parentheses. The parameters are also used to monitor the data quality during the screening campaign and modifications in the formulas that take into account the number or replicate samples have also been introduced (NIH, 2008). After setting the assay conditions, the assay quality parameters are complemented by measurements of plate-to-plate and day-to-day variation calculated from the maximal and mid signals as well as by determination of a minimum significance ratio (MSR). The MSR value is calculated from the deviation in the EC/IC_{50} value of a selected standard compound in dose-response experiments carried out on separate days, and the value is used to define the smallest potency difference that the assay can recognise (Brian et al., 2006).

Compound interference. Even when the assay procedure can be optimised and validated to yield reliable data, properties of the screening compounds may give rise to unpredictable false positive results in the assay implementation phase. For instance, the fluorescent nature of some organic small molecules is likely to cause problems in assays using fluorescent intensity as the readout. Many of the fluorescent probes utilise the blue end (450 – 570 nm) of the visible light spectrum (for example, GFP has excitation and emission maximums at 395 nm and 509 nm, respectively); screening libraries are rich in heterocyclic compounds that tend to absorb and emit light within these same wavelengths, and besides the actual screening compounds, the impurities present in chemical libraries are a remarkable source of interfering autofluorescence (Simeonov et al., 2008). Compromised assay sensitivity due to autofluorescence may force the introduction of additional washing steps in the assay protocol, or the problem can be avoided by the use of alternative detection modes such as time-resolved fluorescence (TRF). TRF probes are lanthanide chelates that show an extended delay between the excitation and emission phase, which allows for postponing of the fluorescent readout by 0.1 - 0.2 ms, which is long enough to quench the short-lived background autofluorescence (Gribbon et al., 2003).

Screening hit lists can also be dominated by false positives generally referred to as promiscuous binders. These compounds typically exhibit steep dose-response relationships and show inhibition that is highly sensitive to assay conditions such as ionic strecgth, pH changes or protein concentration. The underlying mechanism leading to promiscuous inhibition is thought to involve aggregate formation by the screening compounds as the formation of spherical aggregates of 30 – 200 nm in diameter from drug-like small molecules has been demonstrated by dynamic light

scattering and electron microscopy. Such particles have been shown able to reversibly inhibit several unrelated enzymes (McGovern et al., 2002). Screening hits classified as aggregate formers are generally omitted from further studies and marked in databases as nonleadlike structures to reduce the repeated identification of false positives (Scidler et al., 2003). However, it should be noted that some of the clinically used therapeutics (8% in the study by Scidler et al) show aggregating behaviour in biochemical buffers, which makes the interpretation less obvious.

Aggregates can be formed by compounds with wide structural diversity and despite some efforts no single chemoinformatic model is capable of reliably predicting aggregating behaviour. Aggregation is more likely to occur at higher micromolar compound concentrations, but a high total organic load may also induce aggregating behaviour at low concentrations of well-behaving inhibitors when compound mixtures are screened (Feng et al., 2006). In practical terms, these promiscuous binders can be identified by re-running the screen in the presence of a low concentration of nonionic detergent, which usually prevents aggregate formation and thus abolishes the inhibitory potency of such samples.

False positives in bioactivity screens can also be caused by a more specific interaction of the screening compounds with probes or reporters of the assay. The most studied reporter protein in this respect is firefly luciferase for which competitive and noncompetitive inhibitors have been described with various chemical scaffolds including the widely studied natural product resveratrol (Auld et al., 2008a; Bakhtiarova et al., 2006, Heitman et al., 2008). Many of these compounds inhibit luciferase at a biologically relevant micromolar concentration and thus are often scored as hits in reporter gene assays using this marker. Notably, luciferase inhibitors have also been identified as false positives in assay platforms for reporter gene activators via the formation of stabilised enzyme-inhibitor complex, which diminishes reporter degradation and leads to the accumulation of reporter enzyme within the inhibitor-treated cells (Auld et al., 2008b).

Systematic studies on luciferase inhibition by the compounds in screening collections have been carried out and made available as PubChem entries, show an approximate prevalence of 3% in diverse chemical libraries (Inglese *et al.*, 2007). Similar reports have yet to be published on the specific inhibition of other reporter genes such as *Renilla* luciferase or GFP. Meanwhile, follow-up studies using a different detection system than the primary assay are the method of choice to eliminate false positives due to reporter protein inhibition.

2.2. Alphaviruses

The genus *Alphaviridae* (in the family *Togaviridae*) contains 29 viruses as listed in The VIII report of the International Committee for the Taxonomy of Viruses (ICTV, 2005). Alphaviruses are animal RNA viruses infecting birds, fish, small rodents and/or larger mammals including humans and domestic animals (Griffin, 2001). Except for the two alphaviral species that infect marine organisms (Salmon pancreatic disease virus and Southern elephant seal virus), all species in the genus are arthropod-borne viruses (arboviruses) that circulate between their vertebrate hosts using *Aedes*, *Culex* and *Culiseta* sp. mosquitoes as vectors.

2.2.1. Prevalence and recent outbreaks

Whereas alphaviral species are found on all continents except Antarctica, individual viruses in the genus exhibit limited geographic distributions that are mainly governed by the ecology of their specific vector host species (Weawer, 2005). Table 2 lists the alphavirus species currently regarded as human pathogens together with their geographic distributions and the abbreviations used for each virus. Six virus species in the genus cause human diseases characterised by polyarthritis (Powers et al., 2007). Together with other viruses which are considered asymptomatic or nonpathogenic, they are referred to as Old World alphaviruses because of their classical epidemic sites in Eurasia, Africa and Australia. In contrast, New World alphaviruses circulate on the American continents. New World alphaviruses differ from their Old World counterparts in some aspects of their replicative process and cause diseases that primarily involve encephalitis or other neuronal syndromes (Calisher, 1994; Zacks and Paessler, 2010). However, Mayaro virus makes an exception of the rule as despite its geographic distribution in Southern America it is associated with an arthritogenic disease. The scope of the current study and the main focus of the following sections are on the Old World alphaviruses and the arthritogenic disease.

The first alphaviral species were isolated during the 1940s and 1950s in Africa (e.g. the Sindbis virus (SINV) in 1952 in Egypt and the Chikungunya virus (CHIKV) in 1952 in Tanzania) (Robinson, 1955; Taylor *et al.*, 1953). Small epidemics and individual cases of SFV, SINV, RRV and CHIKV have been reported from the 1960s through to the 1990s at different sites in Africa,

Table 2. Alphavirus species considered to be human pathogens.

Virus	Abbreviation	Distribution
Old World		
Barmah Forest virus	BFV	Australia
Chikungunya virus	CHIKV	Africa, Asia
O'nyong-nyong virus	ONNV	Africa
Ross-River virus	RRV	Australia, Oceania
Semliki Forest virus	SFV	Africa
Sindbis virus	SINV	cosmopolitan
New World		
Eastern equine encephalitis virus	EEEV	North, Central and South America
Mayaro virus	MAYV	South America
Western equine encephalitis virus	WEEV	North and South America
Venezuelan equine encephalitis virus	VEEV	North, Central and South America

Asia and Australia (Fontemille, 1989; Lam et al., 2001; Marhiot et al., 1990; Pialoux et al., 2007; Powers et al., 2007).

The total number of reported alphavirus infections is relatively low compared to other arboviruses, such as yellow fever virus or dengue virus, but it has been suggested that the low number of reported cases is partly due to misdiagnosis of alphaviral infections as dengue fever (Pialoux et al., 2007;). CHIKV and dengue virus use the same vector mosquito species for transmission, and the clinical picture of a mild CHIKV fever may be difficult to distinguish from dengue fever in cases where serology testing is not done (Carey, 1971; Nimmannitya *et al.*, 1969). More recently, documented cases of dengue and chikungunya virus co-infections have been reported (Nayar *et al.*, 2007; Schilling *et al.*, 2009).

In Eastern Finland, a small outbreak of Sindbis virus is seen every autumn causing from only a few to approximately 1,000 cases annually (Laine et al., 2004). Migratory birds are considered the main virus reservoir and different *Culex* and *Culiseta* sp. mosquitoes act as vectors. The disease, which is known as Pogosta disease (Carelian fever), is a typical mild syndrome caused by Old World alphaviruses and is characterised by fever, maculopapular rash or related skin lesions and rarely persistent joint disorders (Kurkela et al., 2005).

Ross-River virus (RRV) disease, the most important arboviral disease in Australia, is caused by infection with the corresponding alphaviral species RRV that was originally isolated near Ross River in Greensland, South Australia (Harley et al., 2001). RRV pools are maintained in local rodent species and the virus causes epizoonotic outbreaks in the wetland areas of Australia mainly during the season of heavy rainfall. A total of 1000 to 7000 people are affected annually by RRV disease. Typically, 80% to 90% of infected individuals experience incapacitating arthritic symptoms.

After several decades of sporadic cases, alphaviruses, especially CHIKV, have emerged to the general knowledge as endemic viruses. In 2005, CHIKV caused a massive outbreak on the islands of the Indian Ocean particularly in La Réunion where 270,000 individuals (more than one third of the island's population) were affected (Renardt et al., 2007). The virus rapidly spread to other islands in the Indian Ocean as well as India where approximately 1.7 million confirmed cases were reported (Kaur et al., 2008; Thavara et al., 2007). The epidemic lasted until 2007 and has been estimated to involve a total of more than 6 million cases worldwide (Figure 1). Imported cases in travellers returning from the infected areas were reported in 14 European countries, USA, Canada and Australia, and an outbreak of approximately 200 confirmed cases arose in Italy in 2007 (Penning et al., 2007; Rezza et al., 2007). In 2008 and 2009, epidemic outbreaks were also reported from Malaysia and Singapore (Leo *et al.*, 2009) . The latter outbreaks raised particular concerns as they stand as proof that the emergence of a tropical disease, such as Chikungunya fever, may be a realistic risk in temperate areas and regions with modern healthcare standards and facilities.

Figure 1. Endemic area of the Chikungunya virus. Information on countries among the endemic are are from the USA Center for Disease Control and Prevention.

Reasons for the re-emergence and the changes in endemic intensity of CHIKV and other arboviruses have been suggested to include virus genome microevolution, the spread of vectors into more temperate areas due to climate change and increased travelling and increasing population and urbanisation (Chretien and Linthicum, 2007; Gubler, 2002; Schuffenecker et al., 2006). In the CHIKV 2005 – 2007 outbreak, a single amino acid change A226V in the viral E1 envelope glycoproteins was identified in 90% of the clinical virus isolates collected after September 2005(Schuffennacker et al., 2006; Tsetsarkin et al., 2007). This change has been linked to improved adaptation of the virus for replication in *Aedes albopictus* mosquitoes. Although this mosquito species was previously seen as a secondary vector for CHIKV, it has emerged as the primary vector in the recent outbreak especially in urban and suburban environments.

2.2.2. Clinical aspects of alphavirus infections

Upon vertebrate transmission via the bite of an infected mosquito, an asymptomatic period of 4 to 7 days is usually observed before the onset of clinical symptoms (Kam et al., 2009). The first replicative cycles of alphaviruses in vertebrate hosts are thought to occur in dermal tissue at the site of the mosquito bite. Alphaviruses such as SINV and VEEV have been shown to infect macrophages and other antigen-presenting cells and promote their migration to the lymph nodes from where the virions are disseminated into the bloodstream (Gardner et al., 2000; McDonald and Johnston, 2000). High viremias are typical in acute alphavirus infections (10^9 to 10^{10} copies of the viral genome per ml of patient sera); the main viral pool being generated in the liver and spleen. While CHIKV and other alphaviruses have been experimentally shown to infect a variety of mammalian cell types, the animal models for CHIKV- and RRV-induced arthritogenic diseases have provided evidence that fibroblasts, epithelial cells and, to lesser extent, macrophages are the main cell types infected *in vivo* (Couderc et al., 2008; Rulli et al., 2005; Zieger et al., 2008).

Most alphaviruses are strong inducers of type I interferons (IFN) and lead to a proinflammatory response including the induction of IL-1α, IL-6 and TNF-α (Kupper and Fuhlbrigge, 2004; Simon et al., 2007). The fever observed in the vast majority of infected individuals is a result of the high levels of proinflammatory cytokines in the bloodstream. The fever is typically high (more than 39

°C) and is often accompanied by maculopapular rash, other skin disorders and gastrointestinal symptoms such as diarrhea and vomiting (Borgherini et al., 2007; Hochedez *et al*, 2006; Robin et al., 2009).

In most patients, the acute viremia is cleared from the bloodstream within 10 days (Penning et al., 2008). Due to the short time scale of acute infection, the adaptive immune response is not raised against CHIKV at this stage (Kam et al., 2009). Anti-CHIKV IgM antibodies are detectable in the blood of most individuals suffering from acute viremia whereas the IgG response is typically seen only after the viremia is cleared. Cross-reactivity of IgG antibodies is usually observed between the viruses of the genus, which may limit the specificity of serological tests as diagnostic tools. The alphaviral species are divided into seven antigenic serocomplexes based on this feature , SFV, CHIKV, RRV and ONNV falling into a same complex (Powers et al., 2001).

Old World alphavirus infections, particularly CHIKV and RRV, are distinguished from other endemic arboviral diseases by the high incidence of myopathy and polyarthritis (Simon et al., 2007; Sissoko et al., 2009). Joint disorders are typically encountered in 10% to 30% of CHIKV-infected individuals and in up to 90% of RRV disease cases. These disorders usually manifest as severe and incapacitating arthralgia that can last up to 1 to 2 years after the acute illness. The arthritic symptoms resemble rheumatoid arthritis, as they are most intense in the joints of extremities, such as fingers, toes, wrists and ankles. The relative importance of the proinflammatory response and direct virus attack in the etiology of connective tissue symptoms is not clear. Alphaviral infection is transient, and chronic forms in these or other tissues are not known to exist. Recent studies on CHIKV mouse models have also demonstrated that depletion of the macrophage-derived proinflammatory factors significantly diminishes pathological changes in the skeletal muscle and joints of infected animals (Lidbury et al., 2008). However, satellite cells and fibroblast cell lines have been shown to be infected by CHIKV *in vitro* (Ozden et al., 2008; Sourisseau et al., 2007). Furthermore, positive immunostaining for CHIKV antigens has been seen in satellite cells and muscle and synovial fibroblasts in CHIKV-infected mice indicating that the symptomatic tissues are also the sites of virus replication (Couderc et al., 2008). Pathology induced by different alphaviral species may not be uniform in this respect as fever indicating the proinflammatory response is reported in only approximately half of Ross-River viral infections even though most of the patients experience long-lasting and incapacitating arthritis and myopathy (Condon and Rouse, 1995).

Even though Old World alphavirus infections have been described as relatively benign, a significant number of neurological symptoms were observed in the most recent epidemic (Das et al., 2010). The CNS symptoms were especially pronounced in small children and led also to deaths and persistent disabilities. In La Réunion approximately 50% of infected pregnant women transmitted the virus to their newborns and more than half of the infected neonates developed CNS symptoms including seizures, encephalopathy and brain oedema (Geradin et al., 2008; Ramful et al., 2007; Robin et al., 2008). Permanent disabilities were detected in 10% to 20% of these cases. Similar CNS symptoms, autism and other behavioural disorders as well as peripheral neuropathy were observed in children of all ages and adults, yet as less severe forms. The ability of recent CHIKV isolates to infect neuronal cells is a matter of debate. Sug gestions have been made to support this view but the mouse models have shown either no CNS staining at all or staining of only cells in the choroid plexus; the latter could be linked with the brain oedema observed in many paediatric CHIKV patients.

Attempts to develop a vaccine against CHIKV have thus far been unsuccessful due to a large number of adverse effects caused by the attenuated virus strains used for this purpose (Kam et al., 2009). Medical treatment for alphavirus infection is currently limited to chemotherapy used for symptomatic relief as no chemical agents that suppress alphavirus replication are approved for clinical use. Most patients are treated with nonsalicylate anti-inflammatory drugs, analgesics and dermatological preparation to alleviate the skin lesions (De Lamballerie *et al.*, 2009).

2.2.3. Alphavirus genome and replication cycle

Alphaviruses have a single-stranded, positive sense RNA genome that includes two open reading frames, a 5’ methyl cap structure and a 3’ poly-A tail (Strauss and Strauss, 1994). The 5’ two-thirds of the genome corresponding to the 42S RNA fraction encodes the four nonstructural proteins (nsP1, nsP2, nsP3 and nsP4) responsible for the activities required for intracellular amplification of the viral RNA. The 3’ one-third of the genome is transcribed from the subgenomic promoter to yield a 26S RNA encoding the viral structural proteins including capsid (C) and envelope glycoproteins E1 and E2 together with residual polypeptides E3 and 6k (Figure 2). Each of the viral proteins carries out its characteristic activities that enable different aspects of the replication cycle

(Kääeiäinen and Ahola, 2002). Table 4 lists the proteins encoded by the alphavirus genome and presents the most essential known features of each protein.

Alphaviruses enter host cells via receptor-mediated endocytosis in a process requiring C-type lectins or in the case of *in vitro* infection of laboratory strains, heparin sulfate (Klimstra et al., 2003), Once entering the endolysosomal vesicle system, the low pH in these vesicles triggers conformational changes in the viral envelope glycoproteins, which allows E1 protein-mediated fusion of the viral envelope with cellular membranes (Helenius et al., 1981; Martin et al., 2009). The viral nucleocapsid is thus released into the cytoplasm and rapidly dissembled via its interaction with the 60S ribosomal subunit to release the single copy of the RNA genome (Ulmanen et al., 1976; Wiggler, 2009). Once the capped RNA is made available to ribosomes, the precursor polyprotein P1234 is translated and processed stepwise into individual nonstructural proteins. The

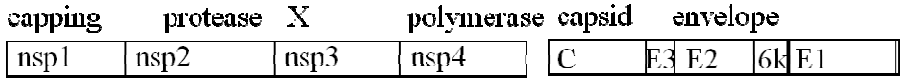

Figure 2. Genomic organisation of alphaviruses.

Table 3. Proteins encoded by the alphavirus genome.

Protein	Size (aa)	Structural elements	Features
nsP1	537	guanosine-7-methyltransferase guanylyltransferase	RNA capping Membrane anchor in RCs
nsP2	799	5'-triphosphatase NTPase helicase papain-like protease	P1234 polyprotein processing Regulation of (-)-strand synthesis Host cell transcriptional silencing
nsP3	482	macrodomain hyperphosphorylated region	Targeting of RCs to endolysosomes Association with neurovirulence
nsP4	614	RNA-dependent RNA polymerase	Production of viral RNAs
C	267	basic domain liner regain trypsin-like protease	Formation of nucleocapsid
E1	438	glycoprotein	Mediation of membrane fusion
E2	422	glycoprotein	Interaction with host cell surface receptors
E3	66	secretory polypeptide	Unknown function
6k	60	residual polypeptide chain	Assists in E1 folding

The presented amino acid numbers correspond to the Semliki Forest virus sequence.

intermediate cleavage products and mature nsPs form the differential stages of replication complexes, which act to multiply the viral RNA.

Early processing of P1234 results in production of the P123 intermediate and nsP4, which combine to use the positive RNA strand as a template for minus strand synthesis (Shuraji et al., 1994). Minus strand RNA is produced only at the early phase of infection and is shut down 4 to 6 h postinfection. Thereafter, the minus strands are used as templates for further plus strand synthesis under both genomic and subgenomic promoters. The virus seems to have developed delicate regulatory machinery to control which RNA polarity and size are preferred by using a single polymerase (nsP4) in each phase of the replication cycle; proteolytic processing of the P1234 polyprotein plays a central role in this regulation. (Salonen et al., 2003; Suioanki et al., 1998; Vasilieva et al., 2003) Based on experimental data from various sources, a model has been built in which RNA synthesis is initiated by the formation of a complex of nsP4 and P123. As discussed above, this complex produces mainly RNAs with negative polarity. The further processing of P123 switches the RNA synthesis to favour plus strand synthesis of mainly genomic (42S) RNA. Final maturation of the replication complexes by cleavage to individual nsPs guides the polymerase to produce subgenomic (26S) RNA, which is ultimately translated into viral structural proteins (Kim et al., 2004; Lemm et al., 1993; Lulla et al., 2008; Sawicki and Sawicji, 1990; Shirako et al., 1994).

As with many RNA viruses, alphavirus RNA synthesis takes place in membranous organelles that are thought to rescue the virus from cellular defence mechanisms induced by foreign RNA (Salonen et al., 2005). Early in infection, replication complexes are found in plasma membrane invaginations that are connected to the cytoplasm by only narrow neck-like structures (Spuul et al., 2010). In the later phase of infection, these structures are internalised asnd fused with acidic vesicles of the endolysosomal membrane compartment to undergo microtubule-mediated trafficking into perinuclear regions of the cell. Further fusion of the vesicles with one another leads to the formation of cytopathic vacuoles (CPVs), a generally known hallmark of alphavirus infection (Grimley et al., 1968).

CPVs are modified organelles derived from the endolysosomal compartment that are 200 to 1000 nm in diameter and serve as sites for plus strand RNA synthesis. The cytoplasmic side of CPVs carries the microstructures known as spherules. These structures are invaginations of 50 nm in

diameter that are identified in electron microscopy as electron-dense areas and are recognised by antibodies against double-stranded RNA (Frohauer et al., 1988). All four nonstructural proteins are present in CPVs; however, the spherules are not the sole cellular location for any of the four proteins (Kujala et al., 2000). Additionally, early minus strand RNA synthesis is thought to occur in membranous organelles, but its exact intracellular localisation has been difficult to determine due to the short half-life of the minus strand RNA polymerase complex.

Once the capsid proteins have been translated from 26S subgenomic RNA the proteins recognise plus-stranded 42S RNA based on a packaging signal in nsP2 encoding region, which leads to the rapid assembly of 240 copies of the protein to form the icosahedral nucleocapsid enclosing a single copy of genomic RNA (Weigler et al., 2009). The nucleocapsid is transported to the plasma membrane where the envelope glycoproteins are also independently transported after proteolytic processing and glycosylation in the trans-Golgi network to allow for the final steps in virus particle maturation. A total of 240 copies of both E1 and E2 membrane glycoproteins first form heterodimers, which further trimerise to generate the 80 protein spike complexes integrated into the envelope of each virion (Mukhopahyay et al., 2006). Finally, the conformational changes involved in assembly trigger the exocytic events resulting in budding of the virions into the extracellular space.

2.3. Antiviral and cell viability screening

2.3.1. Assays for cell viability

In biomolecular screening, the term cytotoxicity and its *in vivo* counterpart acute toxicity are used to indicate the harmful effects on basic cellular functions caused by the presence or nonspecific binding of a reactive chemical structure to cellular macromolecules. The phenotypic nature of cell viability and cytotoxicity assays refers to this range of underlying possibilities that lead to the decrease in cell viability. However, the predictive value of such phenotypic assays was demonstrated in the 1990s by studies in the MEIC (multicentre evaluation of *in vitro* cytotoxicity) framework. Despite the heterogenic nature of the experimental data used in the analyses, a linear correlation between *in vitro* cell viability IC_{50} values and rodent LD_{50} values was reported and the

Table 4. Assay endpoints in cell viability evaluation.

Cytotoxicity and cell viability assay endpoints	Example
Cell number/amount of biomass	
quantification of total protein amount	sulphorhodamin staining
quantification of total DNA amount	DAP staining
Morphological integrity	
enzyme leakage assays	LDH leakage assay
dye exclusion methods	propidium iodide
Metabolic activity	
incorporation of labelled biomolecules	[3H]thymidine incorporation
reduction of fluorescent/colorimetric dyes	resazurin, MTT reduction
detection of oxygen consumption	
determination of intracellular ATP level	

predictive capacity was even better when comparing the *in vitro* IC_{50} values with human plasma LD_{50} concentrations (Clemedson et al., 1991).

Several cell viability assay endpoints are in general use and can be categorised into three main classes: total cell number, morphological integrity and metabolic activity (Table 4). As such, the classification is only indicative and overlap does exist depending on the experimental parameters used. For instance, with short exposure times assay endpoints based on metabolic activity are good sensors of cellular stress (e.g. intracellular ATP levels rapidly decrease in response to chemical stress (Mueller et al., 2004)) whereas with longer exposure times observed changes in the metabolic state of the culture (e.g. total ATP content) rather reflect the total amount of biomass (Crouch et al., 1993).

Experimental artefacts have also been reported in some cell viability assays. The gold standard assay based on MTT tetrazolium dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction has been shown to underestimate the toxicity of chemicals that are substrates and inhibitors of P-glycoprotein, the main efflux pump responsible for xenobiotic exclusion within cells (Vellonen et al., 2004). As MTT itself is a P-glycoprotein substrate, disturbance of the transport function leads to its intracellular accumulation and enhanced reduction of the coloured end product. Given this published example, it is likely that chemical agents affecting membranetransporter proteins may lead to similar problems in other assays dependent on dye localisation such as in dye exclusion methods used to assess plasma membrane integrity.

Another example of a compound-borne artefact in cell viability assays is the interference of antioxidants with the MTT assay. Assays using MTT and other tetrazolium salts are based on spectrophotometric quantification of the reduced dye produced by cellular reductases. However, the same reactions can occur without cells in the presence of redox cycling chemicals. Plant-derived antioxidants and silicon nanoparticles have been shown to lead to enhanced MTT reduction and thus underestimation of the harmful potential of the compounds (Bruggisser et al., 2002; Laaksonen et al., 2007; Peng et al., 2004). As in the case of transporter proteins, similar problems may be present in other dye reduction assay formats such as the widely used resazurin (AlamarBlue) reduction test.

2.3.2. Phenotypic assays for antiviral screening

The most widely used assay for phenotypic antiviral screening is the measurement of virus-induced cytopathic effect (CPE) (as a reference, see the list of assay methods used in reports on alphavirus inhibitors in Table 5). The replicative machinery of alphaviruses as well as several other viruses causes a severe decrease in host cell viability that leads to apoptotic cell death within 24 h of *in vitro* alphavirus infection (Frolova and Schlesinger, 1994; Levine et al., 1993). The cytopathic effect is visually detectable as abnormal cell morphology and detachment of cells from the culture plates. Early reports on the use of CPE as the antiviral endpoint involved reading of the results simply by visual inspection of cultures under a light microscope. Later on more quantitative forms of the assay have been used to increase throughput and diminish user-to-user variation. In this respect, most assay endpoints for cell viability described in the previous section are applicable; however, the same test sample-derived artefacts (effects on plasma membrane transporters and cellular redox state) should be considered. The most popular assay format in this respect is probably the use of tetrazolium salts to monitor host cell metabolic activity. The determination of ATP levels and dye exclusion techniques have also been used to develop HTS assays of CPE reduction (Li et al., 2009; Mo et al., 2008; Schmidtke et al., 2001). The emergence of label-free formats in cell viability assays can also provide new approaches for antiviral assays based on CPE as real-time monitoring of impedance in cell monolayers gives an opportunity for kinetic measurements of cell viability during the course of viral infection (Owens et al., 2009).

Other conventional assay types for virus replication can be used as secondary assays but they may not provide alternatives for HTS environment due to relatively low throughput capacities. A direct measure for viral replication is the quantification of the incorporation of labelled biomolecules, usually [^{3}H]uridine, into newly produced viral RNA (Miiller et al., 1980; Smee et al., 1991). The replicative machinery of alphaviruses efficiently shuts down host cell transcription via the interaction of nsP2 with unidentified cellular factors (Frolova et al., 1999; Gorchakov et al., 2005; Sawicki et al., 2006), and thus the vast majority of RNA produced in infected cells is of viral origin. However, when nsP2 production is turned down by suppression of the infection with an antiviral agent, this transcriptional silencing is less intense and the balance between cellular and viral RNA synthesis is most likely shifted in favour of the host cell. To control this phenomenon, viral RNA labelling experiments require the use of actinomycin D to inhibit host cell RNA synthesis. However, this approach is problematic in some cases as the presence of actinomycin D is known to reverse the antiviral activity of ribavirin and may also give rise to similar problems with other compounds (Liao and Stollar, 1993; Malinoski and Stollae, 1980).

Quantitative measurement of the production of new virus particles in the presence of test compounds can be achieved by the use of plaque titration (Dulbecco and Vogt, 1953 Sabara et al., 2003). The method can be used in antiviral studies in a direct plaque reduction assay format or indirectly to quantify virus yields. In the former case, the test compounds are present in serial virus dilutions used to raise plaques. In the latter case, the virus is exposed to test compounds in a low-multiplicity infection, and the collected viral yield of the infection is subjected to plaque titration.

Alternatively, virus quantification can be achieved by the determination of $CCID_{50}$ (cell culture 50% infective dose) values or the haemagglutination assay. The $CCID_{50}$ (alternatively called the tissue culture 50% infectious dose ($TCID_{50}$)) has classically been used to quantify viruses for which plaque titration is not appropriate (LaBarre and Lowy, 2001; Reed and Muench, 1938). In this case, the virus is quantified based on the cytopathic effect caused by viral dilutions, and the $CCID_{50}$ value is defined as the dilution leading to a 50% decrease in cell viability.

The haemagglutination reaction, on the other hand, was originally described for the influenza virus and based on the ability of viral envelope glycoproteins to react with the acetylneuraminic acid on the surface of erythrocytes, which leads to agglutination or the visually detectable aggregation of cells (Donald and Isaacs, 1954). Suitability of haemagglutination assay for large sample numbers is

seriously limited by the need for primary erythrocytes, but the assay may still be useful in subtracting the properties of antiviral agents. The haemagglutination assay solely measures the number of viral particles in a given sample, whereas in both the plaque assay and $CCID_{50}$ determination, the infectivity of the virions also plays a central role in the results. The distinction between the two aspects may be of particular use when the antiviral effects relate to the maturation step; some maturation inhibitors cause the formation of aberrant virions with decreased infectivity, yet the viral titer in the first infection cycle may not be dramatically decreased.

2.3.3. Use of surrogate viruses

When screening for inhibitors of human pathogens, attenuated virus strains or surrogate virus species are typically used to reconcile safety issues and screening facility requirements (Buckwold et al., 2002; Julander et al., 2008; Neyts et al., 1996). Molecular details of the replication cycle of surrogate virus species are often known in much greater detail than the actual pathogen and suitable research tools such as high-quality monoclonal antibodies are more frequently available. Therefore, the follow-up studies of identified screening hits can be readily conducted for tracking of the mechanisms that underlie the antiviral activity. In this respect, Semliki Forest virus (SFV) is the obvious surrogate virus within the Alphavirus genus. SFV is the most widely studied virus in the genus with regard to the molecular details of replication, is easy to grow in cell culture systems and is considered safe to use. Some strains of SFV are lethal to neonatal mice yet the suitability of the age-dependent encephalitis model for studying the human alphavirus-borne arthritogenic disease has been critically discussed. Instead, the mouse models for RRV- or CHIKV-induced arthritis may be more relevant in this respect (Rulli et al., 2005; Zeiger et al., 2008). A single human death has been reportedly caused by SFV infection; in which the victim was a research scientist suffering from an immunodeficiency syndrome (Willems et al., 1979).

In general, the amino acid sequence identity within the alphavirus genus is least as 60% for the nonstructural proteins and 40% for the structural proteins (Gould et al., 2009). SFV, CHIKV and RRV are members of the same alphavirus antigenic serocomplex indicating that they are closely related in terms of evolution. Viruses of the same serocomplex exhibit antibody cross-reactivity with each other, and the relationships are also consistent with phylogenetic analyses of E1 coding region sequences that show approximately 60% identity of this diverse genomic region within

viruses of the SFV serogroup (Powers et al., 2001). In comparison, sequence identity between the hepatitis C virus and its commonly used surrogate virus bovine viral diarrhoea virus (BVDV) is approximately 20% (Buckwold et al., 2002).

The interest in alphaviruses as expression vectors for gene therapy and other applications has yielded some experience with the insertion of marker genes and other foreign material into alphavirus genomes. Constructs with duplicate subgenomic promoters have been used to deliver the foreign material into cells but material placed as its own transcriptional unit is easily lost during viral life cycles when there is a lack of selective pressure (Hahn et al., 1992; Vähä-Koskela et al., 2003). Virus strains that are genetically more stable and less attenuated have been achieved by placing the markers as part of existing transcriptional units. Both structural protein coding regions (between the capsid and E3 sequences) and a nonstructural protein-coding region (between nsP3 and nsP4) have been used. Additional protease cleavage sites can be utilised to allow for correct processing of the polyprotein precursors (Tamberg et al., 2008; Tgomas et al., 2003). Furthermore, constructs that are more suitable for localisation studies, and other microscopic techniques have been made in which the marker gene is produced as a fusion protein with nsP3 (Frolova et al., 2006).

2.3.4. Replicon cell lines

The use of replicon systems as antiviral screening tools was introduced for common consideration by the description of cell lines carrying subgenomic RNA replicons of the hepatitis C virus (HCV) (Lohmann, et al., 1999; Pietschmann, et al. 2001). The replicon systems revolutionised HCV antiviral screening and other aspects of *in vitro* HCV research, which had previously relied solely on surrogate viruses because HCV is difficult to propagate in any available model system.

In general, RNA replicons are constructs with the viral components required for amplification of the RNA when transfected into suitable host cells but lacking structural protein genes needed for the production of virus particles. The system is thus noninfectious and allows for screening against viruses that would otherwise require BSL-qualified facilities. The antiviral agents identified in replicon assays are naturally limited to target only the virus replication phase, but replicon assays

have been successfully used in antiviral HTS campaigns including that against the West Nile virus (Noueiry et al., 2007; Puig-Basagouti et al., 2006).

To establish stable cell lines that persistently express alphavirus replicons, modifications in the virus replicative machinery are required to diminish the virus-induced cytopathic effect. Noncytotoxic replicons of SFV and SIN have RNA synthesis levels of 5% to 10% of the wild-type virus or wild-type replicons. However, the low level of RNA synthesis is not thought to be the sole reason for the noncytotoxic phenotype as SIN mutants with similar low levels of RNA synthesis are known but still have the cytotoxic phenotype (Fata et al., 2002; Frolova et al., 2002). Instead, the noncytotoxic phenotype of Old World alphavirus replicons relies on adaptive mutations in nsP2, which is the protein responsible for inhibition of host cell transcription that leads to the apoptotic cell death seen in alphavirus infections (Frolova et al., 1999; Frolova et al., 2002). Several mutations in different sites of nsP2 have been shown to result in the noncytotoxic phenotype and despite the distribution of these point mutations throughout distinct sites of this multidomain protein all the studied examples share the same features. In contrast to the wild-type counterparts, the mutant replicons exhibit unstable replication complexes (RC+), and as a result of constant recycling of replication complexes, they demonstrate the continuous synthesis of minus strand RNA (Sawicki et al., 2006).

Differentual replication strategies between in the Old World and New World alphavirus are illustrated, among other things, by the replicon systems studied on these viral species. New World alphaviruses usually do not require mutations in the replicase proteins to gain persistent and well-tolerated phenotypes as they recruit the capsid protein instead of nsp2 for transcriptional silencing of the host cell (Garmashova et al., 2007).

2.4. Known alphavirus inhibitors

The published reports on alphavirus inhibitors are listed in Table 5 in chronological order. The sections below discuss the selected examples of these compounds with the emphasis on their target sites and described properties.

Table 5. Known inhibitors of alphavirus replication.

Compound	Target virus	IC_{50} (µM)	Assay method	Reference
ribavirin	SFV	280*	CPE reduction	Huffman et al., 1973
arildone[1]	SFV	8*	Plaque reduction	Kim et al., 1980
chloroquine	SFV	50	[^{3}H]uridine incorporation	Helenius et al., 1980
(S)-DHPA[2]	SFV	700*	CPE reduction	De Clercq et al., 1985
neplanocin A	SFV	4*	CPE reduction	De Clercq et al., 1985
ribavirin-5'-sulphamate	SFV	10	CPE reduction	Smee et al., 1988
3'-fluoro-3'deoxyadenosine	SFV	14*	CPE reduction	van Aaerschen et al., 1989
carbodine	SFV	13*	CPE reduction	De Clercq et al., 1990
cyclopentyl cytosine	SFV	0.8*	CPE reduction	De Clercq et al., 1991
EICAR[3]	SFV	7*	CPE reduction	De Clercq et al., 1991
VX-497[4]	VEEV	19	CPE reduction	Markland et al., 2000
6-azauridine	SFV CHIKV	2*	CPE reduction	Briolant et al., 2004
(R)-hydroxymethyldioxane	SINV	1.0	virus yield	Kim et al., 2005
glaucogenin C	SINV	0.0015	CPE reduction	Li et al., 2007
(-)-carbodine	VEEV	0.8*	virus yield	Julander et al., 2008
CGC32091[5]	WEEV	9.3	Replicon suppression	Peng et al., 2009

*Recalculated as molar concentration based on molecular weight and the mass per volume concentration given in the original publication.

[1] 4-[6-(2-chloro-4-methoxyphenoxy)hexyl]-3,5-heptane-dione.

[2] (S)-9-(2,3-dihydroxypropyl)adenine.

[3] 5-Etinyyli-1-β-D-ribofuranosyyli-imidatsoli-4-karboksiamidi.

[4] N-[3-[3-[3-Methoxy-4-(5-oxazolyl)phenyl]ureido]benzyl]carbamic acid tetrahydrofuran-3(S)-yl ester, merimepodib.

[5] {[4-(4-fluorobenzyl)-4H-thieno[3,2-b]pyrrol-5-yl]carbonyl}-N-(2-furylmethyl)piperidine-4-carboxamide.

2.4.1. *In vitro* studies on alphavirus inhibitors

Among the earliest reports on alphavirus inhibition by small organic molecules were studies on ribavirin, a guanosine analogue with a broad antiviral spectrum and an IC_{50} value of 70 µg/ml (approximately 280 µM) against SFV (De Clercq et al., 1985; Huffman et al., 1973; Sarver and

Srollar, 1978). Ribavirin has also been reported to act synergistically with the C-nucleoside analogues tiazofurin and selenazofurin against Venezuelan equine encephalitis virus (VEEV) (Huggins et al., 1984) and with interferon α against CHIKV and SFV (Briolant et al., 2004). The latter has been considered particularly useful due to the sensitivity of alphaviruses to interferon, but no *in vivo* studies have been published on this combination thus far.

Since the early reports the mechanistic studies on ribavirin have covered at least four alternatives: inhibition of a cellular inositol monophosphate dehydrogenase (IMPDH) enzyme; inhibition of viral polymerases by ribavirin triphosphate; inhibition of the guanylyltransferase activity required for virus RNA capping and induction of error catastrophe by incorporation of ribavirin triphosphate into newly synthesised RNAs (Cassidy et al., 1989; Craci et al., 2002; Goswami et al., 1979; Toltzis et al., 1988). The relative importance of the alternatives in alphavirus inhibition has not been examined, but studies on flaviviruses indicate that the antiviral IC_{50} values of ribavirin in different cell culture systems are well correlated with the GTP depletion IC_{50} values . (Leyssen et al., 2005). Thus, a generally accepted view regards IMPDH inhibition is the major target that yields the antiviral activity of ribavirin at least *in vitro*. IMPDH is a cellular enzyme catalysing a rate-limiting step in *de novo* guanosine biosynthesis. Inhibition of enzymatic activity leads to a decrease in the cellular GTP pools in situations where GTP is consumed in large amounts, such as during the extensive synthesis of viral RNA (Colby et al., 1999).

While IMPDH inhibition seems to be the main mechanism of action against flaviviruses, overlap between the mechanisms has also been suggested. Decreased GTP levels may promote the incorporation of ribavirin triphosphate into viral RNA and thus expose the virus to the error catastrophe. (Day et al., 2005) In the case of alphaviruses, the mechanistic data on ribavirin are limited to the characterisation of SINV mutants that carry point mutations in the nsP1 coding region and are resistant to ribavirin (Scheidel et al., 1987; Scheidel and Stoilar, 1991). Such mutations have been postulated to lead to an enhanced affinity of the nsP1 protein for guanosine, which would rescue the RNA capping activity of the protein at low GTP concentrations.

In contrast to ribavirin, mycophenolic acid is nonnucleoside structured, irreversible inhibitor of IMPDH clinically approved as an immunosuppressing agent to prevent host rejection complications

in organ transplants including liver transfers in patients with chronic hepatitis C virus infection (Kitchin et al., 1997). Mycophenolic acid is a potent inhibitor of various viruses *in vitro*, and IMPDH is generally considered the underlying factor for the antiviral activity (Diamond et al., 2002). However, the activity of mycophenolic acid in the HCV replicon has been suggested to be guanosine-independent (Henry et al., 2006). As mycophenolic acid does not have nucleoside structure and is thus not likely to incorporate into viral RNA, induction of the error catastrophe does not seem plausible in this case. With regard to alphaviruses, the antiviral efficacy of mycophenolic acid has been shown against SFV and SIN; mutant SINV strains resistant to ribavirin also show cross-resistance to mycophenolic acid (Scheidel et al., 1987).

Also a structurally unrelated IMPDH inhibitor VX-497 has been demonstrated to inhibit VEEV with an IC_{50} value of 19 µM (Markland et al., 2000). This broad-spectrum antiviral agent is under clinical evaluation for the treatment of chronic HCV infections under the generic name merimepodib; it resembles ribavirin in the sense that it does not bind covalently to IMPDH. However, VX-497's high affinity for the enzyme makes it practically noncompetent in nature (Marcelin et al., 2007; Rustgi et al., 2009). Chemical structures of the three IMPDH inhibitors ribavirin, mycophenolic acid and VX-497 are presented in Figure 3.

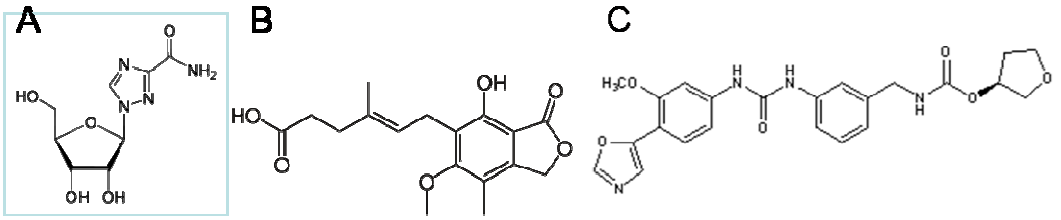

Figure 3. Chemical structures of the IMPDG inhibitors ribavirin (A), mycophenolic acid (B) and VX-497 (C).

Another cellular enzyme described as a target for alphavirus inhibitors is S-adenosylhomocysteine (SAH) hydrolase. SAH hydrolase is responsible for the breakdown of S-adenosylhomocysteine, which is formed as the end product in methyltransferase enzyme reactions using S-adenosylmethionine as a methyl donor (Kloor and Osswald, 2004). Inhibition of SAH hydrolase leads to accumulation of SAH in the cytoplasm and the subsequent suppression of methyltransferase activity. In the context of alphaviruses, the accumulation of SAH suppresses the

methyltransferase activity of nsP1 involved in the capping of viral RNA. The usability of this approach has been demonstrated by the potent inhibition of SFV by the SAH hydrolase inhibitors neplanocin A and, with weaker potency for (S)-DHPA (Table 5) (De Clercq et al., 1985). However, since the publication of these two antiviral agents, no other published reports have emerged on this aspect of alphavirus inhibition.

Cellular enzymes also suggested as targets for alphavirus maturation inhibitors include membranous furin and other related preprotein convertases that were studied for their ability to cleave the E3-E2 intermediate polypeptide into mature structural proteins in the African CHIKV strain (Ozden et al., 2008). Decanoyl-RVKR-chloromethyl ketone (dec-RVKR-cmk), an inhibitor of furin, was found to abolish cleavage of the E3-E2 intermediate polypeptide into the corresponding structural proteins and thus leads to the production of aberrant virions with significantly decreased infectivity. Treatment of myoblast cells with 100 µM dec-RVKR-cmk resulted in a decrease in CHIKV titer by two orders of magnitude. However, the main drawback of this strategy is that the E3E2 cleavage site is not conserved in different CHIKV strains. The authors also observed that a different spectrum of preprotein convertases prefer the cleavage site in Asian CHIKV strains; therefore, the development of a specific and well-tolerated furin inhibitor to be used as an anti-CHIKV agent might be a difficult task.

In 2005, synthetic dioxine derivatives were reported to inhibit SIN by the proposed mechanism of targeting the hydrophobic pocket in the SINV capsid protein and thus preventing virus nucleocapsid assembly and blocking the interaction site of the E2 glycoprotein with the capsid protein (Kim et al., 2005). The active structure was originally found by accident due to cocrystallisation of solvent dioxine molecules during attempts to determine the X-ray structure of SIN capsid protein. However, the study represents the first and thus far the only published work using structure-based drug design for the identification of alphavirus inhibitors as the authors used molecular docking to design and optimise the linker moiety connecting the two dioxine rings and allow for similar positioning as seen with the cocrystallised solvent molecules. The derivatives were assayed for the reduction of SINV yield; in which (R)-hydroxymethyldioxane showed 50% inhibition at a concentration of 1 µM.

Miller and coworkers reported an HTS campaign on New World alphaviruses using a Western equine encephalitis virus (WEEV) replicon cell line in the primary assay (Peng et al., 2009).

CGC32091, a thieno[3,2,b]bipyrrole derivative, was discovered as the lead compound in the screen and the results from structure-activity relationship studies with compounds sharing the same backbone revealed submicromolar IC_{50} values for six of the twenty derivatives studied. The target site of action of CGC32091 and its analogues was not determined against WEEV, but the authors pointed out that the same compound has been recently shown to inhibit the hepatitis C virus RNA-dependent RNA polymerase (RdRp) (Ontoria et al., 2006). In the case of WEEV, the compound was originally found as a hit in a replicon assay indicating that its target site against WEEV lies in the replication phase rather than in virus entry or maturation, which makes RdRp a plausible target candidate for also the WEEV inhibition.

2.4.2. Alphavirus inhibitors with described *in vivo* properties

The carbocyclic cytosine analogue carbodine (cyclopentenyl cytosine) was described in 1990 as a broad-spectrum antiviral agent that inhibits several unrelated virus species including DNA viruses and positive and negative stranded RNA viruses (De Clercq et al., 1990). The molecular target of carbodine was identified as the cellular enzyme cytidine triphosphate (CTP) synthetase, which is responsible for the maintenance of cellular CTP pools by the conversion of uracil triphosphate (UTP) to cytidine triphosphate (CTP). It was later found to be the D-enantiomer of carbodine, (-)-carbodine that exerts the antiviral activity and suppresses CTP production (Julander et al., 2008). (-)-Carbodine exhibited an IC_{50} value of 0.2 µg/ml against VEEV in a virus yield assay. It was demonstrated to reduce VEEV titers in infected mice brain tissue as well as relieve the symptoms and prolong the mean survival days of infected mice. Notably, *in vivo* antiviral activity was observed when (-)-carbodine was administered as late as 4 days postinfection when the VEEV vaccine strain TC-83 was used. However, the dosage required to achieve the antiviral effect was high (200 mg/kg) and achieving similar levels in humans may be difficult due to severe adverse effects.

A recent report on alphavirus inhibitors introduced a novel mechanism of action by *seco*-pregnane steroids isolated from two plants used in traditional Chinese medicine (*Strobilanthes cusia* and *Cynanchum paniculatum*; Li et al., 2007). Glaucogenin C and its glycosides cynatratoside A and paniculatumosides C, D and E were shown to specifically inhibit the production of subgenomic RNA of viruses from the alphavirus-like super family. The authors also reported the protective

effect of one of the compounds in a mouse model of SINV infection. However, the protective effect required the administration of the compound prior to or at the same time as viral inoculation; administration of the compound 4 h postinfection failed to rescue the mice.

Chloroquine is thus far the only chemical agent used in a placebo-controlled clinical trial for treating alphavirus viremias. The antiviral effect of chloroquine against alphaviruses was discovered in the early 1980s as the accumulation of chloroquine and other lipophilic amines into lysosomes was demonstrated to inhibit the SFV uncoating process (Helenius et al., 1982). As a consequence of base accumulation the increased lysosomal pH disturbs the membrane fusion of the viral and lysosomal membranes required for successful release of the viral genome into the cytoplasm. The same phenomenon is seen also with other weak lysosomotropic bases including the well-studied anti-influenza agent amantadine, which inhibits SFV although it is significantly weaker (IC_{50} value of 500 µM). Chloroquine has been introduced as an antiviral agent in clinical trials against HIV because a similar entry route is exploited by most enveloped viruses, and its antiviral activities are currently known to also involve interference with the pH-dependent steps in virus maturation (Savarino et al., 2003).

However, the double blind clinical trial in La Réunion with 54 patients suffering from acute CHIKV viremia during the recent outbreak failed to demonstrate any significant difference between chloroquine and placebo-treated groups in the duration of the febrile illness or decrease the viral titers measured from blood samples (De Lamballerie et al., 2008). Chloroquine has previously shown indications of worsening the condition of SFV-infected mice in an encephalitis-based animal model; and despite its potent *in vitro* alphavirus inhibition, the use of chloroquine in antialphaviral therapy is discouraged (Maheshwari et al., 1991; Savarino et al., 2007).

The raised profile of alphaviruses in recent years has also led to the development of biotechnological products against alphaviruses. Phosphorodiamidate morpholino oligomers (PMO) are antisense oligomers with modified backbones, which have been designed against the 5' noncoding region of VEEV and exhibit potent inhibition of the virus production of multiple VEEV strains in cell culture systems (Paessler et al., 2009). PMO oligomers can be readily administered intranasally to animals, and the antisense agent was shown to yield 100% survival of mice infected with a pathogenic VEEV strain when treated with PMOs before and after infection. When only

postinfection treatment was used, the agent yielded 64% protection. The treatment was well tolerated and resulted in reduced viral titers in the CNS and peripheral tissues of all animals.

To summarise, studies on alphavirus inhibitors have been scarce in the past decades and involved only broad-spectrum antiviral agents that have been reported as inhibitors of alphaviruses as a part of a panel of unrelated viral species. However, the first specific agents have more recently appeared. Additionally, advances have been seen in the field of biotechnological products. Unfortunately, these products are beyond the reach of public health services in most alphavirus epidemic areas and are not likely to change the management of alphaviral diseases in near future.

3. AIMS OF THE STUDY

Antiviral screening relies on robust and reproducible assay methods that allow for rapid evaluation of a large number of samples in a biologically relevant setup. To meet these needs in alphavirus antiviral lead discovery, the aims of the current study were to develop and validate antiviral assays using relevant alphaviral species and to establish reliable counter-screening methods for cell viability evaluation.

To achieve the overall aims, the following approaches were used:

- to validate and compare three cell viability assay methods based on LDH leakage, WST-1 reduction and ATP level determination (**I**);
- to use a marker gene containing a strain of the Semliki Forest virus to develop an antiviral assay covering the complete virus replication cycle as a target for antiviral activity (**II**);
- to develop a screening system for Chikungunya virus using a cell line stably expressing the CHIKV replicon with marker genes inserted (IV);
- to validate and compare the antiviral assays using reference assay methods and standard compounds (**II** and **IV**);
- to demonstrate the performance of the assays in the identification of antiviral agents among small organic molecule collections with diverse chemical structures (**II, III** and **IV**).

4. MATERIALS AND METHODS

The following section provides an overview of the methods used in the study. Detailed experimental protocols are presented in the Materials and Methods sections of each original publication **I-IV**.

4.1. Compound libraries

Four small organic molecule libraries were included in the study and contained natural products, semisynthetic derivatives of natural compounds and synthetic drug molecules. Twenty-nine biocatalytically produced nucleoside analogues were provided by Professor Seppo Lapinjoki (Department of Pharmaceutical Chemistry, University of Kuopio, Finland), and a set of 51 synthetic triterpenoids derived from betulin and betulinic acid were synthesised at the Division of Pharmaceutical Chemistry, University of Helsinki by the research group of Professor Jari Yli-Kauhaluoma. The in-house natural compound library (n = 124) consisted of commercially available pure plant secondary metabolites, which were mainly flavonoids, coumarins and other polyphenols. The collection of pharmaceutical compounds (n = 234) contains clinically approved drugs in different therapeutic areas as well as some metabolites of the drugs. All compounds in the natural and pharmaceutical compound collections were purchased from various commercial sources (see supplementary table in **IV** for complete listing). All libraries were maintained as DMSO stock solutions at 20 mM concentrations except for the betulin-derived compounds for which 10 mM stock solutions were prepared. The chemical libraries were stored at – 20 °C, and each aliquot was subjected to a maximum of 10 freeze-thaw cycles.

4.2. Standard compounds

In I, known cytotoxic agents were used to validate the assays. Gramicidin S, polymyxin B, 5-fluorouracil, camptothecin and Triton X-100 were all purchased from Sigma-Aldrich. Polymyxin B (10 000 IU/ml) was also used as a positive control in all further cell viability studies. Antiviral

standard compounds of ribavirin (II, III and IV), mycophenolic acid, 6-azauridine and chloroquine (IV) were purchased from Sigma-Aldrich.

4.3. Cells and viruses

Baby hamster kidney BHK21 cells were purchased from the American Type Culture Collection (ATCC) and used for all antiviral assays. In the cell viability assays, three human cell lines were used together with BHK21 cells. Human colon carcinoma Caco-2 cells were a kind gift from Professor Arto Urtti (Centre for Drug Research, University of Helsinki), and the Calu-3 cell line was purchased from ATCC. Hepatocellular Huh-7 cells were a gift of Dr. Ralf Bartenschlager (University of Heidelberg, Germany) and used in **I**, **II** and **III**. The BHK-CHIKV-NCT cell line persistently transfected with the CHIKV RNA replicon was selected in the laboratory of Dr. Andres Merits, University of Tarto, Estonia as described in **IV**. The fluorescence excitation and emission maximum of the cell line were defined as 478 nm and 509 nm, respectively, using the Varioskan Flash multimode platereader (Thermo Fischer Scientific Inc.).

All virus stocks were propagated by the electroporation of BHK21 cells with RNA obtained by *in vitro* transcription of cDNA plasmids containing the full-length infectious clones of each virus. Wild-type Semliki Forest virus was obtained from plasmid SFV4 and Sindbis virus from plasmid TOTO1101 (Liljeström et al., 1991; Rice et al., 1987). The cDNA plasmid for SFV-*Rluc*-H2, a full-length infectious Semliki Forest virus inserted with the *Renilla* luciferase coding sequence, was a kind gift from Dr. Andres Merits (University of Tarto, Estonia). The luciferase marker was placed in the SFV-*Rluc* genome in a similar manner as EGFP in Tamberg et al., 2007.

4.4. Antiviral assays

4.4.1. [^{3}H]uridine incorporation assay (II)

Viral RNA synthesis rates were determined by the quantification of [^{3}H]uridine incorporation in infected BHK21 cell cultures. Briefly, confluent cell cultures in 6-well plates were infected with wild-type virus (SFV or SINV), and a 1-h pulse of 15 µCi of [^{3}H]uridine (Amersham) was given to

the cultures at selected time points. Prior to the uridine pulse, cells were treated for 1 h with 5 µg/ml actinomycin D to inhibit host cell RNA synthesis. After the uridine pulse, cultures were washed three times with PBS and lysed with 1% sodium dodecyl sulphate (SDS). The lysate samples were precipitated with 10% trichloroacetic acid (TCA), and the precipitates were collected on glass fibre filters for scintillation counting (MicroBeta Trilux, PerkinElmer Life and Analytical sciences Inc.).

4.4.2. Virus-based reporter gene assay (II, III and IV)

A luciferase-based screening assay was developed by exploiting the SFV strain containing the *Renilla* luciferase gene inserted in its nonstructural protein-coding region. In this assay, confluent BHK21 cell cultures in 96-well plates were infected with SFV-*Rluc*, and the *Renilla* luciferase activity of the samples was determined at selected time points using the Renilla Luciferase assay system kit (Promega). The infection medium was removed, wells were washed with PBS and cell culture lysis reagent was added to the wells. After a 15-min incubation at RT, luciferase substrate solution was added, and the luminometric readout was taken within 5 min in the Varioskan Flash (Thermo Fischer Scientific Inc.).

4.4.3. Plaque assay (II and IV)

In the plaque assay, confluent BHK21 cell cultures in 6-well plates were inoculated with 10-fold serial dilutions of SFV, and the infected cultures were incubated in carboxymethyl cellulose (CMC) medium for 48 h. The cultures were then washed and stained with crystal violet to visualise the plaques. The plaque titration method was used to quantify viral yields either as in the plaque reduction format (**II**) or the virus yield assay (**IV**). In the plaque reduction assay, test compounds were simultaneously administered to the cultures with the virus inocula and remained there throughout the 48-h incubation. In the virus yield assay, BHK21 cell cultures were infected with SFV (multiplicity of infection (MOI) = 0.01) in MEM + 0.2% BSA and the medium was harvested 16 h postinfection. Virus in the harvested samples was quantified by plaque titration.

4.4.4. CPE reduction assay (IV)

CPE reduction was assayed using confluent BHK21 cell cultures in 96-well plates infected with either wild-type SFV or SINV (MOI = 0.01). After optimised infection times (22 h for SFV and 24 h for SINV), the cultures were washed, and host cell viability was determined using the WST-1 reduction method (see Section 4.5.2.).

4.4.5. Guanosine supplementation studies (III and IV)

Antiviral agents ribavirin (**III** and **IV**) and mycophenolic acid (**IV**) were characterised in the SFV-*Rluc* screening assay and in the BHK-CHIKV-NCT replicon cell line by guanosine supplementation experiments during which 50 µg/ml guanosine (Sigma-Aldrich) was administered simultaneously to the cell cultures with each antiviral compound. The detection in both assays was carried out as described in Section 4.4.2.

4.4.6. Administration time experiments (III)

Three betulin-derived compounds and 3'-amino-3'deoxyadenosine were subjected to administration time experiments using an MOI of 10 for the infection of SFV-*Rluc*. After 1 h of virus adsorption, the virus inoculum was removed, and the cultures were washed twice. Luciferase activity was determined 5 h postinfection. Compounds (200 µM) were present in the infected cultures either throughout the infection (0 – 5 h), during viral adsorption (0 – 1 h) or after delayed administration (1 – 5 h or 2 – 5 h).

4.4.7. Synergism studies (III)

To study the potential synergistic inhibition by betulin-derived compounds with 3'-amino-3'-deoxyadenosine, the IC_{50} values of three selected betulin derivatives were determined alone and in the presence of 0.5 µM, 2 µM, 5 µM, 20 µM and 50 µM nucleoside. The IC_{50} values were used to

generate isobolograms as described in Gerco et al., 1995. Here, the NE-SW diagonal of the graph crossing the axes at (0, 1) and (1, 0) represents the Loewe additivity or expected response for the given combination. Bending of the graph below the additivity diagonal is a hallmark of Loewe synergism. To analyse intensity of the synergism in individual concentration combinations, interaction indices (I) were calculated for each normalised surviving fraction that resulted from a given combination using the equation $I = D_1/D_{X1} + D_2/D_{X2}$, where D_1 and D_2 represent the concentrations of compounds 1 and 2 in the combination and D_{X1} and D_{X2} represent the concentrations of drug 1 and 2 alone that yield the same response.

4.5. Cell viability assays

4.5.1. LDH leakage assay (I)

LDH activity in samples of medium collected from cell cultures exposed to test compounds was determined by the method described by Korzeniewski et al. (1983). Briefly, the medium samples were mixed with an equal volume of substrate solution containing lactic acid, iodonitrotetrazolium chloride (INT), nicotinamide adenine dinucleotide (NAD^+) and phenazine methosulphate. After 30 min of shaking, the enzymatic reaction was stopped by adding 1 M acetic acid, and the absorbance resulting from the reduction of INT by NADH was read at 490 nm (Victor Multimode platereader, PerkinElmer Life and Analytical Sciences Inc.), At 24 h of exposure, the assay was utilised to determine total cell content in cultures exposed to test samples. Here, wells were washed with Hank's balanced salt solution (HBSS) and lysed with 9% Triton X-100. The LDH activity of each lysate was assayed as described above.

4.5.2. WST-1 reduction assay (I, II and IV)

WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) is a tetrazolium salt that produces a water-soluble formazan product as a result of its reduction by cellular enzymes (Ishiyama et al., 1993). After exposure to assay samples, cells were washed and incubated in HBSS containing 10 µl of the WST-1 solution (Roche Diagnostics) for 2 hours.

Intensity of the absorbance by the formazan product was read at 440 nm (Varioskan Flash, Thermo Fischer Scientific Inc.).

4.5.3. ATP level determination (I, II, III and IV)

Intracellular ATP levels were determined using the ATP-dependent firefly luciferase reaction (Crouch et al., 1993). The CellTiter-Glo luminometric cell viability assay kit (Promega) was used for this purpose. The one-step assay procedure involves treatment of cell cultures with the assay reagent, which contains lysis buffer and components for the enzymatic reaction and subsequent luminometric readout (Varioskan Flash, Thermo Fischer Scientific Inc.).

4.6. Data analysis

All cell viability and antiviral screening data were normalised using untreated and uninfected control samples to set minimum and maximum values. The assay quality parameters S/B, S/N and Z' (see section 2.1.3. for formulas) were used for assay optimisation. Inter-plate and inter-day variations for each assay were determined as the deviation of the normalised mid-signal that was calculated from cultures treated with the appropriate concentrations of ribavirin (antiviral assays) or polymyxin B (cell viability assays). Relative IC_{50} values for the antiviral and cytotoxic effects were determined by fitting data from dose-response experiments into sigmoidal dose-response curves using nonlinear regression analysis in GraphPad Prism software versions 3.0 and 5.0. Selectivity indices were calculated as the ratio of cell viability and antiviral IC_{50} values. One-way ANOVA and unpaired t-test were used to evaluate the statistical significance of differences in IC_{50} values. For synergism studies (**III**), the Loewe additivity model was used to evaluate the expected antiviral activities of combinations of betulin-derived compounds and 3'-amino-3'-deoxyadenosine.

5. RESULTS

5.1. Validation and comparison of cell viability assays [I]

As selectivity for the target virus is one of the essential features of any antiviral drug, cell viability assays are required as counter-screens in all antiviral screening efforts. Various endpoints are used for cell viability and cytotoxicity assays, which have led to sometimes confusing interpretations of the results. In the first part of this study, three cell viability assays were automated and validated, then compared using the standard compounds gramicidin S, polymyxin B, 5-fluorouracil, camptothecin and Triton x-100.

The assays were established using the Caco-2 cell line to select suitable cell density and buffer conditions. The protocols were taken to the Biomek FX workstation for automated liquid handling, Where the procedures were further optimised for reproducibility by using the assay quality parameters S/B, S/N and Z'. Parameter values for the assays are presented in Table 6, indicating the optimal assay performance.

Table 6. Assay quality parameters of the three cell viability assays.

Assay	S/B	S/N	Z'	Inter-plate variation	Inter-day variation
LDH leakage	7.9	63.9	0.72	8.9	2.4
WST-1 reduction	36.5	104.8	0.54	11.4	18.4
Determination of ATP level	388.6	18.1	0.83	7.1	2.7

The formulas used to calculate assay quality parameters S/B, S/N and Z' are presented in section 2.1.3. Inter-plate and inter-day variation values were calculated using the normalised mid-signal values (samples treated with 6,000 IU/ml polymyxin B) of three plates and two separate days.

To study standard compounds in the assays, two alternative exposure times of 1.5 h and 24 h were chosen. However, the LDH leakage assay was observed to suffer from a compromised signal window during the 24-h exposure probably due to spontaneous LDH leakage. Therefore, the assay was used to determine the total LDH amount as an indicator of cell number with this longer exposure time. Testing for the dose-dependent increase in LDH leakage after 1.5 h of exposure resulted in only a minor effect of gramicidin S and polymyxin B (30% - 40% of the maximal

activity in the collected assay media at the highest concentration used). No LDH activity detectable over the reagent background was measured from media samples exposed to 5-fluorouracil or camptothecin. Only exposure to Triton X-100 yielded a sigmoidal dose-response relationship that reached the maximal LDH activity determined by lysis control samples giving an IC_{50} value of 0.05% (v/v). The two other assays based on WST-1 tetrazolium salt reduction and determination of the cellular ATP level were able to detect decreases in cell viability after 1.5 h of exposure to all five compounds, and IC_{50} values were determined from the dose-response experiments. A statistically significant difference in the IC_{50} values for camptothecin and Triton X-100 was observed between the two assays with the ATP assay being more sensitive in both cases (camptothecin IC_{50} values 21.0 ± 2.1 µM in the WST-1 assay and 6.8 ± 1.5 µM in the ATP assay and Triton X-100 IC_{50} values of 0.02 ± 0.0008% in the WST-1 assay and 0.005 ± 0.002% in the ATP assay). No statistical difference in IC_{50} values was observed between the WST-1 and ATP assay for gramicidin S, polymyxin B and 5-fluorouracil.

The ATP assay was used to evaluate the effects of standard compounds on cell lines of different origins. In addition to the Caco-2 cells used for assay validation, the human bronchial epithelium Calu-3 and human hepatocellular Huh-7 cell lines were used as well as the baby hamster kidney BHK21 cells that were used for antiviral studies. The IC_{50} values determined after 24 h of exposure are presented in Figure 2 in **I**. A statistically significant difference in IC_{50} values between the cell lines was found for camptothecin and 5-fluorouracil using one-way ANOVA whereas gramicidin S and polymyxin B had similar potencies in all cell lines. Pair-wise comparison of the camptothecin and 5-fluorouracil values identified the Huh-7 line as demonstrating differential values whereas no statistically significant differences were seen between Caco-2, Calu-3 and BHK21.

The 24-h exposure time was chosen for antiviral counter-screens based on the results of this study. In addition to the BHK21 cell line used for antiviral assays, Huh-7 cells were selected for hit compound profiling with either 24-h or 48-h exposure times.

5.2. Antiviral assay development

5.2.1. Infection time scale and RNA labelling assay (II)

The replication rate of wild-type SFV was studied by RNA synthesis rate experiments in which incorporation of [^{3}H]uridine into newly synthesised viral genomes was monitored using sequential 1-h labelling pulses to establish a relevant time scale for antiviral assay endpoints. Graphs of the [^{3}H]uridine incorporation rates from experiments with different multiplicities of infection are shown in Figure 4. As indicated by the graphs, each decrease by one order of magnitude in infection multiplicity shifts the time of maximal RNA synthesis onward by two hours (also see Table 1 in **II**). Independent of the MOI used, the same plateau level is finally reached to indicate the maximal RNA synthesis rate. To allow for a setup in which several infection cycles are included, a MOI of 0.001 was selected for the antiviral assay. A detection time point of 14 h was set for all future experiments based on the RNA labelling data as it represented the time at which the RNA synthesis rate is reaching it maximum.

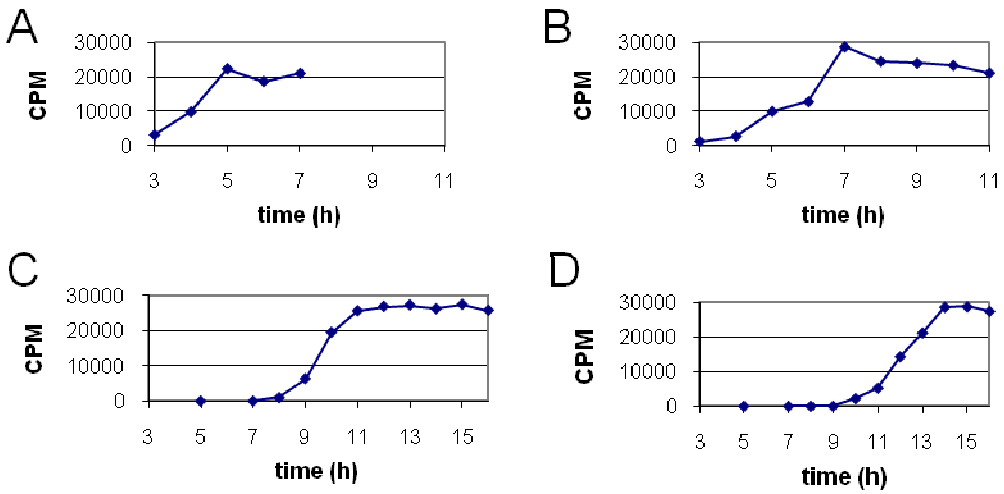

Figure 4. RNA synthesis rate of SFV in BHK21 cell cultures infected with different multiplicities. The time (in hours) given on the X-axis represents the time of culture lysis at the end of each [^{3}H]uridine pulse. A. MOI = 10; B. MOI = 1; C. MOI = 0.01; D. MOI = 0.001.

5.2.2. Luciferase-based screening assay using Semliki Forest virus (II)

A reporter gene-based screening assay was developed to use the surrogate virus approach in the identification of alphavirus inhibitors. To this end, an infectious clone of SFV was used in which the *Rluc* insertion was made into the nonstructural protein-coding region of the SFV genome between the sequences encoding nonstructural proteins 3 and 4. An additional protease cleavage

site was added to allow proteolytic processing by the viral nsP2 protease (Figure 5a). A SFV-*Rluc* virus stock was propagated by *in vitro* transcription and subsequent RNA electroporation into BHK21 cells; and the primary stock collected from electroporated cultures was further used to propagate the P2 working stock. The working stock of SFV-*Rluc* had a titer of 1.5 x 10^9 PFU/ml, which is comparable to the wild-type SFV stocks obtained by similar means (e.g. the wt SFV stock used in these studies has a titer of 4.5 x 10^9 PFU/ml). Proper processing of the viral polyprotein P1234 was confirmed by western blot analysis of lysates from infected cell culture samples using anti-nsP3 antiserum, which resulted in the identification of a protein band of the correct size and increasing intensity during the course of infection.

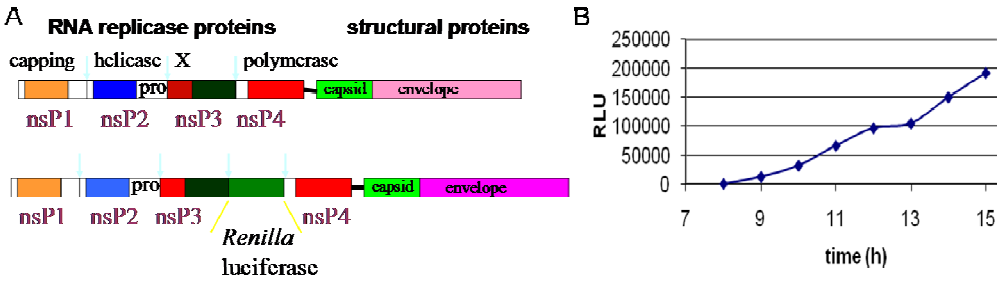

Figure 5. A. Genomic organisation of SFV and SFV-*Rluc*; B. *Rluc* activity of BHK21 culture lysates infected with an MOI of 0.001 SFV-*Rluc*.

Under visual inspection by light microscopy, SFV-*Rluc* had a cytotoxic phenotype at 20 h postinfection (MOI = 0.01), which was indistinguishable from the wt phenotype under these same conditions. [^{3}H]uridine incorporation experiments were also conducted with SFV-*Rluc* in which the virus reached similar maximal uridine incorporation rates as the wt SFV. No delay was observed in reaching the plateau. *Rluc* activity was also analysed in samples collected at sequential time points from cultures infected with SFV-*Rluc* at an MOI of 0.001, and it was found to stay linear over the time scale used (Figure 5b).

5.2.3. Chikungunya virus replicon assay (IV)

To screen for inhibitors of CHIKV, a screening system was built using a BHK21-based replicon cell line that persistently expresses CHIKV replicase proteins. The CHIKV sequence originated from a viral strain isolated in La Réunion during the 2005-2006 outbreak and was modified with adaptive mutations in the nsP2 region. The structural protein-coding region was replaced with the enhanced green fluorescent protein (EGFP) gene fused with the puromycin acetyltransferase sequence and the *Renilla* luciferase gene was fused to the C-terminus of nsP3. The selected cell line was designated BHK-CHIKV-NCT; it was grown under puromycin selection and showed stable marker gene expression at least for 20 passages.

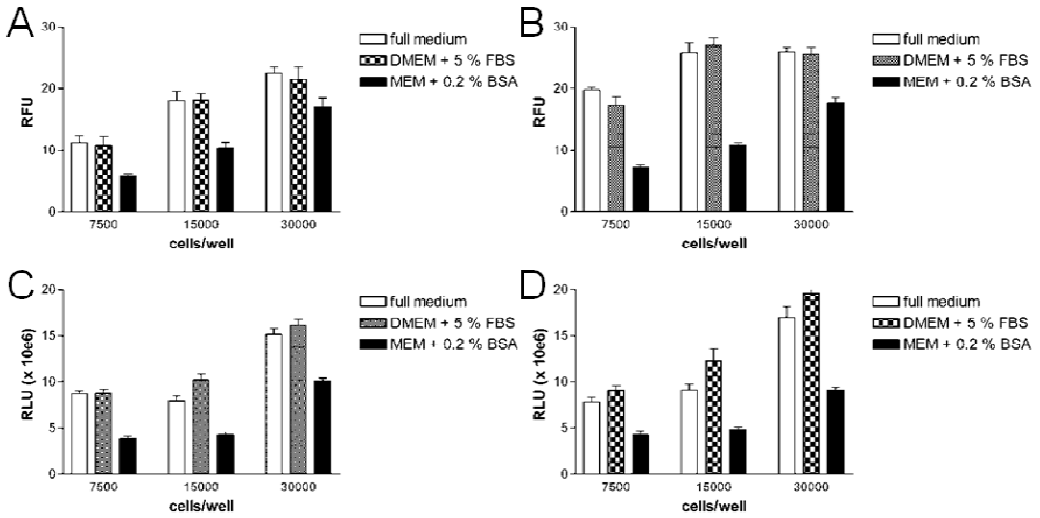

Figure 6. Expression of EGFP and *Rluc* marker genes of the BHK-CHIKV-NCT replicon cell line with different media and cell seeding densities. A. EGFP expression after 24 h of incubation; B. EGFP expression after 48 h of incubation; C. *Rluc* expression after 24 h of incubation; D. *Rluc* expression after 48 h of incubation. All cultures were seeded in complete medium and incubated overnight before the onset of the described experiment.

To establish the antiviral assay, the replicon cells were seeded in 96-well plates and grown in either complete BHK medium, DMEM supplemented with 5% FBS or MEM + 0.2% BSA, and either in the presence or absence of 5 µg/ml puromycin. Different seeding densities of the cells were used to study the expression of replicon marker genes under different assay conditions. As indicated by the graphs presented in Figure 6, the replicon cell line demonstrates high EGFP and *Rluc* signals in both growing (cell seeding densities of 7,500 and 15,000 cells/well) and confluent (seeding density of 30,000 cells/well) cell cultures. Cell cultures grown in MEM + 0.2% BSA show less intense signals due to the slower replication rate of the cells while the difference between the growing

cultures in DMEM supplemented with 5% FBS and those grown in complete medium is small. The presence of puromycin had no effect on EGFP or *Rluc* expression after 24 or 48 h of incubation (data not shown), and therefore, all inhibitor studies were carried out without puromycin.

In preliminary assays with standard compounds, ribavirin and mycophenolic acid reduced ATP levels of the replicon cell line by 40% to 55% when growing cell cultures were exposed to 50 µM concentrations of the compounds. Similar 48-h exposures of the confluent cell cultures did not lead to a decrease in the cellular ATP levels. Because the confluent cultures were shown to maintain high levels of EGFP and *Rluc* expression, they were chosen as the primary alternative for antiviral assays. Studies using the nucleoside analogue 3'-amino-3'-deoxyadenosine (Table 7) indicated that a 48-h exposure is sufficient for the suppression of replicon expression with an antiviral agent. DMEM supplemented with 5% FBS was selected for the assay medium instead of complete BHK medium or MEM + 0.2% BSA to prevent culture overgrowth or the induction of apoptotic pathways during the experiments.

Table 7. Suppression of CHIKV replicon marker genes by 3'-amino-3'deoxyadenosine

Marker	Medium	24 h 7,500 cells/well	24 h 30,000 cells/well	48 h 7,500 cells/well	48 h 30,000 cells/well
EGFP	full medium	64.4	68.0	36.9	33.2
EGFP	DMEM + 5% FBS	63.2	68.7	35.3	38.6
Rluc	full medium	67.1	58.8	23.4	16.7
Rluc	DMEM + 5% FBS	63.9	60.3	21.7	16.4

Values represent the mean of the remaining EGFP and *Rluc* expression as a percentage compared to the untreated controls. All experiments were performed in triplicate.

5.3. Antiviral assay validation

5.3.1. Assay performance (II and IV)

Antiviral assays were optimised to be run in a 96-well plate format and were evaluated for their robustness and reproducibility based on signal windows and signal variation. Assay quality parameters from optimised assay protocols for the SFV-*Rluc* and CHIKV replicon assays are presented in Table 8. The same parameters from the colorimetric CPE reduction assay used in

follow-up studies in **IV** are included for comparison. The threshold value of 0.5 set for optimal robustness was met with the SFV-*Rluc* assay and CHIKV replicon assay as indicated by the Z' values. The CPE assay resulted in a Z' value 0.44, which was lower than that of the three other assays but still close to the 0.5 limit.

Table 8. Assay quality parameters of the miniaturised antiviral assays.

Assay	S/B	S/N	Z'	Inter-plate variation	Inter-day variation
SFV-*Rluc*	152.3	6.4	0.52	8.9	11.6
SFV CPE	4.3	5.8	0.44	12.2	8.5
CHIKV replicon, EGFP	65.3	16.3	0.79	4.7	7.4
CHIKV replicon, *Rluc*	231.4	30.7	0.74	6.2	6.9

Formulas used to calculate the assay quality parameters S/B, S/N and Z' are presented in section 2.1.3. Inter-plate and inter-day variation values were calculated using the mean signal value of the normalised mid-signal (samples treated with 100 µM ribavirin in the SFV-*Rluc* assay and 15 µM ribavirin in the CPE reduction assays and replicon assay) of three plates and two separate days.

5.3.2. Standard compounds (II and IV)

As part of the assay validation process, four chemical agents including ribavirin, mycophenolic acid, 6-azauridine and chloroquine were studied in the developed assays. All four compounds have previously documented activity against alphaviruses (Briolant et al., 2004; Helenius et al., 1982; Huffman et al., 1973). The antiviral IC_{50} values of these compounds in different assays are collected in Table 9. RNA labelling studies were not included in the comparison as this assay requires the use of actinomycin D to block host cell RNA synthesis. Actinomycin D neutralises the antiviral activity of ribavirin via uncharacterised cellular interactions, which makes the use of this combination irrelevant (Malinoski and Stollae, 1980). Chloroquine did not show inhibition of CHIKV replicon marker gene expression, and thus, no IC_{50} value was obtained in this assay. This result is consistent with chloroquine's known mode of action, which is based on the inhibition of virus entry and maturation (Savarino et al., 2003).

Ribavirin and mycophenolic acid exhibited statistically significantly higher IC_{50} values in the SFV-*Rluc* assay as compared to CPE reduction, whereas chloroquine demonstrated similar potency in the two assays. On the other hand, 6-azauridine was found to have only a minor inhibitory potential against SFV in both assays (30% – 40% inhibition at the highest concentration used) but was much

more potent in the CHIKV replicon assay. Ribavirin and mycophenolic acid were also assayed for alphavirus inhibition in the presence of external guanosine supplementation. Both compounds showed no inhibition of SFV-*Rluc* at the highest concentration used (300 µM) when the culture medium was supplemented with 50 µg/ml guanosine. Guanosine supplementation prevented the inhibition by ribavirin treatment in both the EGFP and *Rluc* readings in the CHIKV replicon assay. Inhibition of the replicon marker genes by mycophenolic acid was also reversed by guanosine supplementation, yet the highest mycophenolic acid concentration (200 µM) still resulted in 35% inhibition of the EGFP readout. As discussed in section 2.3.1., no direct evidence on the mechanism of action of alphavirus inhibition is available for ribavirin or mycophenolic acid. However, in light of this evidence, the inhibition of IMPDH is the most prominent player in this respect.

Table 9. The 50% inhibitory concentrations of ribavirin, mycophenolic acid, 6-azauridine and chloroquine in the antiviral assays used.

Compound	**SFV *Rluc* IC_{50} (µM)**	**SFV CPE IC_{50} (µM)**	**SINV CPE IC_{50} (µM)**	**CHIKV EGFP IC_{50} (µM)**	**CHIKV *Rluc* IC_{50} (µM)**
Ribavirin	95.1	28.1	51.8	8.8	25.4
Mycophenolic acid	121.3	39.0	44.4	-1.5	4.1
Chloroquine	13.4	8.2	11.3	-	-
6-azauridine	>200	>200	>200	2.4	3.1

The values represent the means from two individual experiments performed in triplicate.

A recent review introduced the antimalarial drug quinine as a potential candidate for alphavirus antiviral therapy (Gould et al., 2009). Based on unpublished results, quinine was stated to have an IC_{50} value of 0.1 µg/ml (0.3 µM) against CHIKV infection in Vero cells. The authors also indicated that CHIKV strains grown in increasing quinine concentrations accumulate mutations in nsP1 indicating that virus capping is a potential target for antiviral therapy. Despite this observation, quinine showed no effect on reporter gene expression in the CHIKV replicon. Quinine did have an IC_{50} value of 71 µM in the full virus assay using SFV-Rluc (data not included in original publications).

5.4. Screening for alphavirus inhibitors

5.4.1. Nucleoside analogues (II)

Nucleoside analogues have classically been at the centre of antiviral screening as several screening campaigns have been aimed at the identification of structural modifications in the nucleoside skeleton that would lead to selective affinity for viral polymerases or other enzymes responsible for viral DNA or RNA metabolism.

In **II**, a collection of 29 nucleoside analogues was assayed against SFV and SIN in the RNA labelling assay and also studied in the reporter gene-based assay using SFV-*Rluc*. These studies revealed two structural modifications that exert an inhibitory potential against the two viruses: substitutions on C3 of the sugar residue and the introduction of large substituents on N6 of the purine base. The secondary assay of the study (plaque reduction assay) demonstrated that compound 15 (3'-amino-3'deoxyadenosine) was the most efficient among the studied analogues and reduced the SFV yield by 820-fold at a concentration of 100 µM. 3'-amino-3'-deoxyadenosine showed moderate effect on BHK21 cell viability in **II** and was found to have a cell viability IC_{50} value of 187.1 µM in Huh-7 cells as measured by ATP assay after 48 h of exposure (Table 1 in **IV**). A selectivity index in the range of 10 was thus achieved for this nucleoside.

The nucleoside analogues were also assayed against the BHK-CHIKV-NCT cell line for their ability to suppress expression of the CHIKV replicon (data not presented in the original publications). Figure 7 presents the results from these experiments in which the replicon cell line was exposed to 50 µM nucleoside analogues for 48 h. The results obtained in these experiments indicate similar inhibitory activities for each nucleoside as reported against SFV in **II,** derivatives 12, 15 and 16 showing the most intense inhibition.

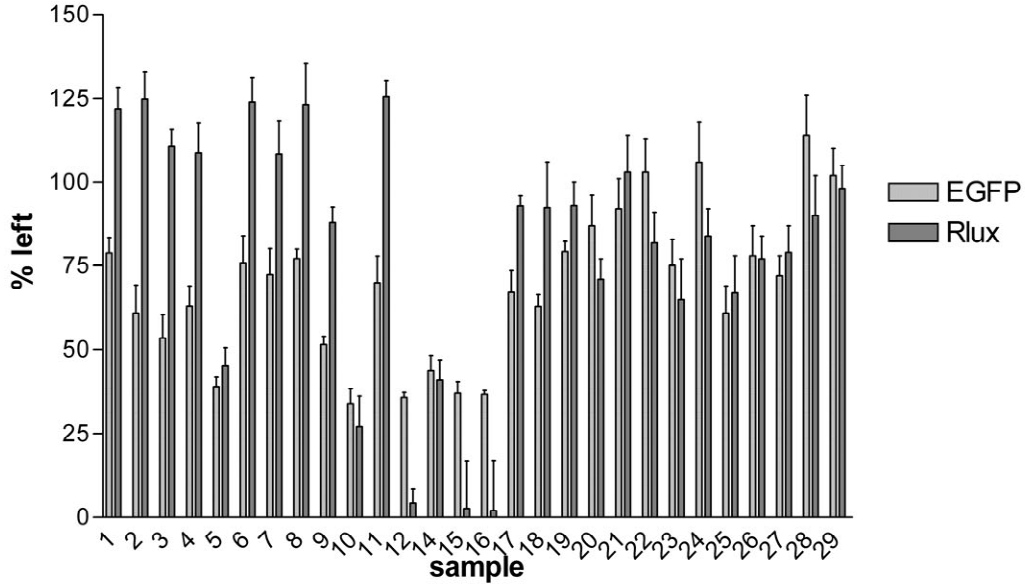

Figure 7. Effects of the nucleoside analogues on expression of the EGFP and *Rluc* reporter genes of the CHIKV replicon in the BHK-CHIKV-NCT cell line. Columns represent the mean and SD values of three replicates. See Figure 1 in **II** for the chemical structures of each nucleoside analogue.

5.4.2. Semisynthetic betulin-derived compounds (III)

The pentacyclic triterpenoid betulin is an abundant natural product that represents up to one-third of the dry weight of the outer bark of white birches (*Betula* sp.). The oxidised form of betulin, betulinic acid, can also be found in birch bark in minor quantities and is present in various unrelated plant species. Betulinic acid and its derivatives are widely studied for their proapoptotic and immunomodulatory properties, and accumulating evidence has also demonstrated their antimicrobial activities (Alakurtti et al., 2004; Cichewicz and Kouzi, 2004).

The third publication of the current study covers the screening of antialphaviral activity of 51 semisynthetic derivatives of betulin and betulinic acid bearing modification of both the C3 and C28 hydroxyl groups and the 20,29 double bond. The activity distribution of the derivatives based on the antiviral and cytotoxic properties is visualised in the scatter plot in Figure 8.

Of the 51 derivatives tested in the primary assay, 19 scored below the hit limit (<20% SFV replication remaining) and met the set criterion for cell viability (>80%). These derivatives were subjected to dose-response experiments and extended cell viability evaluation. 3,28-O-diacetylbetulin (compound **4** in III) had the lowest IC_{50} value among these derivatives (9.1 µM; Table 2 in **III**), but betulonic acid **11** and betulinic acid **13** also had IC_{50} values in the same range (13.3 µM and 14.6 µM, respectively; no statistically significant difference between the three compounds). For further cell viability testing, Huh-7 cells were exposed to 500 µM concentrations prior to determination of the ATP level. With few exceptions, the hit compounds showed no decrease in ATP levels at this concentration. Thus, selectivity indices were not calculated as in the absence of true cell viability IC_{50} values this measurement would not rank the compounds by selectivity but rather would offer just another measure of compound potency.

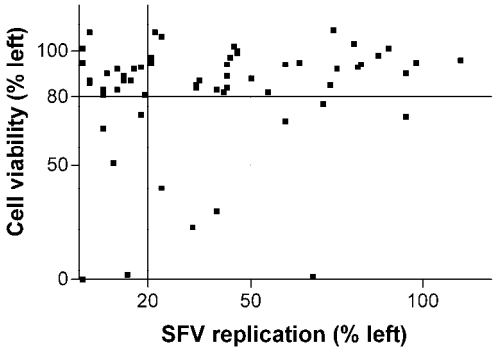

Figure 8. Scatter plot of the antiviral and cytotoxic properties of the betulin-derived compounds. Each point represents one assayed compound; the location of the point is defined by its impact on SFV-*Rluc* replication and Huh-7 cell viability at 50 µM.

Three betulin-derived compounds (betulinic acid, 38-*O*-tetrahydropyranylbetulin and the cycloadduct of 4-phenyl-1, 2, 4-triazolidine-3, 5-dione and 3β, 28-di-O-acetylbetulin-12, 18-diene; whose chemical structures are presented in Figure 9; correspond to compounds 13, 17 and 41 in **III**) were also analysed in administration time experiments using a high multiplicity SFV-*Rluc* infection (10 PFU/cell). The betulin derivatives as well as 3'-amino-3'-deoxyadenosine were subjected to either delayed administration (1 h or 2 h postinfection) or early withdrawal (1 h postinfection) under conditions where expression of the *Rluc* reporter gene is measured after giving time for only a single cascade of virus replicative machinery (detection at 4 h postinfection). Under

such conditions, betulinic acid and 3'-amino-3'-deoxyadenosine decreased *Rluc* activity when administered 1 h or 2 h postinfection but had no effect on luciferase activity in samples where the compounds were washed away at 1 h (Figure 3 in **III**). 28-O-tetrahydropyranylbetulin and the heterocyclic betulin derivative 41 differed from betulinic acid and 3'-amino-3'-deoxyadenosine in the assay as they did not suppress *Rluc* marker gene expression in any of the administration times used for the experiments indicating that the target site of these two derivatives is not likely to involve virus entry or replication. As the experiment time scale covers only a single infectious cycle and the marker gene is produced as a part of the replicase polyprotein, maturation inhibitors do not show suppression of *Rluc* in this setup.

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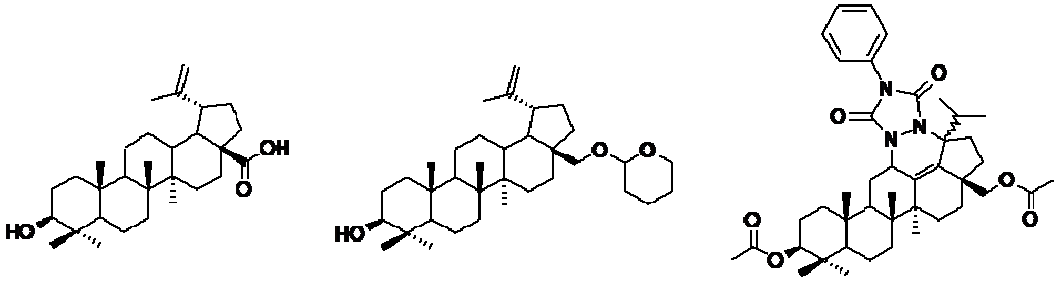

Figure 9. Chemical structures of betulinic acid, 38-*O*-tetrahydropyranylbetulin and the cycloadduct of 4-phenyl-1, 2, 4-triazolidine-3, 5-dione and 3β, 28-di-O-acetylbetulin-12, 18-diene

Table 10. IC_{50} values of betulinic acid, 28-*O*-tetrahydropyranylbetulin and the heterocyclic betulin derivative 41 against SFV, SINV and CHIKV;.

Compound	SFV IC_{50} (µM)	SINV IC_{50} (µM)	CHIKV EGFP IC_{50} (µM)	CHIKV *Rluc* IC_{50} (µM)
Betulinic acid	14.6	0.5	1.5	4.0
28-*O*-tetrahydropyranylbetulin	17.2	1.9	-	-
Cycloadduct of 4-phenyl-1,2,4-triazolidine-3,5-dione and 3β,28-di-O-acetylbetulin-12,18-diene	19-7	6.4	-	-

Values represent the data from two individual experiments performed in triplicate. - , no inhibition observed

The same three betulin derivatives were also tested in the CHIKV replicon system (data not included in the original publications). Consistent with data from the administration time experiments, 28-*O*-tetrahydropyranylbetulin and the heterocyclic betulin derivative 41 did not inhibit expression of the replicon reporter genes while betulinic acid suppressed EGFP and *Rluc* expression with IC_{50} values of 1.5 µM and 4.0 µM, respectively (Table 10). In **III**, the three

compounds were also demonstrated to inhibit SINV replication with IC_{50} values in the low micromolar range (experiments conducted with the RNA labelling assay).

Furthermore, synergism studies were carried out between the three betulin-derived compounds and 3'-amino-3'-deoxyadenosine using the Loewe synergism model. Dose-response curves of each betulin derivative were determined in the presence of varying nucleoside concentrations. I values for the combinations that were calculable (excluding those leading to a response in either the upper or lower plateau of the dose-response curve) are presented in Table 11. As indicated by the values, the observed synergism was concentration-dependent and most intense at low compound concentrations.

Table 11. Interaction indices for betulinic acid (13), 28-*O*-tetrahydropyranylbetulin (17) and the heterocyclic betulin derivative 41 in combination with 3'-amino-3'-deoxyadenosine.

D_1 (µM)	**D_2 (µM)**	**I (13)**	**I (17)**	**I (41)**
10	0.5	0.96	0.27	0.26
50	0.5	1.12	0.51	-
2	2	-	0.90	1.50
10	2	1.27	0.48	0.25
50	2	1.56	0.54	-
0.08	5	0.28	-	-
0.4	5	0.25	1.44	0.27
2	5	0.35	0.24	0.18
10	5	0.52	0.81	-
0.08	20	0.51	0.91	0.82
0.4	20	0.88	-	0.53
2	20	0.99	-	-
0.08	50	1.23	1.28	0.92

D_1 represents the concentration of each betulin derivative and D_2 is the concentration of 3'-amino-3'-deoxyadenosine.

5.4.3. Library of natural products (IV)

In **IV**, a library of 124 natural compounds was screened for inhibition of alphavirus replication using the SFV-*Rluc* assay for the primary screen. The natural product collection consisted mainly of flavonoids, coumarins, gallates and other phenolic compounds, most of which are well-

characterised plant secondary metabolites. Such metabolites have undergone evolutionary selection to serve a role in the defence and interaction paths used by plants; and natural compounds are thus regarded as a rich source for bioactive structures (Koehn and Carter, 2005).

A complete list of screening compounds and primary screening results is presented in Supplementary information for **IV**. The activity distribution of the natural compounds in the primary screen using the SFV-*Rluc* assay is shown in Figure 10A and contains a high proportion of anti-SFV compounds. A confirmatory assay was conducted for all compounds yielding less than 25% remaining SFV replication in the primary screen along with a counter-screen for BHK21 cell viability (ATP assay, 24-h exposure). Fifteen compounds (Table 1 in **IV**) were defined as screening hits (hit rate = 12.1%) after this filtering. Furthermore, a number of compounds in the library showed moderate SFV-*Rluc* inhibition (points between the 25% and 50% lines in Figure 10A).

Confirmed hits of the study were subjected to dose-response experiments of which representative results are presented in Figure 10B. Results from the SFV-*Rluc* screening assay were compared using the ATP cell viability assay involving Huh-7 cells exposed for 48 h, and compounds yielding selectivity indices higher than 10 were selected for further analyses.

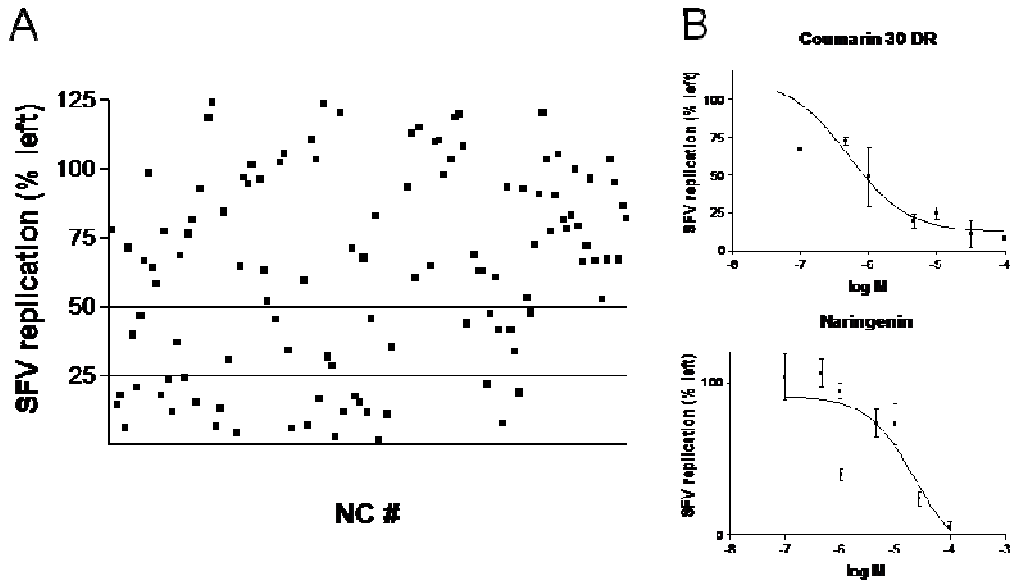

Figure 10. A. Primary screen of natural compounds using the SFV-*Rluc* assay. Results represent the remaining SFV replication in each sample treated with a compound concentration of 50 µM; n = 124. B. Representative examples of dose-response curves of confirmed hits of the NC screen.

A secondary assay on the SFV-induced cytopathic effect was next utilised for the selected hit compounds using BHK21 cell viability quantification by the WST-1 reduction assay. Similar experiments were also carried out using the CPE assay. Further follow-up studies were performed with SFV yield assays where the viral titers were determined from infected cultures treated with compound concentrations of 50 µM. CPE reduction and virus yield assays confirmed the anti-SFV activity of most of the screening hits, and no statistically significant difference in IC_{50} values was found between SFV and SINV for CPE reduction (Table 2 in **IV**) All of the compounds that reduced the SFV- and SINV-induced CPE reduced also SFV titers 10 – 100 fold in the virus yield assay.

The natural compounds were also assayed in the BHK-CHIKV-NCT replicon cell line where the flavonoids apigenin, chrysin, naringenin and silybin suppressed the levels of both the EGFP and *Rluc* markers. The coumarins bergapten and coumarin 30 did not reduce the marker levels. Potency evaluation in dose-response experiments resulted in IC_{50} values of 22.5 µM and 28.3 µM for apigenin and 25.8 µM and 30.0 µm for naringenin (EGFP and *Rluc* readings, respectively), while chrysin and silybin had IC_{50} values ranging from 45.8 µM to 71.1 µM (Table 3 in **IV**). Chemical structures of the four flavonoids are presented in Figure 11, which reveals that all of the compounds have a 5,7-dihydroxyflavone structure.

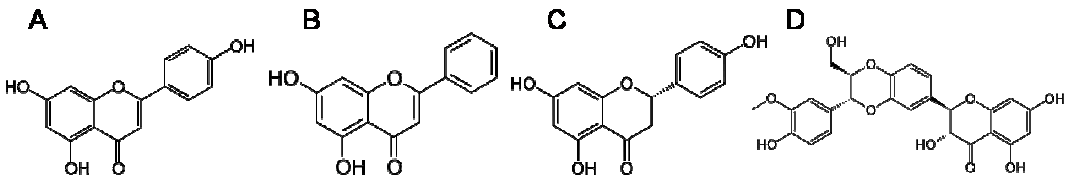

Figure 11. Chemical structures of apigenin (A), chrysin (B), naringenin (C) and silybin (D).

Also screening artefacts arisen by autofluorescent screening compounds were dencountered in the NC screen. Figure 12 shows the fluorometric and luminometric readings of an experiment measuring the effect of coumarin 30 on the CHIKV replicon markers. The fluorescence reading of the assay samples containing 50 µM coumarin 30 (leftmost columns) yielded a signal that was approximately five-fold greater than the untreated replicon samples (columns in the middle) while the luminometric readout gave a signal that was in the same range as the untreated control. The rightmost columns represent the fluorescent and luminescent signals of 50 µM coumarin 30 alone

(diluted in the assay buffer) confirming that the observed bias in the readings was a consequence of the compound's autofluorescence

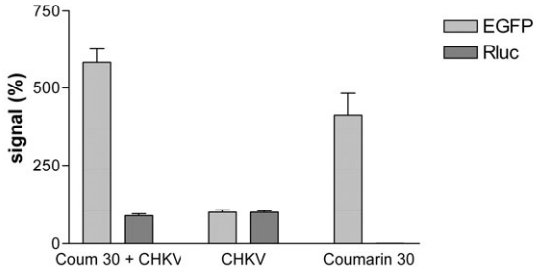

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Figure 12. Fluorescent and luminescent readings from experiments with coumarin 30 on the CHIKV replicon.

5.4.4. Library of pharmaceutical compounds (IV)

An alternative approach to find novel inhibitors of alphavirus replication was applied in **IV** by evaluating a library of drug molecules in a screening assay against SFV-*Rluc*. Finding a chemical agent already approved for clinical use would allow for rapid proceeding to the efficacy evaluation in animal models and possibly in human volunteers as the long-term safety profile of clinically used drugs has already been characterised and approved by authorities for at least some indications. The identification of an inhibitory effect by a molecule with a known pharmacological target may also provide clues for elucidating the pathways and signalling cascades involved in the viral replication process within the cells. Even though the indication for which the drug is approved may not represent the only target site of the compound, it provides a good starting point for studies on the observed inhibitory effects.

The library of pharmaceutical compounds tested in Publication **IV** consisted of 234 bioactive synthetic compounds, most of which are drugs that are or have previously been in clinical use. The compounds represent several therapeutic areas including drugs for cardiovascular and metabolic diseases, antidepressants, antipsychotics and other psychiatric drugs, antibiotics and analgesics.

A similar screen design as described above for the natural compounds was used in the primary screen, the confirmatory assays and the cell viability counter-screen of compounds in the pharmaceutical collection. After the exclusion of compounds yielding non-reproducible results and

those that decreased BHK21 cell viability, 12 compounds were defined as screening hits (hit rate = 5.0%). Furthermore, 24 compounds in the library showed moderate SFV-*Rluc* inhibition (points between the 25% and 50% lines in Figure 13A). Figure 13A represents the activity distribution of the pharmaceutical compounds at a concentration of 50 µM in the primary screen against SFV-*Rluc*; and representative examples of dose-response curves are shown in Figure 13B. Using the same procedure as with the natural compounds, the follow-up studies on SFV- and SINV-induced CPE reduction and the SFV yield assay confirmed the inhibition of two distant members of the alphavirus genus by the hit compounds (Table 2 in **IV**).

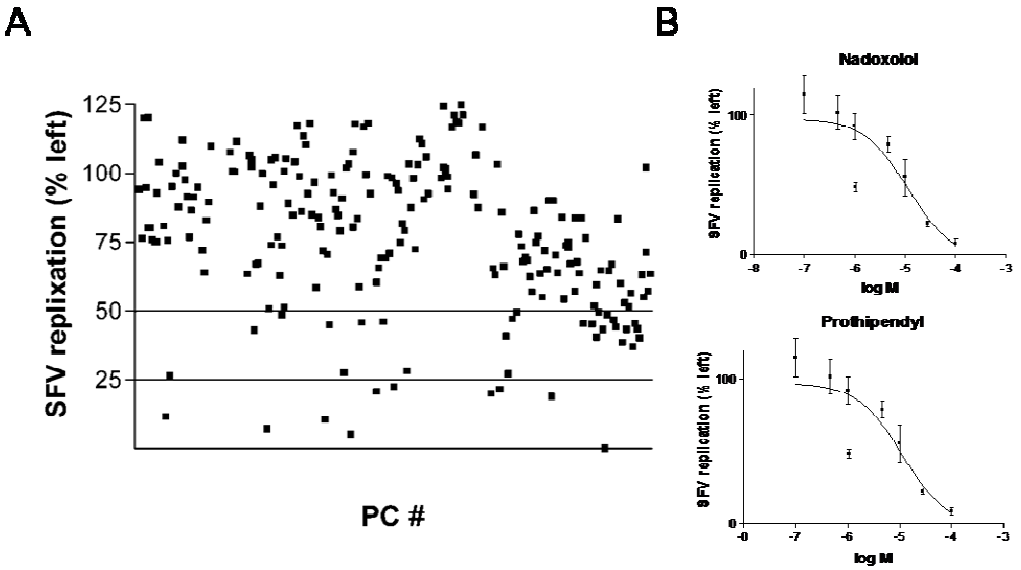

Figure 13. A. Primary screen of pharmaceutical compounds in the SFV-*Rluc* assay. Results represent the remaining SFV replication in each sample treated with a compound concentration of 50 µM; n = 234. B. Representative examples of dose-response curves of confirmed hits among the PC compounds.

The PC screen hit list included the antipsychotic drugs chlorpromazine, perphenazine, thiethylperazine and thioridazine as well as a first-generation antihistaminic agent methdilazine and the antiparkinsonism agent ethopropazine (also known as profenamine). Despite their slightly different indications reflecting the differential affinities for the related plasma membrane receptor proteins, all six of these compounds share the same 10*H*-phenothiazine core structure consisting of three fused rings of which the middle one contains nitrogen and sulphur atoms in *para* positions. Ethopropazine and thioridazine were also shown to reduce SFV- and SIN-induced CPE by the IC_{50}

values of 17.1 µM and 21.4 µM (ethopropazine) and 19.3 µM and 37.3 µM (thioridazine), respectively. Both compounds also showed moderate inhibition of the SFV yield when they were present during the infection at a concentration of 50 µM. None of the six compounds inhibited the CHIKV replicon as measured by expression of the two marker genes, which implies that the target site for the antiviral activity of these compounds lies outside the viral replication phase.

6. DISCUSSION

6.1. Use of cell viability assays

Cell viability assays were originally developed some decades ago mainly for immunological and oncological purposes and were later adapted for bioactivity assay counter-screens and for predictive toxicology. Despite the correlation of cytotoxicity assays with *in vivo* data (see section 2.4.1.), quantitative and even qualitative differences in cytotoxicity assay results between different assay endpoints are not hard to find (Larksome et al., 2007; Mueller et al., 2004; Puttonen et al., 2008). The assay endpoint plays a crucial role in assay sensitivity and governs the exposure time required as indicated by the results of **I** in the current study. The results also indicate that the nature of the test samples should be taken into account in assay selection, which is also emphasised by the experimental artefacts described for transporter proteins and cellular redox state. To allow more reliable conclusions, parallel use of more than one assay endpoint has been suggested (Wilson, 2000). Calculation of selectivity indices in antiviral screening relies on the determination of cell viability IC_{50} values from the selected assay. Therefore, the quantitative precision and reliability of the cell viability assays are as essential as optimisation of the antiviral assays themselves. In the current study, reproducibility of the cell viability data is confirmed by the use of statistical parameters to monitor data homogeneity throughout the screens.

In general, improved sensitivity has been considered a beneficial factor in assay selection. However, definitive answers on optimal sensitivity regarding both predictive toxicology and bioassay counter-screening are yet to be found. According to data presented in this study, the ATP assay is the most sensitive of the tested assays for detecting chemical-induced cellular stress, and similar results have been reported elsewhere (Mueller et al., 2004), When optimised for minimal signal deviation, this assay is capable of reliably detecting 10% to 20% changes in cellular ATP pools. However, it is not clear whether a mild or moderate decrease in intracellular ATP levels would result in any harmful *in vivo* effects, or whether it affects viral replication enough to classify the antiviral result as a false positive. The well-documented examples of resveratrol, curcumin and other phytochemicals with a biphasic dose-response relationship in cell viability illustrate this question. The adaptive stress response induced by low doses of these phytochemicals triggers

changes leading to cytoprotection whereas the decrease in cell metabolism induced by higher concentrations may lead to cell death (Mattson, 2007).

6.2. Antiviral assay development

CPE reduction has been established as the standard endpoint for antiviral screening due to its wide applicability in panels of unrelated viruses. The classical form of the CPE reduction assay involves visual inspection of cell cultures under a microscope to evaluate the extent of the cytopathic effectand it is thus labour-oriented, subjective and only semiquantitative in nature. These technical obstacles can be overcome by using a suitable cell viability assay method, such as a tetrazolium salt. However, the problem of distinguishing between the cytopathy caused by virus and the test compound-borne toxicity remains unsolved. Even though chemical agents affecting the host cell viability were not relevant as lead candidates the dissection into inactive and toxic derivatives would be required for accurate SAR studies. Furthermore, the general endpoint of the assay provides few options for follow-up studies to determine the target site of the antiviral activity.

In **II**, a novel luminescent assay was described for screening for inhibitors of alphavirus replication. One of the key features of luminometric detection is the high dynamic range of the signal, which is beneficial in antiviral screening where linear detection over several orders of magnitude is required. This feature was highlighted by the S/B value achieved with the assay (approximately 150 as compared to 4.3 in the colorimetric CPE reduction assay). The signal shows some deviation presumably due to the relative error in the small original inoculum (100 PFU/well) but the wide signal window compensates for the noise and the achieved Z' value of 0.5 indicates assay robustness. Furthermore, signal deviation does not interfere with assay precision as the normalised mean signal determined as the response of cultures treated with 100 µM ribavirin show only approximately 10% deviation (8.9% between plates and 11.6% between days).

The developed assay utilises a strain of the Semliki Forest virus encoding for the *Renilla* luciferase marker, which is inserted within the nonstructural protein-coding region of the viral genome. Thus, the expression of *Rluc* reflects the production of viral replicase proteins allowing for the monitoring of this essential aspect of viral replication. The luciferase is inserted with an extra protease cleavage site recognised by viral nsP2 protease, which leads to release of the marker protein into

the cytoplasm of infected cells during the course of P1234 polyprotein processing. This feature was confirmed by the correct size of the nsP3 protein as determined by immunoblotting of the lysates of infected cell cultures. The release of the reporter is probably an important contributor to the genetic stability of SFV-*Rluc*; the genome of alphaviruses is extremely condensed (a total of approximately 11,500 – 11,800 base pairs) and any nonessential components that cause delays or not being essential in replication are likely to be rapidly lost when the virus is passaged in cell culture. However, SFV-*Rluc* exhibits over 90% luciferase positivity in a third passage of low multiplicity infections, each of which consists of two to three virus replication cycles.

Use of the luciferase marker virus also allows for follow-up assays in which the infection multiplicity and timescale can be changed to determine the antiviral activities targeting certain steps of the viral replication cycle. By comparing data from low and high multiplicity infections, inhibitors of virus maturation can be identified as agents showing antiviral activity in a low multiplicity infection, but not showing suppression of the marker gene levels at 4 - 5 h in a high multiplicity infection (as done in **III**). On the other hand, the low detection limit of luciferase in high multiplicity infections yields a detectable signal as soon as 30 minutes after inoculum administration. The capped positive-stranded RNA genome of SFV is readily transcribed as soon as it is released from the nucleocapsid into the cytoplasm to yield the initial fraction of nonstructural proteins. Assaying the effect of antiviral hits on such early stage events in the viral replication cycle may provide indicative data on the target site of the antiviral action, but no definitive conclusions can be drawn to distinguish between the entry and replication inhibition. Replication in wt viral infection is initiated as soon as the first P1234 precursors are formed and thus takes place simultaneously with further translation of the initial RNA strand. In this respect, more solid mechanistic information could be achieved by using a system in which replication is restricted, such as a high multiplicity infection of a replication-negative TS mutant virus strain at an elevated temperature. In general, when comparing data from infections with different multiplicities, one should be bear in mind that the antiviral potency of many chemical agents is dependent on infection multiplicity and sufficiently high compound concentrations should be used to avoid false interpretations.

The luminometric antiviral assay based on SFV-*Rluc* was validated using the known alphavirus inhibitors ribavirin, mycophenolic acid, chloroquine and 6-azauridine. Ribavirin and mycophenolic acid showed higher IC_{50} values in the reporter assay than in the CPE reduction assay used for

comparison. Chloroquine, 6-azauridine and the screening hits identified in studies **II**, **III** and **IV** did not show significant differences in IC_{50} values between the two assays. Both ribavirin and mycophenolic acid are inhibitors of inositol monophosphate dehydrogenase (IMPDH), but it is not clear if the observed differences in sensitivity between the two assays are related to this mode of action.

Another new antiviral assay was described in **IV** in the form of a stable cell line expressing CHIKV replicase proteins and two marker genes. The noncytopathic, persistent phenotype of the replicon was achieved by combining a point mutation and a 5 aa insertion into the CHIKV nsP2 sequence. *Renilla* luciferase was placed in the end of nsP3, but with no additional protease cleavage site. EGFP was fused with puromycin acetyltransferase and placed under a subgenomic promoter to maintain replication by selection pressure. Huge dynamic ranges and excellent Z' values were achieved for both the luminometric and fluorometric readings after optimisation steps. Reproducibility was also demonstrated by only approximately 5% deviations in the normalised mid-signals (cultures treated with 15 µM ribavirin).

Alphaviruses replicate efficiently in cells that are in the resting state and do not require cell division. Confluent cell cultures were also selected for antiviral assays with the BHK-CHIKV-NCT replicon cell lines as the cells were shown to maintain marker gene levels in the medium even in the absence of puromycin at least for 48 h. Furthermore, confluent cell cultures were less sensitive to test compound-borne decreases in cellular ATP levels. Although compounds causing slower cell replication rates at high concentrations may not be relevant as future lead compounds, the compromised cell culture viability at higher compound concentrations limits the determination of reliable dose-response curves.

The intracellular dynamics of noncytotoxic alphaviruses and their replicons have been shown to be essentially different from their wild-type counterparts; minus strand synthesis is continuous and replication complexes are unstable and undergo recycling in the persistent viral infection (Sawicki et al., 2003). The causality relationship between the two aspects is not obvious. It is unknown whether instability of the replication complexes allows for the formation of new replication complexes and production of the minus strand RNA by these new replication complexes or whether the failure of viral and host cell factors to stop minus strand synthesis is the factor that destabilises the replication complexes. Notably, despite the continuous minus strand synthesis no accumulation

of total RNA has been seen in the persistent infections. In general, noncytotoxic SFV and SIN replicons produce low levels of RNA rather than cause its accumulation (Sawicki et al., 2006).

Whatever the causal relationship of these two aspects, the continuous minus strand synthesis in the replicon system allows for the identification of screening hits affecting this step of alphavirus replication and is thus considered a benefit for the screening system. The recycling of replication complexes also gives rise to two alternatives concerning the screening hit activities; the compounds can act by inhibiting the production of new replication complexes or by inhibiting the function of established RCs.

In general, the noncytopathic phenotype of alphaviruses is linked to mutated forms of nsP2 but host cell factors also play a role in regulating the viral replication process in this respect. Mouse embryo fibroblasts (MEF) deficient in RNase L, but not thise lacking in protein kinase R or MX-1, exhibit a similar phenotype as noncytotoxic nsP2 mutant viruses (Sawicki et al., 2003). Even though the persistent infection caused by nsP2 mutant viruses and wild-type viruses in RNase L-deficient cell lines have similar phenotypes it has not been elucidated if the two aspects are interconnected and whether they are the only players in the virus-cell interactions responsible for the noncytotoxic phenotype. A simple assay setup to identify the undescribed components in this interplay could be offered by the combination of the primary HTS screen for agents (siRNA or small organic molecules) that reduce wild-type virus CPE with a counter-screen of the hits for viral replication using the reporter gene assay described in **II**. The identification of siRNA or chemical probes causing a noncytotoxic yet replicative viral phenotype would give us new tools for better understanding of this process.

6.3. Aspects of alphavirus antiviral lead discovery

The proinflammatory cytokines IL-1, IL-6 and TNF-α play a central role in the onset of clinical symptoms of the febrile illness caused by CHIKV, RRV and other arthritogenic alphaviruses (Kam et al., 2009, Rulli et al., 2005). High circulating levels of these cytokines during the acute viremia have been suggested to predict disease severity, and the role of proinflammatory cytokines has shown to be central in the development of joint and muscle manifestations (Lidbury et al., 2008; Ng et al., 2008). The relative emphasis on the relationship between viral replication and the

cytokine response is an essential point to consider in the chemotherapy used to treat infected individuals. Even though high viral titers in the bloodstream correlate to some extent with a severe clinical picture, there are currently no data to demonstrate that suppression of viral titers in the blood or acceleration of virus clearance would lead to clinical benefits after the onset of the cytokine storm. However, effective suppression of viral loads by antiviral agents may still be useful for prophylactic medication or for prevention of virus transmission. Evaluation of the role of virus clearance in clinical treatment would require the use of chemical agents that solely target the virus and leave the immune system unmodified, but none of the agents currently in common use are suitable for this task. However, the design and development of inhibitors targeting viral enzymes could provide tools for such studies. In contrast to many other viral species, the nsP4 RNA polymerase may not be the optimal starting point as a screening target given the technical obstacles in assaying this enzymatic activity. The papain-like protease in the C-terminal portion of nsP2 could provide an option; however, the selectivity of potential inhibitors should be considered. On the other hand, targeting the unique capping function of nsP1 could lead to specific inhibition. Additionally, competitive binding of ADP-ribose in the nsP3 macrodomain should be validated as an antiviral target.

The combination of antiviral and immunomodulatory effects is also likely to be the main factor causing different outcomes in *in vitro* and *in vivo* studies of antiviral agents. The small molecule alphavirus inhibitors ribavirin and mycophenolic acid exert their activities via the depletion of cellular GTP pools, which at least in the case of mycophenolic acid, leads to suppression of immune system cells. Mycophenolic acid is clinically approved for immunosuppressive use while most *in vivo* studies on its antiviral activity have been left unpublished due to unsatisfactory results (Diamond, 2009). Ribavirin has also been shown to possess immunomodulatory properties and to modulate the balance between helper T cell populations among its other effects (Hollgren et al., 1998). Ribavirin is used to treat chronic HCV infections in combination with IFN-α; in this combination IFN has been suggested to act as the antiviral agent and ribavirin as the immunomodulatory agent (De Ckercq et al., 2009; Feld and Hoofmagle, 2005). Chloroquine also has well-documented immunomodulatory effects that involve both inhibition of TNF-α production and impairment of TNF-α-induced signalling in its target cells (Jeong et al., 1997; Jeong et al., 2002). The role of unfavourable immunomodulatory effects has been suggested in the failure of the 2007 clinical trial against CHIKV; and an earlier study, chloroquine enhanced SFV replication in mice and was interpreted in the same context (De Lamballerie et al., 2008; Maheshwari et al.,

1991). However, the results are still be regarded as inconclusive due to the small number of patients enrolled into the clinical trial (n = 27 in both the active and control groups) as well as the differences in biology between the animal models.

The use of the animal models to study the antialphaviral effects of any chemical agent should be carefully considered. Classically, the standard animal model used in alphavirus studies has been the age-dependent encephalitis of neonatal infected mice using prolonged survival or protection from death as the endpoint. More recently, adult mice have been infected with CHIKV or RRV to induce an arthritogenic disease better reflecting the pathology seen in human infection with these viruses. Thus far, no published reports on the characterisation of antiviral agents in these models are available. The neurotrophic potential of Old World alphaviruses in neonatal mice is considered a consequence of their immature immune system as most of these viruses are apathogenic in adult mice with a mature interferon response (Muller et al., 1994; Ryman et al., 2000). When administering a drug candidate with immunomodulatory properties, it is not obvious that the infection outcome will be similar in the different models. The proper interpretation of the results is crucial for making the right decisions on the continuation or withdrawal of any drug candidate.

CHIKV and other alphaviruses are sensitive to IFN treatment *in vitro* (Briolant et al., 2004; Sourisseau et al., 2007) and treatment with pegylated IFN-α has also been shown to protect mice against VEEV-induced encephalitis (Lukaszewski *et al.*, 2000). However, no attempt to treat alphavirus infection by commercially available interferon preparates has been published. The underlying reasons presumably include not only the unsuitability of injection therapy in most alphavirus endemic areas but also the uncertainty of the potential risks concerning the worsening of acute symptoms in response to the treatment.

More experimental information on the immunopathogenesis of the arthritogenic infectious disease is required to guide antialphaviral drug discovery in the right direction. Given the well-established role of TNF-α in inflammatory responses and the recent evidence of its role in the arthritic symptoms of CHIKV infections, clinically approved biotechnological drugs targeting this cytokine could be used as an experimental treatment for treating severe alphavirus infections. Monoclonal anti-TNF-α antibodies and soluble chimeric TNF-α receptors have been proven efficacious in many autoimmune diseases including rheumatoid arthritis. The experimental use of these preparations alone or in combination with a suitable antiviral drug, administered either during the acute viremia

or after its clearance in the arthritogenic mouse model would shed more light on the relevance of immunomodulation in the therapy.

6.4. Chemical scaffolds for alphavirus inhibitors

The core structure of 10*H*-phenothiazine was identified in 6 of the 12 pharmaceutical compounds scored as screening hits against SFV in study **IV**. In a recent HTS campaign against Western equine encephalitis virus, Peng et al. (2009) described thieno[3,2,b]bipyrrole as a lead structure for antialphaviral therapy. As indicated by the chemical structures presented in Figure 14, both the structure identified by Peng et al. and the 10*H*-phenothiazine structure are heterocycles containing nitrogen and sulphur atoms in their ring structures.

However, the two structures differ from each others in several key aspects: i. 10*H*-phenothiazines consist of three fused rings whereas in thieno[3,2,b]bipyrroles the third ring is separated from the two other rings by a carbonyl group; ii. thieno[3,2,b]bipyrrole consist of five-atom rings whereas in 10*H*-phenothiazines all rings are six-member; iii. 10*H*-phenothiazines contain nitrogen and sulphur atoms in the same ring whereas thieno[3,2,b]bipyrroles contain heteroatoms in different rings. The

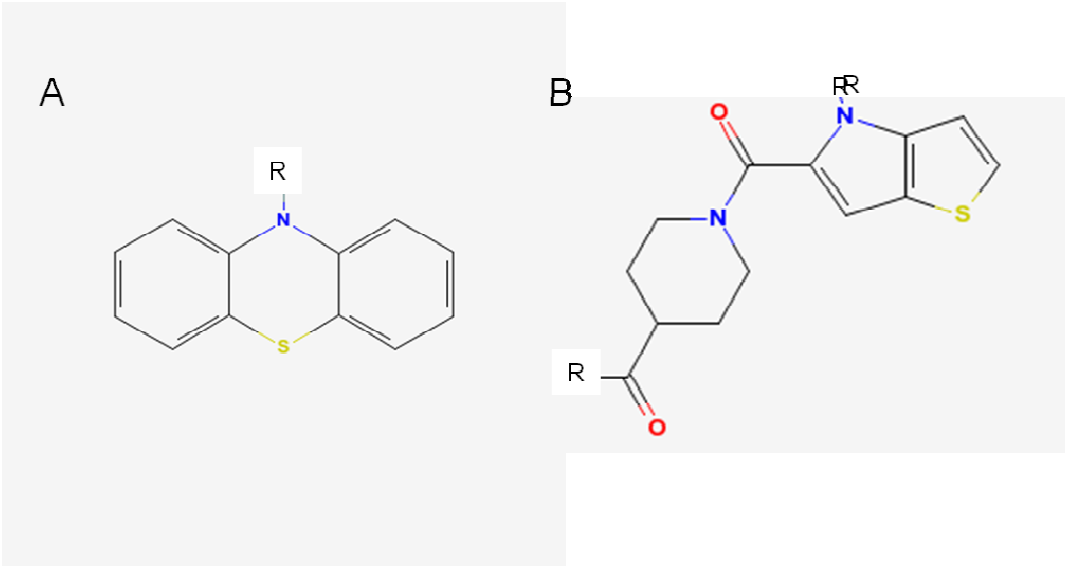

Figure 14. A. 10*H*-phenothiazine structure found in six of the screening hits in **I**V; B. core structure of thieno[3,2,b]bipyrrole derivatives identified as chemical leads against WEEV as reported by Peng et al., 2009.

thienyl pyrroles were identified as alphavirus inhibitors in a replicon-based screen and have been shown in another study to inhibit hepatitis C virus RdRp (Ontoria et al., 2006). On the other hand, none of the six pharmaceutical compounds sharing the 10*H*-phenothiazine structure suppressed expression of the CHIKV replicon in **IV** indicating either a significant difference in sensitivity between SFV and CHIKV or, more likely, the exertion of compound activity via a target site not present in the replicon system. Therefore, the differences in chemical structures between the two lead structures are presumably tied to different modes of antiviral activity.

Four of the compounds sharing the 10*H*-phenothiazinyl group were antipsychotic and two act on histamine and cholinergic receptors. It is worth noting that all six agents target CNS diseases indicating that these drugs are able to cross the blood-brain barrier to reach their target site. This feature is highly beneficial in the treatment of alphavirus infections especially as New World neurotrophic alphaviruses use CNS neurons as host cells in infected individuals leading to encephalitis. Recent CHIKV epidemics have been characterised by an increasing number of neurological symptoms; the ability of CHIKV to infect neurons is currently a matter of debate (Courfier et al., 2008; Das et al., 2010; Sourisseau et al., 2007). However, presence of the virus in the CNS is made obvious by virus isolates collected from the cerebrospinal fluid of infected individuals. The main route of CNS penetration is thought to be the choroid plexus as the blood-brain barrier remains intact in infected mice.

6.5. Natural products as alphavirus inhibitors

The limited number of natural products among the published inhibitors of alphaviral replication is likely due to lack of studies in this area rather than the failure to identify such compounds. The recent study of *seco*-pregnane glaucogenin C and its glycosides (Li et al., 2007) demonstrated that natural products are an important unexplored source of alphavirus inhibitors. The use of natural products as a starting material for bioactivity screening is also considered to offer the best chance to identify compounds with previously indescribed mechanisms of action (Newman and Cragg, 2007). This idea is true for glaucogenin C and its glycosides; they were shown to specifically suppress alphavirus subgenomic RNA synthesis via a yet to be described molecular interaction.

An ethnopharmacological approach for the identification of alphavirus inhibitors was taken in an Australian study on medicinal plants used by aboriginals and revealed the activity of extracts from two local plant species (*Eremophila latrobei* and *Pittosporum phylliraeoides)* against Ross-River virus (Semple et al., 1998). The extracts were found to show no antiviral activity against poliovirus or human cytomegalovirus indicating at least some degree of selectivity for RRV. The maximal antiviral effect observed was approximately 40% inhibition of RRV-induced CPE, but the concentration and the identity of the active ingredients in the extracts were not studied. However, the authors pointed out that the extracts have been reported to contain sesquiterpenoids and triterpenoids, which bear structural similarity to the betulin-derived compounds demonstrated in this work to be alphavirus inhibitors.

Betulinic acid and its esters are widely distributed in the plant kingdom and have also been reported to be present in various unrelated medicinal plants on different continents (Aponte et al., 2008; Nick et al., 1995; Zuo et al., 2008). Many of these areas are also sites of past CHIKV outbreaks, and the majority of these regions still rely on traditional herbal remedies either as their only source of medication or to supplement Western medicine. Given the shortage in alphavirus antivirals and the unlikelihood of the appearance of new licensed chemical entities in this respect, the rational use of locally available plant- or marine-derived preparations should be promoted by investigating the antiviral activities of these secondary metabolites and their combinations.

In addition to the betulin-derived compounds, the natural products studied in the current work included flavonoids and other polyphenolic compounds. Flavonoids have been extensively studied in anti-HIV drug discovery as HIV-I integrase inhibitors, reverse transcriptase inhibitors and for their ability to downregulate the expression of chemokine receptors required for HIV entry (Asres et al., 2005). Various flavonoids are also known to inhibit RNA viruses from the rhinovirus and picornavirus families. These studies have focused on virus entry as the antiviral target indicating that flavonoids may target the viral capsid proteins to prevent the uncoating process (Jassim and Naji, 2003). Studies presented in this work (**IV**) identified several flavonoids as inhibitors of alphavirus replication with apigenin being the most consistent inhibitor in the assays used. Apigenin and three other 5,7-dihydroxyflavones (chrysin, naringenin and silybin) also showed inhibition in the CHIKV replicon cell line indicating that they exert activities related to the viral replication phase rather than entry or maturation.

Data from other sources are available concerning the immunomodulatory activities of both the flavonoids and betulin-derived compounds described as alphavirus inhibitors in the current study. Flavonoids are widely studied *in vitro* and *in vivo* for their anti-inflammatory activities, which include the direct antioxidative effect via free radical scavenging but also more specific activities such as inhibition of the proinflammatory enzymes involved in eicosanoid biosynthesis (phospholipase A2, cyclooxygenase 1 and lipoxygenase), downregulation of leukocyte adhesion molecules in endothelial cells and inhibition of the production and/or secretion of proinflammatory cytokines (Garcia-Lafnente et al., 2009). Hydroxyflavones such as luteolin and apigenin have been reported to efficiently inhibit TNF-α-stimulated IL-6 production as well as IL-1-induced prostaglandin production in human endothelial cells. Luteolin and the isoflavone genistein are also potent inhibitors of IL-1α, IL-6 and TNF-α production in LPS-stimulated macrophages (Gerritsen et al., 1995; Xagorari et al., 2001).

Betulin and betulinic acid like many other triterpenoids play a dual role in modulating the functions of the immune system. Betulinic acid has been shown to stimulate interferon secretion in LPS-induced macrophages but has also been shown to inhibit phospholipase A2 and reduce nitric oxide production in stimulated leucocytes (Honda et al., 2006; Tseng and Liu, 2004; Yun et al., 2003). *In vivo* data on the immunomodulatory effects of pure betulinic acid are not available. However, the seed extract of an Indian fluid *Ziziphus mauritiana* in which one of the major constituents is betulinic acid was recently demonstrated to induce interferon production and promote the Th1 lymphocyte-mediated immune response in mice (Mishap et al., 2010). As discussed in previous sections, the optimal combination of antiviral and immunomodulatory activities in the treatment of alphaviral diseases remains to be found. *In vivo* studies on these and other chemical agents would provide evidence to support taking further steps towards the relevant therapy.

6.6. Concluding remarks and future prospects

Success in the early phases of drug discovery is governed by an interplay of understanding the target biology, the choice of screening material and the ability to build biologically relevant and robust assay systems for screening campaigns. The rational drug discovery process is based on the selection of validated target molecules for chemical intervention in the given condition and the small molecule ligand indentified in screening campaigns is expected to prove its efficacy in a

suitable *in vivo* model within the limits of its kinetic properties. Accumulating structural information on the target proteins has allowed the use of virtual screening and *in silico* ligand design tools to promote the shift from diverse to more focused screening libraries and has allowed for educated guesses in the design and identification of more specific and more potent ligands. Whether the chemical leads identified in these processes truly represent more specific modulators of biological targets or an artificial specificity due to the lack of long-term studies remains to be seen. While specific, high-affinity ligands certainly contribute to target validation, the ultimate question concerning medical treatment is whether a specific inhibitor of a single target provides the best medical advantage given the complex nature of physiological systems rich in homeostatic and pleiotropic mechanisms. Examining older drugs reveals that it is the rule rather than exception that these agents have moderate but wider affinity profiles or even several distinct modes of action. Though the lack of specificity may obviously manifest itself as a risk for adverse drug effects, the superior *in vivo* antiviral activity of ribavirin as compared to a more potent IMPDH inhibitor mycophenolic acid for example implies that the different target sites essentially contribute to the final clinical benefits achieved by the medical treatment.

In addition to questions related to the search for selectivity and specificity, the field of bioactivity screening is facing the challenge of less-validated targets and interest in targeting biological processes of a poorly characterised nature. Rather than being a step in a linear process from target selection to lead discovery, bioactivity screening is contributing more and more to the identification and validation of target proteins. In this respect, phenotypic assays are the intuitive screening strategy and more consideration must be given to the selection of screening libraries that confer blind screening to the approaches allowing rapid follow-up studies.

The recently increased interest in alphaviruses and the antiviral therapies targeting them is still waiting to be turned into advances in the clinical treatment of the diseases caused by these pathogens. Studies with classical chemical inhibitors, such as chloroquine and ribavirin, indicate that more detailed understanding of the virus-host cell interactions and the role of immunomodulation in antialphaviral chemotherapy is required for rational design and screening of potential antiviral compounds. While more complex high-content screening systems and *in vitro* models using tools such as co-cultures of different cell populations are yet to be built, the antiviral assays described in the current study provide robust tools for the screening of alphavirus inhibitors. Combined with pathway biology, assays for specific target molecules, virtual screening and other

tools these assays will hopefully guide us closer to effective strategies for fighting Chikungunya and other alphaviral pathogens.

The rapid mutation rate of RNA viruses is likely to lead in the emergence in still new pathogens and epidemic outbreaks in future. While investigation of the molecular details of RNA virus replication is essential for providing validated drug targets, this process may not provide the rapid response required to fight emerging pathogens. Therefore, access to efficient phenotypic screening strategies using robust assay formats is the first line of defence when combined with careful selection of the screening material. The chance for off-label use of readily licensed medicines is a choice for serious consideration yet rhis strategy requires the frequently cited serendipity. In the long run, the combination of phenotypic assays with diverse natural product collections may prove invaluable in the identification of the elegant starting points for future target-based lead discovery campaigns.

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