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Screening for marine-derived antivirals

Katja-Emilia Lillsunde

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Supervisors

Professor Päivi Tammela
Division of Pharmaceutical Biosciences
Faculty of Pharmacy
University of Helsinki

Docent Tero Ahola
Department of Microbiology
Faculty of Agriculture and Forestry
University of Helsinki

Reviewers

Professor Jeanette Hammer Andersen
The Arctic University of Norway, Tromsø
Norway

Tenure Track Assistant Professor Leen Delang
Rega Institute for Medical Research, KU Leuven
Belgium

Opponent

Professor Judith Rollinger
Department of Pharmacognosy
Faculty of Life Sciences, University of Vienna
Austria

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ABSTRACT

Viruses are accountable for numerous diseases that form an immense threat to public health worldwide. The capability of viruses to continuously adapt to a changing environment makes the discovery of new treatments for viral diseases crucial. Natural products play an important role in the discovery of new drug candidates, essentially as a source of lead compounds that can be chemically optimized to achieve improved drug properties, such as safety and efficacy.

In natural product drug discovery, the marine environment offers outstanding potential to discover new compounds with interesting bioactive properties. Marine species and their unique metabolites are still only partly discovered, and at the same time, vulnerable marine environments are threatened by human activities through pollution, overexploitation and climate change. The protection of marine biodiversity and sustainable use of marine resources is therefore a vital part of the study of marine organisms and their metabolites.

This dissertation focuses on studying the antiviral properties of marine-derived compounds and their synthetic derivatives. The first part of this study covers screening of crude extracts from the Indian Ocean soft coral *Sinularia kavarattiensis* in a chikungunya virus replicon model followed by bioactivity-guided isolation and further study of the purified compounds. This study led to the isolation of six known norcembranoid compounds and the isolation and characterization of one novel compound, kavaranolide. Two of the isolated compounds were moderately active in the chikungunya replicon model, but also showed cytotoxic properties.

The second part of the research focuses on studying the antiviral potential of synthetic compounds inspired by the marine sponge-derived alkaloids clathrocin and oroidin. In the screening of a compound library of 157 clathrocin and oroidin analogues in chikungunya virus and hepatitis C virus replicon models, four compounds were discovered to selectively inhibit the hepatitis C replicon with IC_{50} -values ranging from 1.6 to 4.6 μ M. Interaction with the cellular chaperone Hsp90 was proposed as the mechanism of action underlying the activity, and this hypothesis was supported by the results from molecular modelling and microscale thermophoresis interaction studies.

Based on the study of clathrocin and oroidin analogues, 12 new compounds with a 4,5,6,7-tetrahydrobenzo[1,2-*d*]thiazole structure were synthesized, in order to obtain improved binding to Hsp90 and improved

antiviral properties. Three of the synthesized compounds showed improved binding to Hsp90 and specific inhibition in hepatitis C genotype 1b and 2a replicon models and moreover, inhibited the replication of full-length hepatitis C genotype 2a virus in a reporter virus RNA assay (IC_{50} -values 0.03–0.6 μ M). As Hsp90 is a host protein utilized by the viral replication machinery, antiviral activity achieved through inhibition of Hsp90 could be an attractive strategy to combat resistant viral strains.

CONTENTS

ABSTRACT	3
CONTENTS	5
LIST OF ORIGINAL PUBLICATIONS	7
CONTRIBUTIONS	8
ABBREVIATIONS	9
1 INTRODUCTION.....	10
2 REVIEW OF THE LITERATURE.....	12
2.1 Marine natural products in drug discovery	12
2.2 Marine-derived antivirals	17
2.2.1 Vidarabine	18
2.2.2 Iota-carrageenan	18
2.2.3 Bryostatins	19
2.2.4 Griffithsin	20
2.3 Hepatitis C virus (HCV)	20
2.3.1 HCV classification and characteristics.....	21
2.3.2 HCV replication and host response.....	21
2.3.3 Model systems in HCV research	22
2.3.4 Current HCV treatment.....	22
2.4 Chikungunya virus (CHIKV).....	24
2.4.1 CHIKV classification and characteristics.....	25
2.4.2 CHIKV transmission, replication and epidemics.....	26
2.4.3 Drug development strategies against CHIKV	26
2.5 Inhibition of Hsp90 as an antiviral strategy	28
3 AIMS OF THE STUDY	32
4 MATERIALS AND METHODS	33
4.1 Studied marine-derived material.....	33
4.2 Reference compounds.....	33
4.3 Cell-based methods.....	34
4.3.1 Cell lines and culture conditions.....	34
4.3.2 Screening for CHIKV replication inhibitors	35
4.3.3 Screening for HCV replication inhibitors	36
4.3.4 Cytotoxicity evaluation.....	36

4.3.5	Live-cell imaging	37
4.3.6	Data analysis and assay quality in cell-based experiments	38
4.4	Microscale thermophoresis	39
5	RESULTS.....	40
5.1	Antiviral, cytotoxic and anti-inflammatory properties of <i>S. kavarattiensis</i> (I)	40
5.1.1	Primary screening and bioactivity-guided fractionation ...	40
5.1.2	Primary evaluation and imaging studies of isolated compounds	41
5.1.3	Anti-inflammatory and immunomodulatory activity of purified compounds	45
5.2	Antiviral potential of synthetic clathrocin and oroidin analogues and their derivatives (II, III)	45
5.2.1	Primary evaluation, hit selection and optimization.....	45
5.2.2	Mode-of-action studies	49
5.2.3	Antiviral activity against full-length HCV.....	51
6	DISCUSSION.....	53
7	CONCLUSIONS AND FUTURE PROSPECTS	57
	REFERENCES.....	59

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text by Roman numerals **I-III**:

- I** Lillsunde, K.-E.; Festa, C.; Adel, H.; De Marino, S.; Lombardi, V.; Tilvi, S.; Nawrot, D.A.; Zampella, A.; D'Souza, L.; D'Auria, M.V.; Tammela, P. Bioactive Cembrane Derivatives from the Indian Ocean Soft Coral, *Sinularia kavarattiensis*. *Mar. Drugs* **2014**, *12*, 4045-4068.
- II** Lillsunde, K.-E.; Tomašič, T.; Kikelj, D.; Tammela, P. Marine alkaloid oroidin analogues with antiviral potential: A Novel Class of Synthetic Compounds Targeting the Cellular Chaperone Hsp90. *Chem. Biol. Drug Des.* **2017**, *90*, 1147-1154.
- III** Lillsunde, K.-E.; Tomašič, T.; Schult, P.; Lohmann, V.; Kikelj, D.; Tammela, P. Inhibition of Hepatitis C Replication by Targeting the Molecular Chaperone Hsp90: Synthesis and Biological Evaluation of 4,5,6,7-Tetrahydrobenzo[1,2-*d*]thiazole Derivatives. *Chem. Med. Chem.* **2019**, *14*, 334-342.

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CONTRIBUTIONS

- I** K-E Lillsunde planned and performed the CHIKV replicon screening assays and cytotoxicity testing and analyzed the results. K-E Lillsunde planned, optimized and performed the live-cell imaging assay and analyzed the results in collaboration with co-author Dorota Nawrot. Lisette D'Souza, Harshada Adel and Supriya Tilvi were responsible for the collection, taxonomic identification and preliminary extraction of the soft coral *S. kavarattiensis*. Simona De Marino and Carmen Festa performed compound isolation, NMR analysis and structure investigation. Valter Lombardi designed and performed the anti-inflammatory experiments and analyzed the results. K-E Lillsunde wrote the publication with contributions from co-authors.
- II** K-E Lillsunde performed the biological assays (cell-based assays and microscale thermophoresis testing) and analyzed the results. Assay design and result analysis were performed in collaboration with co-author Päivi Tammela. Co-authors Danijel Kikelj and Tihomir Tomašič were responsible for compound design and synthesis. K-E Lillsunde wrote the publication with contributions from co-authors.
- III** K-E Lillsunde performed the HCV replicon (genotype 1b) assays and microscale thermophoresis testing and analyzed the results. Assay design and result analysis were performed in collaboration with co-author Päivi Tammela. The screening against HCV replicon genotype 2a and full-length HCV was performed by co-author Philipp Schult. Co-authors Danijel Kikelj and Tihomir Tomašič were responsible for compound design and synthesis. K-E Lillsunde wrote the publication with contributions from co-authors.

ABBREVIATIONS

ATP	Adenosine triphosphate
CC ₅₀	50% cytotoxic concentration
CHIKV	Chikungunya virus
DAA	Direct-acting antiviral
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EGFP	Enhanced Green Fluorescent Protein
Fluc	Firefly luciferase marker protein
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HPV	Human papilloma virus
Hsp90	Heat shock protein 90
IC ₅₀	50% inhibitory concentration
IL	Interleukin
IRES	Internal ribosome entry site
JEV	Japanese encephalitis virus
K _d	Dissociation constant
MST	Microscale thermophoresis
NS/nsP	Non-structural protein
Rluc	<i>Renilla</i> luciferase enzyme
RNA	Ribonucleic acid
SARS-CoV	Severe acute respiratory syndrome-related coronavirus
S/B	Signal to background ratio
SI	Selectivity index
siRNA	small interfering RNA
S/N	Signal to noise ratio
SVR	Sustained virological response
TNF- α	Tumor necrosis factor alpha
Z'	Z' factor, screening window coefficient, parameter used to evaluate screening assay quality
17-AAG	17-N-allylamino-17-demethoxygeldanamycin
17-DMAG	17-dimethylaminoethylamino-17-demethoxygeldanamycin

1 INTRODUCTION

In an era where modern medicine has enabled remarkable control of infectious diseases worldwide, the global community continues to face severe challenges posed by longstanding, emerging and re-emerging infectious diseases, many of which are caused by viruses.¹ Despite that hundreds of viral pathogens known to cause human disease have been identified, approved antiviral therapies are available only for few.² Researchers are challenged by the outstanding ability of pathogens to adapt to an ever-changing environment, leading to drug resistance and changing patterns in spread of diseases.

The utilization of natural products in drug discovery keeps playing an important role in the development and discovery of new pharmaceuticals.³ In natural product drug discovery, marine natural products have gained increasing interest over the past decades.⁴ Oceans cover a majority of the earth's surface, but only part of the massive biodiversity that these unique environments represent have been studied.⁵ Even though our understanding of the expanse of marine species and the richness of underwater habitats is increasing, marine flora and fauna is threatened by multiple factors tracing back to human activities. Protection activities fail to keep up with the emerging threats such as microplastic pollution and alarming outcomes of global climate change, most importantly sea level rise, acidification, stratification, habitat destruction and changes in ocean circulation.⁶⁻⁸

While some natural products used in medicine are isolated from their natural origin or produced through full or partial chemical synthesis, natural products are especially important as a source of chemical leads.³ The drug properties of these lead compounds can be chemically optimized to improve aspects such as efficacy, safety and pharmacokinetic profile.

This thesis focuses on the discovery of compounds with antiviral activity based on marine natural products. By using viral replicon-based cell models, diverse sets of compounds can be screened for their potential to interfere with the replication of the target virus. The use of replicon models provides a safe, efficient and user-friendly means

for identification of compounds and marine-derived compound mixtures with interesting bioactive properties. In this work, the results of the cell-based replicon screens were utilized as a base for bioactivity-guided compound purification and characterization, successful chemical optimization of the identified hit compounds, characterization of hit compound properties and analysis of the potential mechanism of action.

2 REVIEW OF THE LITERATURE

2.1 Marine natural products in drug discovery

A majority of the planet's surface is covered by water, and oceans represent an enormous biodiversity that holds outstanding opportunities to find unique pharmacologically active substances.^{4,9} The biodiversity of the deep sea is among the highest on the planet, yet it has been estimated that less than 5% of the deep sea has been explored and less than 0.01% of the deep sea floor has been studied in detail.⁵ This suggests that much of the potential of marine organisms as sources of new drugs still remains undiscovered.

The use of marine-derived products has been present in traditional medicine in China and Japan for centuries, but the systematic exploring of marine sources for new drug candidates began in the 1960's and has raised interest in academia and the pharmaceutical industry ever since.⁴ As a result of intensive research in the area, hundreds of new marine-derived compounds are described every year.¹⁰ These compounds often represent unique and diverse chemotypes with interesting biological activities.^{10,11}

During the first decades of marine drug discovery, a great proportion of the new compounds isolated from marine organisms originated from marine invertebrates – up to 2008, approximately 75% of marine bioactive compounds were isolated from marine invertebrate phyla *Porifera* and *Coelenterate*.¹² The interest in bioactive compounds produced by marine micro-organisms is, however, steadily increasing. A recent review by Carroll and coworkers¹³ shows that since 2014, the number of new marine compounds isolated from marine microbes has exceeded the number of compounds isolated from invertebrates. Figure 1 visualizes this ongoing change in marine drug discovery. The number of new compounds from other sources than micro-organisms and invertebrates is comparably low and has stayed somewhat stable over the past years.¹³

It is also becoming evident that numerous bioactive natural products are not produced by the organism from which their isolation was originally described, but in fact by microbes living in symbiotic relationships with the eukaryotic host organism.¹⁴ An excellent example of such a compound is the marine-derived anticancer agent trabectedin (ET-743, Yondelis®) from the Caribbean mangrove tunicate *Ecteinascidia turbinata* which, based on genome sequencing data, is suggested to be a product of a symbiotic micro-organism of the tunicate.¹⁵

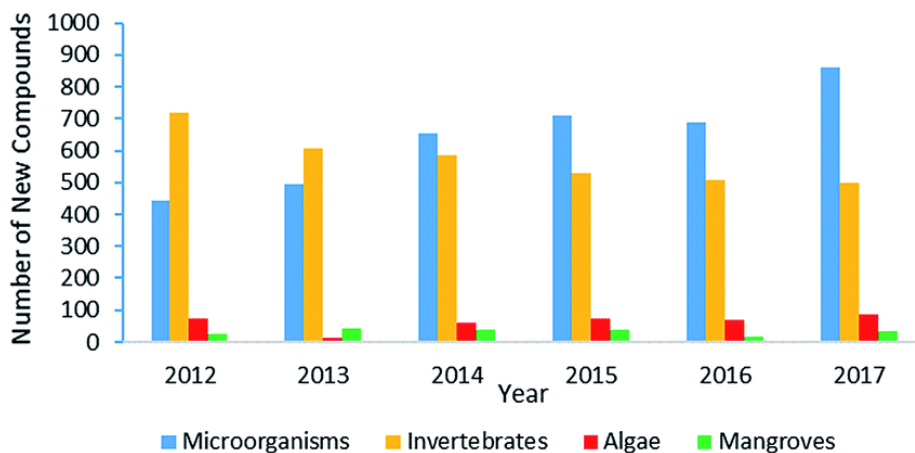


Figure 1. Sources of new marine compounds from year 2012 to 2017 (adapted from Carroll et al. 2019 ©The Royal Society of Chemistry).¹³

When analysing the chemical structures of the compounds isolated from marine sources, alkaloids and terpenes are the most abundant compound classes representing 23.66% and 22.46%, respectively, of the marine-derived compounds described in literature up to year 2012.¹⁰ They are followed by ethers and ketals (11.89%), sterides (9.76%), lactones (9.68%), peptides (8.80%) and hydroxybenzenes and chinones (6.99%).¹⁰

Anticancer drug development has historically been the core of marine drug discovery, which mainly has its roots in the allocation of research funding that has favored anticancer research over other targets.^{4,10} A review of biologically active compounds derived from marine sources between years 1985 and 2012 shows that more than

half of the new compounds were described to possess anticancer activity (Figure 2).¹⁰ Other bioactive properties reported for marine-derived compounds are antibacterial, antifungal, antiviral, pest resistance, prevention of cardiovascular disease and neuroprotection (Figure 2).

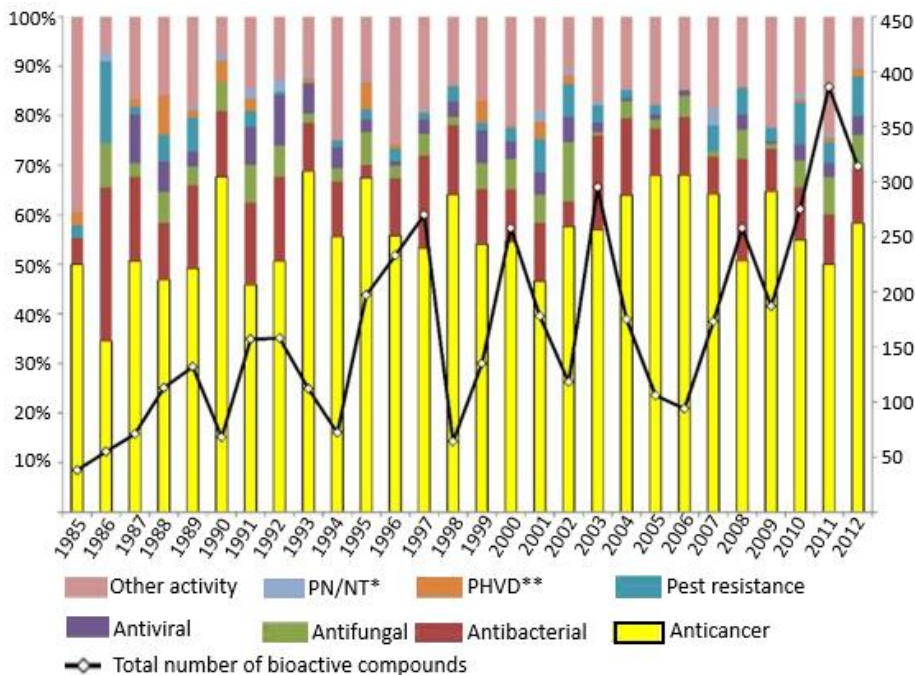


Figure 2. The proportion and amount of different bioactivity classes in marine-derived compounds discovered between years 1985 and 2012. *PN/NT: protection of neurons/neurotoxicity. **PHVD: prevention of heart and vascular disease. Figure adapted from Hu et al. 2015 © MDPI.¹⁰

The effort put on anticancer drug discovery over the past decades is also reflected by the fact that out of 11 marine-derived compounds approved for clinical use, 5 are anticancer drugs (Table 1).¹¹ The marine-derived drugs approved for clinical use represent a plethora of structurally and chemically diverse compounds. It shall also be noted, that not all compounds presented in Table 1 are registered as drugs, but as medical devices. For instance, the antiviral compound Carragelose® is marketed as a medical device.

Table 1. Marine-derived drugs and medical devices in clinical use.

Drug and trade name	Chemical class	Indication	Source	Reference
Brentuximab vedotin (Adcetris®)	Antibody-drug-conjugate consisting of monoclonal antibody cAC10 and cytotoxic agent MMAE	Anticancer	The cytotoxic agent MMAE is a synthetic derivative of dolastatin 10, a pseudopeptide from mollusc <i>Dorabella auricularia</i>	[16]
Cytarabine, Ara-C (Cytosar-U®)	Nucleoside	Anticancer	Synthetic derivative inspired by the arabinose-containing nucleosides spongothymidine and spongouridine from the Caribbean sponge <i>Tethya crypta</i>	[4]
Eribulin mesylate (Halaven®)	Macrocyclic ketone	Anticancer	Synthetic derivative of halichondrin B originally isolated from marine sponge <i>Halichondria okadae</i>	[17]

Drug and trade name	Chemical class	Indication	Source	Reference
Ethyl eicosapentaenoic acid (Vascepa®)	Fatty acid	Anti-hypertriglyceridemia	Fish oil-derived product	[11]
Iota-carrageenan (Carragelose®)	Sulphated galactose polymer	Antiviral	<i>Rhodophyceae</i> seaweeds	[18]
Omega-3-acid ethyl esters (Lovaza®)	Fatty acid	Anti-hypertriglyceridemia	Fish oil-derived product	[11]
Omega-3 carboxylic acids (Epanova®)	Fatty acid	Anti-hypertriglyceridemia	Fish oil-derived product	[11]
Polatuzumab vedotin (Polivy®)	Antibody-drug-conjugate consisting of anti-CD79 monoclonal antibody and cytotoxic agent MMAE	Anticancer	The cytotoxic agent MMAE is a synthetic derivative of dolastatin 10, a pseudopeptide from mollusc <i>Dorabella auricularia</i>	[4,19]
Trabectedin, ET-743 (Yondelis®)	Tetrahydro-isoquinoline alkaloid	Anticancer	Mangrove tunicate <i>Ecteinascidia turbinata</i> (Symbiotic micro-organism <i>Candidatus</i>	[15,20]

Drug and trade name	Chemical class	Indication	Source	Reference
			<i>Endoecteinascidia frumentensis</i>)	
Vidarabine, Ara-A (Vira-A®)	Nucleoside	Antiviral	Synthetic analogue of spongouridine isolated from Caribbean sponge <i>Tethya crypta</i>	[21]
Ziconotide (Prialt®)	Cyclic peptide	Analgesic	Synthetic version of peptidic neurotoxin produced by marine snail <i>Conus magus</i>	[22]

As shown in Table 1, nearly all clinically approved compounds result from chemical optimization of a marine lead structure. Indeed, natural products form only a small portion (6%) of new chemical entities introduced to the clinic, as synthetic derivatives of natural products or compounds inspired by natural products, are predominant.³ In addition to currently approved compounds, several marine-derived compounds are being investigated in clinical trials.^{4,11}

2.2 Marine-derived antivirals

This section discusses the antiviral compounds of marine origin that have been developed into clinical use. Additionally, an overview of the marine-derived antivirals bryostatins and griffithsin with potential for future clinical use in treatment and prevention of viral diseases is presented.

2.2.1 Vidarabine

Vidarabine (Ara-A; Vira-A®), Figure 3), a synthetic analogue of the nucleoside spongouridine, was the first marine-derived antiviral agent taken into clinical use.²¹ The isolation of spongouridine from the Caribbean sponge *Tethya crypta* was reported by Bergmann and Feeney already in 1951.²³ The peculiarity of spongouridine and concomitantly isolated spongothymidine is that the sugar unit in these nucleosides is arabinose instead of the more commonly occurring ribose. Vidarabine is active against herpes simplex virus and varicella-zoster viruses, although its use has largely ceased due to availability of more potent antivirals, such as acyclovir.^{21,24}

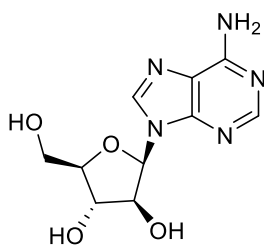


Figure 3. Structure of vidarabine.

2.2.2 Iota-carrageenan

Iota-carrageenan (Figure 4) is derived from red algae (*Rhodophyceae*).¹⁸ It is a sulphated galactose polymer commonly used in nutritional preparations and topical products¹⁸, marketed as an over-the-counter product under trade name Carragelose®.²⁵ Carrageenan polymers are classified into Iota-, Kappa- and Lambda-polymers depending on the amount and location of the sulphate moieties in the polymer structure.²⁶ In clinical evaluation of Iota-carrageenan nasal spray, a 0.12% saline solution administered three times a day upon early onset of common cold symptoms reduced the symptoms, the viral load of nasal lavages and levels of pro-inflammatory mediators.¹⁸ Administration of Iota-carrageenan nasal spray has been shown to reduce the duration of disease and reduce relapses of common cold caused by human rhinovirus, human coronavirus and influenza A virus.²⁷

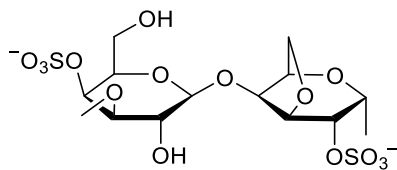


Figure 4. Structure of iota-carrageenan.

2.2.3 Bryostatins

Bryostatins are a class of more than 20 naturally occurring macrolidic cytotoxins originally isolated from marine bryozoan species *Bugula neritina*.⁴ Bryostatins occur in *B. neritina* in very low amounts, and it is likely that their actual producer is an uncultivated bacterial symbiont of the invertebrate.²⁸ Total synthesis for bryostatin 1 (Figure 5) has been developed and synthetic ring-expanded derivatives have also been reported.²⁹⁻³¹ Bryostatins and their analogues act mainly through modulation of protein kinase C activity and have been studied extensively as potential drugs against numerous targets, most notably Alzheimer's disease, cancer and HIV.^{4,32} In a clinical trial in HIV-infected patients, bryostatin 1 was well tolerated but did not affect protein kinase C activity or transcription of latent HIV.³³

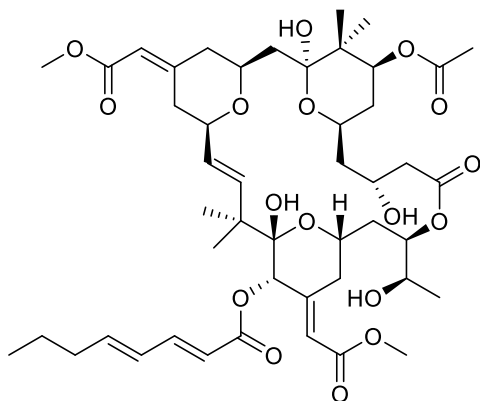


Figure 5. Structure of bryostatin 1.

Based on the assumption that only part of the bryostatin 1 structural moieties are accountable for its biological activity, a pharmacophore model has been used to guide the synthesis of

simplified bryostatin analogues, “bryologs” that *in vitro* induce the activation of latent HIV.³⁴ The reversion of HIV from latency while administering antiretroviral therapy would allow eradication of the reservoir cells. ³⁴ An *in vivo* study conducted in humanized mice showed that the bryostatin analog SUW133 was better tolerated than naturally occurring bryostatin 1 and it potently induced HIV expression from latency.³⁵ Besides HIV, bryostatin analogs might be promising candidates in the treatment of other latency-exhibiting viral infections, such as infection by herpes simplex viruses.⁴

2.2.4 Griffithsin

Griffithsin is a protein isolated from red alga *Griffithsia sp.* that shows interesting potential for drug development against numeral viral infections, including HIV, SARS-CoV and other related *Coronaviridae* viruses, HCV, HPV and JEV.³⁶ Griffithsin acts as a viral entry inhibitor that prevents the cell-to-cell transmission of the virus.^{36,37} Scalable manufacturing of griffithsin has been achieved in tobacco plants through the use of a tobacco mosaic virus vector expressing the protein.³⁷

In the study of plant-produced griffithsin on human cervical explants, griffithsin potently prevented infection of the explants by HIV-1.³⁷ In the same study, griffithsin was shown to be non-irritant and non-inflammatory in human cervical explants and in an *in vivo* rabbit model, while showing no detectable mitogenic activity on human peripheral mononuclear cells.³⁷ The beneficial safety profile of griffithsin has been further demonstrated in study of its effects on the rectal mucosal microbiome and proteome in rhesus macaques.³⁸ Furthermore, the clinical safety of vaginally and rectally administered griffithsin is currently being studied.^{39,40}

2.3 Hepatitis C virus (HCV)

The World Health Organization estimates that 71 million people globally are infected with hepatitis C virus (HCV) and that a significant proportion of infected patients develop liver cirrhosis or liver cancer.⁴¹ In 2016, HCV and its associated diseases led to the death of approximately 390 000 people.⁴¹ HCV is transmitted by exposure to

contaminated blood either percutaneously, from mother to infant, or by sexual intercourse.⁴²

2.3.1 HCV classification and characteristics

HCV is a positive-strand enveloped RNA virus that belongs to the genus *Hepacivirus* in the *Flaviviridae* family.⁴² HCV is a genetically heterogeneous group of viruses classified into seven genotypes that are distinguished by differences in genome, geographical distribution, progression of disease and treatment response.⁴³ Furthermore, the different genotypes are divided into subtypes. On the nucleotide level, the HCV genotypes differ from each other by 31-33%, whereas subtypes show 20-25% divergence.⁴⁴ Also intergenotypic and intersubtype HCV genomes do occur.^{45,46}

The HCV virion is approximately 45-75 nm in size and consists of the positive-strand RNA, the core protein that forms the viral nucleocapsid and two envelope glycoproteins, E1 and E2.^{47,48} The RNA genome contains almost 10 000 nucleotides and consists of a 5' non-translated region that acts as internal ribosome entry site (IRES), an open-reading frame that encodes the structural and non-structural proteins and a 3' non-translated region.^{47,49} The structural proteins consist of the core protein, the envelope glycoproteins E1 and E2 and viroporin p7, which is essential for production and release of new infectious virus particles from infected cells.^{48,50} The six non-structural proteins (NS) responsible for the viral replication process are the NS2-3 protease, the NS3 serine protease, the NS4A polypeptide, the NS4B and NS5A proteins and the NS5B RNA-dependent RNA polymerase.⁴⁷

2.3.2 HCV replication and host response

HCV replication mainly takes place in the liver parenchymal cells, the hepatocytes, which the virus enters through receptor-mediated endocytosis.⁴⁸ The envelope glycoproteins E1 and E2, which together are suggested to form a disulfide-bridge stabilized heterodimer mediate the receptor binding and cellular entry of the virus particle.⁴⁸ After cell entry the translation of the genomic RNA is directly initiated with mediation of the IRES.⁴⁹ The HCV half-life in the body of the infected individual is less than 3 hours and the replication process is

highly efficient with production and clearance rate of 10^{12} viral particles per day.⁵¹ Typically, the acute phase of HCV infection is asymptomatic, and without treatment, can lead to later onset of liver complications such as cirrhosis and hepatocellular carcinoma.⁵²

The immune response to HCV infection involves both innate and adaptive immunity and affects the progression of the infection in the acute and long-term phase.⁵³ Even within one host, HCV exists as a genetically diverse viral population, which makes tackling the virus challenging for the innate immune system and may lead to establishment of widespread infection before the innate immune response is activated.^{53,54} Despite this, approximately one out of four HCV patients spontaneously clear the virus, with a higher likelihood for viral clearance in female patients compared to men.⁵⁵

2.3.3 Model systems in HCV research

The study of the HCV life cycle and potential replication inhibitors took a leap forward in 1999 upon creation of a cell culture system utilizing Huh-7 hepatoma cells that express a subgenomic HCV replicon and a selectable marker.⁵⁶ In 2005, complete *in vitro* replication in cell culture of HCV genotype 2a, cloned from an individual with fulminant hepatitis, was achieved.^{57,58} The secreted virus, infectious for Huh-7 cells, was shown to be neutralized by antibodies and immunoglobulins from patients chronically infected with HCV.⁵⁷ Furthermore, the use of HCV pseudoparticles, related viruses such as the bovine viral diarrhoea virus, as well as chimpanzee and mouse animal models have been utilized in the study of progression of HCV infection, viral replication and host-HCV interactions.⁴⁷

2.3.4 Current HCV treatment

The treatment options for HCV infection have evolved rapidly in recent years upon the introduction of direct-acting antiviral (DAA) therapies.⁵⁹ The aim of HCV treatment today is to cure the infection, which means achieving a sustained virological response (SVR) defined as undetectable HCV RNA 12 weeks or 24 weeks after completing treatment.⁶⁰

Prior to the introduction of DAAs, HCV treatment was limited to interferon therapy, often applied in combination with the antiviral agent ribavirin⁶¹, a nucleoside analogue assumed to have multiple modes of action: mainly direct inhibition of HCV replication, inhibition of host inosine monophosphate dehydrogenase enzyme, induction of viral mutagenesis and induction of host immune response.⁶² Interferon therapy comes with the risk of adverse effects such as bone-marrow depression, flu-like symptoms, neuropsychiatric conditions and autoimmune disorders, whereas ribavirin treatment is associated with hemolytic anemia.⁶³ These issues led to problems with adherence to therapy as well as premature therapy withdrawals.

The first DAAs taken into clinical use in 2011 were NS3 protease inhibitors boceprevir and telaprevir that were used in triple therapy together with pegylated interferon alpha and ribavirin.^{64,65} The introduction of these DAAs to the treatment regimen improved the treatment outcomes of HCV as measured by the SVR, which was approximately 75% in patients infected with HCV genotype 1 and treated with triple therapy containing either boceprevir or telaprevir.^{64,65} After the introduction of the first NS3 protease inhibitors, numerous DAAs have been developed and many are in clinical trials, with treatment outcomes exceeding 95% SVR.^{59,66} The main targets in development of DAAs are HCV NS3, NS5A and NS5B.⁶⁶

Due to the fast and error-prone replication of HCV, resistance development towards DAAs is a critical issue in treatment of the virus despite the development of new efficient antivirals.^{66,67} To prevent occurrence of resistant mutations, most of the current treatment regimens involve dual or triple therapy combining DAAs with different mechanisms of action.⁶⁶ An overview of the DAAs and their combinations recommended for the treatment of HCV in the clinical practice guideline of the European Association for the Study of the Liver,⁶⁰ is provided in Table 2. As reflected by Table 2, the NS5B polymerase inhibitor sofosbuvir is nowadays the cornerstone of HCV treatment.⁶⁰ The current treatment options enable achieving a SVR that corresponds to cure of the infection with low risk of relapse. Long-term follow-up studies show that definitive cure of HCV is achievable in a vast majority of cases.⁶⁰

Table 2. Drugs and drug combinations recommended for clinical treatment of HCV in Europe.^{60,68-75}

Drug or drug combination	Mechanism of action
Sofosbuvir	NS5B polymerase inhibitor
Sofosbuvir/ velpatasvir	NS5B polymerase inhibitor/ NS5A inhibitor
Sofosbuvir/ velpatasvir/ voxilaprevir	NS5B polymerase inhibitor/ NS5A inhibitor/ NS3-NS4A protease inhibitor
Glecaprevir/ pibrentasvir	NS3-NS4A protease inhibitor/ NS5A inhibitor
Sofosbuvir/ ledipasvir	NS5B polymerase inhibitor/ NS5A inhibitor
Paritaprevir/ ombitasvir/ ritonavir	NS3-NS4A protease inhibitor/ NS5A inhibitor/ HIV protease inhibitor
Dasabuvir	NS5B polymerase inhibitor
Grazoprevir/ elbasvir	NS3-NS4A protease inhibitor / NS5A inhibitor

2.4 Chikungunya virus (CHIKV)

Chikungunya virus (CHIKV) causes a disease called chikungunya fever for which the characteristic acute symptoms are high fever, joint pain, myalgia, nausea, headache and rash.⁷⁶⁻⁷⁸ Besides the symptoms caused by acute infection, chikungunya fever typically leads to a chronic disease stage with relapsing rheumatic manifestations that

can persist for months or even years after the initial CHIKV infection.^{79,80} Asymptomatic infections have also been reported and estimated to occur in approximately 15% of the infected individuals.⁸¹ Herein, the features of the virus, its spread and applied strategies for antiviral drug development against CHIKV are discussed. As vaccine development is not in the scope of this thesis, the vaccine strategies are not included.

2.4.1 CHIKV classification and characteristics

CHIKV is classified as an alphavirus and it belongs to the family *Togaviridae*.⁸² The alphaviruses are positive-strand RNA viruses spread by arthropods that characteristically use vertebrates, including humans, as hosts. Alphaviruses are further classified into Old World and New World alphaviruses based on geographical distribution and infectious properties and, based on antigenic classification, into at least eight different categories.^{82,83} CHIKV is an Old World alphavirus that belongs to the Semliki Forest complex.⁸⁴

The alphavirus particles are 60-70 nm in diameter and their genetic material is protected by a capsid formed by a single protein.⁸² The alphavirus nucleocapsid is surrounded by a lipid envelope that contains two virus-encoded glycoprotein components, E1 and E2, which play an essential role in the attachment and entry of the virus to its host cell. In addition, a third surface protein E3, which has the same precursor as E2, is present on the virion surface.⁸⁵

The alphavirus genome is a single-stranded positive-sense RNA composed of 11 000 to 12 000 nucleotides with two open-reading frames and encodes for the structural and non-structural proteins.⁸² The non-structural protein open-reading frame is located at the 5' end of the genome and encodes for four proteins that the virus uses for transcription and replication of viral RNA, polyprotein cleavage and RNA capping.^{82,86} The structural open-reading frame encodes for the precursors of the three envelope and capsid proteins.⁸⁶ nsP1 is responsible for negative-strand RNA synthesis and RNA capping, nsP2 has NTPase, RNA helicase, RNA triphosphatase and thiol protease activities, nsP3 participates in RNA synthesis and nsP4 is a RNA-dependent RNA-polymerase.⁸⁶

2.4.2 CHIKV transmission, replication and epidemics

CHIKV is transmitted to the vertebrate host through the bite of an infected mosquito, most commonly belonging to the genus *Aedes*.⁸⁷ The alphavirus virions enter their host cell via receptor-mediated endocytosis, after which the virion, surrounded by an endosome, is released to the cytoplasm.⁸⁴ Following this, the viral nucleocapsid binds to the host ribosomes and the viral genomic RNA is released for translation.⁸⁴ After infection through a mosquito bite, CHIKV replicates in the skin fibroblast cells and spreads to the liver and joints.⁸⁸⁻⁹⁰

A CHIKV epidemics resulting in massive spread of the virus broke out in 2005 as the result of a single mutation in the viral genome.⁹¹ The mutation enabled the adaptation of the virus to the geographically widespread mosquito species *Aedes albopictus*, which is a common vector for arthropod-borne diseases.⁹² Due to adaptation to the *A. albopictus* vector, CHIKV epidemics are no longer restricted to tropical areas, but have been reported in regions with temperate climate, such as Italy and the south-eastern parts of France.⁹³⁻⁹⁵ In the Americas, CHIKV was first reported in 2013, and has since then spread into all Central American countries, northern Mexico and most of South America.⁹⁶⁻⁹⁸

2.4.3 Drug development strategies against CHIKV

The current treatment of chikungunya fever is symptomatic, including the use of analgesics, anti-inflammatory agents including corticosteroids, and sometimes amitriptyline or gabapentin, in case the patient suffers from neuropathic pain.⁹⁹⁻¹⁰⁰ Disease-modifying anti-rheumatic therapy such as methotrexate may be initiated at earliest 8 weeks after the acute phase of the disease.¹⁰⁰ Like in the acute phase, non-steroidal anti-inflammatory drugs and corticosteroids may also be used in the chronic phase. Since the massive epidemic that started in 2005, there has been a growing interest in studying vaccines and antivirals that could be used in prevention and treatment of CHIKV infection.¹⁰¹

An advantageous treatment option would be the use of an antiviral that has already been approved for clinical use in another indication,

as the safety profile of such an agent would already be well-characterized. The antiviral agent ribavirin, a guanosine analogue, has been shown to be effective against CHIKV *in vitro* in combination with interferon-alpha.^{102,103} In another study ribavirin was administered in combination with the tetracycline antibiotic doxycycline in mice animal models, and effective inhibition of CHIKV replication together with attenuated infectivity was achieved.¹⁰⁴ Despite the rather promising results achieved *in vitro* using interferon against CHIKV, the success in clinical use of interferons against other viruses than HCV has been modest.¹⁰⁵

The nucleobase mimetic favipiravir, originally discovered as an influenza A replication inhibitor, was shown to inhibit replication of laboratory strains and clinical isolates of CHIKV as well as other related alphaviruses *in vitro*.¹⁰⁶ Favipiravir also reduced CHIKV-induced disease in a mouse model compared to placebo treatment.

Various natural product-derived compounds have been studied in the search for potential anti-CHIKV activity. The natural product curcumin has been demonstrated to be active against CHIKV through blocking cell entry *in vitro*.^{107,108} Berberin, a plant-derived alkaloid compound, has been shown to inhibit CHIKV replication dose-dependently *in vitro*, to possess broad antiviral activity against related alphaviruses Semliki forest virus and Sindbis virus and to reduce CHIKV-induced inflammatory disease *in vivo* in a mouse model.^{109,110}

An alternative approach for identifying potential CHIKV inhibitors is application of computer-aided (*in silico*) techniques in screening of compound libraries, often accompanied by synthetic chemistry to optimize identified hits.¹⁰¹ Such techniques have been applied successfully to identify compounds that target the CHIKV nsP2, for which the three-dimensional structure is known.^{111,112} Furthermore, automated high-throughput screening can be applied to efficient studying of large compound libraries against CHIKV.¹¹³

Targeting the CHIKV viral genome directly has been studied in applications using small interfering RNAs, microRNAs and small hairpin RNAs.¹¹⁴⁻¹¹⁷ In a study where four artificial microRNAs were designed to target different regions of the CHIKV genome, the concomitant use of these microRNAs resulted in over 99% inhibition of CHIKV in a plaque reduction assay in Vero cells.¹¹⁷ In another study, a plasmid-based small hairpin RNA, targeted for CHIKV capsid

structural protein E1 and nsP1 genes, inhibited CHIKV replication in cell culture and in a mouse model of CHIKV infection *in vivo*.¹¹⁵

Neutralizing antibodies targeting the structural E2 protein of CHIKV have been shown to protect mice from infection *in vivo*.^{118,119} Interestingly, a mouse monoclonal antibody targeted for structural protein E1 showed reduction of the virus progeny in cells infected with CHIKV whilst the virus replication was not affected.¹²⁰ The further observation that the virus accumulates in the cells led the researchers to suggest the inhibition of virus release as the mechanism of action. Furthermore, monoclonal antibodies in combination with disease-modifying antirheumatic drug abatacept efficiently controlled foot swelling and reduced the levels of inflammation markers in a disease mouse model.¹²¹

Another interesting approach to CHIKV drug development is targeting the host cell factors and pathways that interfere with the life cycle of the virus. Protein kinase C is a family of kinases that play many roles in cellular functions and has been proposed as a potential host target in inhibition of CHIKV replication by Abdelnabi and coworkers.¹²² Prostatin, a protein kinase C activator, was shown to dose-dependently reduce the production of infectious CHIKV particles and halt the accumulation of viral proteins in human skin fibroblast cells.¹²²

Targeting polyamines, small molecules required for diverse cellular processes has been suggested as another possible method to inhibit CHIKV replication through a non-viral target.¹²³ CHIKV replication has been shown to be regulated by depletion of the polyamines spermidine and spermine as the virus requires these polyamines for genome transcription and translation.

2.5 Inhibition of Hsp90 as an antiviral strategy

This section reviews the role of Hsp90 in the viral life cycle with focus on HCV and CHIKV, which are used in the antiviral screening described in this work.

Heat shock protein 90 (Hsp90) is a family of molecular chaperones and one of the most common protein types encountered in cytoplasm with many essential cellular functions.¹²⁴ The vital role

of chaperones is explained by their ability to support other proteins in the cell through stabilizing otherwise unstable protein conformers and facilitating their function.¹²⁵ Hsp90 is a highly conserved protein found in organisms from bacteria to mammals.¹²⁴ Hsp90 exists in the cytoplasm in two main forms, constitutively expressed Hsp90 β and the inducible form Hsp90 α .¹²⁶ Cellular stress causes induction in the transcription of Hsp90 leading to approximately a doubled expression rate in cells that are subject to stress.¹²⁴

Viruses rely on the replication machinery of the host cell, and therefore viruses are as dependent on the chaperone functions as are the host cell proteins.¹²⁷ Numerous viral proteins are client proteins of Hsp90, and also some host factors participating in the progression of viral infection require Hsp90 for their processing.¹²⁸ Hsp90-dependent processes in the life cycle of viruses are mainly the entry of the virus to the host cell, intracellular trafficking, the expression of viral genes, genome replication, viral protein stabilization, the maturation of the virion and viral infectivity.¹²⁸

Hsp90 plays several roles that are essential for the lifecycle of HCV in the infected cell. An overview of the Hsp90 functions relevant for successful HCV propagation is presented in Table 3. As demonstrated in Table 3, Hsp90 has been identified to participate in HCV replication through interaction with NS3 and NS5.¹²⁹⁻¹³⁵ Furthermore, Hsp90 affects the expression of viral genes through interaction with the host cell protein argonaute2.¹³⁶ Inhibiting the function of Hsp90 thus has impact on several aspects of the progression of HCV infection in the host cell.

Table 3. The roles of Hsp90 in the HCV lifecycle.

Hsp90-dependent process	Hsp90 client protein	Role of Hsp90	Reference
Genome replication	NS3	Cleavage at NS2/3 junction, function of NS3	[129, 134,135]
Genome replication	NS5	Function of NS5, replication complex formation/genome replication	[130-133]
Stabilization of viral proteins	NS3	Stabilization of NS3	[134]
Expression of viral genes	Host cell argonaute2	Hsp90 stabilizes argonaute protein 2. Argonaute proteins bind small RNA components and coordinate expression of genes through interaction with other proteins.	[136]

The functions of Hsp90 in the life cycle of CHIKV have not been extensively studied, however, there is evidence that Hsp90 participates in CHIKV propagation. Rathore and co-workers demonstrated that CHIKV nsP3 and nsP4 are likely to be Hsp90 client proteins and that CHIKV replication in cell culture is disrupted by siRNA-mediated silencing of Hsp90 transcripts.¹³⁷ In addition, Hsp90 inhibitors were shown to reduce viral titres and reduce inflammation *in vivo* in a murine CHIKV model. Interestingly, both Hsp90 α and Hsp90 β isoforms interact with CHIKV non-structural proteins. In another study, Das and coworkers showed through immunoprecipitation studies that Hsp90 interacts with CHIKV nsP2

and that the translation of viral and cellular mRNA may be facilitated through a Hsp90-associated signaling pathway, suggesting that Hsp90 is required for the replication and production of viruses in CHIKV-infected cells.¹³⁸

The N-terminal ATP-binding domain of Hsp90 is the target site of most Hsp90 inhibitors, such as 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) and 17-*N*-allylamino-17-demethoxy-geldanamycin (17-AAG), which are commonly used as model compounds for Hsp90 inhibition.¹³⁹ Binding of compounds to this domain results in malformation and/or degradation of the Hsp90 client protein. Inhibitors targeting the C-terminal and middle regions of Hsp90 have also been described but are less common.

Despite the prospects of developing antivirals targeting Hsp90, the largest efforts have so far been put in developing Hsp90 inhibitors for the treatment of cancer. The role of Hsp90 in the life cycle of cancer cells is well characterized, and since the discovery of the first Hsp90 inhibitor in 1994, intensive research has been ongoing in order to develop anticancer agents that act through Hsp90 inhibition.¹⁴⁰

The clinical development of Hsp90 inhibitors has been hindered by the lack of target specificity, off-target effects and insufficient validation for the mechanism of action.¹⁴¹ The *in vivo* toxicity hampering the clinical development of Hsp90 inhibitors is likely related to the abundance, multiple roles and numerous client proteins of Hsp90 in cells, which makes targeting specific viruses and controlling cytotoxicity main priorities in developing Hsp90-targeted antivirals.¹²⁸

3 AIMS OF THE STUDY

This study aimed to identify novel marine-derived antiviral compounds, both by studying compounds isolated from marine organisms (**I**) and by exploring synthetic compounds inspired by marine lead structures (**II, III**). The aim was first to evaluate antiviral potential through simple and efficient primary screening methods to identify hit compounds. Moreover, the study aimed to characterize the hit compounds from the primary screening phase to evaluate the properties relevant for further drug development potential, such as specificity, toxicity and mode of action.

4 MATERIALS AND METHODS

4.1 Studied marine-derived material

The study covered screening for antiviral compounds from marine sources and included the study of both marine extracts and compounds isolated thereof as well as synthetic compounds inspired by marine lead structures. In publications **I – III**, the following types of samples were studied:

- **(I)**: Crude methanol extract of Indian Ocean soft coral *S. kavarattiensis*, chloroform- and ethyl acetate-enriched extracts and 7 purified compounds isolated from the chloroform-enriched extract. Soft coral species *S. kavarattiensis* was collected off the coast of Rameshwaram, Tamil Nadu, India (Latitude: 9°16'60''N Longitude: 79°17'60''E) in December 2010 by collaborators at CSIR-National Institute of Oceanography, India.
- **(II)**: Set of 157 analogues of the marine compounds clathrocin and oroidin, designed and synthesized by collaborators at the University of Ljubljana, Slovenia.
- **(III)**: Set of 12 synthetic compounds based on the 4,5,6,7-tetrahydrobenzo[1,2-*d*]thiazole-2-amine structure, designed and synthesized by collaborators at the University of Ljubljana, Slovenia.

Compound stock solutions were prepared in DMSO or ethanol and stored at -20° C.

4.2 Reference compounds

The antiviral agent 6-azauridine was used as reference compound in the CHIKV replicon screening. 6-azauridine was used also for assay validation and optimization prior to compound screening for both Rluc (luminescence) and EGFP (fluorescence) detection. 6-azauridine

exhibited dose-dependent inhibition during 48-hour treatment of cells, inhibiting the expression of the Rluc marker with an IC₅₀-value of 2 µM. 6-azauridine was obtained from Sigma-Aldrich Co. (Saint Louis, MO, USA), and the compound stock solution was prepared in DMSO.

Ribavirin obtained from Sigma-Aldrich Co. (Saint Louis, MO, USA) was used for optimization and validation of the HCV replicon assay and as antiviral control compound in the HCV replicon screening. Ribavirin stock solution was prepared in DMSO. In the 24-hour experiment setting, ribavirin inhibited expression of the HCV replicon with an IC₅₀-value of 110 µM. In the 48-hour experiment, ribavirin had an IC₅₀-value of 64 µM.

In cytotoxicity testing in CHIKV and HCV replicon cells, the cytotoxic agent polymyxin B (Sigma-Aldrich Co. Saint Louis, MO, USA) was used as control compound. Polymyxin B was also used for the validation and optimization of the cytotoxicity assays. Polymyxin B stock solutions were prepared in DMSO. In 24-hour treatment of HCV replicon cells, polymyxin B had a CC₅₀-value of 7900 IU/ml and in 48-hour exposure of BHK-CHIKV-NCT cells to polymyxin B the CC₅₀-value was 5900 IU/ml.

The characterized Hsp90 inhibitor 17-DMAG was used as reference compound for Hsp90 inhibition in cell-based studies and MST. 17-DMAG was obtained from InvivoGen (San Diego, CA, USA). In the BHK-CHIKV-NCT cell line, the IC₅₀-value of 17-DMAG was 0.14 µM. The IC₅₀-value in the HCV replicon cell line was 0.06 µM (exposure time 48 h). In MST experiments, the K_d for 17-DMAG was 0.27 µM. The compound stocks of 17-DMAG were prepared in ethanol.

4.3 Cell-based methods

4.3.1 Cell lines and culture conditions

A baby hamster kidney cell line (BHK-21) harboring a CHIKV replicon (BHK-CHIKV-NCT) was used in screening of potential CHIKV replication inhibitors. This CHIKV replicon cell line, provided and developed by Pohjala and co-workers¹⁴², continuously expresses two marker proteins, enhanced green fluorescent protein (EGFP) and *Renilla* luciferase (Rluc) along with the selection marker puromycin

acetyltransferase. EGFP and Rluc marker proteins can be used as optional indicators of viral replicon expression.

The CHIKV replicon cells were cultured in high-glucose Dulbecco's Modified Eagle's Medium with *L*-glutamine supplemented with 7.5% fetal bovine serum, 2% tryptose-broth phosphate, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 µg/ml streptomycin and 5 µg/ml puromycin. The cells were maintained aseptically at 37 °C with 5% CO₂ and 95% humidity and subcultured three times a week.

The cell line used for the screening of potential HCV inhibitors was a human liver cancer Huh-7 cell line expressing a subgenomic replicon of HCV genotype 1b when exposed to continuous geneticin selection.¹⁴³ Similarly to the CHIKV replicon cell line, the HCV replicon cell line expresses a marker protein (firefly luciferase, Fluc) that enables convenient detection of viral replicon inhibition. The cell line was kindly provided by Prof. Ralf Bartenschlager (University of Heidelberg, Germany).

The HCV replicon cell line was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, 292 µg/ml *L*-glutamine, 1% non-essential amino acids, and 250 µg/ml geneticin. The cells line was maintained aseptically at 37 °C with 5% CO₂ and 95% humidity and subcultured 2-3 times per week.

4.3.2 Screening for CHIKV replication inhibitors

For the CHIKV replicon inhibition assay (studies **I** and **II**), CHIKV replicon cells were seeded onto opaque-white, clear-bottomed 96-well plates (PerkinElmer Inc., Waltham, MA, USA). The cell-density used for the experiments was 40 000 cells/well. The plated cells were allowed to incubate for 24 hours at 37 °C.

The studied samples (dissolved compounds and soft coral extracts and extract fractions) and were diluted into assay medium that consisted of high-glucose Dulbecco's Modified Eagle's Medium and *L*-glutamine supplemented with 5% fetal bovine serum (FBS), 1 mM sodium pyruvate, 100 IU/ml penicillin and 100 µg/ml streptomycin. Each sample was tested in triplicate. Cells were exposed to compounds for 48 hours.

After exposing the cells to compounds, the Rluc marker expression was determined with a *Renilla* luciferase assay kit from Promega

(Madison, WI, USA) following the manufacturer's instructions. Varioskan Flash plate reader (Thermo Fischer Scientific, Vantaa, Finland) was used for luminescence detection. In the luminescence measurement, automatic dynamic range settings and a measurement time of 1 s was applied. In primary screening, each sample was tested in triplicate.

4.3.3 Screening for HCV replication inhibitors

For evaluation of HCV replicon inhibition in studies **II** and **III**, HCV replicon cells were seeded onto opaque-white, clear-bottomed 96-well microplates from PerkinElmer Inc (Waltham, MA, USA) using a cell density of 30 000 cells/well. The cells were allowed to incubate overnight at 37°C before starting the experiment.

Dissolved compounds were diluted in assay medium consisting of Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, 292 µg/ml *L*-glutamine and 1% non-essential amino acids. Each compound was tested in triplicate.

After exposure to compounds, the inhibition of the HCV replicon was measured by detection of the Fluc marker using a Luciferase Assay System kit from Promega Co. (Madison, WI, USA). In the primary screening of the compound library of clathrocin and oroidin analogues (study **II**), inhibition of the HCV replicon was evaluated after 24-hour exposure. In the next screening phases (studies **II** and **III**) a prolonged exposure time of 48 hours was applied. A Varioskan Flash plate reader (Thermo Fisher Scientific, Finland) was used for luminescence detection. In plate readings, automatic dynamic range settings and a measurement time of 1 s was utilized.

4.3.4 Cytotoxicity evaluation

The evaluation of compound cytotoxicity was performed in HCV and CHIKV replicon cell lines by ATP quantitation. The assay conditions, cell density and exposure time to compounds was the same as in the anti-replicon assays. Each compound was tested in triplicate. The composition of the assay medium used for HCV and CHIKV replicon cells is described in 4.3.2 and 4.3.3. Opaque-white,

clear-bottomed 96-well microplates (PerkinElmer Inc., Waltham, MA, USA) were used in all cytotoxicity assays.

ATP quantitation was carried out using a CellTiter GLO® Luminescent Cell Viability Assay kit (Promega, Madison, WI, USA). After exposing the HCV or CHIKV replicon cells to compounds for 24 or 48 hours, cells were allowed to equilibrate at room temperature, then washed with phosphate buffered saline solution before adding 50 µl CellTiter GLO® Reagent and 50 µl assay medium to plate wells. Cell lysis was induced by shaking the plates for 2 minutes, and after 10 minutes at room temperature, the luminescence signal was recorded with a Varioskan Flash plate reader (Thermo Fisher Scientific, Finland) as described in sections 4.1.2 and 4.1.3.

4.3.5 Live-cell imaging

A continuous cell culturing platform (Cell-IQ® Fluorescence, Chip-Man Technologies Ltd., Tampere, Finland) was used in study I. This method enabled simultaneous analysis of cell proliferation, morphology and expression of EGFP marker in CHIKV replicon cells. Clear-bottomed, black-framed 96-well plates from PerkinElmer Inc. (Waltham, MA, USA) were used in the experiment. 3000 cells/well were seeded onto plates and incubated for 24 hours at 37 °C before adding the compounds diluted in assay medium (for composition see section 4.3.2). All assays were performed in triplicate.

After exposing the cells to the compounds, the assay plate was equipped with a Cell-Secure Lid (Chip-Man Technologies, Tampere, Finland) with gas input connector and a sterile 0.2 µm filter. The imaging study was carried out for 48 hours by taking phase-contrast and fluorescent images every 30 minutes using a 10× objective. Incubation was carried out at 37 °C using the equipment manufacturer's default CO₂ flow settings. In phase-contrast imaging, exposure time was 10 ms and z-stack 17.60 µm. In fluorescent imaging, the manufacturer's default settings for green fluorescent protein with exposure time of 200 ms and binning 2 × 2 were used.

4.3.6 Data analysis and assay quality in cell-based experiments

In the calculation of CHIKV and HCV replicon inhibition based on luminescent marker protein detection, the luminescence signal for DMSO vehicle in assay medium was used as maximum signal, to which the luminescence signal of cells treated with compounds was compared. The inhibition of viral replicon was expressed as percentage compared to the maximum signal. The cytotoxic effect of compounds expressed as a percentage was calculated by using the signal from cells treated with DMSO vehicle only as maximum signal and wells with reagent only as background value.

In dose-response analyses, the dose-response data was fitted to sigmoidal dose-response curves by using the OriginPro 8.6 software (OriginLab, Northampton, MA, USA). Based on sigmoidal fitting, IC_{50} and CC_{50} -values were calculated using the software. Selectivity indices (SI) were obtained by dividing the CC_{50} with the IC_{50} .

Throughout the cell-based experiments based on luminescence detection, assay quality was monitored for each individual assay plate. The used quality parameters were signal-to-background ratio (S/B), signal-to-noise ratio (S/N) and screening window coefficient (Z'-factor).¹⁴⁴

6-azauridine (Sigma-Aldrich Co., Saint Louis, MO, USA) was used as control compound for the assay optimization and validation of the live-cell imaging assay. The imaging results were analyzed using the Cell-IQ Analyser® software (Chip-Man Technologies, Tampere, Finland) by creating analysis protocols following instructions from the software supplier. The cell counting protocol was created and optimized by defining segmentation parameters and building a sample cell library allowing the software to automatically classify cells as living, dead or dividing based on the cell morphology. The fluorescence intensity protocol was created and optimized for the assay conditions by adjusting brightness, contrast, gamma, background correction and fluorescence threshold. The live-cell imaging assay and analysis protocols were validated by running 48-hour tests for DMSO vehicle, control antiviral agent 6-azauridine and control cytotoxic agent polymyxin B.

4.4 Microscale thermophoresis

Microscale thermophoresis (MST) was used to study compound binding to Hsp90. Full-length human Hsp90 β (GenBank Accession No. AY359878) with C-terminal His tag) MW = 83 kDA, expressed in an *E. coli* expression system was obtained from Sigma- Aldrich Co. (Saint Louis, MO, USA). A Monolith NTTM Protein Labeling Kit RED-NHS (NanoTemper Technologies GmbH, Munich, Germany) was used for Hsp90 β labelling.

The dilution series of test compounds (16 concentrations, 0.003 to 100 μ M) and control compound 17-DMAG (14 concentrations, 0.0015 to 12.5 μ M) dissolved in ethanol were prepared using a buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM MgCl₂, 0.05% Tween-20 and 5% (V/V) ethanol. Three independent measurements were run for each compound.

Compounds dissolved in buffer were mixed with labeled Hsp90 β at a final concentration of 35 nM and transferred into Monolith NT.115 premium- coated capillaries (NanoTemper Technologies GmbH). A NanoTemper Monolith NT.115 instrument (NanoTemper Technologies GmbH) was used for MST measurements using 20% LED power and 20% MST power. Analysis of the results and calculation of dissociation constants was performed by using the NT Analysis software (NanoTemper Technologies GmbH) based on thermophoresis and temperature jump data from the three separate measurements.

5 RESULTS

This chapter summarizes the results of the original publications **I-III**. Publication **I** describes the study of Indian Ocean soft coral *Sinularia kavarattiensis*. The starting point of the study of *S. kavarattiensis* was bioactivity testing of methanol and methanol-chloroform extracts, followed by chemical fractionation and compound isolation. The isolated compounds' antiviral potential and cytotoxic properties were evaluated in a CHIKV replicon cell model using luminescence-based detection techniques and live-cell imaging with integrated fluorescence detection.

Publications **II** and **III** focused on exploring the antiviral potential of synthetic compounds that are based on a marine lead structure. Publication **II** covers the screening of a library of synthetic compounds inspired by alkaloid compound oroidin in CHIKV and HCV replicon models, along with the mode of action-studies based on the assumed target in the HCV replication cycle. Based on the results of study **II** and the assumed target protein, a series of novel synthetic compounds was designed. This compound series was studied for antiviral potential and potential mode of action against HCV (**III**).

5.1 Antiviral, cytotoxic and anti-inflammatory properties of *S. kavarattiensis* (I)

5.1.1 Primary screening and bioactivity-guided fractionation

The Indian Ocean soft coral *S. kavarattiensis* was studied as part of a larger bioactivity screening project and selected for further exploration due to promising results in the primary screening phase. The methanol extract of *S. kavarattiensis* decreased the level of Rluc marker in the BHK-CHIKV-NCT replicon cell line by 71%, whereas the methanol-chloroform extract decreased marker levels by 72%. Both extracts were tested at a 100 µg/ml concentration. In primary cytotoxicity testing, the methanol extract caused a 15% reduction in

ATP levels compared to the control, whereas a 17% reduction was observed for the methanol-chloroform extract. Both extracts inhibited the Rluc marker expression in a dose-dependent manner.

Following the bioactivity studies on the crude extracts, a fractionation was performed for the methanol extract and the same bioactivity assays were performed for the obtained enriched extracts. The chloroform-enriched extract decreased Rluc marker levels by 47% and showed 24% cytotoxicity as measured by reduction in ATP levels at concentration 100 $\mu\text{g/ml}$. The ethyl acetate-enriched extract (100 $\mu\text{g/ml}$) decreased Rluc marker levels by 65% and caused 20% reduction in ATP levels. The chloroform-enriched extract was selected for further fractionation, resulting in isolation of six known norcembranoid compounds **1-6** (**I**) and one new compound, kavaranolide (**7**, **I**). The chemical structures of compounds **1-7** (**I**) are shown in Figure 6.

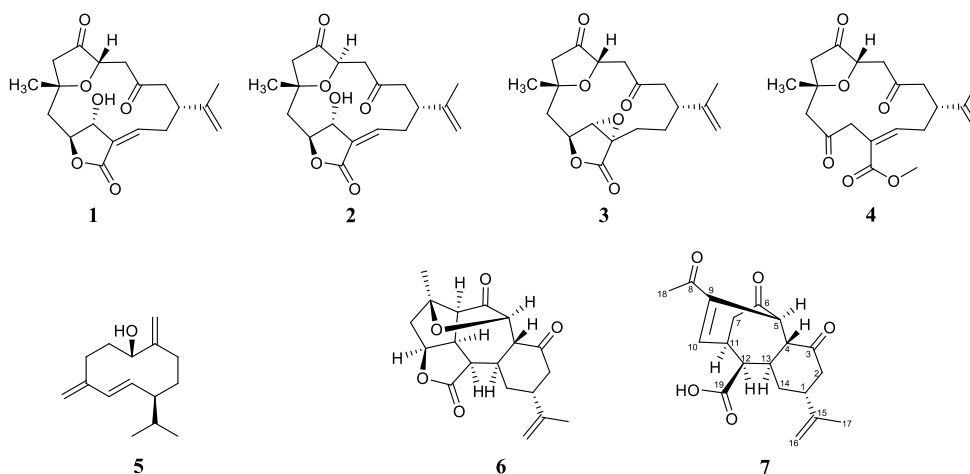


Figure 6. Compound structures **1-7** (**I**).

5.1.2 Primary evaluation and imaging studies of isolated compounds

In the primary evaluation phase, isolated compounds **1-3** (**I**) and **5-7** (**I**) were tested at a single 100 μM concentration for inhibition of the CHIKV replicon and cytotoxicity. The compounds caused an inhibition of the CHIKV replicon between 0.5% and 63% as shown by decrease of Rluc marker expression (Figure 7) and only slight cytotoxicity (<6%). Compounds **1** and **2** (**I**) showed the strongest

inhibition, with more than 60% reduction of Rluc marker expression compared to the vehicle control.

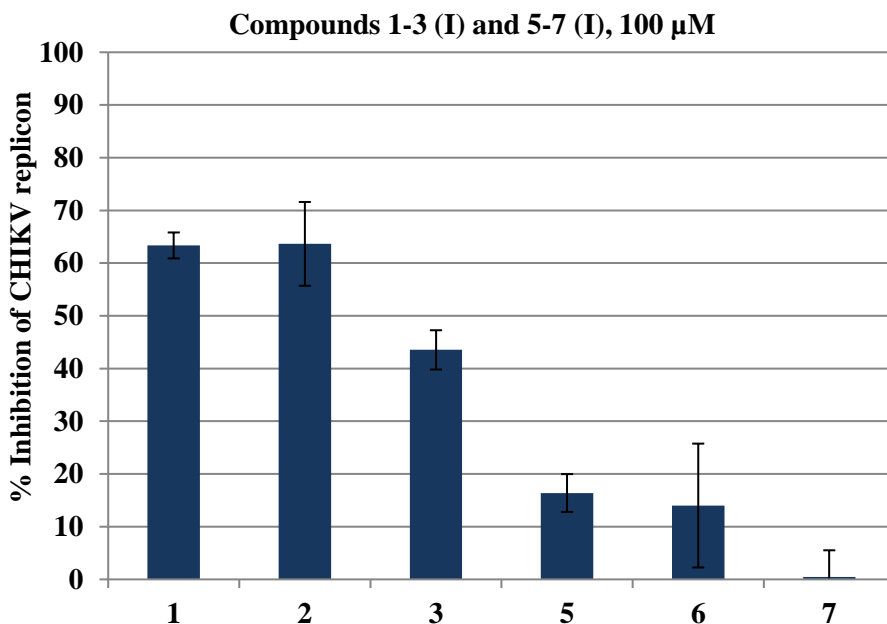


Figure 7. CHIKV replicon inhibition of compounds **1-3 (I)** and **5-7 (I)**, determined by the measurement of Rluc marker after 48 h. Inhibition percentages are expressed as the average of three replicates. Error bars represent standard deviation. The positive control, 6-azauridine, inhibited the replicon with an average IC_{50} value of 2 μ M.

As follow-up, a live-cell imaging study with integrated fluorescence intensity measurement for the detection of EGFP marker protein was conducted for BHK-CHIKV-NCT cells treated for 48 h with compounds **1-3 (I)** and **5-7 (I)**. The imaging study confirmed the results of the Rluc marker experiment, showing that compounds **1** and **2 (I)** decreased the fluorescence marker protein levels more than compounds **5**, **6** and **7 (I)**, that caused only minor decrease of the fluorescence intensity (Figure 8). The EGFP expression inhibition by compound **1 (I)** was, however, not as prominent as the Rluc marker decrease observed previously. Likewise, compound **3 (I)** did not decrease fluorescence intensity in the imaging experiment at the

tested concentrations 50 and 100 μM , despite that a 44% decrease of Rluc marker was observed by luminescence detection.

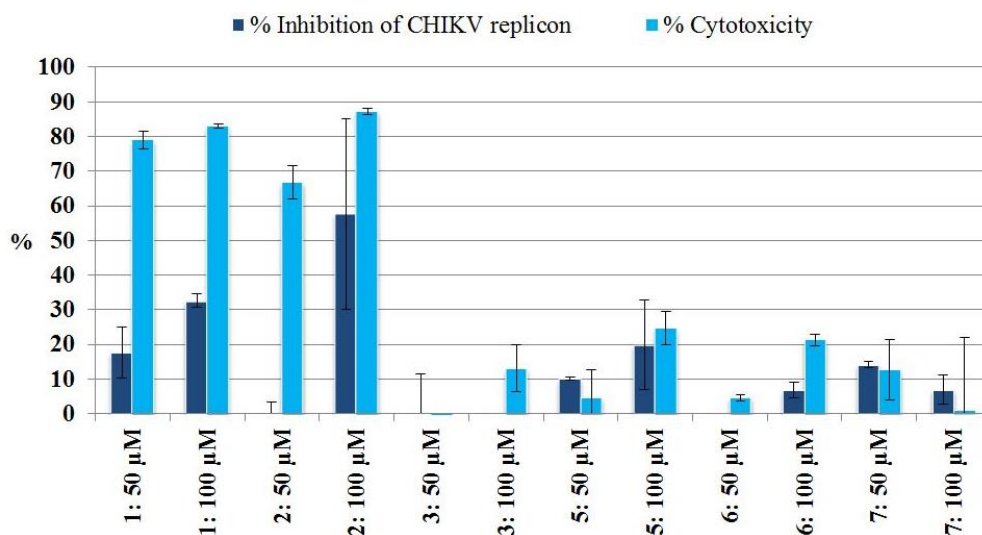


Figure 8. Inhibition of CHIKV replicon (%) and the cytotoxicity (%) of compounds **1-3 (I)** and **5-7 (I)** measured by Cell-IQ® live cell imaging in the BHK-CHIKV-NCT cell line after 48 h exposure. Results are presented as the average of two independent assays with three replicates each; error bars represent standard deviation.

An additional benefit of the imaging study was that it enabled a more thorough study of the compounds' cytotoxic properties. Live-cell imaging revealed the cytotoxic properties of compounds **1** and **2 (I)** that were not observed in the ATP quantitation experiment. In the imaging of cells treated with 100 μM of **1** and **2 (I)**, the number of cells classified as living or dividing decreased by >80%, even though the decrease of fluorescence intensity was not as dramatic. Figure 9 shows images of changes in morphology and expression of EGFP in cells treated with 50 μM and 100 μM of compounds **1-3 (I)**, **5-7 (I)**, control 6-azauridine and the vehicle DMSO captured after 48 h exposure.

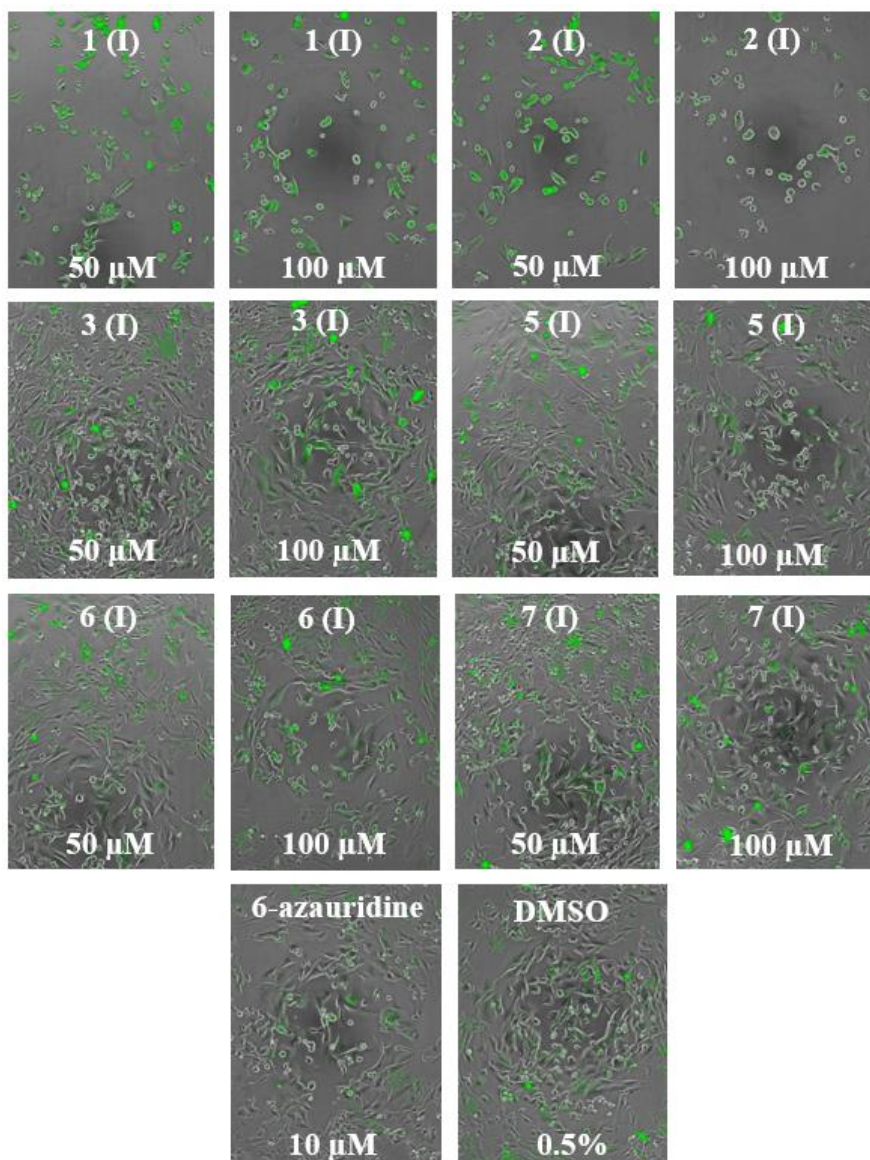


Figure 9. Photomicrographs captured using Cell-IQ® after 48 h exposure of BHK-CHIKV-NCT cells to **1-3 (I)**, **5-7 (I)**, control compound 6-azauridine and vehicle DMSO.

The cell density used in experiments was hypothesized to be an underlying reason behind the observed cytotoxicity of compounds **1** and **2 (I)**. To further study the impact of cell density, additional ATP

quantitation experiments were performed for compounds **1** and **2 (I)** by using the same, lower, cell density (3000 cells/well) as in the imaging studies. These experiments showed that the cell density indeed plays a major role in the resistance to toxic effects of the compounds. At the lower cell density of 3000 cells/well, a 100 μM concentration of compounds **1 (I)** and **2 (I)** reduced ATP levels by 39% and 44%, respectively.

5.1.3 Anti-inflammatory and immunomodulatory activity of purified compounds

The anti-inflammatory activity of compounds **1–4** and **6 (I)** was evaluated in primary microglial cell cultures. In this study, compound **2 (I)** induced a shift from amoeboid microglia to ramified microglia shapes, which indicates that **2 (I)** shows potential to modulate the cellular immune response. In addition to the observed effect on cell morphology, decreased levels of pro-inflammatory cytokines IL-1 β , IL-6, IL-8, IL-18 and TNF- α and increased levels of anti-inflammatory cytokines IL-4 and IL-10 were observed in cell cultures treated with 2.5 $\mu\text{g/ml}$ (7.2 μM) 5.0 $\mu\text{g/ml}$ (14.4 μM) of compound **2 (I)**.

Compound **2 (I)** also effectively and dose-dependently inhibited cytokine release in dose-response experiments. The IC_{50} -values for cytokine release inhibition ranged from 0.5 $\mu\text{g/ml}$ to 2.9 $\mu\text{g/ml}$ for the tested cytokines, being considerably lower than the corresponding values for the positive control prednisolone (IC_{50} range 10–20 $\mu\text{g/ml}$). The IC_{50} -value for cytotoxic activity was higher than the maximum test concentration 100 $\mu\text{g/ml}$, thus cytotoxic effects did not impact the results.

5.2 Antiviral potential of synthetic clathrocin and oroidin analogues and their derivatives (II, III)

5.2.1 Primary evaluation, hit selection and optimization

A library of 157 analogues of the marine compounds clathrocin and oroidin was screened for potential antiviral activity in BHK-

CHIKV-NCT and HCV-Huh-7 replicon cell lines. The compound concentration in the initial testing was 50 μM . 15 compounds inhibited the Rluc marker expression in the CHIKV replicon cell line by more than 50% (hit rate 9.6%) and 30 compounds inhibited the Fluc marker expression in HCH-Huh-7 cells by more than 50% (hit rate 19%).

The hit compounds from the primary screens were tested for cytotoxicity by ATP quantitation and the dose-response was evaluated in the viral replicon models. A majority of the primary hits were excluded in this phase because of toxic effects, lack of clear dose-response correlation or lack of efficacy.

Four compounds from the initial library (Figure 10) inhibited the HCV replicon marker expression in a dose-dependent manner with IC_{50} -values ranging from 1.6 to 4.6 μM and selectivity indices in the range of 22 to 61. Out of the four hits, compound **2** (**II**) inhibited HCV replicon expression with the lowest IC_{50} -value (1.6 μM) and highest selectivity (SI 60).

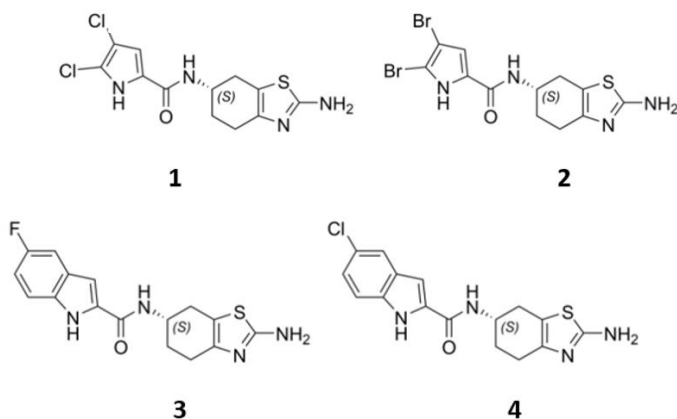
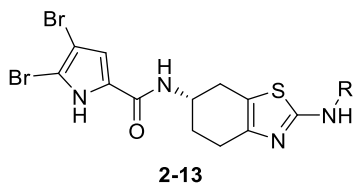


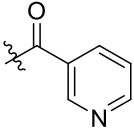
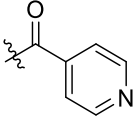
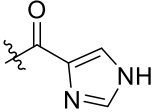
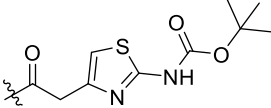
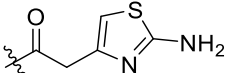
Figure 10. Compound structures **1-4** (**II**).

To achieve improved binding to Hsp90, a series of 12 new structurally optimized compounds was designed and synthesized based on compound **2** (**II**), which showed the highest potential for HCV inhibition in the initial series of clathrocin and oroidin analogues. In the novel series of compounds, the 2-amino group of the 4,5,6,7-tetrahydrobenzo[1,2-*d*]thiazole-2-amine was substituted via amide bond with side chains containing heteroaromatics, amino groups, or carbamates for improved interaction with Hsp90 (Table 4).

Table 4. Compound structures **2-13 (III)**.



Compound	R
2	
3	
4	
5	
6	
7	
8	

9	
10	
11	
12	
13	

The 12 new compounds were initially screened in the HCV replicon cell model at a concentration of 50 μM . Based on the primary evaluation and initial cytotoxicity studies using ATP quantitation, compounds **2**, **5**, **6**, **8**, **10** and **11** (**III**) were selected for further assessment of dose-response and cytotoxicity. The selected compounds inhibited the HCV replicon dose-dependently without inducing toxic effects at replicon-inhibiting concentrations (Table 5). Compounds **2**, **5** and **8** (**III**) were finally selected for further assessment, as they inhibited the HCV replicon selectively at low micromolar concentrations. Compound **5** (**III**) inhibited the HCV replicon marker expression with an IC_{50} -value of 1.3 μM and possessed the highest selectivity ($\text{SI}=98$), showing clear improvement in selectivity compared to compound **2** (**II**) and thus proving the optimization strategy successful (for all bioactivity results, see Table 5).

5.2.2 Mode-of-action studies

Each hit compound **1-4 (II)** from the primary screening of the full compound library had originally been designed to target the ATP-binding site of bacterial DNA gyrase and shared the common structural feature of an unsubstituted 2-amino group on a 4,5,6,7-tetrahydrobenzo[1,2-*d*]thiazole core and a hydrophobic moiety either as a substituted pyrrolamide or indolamide (Figure 10). As bacterial DNA gyrase and the cellular chaperone Hsp90 share a structurally similar ATP-binding pocket¹⁴⁵, Hsp90 was considered a potential target for the compounds.

The effect of a known Hsp90 inhibitor, 17-DMAG, was evaluated in the HCV-Huh-7 cell line to assess the effect of Hsp90 inhibition on the HCV replicon marker expression. In dose-response experiments, 17-DMAG inhibited the expression of the HCV replicon with an IC₅₀-value of 0.06 μM, thus showing that HCV replicon inhibition caused by compounds **1-4 (II)** could be associated with binding to Hsp90.

To further evaluate Hsp90 as a target, the potential binding modes of compounds **1-4 (II)** to Hsp90 were studied by molecular docking, which supported the possible interaction with this target. This potential mode of action was therefore studied by means of microscale thermophoresis (MST). The obtained *K_d*-values for the interaction of compounds **1-4 (II)** with Hsp90 isoform β were in the range of 18-79 μM (Table 5), thus confirming the possible interaction, which was, however, considerably weaker than that observed for the positive control 17-DMAG (*K_d* 0.27 μM).

To assess the applied optimization strategy, the interaction of structurally optimized 4,5,6,7-tetrahydrobenzo[1,2-*d*]thiazole derivatives with Hsp90β was also studied by MST. Based on the results of the HCV replicon (genotype 1b) screen, the binding properties of compounds **2, 5, 6, 8, 11** and **13 (III)** were assessed. Compounds **8** and **13 (III)** showed the strongest interaction with the assumed target protein, with *K_d* values of 14±2.4 μM and 12±1.9 μM and slightly improved binding compared to compounds **1-4 (II)** (Table 5).

Table 5. Bioactivity of compounds **1-4 (II)** and **2, 5, 6, 8, 10, 11, 13 (III)** and control compound 17-DMAG in HCV replicon models, against full-length HCV (**2, 5** and **8, III**) and affinity to human Hsp90 β of compounds **1-4 (II)** and **2, 5, 6, 8, 11, 13 (III)** and 17-DMAG determined by MST. The IC₅₀ and CC₅₀ values are reported as average +/- standard deviation of two (genotype 2a) or three (genotype 1b, full-length virus assay) replicates. MST results are presented as dissociation constant $K_d \pm$ standard deviation based on three separate measurements.

Compound	IC ₅₀ HCV geno- type 1b (μ M)	CC ₅₀ HCV geno- type 1b (μ M)	SI geno- type 1b	IC ₅₀ HCV genoty- pe 2a (μ M)	IC ₅₀ full- length HCV (μ M)	K_d Hsp90 (μ M)
1 (II)	4.6 \pm 0.43	101 \pm 7.9	22	<i>nd</i>	<i>nd</i>	19 \pm 5.7
2 (II)	1.6 \pm 0.08	98 \pm 8.7	61	<i>nd</i>	<i>nd</i>	39 \pm 14
3 (II)	3.1 \pm 0. 8	84 \pm 1.8	27	<i>nd</i>	<i>nd</i>	79 \pm 17
4 (II)	2.8 \pm 0.35	78 \pm 4.0	28	<i>nd</i>	<i>nd</i>	18 \pm 3.4
2 (III)	2.5 \pm 0.25	154 \pm 2.9	62	1.52 \pm 0.15	0.60 \pm 0.1	28 \pm 5.0
5 (III)	1.3 \pm 0.23	128 \pm 6.6	98	0.49 \pm 0.06	0.03 \pm 0.006	16 \pm 5.1
6 (III)	22 \pm 2.8	67 \pm 2.0	3.0	<i>nd</i>	<i>nd</i>	27 \pm 2.2
8 (III)	1.2 \pm 0.41	48 \pm 2.5	40	1.32 \pm 0.1	0.10 \pm 0.02	14 \pm 2.4

Compound	IC ₅₀ HCV geno- type 1b (μ M)	CC ₅₀ HCV geno- type 1b (μ M)	SI geno- type 1b	IC ₅₀ HCV genoty- pe 2a (μ M)	IC ₅₀ full- length HCV (μ M)	K _d Hsp90 (μ M)
10 (III)	5.5 \pm 1.8	204 \pm 4.9	37	<i>nd</i>	<i>nd</i>	<i>nd</i>
11 (III)	21 \pm 2.2	86 \pm 5.2	4.1	<i>nd</i>	<i>nd</i>	40 \pm 4.9
13 (III)	7.2 \pm 0.46	72 \pm 4.2	10	<i>nd</i>	<i>nd</i>	12 \pm 1.9
17-DMAG	0.06 \pm 0.01	1.7 \pm 0.25	28	0.04 \pm 0.006	*	0.27 \pm 0.02

nd = not determined

* no effect in the tested concentration range (IC₅₀>5 μ m) or time frame

5.2.3 Antiviral activity against full-length HCV

Compounds **2**, **5** and **8 (III)** were selected as the most promising HCV inhibitor candidates for further evaluation based on the highest selectivity and lowest IC₅₀-values in the HCV genotype 1b replicon studies. These compounds were tested for antiviral activity against HCV by using a method based on the electroporation of full-length reporter virus RNA into Huh7 Lunet cells.

As replicating full-length reporter viruses are derived from HCV genotype 2a, the compounds were first evaluated in a HCV genotype 2a replicon cell line, similarly to the HCV replicon genotype 1b evaluation. Based on determination of IC₅₀-values of the test compounds and the 17-DMAG control, HCV genotype 2a turned out to be equally susceptible to Hsp90 inhibition as genotype 1b. Compounds **2**, **5** and **8 (III)** inhibited the HCV genotype 2a replicon with IC₅₀-values of 1.52 μ M, 0.49 μ M and 1.32 μ M, respectively. Also, the 17-DMAG control was equally effective against genotype 2a (IC₅₀ = 0.04 μ M).

All three compounds **2**, **5** and **8 (III)** were also effective in the full-length reporter virus assay. Compound **5 (III)** had the lowest IC₅₀-value of 0.03 μ M, whereas the IC₅₀-values for **8** and **2 (III)** were 0.05

μM and $0.6 \mu\text{M}$, respectively (Table 5). On the contrary, 17-DMAG did not inhibit the replication of the full-length virus in the tested concentration range and time frame. In a study by Kim and colleagues¹⁴⁶ Huh7 cells infected with cell-culture-generated HCV genotype 2a (clone JFH1) were treated with 17-DMAG for 72 hours. In their study, 17-DMAG decreased HCV RNA levels with an IC_{50} value of $0.071 \mu\text{M}$. In our experimental setting, the exposure time to the compounds was only 24 hours, which might be a reason underlying the lack of efficiency of 17-DMAG.

6 DISCUSSION

The studies **I-III** cover two different approaches to marine drug discovery. On one hand, we studied a marine soft coral extract, which led to bioassay-guided fractionation, compound purification and bioactivity studies of the purified compounds. On the other hand, we utilized an approach based on bioactivity screening of a synthetic compound library inspired by marine structures, followed by chemical optimization of one of the hit compounds.

In both of these approaches, the aim was the discovery of potential new antiviral agents. Reflecting the results towards this goal, study **I** led to the discovery of crude extracts and enriched fractions of *S. kavarattiensis* with promising activity in the CHIKV replicon cell line, but the study of purified compounds showed cytotoxic effects and lack of specificity, leading to the conclusion that the compounds would likely not be of interest as potential antivirals. The modest activity of purified compounds in a specific bioactivity assay compared to the promising activity observed for extracts is a well-known phenomenon in natural product drug research.¹⁴⁷

Natural product extracts are mixtures of hundreds, or sometimes thousands, of individual chemical constituents at different concentrations, and it is frequently challenging to assign the activity of an extract to one or few compounds in the mixture.¹⁴⁸ The reason behind the higher biological activity of extracts is often claimed to be the beneficial synergistic interactions of the compounds in the mixture. Also antagonism, where the effects of one active constituent in a mixture are masked by another component, can affect the bioactivity studies of mixtures, which constitutes another challenge to natural product-based drug discovery.¹⁴⁷

In the study of marine bioactive compounds derived directly from the marine source organism, such as the work described in publication **I**, supply issues must be carefully considered. Various strategies can be applied to overcome supply challenges, ranging from compound isolation from the source organism to utilizing microbial metabolites and applying total synthesis.¹⁴⁹ Both for ecological and economic

reasons it is oftentimes favorable to explore the potential synthetic routes instead of collecting and processing the source organism.

Despite the drawback of not identifying remarkable antiviral activity for any of the purified compounds of *S. kavarattiensis*, one previously undescribed compound, kavaranolide, was identified in study **I**, and compound **2 (I)** was shown to possess promising anti-inflammatory and immunomodulatory potential. Thus, the results contributed to a better understanding of the soft coral species *S. kavarattiensis* and its bioactive potential. The results also highlight the importance of early cytotoxicity-profiling of studied extracts and isolated compounds. Taking into consideration that the plethora of marine diversity to date is only partly explored, numerous opportunities remain to discover potent bioactive compounds through exploration of natural resources.

Studies **II** and **III** on synthetic analogues of marine natural products enabled the exploration of the individual compounds for which the chemical structure responsible for the observed bioactivity was known. Furthermore, the structure-activity relationships could be studied, and chemical optimization opened new pathways to improve the bioactivity. The study of synthetically obtained marine compounds or their derivatives as exemplified in publications **II** and **III** is, unlike the study of compounds isolated from the marine source organism, not limited by sufficient compound supply or challenges in the sustainable exploitation of natural resources.

From a methodological point of view, studies **I** to **III** include a selection of methods well-adapted for the study of potential antiviral properties of diverse compound libraries. Each of these studies also demonstrate the strength of a close multiprofessional collaboration that covers chemical characterization and synthesis combined to bioactivity profiling. In studies **I** to **III**, knowledge of the compound chemistry that was brought to each project through close collaboration with scientists experienced in chemical synthesis and analytics enabled coherent directing and planning of the bioactivity assays.

Replicon-based screening approaches are an excellent tool for primary study of potential viral replication inhibitors, as they are safe, user-friendly and easily adaptable to a multiwell-plate format. Replicon-based screening methods, similar to those described in this

work, have been used in antiviral screening against various other human pathogens, such as Dengue virus and West Nile virus.^{150,151} In replicon-based screening methods, fluorescent and luminescent proteins are often used as markers, as they enable efficient and robust result detection. While being accessible and convenient, the viral replicon-harboring cell lines are limited in terms of correspondence to all aspects of the clinical virus infection, as inhibitors of the entry and maturation phases in the viral life cycle cannot be identified by using this method.

Study **II** resulted in the identification of hit compounds with selective activity and low micromolar IC₅₀-values in the HCV replicon model. Compound **2 (II)**, which inhibited the HCV replicon expression with the lowest IC₅₀-value (1.6 μM) and highest selectivity (SI 60) was selected as the lead compound for further structural optimization, which resulted in the synthesis of 12 new compounds bearing the 4,5,6,7-tetrahydrobenzo[1,2-*d*]thiazole structure. The optimization strategy turned out to be successful considering the improved selectivity (SI 98) of compound **5 (III)** compared to the lead compound (**2 [II]**, SI 60). The low cytotoxicity of compound **2 (II)** derivatives is especially outstanding when comparing to the reference compound 17-DMAG. Compounds **2 (III)** and **5 (III)** did not show cytotoxic effects at replicon-inhibiting concentrations in the HCV genotype 1b replicon cells (CC₅₀ values >100 μM), whereas the CC₅₀ value for the reference compound 17-DMAG was 1.7 μM with a selectivity index of 28.

The structural optimization of compound **2 (II)** did not result in major improvement in the affinity to the assumed target protein Hsp90. The binding affinity of the positive control 17-DMAG to Hsp90 was approximately 50-fold to that of compounds **8** and **13 (III)**, which were the compound **2 (II)** derivatives with the strongest affinity to Hsp90. There is also no clear correlation between the replicon-inhibiting activity and affinity to Hsp90 of the studied compounds **1-4 (II)** and **2, 5, 6, 8, 10, 11** and **13 (III)**. Therefore, it is possible that, in addition to Hsp90, there are other cellular targets underlying the activity. This could also contribute to explaining why the control Hsp90 inhibitor 17-DMAG was active in the HCV replicon assays, but inactive in the full-length reporter virus assay.

Hsp90 plays several roles in the HCV and CHIKV lifecycles. Hsp90 enables HCV replication through interaction with two viral non-structural proteins, NS3 and NS5¹²⁹⁻¹³⁵ and impacts the expression of viral genes through interaction with argonaute2, a host cell protein.¹³⁶ CHIKV nsP3 and nsP4 are probable Hsp90 client proteins and Hsp90 inhibitors reduce viral titres and inflammation *in vivo*.¹³⁷ Hsp90 also interacts with CHIKV nsP2, and the translation of viral and cellular mRNA may be facilitated through a Hsp90-associated signaling pathway.¹³⁸ Inhibiting the function of Hsp90 thus interferes with several aspects of the progression of HCV and CHIKV infection in the host cell.

Targeting host factors such as Hsp90 in antiviral drug development could be an advantageous strategy in combatting drug-resistant viral strains. Because of the multiple roles of Hsp90 in the cellular life cycle, the foremost challenge in the clinical development of Hsp90 inhibitors is the *in vivo* toxicity related to the off-target effects and challenges with target specificity, resulting in the failure and termination of many Hsp90 inhibitor-associated clinical trials.¹⁴¹ Study **III** demonstrated that compounds **2 (III)** and **5 (III)** did not show considerable cytotoxicity *in vitro*. These results are especially encouraging when comparing compounds **2 (III)** and **5 (III)** to the reference Hsp90 inhibitor 17-DMAG, which is potent, but also cytotoxic at low concentrations, indicating a narrow therapeutic window.

7 CONCLUSIONS AND FUTURE PROSPECTS

The main findings of the work presented herein are:

1. The results of study **I** contributed to a deeper understanding of the chemical constituents and bioactive properties of soft coral species *S. kavarattiensis*. The live-cell imaging -based method developed and used in the study enabled concurrent analysis of the compounds' inhibition of the CHIKV replicon and effects on cell morphology and proliferation.
2. Bioactivity screening of a library of synthetic clathrocin and oroidin analogues led to the identification of four hit compounds with potent and selective activity in a HCV replicon model (**II**). The study of the assumed mechanism of action resulted in the synthesis of a targeted set of 12 novel compounds based on the 4,5,6,7-tetrahydrobenzo[1,2-*d*]thiazole structure (**III**). Improved selectivity in the HCV replicon genotype 1b model and slightly improved binding to the proposed target protein Hsp90 was observed, showing that the selected optimization strategy was successful. Furthermore, three compounds were shown to specifically inhibit the replication of full-length HCV genotype 2a in a reporter virus RNA assay.

In conclusion, this thesis shows substantial examples of two different approaches to marine drug discovery: a bioactivity study of marine extracts followed by the isolation and bioactivity studies of purified compounds, and a marine lead compound-based synthetic approach covering chemical optimization of identified hit compounds and study of the plausible mechanism of action.

A broader knowledge of the role of Hsp90 in the replication and infectivity of viruses is vital for achieving success in antiviral Hsp90 inhibitor development. Improved insight into the intricate role played by Hsp90-type proteins in the cellular life cycle is the key for tackling the issues faced with off-target effects and lack of specificity that have

hampered the clinical development of Hsp90 inhibitors. The results of publications **II** and **III** imply that Hsp90 is a potential target of the studied compounds and can guide further development of antiviral agents targeting this cellular protein.

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