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Bioactivity profile of barettin

With special focus on anti-inflammatory, antioxidant and anticoagulant activities

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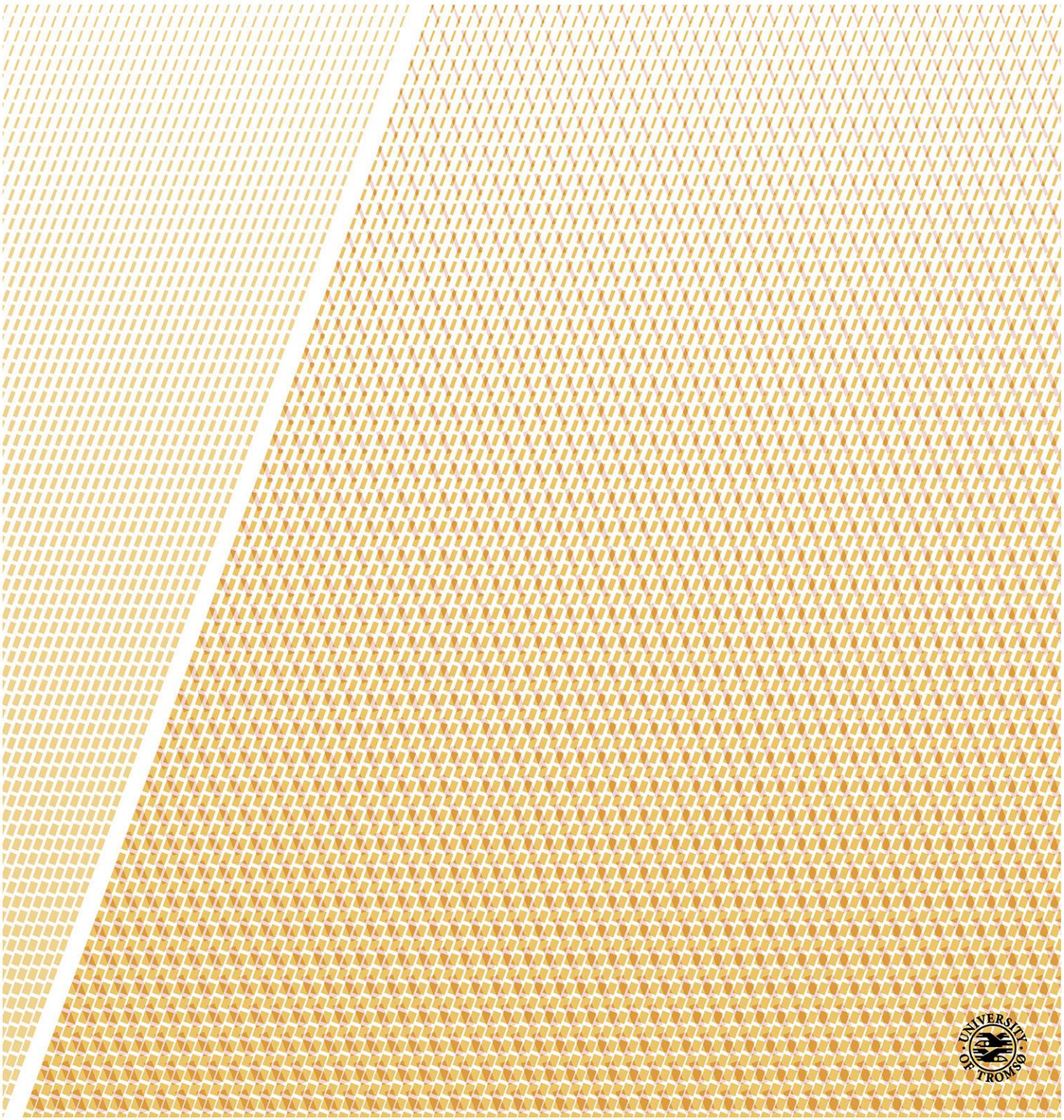
A dissertation for the degree of Philosophiae Doctor – April 2015



Summary

The diversity of the marine species inhabiting the oceans makes them highly interesting as potential sources of bioactive molecules for various applications. The extreme environment in the Barents Sea, in terms of temperature and light availability, have forced its organisms to adapt to their surroundings by developing unique biomolecules, and Arctic marine organisms are therefore an excellent starting point when searching for compounds with novel bioactivities.

In this thesis, the focus has been on revealing potential antioxidant and anti-inflammatory effects of barettin, a compound originally isolated and described from the marine sponge *Geodia barretti* in 1986. Several papers have previously highlighted the strong anti-fouling properties of the molecule. In paper I, we presented novel antioxidant and anti-inflammatory properties of barettin. We found that barettin had strong dose-dependent antioxidant effect in both biochemical and cellular assays and follow-up experiments in an inflammatory cell model showed that barettin inhibited LPS-induced production of IL-1β and TNFα. The combination of the antioxidant and anti-inflammatory effects led us to explore barettin as a potent inhibitor of atherosclerosis development. In paper II, barettin was found to also inhibit LPS-induced production of MCP-1 in THP-1 cells, a chemokine strongly linked to monocyte recruitment and atherosclerosis. When THP-1 cells were co-stimulated with LPS and IL-4, IL-4 being a cytokine with pleiotropic effects, barettin increased the anti-inflammatory effect of IL-4. In paper II, we also presented a biochemical kinase inhibitory profile of barettin, showing inhibitory effect of three kinases, namely salt-inducible kinase 2 (SIK2), calcium/calmodulin-dependent kinase 1α (CAMK1α) and receptor-interacting protein kinase 2 (RIPK2), the two latter being involved in inflammatory processes. The inhibition observed on IL-1β production could be explained by the ability of barettin to inhibit RIPK2, as this kinase have a role in NF-κB activation and possibly also IL-1β maturation. The inhibitory effect of barettin on CAMK1α, which is involved in IL-10 regulation, probably result in an observed reduction of the anti-inflammatory cytokine IL-10 in THP-1 cells.

In an *ex vivo* whole blood model stimulated with LPS, the effect of barettin on tissue factor (TF), TNFα, MCP-1, IL-10, TxB2, LTB4 and HMGB1 was evaluated (paper III). Barettin reduced TF, TNFα, TxB2, LTB4, MCP-1 and IL-10, whereas the effect on HMGB1 was modest. In summary, the overall results show that barettin affects several cellular processes and the effect of barettin should be further evaluated both *in vitro* and *in vivo* to find the true potential of the molecule.

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Tromsø, April 2015

List of publications

This thesis is based on the following publications. They are referred to by their roman numerals in the text.

Paper I

Lind KF, Hansen E, Osterud B, Eilertsen KE, Bayer A, Engqvist M, et al. *Antioxidant and anti-inflammatory activities of barettin*. Mar Drugs. 2013;11:2655-66.

Paper II

Lind KF, Osterud B, Hansen E, Jørgensen TØ, Andersen JH. *The immunomodulatory effects of barettin and involvement of the kinases CAMK1α and RIPK2.* Manuscript submitted and under revision

Paper III

Lind KF, Olsen JO, Hansen E, Jørgensen TØ, Andersen JH, Osterud B. *Barettin: A marine natural compound with potent anticoagulant and anti-inflammatory properties*. Manuscript

Abbreviations

5-HT$_{2C}$	5-hydroxytryptamine; subtype 2c
AAPH	2,2'-azobis-2-methyl-propanimidamide dihydrochloride
AIF	Anti-inflammatory
ATP	Adenosintriphosphate
Br	Bromine
CAA	Cellular Antioxidant Activity
CAMK1α	Calcium/calmodulin-dependent protein kinase 1α
CLPAA	Cellular Lipid Peroxidation Antioxidant Activity
CumOOH	Cumene hydroperoxide
CVD	Cardiovascular disease
DAMP	Damage-associated molecular pattern molecules
DCFH-DA	2′,7′ dichlorofluorescin diacetate
DKPs	Diketopiperazines
DNA	Deoxyribonucleic acid
ED	Endothelial dysfunction
ELISA	Enzyme-linked immunosorbent assay
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
FRAP	Ferric Reducing Ability of Plasma
H_2O_2	Hydrogen Peroxide
HepG2	Human liver carcinoma cell line
HMGB1	High mobility group protein B1
HMG-CoA	Hydroxymethylglutaryl-coenzyme A
HTS	High-throughput screening
HUVEC	Human Umbilical Vein Endothelial Cells
IL	Interleukin
JAK	Janus kinase
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
LTB	Leukotriene B4
MabCent	Centre for Research-based Innovation on Marine Bioactivities and Drug Discovery
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic protein-1
MNK	Mitogen-activated protein kinase (MAPK) interacting protein kinase 1
MNP	Marine natural product
MRC-5	Human Fetal Lung Fibroblast Cell line
MS	Mass spectrometry
MW	Molecular weight
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NP	Natural product
NSAID	Non-steroidal anti-inflammatory drugs
O_2^-	Superoxide
OH-	Hydroxyl
oxLDL	Oxidized low-density lipoprotein
$ONOO^-$	Peroxynitrite
ORAC	Oxygen radical absorbance capacity
OS	Oxidative stress
PAI	Plasminogen activator inhibitor
PAMP	Pattern-associated molecular pattern molecules
PTP1B	Protein tyrosine phosphatase 1B
RIPK2	Receptor-interacting serine/threonine kinase 2
ROS	Reactive oxygen species
SIK2	Salt-inducible kinase 2
TE	Trolox equivalents
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
THP-1	Human monocytic leukemia cell line
TLR4	Toll-like receptor 4
TNFα	Tumor necrosis factor α
TxB2	Thromboxane B2
VCAM-1	Vascular cell adhesion molecule-1
WB	Whole blood
WHO	World Health Organization

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Paper I, II and III

1 INTRODUCTION

1.1 Bioprospecting

Man has always harvested from nature not only for nutrition but also for plants and animals that could relief pain and cure disease. The term “Biodiversity prospecting”, currently known as “bioprospecting”, first appear in PubMed in publications from the early 1990s. The Encyclopedia of Biodiversity defines bioprospecting as “the systematic search for genes, natural compounds, designs, and whole organisms in wild life with a potential for product development by biological observation and biophysical, biochemical, and genetic methods, without disruption to nature.”[1]. The definition covers a wide variety of organisms from microbes to larger and more complex organisms like algae, plants and fish and aims at commercializing biodiversity. By utilizing a compound in its unmodified state or by modifications, natural product (NP) drug discovery aims at finding novel treatment against various diseases[2].

1.2 Natural products in history

The medicinal use of natural products goes back thousands of years[3]. The earliest records referring to the utilization of natural resources as medicine can be traced back to the ancient Egyptians but there are also archaeological findings revealing that even older cultures found herbs and plants useful for treatment of diseases and discomforts[4]. Also in the ancient Greece as well as in Asia and South America, herbal remedies were important. In some cultures, for instance in China, traditional medicine still has a significant position in the modern civilization[5].

Availability and accessibility has made plants the preferred choice and main source in traditional medicine. Macroalgae are one of the few marine organisms that have been used in traditional medicine as many are easily collectable[6]. However, from the 20th century the

strategies and methods in the field of drug discovery greatly advanced and so did the availability of previously inaccessible sources[7]. With the improvements in diving techniques and remotely operated vehicles, the oceans were within reach and over the last 50 years the number of bioactive marine natural products (MNPs) has increased as we continue to explore the seas[8]. Although much of the resources in bioprospecting is spend on drug discovery, the industry also has a growing interest in NPs for other applications. Antifouling agents for ships, enzymes for biotechnology as well as nutraceuticals and cosmeceuticals are just a few examples of how NPs can be developed into new products.

In modern science, the active substances of many plants used in traditional medicine have been identified, although many molecules responsible for the pharmacological activities are still unknown[6]. The new era started in 1805, when morphine (from *Papaver somniferum*) became the first active NP to be isolated[9]. Later, salicin from the white willow tree (*Salix alba*) was the precursor of acetylsalicylic acid, digoxin was isolated from *Digitalis lanata* and taxol from the yew tree *Taxus brevifolia*[10]. They have anti-inflammatory, cardioactive and antitumor activity, respectively. NPs have continued to play a major part in modern drug chemistry and currently, more than 260 000 compounds isolated from nature, both marine and terrestrial, are listed in the Dictionary of Natural Products.

1.3 Organized drug discovery

Natural products like the aforementioned morphine and salicylic acid are secondary metabolites. These metabolites are not involved in primary functions like growth, development or reproduction and is often unique to an organism or species[6]. Especially sessile organisms and organisms lacking an immune system are rich in secondary metabolites, utilizing them as chemical defense mechanisms as for example keeping predators away, paralyze prey or protect from bacteria, fungi and parasites[6, 11]. With the realization that nature could hide valuable compounds, the organized search for novel compounds began and throughout the twentieth century nature has served as a valuable source for novel drug discovery.

1.3.1 Chemical diversity

Along with the growing interest in NPs, technology developed and with it came high-throughput screening (HTS) and the ability to rapidly screen large number of samples in a short time. This technology demanded larger screening libraries than what was possible to generate by isolating natural compounds and as a consequence, large chemical libraries containing up to millions of compounds were developed using combinatorial chemistry[12-14]. In addition to the limited supply of screening material, NP drug discovery demanded resources to characterize and determine novelty of a hit and also synthesis of material to enable further testing. The expectations to combinatorial synthesis were therefore high. But in combinatorial synthesis a high number of compounds are produced from a limited set of chemical scaffolds, so even though the synthetic libraries are large, their chemical diversity is often low. The overall results ended up with being a disappointment as the hit rate of these libraries were limited.

The attention then turned to NPs, as these molecules showed higher chemical diversity. During evolution, secondary metabolites have evolved to bind to a range of specific receptors in other

organisms[12, 15]. These proteins or enzymes are similar to targets relevant for drug discovery, and many NPs also show drug-like properties in size, shape and solubility[12, 16]. In addition to the high chemical diversity seen in NPs the number of known protein folds is limited, increasing the probability that an NP will have a biological target[17]. In their 2012 review, Newman and Cragg reported that only 36 percent of small-molecule approved drugs were truly synthetic and devoid of natural inspiration[18]. With time, technology has allowed for a more efficient testing of natural samples and several large screening programs have been initiated to find novel compounds and bioactivities.

1.3.2 Natural products from marine sponges

Sponges (phylum Porifera) are multi-cellular, filter-feeding organisms living their adult life as sessile animals. In the 1950s, Bergmann and Feeney isolated two new nucleosides (spongothymidine and spongouridine) from the Caribbean sponge *Tethya crypta*, which resulted in Ara-C (synthetic derivative), the first approved marine-derived anti-cancer drug and Ara-A (Vidarabine), an antiviral drug [19-22]. They were approved in 1969 and 1976, respectively, but Vidarabine has later been withdrawn from the marked[23]. Since then, sponges have been one of the richest source of novel marine natural compounds[24, 25]. Blunt et al reported in their annual review for 2012 that 355 novel compounds had been isolated from marine sponges, an increase of 20 percent from the previous year[26]. Metabolites isolated from sponges have activities ranging from antiviral, anti-inflammatory, antibacterial, immunosuppressive to anticancer and neuroprotective[27] (see examples in Table 1). The secondary metabolites are varied and include the previously uncommon nucleosides (like spongothymidine in Ara-C) to sterols, fatty acids, peroxides, cyclic peptides and alkaloids. Often, it is not the sponge itself but rather sponge-associated microorganisms that turn out to be the actual producers of the bioactive metabolites[28].

Table 1. Bioactive compounds isolated from marine sponges.

Compound	Species	Mechanism	Reference
Manoalide	*Luffariella variabilis*	Phospholipase A2 inhibitor	[29]
Halichondrin B	*Halichondria okadai*	Tubulin polymerization inhibitor	[30]
Halichlorine	*Halichondria okadai*	VCAM-1 inhibitor	[31]
Barettin	*Geodia barretti*	Antifouling	[32]

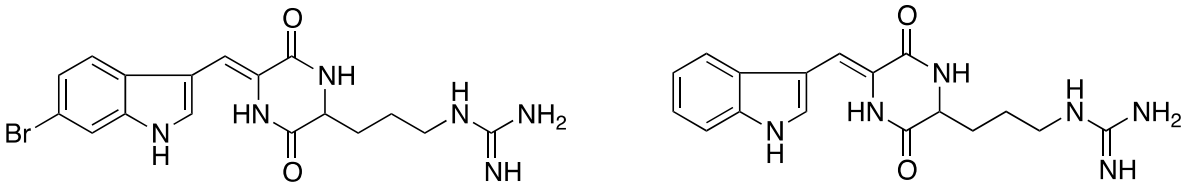
Barettin

Barettin (cyclo-[6-bromo-8-en-tryptophan]-arginine], MW= 419.2758 g/mol) was first isolated and described in 1986 from the marine sponge *Geodia barretti*[32](Figure 1) and in 2002, Sölter et al published data suggesting a slight structure modification of the originally proposed molecule[33]. This revised structure was later confirmed by Johnson et al when they successfully synthesized barettin[34] (Figure 2 (**1**)). Barettin is presumably the result of symbiosis between *G. barretti* and a so far unidentified microorganism, as animals lack the Shikimate pathway necessary for production of tryptophan and other aromatic amino acids[35].

Papers have previously been published on the anti-fouling properties of barettin [36-39]. Both in laboratory experiments and in field tests using marine coatings mixed with barettin, there was a reduced recruitment of barnacle larvae from *Balanus improvisus* compared to controls. The effect was concentration-dependent and the authors suggested that barettin targeted a specific molecule within the barnacle[36]. The serotonin receptor 5-HT_{2C} was later identified as a target, probably due to the serotonin-like structure of barettin[40, 41].

Figure 1. Specimen of *Geodia barretti*. Photo: Sten-Richard Birkely

Barettin is a diketopiperazine (DKP), a class of cyclic dipeptides isolated from a variety of species including marine organisms[42]. Their structure allows the DKPs to bind to a broad range of receptors, resulting in a diverse set of activities, like cytotoxicity, antiviral, antifungal and ability to interact in cardiovascular functions[43-45]. The structure of the DKPs allow for side chain substitutions, which makes them interesting as scaffolds for drug development.



(1) **(2)**

Figure 2. Barettin (**1**) and debromobarettin (**2**).

1.4 Marine-derived drugs

The great biodiversity of marine organisms have made them a rich source of secondary metabolites and because of the wide thermal ranges, light and nutrient availability, pressure and other external mechanical factors, the oceans are suppliers of a library of compounds more diverse than can be created in a lab[11]. The result is not only seasonally variations in metabolites but also unique secondary metabolites from two individuals of the same species collected at different geographic locations[46].

MNPs have resulted in some novel drugs and many new drug candidates and show a wide range of bioactivities, like anti-tumor, anti-microbial, antioxidant, anti-infective and anti-inflammatory[8, 18, 47, 48]. In 2004, the first MNP, ziconotide (Prialt) from the cone snail *Conus magus* was approved as a drug in the United States[7, 47]. It is prescribed to treat chronic pain in spinal cord injury by acting on voltage-gated Ca^{2+}-channels[49]. Trabectedin (Yondelis), originally isolated from a tunicate but now semi-synthesized, was the first marine anticancer drug on the market[7]. By binding to the minor groove of the DNA double helix, trabectedin interferes with gene transcription, cell division and repair mechanisms of DNA[7, 11]. There are eight marine-derived drugs available on the marked today[47], and several are in clinical trials, the majority are being tested for treatment of different cancers.

1.5 Inflammation

Inflammation is a part of the body's response to pathogens and tissue injury but when the inflammatory response is dysregulated, it is the underlying cause of several pathological conditions. Treating inflammation is therefore important to control several diseases. Today, many inflammation-related diseases are treated with non-steroidal anti-inflammatory drugs (NSAIDs) or glucocorticoids. Treatment with these drugs are usually efficient but can result in serious side effects, like osteoporosis, impaired wound healing and ulcers[50, 51]. Finding new, less problematic anti-inflammatory agents is therefore of importance.

Inflammation is a controlled response of the innate immune system with several cell types and mediators involved and precise orders of cell activation. Circulating monocytes, resident tissue macrophages and dendritic cells respond to pattern-associated molecular pattern molecules (PAMPs), like lipopolysaccharides (LPS), peptidoglycan and viral nucleic acids by secreting cytokines and chemokines[52]. These mediators start a cascade of events that recruits more immune cells to the site of inflammation. Recruited neutrophils induce several changes in the endothelium, facilitating their movement into the sub-endothelium where they release granule proteins and phagocytize microbes[53]. Circulating monocytes move into the endothelium in response to neutrophil products and differentiate into tissue macrophages. Together, macrophages and neutrophils collaborate to remove the pathogen by activating an array of antimicrobial mechanisms[54]. Both monocytes and neutrophils respond quickly to stimuli but in contrast to neutrophils, macrophages live longer and have a central role in regulating both innate and adaptive immune response[54]. When the pathogens have been removed and tissue homeostasis restored, the inflammatory response ends and signals prevent more cells to be recruited[55]. In case of a sterile inflammation, caused by trauma or chemically induced, damage-associated molecular pattern molecules (DAMPs) cause the cells to release the same mediators

as in infection[56]. Examples of DAMPs are cytoplasmic components of necrotic cells and HMGB1[52].

Acute inflammations are restricted to a specific area and reduced after a period of time but occasionally a sterile inflammatory response can trigger development of a chronic inflammation[57]. Overproduction of cytokines, defects in regulation of inflammatory pathways or failed removal of the primary stimulus caused by impaired down-regulation of the inflammatory response can result in chronic diseases like Alzheimer's, asbestosis, cancer and cardiovascular diseases (CVDs) including atherosclerosis and ischemia[56-58].

1.5.1 Cardiovascular diseases

CVDs are disorders affecting the heart and blood vessels. According to the World Health Organization (WHO) CVDs were the leading causes of non-communicable disease in 2012 and resulted in 17.5 million deaths[59]. WHO estimates that more than 30 million people will die annually due to cardiovascular-related diseases by 2030. Defects present at birth lead to some of these deaths but most are lifestyle diseases. CVDs does not only have impact on the individual level but also affects the general public by being an immense financial burden.

Atherosclerosis

Atherosclerosis is the underlying disorder of many CVDs. The disorder is a chronic inflammatory disease leading to thickening of the artery wall, which can result in thrombosis[60, 61]. The thickening is a consequence of a growing plaque formed by accumulation of lipids and recruited immune cells in the artery wall. Covering the plaque is a cap of collagen made by smooth muscle cells. The plaque grows slowly over time, eventually containing both apoptotic and necrotic cells as well as cell debris and cholesterol crystals[61]. The immune cells in the

plaque continuously secrete enzymes and pro-inflammatory mediators, which upon plaque rupture contribute to platelet aggregation and coagulation. The formed thrombus can block the blood vessel in near proximity to the site of formation or be carried with the circulation to more distant parts of the body. The outcome is atherothrombosis, which in worst case ends with ischemia or myocardial infarction.

One of the underlying causes of atherosclerosis is oxidative stress (OS). OS follows an imbalance in the body's production of free radicals and the cellular antioxidant defense system[62, 63]. Examples of free radicals include superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl (OH^-) and reactive nitrogen species like peroxynitrite ($ONOO^-$). ROS follows reactions of normal cellular processes and take part in homeostasis and cellular signaling. They participate in processes like oxygen sensing, immune functions and responses, apoptosis and blood pressure[64, 65]. OS does not necessarily cause oxidative damage, as the body is able to compensate for the oxidative burden with a number of cellular antioxidants and regain homeostasis[66]. Examples of endogenous antioxidants are superoxide dismutase, catalase, thioredoxin and glutathione peroxidase. Dysregulated OS on the other hand, have been coupled to several diseases, like hypertension, cancer, diabetes, neurodegenerative diseases and as mentioned, atherosclerosis[67].

Factors like smoking, age, hypertension and diabetes type 2 increase the risk of atherosclerosis[68]. These factors amplify the production of ROS, which have been shown to induce endothelial dysfunction (ED)[69]. The endothelium is not only a barrier keeping the blood in a separate system as initially thought but actively contributes to vascular homeostasis [69]. The healthy endothelium participates in coagulation control, regulation of vascular tone, immune response and has anti-oxidative effects. Disturbance to this tightly regulated tissue causes the

endothelium to alter properties and become dysfunctional. Markers of ED are impaired vasodilation and up-regulation of adhesion molecules and pro-inflammatory mediators, all of which contribute to the onset of atherosclerosis[70].

1.5.2 Current and potential atherosclerosis treatment

Besides reducing the atherosclerotic risk through diet and exercise, medical treatment is available to reduce the chance of atherosclerotic complications. The most common is the use of statins, hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors that, when they were first isolated from a fungal growth media, were found to reduce cholesterol levels[71]. Later, anti-inflammatory properties of statins were reported and are perhaps the most important effects of statin treatment[72]. These effects are in part caused by improvement of the endothelial function and reduction in anti-inflammatory cytokines[73]. Despite the success with statins, a treatment that usually require life-long medication, some people develop statin intolerance, manifesting itself as myopathy[74]. Finding alternatives with less serious side effects will be of great value to those who cannot tolerate or do not respond well to statins.

As atherosclerosis is a multifactorial disease, focusing on multiple targets could be the solution to treat the disease. Both enhanced ROS production and inflammation are central in the disease development and could be suitable targets[75, 76].

Cytokines

Cytokines act as key modulators in inflammation and participate in both innate and adaptive immunity[77]. Some cytokines have a larger impact on atherosclerosis than others, like tumor necrosis factor alpha (TNFα) and interleukin-1β (IL-1β). TNF was initially identified as responsible for necrosis in certain tumors but is today mostly recognized for its role as a pro-

inflammatory mediator in innate immunity[77]. TNFα is predominately, but not exclusively, expressed by activated macrophages and other immune cells and due to its important role in inflammation has been proposed as a target in the atherosclerosis development[78]. Production of TNFα initiates a cascade of other cytokines involved in the inflammatory response, activation of adhesion molecules and growth stimulation[79]. TNFα has been successful as a therapeutic target and TNFα-blockers like Infliximab and Golimumab are in use today in treatment of diseases including psoriasis, Crohn's disease and rheumatoid arthritis[80].

Another key cytokine in atherosclerosis is IL-1β, which is expressed by many cell types and has pro-inflammatory properties[81]. It is produced as an unfinished product (pro-IL-1β) and must be cleaved by caspase-1 to be activated. In addition to share many of the pro-inflammatory properties of TNFα, IL-1β acts as a chemoattractant on neutrophils[79, 82]. As a therapeutic target, IL-1β is effective, and treatment with Anakinra, an IL-1 receptor antagonist, reduces the severity of several inflammatory diseases[83].

Monocyte chemotactic protein-1 (MCP-1/CCL2) is a chemokine expressed by immune cells and activated endothelial cells and has been isolated from atherosclerotic lesions[84]. This chemokine is essential for recruiting monocytes to sites of inflammation thereby increasing the number of macrophages in the vessel wall[82]. Studies show that deletion of either MCP-1 or its receptor CCR2 recruits fewer monocytes to the site of inflammation and reduces arterial plaque formation[85].

Tissue factor

A dysfunctional endothelium has reduced ability to maintain an antithrombotic environment and increases the production of thrombotic factors including tissue factor (TF) and plasminogen

activator inhibitor (PAI)[69]. In a healthy artery, pro-thrombotic and anti-thrombotic factors tightly regulate the coagulation cascade, but an imbalance in this regulation can lead to thrombosis or bleeding, depending on affected mechanisms. TF is the major initiator of coagulation, normally inhibited by the tissue factor pathway inhibitor (TFPI)[86]. Upon activation, TF triggers a cascade by binding to plasma factor VIIa, an alliance ultimately leading to thrombin formation and fibrin deposition. In a normal tissue exposed to injury this activation enables a bleeding to stop and a wound to heal. However, in a pathophysiological situation where the regulation is ineffective, the result might be serious as it can lead to thrombus formation and blood clotting. Elevated levels of TF are observed in the atherosclerotic plaques and upon plaque rupture, TF leaks into the circulation and can cause thrombosis[87]. A coagulation cascade induced by TF can start by vessel wall injury and leaking of TF into the blood, or when blood cells themselves produce TF after being activated.

Kinases and inflammation

Kinases are proteins that by catalyzing the transfer of phosphate groups from (usually) ATP to a protein substrate activate the substrate and trigger signal transfer further down the pathway. Kinases were discovered in the 1950s and have later been found to be involved in many signal transduction pathways[88]. Their important role in cell regulation and therefore in disease have made them excellent targets for drugs. As natural scaffolds tend to be a better starting point to drug design than synthetic drugs, searching among NPs to find potential kinase inhibitors could be a solution[12]. More than 160 kinases have been associated with inflammation and increased attention has been given the use of kinase inhibitors in treatment of inflammation and autoimmune diseases.

2 AIM OF THESIS

The overall aim of this work was to characterize the biological properties of barettin with a special focus on mediators involved in inflammation and cardiovascular disease.

The main objectives of this study were to:

- Characterize the anti-inflammatory properties of barettin with a special focus on atherosclerosis-related cytokines and chemokines using cell lines and an *ex vivo* whole blood model
- Characterize the antioxidant potential of barettin using biochemical and cellular assays
- Characterize the anticoagulant properties of barettin using an *ex vivo* whole blood model

3 SUMMARY OF PAPERS

Paper I

Karianne F. Lind, Espen Hansen, Bjarne Østerud, Karl-Erik Eilertsen, Annette Bayer, Magnus Engqvist, Kinga Leszczak, Trond Ø. Jørgensen, Jeanette H. Andersen

Antioxidant and Anti-Inflammatory Activities of Barettin

Marine Drugs, 2013, 11(7), 2655-2666

The known anti-fouling compound barettin, isolated from the marine sponge *Geodia barretti*, was tested in biochemical and cellular assays to evaluate its antioxidant and anti-inflammatory potential. Barettin showed strong antioxidant properties in the biochemical antioxidant assays FRAP and ORAC, data that was supported in a cellular lipid peroxidation assay (CLPAA) on liver HepG2 cells. In the CLPAA assay, barettin inhibited membrane lipid peroxidation in a dose-dependent manner, but failed to reduce intracellular ROS formation when measured in the CAA assay. The bromine present on the tryptophan residue of barettin was believed to be important for the activity, as a de-brominated analogue failed to show the same antioxidant properties. We also found that barettin had anti-inflammatory properties and was able to reduce both LPS-induced production of TNFα and IL-1β in THP-1 immune cells. The combined antioxidant and anti-inflammatory properties of barettin makes it a good candidate to test further for other atheroprotective properties.

Paper II

Karianne F. Lind, Bjarne Østerud, Espen Hansen, Trond Ø. Jørgensen, Jeanette H. Andersen

The immunomodulatory effects of barettin and involvement of the kinases CAMK1α and RIPK2

Our group has previously reported novel anti-inflammatory and antioxidant properties of the marine compound barettin. In this study we present data that show the ability of barettin to reduce the secretion of monocyte chemotactic protein-1 (MCP-1) from immune cells, an important feature for a potential atheroprotective agent. We also present biochemical data showing that barettin has inhibitory activity against two protein kinases related to inflammation, namely the receptor-interacting serine/threonine kinase 2 (RIPK2) and calcium/calmodulin-dependent protein kinase 1α (CAMK1α). The previously reported dose-dependent inhibition of pro-inflammatory IL-1β production is possibly linked to the inhibition of RIPK2, as this kinase has been linked to NF-κB activation. CAMK1α have been demonstrated to be involved in LPS-induced IL-10 production and we found dose-dependent inhibition of IL-10 production when THP-1 immune cells were treated with barettin.

The earlier data from HepG2 cells, showing that barettin reduces lipid peroxidation induced by CumOOH, was confirmed in endothelial cells (HUVEC) and strongly indicates that the antioxidant effect of barettin is not cell specific but a general ability.

The novel activities of barettin, linked to regulation of inflammatory mediators possibly through inhibition of the kinases RIPK2 and CAMK1α, supports previous data and show that barettin should be further studied in an atheroprotective perspective.

Paper III

Karianne F. Lind, Jan Ole Olsen, Espen Hansen, Trond Ø. Jørgensen, Jeanette H. Andersen, Bjarne Østerud

Barettin: A marine natural compound with potent anticoagulant and anti-inflammatory properties

The anti-inflammatory and anticoagulant properties of barettin was studied in an *ex vivo* whole blood model stimulated with LPS. Tissue factor (TF) activity, measured in isolated, frozen and thawed LPS-stimulated mononuclear cells, was attenuated in a dose-dependent manner by barettin. ELISA was used to evaluate the effects on the production of the inflammatory cytokines/chemokines TNFα, IL-10 and MCP-1 as well as the eicosanoids TxB_2 and LTB_4. Barettin was able to dose-dependently reduce TNFα and also reduced the production of TxB_2 and LTB_4. Both the pro-inflammatory chemokine MCP-1 and the anti-inflammatory cytokine IL-10 were reduced by barettin, the latter possibly through inhibition of the kinase CAMK1α. HMGB1 is secreted as an inflammatory mediator by activated monocytes/macrophages and has been linked to CAMK1α. In contrast to the observed inhibition of IL-10 production, barettin only moderately reduced HMGB1. Overall, the results show that barettin has potent anticoagulant and anti-inflammatory properties, and supports previous *in vitro* findings on its potential in prevention of atherosclerosis.

4 GENERAL DISCUSSION

In this thesis, the *in vitro* antioxidant and anti-inflammatory properties of barettin and debromobarettin were evaluated in selected biochemical and cellular assays (paper I/II). In a follow-up *ex vivo* whole blood model the anti-inflammatory as well as anticoagulant properties of another barettin isomer (E-barettin) were evaluated (paper III). Before identifying barettin, fractions from other marine species were tested for their anti-inflammatory properties. A summary of these results are presented in appendix 3.

4.1 Screening of marine extracts

Even with increased knowledge and progression in technology, NP drug discovery is still comprehensive and time-consuming, challenges are yet to be solved and most samples will not advance from the primary screening to follow-up assays due to lack of activity. Crude extracts are complex, containing hundreds of compounds with an abundance of different properties, for instance fluorescence that may interfere with the bioassays[15]. Factors like stability and concentration often determines the probability of a metabolite being detected. Extracts are in general partially purified into fractions before screening, each fraction a cocktail of many compounds with similar chemical properties. When these fractions are further purified and two or more compounds acting in synergy to exert bioactivity are separated, bioactivity is often lost. In cases were bioactivity is successfully linked to a specific compound, structure elucidation is essential. As more of nature is explored by bioprospecting, the chance of finding a known compound increases, but the bioactivity could be novel and of both scientific and commercial interest.

Other factors like supply issues, seasonal variations and costs of collecting contribute to make NP drug discovery challenging. To enable structure elucidation and thorough biological testing it is essential to have sufficient amount of compound. The list of potentially good drug candidates that never reached the market due to either unethical supply issues or difficulties with the chemical synthesis is long. Being able to synthesize potential drug candidates is therefore important. In 1969, ecteinascidin-743 (trabectedin, Yondelis) was isolated from the tunicate *Ecteinascidia turbinata* and showed anti-cancer activity[7]. One ton of animal was necessary to yield 1g trabectedin, so harvesting from the oceans for further work was therefore not an alternative. The supply issues delayed the biological profiling and the structure was not published until 1990. Aquafarming of *E. turbinata* partially solved the supply problem but yields were still low. In the end, the solution was semi-synthesis with the antibiotic cyanosafracin B as starting material and Yondelis is currently being used to treat soft tissue sarcoma and ovarian cancer[89, 90]. This example illustrates the importance of chemical synthesis for drug development. Of the lead compounds reaching clinical trials, many are discontinued due to toxicity or lack of efficiency. From the discovery of an active molecule, it is estimated that it will take approximately 10-20 years and more than 2,5 billion dollars until it reach the marked[91].

4.2 Bioactivity profile of barettin

4.2.1 Isolation and identification

Fractions of a *G. barretti* sample collected in the Barents Sea showed strong antioxidant activity in biochemical assays and barettin was identified as the active component using MS. Two reported stereoisomers of barettin were isolated (*Z/E*)[36]. Together with the previously published anti-fouling activity of barettin[37, 39], this novel antioxidant activity led to the further examination of barettin properties. Unless otherwise stated, barettin refers to *Z*-barettin.

Secondary metabolites from marine organisms are often halogenated and although chlorine is more abundant in the marine environment, bromine is the most commonly incorporated halogen, probably due to the broad presence of bromoperoxidases in marine invertebrates[92, 93]. Halogenation of NPs is seen in many marine species, like sponges, worms, mollusks, bacteria and algae[94] and is also a well-established method in drug design, as halogenation improves membrane permeability and drug uptake[95, 96]. In addition to the isolated barettin isomers, which has a bromine in the tryptophan residue, we also synthesized and tested (in selected assays) debromobarettin (Figure 2(**2**)) to determine the relevance of bromination for the bioactivity.

As described in paper I and II, cytotoxicity of barettin was evaluated on the main cell lines used throughout the project (THP-1 and HUVEC) in addition to cell lines commonly used in *in vitro* cytotoxicity testing (HepG2 and MRC-5)[97]. At concentrations up to 100 µM for 72 h, barettin was not cytotoxic, supporting the results from Sjögren et al. who found the anti-fouling activity of barettin to be non-toxic[36].

4.2.2 Antioxidant activity

In paper I, the antioxidant potential of barettin and debromobarettin was established using the biochemical antioxidant assays FRAP (ferric reducing antioxidant power) and ORAC (oxygen radical absorbance capacity). We found that barettin had a FRAP value of 77 μM trolox equivalents (TE) when tested at 30 μg/ml (71.6 μM) and an ORAC value of 5.5 μM TE at the same concentration. The effect in both assays was dose-dependent. In the FRAP assay, the antioxidant effect of barettin was slightly better than that of debromobarettin, in contrast to the ORAC assay where barettin and debromobarettin had the same effect.

Biochemical assays like FRAP and ORAC are useful when screening large sample sets for antioxidant potential, as they are fast and cost-effective and require a smaller amount of test compound than cellular assays. It is important though to have in mind that such assays should only serve as a starting point and that results should be followed up with cell-based assays. The FRAP assay was originally developed for biological fluids but has become a method often applied to study the antioxidant potential of natural compounds. The assay measures the potential of an antioxidant to reduce Fe^{3+} to Fe^{2+}, but at a non-physiological pH and without the production of ROS[98]. The ORAC assay is a well-established method detecting decomposition of a fluorescent probe exposed to peroxyl radicals. Antioxidants will protect the probe and delay or prevent loss of fluorescence. This method is considered more relevant than FRAP but being a pure biochemical assay it has its limitations. Cellular assays, despite being more time-consuming, offer more biological relevant information as they take into account bioavailability and toxicity of a compound. The antioxidant potential of barettin was confirmed in the cellular lipid peroxidation antioxidant activity (CLPAA) assay presented in paper I and II, and showed that barettin is able to dose-dependently reduce cumene hydroperoxide (CumOOH)-induced lipid peroxidation in cellular membranes of liver HepG2 cells and the endothelial HUVEC.

HepG2 is a cell line frequently used to study the antioxidant effect of natural products[99] while HUVEC are often used for studying atherosclerosis and diseases of the vascular system[100]. The results from the lipid peroxidation assay indicate that this antioxidant effect of barettin is a general property and not cell line-specific.

When membrane lipids are exposed to free radicals, a chain reaction with reactive aldehydes as end-products is initiated, which can lead to ED and cardiovascular disease[75, 101]. The endothelium goes from releasing mediators that maintain homeostasis to express higher levels of adhesion molecules and pro-inflammatory factors and in addition, its vessel dilatory capacity is reduced. Further, lipid peroxidations alter the membrane physiology and change the fluidity and permeability of the membrane as well as cause damage to cell membrane-associated proteins[102, 103]. Overall, the endothelium is more prone to vascular inflammation and subsequent atherosclerosis[70]. The ability of barettin to reduce lipid peroxidation in the CLPAA assay and thus stabilize cell membranes makes it a good candidate for evaluation as an atheroprotective compound. In this context, it was also of interest to test the ability of barettin to reduce formation of intracellular peroxyl radicals. To do this, DCFH-DA-treated HepG2 cells were incubated with barettin before the ROS formation was induced with the free radical initiator AAPH (CAA assay). But in contrast to the results obtained from the lipid peroxidation experiments, barettin was not able to prevent formation of peroxyl radicals neither in HepG2 cells nor HUVEC. As the CLPAA assay measures lipid peroxidation in cellular membranes whereas the CAA assay measures intracellular fluorescence of a ROS-sensitive probe, the results indicate that barettin incorporates into cellular membranes and protects the C11-BODIPY probe from CumOOH oxidation but is not able to protect the CAA probe (DCFH-DA) or prevent the intracellular ROS formation induced by AAPH (Figure 3).

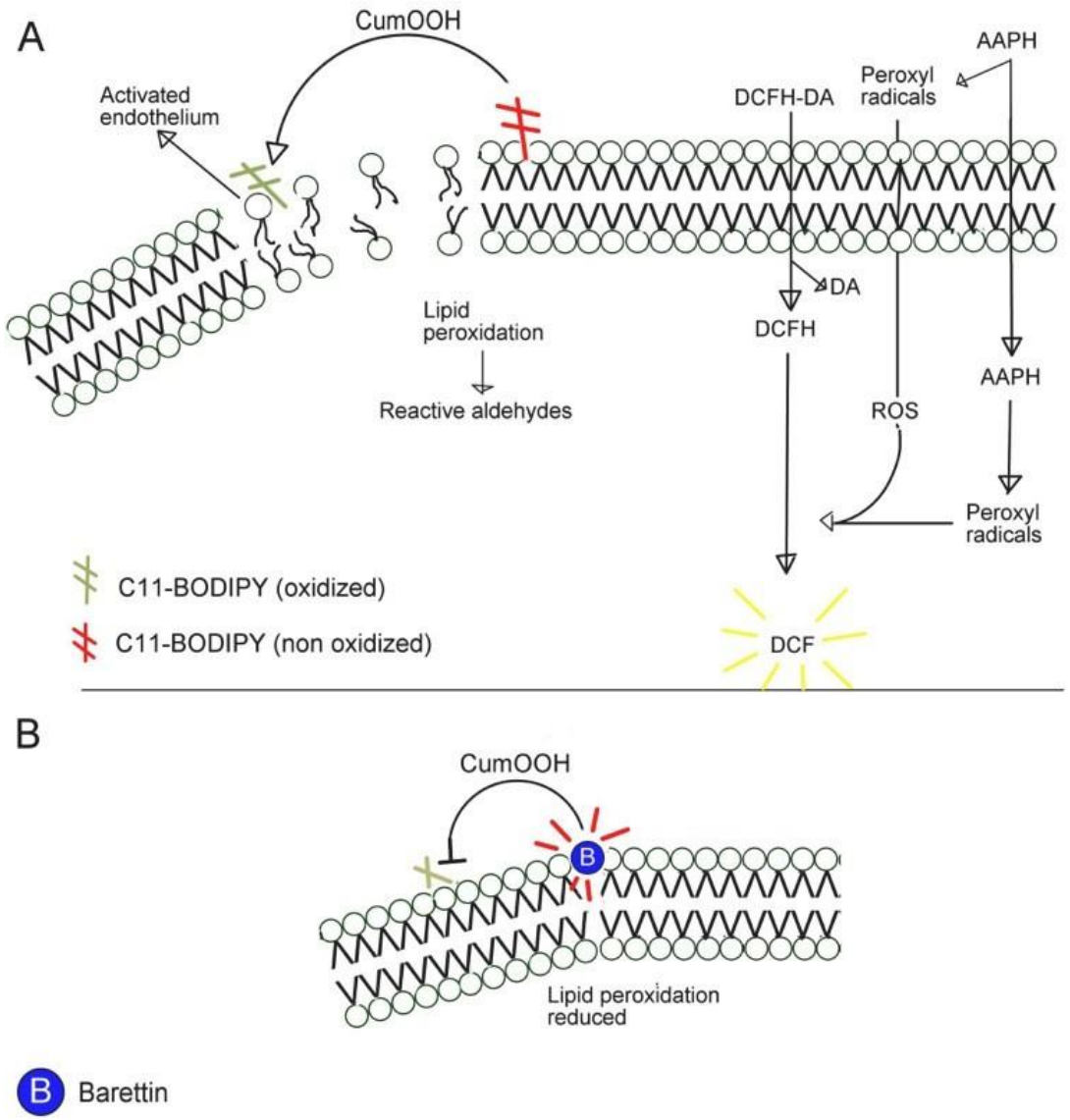


Figure 3. Possible antioxidant mechanism of barettin in a lipid bilayer. (A) The radical initiator CumOOH will initiate lipid peroxidation in a lipid bilayer, resulting in reactive aldehydes as well as an activated endothelium. The lipophilic C11-BODIPY, which shifts from red to green when oxidized, can be used to measure the rate of oxidation. Intracellular ROS formation can be detected with the hydrophilic probe DCFH-DA, which crosses the cell membrane and is cleaved by cellular esterases into DCFH. The free radical initiator AAPH will cause ROS formation and the free radicals will cleave the DCFH into fluorescent DCF which can be measured.
(B) Barettin localizes to the lipid bilayer of cellular membranes and protects the C11-BODIPY as well as membrane lipids from oxidation by CumOOH. Barettin will not protect the hydrophilic DCFH from oxidation by cytoplasmic free radicals.
Figure modified from Wolfe[104].

When low-density lipoprotein (LDL), the carrier of cholesterol and fatty acids in the blood, is oxidized by free radicals, the formed oxLDL is a strong inducer of ED[105]. In paper II, we tested if barettin was involved in the oxLDL pathway by testing its ability to prevent oxLDL-induced cell death in HUVEC. The lack of effect on cell viability observed after pre-incubation with barettin before addition of oxLDL indicates that barettin will probably not have an effect in signaling involving oxLDL.

4.2.3 Immunomodulatory and kinase inhibitory activity in cell models

Marine sponges have been the source of many marine-derived compounds with several reported bioactivities[106]. Even so, few of the NP-derived approved drugs on the market today are used as treatments against inflammations. The strong antioxidant properties of barettin led us to examine the potential anti-inflammatory effects as well, as atherosclerosis is a result of both oxidation and inflammation[105].

In paper I, we tested the effect of barettin on LPS-induced IL-1β and TNFα production in THP-1 cells, two important pro-inflammatory cytokines. At concentrations ranging from 50-100 μg/mL, barettin inhibited TNFα production modestly, whereas the effect on IL-1β was much better, as barettin inhibited LPS-induced IL-1β production dose-dependently from 2.5 to 100 μg/mL. The dose-dependent effect of barettin could indicate inhibition of a specific target involved in regulation or maturation of IL-1β. Since kinases regulate several pathways involved in inflammation, barettin was tested against a panel of 140 kinases (at The International Centre for Kinase Profiling in Dundee, Scotland) to determine its kinase inhibitory potential (appendix 1). As presented in paper II, two of the three kinases most inhibited by barettin at 50 μM, namely receptor-interacting serine/threonine kinase 2 (RIPK2/RIP) and calcium/calmodulin-dependent protein kinase 1a (CAMK1α), have both been linked to inflammation[107].

RIPK2 and IL-1β

RIP kinases are involved in both innate and adaptive immunity, and they are induced by LPS, cytokines and peptidoglycans. RIPK2 has been proposed as a potential target in inflammatory treatment, including atherosclerosis[108-113]. The activities of RIPK2 include signal transfer from LPS via toll-like receptor 4 (TLR4) and involvement in NF-κB activation and IL-1β maturation[114-116]. In paper I we showed a dose-dependent reduction of IL-1β production from LPS-induced THP-1 cells treated with barettin. A possible explanation for this inhibition was discussed in paper II when biochemical kinase data showed that barettin has an IC_{50} value of 8.0 μM on RIPK2 activity (appendix 2). Data on cell signaling and the exact role of RIPK2 and its active site in inflammation is still debated[117-119]. The results presented in paper I and II on the anti-inflammatory effect of barettin indicates a connection between LPS, RIPK2, NF-κB activation and IL-1β maturation. One possible explanation is that RIPK2 acts as an adaptor molecule rather than an active kinase[117] and that the inhibition of RIPK2 by barettin lead to impaired signal transduction and downstream effects on NF-κB activation. In agreement with our results from THP-1 cells, studies have shown that both local and systemic immune signaling in macrophages from $RIPK2^{-/-}$ mice was reduced[113]. It should be noted that this study did not look at IL-1β production but at other cytokines, including TNFα and MCP-1. Results presented in paper II indicate that the effect of barettin on LPS-induced MCP-1 from THP-1 cells was similar to the effect observed on TNFα in paper I. This reduction in MCP-1 and TNFα was confirmed in an *ex vivo* whole blood model where *E*-barettin was able to dose-dependently reduce production of these cytokines after LPS-stimulation (paper III). The lack of a true dose-response effect of barettin on TNFα and MCP-1 in THP-1 cells argues for a mechanism that don't directly target NF-κB activation but rather IL-1β maturation and other pro-inflammatory pathways.

CAMK1α and IL-10

The CAMK1 family members are involved in gene expression, cell cycle regulation and differentiation and CAMK1α mediates IL-10 production in LPS-stimulated macrophages via p38 MAPK[120]. The dose-dependent reduction of LPS-induced IL-10 observed after barettin treatment in THP-1 cells (paper II) could be linked to the inhibitory effect of barettin on CAMK1α, as barettin has an IC_{50} value of 5.7 μM on the CAMK1α activity (appendix 2). IL-10 was also reduced in whole blood plasma after treatment with *E*-barettin (paper III), possibly by the same mechanism. IL-10 is an anti-inflammatory cytokine produced by macrophages and lymphocytes and participate in resolution of inflammation as well as reducing levels of pro-inflammatory cytokines[79, 82]. Overexpression of IL-10 is efficient in preventing atherosclerosis, whereas development of atherosclerosis has been linked to IL-10 deficiency[121-123]. Reduced levels of IL-10 would therefore be unfavorable in the context of atherosclerosis. However, the reduction of several pro-inflammatory mediators observed after barettin treatment in both THP-1 cells and WB, may be more important than levels of IL-10[120]. Further, at low concentrations (<25 μM), barettin was able to upregulate IL-10 production in LPS/IL-4 co-stimulated THP-1 macrophages but this increase was suppressed when concentrations exceeded 25 μM. The explanation for this biphasic effect is not known yet, but other compounds have previously been reported to act in a similar manner[124, 125]. Today there are not many known CAMK inhibitors of marine origin and no compounds appear to be reported that specifically inhibit CAMK1α[126]. Of the few CAMK inhibitors that have been identified, leptosin M, which was isolated from an algae-associated fungi, inhibits CAMKIII[127], whereas staurosporine, originally isolated from a bacterium growing on an ascidian, inhibits CAMKII[128].

The kinase screening also showed that barettin is able to inhibit other kinases as well, although with less efficiency. Of these, the mitogen-activated protein kinase (MAPK) interacting protein

kinase 1 (MNK1), is another serine/threonine kinase involved in inflammation and immune regulation[129]. It can therefore not be ruled out that the observed anti-inflammatory effect of barettin could be caused by inhibition of other kinases than RIPK2 and CAMK1α.

Barettin is a NP that strongly inhibits a limited number of kinases, but these kinases are involved in more than one pathway. For instance, in addition to inhibiting RIPK2 and CAMK1α, barettin also inhibits salt-inducible kinase 2 (SIK2) with an IC_{50} value of 6.1 μM, as presented in paper II (see also appendix 2). SIK2 is involved in several pathways, including metabolism, cancer and melanogenesis[130-132]. Studies show that SIK2 has anti-lipogenic effect and also phosphorylates a specific serine residue in insulin receptor substrate-1 in adipocytes; a receptor connected to insulin resistance[131, 133]. Further studies should therefore be performed to evaluate the effect of barettin on SIK2 in relation to CVDs.

The observed inhibitory effect of barettin on the production of both pro- and anti-inflammatory cytokines, possibly through inhibition of kinases, illustrates the complexity of kinase signaling and why it is challenging to develop kinase inhibitors into drugs. A study by Levin et al. demonstrates the link between inflammation and lipid accumulation and how targeting kinases in drug development can give unexpected results as they observed that atherosclerotic lesions were greater in $RIPK2^{-/-}$ mice than in the control group despite reduced immune signaling[113]. Moreover, the high degree of similarity in the active site of many kinases means that an inhibitor will likely affect more than one target. There is also a risk of development of drug resistance which results in activation of alternative pathways and reduced treatment efficiency[107, 134]. This challenge can be illustrated with the Janus kinases (JAKs) as they have a central role in cell activation induced by cytokines and participate in regulation of several cellular functions for instance transcription of pro-inflammatory genes[134, 135]. Previously, JAK3 was considered an

excellent target due to very specific activity in the immune system, but homology between the catalytic domains of the JAKs have made it more of a challenge than expected to find selective JAK3 inhibitors[107, 136].

4.2.4 Whole blood model

To follow-up on the *in vitro* studies of barettin, the effect of the barettin was also tested in an *ex-vivo* whole blood (WB) model, and results are presented in paper III. The WB model takes into account that all blood components and plasma proteins are intact and that the different cell types are present. It does however not reflect the true environment as blood flow and interactions with for instance endothelial cells are not taken into consideration. The method is also subject to interindividual variations and data should be carefully interpreted. The model will nevertheless give useful information about the effect of a compound on stimulated and non-stimulated blood cells.

In the WB model, only the *E*-barettin showed activity. In WB, we found that in LPS-stimulated samples, 20 μg/ml barettin was able to reduce the expression of TF measured in isolated frozen and thawed mononuclear cells with 60.7 % compared to an untreated control. As previously described, the expression of TNFα, MCP-1 and IL-10 was also reduced, when measured in plasma. In addition, at 10 μg/ml, barettin reduced the expression of TxB_2 and LTB_4 as well by 50.6 and 26.7 percent, respectively. The relevance of TNFα, MCP-1 and IL-10 reduction have already been discussed. TxB2, the stable product of TxA2, and LTB4 are both linked to development of atherosclerosis and potential therapeutic targets[137, 138]. The effect of barettin on TF activity as well as on TxB_2 and LTB_4 demonstrates interesting anticoagulant properties as coagulation is a serious outcome of plaque rupture and the ultimate thrombus formation.

The high mobility group box 1 (HMGB1) protein is secreted by monocytes/macrophages as an inflammatory mediator and has been linked to development of atherosclerosis[139]. As the CAMK1α, inhibited by barettin (see above), is involved in HMGB1 regulation and IL-10 production, it was of interest to study the effect of barettin on HMGB1 production. At 10 μg/ml a small reduction in HMGB1 levels in plasma samples from LPS-stimulated blood was seen, but this reduction was less pronounced than the other test parameters mentioned. The effect of barettin and the debrominated analogue on HMGB1 in THP-1 cells was evaluated but no reduction was observed (unpublished data).

4.2.5 Structure-activity relationship and choice of test system

The small structural variances between the two barettin stereoisomers resulted in interesting bioactivity differences when comparing results from the THP-1 cell line and the WB model. In THP-1 cells, only *Z*-barettin and not *E*-barettin showed anti-inflammatory activity while the opposite was observed in WB samples. In fact, in the WB model, *Z*-barettin acted in a pro-inflammatory manner (data not published). The observed differences between WB and cell culture is possibly caused by different monocyte behavior in different test systems. Studies on adherent and non-adherent alveolar macrophages show that upon adherence, the cells are primed for activation and will produce inflammatory mediators after LPS stimulation in contrast to non-adherent cells[140]. Østerud et al. has also showed in several studies that macrophages isolated from whole blood respond differently to treatment than monocytes in cell cultures[141]. The effect observed by barettin *in vitro* (THP-1) could very well represent the effect of barettin on cells in the arterial wall, as the monocytes differentiate into macrophages when leaving the vessel and enter the intima.

The differences between the stereoisomers were also demonstrated in the biochemical and the cellular antioxidant assays, where only *Z*-barettin and not *E*-barettin showed antioxidant potential. The discrepancy in bioactivity between the two isomers demonstrates not only structure-activity relationship (SAR) but also that the choice of test system is of critical importance to outcome of analysis.

In contrast to the results from the biochemical antioxidant assays, debromobarettin did not reduce lipid peroxidation in neither HepG2 nor HUVEC, indicating that the bromine is important for its *in vitro* antioxidant activity. In the *in vitro* anti-inflammatory assays, debromobarettin had no or moderate effect on cytokine production and similar, when tested in the whole blood model, debromobarettin did not reduce cytokine production or the TF activity (unpublished). Overall, bromine seems to be important for the biological properties of barettin, a feature also affecting its anti-fouling properties[37]. Studies with serotonin analogues, a compound with structural similarities to barettin, showed that by introducing a halogen to serotonin receptor antagonists, both the affinity and selectivity to serotonin receptors were improved[142, 143]. The hypothesis is that by inserting a halogen atom, the compound will be bigger and able to fill the active pocket in its target better than the dehalogenated analogue[95].

4.3 Further work

The results presented and discussed in this thesis show that barettin is a compound with anti-inflammatory, antioxidant and anticoagulant effects which should be further explored to find if barettin is a potential drug for atherosclerosis treatment. Barettin has a positive impact on many inflammatory mediators and it would therefore be of interest to expand the test panel to include more cytokines and relevant cell lines. The significance of the reduced IL-10 production after barettin treatment, possibly by CAMK1α inhibition, also needs to be further examined as well as how barettin affects mechanisms controlled by other kinases.

In addition to the mode-of-action studies, the effect of barettin as an anti-inflammatory, anti-oxidative and anti-coagulant drug has to be verified in relevant *in vivo* models. There are many examples of compounds that have good effects in *in vitro* systems, but when they are tested in animal models they fail to produce the same effects. Thus, appropriate animal models are necessary in order to establish if barettin is truly able to reduce atherosclerosis development and lesion formation *in vivo*.

5 CONCLUDING REMARKS

History shows that natural products have played a significant role in development of many of the drugs currently on the marked. I am certain that the attention given natural products in drug development today, and in particular marine natural products, will result in more efficient as well as new drugs for many different indications, including cancer and inflammations.

Despite major efforts to reduce the number of CVDs, there is a global increase in diseases affecting the heart and blood vessels. It is becoming more important to find new efficient treatment to address this problem, as it is not merely a health issue but also has great socioeconomically impact. Barettin is a promising candidate for treatment of atherosclerosis, as it shows both antioxidant, anti-inflammatory and anticoagulant properties.

Appendix 1

Group	Family	Kinase	Remaining aktivity (%)
AGC	PKC	PKCa	92
		PKCγ	101
		PKCz	69
	PKA	PKA	87
	PKB	PDK1	95
	AKT	PKBa	78
		PKBb	42
	RSK	S6K1	29
		MSK1	55
		RSK1	32
		RSK2	37
	PKN	PRK2	60
	DMPK	ROCK 2	72
	SGK	SGK1	38
CK1	CK1	CK1γ2	89
		CK1d	88
	TTBK	TTBK1	92
		TTBK2	71

Group	Family	Kinase	Remaining activity (%)
CMGC	DYRK	DYRK1A	33
		DYRK2	80
		DYRK3	63
		HIPK1	60
		HIPK2	58
		HIPK3	78
	CLK	CLK2	25
	SRPK	SRPK1	86
	GSK	GSK3b	83
	MAPK	ERK1 (MAPK3)	93
		ERK2 (MAPK1)	86
		ERK5 (MAPK6)	47
		ERK8 (MAPK15)	35
		p38a MAPK	94
		p38b MAPK	85
		p38g MAPK	73
		p38d MAPK	86
		JNK1	90
		JNK2	67
		JNK3	84
	CDK	CDK2 - Cyclin A	89
		CDK9 - Cyclin T1	71

Group	Family	Kinase	Remaining activity (%)
STE	STE7	MKK1	69
		MKK2	79
		MKK6	114
	STE20	PAK 2	63
		PAK 4	60
		PAK 5	73
		PAK 6	102
		TAO1	92
		MINK1	49
		MST2	56
		MST3	74
		MST4	53
		GCK	65
		MAP4K3	61
		MAP4K5	75
		OSR1	101
	STE 11	MEKK1	78
		ASK1	71
TKL	STKR	TGFBR1	108
	MLK	MLK1	45
		MLK3	36
		TAK1	28
	LISK	TESK1	78
	RIPK	RIPK2	16
	IRAK	IRAK1	89
		IRAK4	68

Group	Family	Kinase	Remaining activity (%)
TK	JakA	JAK2	91
	EGFR	HER4	93
	Tie	TIE2	75
	PDGFR	PDGFRA	58
	VEGFR	VEG-FR	50
	FGFR	FGF-R1	95
	InsR	IGF-1R	78
		IR	69
		IRR	73
	DDR	DDR2	87
	Trk	TrkA	81
	Syk	SYK	68
		ZAP70	95
	Eph	EPH-A2	81
		EPH-A4	90
		EPH-B1	109
		EPH-B2	73
		EPH-B3	121
		EPH-B4	93
	Src	Lck	27
		YES1	77
		BRK	122
		Src	90
	Tec	BTK	42
	Csk	CSK	84
	Abl	ABL	60

Group	Family	Kinase	Remaining activity (%)
Other	NFK2	PINK	93
	IKK	IKKb	62
		IKKe	47
		TBK1	78
	NAK	MPSK1	104
	CAMKK	CAMKKb	68
	ULK	ULK1	101
		ULK2	98
	NEK	NEK2a	65
		NEK6	99
	Aur	Aurora A	111
		Aurora B	68
	TLK	TLK1	92
	PLK	PLK1	110
	Wnk	WNK1	99
	PEK	EIF2AK3	110
	TTK	TTK1	76
	CK2	CK2	82
Atypical	Alpha	EF2K	26

Appendix 2

IC_{50} values (μM) of barettin against selected kinases.

Kinase target	**IC_{50}**	**s.d.***
SIK2	6.1	0.3
CAMK1	5.7	1.5
RIPK2	8.0	0.3

* Standard deviation

Appendix 3

Examples of species tested for anti-inflammatory activity

Species	Common name	Assay(s)
Molpadia borealis	Sea cucumber	THP-1 cells; TNFα
Crangonidae indet	Shrimp	Whole blood; TF activity,
Sabinea septemcarinata	Shrimp	TNFα in plasma
Ctenodiscus crispatus	Starfish	

Initially, fractions from different species reduced TNFα production in THP-1 immune cells. The samples were tested in a follow-up *ex vivo* whole blood model to establish their ability to reduce TF activity and TNFα-production. A limited number of fractions were able to both reduce TF and TNFα, but samples were terminated due to varying repeatability. The results did however demonstrate that marine organisms are an interesting source of secondary metabolites.

Appendix 4

In addition to testing barettin in assays related to inflammation and antioxidant activity, the compound was tested in several other assays to evaluate the biological profile. Due to the excellent ability of barettin to inhibit biofouling in a non-toxic manner, we tested the ability of barettin and debromobarettin to inhibit biofilm formation by *Staphylococcus epidermidis*. Only barettin at concentrations above 60 μM inhibited biofilm formation. The lack of effect in the biofilm assay can possibly be explained by the fact that the biofilm has a microbial target while the anti-fouling assay targets multicellular organisms.

Neither barettin nor debromobarettin was able to reduce PTP1B, which is linked to diabetes, or enhance collagen secretion in human dermal fibroblasts.

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