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Bioactivity profile of barettin

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

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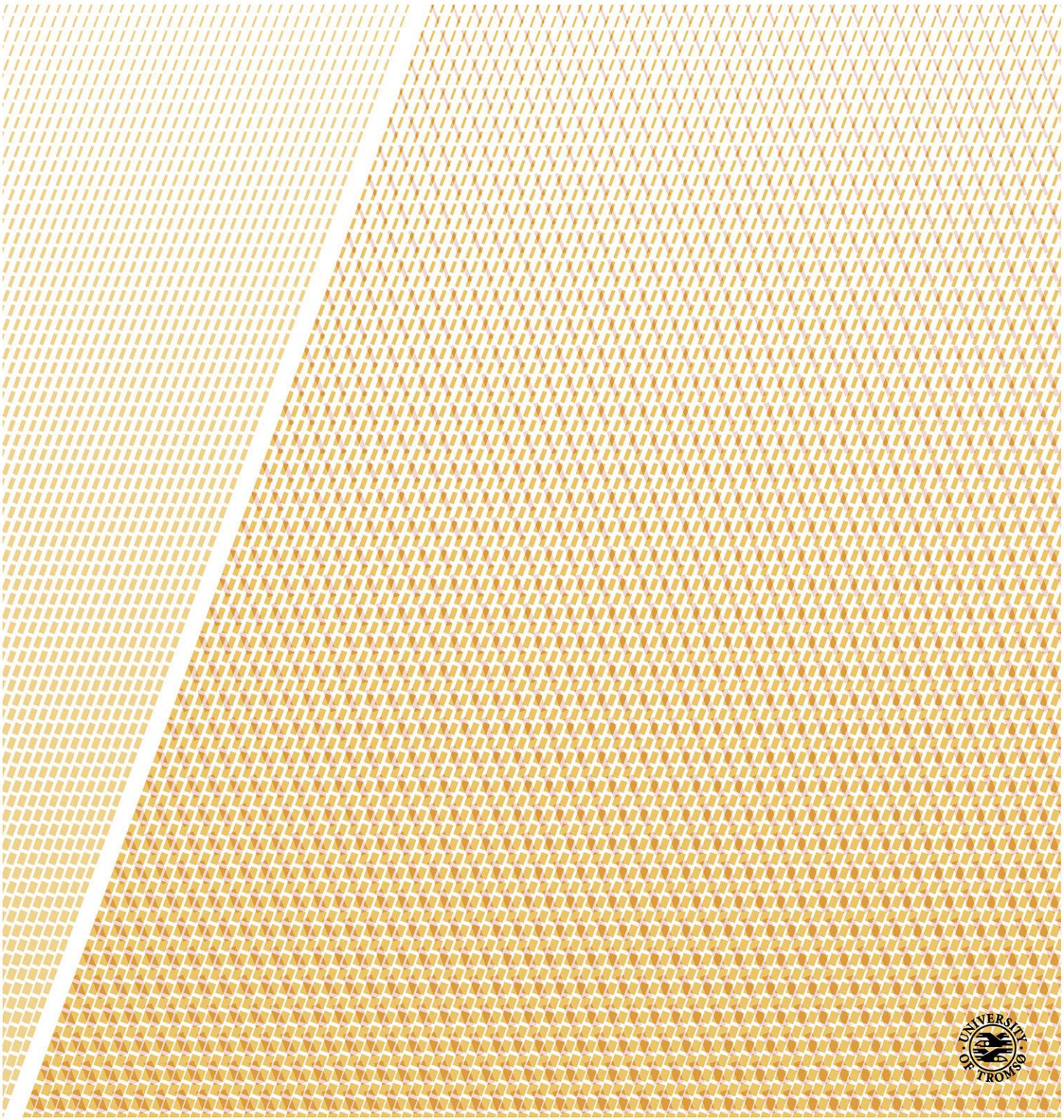
A dissertation for the degree of Philosophiae Doctor – April 2015



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]

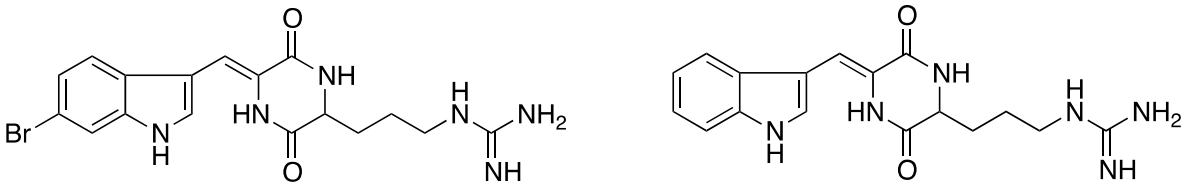
Barettin

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).

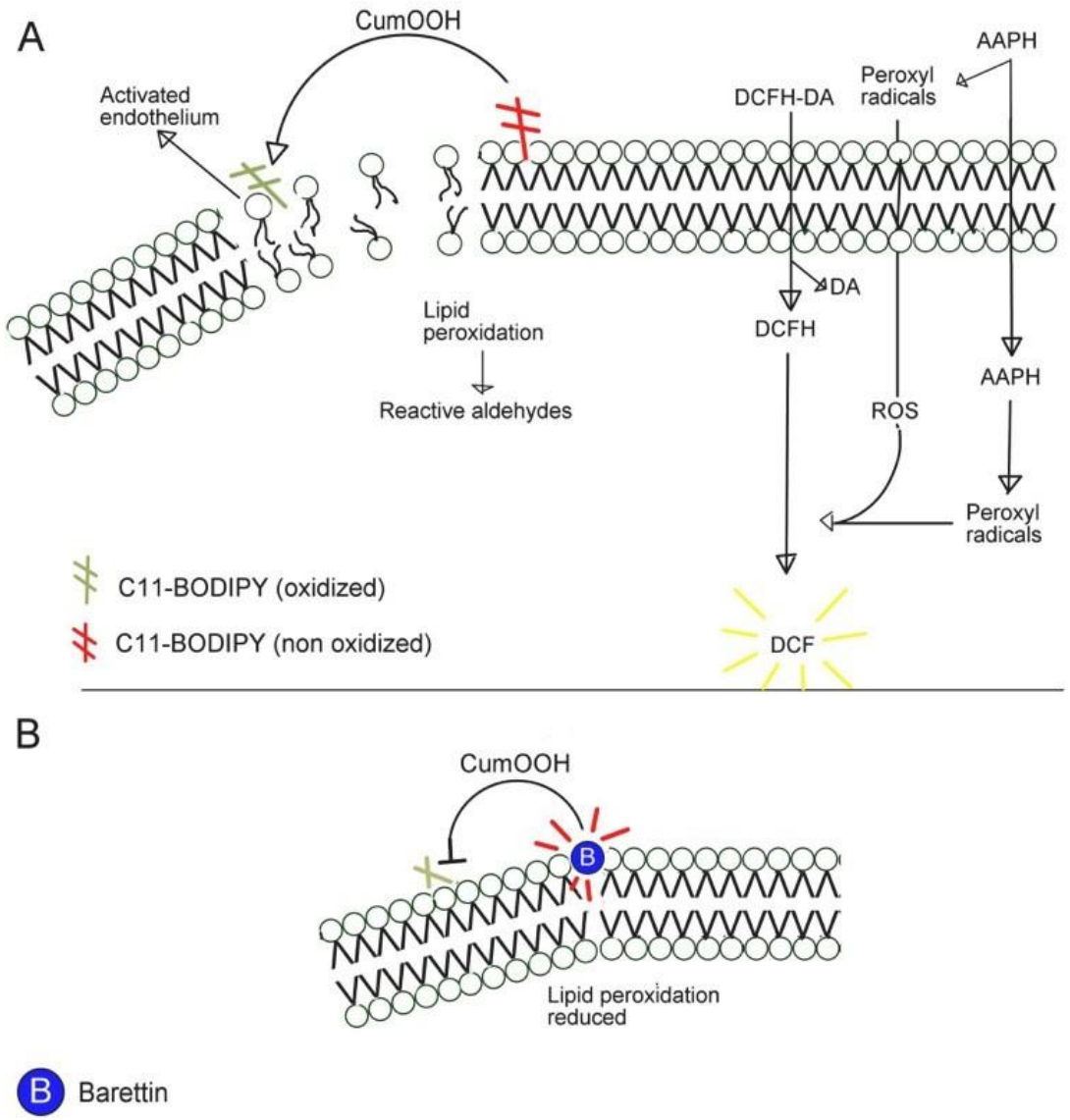


Figure 3. Possible antioxidant mechanism of barettin in a lipid bilayer. (A) The radical initiator CumOOH will initiate lipid peroxidation in a lipid bilayer, resulting in reactive aldehydes as well as an activated endothelium. The lipophilic C11-BODIPY, which shifts from red to green when oxidized, 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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Article

Antioxidant and Anti-Inflammatory Activities of Barettin

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Abstract: In this paper, we present novel bioactivity for barettin isolated from the marine sponge *Geodia barretti*. We found that barettin showed strong antioxidant activity in biochemical assays as well as in a lipid peroxidation cell assay. A de-brominated synthetic analogue of barettin did not show the same activity in the antioxidant cell assay, indicating that bromine is important for cellular activity. Barettin was also able to inhibit the secretion of the inflammatory cytokines IL-1β and TNFα from LPS-stimulated THP-1 cells. This combination of anti-inflammatory and antioxidant activities could indicate that barettin has an atheroprotective effect and may therefore be an interesting product to prevent development of atherosclerosis.

Keywords: anti-inflammatory; antioxidant; barettin; natural product

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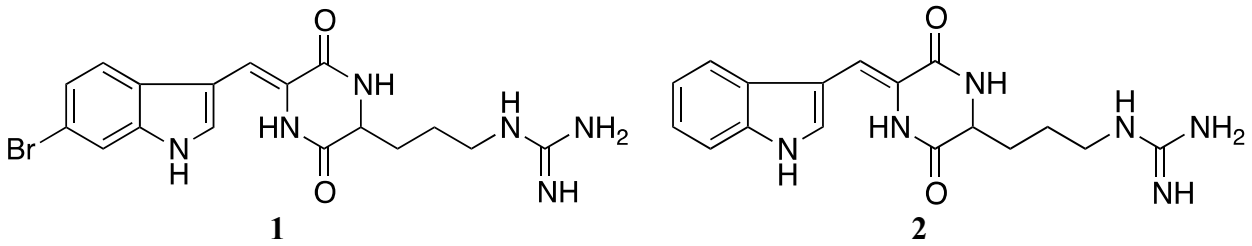
1. Introduction

It is a well-known fact that sponges are rich sources of bioactive compounds. Barettin (cyclo-[6-bromo-8-en-tryptophan]-arginine]) was first isolated and described in 1986 [1] from the marine sponge *Geodia barretti* and in 2002, Sölter *et al.* published data suggesting a slight structure modification of the originally proposed molecule [2]. This revised structure (Figure 1(**1**)) was later confirmed by Johnson *et al.*, when they successfully synthesized barettin [3].

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



Previously, barettin was proven to have anti-fouling properties [4–7] and these properties are thought to be caused by the serotonin-like structure (Figure 1(**1**)) [8,9]. The identification of barettin from *G. barretti* collected outside Northern Norway was the result of the antioxidant screening project at MabCent-SFI [10]. The compound showed strong antioxidant activities in biochemical assays, and we decided to further investigate the possibility of new biological activities of this molecule. These studies included cellular antioxidant assays and anti-inflammatory assays. As the antioxidant defence system and the immune system are closely linked in diseases like arthritis, diabetes, asthma and coronary heart diseases, we were interested in examining whether barettin also has an anti-inflammatory effect. Several natural products which have combined antioxidant and anti-inflammatory effects have previously been described, especially from fruits and plants [11,12]. Of the more well-known are fucoxanthin and resveratrol. Fucoxanthin exerts many effects, including radical scavenging and inhibition of several inflammatory cytokines and mediators [13] whereas resveratrol induces antioxidant enzymes as well as reduce atherosclerotic lesions [14].

The purpose of this work was to study the antioxidant and anti-inflammatory effects of barettin. Originally, less than 13 mg barettin was isolated from *G. barretti*. In order to perform bioactivity studies and confirm the structure, we synthesized barettin and also a de-brominated analogue (Figure 1(**2**)), which were both tested in biochemical and cellular assays. Thus, in this paper we will present novel antioxidant and anti-inflammatory activities for barettin and debromobarettin.

2. Results and Discussion

2.1. Bioactivity Testing

2.1.1. Antioxidant Activity

We used the biochemical assays FRAP (ferric reducing antioxidant power) and ORAC (oxygen radical absorbance capacity) to obtain an indication of the antioxidant potential of barettin and the

synthetic de-brominated analogue. A dose-response activity was observed for both compounds (Figures 2 and 3). At a concentration of 30 µg/mL (71.6 µM) barettin had a FRAP value of 77 µM trolox equivalents (TE) whereas the ORAC value was 5.5 µM TE.

Figure 2. Both barettin and the debromobarettin act in a dose-dependent manner in the oxygen radical absorbance capacity (ORAC) assay to protect fluorescein from degradation ($n = 2$).

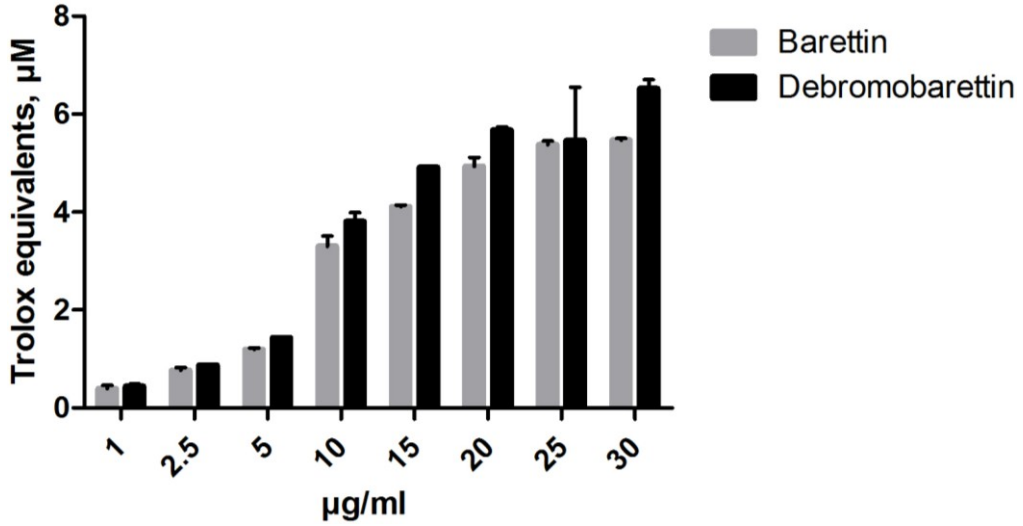
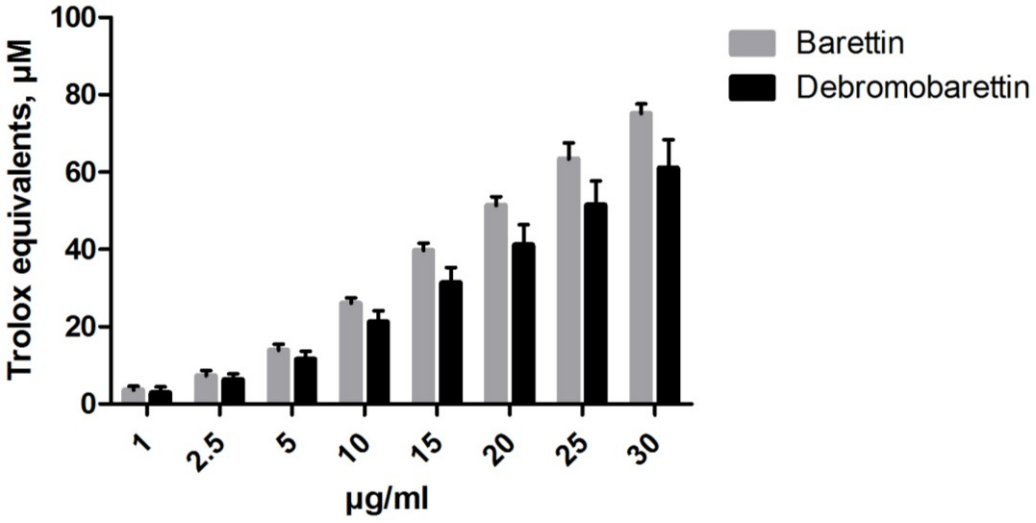


Figure 3. The antioxidant effect of barettin and debromobarettin tested in the ferric reducing antioxidant power (FRAP) assay. Both molecules reduce iron in a dose-dependent manner ($n = 2$).



Biochemical assays are fast, cost-effective and can offer useful information of antioxidant activity. Cellular assays can offer more biological relevant information since they take into account the bioavailability and metabolism of the tested compound [15,16]. To further explore the antioxidant potential of barettin we tested the compounds in two cellular antioxidant assays using HepG2 cells, a human liver hepatocellular carcinoma cell line often used to study the antioxidant effect of natural products [17]. Murakami *et al.* for instance used HepG2 to study the effect of catechins on cellular

antioxidant systems [18] and Alia *et al.* showed that quercetin could protect these cells from oxidative stress by *tert*-butyl hydroperoxide [19].

We used the cellular antioxidant activity assay (CAA, Figure 4) and the cellular lipid peroxidation antioxidant activity (CLPAA, Figure 5) assays [20,21] to measure the intracellular reactive oxygen species (ROS) and the lipid membrane antioxidant activity, respectively. None of the molecules had any effect in the CAA assay (Figure 4). Barettin gave a 55% reduction in lipid peroxidation compared to the control in the CLPAA assay (Figure 5), whereas debromobarettin did not show any activity.

Figure 4. Cellular Antioxidant Activity (CAA) results for barettin and debromobarettin. Neither compound reduced the intracellular oxidation in HepG2 cells. Luteolin (10 μM) was used as a comparative control. Results are normalized to a positive control 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) (n = 3).

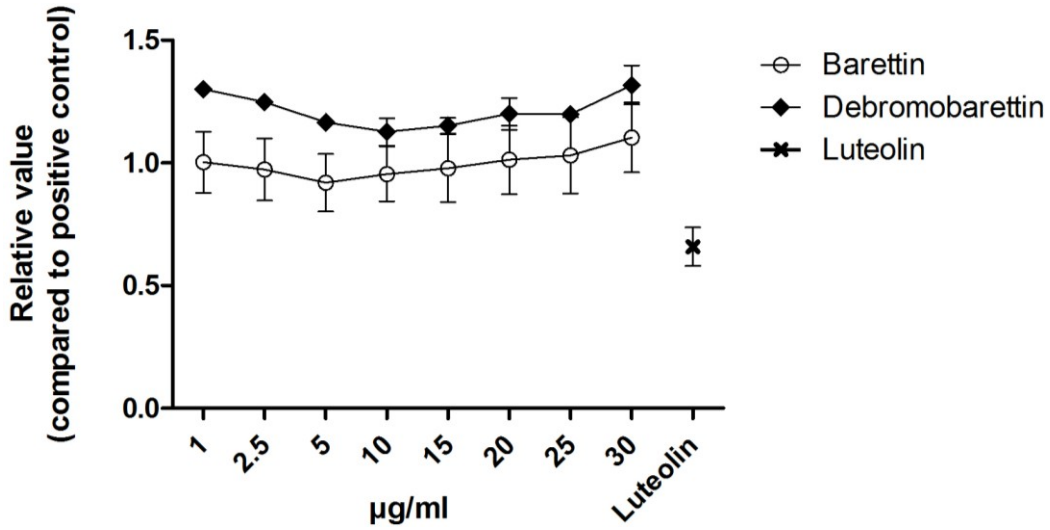
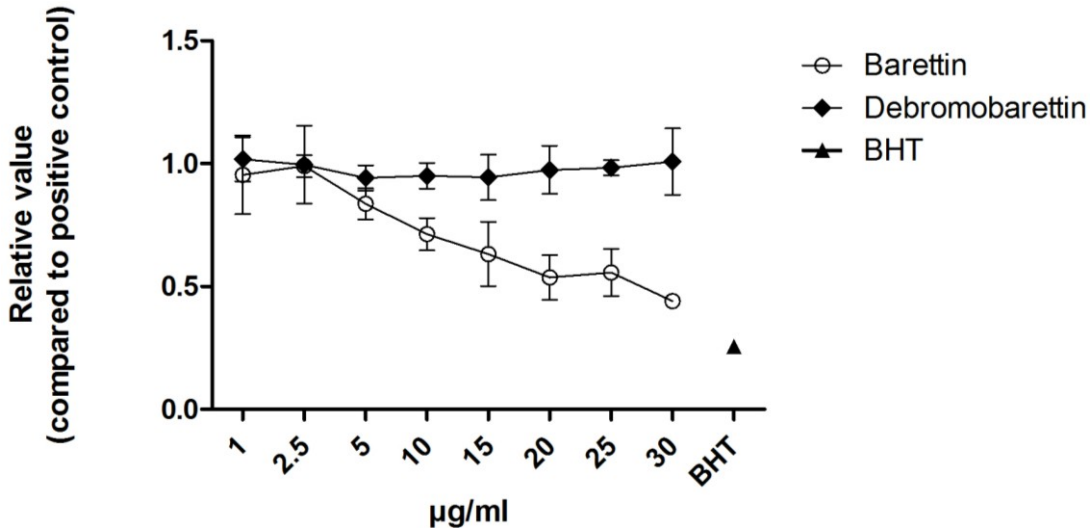


Figure 5. Cellular Lipid Peroxidation Antioxidant Activity (CLPAA) results for barettin and debromobarettin. Barettin acts in a dose-dependent manner to reduce lipid peroxidation in HepG2 cells. This effect was not seen with the de-brominated molecule. Butylated hydroxytoluene (BHT; 10 μM) was used as a comparative control. Results are normalized to positive control with cumene hydroperoxide (CumOOH) (n = 3).



The cellular assays give more information about the antioxidant activity where the CAA indicates the overall oxidative status of the cell by monitoring the decomposition of the water-soluble probe 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) into the fluorescent dichlorofluorescein (DCF) [20,22]. The CLPAA assay on the other hand detects antioxidants preventing lipid peroxidation in cellular membranes by monitoring the increase of a green fluorescence product produced by the lipophilic probe C-11-BODIPY after addition of cumene hydroperoxide (CumOOH) [21].

The lack of response in the CAA assay and the inhibition observed in the CLPAA assay indicates that barettin is not able to prevent formation of the fluorescent DCF by reactive oxygen species (ROS) but inhibits radicals from oxidizing the C-11-BODIPY and membrane lipids.

The barettin molecule has a polar arginine and a non-polar brominated tryptophan end (Figure 1(**1**)) [2,3]. The bromine present in barettin is the only feature distinguishing it from the de-brominated synthetic analogue (Figure 1). Several research groups have proved that halogens are important for cellular antioxidant activity such as Gentry *et al.* who studied the effect of inserting halogen atoms in the DPLPE-Phe enkephalin([D-Pen^2-,L-Pen^5,Phe^6]) [23]. They observed that the lipophilicity and the cell membrane solubility of the CNS-acting drug are dependent on halogenation. Also Gerebtzoff *et al.* used parameters like surface activity and permeability coefficient to show that halogenation improves the drug membrane binding and diffusion in general [24]. Thus we believe that the absence of bromine in the de-brominated analogue reduces the bioavailability and explains the lack of inhibition of the lipid peroxidation seen in the HepG2 cells used in the CLPAA assay.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) can oxidize lipids in low-density lipoprotein (LDL) and cell membranes and lead to conditions like atherosclerosis and chronic inflammation [25,26]. The oxidized lipids are recognized by pattern recognition receptors on immune cells and elicit an immune response by attracting monocytes [27]. In atherosclerosis, foam cell formation initiated by activated macrophages by uptake of oxidized LDL generate plaque development in the blood vessel intima [25]. In this scenario, antioxidants are thought to prevent LDL and cell membrane lipids from being oxidized and thus inhibit development of oxidative stress related diseases, including plaque development and atherosclerosis [28].

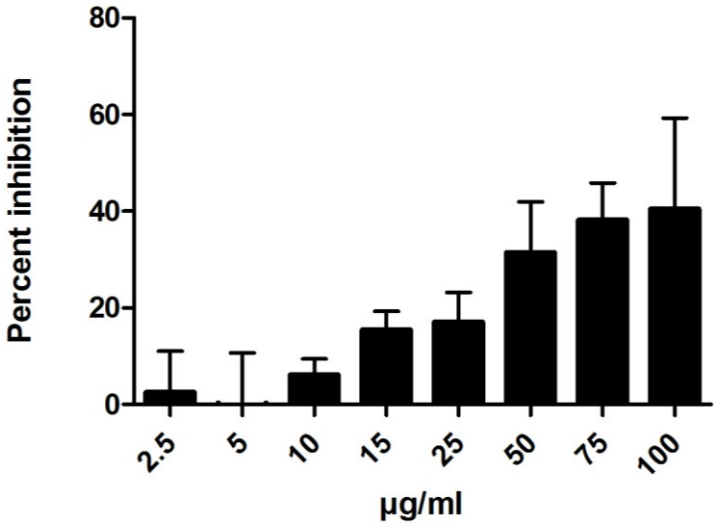
2.1.2. Anti-Inflammatory Activity

Atherosclerosis is recognized as a chronic inflammatory disease where oxidative stress is involved in the onset and progression [29]. We wanted to study whether barettin could also have an anti-inflammatory effect in addition to the lipid peroxidation inhibition observed, making it highly interesting as a possible atheroprotective compound. Using the human acute monocytic leukemia cell line (THP-1) and ELISA, we monitored the tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β) production. We found a dose-dependent inhibition of IL-1β production with increasing concentration of barettin (up to 100 μg/mL, Figure 6). At concentrations ranging from 50 to 100 μg/mL TNFα secretion was also inhibited (data not shown).

As the anti-inflammatory cell system is very sensitive to endotoxin contaminants, LPS present in the environment as endotoxin can in even small amounts affect any bioassay [30,31]. It is therefore essential to avoid such contamination in immune assays. In order to obtain endotoxin-free samples we

used polymyxin B based gel-packed columns to remove pyrogens. The pyrogen-free sample was then used in the anti-inflammatory assay.

Figure 6. Interleukin-1β secretion from THP-1 cells were inhibited by barettin in a dose-dependent manner (n = 3).



2.2. Cytotoxicity

The previously reported anti-fouling properties of barettin and the presented cellular effects could be caused by a general toxicity with the bromine as a cytotoxic "inducer". Hepatocytes are good models for studying toxicity since the liver is the primary site for drug metabolism and biotransformation [32,33]. In addition to the hepatocyte cell line HepG2 we also included normal lung fibroblasts (MRC-5) and THP-1 cells when testing for cytotoxicity.

Cytotoxicity was tested using the CellTiter 96 AQueous One Solution Assay (Promega). In the CAA and CLPAA assays the cells were exposed to the test compounds for 1 h before washing. Thus a 2 h exposure on HepG2 cells should detect whether barettin and/or debromobarettin are likely to cause cell death and false results in these assays. The three cell lines were also exposed to the compounds for 24 h in a separate testing. This would reveal more long-term damage or whether any toxicity was caused by something other than membrane lysis. As can be seen from Figure 7, neither barettin nor debromobarettin were toxic to the HepG2 after 24 h in the concentrations tested. Toxicity was neither detected in THP-1 (after 6 and 24 h) nor MRC-5 cells (data not shown).

Barettin was also tested for cytotoxicity on HepG2 and MRC-5 after 72 h exposure (data not shown), and the compound did not show cytotoxicity until concentrations reached 100 µg/mL, a concentration well above the maximum of 30 µg/mL used in the CAA/CLPAA assays.

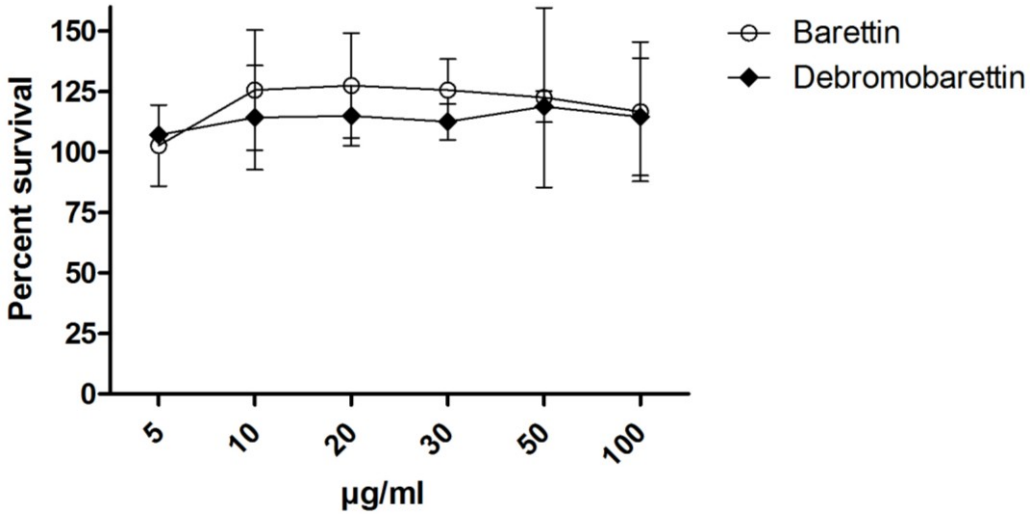
3. Experimental Section

3.1. Purification, Isolation and Identification

The sponge was collected by bottom trawling at 390 m depth in the Barents Sea. The lyophilized material was extracted twice with ultrapure water (2 × 1000 mL) at 4 °C and centrifuged at 4500× *g*

and 5 °C for 30 min before the pellet was freeze dried. The lyophilized pellet was extracted twice with 1000 mL dichloromethane:methanol (1:1, v:v) at 4 °C and subsequently filtered through a Whatman No3 filter. The filtrate was reduced to an orange oily liquid at 40 °C and reduced pressure in a rotary evaporator giving 9 g of organic extract. The organic extract was further chromatographed on a HP20-resin using a solvent step-gradient system of 5%, 25%, 50% and 75% aqueous methanol and two last steps of 100% methanol and 100% acetone. The fraction eluted with 50% methanol was reduced to dryness and dissolved in 1 mL 50% aqueous acetonitrile. Barettin was isolated using a Waters HPLC auto-purification system equipped with a Waters XTerra C18 column (10 × 300 mm, 10 µm) and eluted with a gradient from 25% to 35% of acetonitrile (ACN) and water, both containing 0.1% formic acid and at a flow rate of 6 mL/min. Two isomers of barettin eluted as two peaks giving 12.9 and 3.9 mg pure compound (retention time 5.1 and 6.3, respectively). High-resolution ESIMS gave *m/z* 419.0830 $[M + H]^+$, as the calculated *m/z* for $C_{17}H_{19}BrN_6O_2$ ($[M + H]^+$) is 419.0826.

Figure 7. Results from cytotoxicity testing of barettin and debromobarettin using HepG2 cells**.** Results are expressed as percent survival after 24 h exposure ($n = 2$).



3.2. Synthesis

Barettin and debromobarettin were synthesized according to the published procedure for barettin [3]. The (*L*)-form of N^{α}-(*tert*-butoxycarbonyl)-N^{ω}, $N\omega'$-bis(*tert*-butoxycarbonyl)-arginine was used as starting material (Bachem, Switzerland). The ^{1}NMR and HRMS data were in agreement with published data [2,5]. The crude synthetic products were purified on a Waters HPLC auto-purification system using a Waters XTerra C18 column (10 × 300 mm, 10 µm) and ACN and water (both containing 0.1% formic acid) as mobile phase at a flow rate of 6 mL/min. Barettin was isolated using a gradient from 20% to 40% ACN over 10 min (R_t 7.0 min), and debromobarettin was isolated using a gradient from 15% to 25% ACN over 15 min (R_t 7.2 min).

3.3. Biochemical Assays

FRAP assay. Reagents were prepared according to Benzie and Strain [34] and carried out in a DTX 880 Multimode Detector (Beckman Coulter, CA, USA) at 595 nm. Trolox (Sigma-Aldrich, St. Louis,

MO, USA) was used to prepare the standard curve (0–250 μM; working concentration). The FRAP reagent (TPTZ (2,4,6-Tris(2-pyridyl)-*s*-triazine): Sigma-Aldrich; Fe: Merck, Darmstadt, Germany) was prepared daily. The assay was carried out in clear 96-well plates with 20 μL sample and 150 μL FRAP-reagent added to each well in duplicates. Water was used as a blank. Samples were incubated at 37 °C for 30 min before reading the plate. The blank was subtracted from each sample and a standard curve was created from the average absorbance of the duplicated trolox samples. The equation generated from the standard curve was used to calculate the trolox equivalents (TE) from each sample. Results were expressed as μM TE.

ORAC assay. The method has been modified from Huang *et al.* [35]. The assay was carried out in black 96-well plates (Nunc) using a Victor3 Plate Reader (Perkin Elmer, MA, USA) at 37 °C (excitation 486 nm, emission 520 nm). All reagents were dissolved in 75 mM phosphate buffer (PB, pH 7.4). Diluted concentrations of barettin and debromobarettin were added in duplicates followed by addition of 125 μL fluorescein (52 nM final concentration, Sigma-Aldrich). After a 15 min incubation at 37 °C, 60 μL AAPH (2,2′-Azobis(2-methylpropionamidine) dihydrochloride; Sigma-Aldrich) was quickly added to each sample (44 mM final concentration). Fluorescence was recorded 25 times at 37 °C with a 70 s. delay between repeats. Trolox (0–25 μM working concentration) was included in each run to make a standard curve. PB was used as a blank and for the 0 μM Trolox sample. Area under the curve (AUC) was calculated by subtracting the AUC_{Blank} values. A standard curve was created using the trolox values and trolox equivalents of the samples were calculated from the resulting equation. Results were expressed as μM TE.

3.4. Cellular Assays

HepG2 and MRC-5 cells were grown in MEM Earle's medium (F0325) supplemented with gentamycin (10 μg/mL), non-essential amino acids (1%), sodium pyruvate (1 mM), L-alanyl-L-glutamine (2 mM) and fetal bovine serum (FBS, 10%) and incubated at 37 ºC with 5% CO_2. Media and supplements were from Biochrom (Berlin, Germany). THP-1 cells were grown in RPMI-1640 (Biochrom) supplemented with gentamycin and FBS and incubated at 37 °C with 5% CO2. To differentiate the monocytes into macrophages, 50 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich) was added.

3.4.1. Cellular Lipid Peroxidation Antioxidant Activity (CLPAA) Assay

Approximately 80,000 HepG2 cells per well were seeded in black 96 well plates with clear bottom (Corning, NY, USA) and incubated overnight. Cells were washed with PBS between additions of new reagents. Total reaction volume was 100 μL. All incubations were carried out at 37 °C with 5% CO_2. The cells were labelled with C11-BODIPY (Invitrogen, Eugene, OR, USA) for 30 min and incubated for 1 h with various concentrations of the test compounds. Cumene hydroperoxide (CumOOH, Sigma-Aldrich) was added to initiate lipid peroxidation and the plate was immediately installed in a Victor3 Plate Reader. Both red (590/7 nm (excitation), 632/45 nm (emission)) and green (485/14 nm, 520/10 nm) fluorescence were recorded. Percent inhibition was calculated relative to the positive control (CumOOH without test compound).

3.4.2. Cellular Antioxidant Activity (CAA) Assay

The CAA assay was modified from Wolfe and Liu [20]. HepG2 cells were seeded and grown as described for the CLPAA assay. Cells were washed with PBS between additions of new reagents. Total reaction volume was 100 µL. The incubations were at 37 °C, 5% CO_2. Cells were then incubated with 25 µM DCFH-DA (Sigma-Aldrich) and 20 µL test compound for 1 h. After incubation, 600 µM AAPH was added and the plate immediately placed in in a Victor3 Plate Reader (excitation/emission; 485/520 nm). The plate was incubated for 1 h before the second reading. Results are presented as relative values compared to the AAPH control.

3.4.3. Cytotoxicity

Cytotoxicity was studied in HepG2, MRC-5 cells (normal human lung fibroblasts) and THP-1 cells for 2 h (HepG2), 6 h (THP-1) and 24 h (HepG2, MRC-5 and THP-1). For the 2 h study, 80,000 HepG2 cells were seeded per well. For the 24 h study, 50,000 HepG2 cells, 7500 MRC-5 cells and 10,000 THP-1 cells were used. HepG2 and MRC5 were grown over night, then washed with PBS and added 50 µL test compound at various concentrations diluted in MEM Earle's supplemented as above but without FBS. THP-1 cells were grown as described in the immune assay. After incubation, 10 µL of CellTiter 96® AQ_{ueous} One Solution Reagent (Promega, Madison, WI, USA) was added and plates were then incubated further for 1 h. Absorbance was measured at 485 nm in a DTX 880 Multimode Detector. Results were calculated as % survival compared to negative (assay media) and positive (Triton X-100; Sigma-Aldrich) control.

3.4.4. Anti-Inflammatory

Approximately 10^5 THP-1 cells supplemented with 50 ng/mL PMA were seeded in 96 well plates and incubated at 37 °C, 5% CO_2 for 48 h. The cells were controlled after 24 h by microscopy to make sure they had started to differentiate. After 48 h, the cells were washed and new RPMI (w/o PMA) added before 24 h incubation.

The cells were then added 90 µL fresh medium and 10 µL test compound at various concentrations in duplex. Controls were included in every test run. After incubation for 1 h, all samples except negative cell controls were incubated with 1 ng/mL lipopolysaccharide (LPS; end concentration) for another 6 h at 37 °C. The reactions were stopped by freezing the plates at −80 °C immediately after incubation.

One day prior to the ELISA testing of IL-1β and TNFα secretion, MaxiSorp 96F-well plates (Nunc) were coated with 2 µg/mL capture antibody (eBioscience, San Diego, CA, USA) and placed in the refrigerator overnight.

Between every step, plates were washed with TBS (pH 7.4, with 0.05% Tween-20). All incubations were at room temperature with shaking. A volume of 200 µL blocking buffer (TBS w/2% BSA) was added to the plates and incubated for 1 h. TNFα samples were diluted 1:4 or 1:10 and IL-1β samples diluted 1:2. Standard concentrations of IL-1β and TNFα were added to each plate before incubation for 2 h. Biotin coupled anti-human antibody (eBioscience) was diluted in assay diluent (TBS with 1% BSA) to 3 µg/mL and added to each well and incubated for 1 h. Diluted ExtrAvidin®-Alkaline Phosphatase

(Sigma-Aldrich) was added and plates incubated for 30 min. 100 μL pNPP substrate (Sigma-Aldrich, 1 mg/mL in 1 M diethanolamin buffer pH 9.8) was added to each well, incubated for 45 min and results read at 405 nm.

3.4.5. Endotoxin Removal

We used Detoxi-Gel Endotoxin Removing Columns from Thermo Scientific (Waltham, MA, USA) according to the manufacturers' description. Synthesized barettin was dissolved in DMSO to 100 mg/mL. The sample was further diluted in pyrogen-free water to 5 mL. After regeneration and equilibration of the column the sample was applied and the flow-through collected. The sample was then freeze-dried overnight. The test tube with barettin was weighed before and after elution to estimate the sample weight.

4. Conclusions

Compounds with combined antioxidant and anti-inflammatory properties are of interest for treatment of for instance cardiovascular diseases such as atherosclerosis. Atherosclerosis is a widespread disease, especially in the western world and today there are no therapeutic treatment directly targeting this disease except cholesterol lowering drugs (statins). We found that barettin possess both anti-inflammatory and antioxidant properties making it a candidate for further studies as an atheroprotective compound. We also found interesting differences between barettin and debromobarettin, indicating that bromine could be important for *in vivo* activity of the compounds. Both compounds are active in the biochemical antioxidant assays but only barettin is active in the cellular CLPAA assay where it reduced lipid peroxidation in HepG2 cells. Barettin also inhibited IL-1β and TNFα reduction in THP-1 immune cells. Based on these results barettin is interesting as a lead compound for further structure-activity studies to elucidate the modes of action and any clinical potential.

Acknowledgement

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

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Antioxidant, cytokines, IL-1β, IL-10, marine natural compound

Abstract

Barettin is a marine natural compound with reported anti-inflammatory and antioxidant properties. In this study we show that barettin can reduce the secretion of monocyte chemotactic protein-1 (MCP-1) from immune cells, adding to its potential as an 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). Here, we also demonstrate that barettin reduce the production of the anti-inflammatory cytokine interleukin-10 (IL-10) in a dose and time-dependent manner, possibly by inhibiting CAMK1α. The activities of barettin presented here are linked to regulation of inflammatory mediators, possibly through inhibiting the activity of kinases. These properties should be further explored to determine the potential of barettin in atherosclerosis treatment.

Introduction

Barettin was isolated from the marine sponge *Geodia barretti* collected in the Barents Sea and we have previously shown that barettin is able to reduce lipid peroxidation and production of pro-inflammatory cytokines (1). Both oxidation and inflammation have been linked to development of atherosclerosis and in this paper we have further evaluated the effects of barettin on atherosclerotic mediators.

Inflammation, caused by cells of the innate immune system as monocytes and macrophages, is an important part of the body's defense against tissue damage and harmful pathogens with the main task of removing these pathogens and restore homeostasis. One of the most important roles of macrophages is to release pro-inflammatory mediators which subsequently activates appropriate signaling pathways aimed at the specific threat. But it is equally important that cells of the immune system are able to switch to an anti-inflammatory

mode after a period of activation in order to prevent an excess of pro-inflammatory mediators which will cause tissue destruction. Continuously activated macrophages and other pro-inflammatory cells can result in chronic or autoimmune diseases, like atherosclerosis, rheumatoid arthritis, asthma and type 2 diabetes (2).

Atherosclerosis is a common chronic disease. Its exact mechanism and causes are still debated, although it is widely recognized that it involves inflammatory mediators and endothelial activation (3). Oxidized low-density lipoprotein (oxLDL) is a strong inducer of endothelial dysfunctions and levels of plasma oxLDL are higher in patients with cardiovascular disease than in healthy subjects (4). When oxLDL is taken up by the scavenger receptor lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), the endothelium is activated and initiates a cascade of inflammation and atherosclerotic plaque formation events (5-7). Adhesion molecules on the activated endothelium binds circulating monocytes and several inflammatory factors are secreted by cells in the intima. One is monocyte chemotactic protein-1 (MCP-1), which attracts even more monocytes to the site of inflammation. The following cascade of events leads to macrophage differentiation, foam cell formation by macrophage oxLDL uptake and pro-inflammatory cytokine secretion, all of which amplifies the atherosclerotic inflammation. By inducing cell death in human umbilical vein endothelial cells (HUVEC), we wanted to investigate if barettin is able to prevent oxLDL-uptake and thus reduce endothelial activation. Activated endothelial cells are able to secrete MCP-1 and we also tested the effect of barettin on MCP-1 production on tumor necrosis factor α (TNFα)-activated HUVEC as well as from lipopolysaccharide (LPS)-stimulated THP-1 macrophages.

Cellular signaling is often controlled by kinases, and kinase-targeted therapy is an emerging field in drug discovery. However, the complexity of cell signaling makes it challenging to develop kinase inhibitors into drugs. In general, kinase-targeted drugs prescribed to treat inflammatory diseases are often either not sufficiently specific as they can inhibit

structurally similar kinases, or drug resistance is developed as compensatory pathways are activated (8, 9). Even though a high number of inflammation-associated kinases have been identified there are just a few drugs on the market targeting kinases for diseases involving the immune system (9, 10). Rapamycin (Sirolimus), originally isolated from a soil bacterium, inhibits the mTOR kinase, and it was the first kinase inhibitor approved as an immunosuppressant (9, 11, 12). Because inhibition of mTOR also leads to activation of NF-κB and an increased pro-inflammatory status, the use of this drug as an immunosuppressant is controversial (9). Today, rapamycin is mainly used to treat cancers. To investigate if the observed anti-inflammatory effects of barettin are exerted through inhibition of kinases related to inflammation, we screened barettin against a panel of 140 different kinases.

The properties of interleukin-4 (IL-4) are both pleiotropic and time/cell specific. This cytokine reduces expression of tumor necrosis factor alpha (TNFα) and interleukin-1β (IL-1β) in LPS-stimulated human monocytes but up-regulates MCP-1 and other pro-atherogenic modulators in endothelial cells (13-15). Moreover, in human monocytes IL-4 enhances the LPS-induced production of IL-10, an anti-inflammatory cytokine able to inhibit the production of several pro-inflammatory mediators, and an elevated level of IL-4 is detected in atherosclerotic lesions (16, 17). Here, we have studied the effect of barettin on LPS/IL-4-coactivated macrophages at a cytokine level.

Material and methods

Barettin

Two forms of barettin (*Z*-barettin and *E*-barettin) were isolated from the sponge *G. barretti* as described in a previous article (1). In the present study, we have examined the effect of *Z*-barettin.

HUVEC and THP-1

For the cytokine assays, HUVEC (CRL-1730) were seeded at a concentration of 5 000 cells per well in F-12K medium (ATCC #30-2004) supplemented with 10 µg/ml gentamycin (Biochrom, Berlin, Germany), 0.03 mg/ml endothelial cell growth supplement (ECGS, #E-2759, Sigma-Aldrich, St. Louis, MO, USA), 0.1 mg/ml heparin (Sigma-Aldrich) and 10 % fetal bovine serum (FBS, Biochrom) and incubated at 37 ºC. After 24 h, fresh media with 2 % FBS was added and the cells incubated for another 24 h. The cells were washed with phosphate buffered saline (PBS) (pH 7.2) before serum-free medium and test material were added to a total volume of 100 µl. The cells were then allowed to incubate at 37 ºC for 1 h before 1 ng/ml TNF (eBioscience, San Diego, CA, USA) was added. After a 6 h incubation at 37 ºC the reactions were stopped by freezing the plates at -80 ºC and they were kept at -80 ºC until the ELISA was performed. For the antioxidant assays, HUVEC were seeded at concentrations of 10^4 cells per well as described above.

THP-1 cells were seeded in 96 well plates at 10^5 cells per well in RPMI-1640 (BioChrom, FG 1385) supplemented with gentamycin and phorbol 12-myristate 13-acetate (PMA, 50 ng/ml). Plates were incubated for 48 h at 37 °C, 5% CO_2 and inspected after 24 h by microscopy to make sure they had started to differentiate. After the 48 h incubation, the cells were washed and added new RPMI (w/o PMA) before a further 24 h incubation. The cells were subsequently washed with PBS (pH 7.2) before serum-free medium and concentrations of

barettin from 3.125 to 100 μM were added and incubated 1 h before LPS (1 ng/ml) and IL-4 (10 ng/ml) were added to a total volume of 100 μl. The cells were then incubated for another 1, 2, 3 or 24 h. The reactions were stopped by freezing at −80 °C immediately after incubation, and kept at this temperature until performing the ELISA.

Cytotoxicity

The cytotoxicity of barettin in HUVEC cells was tested using 100 μM compound and measuring cell viability after 6 and 24 h with the CellTiter 96 AQueous One Solution (Promega, Madison, WI, USA) as previously described for other cell lines (1). In brief, 10^4 cells per well were seeded in 96 well plates and incubated with barettin for the time periods indicated. Then, 10 μl Aqueous One Solution was added each well and incubated further for 1 h. Absorbance was measured at 485 nm in a DTX 880 Multimode Detector and samples were compared to a control treated with Triton X-100 (0.01 percent) to induce complete cell death.

Antioxidant assays

The Cellular Antioxidant Activity (CAA) assay and the Cellular Lipid Peroxidation Antioxidant Assay (CLPAA) were performed with barettin at concentrations up to 100 μM as previously described on HepG2 cells to determine if barettin was able to inhibit cellular reactive oxygen species (ROS) formation or lipid peroxidation, respectively (1). In the CAA assay, the HUVEC were incubated with the test compound and 25 μM DCFH-DA (Sigma-Aldrich) for 1 h before the free radical initiator AAPH (2,2′-Azobis(2-methylpropionamidine) dihydrochloride; Sigma-Aldrich) was added. Sample readings were recorded immediately after addition of AAPH and after 1 h incubation with AAPH. Results were compared to an AAPH control.

In the CLPAA assay, C_{11}-BODIPY-labeled cells were treated for 1 h with the test compounds. Cumene hydroperoxide (CumOOH) was added to initiate lipid peroxidation and

green fluorescence was recorded over a period of 60 min (excitation/emission: 485/520 nm). Results were compared to a positive control with CumOOH alone.

OxLDL-induced cell death in HUVEC

HUVEC (10^4 cells per well) were seeded in 96 well plates as described above and incubated at 37 ºC overnight. The cells were allowed to incubate with 50 and 100 µM barettin for 1 h before adding 50-150 µg/ml oxLDL (Cell Biolabs, San Diego, CA, USA) and incubated further at 37 ºC for 24 h. The cell viability was measured using the CellTiter 96 AQueous One Solution as described above.

MCP-1 and IL-10 ELISA

ELISA was performed as described in the kit protocol using kits from eBioscience. The day prior to ELISA testing, MaxiSorp 96F-well plates (Nunc) were coated with MCP-1 or IL-10 capturing antibody and incubated overnight at 4ºC. Between each step, plates were washed with PBS (pH 7.2, with 0.05% Tween-20). All incubations were at room temperature with gentle shaking and unless otherwise stated, total reaction volume was 100 µl/well. After the overnight coating, 200 µL assay diluent was added and plates were incubated further for 1 h. MCP-1 samples were diluted 1:10 and IL-10 samples 1:5 in assay diluent and incubated for 2h. Diluted detection (secondary) antibody was next incubated with the samples for 1 h. Avidin-HRP was then added and plates were incubated further for 30 min. Finally, 100 µL substrate solution was added and incubated at room temperature for up to 15 min before 50 µl stop solution (2N H_2SO_4) was added. Absorbance was read at a DTX 880 Multimode Detector at 450 nm. Standard concentrations of MCP-1 and IL-10 was included in the setup to create a standard curve.

Kinase screening

Samples of barettin was sent to the International centre for kinase profiling (Dundee, UK) for kinase-screening against their panel of 140 kinases (see the appendix). The initial screening was performed using 50 μM barettin, as barettin at this concentration has both antioxidant and anti-inflammatory effect (1). An IC_{50} determination was performed against the three kinases best inhibited by barettin at concentrations from 8.3 nM to 250 μM.

Results

Antioxidant activity in HUVEC

When lipids in cell membranes are peroxidized the cell surface is altered and activates innate immune functions (18). An activated endothelium is an initiator of atherosclerosis (19) and by preventing lipid peroxidation of the vascular endothelial cells, we believe this can reduce atherosclerosis development. As previously reported, barettin reduced intracellular lipid peroxidation but not intracellular ROS formation in liver HepG2 cells (1). HUVEC is a model system often used to study endothelial cell functions and their role in atherosclerosis development (20). We therefore tested the effect of barettin as a functional antioxidant in these cells. As seen from Figure 1, the lipid peroxidation was reduced in a dose-dependent manner (down to 10 μM) although ROS formation was not prevented as measured by CAA (data not shown). Barettin did not reduce the cell viability in HUVEC (100 μM for 24h, data not shown).

OxLDL-induced cell-death in HUVEC

The effect of barettin on oxLDL-induced cell death in HUVEC was tested as oxLDL uptake is coupled to atherosclerosis (19). Barettin at 50 μM was not able to prevent the effect of 50-150 μg/ml oxLDL on cell viability (data not shown).

Effect of barettin on MCP-1 in HUVEC and THP-1 cells

MCP-1 is a chemokine that has been coupled to atherosclerosis development (21, 22) and IL-4 is known to up-regulate MCP-1 in HUVEC (23). To study whether barettin could have an effect on the MCP-1 production, both HUVEC and THP-1 cells were treated with an appropriate inducer (HUVEC with TNF, THP-1 with LPS or LPS+IL-4) and production of MCP-1 protein was measured using ELISA. IL-4 alone up-regulated LPS-induced MCP-1 production in THP-1 cells compared to cells stimulated with LPS alone (Figure 2A). When THP-1 cells were

treated first with barettin and then stimulated with LPS or LPS/IL-4 for 24 h, barettin at concentrations above 75 μM inhibited LPS as well as LPS/IL-4-induced production of MCP-1. The barettin inhibition of MCP-1 was slightly stronger in LPS/IL-4 co-stimulated cells compared to cells stimulated with LPS alone. IL-4 alone stimulated production of MCP-1 but less than LPS alone. Barettin failed to reduce the TNFα-induced MCP-1 in HUVEC (Figure 2B).

Kinases inhibitions

Kinases are central in regulation of inflammatory processes. To examine the involvement of kinases in the observed anti-inflammatory effect of barettin, barettin was screened against a panel of 140 kinases from several families (Appendix). Kinases with a residual activity below 40 % at 50 μM barettin are presented in Table 1. Barettin inhibited three kinases in particular, namely receptor-interacting serine-threonine kinase 2 (RIPK2), calcium/calmodulin-dependent protein kinase 1a (CAMK1α) and salt-inducible kinase 2 (SIK2) (Table 1). IC_{50} values for these three kinases were determined and they all had values ranging from 5.7 to 8.0 μM (Table 2).

LPS and LPS/IL-4-induced production of IL-10 in THP-1

CAMK1α mediates LPS-induced IL-10 secretion in macrophages (24). IL-10 is a cytokine with anti-inflammatory properties and since barettin strongly inhibits CAMK1α activity at 50 μM we also tested the effect of barettin on IL-10 production in THP-1 cells. Barettin inhibited IL-10 secretion from LPS-stimulated THP-1 macrophages in a dose and time-dependent manner (Figure 3). After 1 and 2 h of LPS-stimulation, no IL-10 was detected, after 3 h less than 50 pg/ml of the cytokine was secreted. After 24 h there was a marked increase in IL-10 production and barettin inhibited this in a dose-dependent manner.

LPS induces IL-10 production in monocytes and IL-4 further enhances the IL-10 expression (17). As seen in Figure 4, after 24 h, IL-4 upregulated LPS-induced IL-10 production significantly, in contrast to the more moderate up-regulation after 3 h. The dose-dependent inhibition of barettin on LPS-induced IL-10 (Figure 3) was also present when THP-1 cells were co-stimulated with LPS/IL-4 (Figure 4). However, when the co-stimulant IL-4 was present, barettin was able to up-regulate IL-10 at concentrations below 25 μM. IL-4 alone induced IL-10 at the same level as LPS.

Discussion

The previously reported antioxidant and anti-inflammatory properties of barettin led us to study the effect of the compound on other atherosclerosis-related mediators (1). Peroxidation of cell membrane lipids caused by oxidative stress changes the physiology of the cell membrane and results in cell membrane modifications as well as damage to cell membrane-associated proteins (25, 26). Thus, a reduction in lipid peroxidation would be beneficial to improve endothelial function as it reduces the harmful effects of ROS on the cell membrane. We found that barettin reduced lipid peroxidation in HUVEC and HepG2 cells, indicating that the antioxidant properties of barettin is a general property and not cell specific. Lack of effect on intracellular ROS formation in HUVEC confirmed the results from previous testing on HepG2 cells (1) suggesting that barettin is unlikely to interfere with ROS-mediated cellular signaling. Barettin was neither able to increase cell viability when cells were exposed to oxLDL for 24 h (data not shown), indicating that the mechanism of barettin does not involve oxLDL-pathways.

Since the early 1990's, MCP-1 has been considered an important mediator in several diseases, especially cardiovascular diseases and the atherosclerosis process (27-29). A reduction of MCP-1 production in endothelial cells and macrophages could prevent recruitment of monocytes to the arterial wall and to the arterial intima. Thus, the reduced MCP-1 expression

observed in THP-1 cells after barettin treatment could decrease the number of monocytes recruited and differentiated into macrophages and thereby suppress inflammation and development of atherosclerosis in the intima.

Interleukin-4 (IL-4) has been shown to upregulate MCP-1 production in endothelial cells (32), and since barettin reduced LPS-induced MCP-1 production in THP-cells, it was interest to examine the effect of barettin on THP-1 cells co-stimulated with LPS and IL-4. Interestingly, the effect of barettin on MCP-1 production in THP-1 cells was stronger on co-stimulated cells compared to cells stimulated with LPS alone (Figure 2). This could mean that barettin has an unknown target involved in IL-4 mediated signaling.

As kinases regulate several pathways involved in inflammation, barettin was screened against a panel of 140 kinases and the strongest inhibitory activity was found against CAMK1α, SIK2 and RIPK2 (Table 1), of which CAMK1α and RIPK2 have been linked to inflammation (9). Both RIPK2 and CAMK1α are serine/threonine kinases. The RIP kinases are involved in both innate and adaptive immune processes and can be induced by cytokines, LPS stimulation and peptidoglycans (30-34). RIPK2 is involved in transferring signals from LPS via toll-like receptor 4 (TLR4), and has been coupled to NF-κB activation and possibly also IL-1β maturation (35-38). Our findings that barettin has a potent inhibitory effect on RIPK2, might explain our previous results of dose-dependent inhibitory effect of barettin on LPS-induced IL-1β production in THP-1 cells (1). The exact role of RIPK2 in the aforementioned activities is however debated, as the active site of RIPK2 does not seem to be necessary for all functions of the molecule. For instance, RIPK2 kinase activity was not required for LPS-induced NF-κB activation or cytokine production but these processes were impaired in RIPK2-deficient mice after LPS activation (38, 39). Lu et al. suggest that RIPK2 may act as an adaptor molecule rather than an active kinase. In that case, the inhibition observed by barettin on IL-1β production could

be caused by inhibition of RIPK2 leading to impaired signal transduction and downstream effects on NF-κB activation.

CAMK1α is sensitive to Ca^{2+} and is together with its family members involved in gene expression, cell cycle regulation and differentiation (24). Zhang et al. reported that CAMK1α mediates LPS-induced IL-10 release in macrophages (24) while Woodward et al. reported an up-regulation of IL-10 in LPS-stimulated human monocytes isolated from blood(17). When these cells were co-stimulated with LPS/IL-4, the IL-10 production was further augmented (17). These findings are in accordance with our own results (Figure 4), on THP-1 cells showing that at low concentrations (below 25 μM), barettin was able to enhance the anti-inflammatory effect of IL-4 and further increased the secretion of IL-10. In contrast, when higher concentrations of barettin were used, the effect of LPS and IL-4 on IL-10 production was almost completely abolished. The dose-dependent inhibition of IL-10 production may be caused by the inhibitory effect of barettin on CAMK1α. Why barettin at low concentrations is able to stimulate the IL-10 production in the presence of IL-4 needs further investigation. As IL-10 is produced in the mid and late stages of inflammation by macrophages and lymphocytes, and because of the anti-inflammatory properties, it is considered atheroprotective as well (40). As IL-10 is an important mediator in inhibition of inflammation, a reduction might not always be favorable but high levels of IL-10 have been linked to severe outcome in patients with sepsis (41, 42). However, as levels of IL-1β, TNFα and MCP-1 are all reduced by barettin in THP-1 cells, the IL-10 inhibition does not augment the inflammatory situation. Currently we do not know how other important inflammatory mediators are affected by the reduced levels of IL-10.

Kinases are involved in complex signal transduction pathways, and even a selective kinase inhibitor will affect an array of signaling pathways (43). The kinase screening shows that barettin is a molecule with effect against a limited number of kinases, but these kinases are however involved in more than one pathway, which causes both positive and negative effects.

For instance, RIPK2 has been suggested to be a potential target in inflammatory treatment, including atherosclerosis (32). Studies have shown that both local and systemic immune signaling in macrophages from RIPK2$^{-/-}$ mice was reduced (44). However, the atherosclerotic lesions were greater in the RIPK2$^{-/-}$ mice than in the control group. This shows how inflammation and lipid accumulation is linked in development of atherosclerosis but also demonstrate the complexity of kinase signaling and inhibition.

Moderate inhibitory activity (between 20 and 30 percent remaining kinase activity) was found against eight kinases other than the three mentioned initially, among them MAPK interacting protein kinase (MNK) which have been proposed as a potential target in inflammation therapy since it is involved in several cellular functions like production of pro-inflammatory cytokines (45, 46). We can therefore not rule out that some of the observed effects are caused by additional inhibition of other kinases like the MNK.

Conclusion

Our study supports previous results demonstrating that barettin has potent anti-inflammatory and antioxidant effects that may be beneficial in treatment of atherosclerosis. Barettin reduced MCP-1 in THP-1 cells stimulated with LPS both in the absence and presence of IL-4. At low concentrations barettin enhanced the production of the anti-inflammatory cytokine IL-10 in the presence of IL-4, whereas higher concentrations of barettin (above 25 μM) inhibited IL-10 production. In the absence of IL-4, barettin had a strong inhibitory effect on IL-10 production. Previously, we have demonstrated that barettin dose-dependently inhibit IL-1β production in LPS-stimulated THP-1 cell. These results may be explained by the fact that barettin inhibited the kinases RIPK2 and CAMK1α, which both have been linked to inflammation.

Declaration of Interest

The authors report no declarations of interest.

Appendix

Kinase	Remaining activity	STDEV	Kinase	Remaining activity	STDEV	Kinase	Remaining activity	STDEV
MKK1	69	7	**HIPK3**	78	21	**NUAK1**	62	5
MKK2	79	5	**CLK2**	25	4	**SIK2**	8	1
MKK6	114	19	**PAK2**	63	1	**SIK3**	26	2
ERK1	93	16	**PAK4**	60	2	**TSSK1**	72	3
ERK2	86	2	**PAK5**	73	11	**CK1γ2**	89	4
ERK5	47	1	**PAK6**	102	13	**CK1δ**	88	7
JNK1	90	0	**MST2**	56	1	**CK2**	81	5
JNK2	67	8	**MST3**	74	10	**TTBK1**	92	12
JNK3	84	2	**MST4**	53	1	**TTBK2**	71	10
p38a MAPK	94	3	**GCK**	65	4	**DYRK1A**	33	3
p38b MAPK	85	4	**MAP4K3**	61	4	**DYRK2**	80	13
p38g MAPK	73	3	**MAP4K5**	75	1	**DYRK3**	63	1
p38d MAPK	86	8	**MINK1**	49	1	**NEK2a**	65	12
ERK8	35	0	**MEKK1**	78	7	**NEK6**	99	4
RSK1	32	4	**MLK1**	45	6	**MPSK1**	104	23
RSK2	37	2	**MLK3**	36	1	**WNK1**	99	5
PDK1	95	7	**TESK1**	78	25	**ULK1**	101	1
PKBa	78	3	**TAO1**	92	14	**ULK2**	98	8
PKBb	42	9	**ASK1**	71	3	**TGFBR1**	108	24
SGK1	38	9	**TAK1**	28	4	**Src**	90	19
S6K1	29	4	**IRAK1**	89	7	**Lck**	27	1
PKA	87	8	**IRAK4**	68	7	**CSK**	84	2
ROCK 2	72	3	**RIPK2**	16	3	**YES1**	77	6
PRK2	60	11	**OSR1**	101	3	**ABL**	60	1
PKCa	92	4	**TTK**	76	8	**BTK**	42	59
PKCγ	101	13	**CAMK1**	1	0	**JAK2**	91	7
PKCz	69	7	**SmMLCK**	28	2	**SYK**	68	7
PKD1	45	5	**PHK**	38	1	**ZAP70**	95	12
STK33	83	9	**DAPK1**	80	16	**TIE2**	75	2
MSK1	55	2	**CHK1**	69	7	**BRK**	122	15
MNK1	20	2	**CHK2**	64	0	**EPH-A2**	81	3
MNK2	60	3	**GSK3b**	83	6	**EPH-A4**	90	2
MAPKAP-K2	65	1	**CDK2-Cyclin A**	89	10	**EPH-B1**	109	4
MAPKAP-K3	77	5	**CDK9-Cyclin T1**	71	25	**EPH-B2**	73	11
PRAK	79	6	**PLK1**	110	4	**EPH-B3**	121	7
CAMKKb	68	1	**Aurora A**	111	6	**EPH-B4**	93	7
IKKb	62	2	**Aurora B**	68	3	**FGF-R1**	95	9
IKKe	47	4	**TLK1**	92	19	**HER4**	93	1
TBK1	78	7	**LKB1**	79	4	**IGF-1R**	78	14
PIM1	55	11	**AMPK (hum)**	118	8	**IR**	69	6
PIM2	77	5	**MARK1**	85	7	**IRR**	73	1
PIM3	70	9	**MARK2**	84	3	**TrkA**	81	5
SRPK1	86	0	**MARK3**	78	20	**DDR2**	87	7
EF2K	26	3	**MARK4**	80	3	**VEG-FR**	50	8
EIF2AK3	110	5	**BRSK1**	77	15	**PDGFRA**	58	6
HIPK1	60	7	**BRSK2**	81	7	**PINK**	93	5
HIPK2	58	3	**MELK**	60	2			

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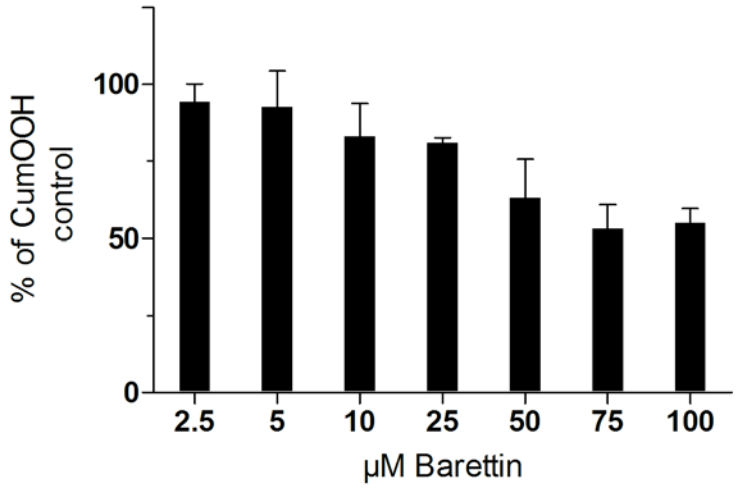

Figure 1. Cellular Lipid Peroxidation Antioxidant Activity (CLPAA) results for barettin. Barettin reduced cumene hydroperoxide-induced lipid peroxidation in endothelial HUVEC in a dose-dependent manner. The results are presented as mean ± SD compared to positive control with CumOOH (n=2).

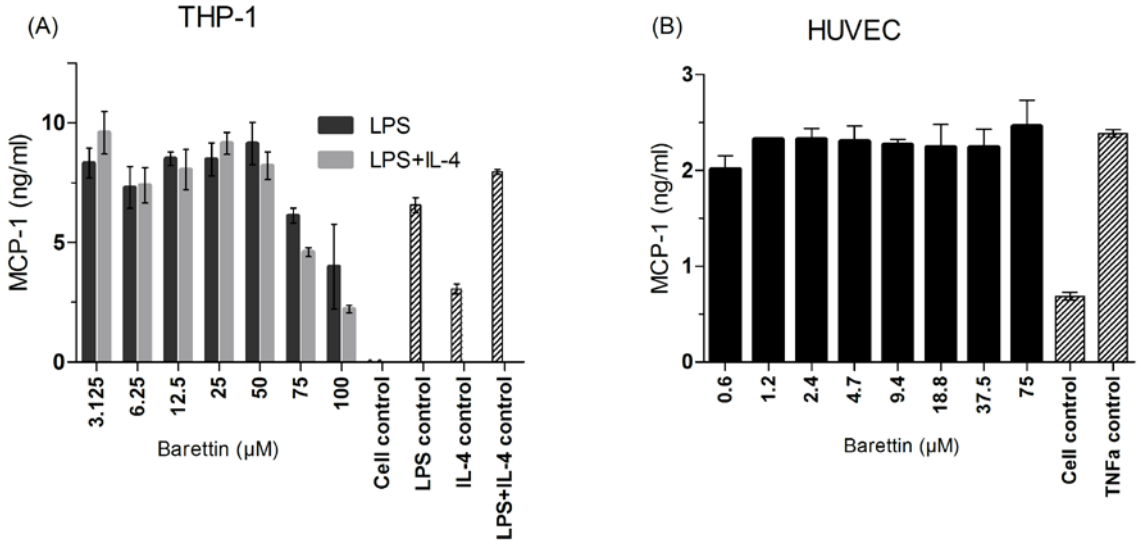

Figure 2. Effect of barettin on MCP-1 production. (A) Barettin inhibited MCP-1 production in LPS/IL-4 co-stimulated THP-1 cells at concentrations above 75 μM. (B) Barettin did not reduce the production of MCP-1 from TNFα-stimulated HUVEC. Results shown are mean ± SD from one representative experiment (n=2).

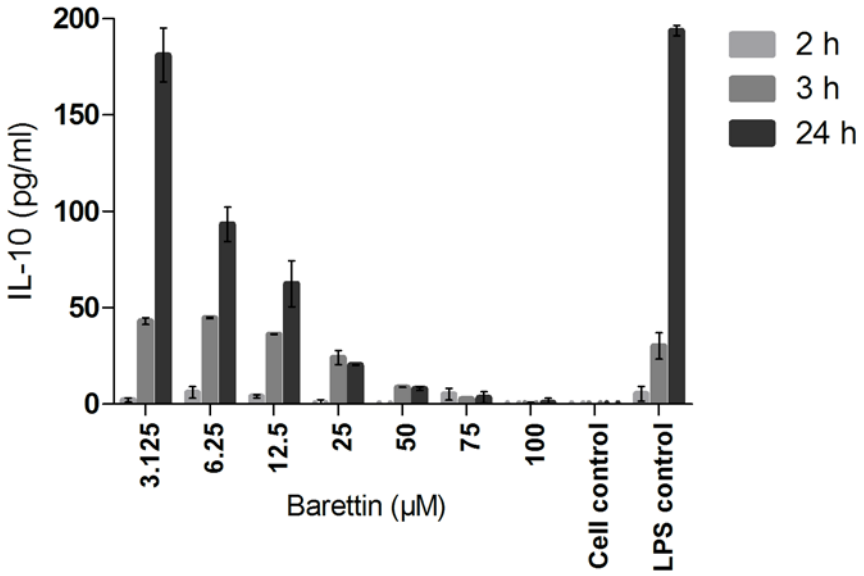

Figure 3. Barettin inhibited LPS-induced IL-10 from THP-1 macrophages in a time and dose-dependent manner. Only minor increases in IL-10 production was observed after 2 and 3 h of LPS-stimulation. After stimulating with LPS for 24 h, there was a considerable increase in IL-10 production and barettin dose-dependently reduced this. Results shown are mean ± SD from one representative experiment (n=2).

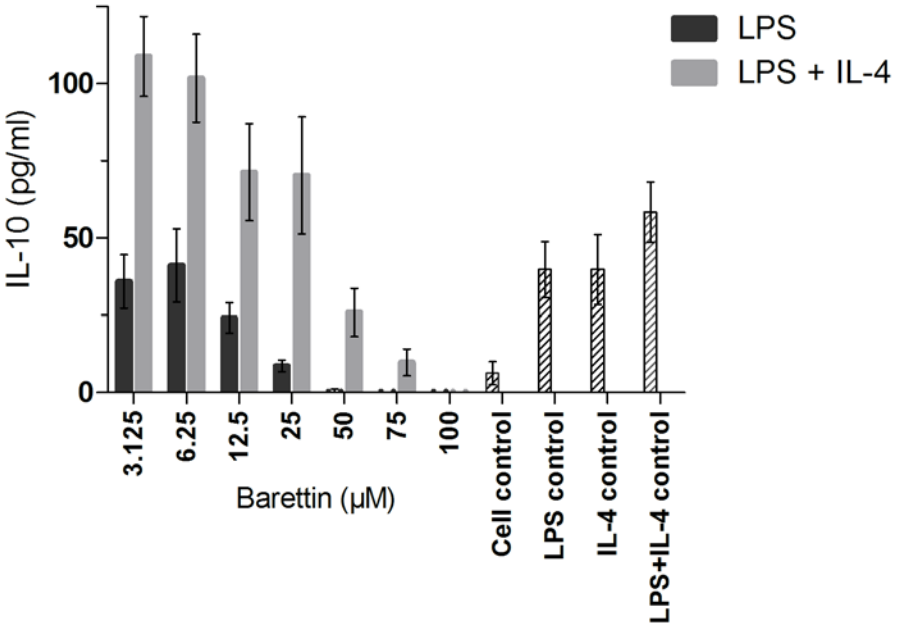

Figure 4. The effect of IL-4 and barettin on IL-10 from THP-1 cells after 24 hours. Barettin inhibited IL-10 from LPS-stimulated cells in a dose-dependent manner. At concentrations below 25 µM, barettin up-regulated IL-10 in LPS/IL-4 co-stimulated THP-1 cells compared to the LPS/IL-4 control. At higher concentrations (>50 µM), barettin inhibited the IL-10 secretion in these cells. Results shown are mean ± SD from two independent experiments (n=2).

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

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Summary

Background: Vascular inflammation is the prominent feature of atherosclerotic lesion formation. In search of bioactive products from the sea, we found barettin, isolated from the marine sponge *Geodia barretti*, to possess potent bioactive immunomodulatory effects. *Objectives:* The aim of this study was to examine the biological functions of barettin using an ex-vivo whole blood model stimulated with LPS. *Methods:* Blood was collected in Fragmin (10 U/ml). Aliquots were immediately stimulated with LPS (5 ng/ml) for 2 hours at 37^{o}C in a rotary incubator (190 rpm) in the presence and absence of barettin. TF activity was measured in isolated, frozen and thawed mononuclear cells, and plasma samples were assessed for TNFα, MCP-1, IL-10 and high mobility group box protein 1 (HMGB1) by ELISA . *Results:* Barettin was recently found by our group to selectively inhibit protein kinases, in particular receptor-interacting serine-threonine kinase 2 (RIPK2) and calcium/calmodulin-dependent protein kinase 1α (CAMK1α). Barettin attenuated LPS-induced TF activity and TNFα in a dose-dependent manner. At 5, 10 and 20 µg/ml, TF activity was reduced 34.8, 36.6, and 60.7 % and TNFα 35.1, 63.0 and 89.2%, respectively. Similarly, LPS-induced MCP-1 (CCL2) was inhibited 46.6, 55.5 and 46.1 % by barettin at 2.5, 5.0 and 10.0 µg/ml. The production of the anti-inflammatory IL-10, which preferably should be high, was also reduced by barettin, (45.6, 54.5 and 34.6 % at 2.5, 5.0 and 10 µg/ml, respectively), probably through the inhibition of CAMK1α. Activated monocytes/macrophages secrete HMGB1 as a cytokine mediator of inflammation. There was a non-significant reduction of HMGB1 by barettin in LPS-stimulated blood, but much less pronounced (21.3 % at 10 µg/ml) compared to the other test products. *Conclusions:* Barettin has a very potent anticoagulant and anti-inflammatory effect with the potential to prevent inflammatory based diseases including atherosclerosis.

Introduction

Atherosclerosis is a pro-inflammatory disease, and it is well known that the pro-inflammatory function of circulating monocytes is associated with enhanced risk of coronary heart disease (CHD). For many years, we have observed that the reactivity of monocytes, as judged by production of tissue factor in lipopolysaccharide (LPS) stimulated blood, is very different between individuals, ranging from low activity to very high activity (high responders) (1). This property of the monocytes appears to be inherited, and hyperactive peripheral blood monocytes were suggested to be associated with a significant risk factor in developing coronary heart disease (CHD) (2). Support for an important role of monocytes in atherosclerosis was reported several years ago as monocyte count was found to be a predictor for novel plaque formation (3, 4).

The emerging notion that chronic infections may unleash atherogenic trigger mechanisms is suggestive of a very important role of monocytes in lesion formation by the way of their proficiency in generating pro-inflammatory products. This warrants focus on the cellular signal transduction network of monocyte activation and potential products capable of suppressing the expression of pro-inflammatory products in blood cells, particularly monocytes. Low-grade inflammation can become detrimental if it persists for a prolonged period of time (chronic inflammation), and this occurs not only in atherosclerosis, but in a variety of chronic illnesses, including diabetes, cancer, obesity, asthma, Alzheimer's disease, Parkinson's disease and autoimmune disorders (5).

Monocytes are the major source of cytokines in blood, but also the only cells in the circulation synthesizing tissue factor (TF) (5). TF is a major initiator of blood coagulation. Being a transmembrane glycoprotein, TF binds to Factor VII (FVII)/FVIIa. The complex between TF and FVIIa activates FIX and FX leading to generation of thrombin, and subsequent activation of platelet and fibrin deposition (6). In addition to its role in coagulation, TF–FVIIa-dependent signaling pathways contribute to a variety of pathologic processes, including inflammation, atherosclerosis, angiogenesis and tumor metastasis (7, 8).

Obviously, products possessing anti-inflammatory properties may have the potential to prevent diseases associated with aging and other diseases connected to lifestyle and diet. In a search for such compounds, barettin isolated from the marine sponge *Geodia barretti*, was found to have

strong antioxidant activities in biochemical assays (9). Since we also have seen that barettin reduced LPS-induced IL-1β in THP-1 cells and inhibited particularly three kinases, RIPK2, CAMK1α and SIK2 (10), it was of great interest to examine its effect in a more physiological test-system. From earlier experience, we have observed that isolated monocytes in cell cultures or monocyte cell line cells behave differentially from monocytes in whole blood. Thus, PMA, TNFα, IL-6 and several other cellular activation products are inducing tissue factor (TF) and cytokine expression in isolated monocytes in cell cultures (11), whereas monocytes of heparinized (hirudinized) whole blood do not express these cell activation products after being exposed to the agonists (14). Further discrepancies between these cell systems have been observed as several hormones/drugs had the opposite effects in whole blood compared to cell cultures (11). This study was therefore undertaken to assess the effect of a barettin product on the production of TF and cytokines, relevant to the development of atherosclerosis, in LPS-stimulated whole blood. Since activated monocytes and macrophages secrete HMGB1 as a cytokine mediator of inflammation (12), it was also of interest to examine the effect of barettin on the production of HMGB1 in LPS-stimulated blood.

Materials and methods

Escherichia coli serotype 026-B6 LPS was obtained from Difco (Detroit, Mi, USA). Lymphoprep was purchased from Amersham (Oslo, Norway). The chromogenic substrate-peptide Pefachrome TH (Pefa-5114) for the determination of thrombin in the TF assay was obtained from Pentapharm (Basel, Switzerland). PMA was from Sigma Chemical Company (St Louis, MO, USA).

Barettin

Two forms of barettin (*Z*-barettin and *E*-barettin) were isolated from the sponge *G. barretti* as described in a previous article (9, 13). In the present study, we have examined the effect of *E*-barettin.

Blood samples

Venous blood was withdrawn from healthy volunteers using a plastic syringe with a 19 G needle. The blood samples were immediately dispensed into 5 ml polycarbonate tubes containing Fragmin at a final concentration of 10 U/ml blood. The study was approved by the Regional Committee for Medical Health Research Ethics in Norway.

Ex vivo stimulation of whole blood.

Aliquots of Fragmin anticoagulated blood (1 ml) were immediately after collection dispersed into polycarbonate tubes and added the various reagents.

Ex vivo stimulation of whole blood with lipopolysaccharide (LPS)

Aliquots of Fragmin anticoagulated blood (1 ml) were placed in polycarbonate tubes and stimulated with LPS (5 ng/ml) in the presence and absence of barettin at various concentrations for 2 hours incubation at 37^{o}C in a rotary incubator (190 rpm). Thereafter, the LPS-stimulation was stopped by adding 100 μl 0.005 M EDTA. Parts of the blood aliquots were centrifuged at 1200 g for 10 min, and the plasma obtained was stored frozen at –70^{o}C until ELISA testing/cytokine testing. The remaining aliquots were diluted 1:1 with saline, and mononuclear cells were then isolated as described earlier (14). The mononuclear cell samples were frozen at –20^{o}C until being thawed for testing the TF activity. The highest concentration of barettin used (20 μg/ml) had no adverse effect on the viability of the monocytes.

Measurement of TF activity

TF was quantified in frozen and thawed mononuclear cells in a two-stage amidolytic assay based on the ability of TF to accelerate the activation of FX by FVIIa, followed by the FXa conversion of prothrombin to thrombin in the presence of activated FV (15). Thrombin was quantified using the Th-1 substrate, and the amount of color generated was determined spectrophotometrically at 405 nm using a microplate reader. Crude rabbit brain extract was used as a standard for TF activity, with an undiluted activity assigned at 1 U/ml. Details of the assay have previously been described (15)

TNFα, MCP-1, IL-10, HMGB1, TxB_2 and LTB_4 measurements in plasma samples

TNFα was measured by using an ELISA system with anti-human TNFα and anti-human TNFα Biotin from eBioscience, whereas MCP-1 was tested in an ELISA kit named Human CCL2 (MCP-1) ELISA Ready-Set-Go, also from eBioscience. TxB_2, the stable product of TxA_2, and LTB_4 were determined by means of enzyme immunoassays (Amersham Pharmacia Biotech, Buckinghamshire, England). The detection limits of the assays for the quantification of TNFα, IL-6, TF antigen, LTB_4 and TxB_2 were 1.0 pg/ml, 0.2 pg/ml, 10.0 pg/ml, 6 pg/ml, and 3.4 pg/ml, respectively.

Cell count

Cell counts were performed in a Sysmex K 1000 (TOA Medical Electronics Co. Ltd, Kobe, Japan).

Statistical analysis

All results are reported as means ± SEM. The statistical analysis was performed by Student's t-test. Results were considered statistically different in the case of $P<0.05$.

Results

The inhibitory effect of barettin on LPS-induced TF activity and TNFα in monocytes from whole blood.

To investigate the effect of barettin on LPS-induced TF and TNFα in monocytes, whole blood anticoagulated with Fragmin was incubated with various concentrations of barettin as indicated and then stimulated with 5 ng LPS/ml for 2 hours at 37ºC in a rotary shaker. After stopping the incubation with EDTA, mononuclear cells were isolated, frozen and thawed before measuring TF activity. Separately incubated test samples were subjected to centrifugation, and the collected plasma was used for measurement of LPS-induced TNFα and IL-10. Fig 1 shows that barettin dose dependent reduced LPS-induced TF activity in monocytes of two different persons tested repeatedly. Whereas the average reduction of LPS-induced TF activity at 5 μg/ml barettin was 34.8 %, it was reduced by 60.7 % at 20 μg/ml ($p<0.01$). Similarly, TNFα was reduced by 35.1 % when blood was challenged with LPS in the presence of 5 μg /ml barettin and by 89.2 % at 20 μg/ml ($p<0.001$) (Fig. 2).

Reduction of LPS-induced TxB_2 and LTB_4 by barettin in whole blood

TxB_2, the stable product of TxA_2, is mainly produced by platelets in LPS-stimulated blood, whereas LTB_4 is produced both in monocytes and neutrophils. Barettin reduced the production of TxB_2 by 32.8 % at 5ug/ml and by 50.6 % at 10 ug/ml barettin ($p<0.05$) (Fig. 3), whereas the production of LTB_4 was reduced respectively 24.5 % and 26.7 % ($p<0.01$) (Fig.4).

*Inhibition of IL-10 synthesis by barettin*IL-10 is a cytokine that has anti-inflammatory effect by its inhibitory effect on LPS-activation of monocytes. Thus, a reduction in IL-10 does outweigh some of the beneficial anti-inflammatory effect of barettin. As shown in Fig. 5, barettin reduced LPS-induced IL-10 in the whole blood regime by 45.6 %, 54.5 % and 34.6 % at 2.5, 5.9 and 10.0 μg/ml, respectively ($p<0.05$).

The effect of barettin on MCP-1 production in monocytes

Since the production of MCP-1 is slower in LPS-induced monocytes of whole blood than for the products tested above, blood was stimulated with LPS ±barettin for 4 hours before plasma was prepared. As observed with the other activation products from monocytes, LPS-induced MCP-1 was reduced by 47.6 % at 2.5 µg/ml barettin and by 55.4 % at 5µg/ml ($p<0.06$) (Fig.6).

The effect of barettin on LPS-induced HMGB1

HMGB1, which is a mediator of inflammation and recently shown to be implicated in the development of atherosclerosis (12), is known to be mainly produced in monocytes/macrophages. The observation that Barettin inhibited CAMK1α, a kinase that has been shown to regulate IL-10 as well as HMGB1 (16), it was of interest to see whether barettin had any effect on this product. There was some reduction, but the effect on the concentration of HMBG1 in plasma of LPS-stimulated blood was less pronounced compared to the other test parameters above as the reduction was 21.3 % at 10 µg/ml barettin ($p<0.05$) (Fig. 7).

Discussion

In an ex vivo whole blood model we show that barettin has a remarkable anti-inflammatory effect by inhibiting LPS-stimulation of monocytes and thereby attenuate TNFα, MCP-1, TxB_2 and LTB_4 as well as TF activity in LPS-stimulated blood at a level which we never have seen before. These are all cellular activation products from monocytes known to play a central role in several inflammatory-induced diseases, including the development of atherosclerosis where MCP-1, TNFα and TF are important parts of the reactions leading to lesion formation and CHD (16). Although IL-10, an anti-inflammatory cytokine, is also significantly reduced by barettin, the overall reduction in several of the pro-inflammatory products may be more significant for the suppression of inflammation-mediated diseases (17). *Z*-barettin was also tested, but in contrast to results from studies on THP-1 cells (9), no effect or only a trend of enhanced LPS-induced monocyte activation was observed in the whole blood system by this barettin isomer.

TF, which is the physiological trigger of blood coagulation, is expressed by activated monocytes in whole blood through an interaction between monocytes, neutrophils and activated platelets in a P-selectin-PSGL-1-dependent reaction (14). In addition, we have previously shown that the TF activity expression of LPS-stimulated monocytes is mediated through signaling by thromboxane receptor, PAF-receptor and a protease (18), whereas at the gene level TF synthesis is regulated by NF-κB and AP-1 (19). TF, in addition to being mandatory for

initiating activation of the coagulation cascade, has important signaling effects on inflammatory reactions. Thus a potent inhibitor of the expression of TF may be of great interest not only for suppressing expression of TF under pathophysiological conditions, e.g. diseases associated with monocyte activation, but also TF-mediated pro-inflammatory reactions as seen in diabetes and obesity (20). The relatively weaker effect observed of barettin on LPS-induced TxB_2 and LTB_4 might stem from the long incubation of 2 hours, as both of them are synthesized quite rapidly and probably bind to the blood cells.

Compared to other anti-inflammatory products, barettin appears to be a very potent drug and more efficient than any other product we have tested previously in the whole blood model, e.g. prednisone, estrogen, melatonin, histamine and aspirin, where the latter product in whole blood upregulated TF activity in LPS-stimulated monocytes in contrast to its inhibitory effect in monocyte cell cultures.

As referred to above, protease(s) appeared to be involved in the expression of TF in monocytes of blood stimulated with LPS. It was therefore not surprising, as reported previously, that when screening barettin for inhibition of a large number of kinases, it showed a significant inhibition of particularly three kinases, RIPK2, CAMK1 and SIK2. Both CAMK1α and RIPK2 have regulatory effects on the innate immune system (16, 21, 22) and may therefore play an important role in the inhibitory effect of barettin of the production of inflammatory products in LPS-stimulated monocytes. Earlier studies showed that RIPK2 deficient mice has impaired activation of the transcription factor NF-κB in response to TLR signaling and are more resistant to LPS-induced lethal sepsis (23, 24). Thus, a study documented that RIPK2 interacts with the kinase TAK1, which leads to activation of NF-κB and mitogen-activated protein kinases (MAPKs) and induction of the expression of pro-inflammatory cytokines. Interestingly, RIPK2 has also been demonstrated to contribute to the induction of pro-inflammatory responses and susceptibility to gram-negative bacteria after exposure to LPS, a condition that was suggested to be associated with reduced Toll-like receptor signaling (25). Apparently, the RIPK2 pathway has likely key roles in the increased lethality and morbidity that clinically is observed in secondary bacterial infections.

Although inhibition of CAMK1α did not alter LPS-induced TNFα release in murine macrophages, it was shown that in a CLP model of sepsis, NT^{RNAi} mice displayed significant elevation of IL-10, IL-6, TNFα and HMGB1 concentrations, which were attenuated in

CaMK1α^{RNAi} mice (17). It was concluded that in a poly-microbial sepsis model, inhibition of CaMK1α had a broader effect with reductions in cytokines, including IL-10. The discrepancy of *in vitro* and *in vivo* was suggested to be attributed to the additional microorganisms, processes, cells and organs involved in the *in vivo* response to poly-microbial infection, in contrast to the LPS alone. However, it might also be argued that in contrast to the cell culture system, *in vivo* environment also includes all blood cells and thereby possible regulatory effects through interactions between the blood cells.

In the present study, we have seen a striking inhibitory effect of *E*-barettin, whereas it had no or only weak effect on LPS-induced TNFα in THP-1 cells (data not reported). In contrast, *Z*-barettin was found to have weak pro-inflammatory effect in the whole blood system (data not shown), but exerted significant inhibition of TNFα, IL-1β, MCP-1 and IL-10 in LPS-stimulated THP-1 cells subjected to adherence by incubation with PMA prior to LPS stimulation (10). This confirms our earlier observations of differential behavior of pro- and anti-inflammatory products tested in the whole blood model (11, 26, 27). Furthermore, in whole blood stimulated with the combination of LPS and PMA, the *E*-barettin tested in this study enhanced the production of monocyte activation products in a similar way as we have observed for melatonin (25) and histamine (26). This phenomenon has previously been demonstrated in studies where rabbit alveolar macrophages were stimulated with LPS. Whereas adherent cells treated with LPS (10 ng/ml) elicited a 26-fold increase in TNFα production, non-adherent cells did not elicit significant TNFα in response to LPS (28). It was concluded that adherence primes monocytes/macrophages for activation and regulation of MAPK signal transduction pathways. This could at least partially account for our observations of the barettin behavior.

In conclusion, this study demonstrates that barettin has a very potent anticoagulant and anti-inflammatory effect in an ex vivo whole blood model with probably great potential in prevention of e.g. atherosclerosis. This study needs to be followed up by experiments exploring the biochemical and cellular mechanisms involved, including studies on lesion formation in transgenic mice.

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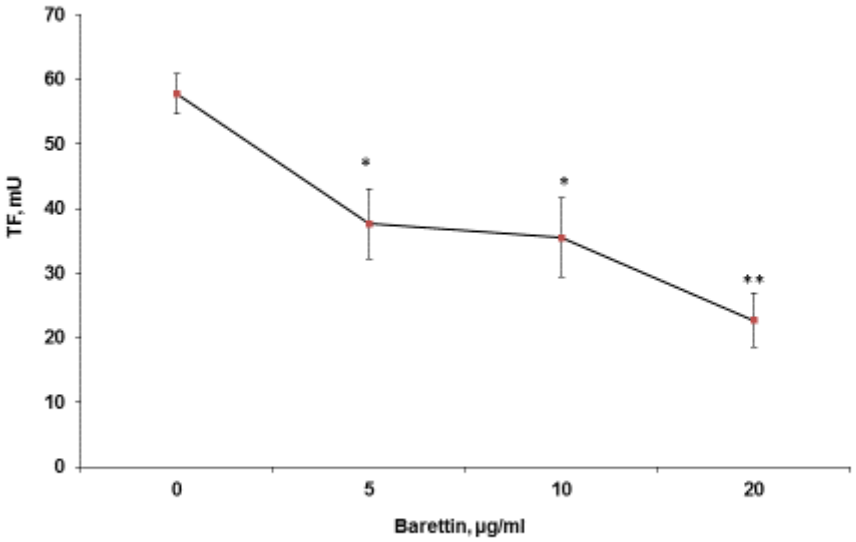

Figure 1

Dose-dependent effect of barettin on LPS-induced TF activity in monocytes of whole blood. Heparinized plasma was incubated with increasing concentrations of barettin, LPS (5 ng/ml) for 2 hours at 37 °C. The isolated mononuclear cells were frozen and thawed before testing for TF activity. Results from two individuals tested separately on four occasions are presented as mean ± SEM, *$p < 0.05$ and **$p < 0.01$. The values are respectively compared to the TF activity obtained without any barettin added.

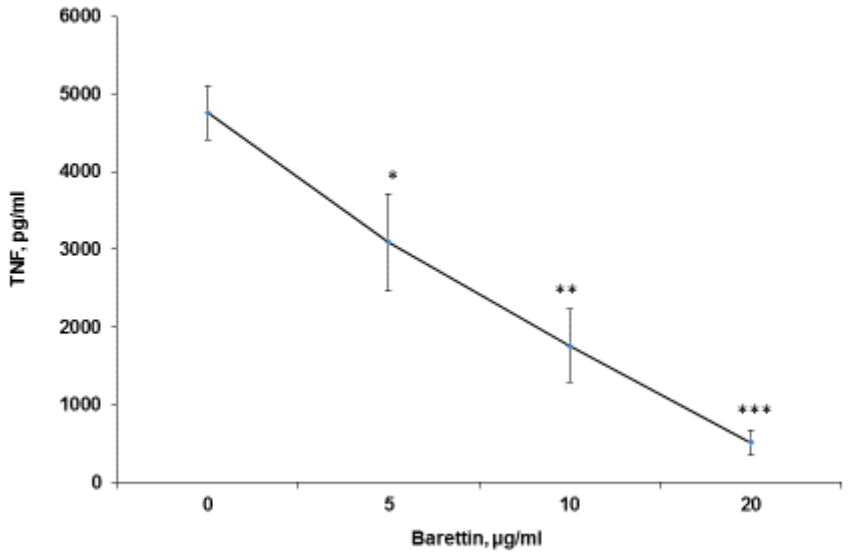

Figure 2

Dose-dependent effect of barettin on LPS-induced TNFα in monocytes of whole blood. The incubation was as described in Figure 1. The results are presented as mean ± SEM from two individuals tested on four different occasions, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. The values are respectively compared to the TNFα obtained without any barettin added.

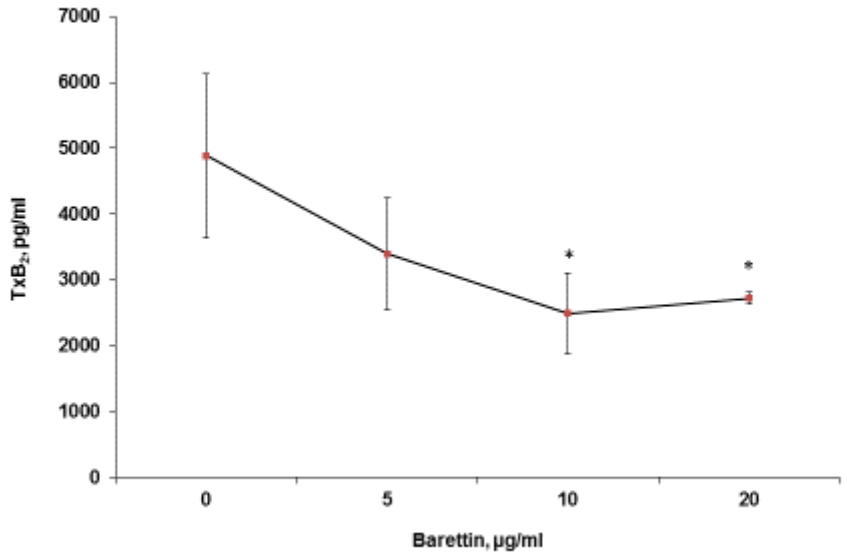

Figure 3

Dose-dependent effect of barettin on LPS-induced TxB_2 in monocytes of whole blood. The incubation was as described in Figure 1. The results are presented as mean ± SEM from two different individuals tested twice, *$p < 0.05$. The values are respectively compared to the TxB_2 obtained without any barettin added.

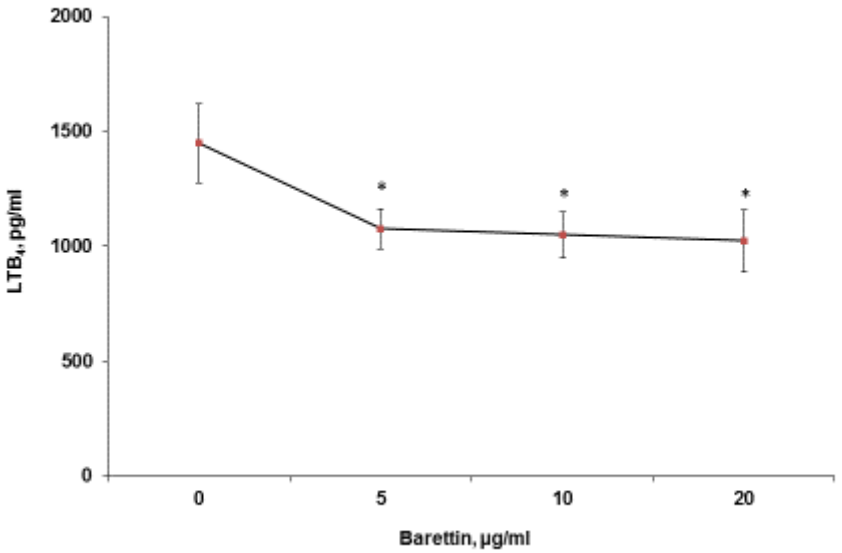

Figure 4

Dose-dependent effect of barettin on LPS-induced LTB4 in monocytes of whole blood. The incubation was as described in Figure 1. The results are presented as mean ± SEM from 2 different individuals tested twice, *$p < 0.05$. The values are respectively compared to the LTB_4 obtained without any barettin added.

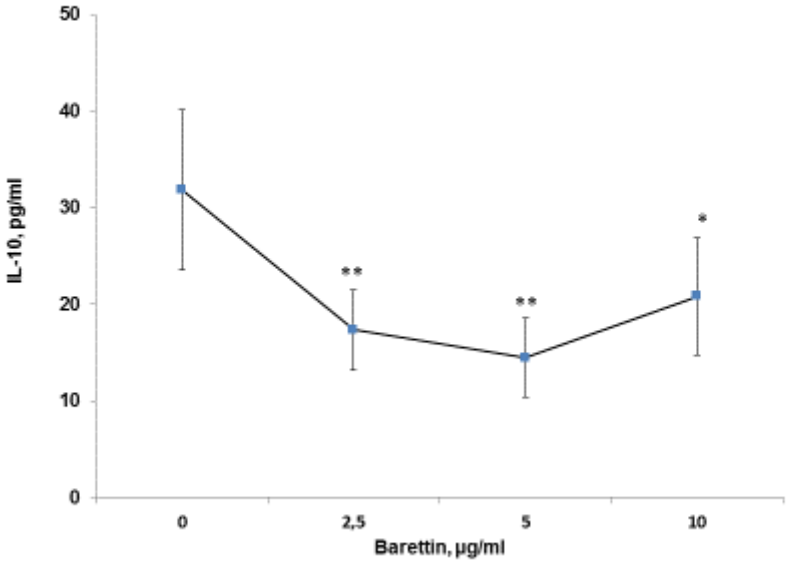

Figure 5

Dose-dependent effect of barettin on LPS-induced IL-10 in monocytes of whole blood. The incubation was as described in Figure 1. The results are presented as mean ± SEM from 2 different individuals tested twice in duplicates, *$p < 0.05$, **$p < 0.01$. The values are respectively compared to the IL-10 obtained without any barettin added.

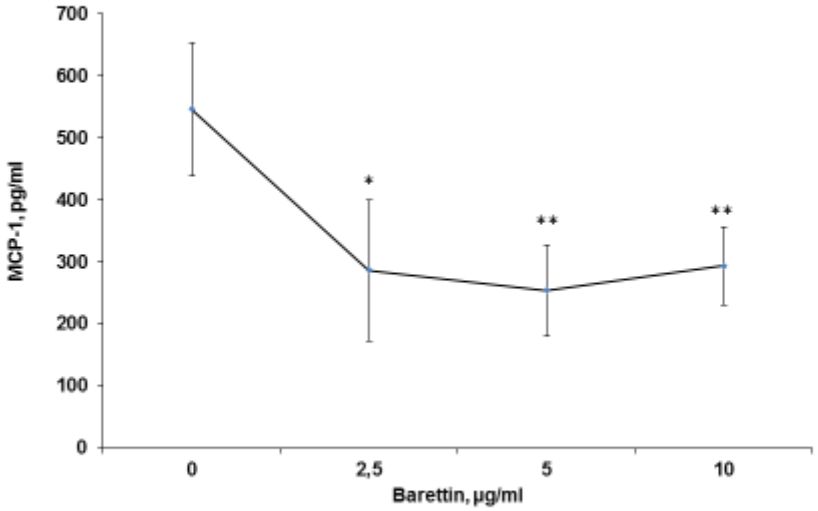

Figure 6

Dose-dependent effect of barettin on LPS-induced MCP-1 in monocytes of whole blood. The incubation was as described in Figure 1 except that the incubation time was 4 hours. The results are presented as mean ± SEM from 2 different individuals tested twice in duplicates, *$p < 0.05$, **$p < 0.01$. The values are respectively compared to the MCP-1 obtained without any barettin added.

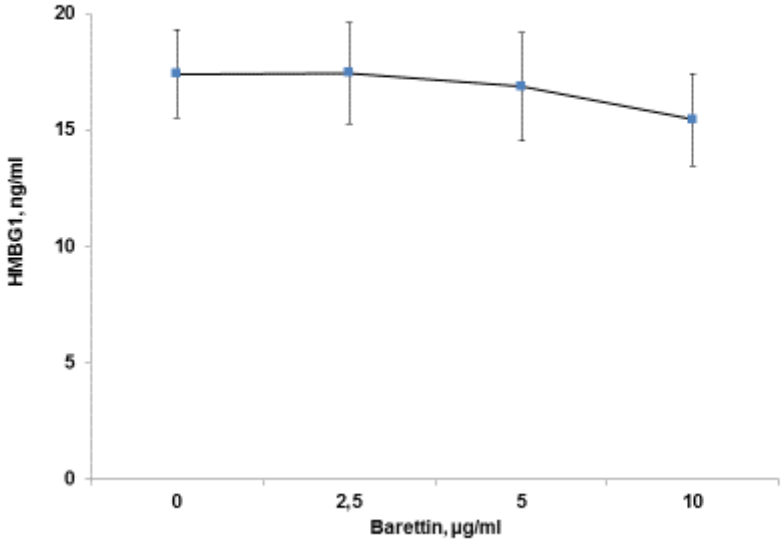

Figure 7

Dose-dependent effect of barettin on LPS-induced HMGB1 in monocytes of whole blood. The incubation was as described in Figure 1 except the incubation time was 4 hours. The results are presented as mean ± SEM from 2 different individuals tested on three different occasions (non significant). The values are respectively compared to the HMGB1 obtained without any barettin added.