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

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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 °C. 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 °C for 1 h before 1 ng/ml TNF (eBioscience, San Diego, CA, USA) was added. After a 6 h incubation at 37 °C the reactions were stopped by freezing the plates at -80 °C and they were kept at -80 °C 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% CO₂ 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 AQeuous 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 H₂SO₄) 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 1α (CAMK1α) and salt-inducible kinase 2 (SIK2) (Table 1). IC₅₀ 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<sup>−/−</sup> mice was reduced (44). However, the atherosclerotic lesions were greater in the RIPK2<sup>−/−</sup> 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.
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References


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).