Evaluation of Hyperforin Analogues for Inhibition of 5-lipoxygenase

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Abstract: The acylphloroglucinol hyperforin, a constituent of the herb *Hypericum perforatum* (St. John´s wort), was recently identified as potent and direct inhibitor of 5-lipoxygenase (5-LO), the key enzyme in the biosynthesis of proinflammatory leukotrienes. In this study, naturally occurring analogues of hyperforin, isolated from *H. perforatum*, as well as a series of synthetic derivatives obtained by chemical modification of hyperforin by acylation, alkylation or oxidation, were analysed for the inhibition of 5-LO. The efficacies of these compounds were evaluated in intact human polymorphonuclear leukocytes, but also the inhibitory effects on isolated recombinant human 5-LO were investigated. Our data show that some of the oxidised hyperforin derivatives possess even improved efficacy, whereas alkylation and acylation have detrimental effects.

Key Words: Hyperforin, St. John´s wort, *Hypericum perforatum*, 5-lipoxygenase, leukotriene, inflammation, polymorphonuclear leukocyte.

INTRODUCTION

The enzyme 5-lipoxygenase (5-LO) initialises the formation of the biologically active leukotrienes (LTs) by oxygenation of arachidonic acid (AA) and subsequent dehydratisation, leading to the unstable epoxide LTA_4 [1]. Due to their proinflammatory and allergic actions, LTs have been implicated in various allergic and inflammatory disorders, such as asthma, rhinitis, rheumatoid arthritis, chronic bowel diseases [1], and quite recently also in atherosclerosis and other cardiovascular diseases [2-4] as well as in cancer [5]. Inhibition of 5-LO enzyme activity is one pharmacological strategy for intervention with diseases related to LTs, but despite the need of efficient and selective 5-LO inhibitors, such drugs are still scarcely available.

St. John´s wort (*Hypericum perforatum*) extracts are widely used for the treatment of depression and anxiety, and hyperforin is assumed as the major component responsible for the antidepressive action [6]. *H. perforatum* extracts or hyperforin have also been reported to exert beneficial effects for the topical treatment of superficial wounds, burns and dermatitis [7, 8]. These anti-inflammatory implications could be confirmed in pharmacological *in vitro* studies, by the ability of hyperforin to suppress the release of reactive oxygen species, elastase, and AA metabolites from activated phagocytes [9-11]. At the molecular level, cyclooxygenase-1 and 5-LO were identified as direct targets of hyperforin [11].

Hyperforin is an unstable prenylated phloroglucinol derivative, which is prone to oxidative degradation. Apparently, the enolized β-dicarbonyl system, leading to two interconverting tautomers, is the critical moiety responsible for the instability of hyperforin. Various oxidised forms of hyperforin were isolated from *H. perforatum* extracts or obtained synthetically [12-16]. Biological evaluation (inhibition of synaptosomal accumulation of [^{3}H]serotonin) of hyperforin derivatives obtained by alkylation, acylation, and oxidation of the β-dicarbonyl moiety revealed that these chemical modifications are detrimental for neurotransmitter reuptake [14, 15].

We could previously show that hyperforin inhibits 5-LO in an uncompetitive manner, but the precise mode of action has not been elucidated yet. Interestingly, equimolar amounts of *H. perforatum* extracts, calculated for hyperforin, were much more potent to inhibit 5-LO *in vitro* as compared to hyperforin itself, and it appeared reasonable that (oxidised) degradation products of hyperforin may contribute to the efficient 5-LO inhibition. Therefore, and in order to determine possible structure-activity relationships, we evaluated the efficacies of hyperforin derivatives with respect to inhibition of 5-LO enzyme.

RESULTS AND DISCUSSION

Previous studies showed that hyperforin and extracts of *H. perforatum* inhibit 5-LO product synthesis in intact human polymorphonuclear leukocytes (PMNL) and suppress the activity of isolated recombinant 5-LO in an uncompetitive manner [11]. The IC_{50} values of hyperforin were determined in the range of 0.09 to 1.2 μM, which fit to the plasma levels of hyperforin (0.2 to 0.38 μM), attained after oral intake of *H. perforatum* extracts at standard dosage [17].

In order to determine possible structure-activity relationships, the enolized β-dicarbonyl system of hyperforin **1** was modified by oxidation, acylation, and alkylation [15], leading to a series of analogues (Fig. (**1**)), some of them present in the plant as genuine constituents, that were

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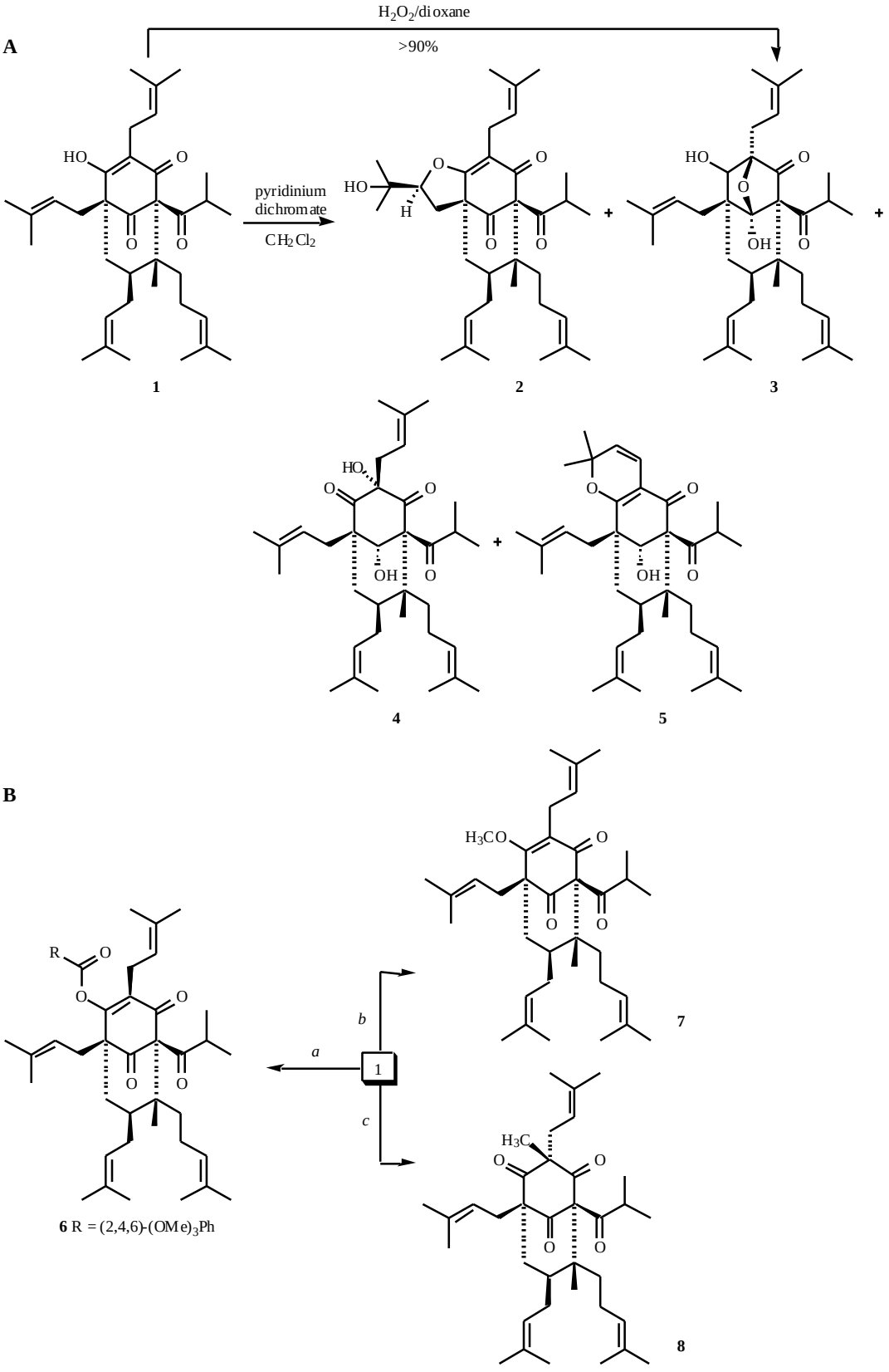

Fig. (1). Synthetic schemes and chemical structures of hyperforin and its oxidised (A) and alkylated or acylated (B) analogues a) Trimethoxybenzoyl chloride/py; b) CH_2N_2/Et_2O; c) CH_3I/NaH-DMSO.

evaluated for inhibition of 5-LO. Freshly isolated PMNL from peripheral blood were preincubated with the test compounds at a final concentration of 10 μM. 5-LO product formation was induced by stimulation with 1 μM ionomycin plus 20 μM AA and analyzed by HPLC. As shown in Fig. (**2A**), hyperforin **1** potently suppressed 5-LO product formation by 93 ± 2.5 %, which is in agreement with our previous studies [11]. Alkylated and acylated analogues were virtually not active. Thus, methylation of the C7-OH group (**7**) or methylation of C8 (**8**) within the β-dicarbonyl system abolished 5-LO inhibition. Similarly, acylation of the 7-enol tautomer (**6**) led to a loss of inhibitory activity. However, the oxidized analogues **2** and **3** caused inhibition of 5-LO by 61 ± 5 and 53 ± 2 %, whereas **4** and **5**, which are also oxidized derivatives of hyperforin **1**, were not effective. It should be noted that **2** and **3** are thought to be natural occurring metabolites of hyperforin **1**, which had been isolated from *H. perforatum* extracts [13, 14]. Thus, **3** was found to be one of the main product of spontaneous degradation of hyperforin to light and oxygen and is also the main product obtained after hyperforin treatment with hydrogen peroxide [18]. Concentration-response experiments for hyperforin **1**, **2**, and **3** were performed; the IC_{50} values were determined at 1.3, 8.1, and 9.4 μM, respectively (Fig. (**2B**)). Accordingly, hyperforin **1** is the most potent 5-LO inhibitor in intact cells, whereas oxidative modifications of the β-dicarbonyl moiety decrease the efficacy.

Pharmacological 5-LO inhibitors block 5-LO catalysis by reducing and/or chelating the active-site iron, but also act in a nonredox fashion by competing at fatty acid binding-sites of 5-LO [1]. Hyperforin possesses no iron-chelating or reducing properties [9, 10] and the mode of action of how hyperforin inhibits 5-LO is unknown. The efficacy of hyperforin analogues to inhibit the enzymatic activity of isolated recombinant 5-LO, was studied. In analogy to the effects of the compounds on 5-LO product synthesis in intact cells, hyperforin **1**, **2**, and **3** potently inhibited 5-LO at 10 μM (Fig. (**3A**)). Neither the acylated nor the alkylated analogues were effective. However, the activity of isolated 5-LO was also efficiently blocked by **5**, which for unknown reasons failed to suppress 5-LO product formation in intact PMNL. Detailed analysis of the potencies of these active compounds (Fig. (**3B**)) determined the IC_{50} values for hyperforin **1**, **2**, **3**, and **5** at 190 nM, 900 nM, 40 nM, and 170 nM, respectively, indicating that purified 5-LO enzyme is highly susceptible for these oxidized analogues. Compared to the parental hyperforin **1**, **3** is almost 5-fold more potent in inhibition of isolated 5-LO, and **5** is about equipotent with hyperforin **1**. In contrast, when these oxidised analogues were assayed for reuptake inhibition of 5-HT in synaptosomes, the potency was strongly reduced as compared to hyperforin **1** [14, 15], and also alkylated and acylated analogues were less potent under these experimental settings [15].

We found in our previous study that equimolar amounts of extracts of *H. perforatum*, calculated for hyperforin, were equipotent with hyperforin in inhibition of 5-LO in intact PMNL [11]. However, for isolated 5-LO *in vitro*, *H. perforatum* extracts were much more potent as compared to hyperforin. In accordance with the data obtained in the present study, the oxidised analogue **3** present in *H. perforatum* extracts may be responsible, or at least contributes, to the efficient enzyme inhibition. Another interesting phenomenon in our previous study was, that hyperforin is about 14-fold more potent in inhibition of isolated 5-LO as compared to 5-LO in intact cells [11]. Similarly, the oxidized analogues **2** and **3** showed about 10- and 230-fold higher efficacy for isolated than for cellular 5-LO, respectively, and **5** was active only on isolated 5-LO enzyme. The question remains, why hyperforin or its oxidised analogues substantially lose efficacy in intact cells. Possibly, in intact cells endogenous components, generated during cell stimulation, compete with hyperforin (or its oxidised analogues) at a common site of 5-LO, thereby impairing the potency. Alternatively, the intracellular uptake

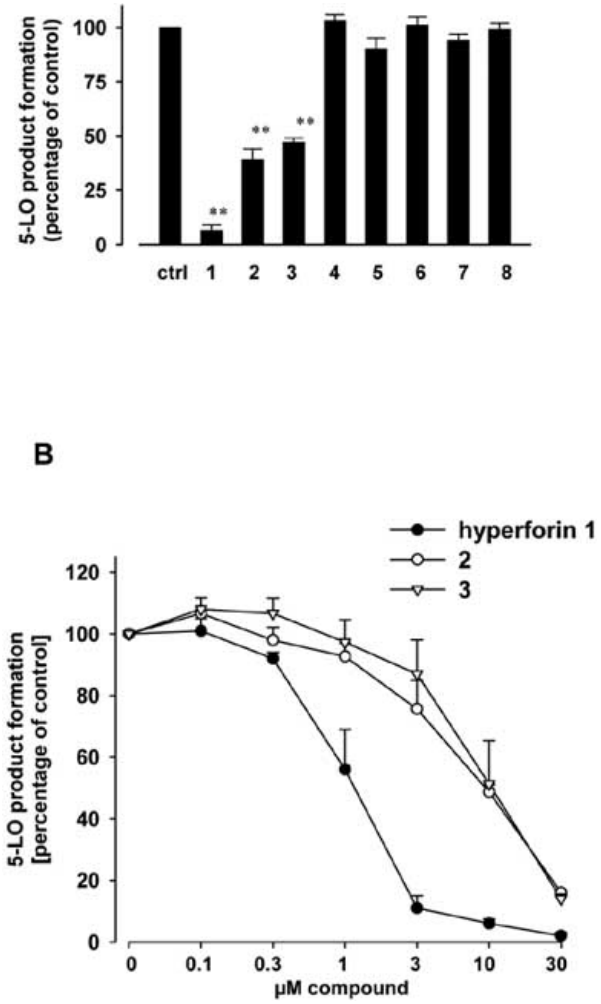

Fig. (2). Effects of hyperforin analogues on 5-LO product formation in intact cells. (**A**) PMNL were preincubated with the indicated compounds at 10 μM, stimulated with 1 μM ionomycin plus 20 μM AA and 5-LO products formed were extracted and determined by HPLC as described in the Experimental Section. (**B**) Cells were preincubated with the indicated concentrations of hyperforin 1, 2, and 3 and stimulated for 5-LO product formation as described above. Results are given as mean + S.E., n = 3-4. Student *t* test; $^{**}P < 0.01$.

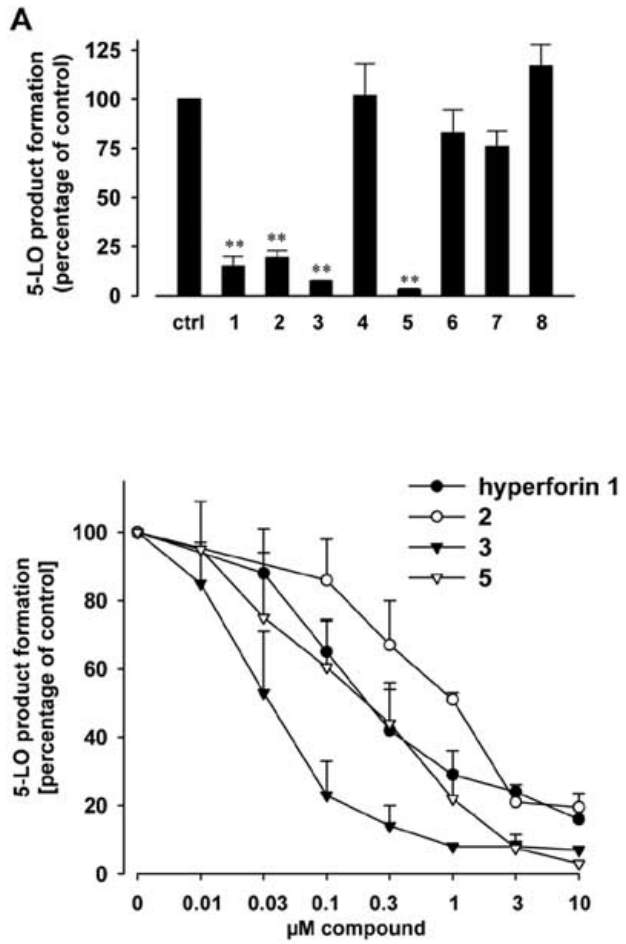

Fig. (3). Effects of hyperforin analogues on the activity of isolated recombinant 5-LO.

Human recombinant 5-LO was expressed in E.coli and partially purified as described. (**A**) 5-LO was added to a 5-LO reaction mix containing 10 µM of the indicated compounds and 5-LO product formation was determined by HPLC as described in the Experimental Section. (**B**) 5-LO was added to a 5-LO reaction mix containing the indicated concentrations of hyperforin **1**, **2**, and **3** and **5**, and 5-LO product formation was determined. Results are given as mean + S.E., n = 3. Student *t* test; ***P*< 0.01.

and availability of the compounds are poor, or the compounds are transported and located in compartments different from those where 5-LO resides. Finally, rapid degradation of the pharmacophore inside the cell is plausible.

In conclusion, hyperforin but also naturally occurring oxidised degradation products are potent inhibitors of isolated 5-LO *in vitro*. Future analysis of the binding-site of these compounds will unravel the precise mode of action. Knowledge of such a regulatory binding-site for pharmacological inhibitors may help to develop novel, selective and effective types of 5-LO inhibitors, capable to intervent with diseases related to LTs.

EXPERIMENTAL SECTION

Materials and Chemistry

Hyperforin was a generous gift from Schwabe AG (Karlsruhe, Germany). Synthetic and natural occurring derivatives of hyperforin were prepared as described previously [15]. AA, ionomycin, and prostaglandin B_1 were obtained from Sigma (Deisenhofen, Germany).

Cells

Human PMNL were freshly isolated from leukocyte concentrates obtained at St Markus Hospital (Frankfurt, Germany). In brief, venous blood was taken from healthy adult donors and subjected to centrifugation at 4000 × g for 20 min at 20°C for preparation of leukocyte concentrates. PMNL were promptly isolated by dextran sedimentation, centrifugation on Nycoprep cushions (PAA Laboratories, Linz, Austria), and hypotonic lysis of erythrocytes as described previously [19]. PMNL (7.5×10^6 cells/ml; purity > 96-97%) were finally resuspended in phosphate-buffered saline pH 7.4 (PBS) plus 1 mg/ml glucose.

Determination of 5-lipoxygenase Product Formation in Intact Cells

Freshly isolated PMNL (7.5×10^6/ml PBS pH 7.4 containing 1 mg/ml glucose and 1 mM $CaCl_2$ (PGC buffer)) were preincubated with the indicated compounds at RT for 15 min. Then, 5-LO product formation was started by addition of 1 µM ionomycin plus 20 µM AA. After 10 min at 37°C, the reaction was stopped with 1 ml of methanol and 30 µl of 1 N HCl, 200 ng prostaglandin B_1 and 500 µl of PBS were added. Formed 5-LO metabolites were extracted and analyzed by HPLC as described [20]. 5-LO product formation is expressed as ng of 5-LO products per 10^6 cells which includes LTB_4 and its all-*trans* isomers, 5(S),12(S)-di-hydroxy-6,10-*trans*-8,14-*cis*-eicosatetraenoic acid (5(S),12(S)-DiHETE), and 5(S)-hydro(pero)xy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-H(p)ETE). Cysteinyl LTs (LTC_4, D_4 and E_4) were not detected and oxidation products of LTB_4 were not determined.

Expression and Purification of Human Recombinant 5-lipoxygenase

Human recombinant 5-LO was expressed in *E. coli* JM 109 cells, transformed with pT3-5LO, and purified as described previously [21]. In brief, cells were lysed by incubation in 50 mM triethanolamine/HCl pH 8.0, 5 mM EDTA, soybean trypsin inhibitor (60 µg/ml), 1 mM phenylmethylsulphonyl fluoride (PMSF) and lysozyme (500 µg/ml), homogenized by sonication (3 × 15 s) and centrifuged at 19,000 × g for 15 min. Proteins were precipitated with 50 % saturated ammonium sulphate during stirring on ice for 60 min. The precipitate was collected by centrifugation at 16,000 × g for 25 min and the pellet was resuspended in 20 ml PBS containing 1 mM EDTA and 1 mM PMSF. After centrifugation at 100,000 × g for 70 min at 4°C, the 100,000 × g supernatant was applied to an ATP-agarose column (Sigma A2767), and the column was eluted as described previously [22]. Partially purified 5-LO was immediately used for *in vitro* activity assays.

Determination of 5-lipoxygenase Product Formation in Cell-free Systems

Isolated human recombinant 5-LO (0.5 µg in 5 µl) was added to 1 ml of a 5-LO reaction mix (PBS, pH 7.4, 1 mM EDTA, 1 mM ATP, and 25 µg/ml γ-globulin). The samples were preincubated with the indicated compounds for 5 – 10 min at 4°C. Samples were then prewarmed for 30 sec at 37°C and 2 mM $CaCl_2$ and 20 µM AA were added to start 5-LO product formation. The reaction was stopped after 10 min at 37°C by addition of 1 ml ice-cold methanol and the formed metabolites were analysed by HPLC as described for intact cells.

STATISTICS

The statistic program "GraphPad PRISM 3.0" was used for statistical comparisons. Statistical evaluation of the data was performed using Student´s *t* test for unpaired observations. A *P* value of < 0.05 was considered significant.

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