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Inhibitory effects of helenalin and related compounds on 5-lipoxygenase and leukotriene C_4 synthase in human blood cells☆

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Abstract

The sesquiterpene lactone helenalin, which can be isolated from several plant species of the Asteraceae family, is a potent anti-inflammatory and antineoplastic agent. In agreement, alcohol extracts of these plants are used for local external treatment of inflammatory conditions. Since leukotrienes are important mediators in inflammatory processes, the inhibitory effects of helenalin and some derivatives on leukotriene (LT) biosynthesis were studied. Treatment of human platelets with helenalin provoked irreversible inhibition of LTC_4 synthase in a concentration- and time-dependent manner with an IC_{50} of 12 μM after a 60 min preincubation. 11α,13-Dihydrohelenalin acetate was less potent. Interestingly, individual donors could be divided into two distinct groups with respect to the efficacy of helenalin to suppress platelet LTC_4 synthase. In human granulocytes, helenalin inhibited both the 5-lipoxygenase (IC_{50} 9 μM after 60 min preincubation) and LTC_4 synthase in a concentration- and time-dependent fashion. In contrast, the drug was without effect on LTA_4 hydrolase. The GSH-containing adducts (2β-(*S*-glutathionyl)-2,3-dihydrohelenalin and 2β-(*S*-glutathionyl)-2,3,11α,13-tetra hydrohelenalin acetate) did not significantly inhibit LTC_4 synthase. The present results indicate a mechanism for the anti-inflammatory effect of helenalin and related compounds.

Keywords: Leukotrienes; Leukotriene C_4 synthase; 5-Lipoxygenase; Helenalin; Sesquiterpene lactones

1. Introduction

STLs are secondary plant metabolites accumulated by many members of the Asteraceae and some other plant families [1,2]. Several compounds of this class possess a broad variety of conspicuous biological activities, some of which are of high interest with respect to potential medical utilisation [1,2]. The effects of STLs are mainly due to the capability of these compounds to inhibit the activity of enzymes and other functional proteins in living cells. These effects are mediated via a common chemical mechanism: formation of covalent bonds with free cysteinyl residues [1,2]. It has been demonstrated in many studies that STLs react spontaneously with low molecular weight thiols, such as cystein and glutathione (γ-glutamyl-cysteinyl-glycine, GSH) [3] by a Michael-type addition of thiol sulphur to α,β-unsaturated structural elements of the STLs (for review see [2]). Details on the stereochemistry and kinetics of such reactions induced by helenalin (which is present in the flowers of Arnica montana and A. chamissonis subsp. foliosa and a number of additional Asteraceae species) and some related compounds have recently been published [4,5].

Helenalin and certain derivatives, such as 11α,13-dihydrohelenalin acetate (Fig. 1), are of potential medicinal interest, since they are potent anti-inflammatory agents *in vitro* as well as *in vivo*. Thus, helenalin has been reported to alleviate carageenan-induced edema and chronic adjuvant-provoked arthritis in the rat [6]. Furthermore, helenalin and related STLs inhibited human neutrophil migration and chemotaxis [7] as well as platelet activation *in vitro*, possibly

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Abbreviations: STL, sesquiterpene lactones; NF, nuclear transcription factor; LT, leukotriene; and 5-HETE, 5-hydroxyeicosatetraenoic acid.

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via inhibition of phospholipase A_2 activity, an effect leading to reduced arachidonic acid liberation [8]. Most importantly with respect to their anti-inflammatory mechanism of action, helenalin, 11α,13-dihydrohelenalin, and chamissonolid in micromolar concentrations were recently shown to inhibit the function of the transcription factor NF-κB [9], which specifically up-regulates certain genes encoding inflammatory cytokines, immunoreceptors, cell adhesion molecules, and growth factors [10]. It has been reported that helenalin selectively modifies the p65 subunit of NF-κB [11], most probably by bifunctional alkylation of two specific cysteinyl residues in the DNA-binding region, thereby inhibiting DNA recognition and binding [12]. Considering the high concentration of reduced GSH present in most cells and the ease with which STLs react with such thiols, it was an open question as to how these compounds could reach their macromolecular target without being deactivated. However, recent studies have demonstrated that the conjugation with GSH is reversible under physiological conditions, so that a fraction of STL molecules is available under equilibrium conditions to form more stable protein adducts [5]. In addition to the effects caused by unconjugated STLs, it has been demonstrated that glutathione adducts of helenalin and 11α,13-dihydrohelenalin acetate are potent inhibitors of equine hepatic glutathione transferase [13]. This effect was not provoked by free helenalin, demonstrating that the glutathione adducts of STLs possess their own biological activity. This action is most probably mediated by the glutathionyl residues that are likely to enhance the affinity of these compounds to GSH-binding sites [13].

The leukotrienes are potent proinflammatory agents [14]. Upon cell activation, leukotriene biosynthesis is initiated by phospholipase A_2-dependent release of arachidonic acid from membrane phospholipids [15]. The liberated arachidonic acid binds to the integral perinuclear membrane 5-lipoxygenase-activating protein (FLAP), which makes the fatty acid available to 5-lipoxygenase [16]. After translocation to the nuclear membrane this enzyme catalyses both the dioxygenation of arachidonic acid to 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and the following dehydration, yielding the unstable epoxide LTA_4 [17,18]. The further metabolism of LTA_4 to biologically active leukotrienes proceeds via two different routes. Thus, LTA_4 may be converted to the inflammatory mediator LTB_4 by a cytosolic LTA_4 hydrolase [19] or metabolised by a membrane-bound LTC_4 synthase [20]. The latter enzyme specifically catalyses conjugation of LTA_4 with the tripeptide GSH, forming LTC_4. This compound is actively exported to the extracellular space [21,22], where it is enzymatically converted to LTD_4 and further to LTE_4. The cysteinyl leukotrienes (LTC_4, D_4, and E_4) bind to specific receptors and mediate a wide variety of inflammatory responses [23]. In particular, these compounds have been demonstrated to be of importance in bronchial asthma, and cysteinyl leukotriene antagonists are now in clinical use for pharmacological treatment of this disease [24,25].

In the light of the anti-inflammatory activities of helenalin and other STLs and the inhibitory effect of STL–GSH adducts on GSH *S*-transferase [13], it was of interest to investigate the effects of these compounds on LTC_4 synthase and other enzymes in the 5-lipoxygenase pathway. In the present study, we have determined the effect of helenalin and some analogues on the LTC_4 synthase activity in platelets. In addition, we have investigated the effect of these compounds on leukotriene formation from endogenous and exogenous substrate in human polymorphonuclear granulocyte suspensions.

2. Material and methods

2.1. Materials

Vacutainer® blood collection tubes were purchased from Becton Dickinson and sodium metrizoate (Lymphoprep®) was from Nyegaard & Co. Ionophore A23187 was obtained from Calbiochem-Boehring. Leukotriene A_4 methyl ester was a gift from Dr. Robert Zipkin, Biomol Research Laboratories and was saponified as described [26]. Leukotrienes B_4 and C_4 as well as prostaglandin B_2 were purchased from Biomol Research Laboratories and fatty acid-free human serum albumin (HSA) from Sigma Chemical Co.

2.2. Sesquiterpene lactones, natural products and semisynthetic derivatives

Helenalin and 11α,13-dihydrohelenalin acetate were isolated from Arnica species [27], whereas 2β-(*S*-glutathionyl)-2,3-dihydrohelenalin and 2β-(*S*-glutathionyl)-2,3,11α,13-tetrahydrohelenalin acetate (Fig. 1) were prepared from the respective natural products, as previously described [4,5]. The purity of all compounds was >95% as determined by ^{1}H-NMR.

2.3. Preparation of cell suspensions and sonicates

Peripheral venous blood from healthy volunteers was drawn into EDTA-containing blood collection tubes. The blood was centrifuged at 200 $\times$ *g* for 15 min and the platelet-rich plasma was collected. Thereafter, the granulocyte fraction was isolated by dextran sedimentation, hypotonic ammonium chloride lysis and Lymphoprep® centrifugation. Finally, the granulocytes were resuspended in phosphate-buffered saline (PBS, 0.9 mM Ca^{2+}, pH 7.4) at a concentration of 15 $\times$ 10^6 granulocytes/mL.

The platelet-rich plasma was further centrifuged at 650 $\times$ *g* for 20 min and the platelets were washed twice in 0.15 M NaCl buffered with 12 mM Tris/HCl, pH 7.4, containing 1.5 mM EDTA. Thereafter, the platelets were resuspended in PBS to a final concentration of 400 $\times$ 10^6 platelets/mL.

For experiments with cell sonicates, platelet suspensions

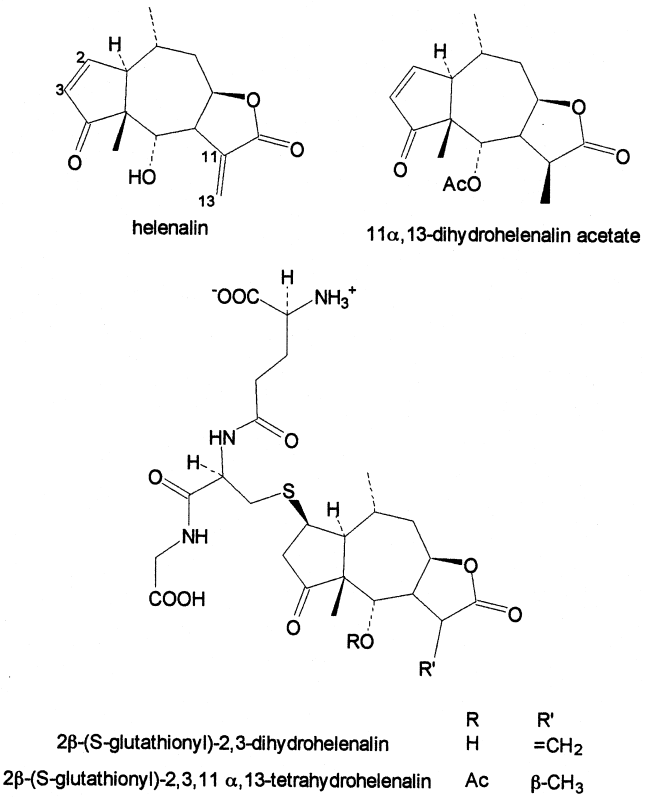

Fig. 1. Structure of the four sesquiterpene lactones: helenalin, 11α,13-dihydrohelenalin acetate, 2β-(*S*-glutathionyl)-2,3-dihydrohelenalin, and 2β-(*S*-glutathionyl)-2,3,11α,13-tetrahydrohelenalin acetate.

were sonicated (Ultrasonic disintegrator Mk2; power output, 50–150 W) in PBS without Ca^{2+} with or without 4 mM gluthathione (GSH) in the presence or absence of the STL derivatives at 0° for 3 × 5 sec. Prior to incubation, 2 mM $CaCl_2$ was added to the platelet sonicates.

Granulocyte suspensions were sonicated in PBS without Ca^{2+} in the presence of 1 mM EDTA at 0° for 3 × 5 sec. Prior to incubation, 2 mM $CaCl_2$ and 1 mM ATP were added to the granulocyte sonicates.

2.4. Incubation procedures

Cell suspensions or sonicates (0.5 mL) were preincubated at 37° for 1–60 min in the presence of HSA (0.3 mg/mL) with or without 1–600 μM of helenalin, 2β-(*S*-glutathionyl)-2,3-dihydrohelenalin, 11α,13-dihydrohelenalin acetate, or 2β-(*S*-glutathionyl)-2,3,11α,13-tetrahydrohelenalin acetate. Thereafter, 10 μM of LTA_4, 1 μM A23187, or 20 μM arachidonic acid was added and the suspensions were incubated for another 5 or 10 min. Reactions were stopped by addition of 5 vol. ethanol containing prostaglandin B_2 as internal standard.

2.5. Analysis of leukotrienes and 5-HETE

Before HPLC, the samples were centrifuged, evaporated, dissolved in the mobile phase, and re-centrifuged. The samples were analysed by RP–HPLC as described [26], using a Nova-Pak C_{18} column (3.9 mm × 150 mm, Water Associates) eluted with acetonitrile/methanol/water/acetic acid (27:18:54:0.8, by vol., apparent pH 5.6, for leukotriene analysis) or methanol/water/acetic acid (73:27:0.01, for HETE analysis) at a flow rate of 1 mL/min, and a variable wavelength UV detector (LDC Spectromonitor III) at 280 nm (leukotrienes) or 236 nm (HETEs), connected to an integrator (EZ Chrom™ chromatography data system).

3. Results

3.1. Effect of helenalin, 2β-(S-glutathionyl)-2,3-dihydrohelenalin, 11α,13-dihydrohelenalin acetate, and 2β-(S-glutathionyl)-2,3,11α,13-tetrahydrohelenalin acetate on LTC_4 synthase activity in human intact platelets

Helenalin, 11α,13-dihydrohelenalin acetate, 2β-(*S*-glutathionyl)-2,3-dihydrohelenalin, and 2β-(*S*-glutathionyl)-2,3,11α,13-tetrahydrohelenalin acetate were tested at 100 μM (5 min preincubation) for their effects on LTC_4 synthase activity in human platelet suspensions incubated with LTA_4. In control incubations (without sesquiterpene lactone), 812 ± 408 (mean ± SD, N = 9) pmol LTC_4/mL were formed. Helenalin caused 50 ± 19% (N = 6) inhibition of the LTC_4 synthase activity, whereas 11α,13-dihydrohelenalin acetate was less potent (26 ± 5% inhibition, N = 4). Under these conditions, the two glutathionyl-helenalin derivatives were without effect on LTC_4 synthase activity in intact platelets. Based on these findings, the effects of helenalin and 11α,13-dihydrohelenalin acetate on platelet LTC_4 synthase were further investigated.

Dose–response experiments (1–300 μM) demonstrated that helenalin and 11α,13-dihydrohelenalin acetate inhibited platelet LTC_4 synthase activity in a concentration-dependent manner (Fig. 2). The IC_{50} values for helenalin and 11α,13-dihydrohelenalin acetate after 5 min preincubation were 100 (N = 6) and 275 μM (N = 4), respectively. However, when the preincubation was increased to 60 min, the IC_{50} value for helenalin decreased to 12 μM (N = 6).

Time-course studies with helenalin and 11α,13-dihydrohelenalin acetate demonstrated that both substances inhibited the LTC_4 synthase activity in a time-dependent manner. Platelet suspensions were preincubated for various times with 30 μM helenalin or 11α,13-dihydrohelenalin acetate prior to incubation with 10 μM LTA_4. The inhibition induced by helenalin increased from 20 ± 10 to 60 ± 17% (N = 10) when the preincubation time was increased from 1 to 30 min. In addition, the inhibitory effect of 11α,13-dihydrohelenalin acetate increased from 12 ± 4% to 29 ± 2% (N = 7). Interestingly, the effect of helenalin on the LTC_4 synthase activity in human platelets varied among different donors. Thus, the individual donors could be di-

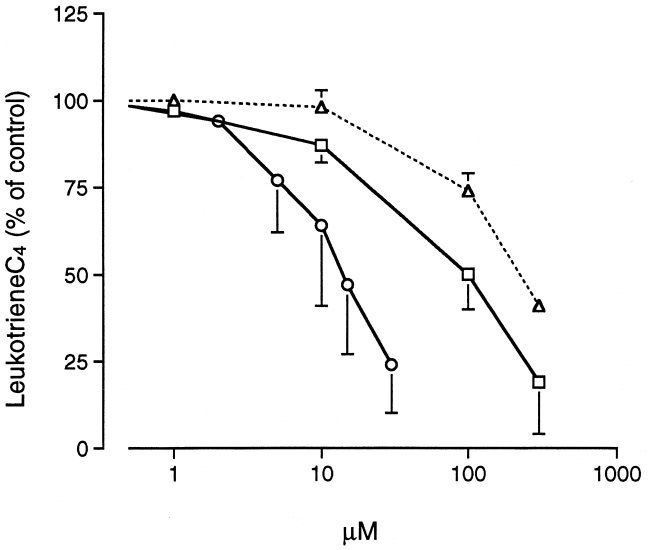

Fig. 2. Dose–response curves of the effect of helenalin and 11α,13-dihydrohelenalin acetate on leukotriene C_4 synthase activity in human platelets. Platelet suspensions were preincubated at 37° for 5 min in the presence of 1–300 μM of helenalin (squares) or 11α,13-dihydrohelenalin acetate (triangles) or 60 min in the presence of 2–30 μM helenalin (circles) prior to incubation with 10 μM LTA_4 for another 5 min. Values are expressed relative to the basal LTC_4 synthase activity in control incubations. Each value represents the mean of 4–6 experiments performed in duplicate. Error bars indicate standard deviation.

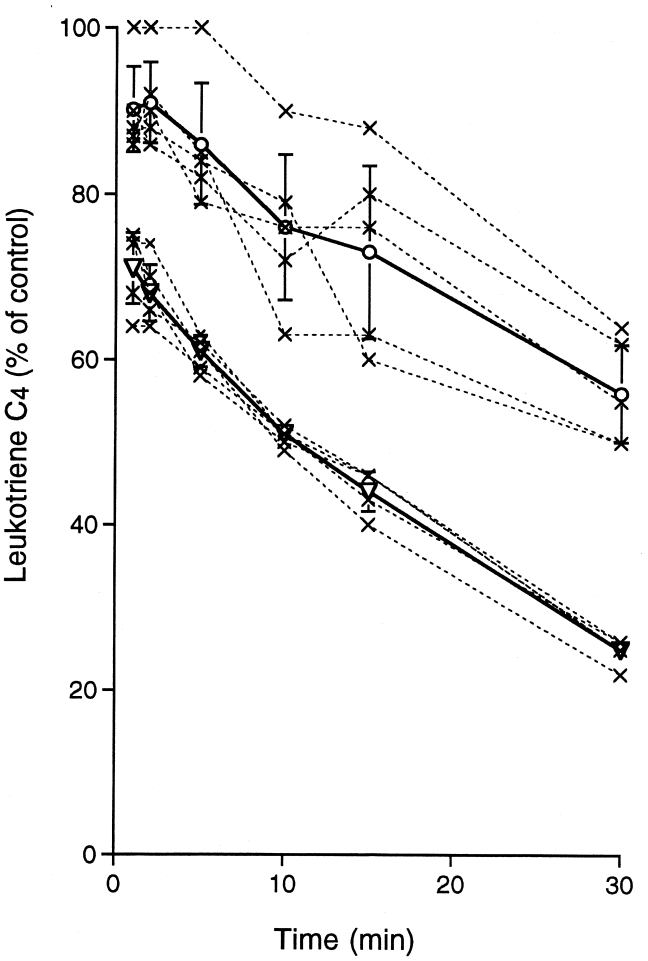

Fig. 3. Time-course of the effect of helenalin on leukotriene C_4 synthase activity in human platelets. Platelet suspensions were preincubated at 37° for 1–30 min in the presence of 30 μM helenalin prior to incubation with 10 μM LTA_4 for another 5 min. Data are presented both as time-courses from ten individual subjects (cross) and as average values obtained from high (N = 5) and low (N = 5) responders (triangles and circles), respectively. Error bars indicate standard deviation. $P < 0.001$ between high and low responders at all times.

vided into two distinct groups with respect to the response of the platelet LTC_4 synthase activity to helenalin. In one group, helenalin provoked 50% inhibition of platelet LTC_4 production after 10 min, whereas only 44 ± 6% inhibition could be observed after 30 min in the other group (Fig. 3). Among platelet preparations from ten randomly tested donors, five individuals displayed the more sensitive profile. Furthermore, repeated experiments with platelets from the same donors showed that the individual sensitivity to helenalin was reproducible (results not shown).

3.2. Effect of helenalin on LTC_4 formation in platelet sonicates

Basal LTC_4 formation in platelets sonicated in the presence of glutathione and incubated with 10 μM LTA_4 for 5 min was 747 ± 352 pmol/mL (N = 11). Preincubation of platelet sonicates with 100 μM helenalin, 11α,13-dihydrohelenalin acetate, 2β-(*S*-glutathionyl)-2,3-dihydrohelenalin, or 2β-(*S*-glutathionyl)-2,3,11α,13-tetrahydrohelenalin acetate for 5 min in the presence of 4 mM GSH prior to addition of LTA_4 did not affect LTC_4 synthase activity. However, when GSH was added simultaneously with LTA_4, 5 min after helenalin, 26 ± 9% (N = 3) and 60 ± 7% (N = 3) inhibition of LTC_4 synthase activity was observed with 100 and 300 μM helenalin, respectively. When low amounts of GSH (50–1200 μM) were added to the sonicates at the same time as helenalin (300 μM), the inhibitory effect was attenuated in a concentration-dependent manner (Fig. 4).

In addition, decreased LTC_4 synthase activity in platelet sonicates was observed, when helenalin was added to the intact platelets before sonication. Interestingly, this effect remained even after washing the cells twice prior to sonication. Thus, incubation with 300 μM helenalin provoked 64 ± 7% (N = 5) and 45 ± 6% (N = 3) inhibition of LTC_4 synthase activity in non-washed and washed cells, respectively. The other investigated compounds were less potent with 33 ± 6% (N = 4), 28 ± 8% (N = 4), and 30 ± 15% (N = 3) inhibition of LTC_4 synthase activity in non-washed cells provoked by 300 μM 11α,13-dihydrohelenalin acetate, 2β-(*S*-glutathionyl)-2,3-dihydrohelenalin and 2β-(*S*-glutathionyl)-2,3,11α,13-tetrahydrohelenalin acetate, respectively.

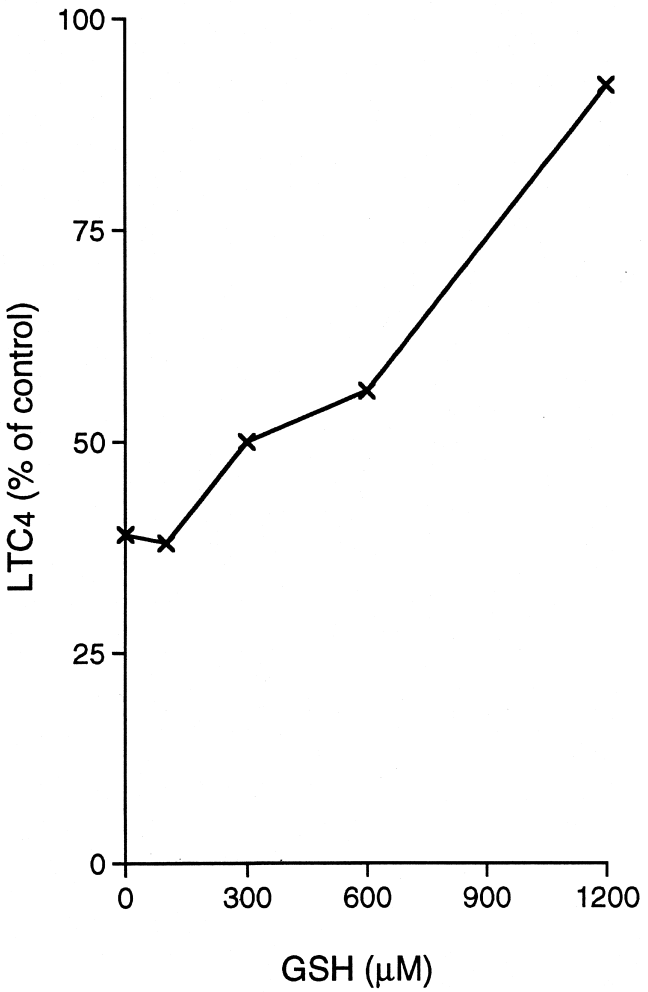

Fig. 4. Dose–response curve of the effect of GSH on helenalin-induced inhibition of platelet leukotriene C_4 synthase activity. Human platelet sonicates were preincubated at 37° for 5 min in the presence of 300 μM helenalin and various concentrations of GSH (0–1.2 mM) prior to incubation for another 5 min with 10 μM LTA_4 and 4 mM GSH. Values are expressed relative to the basal LTC_4 synthase activity in control incubations without helenalin. Each value represents the mean of duplicate determination from one representative experiment out of three.

3.3. Effect of helenalin and 11α,13-dihydrohelenalin acetate on ionophore A23187- or LTA_4-induced leukotriene formation in human granulocyte suspensions

The effect of 5 min preincubation with helenalin or 11α,13-dihydrohelenalin acetate (30–300 μM) on ionophore A23187-induced leukotriene formation in human granulocyte suspensions was investigated. In control incubations performed in the absence of sesquiterpene lactones, 137 ± 101 (mean ± SD, N = 9) pmol LTC_4/mL and 579 ± 148 (N = 8) pmoL LTB_4/mL were produced after 5 min. Helenalin concentration-dependently inhibited the formation of both LTC_4 and LTB_4 (Fig. 5) with an IC_{50} of approximately 70 μM, whereas 11α,13-dihydrohelenalin acetate inhibited the formation of LTC_4 with an IC_{50} of 300 μM. In contrast, the latter compound induced only 13 ± 6% (N = 6) inhibition of LTB_4 formation.

The effects of helenalin or 11α,13-dihydrohelenalin acetate (30–300 μM) on LTA_4 hydrolase and LTC_4 synthase activity in intact granulocytes were also investigated. In control incubations (without STLs), exogenously added LTA_4 (10 μM) was converted to 552 ± 210 (N = 8) pmol LTB_4/mL and 172 ± 109 (N = 9) pmol LTC_4/mL. Helenalin inhibited the LTC_4 synthase activity in a concentration-dependent manner (Fig. 5) with an IC_{50} of approximately 200 μM. In contrast, no significant inhibition of 11α,13-dihydrohelenalin acetate on the LTC_4 synthase activity was observed (13 ± 10% inhibition, at 300 μM; N = 6). The LTA_4 hydrolase was not inhibited by helenalin or 11α,13-dihydrohelenalin acetate (30–300 μM).

3.4. Effect of helenalin and 11α,13-dihydrohelenalin acetate on 5-lipoxygenase activity after incubation of human granulocyte sonicates with exogenous arachidonic acid

Human granulocyte sonicates were incubated for 5 min prior to incubation for another 10 min with 20 μM arachidonic acid. In control incubations, 1528 ± 630 (N = 4) pmol 5-HETE/mL was formed. In the presence of helenalin or 11α,13-dihydrohelenalin acetate, a concentration-dependent attenuation of 5-HETE formation was observed with approximately 50% inhibition at 75 and 200 μM, respectively. When the preincubation time was increased to 60 min, the IC_{50} value for helenalin decreased to 9 μM (Fig. 6).

In time-course studies, granulocyte sonicates were also incubated with 30 μM of the compounds for various times (1, 2, 5, 10, 15, and 30 min) prior to addition of arachidonic acid (20 μM). In the absence of the compounds, 1906 ± 1022 (N = 4) pmol 5-HETE/mL was formed. Time-dependent inhibition of 5-HETE formation was observed in the presence of helenalin or 11α,13-dihydrohelenalin acetate with 50% inhibition obtained after 7 and 10 min, respectively (Fig. 7). After 30 min, 82 ± 8% (N = 4) and 69 ± 9% (N = 4) inhibition was observed in the presence of helenalin and 11α,13-dihydrohelenalin acetate, respectively.

4. Discussion

In the present investigation, we report that helenalin and 11α,13-dihydrohelenalin acetate inhibited LTC_4 synthase activity in human platelets in a concentration-dependent manner. After preincubation for 5 min (Fig. 2), the IC_{50} values were 100 and 275 μM, respectively. However, time-course studies demonstrated that the inhibitory effects of both compounds were time-dependent, with considerably lower IC_{50} values after longer preincubation times. Interestingly, the efficiency of helenalin to inhibit platelet LTC_4 synthase activity was similar to the inhibitory potency of

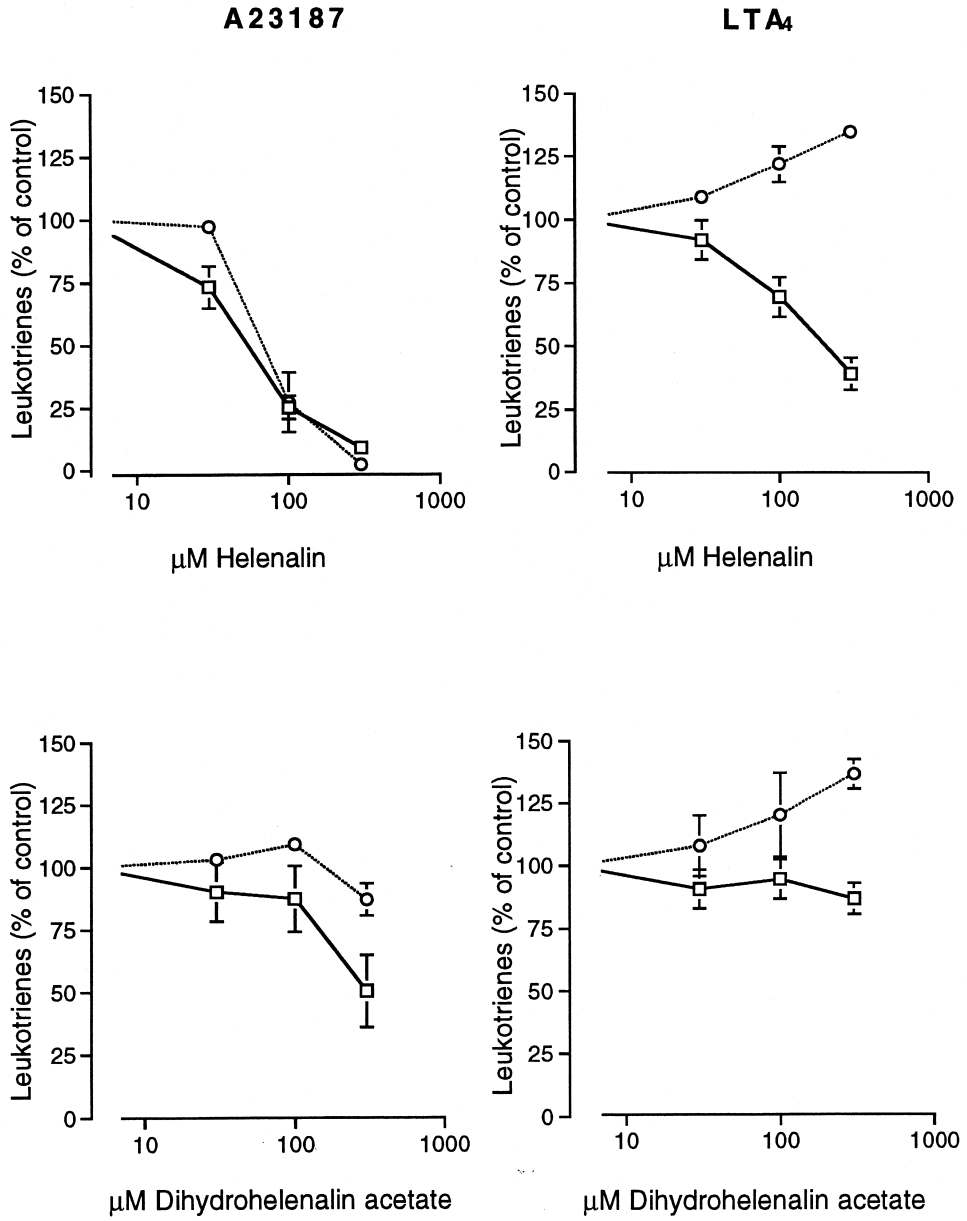

Fig. 5. Dose–response curves of the effect of helenalin and 11α,13-dihydrohelenalin acetate on leukotriene C_4 and B_4 formation after incubation of human granulocyte suspensions with A23187 or exogenous LTA_4. Human granulocyte suspensions were preincubated at 37° for 5 min in the presence of 30, 100, or 300 μM of helenalin or 11α,13-dihydrohelenalin acetate prior to incubation for another 5 min with 1 μM ionophore A23187 (left panels) or 10 μM LTA_4 (right panels). The formation of LTC_4 (squares) and LTB_4 (circles) is expressed relative to the basal formation in control incubations without helenalin or 11α,13-dihydrohelenalin acetate. Each value represents the mean of six experiments performed in duplicate. Error bars indicate standard deviation.

this drug on NF-κB activity (IC_{50} 12 and 5–10 μM, respectively, after 60 min preincubation) [9]. This suggests that inhibition of leukotriene formation could significantly contribute to the anti-inflammatory activity of helenalin.

In contrast to the two unconjugated STLs, their GSH adducts, 2β-(*S*-glutathionyl)-2,3-dihydrohelenalin, and 2β-(*S*-glutathionyl)-2,3,11α,13-tetrahydrohelenalin acetate were without effect. Since the cell membrane permeability can be expected to be considerably lower for these glutathione-containing derivatives, the effect of the four compounds on LTC_4 synthase was also investigated in disrupted platelet preparations. When platelet sonicates were preincubated for 5 min with helenalin in the absence of GSH before the simultaneous addition of 4 mM GSH and LTA_4, LTC_4 synthase activity was inhibited in a concentration-dependent manner. However, none of the four tested STL compounds displayed significant inhibitory effect on the LTC_4 formation at 100 μM when excess GSH (4 mM) was present in the preincubation buffer. This indicates that rapid conversion of free STL to GSH adducts, as previously reported [4,5], can lead to deactivation of the structural elements responsible for the inhibitory effect. It has been suggested that the anti-inflammatory activity of the STLs is mediated chemically by reaction of their α,β-unsaturated carbonyl

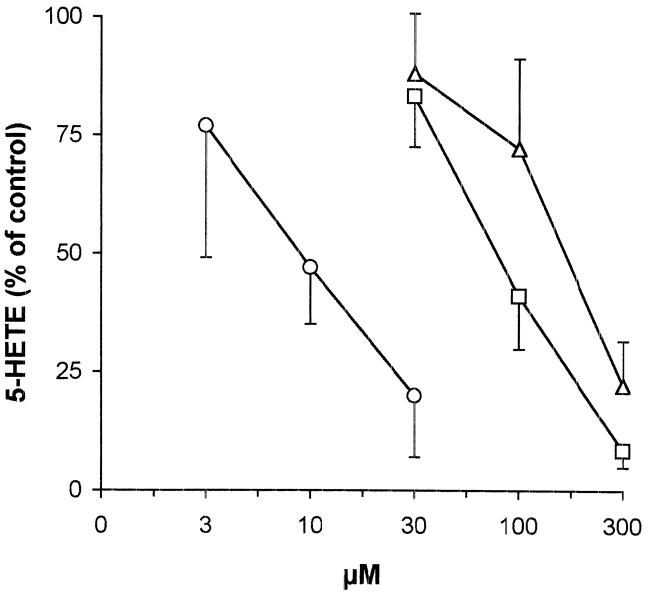

Fig. 6. Dose–response curves of the effect of helenalin and 11α,13-dihydrohelenalin acetate on 5-lipoxygenase activity after incubation of human granulocyte sonicates with exogenous arachidonic acid. Human granulocyte sonicates were preincubated at 37° for 5 min with 30, 100, or 300 μM helenalin (squares) or 11α,13-dihydrohelenalin acetate (triangles) or 60 min in the presence of 3, 10, 30 μM helenalin (circles) prior to incubation for 10 min with 20 μM arachidonic acid. Each value represents the mean of 3–4 experiments performed in duplicate. Error bars indicate standard deviation.

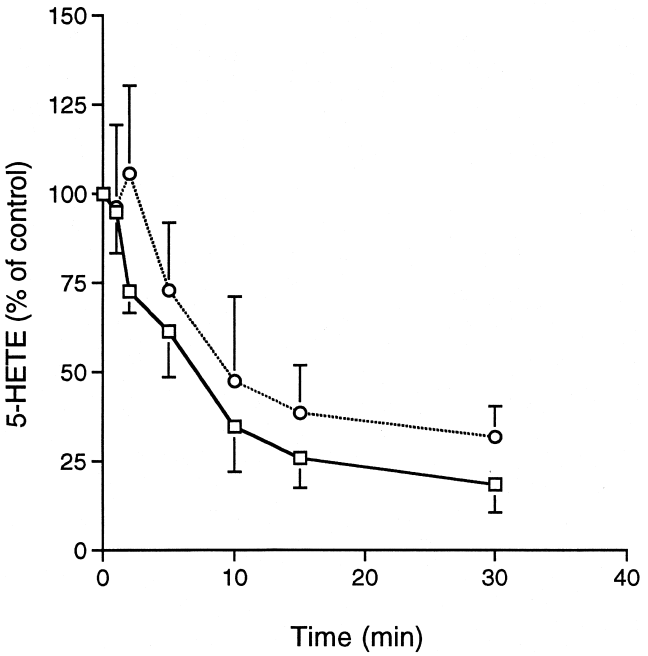

Fig. 7. Time-courses of the effect of helenalin and 11α,13-dihydrohelenalin acetate on 5-lipoxygenase activity in human granulocyte sonicates. Granulocyte sonicates were preincubated for 1–30 min with 30 μM helenalin (squares) or 11α,13-dihydrohelenalin acetate (circles) prior to incubation for 5 min with 20 μM arachidonic acid. Each value represents the mean of four experiments performed in duplicate. Error bars indicate standard deviation.

structures with macromolecular thiol groups and that modification of these structures leads to a loss of anti-inflammatory activity [6]. On the other hand, it has recently been shown that the mono- and bis-GSH adducts of helenalin are almost as active as free helenalin in inhibiting NF-κB activity in a cellular assay. This finding was explained by the observation that the GSH addition is reversible so that an equilibrium between STL–GSH adducts and free STLs is created in the presence of GSH [5]. Our result, demonstrating that the glutathionyl adducts possess some (although weak) activity towards LTC_4 synthase in platelet sonicates, is in agreement with this finding. To further investigate the way in which deactivation of STLs by GSH occurred, low amounts of GSH (50 μM–1.2 mM) were added to the preincubation buffer at the same time as helenalin (300 μM). In these experiments, GSH attenuated the inhibitory effect of helenalin on the LTC_4 synthase in a concentration-dependent manner (Fig. 4).

It has been demonstrated that some STLs interfere with the intracellular GSH balance. This leads to seriously affected cell function and can probably explain some of the effects exerted by these compounds [28]. To exclude that the inhibition of LTC_4 synthase activity in intact platelets was due to a lowering of the free concentration of reduced GSH, intact cells were incubated with STLs prior to sonication. Thereafter, LTC_4 synthase activity was assayed in the sonicated cell suspensions by incubation with LTA_4 and an excess of GSH. Also in these, experiments helenalin concentration-dependently inhibited the LTC_4 synthase activity, excluding that the effect was due to GSH depletion. Interestingly, the inhibition also remained when the platelets were washed before sonication, indicating an irreversible effect as earlier reported for the interaction between STLs and platelet sulfhydryl groups [8].

The inhibitory effect of helenalin on the LTC_4 synthase activity in human platelets varied between different donors. Furthermore, the donors could be divided into two separate groups according to the sensitivity of the platelet LTC_4 synthase to helenalin (Fig. 3). These results may indicate a genetic polymorphism with respect to LTC_4 synthase or transport/metabolism of the drug. Interestingly, platelet sulfotransferase enzyme activity has been reported to vary among individuals due to genetic polymorphism [29]. These cytosolic enzymes conjugate sulphate to a structurally diverse group of catechols, phenols, alcohols, and hormones and are considered to play an important role in the drug response.

To elucidate whether helenalin or 11α,13-dihydrohelenalin acetate had additional inhibitory effects on other enzymes involved in leukotriene biosynthesis, the effects of these compounds were also investigated in human granulocyte suspensions (Fig. 5). Helenalin concentration-dependently inhibited leukotriene formation in human granulocyte suspensions stimulated with ionophore A23187 with an IC_{50} of approximately 70 μM. However, when LTA_4 was used as substrate, the IC_{50} on LTC_4 synthase was 200 μM,

whereas the formation of LTB_4 was not inhibited. The inability of the drug to inhibit the conversion of LTA_4 to LTB_4 excluded an effect on LTA_4 hydrolase. Furthermore, the data revealed inhibitory effects both on the LTC_4 synthase and the LTA_4 formation. Moreover, the inhibition of LTA_4 formation was more potent than the effect on LTC_4 synthase. To further elucidate whether this inhibitory effect was exerted on 5-lipoxygenase, FLAP (5-lipoxygenase activating protein), or phospholipase A_2, human granulocyte sonicates were incubated with exogenous arachidonic acid and 5-HETE formation was measured in order to determine the effect on the 5-lipoxygenase activity. It has been demonstrated that FLAP is not needed for 5-lipoxygenase activity in disrupted cells [30]. Helenalin concentration- and time-dependently inhibited 5-HETE formation (IC_{50}: 75 and 9 μM after 5 and 60 min preincubation, respectively), indicating that this compound is a 5-lipoxygenase inhibitor. However, an additional inhibitory effect at the phospholipase level, as has been reported previously [8], cannot be excluded. Theoretically, inhibition on the 5-HETE formation could be due to an unspecific antioxidant function. However, helenalin does not possess any structural element typical for an antioxidant and has been reported not to affect lipid peroxidation *in vitro* [28]. It may therefore be speculated that helenalin is a non-redox 5-lipoxygenase inhibitor. The inhibitory effect of helenalin on 5-lipoxygenase formation could explain the reported suppression of neutrophil migration and chemotaxis induced by this drug [7]. 11α,13-Dihydrohelenalin acetate also inhibited the 5-lipoxygenase and LTC_4 synthase activity, but with less potency (Figs. 5 and 6). Interestingly, however, this compound has been reported to be less cytotoxic than helenalin by a factor of about 10 [31].

In conclusion, the present study demonstrates that helenalin and 11α,13-dihydrohelenalin acetate inhibited the formation of leukotrienes in human blood cells. The inhibitory effect was dose- and time-dependent and was exerted both on the 5-lipoxygenase and LTC_4 synthase. The inhibition of LTC_4 synthase was provoked by the unconjugated STLs with an intact cyclopentenone ring and not the C-2–GSH adducts. Furthermore, the effects were not related to decreased GSH levels or cytotoxicity. The present findings indicate that the anti-inflammatory effect of helenalin and related compounds is at least partially exerted via inhibition of leukotriene biosynthesis.

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