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Inhibition of COX-1 activity and COX-2 expression by 3-(4′-geranyloxy-3′-methoxyphenyl)-2-*trans* propenoic acid and its semi-synthetic derivatives

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ABSTRACT

Oxyprenylated naturally occurring cinnamic acids displayed efficient and promising biological activities. Aim of this study was to characterize the effects of 3-(4′-geranyl-3′-methoxy)phenyl-2-*trans* propenoic acid and its selected semi-synthetic analogues, on COX-2 expression and activity, and on COX-1 activity, in purified systems or in whole cell systems. The anti-inflammatory activity of title compounds (**1**) was tested as inhibition of COX-2 on isolated monocytes stimulated with LPS (10 μg/ml). COX-2 expression was completely suppressed when monocytes were incubated with 100 μM of 3-(4′-geranyl-3′-methoxy)-phenyl-2-*trans* propenoic acid (**1**) or 3-(4′-isopentenyloxy)phenyl-2-*trans* propenoic acid (**4**). Moreover compounds (**1**) and (**4**) inhibit dose-dependently LPS-induced COX-2 expression.

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Different functional cycloxygenase (COX) isoforms exist: COX-1 which is assumed to be constitutively expressed in almost all tissues, and COX-2, that is inducible in many tissues by inflammatory stimuli and may be elicited in vitro in human monocytes by LPS, some cytokines, and other stimuli.[1–3] COX isoforms are encoded by different genes with distinct pattern of expression,[4] but share the capacity to transform arachidonic acid into prostaglandins (PGs) and thromboxanes (TXs) in a two-step process involving peroxidation and reduction.[5] COX activity is inhibited by aspirin and related non-steroidal anti-inflammatory drugs (NSAID) and has a role in the production of gastro-protective prostaglandins, in the regulation of platelet and in kidney function.[6] COX-2 and its related products have renal and cardiovascular functions, are implicated in the pathogenesis of several inflammatory disorders, and in the pathogenesis of atherosclerosis and tumour formation. In particular, recent studies have suggested that the overexpression of COX-2 and resultant overproduction of prostaglandins may be involved in the development of colon cancer. Selective COX-2 inhibition has thus been actively pursued as a means to obtain anti-inflammatory agents devoid of gastric and renal toxicity,[7] and to obtain candidates agents against the development of cancer of the colon and other organs.[8]

However, clinical use of selective COX-2 inhibitors has revealed unexpected increased incidence of ischemic cardiovascular events in patients at risk and treated for prolonged periods. One possible explanation is the unbalance in prothrombotic and antithrombotic prostanoids generated by COX-2 inhibitors, because these agents inhibit the production from endothelium of COX-2-generated antithrombotic PGI_2, and do not suppress prothrombotic TXA_2, derived from platelet COX-1.[9]

Oxyprenylated secondary metabolites have been considered for decades merely as biosynthetic intermediates of C-prenylated compounds and only in the last 15 years were characterized as phytochemicals exerting interesting and valuable biological activities. Considering the length of the carbon chain, three types of oxyprenyl skeletons can be identified: C_5 (isopentenyl), C_{10} (geranyl) and C_{15} (farnesyl). Isopentenyloxy and geranyloxy chains are quite abundant in nature, while farnesyloxy ones are less common. To date about 300 oxyprenylated derivatives were isolated and/or synthesized and shown to possess a wide variety of valuable and promising pharmacological activities.[10] Among these 3-(4′-geranyloxy-3′-methoxyphenyl)-2-*trans* propenoic acid (**1**), also known as 4′-geranyloxyferulic acid, is a prenyloxycinnamic acid biosynthetically related to ferulic acid in which a geranyl chain is attached to the phenolic group. It was isolated in 1966 from the bark of *Acronychia baueri* Schott, an Australian small tree belonging to the family of Rutaceae (Curini, 2006).[11] Although known for more than four decades, only in the last years some of

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the pharmacological properties of this compound began to be characterized. Since its discovery, this natural product showed valuable properties, including an anti-inflammatory activity, that have been recently reviewed.[12]

The first report about the anti-inflammatory properties of (**1**) was reported in 2000 by Koshimizu and co-workers.[12] These authors tested the methyl and ethyl ester of compound (**1**) and found that both attenuated the expression of *i*NOS and to a lesser extent of COX-2, in RAW 264.7 cells stimulated by LPS and IFN-γ. Soon after, both derivatives were found also to inhibit TPA-induced Epstein-Barr virus activation in Raji cells without notable cytotoxicity (81% and 78% inhibition, respectively), but were by far less effective in attenuating superoxide radicals generation (35% and 45% inhibition, respectively) in HL-60 cells.[13] In DLD-1 cells the methyl ester of 4′-geranyloxyferulic acid was shown to suppress the COX-2 promoter activity at a concentration of 100 μM with a 50% decrease without marked cytotoxicity in a way similar to natural polyphenolic compounds such quercetin, genistein, and resveratrol.[13,14]

More recently detailed studies on the inflammatory properties were carried out by Epifano and co-workers using the Croton-oil-induced ear oedema formation model in mice.[15] These authors showed that that 4′-geranyloxyferulic acid (**1**) inhibited by 41% oedema formation at the dose of 0.3 μmol/cm^2, while the reference anti-inflammatory drug used, indomethacin, gave a 62% inhibition.

On the basis of the reported properties of (**1**), further evaluation and deeper insights on the mechanism of the anti-inflammatory action of 4′-geranyloxyferulic acid, of its novel natural and semi-synthetic derivatives as well as the search of new alternatives to steroids and non-steroidal anti-inflammatory drugs (NSAIDs) are fields of current and growing interest. The identification of new pharmacological agents able to modulate COXs activity and/or expression might represent a way to obtain an anti-inflammatory effect without incurring in unwanted adverse events in the cardiovascular system. Aim of this study was to characterize the effects on COX-1 and COX-2 activity and expression, in a purified system or in human monocytes, of 3-(4′-geranyl-3′-methoxy)-phenyl-2-*trans* propenoic acid (**1**) and some of its selected natural and semi-synthetic analogues (**2–8**) (Fig. 1).

We chose to synthesize and test structural analogues deriving from shortening the *O*-side chain from geranyl to isopentenyl and from substitution of the methoxy group with groups with similar steric hindrance but different electronic effects, like methyl and chlorine. 3-(4'-Geranyloxy)-phenyl-2-trans propenoic acid from Acronychia baueri (Rutaceae)[16] (2) and boropinic acid from *Boronia pinnata* Sm. (*Fam. Rutaceae*),[17] and the semi-synthetic compounds, 3-(4′-isopentenyloxy)phenyl-2-*trans* propenoic acid (**4**) were synthesized as already reported.[18] The synthesis of the other semi-synthetic 3′-chloro and 3′-methyl-4′-prenyloxycinnamic acids (**5**–**8**) was accomplished starting from commercially available 3-chloro-4-hydroxybenzaldehyde and 3-methyl-4-hydroxybenzaldehyde, respectively.

These were submitted to a Wittig–Horner olefination by reaction with methyl trimethylphosphonacetate and Na in MeOH at 70 °C for 24 h to obtain the corresponding cinnamic acid methyl esters (X = –Cl, 99% yield), (X = –Me, 89% yield). [19]All these derivatives were in turn alkylated in position 4′ by reaction respectively with 3,3-dimethylallyl and geranyl bromide promoted by K_2CO_3 as the base in acetone at 80 °C for 2 h, followed by basic hydrolysis in the same vessel and acid/base workup to afford pure isopentenyl or geranyloxycinnamic acids (**5** and **6**) (X = –Cl, 99% and 97% yields) and (**7** and **8**) (X = –Me, 87% and 92% yields), (Scheme 1).

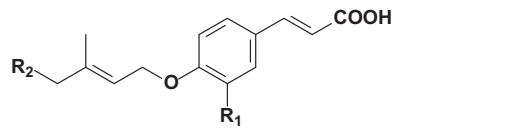
The anti-inflammatory activity of compound (**1**) and its derivatives (**2**–**8**) was tested as inhibition of COX-2 on isolated monocytes stimulated with LPS (10 μg/ml). We used rabbit polyclonal antibodies directed against a unique amino acid sequence present in human COX-2, but not in COX-1, to detect COX-2-like immunoreactivity in isolated monocytes incubated for 20 h in the absence or in the presence of LPS (10 μg/ml). As shown in Figure 2, the anti-COX-2 serum did not recognize any protein in extracts of monocytes incubated for 20 h in the absence of LPS (Ctrl, lane b). After preincubation with LPS, a band of approximately 72 kDa, exhibiting the same electrophoresis migration of standard purified COX-2, was evident in monocyte lysates (lane a). As expected, LPS-induced COX-2 expression was completely suppressed by 5 μM dexamethasone (lane e) (an inhibitor of cycloxygenase-2 [COX-2] induction) used as reference drug.

COX-2 expression was completely suppressed when monocytes were incubated with 100 μM of 3-(4′-geranyl-3′-methoxy)phenyl-2-*trans* propenoic acid (**1**) or 3-(4′-isopentenyloxy)phenyl-2-*trans* propenoic acid (**4**) (lane c and f, respectively). COX-2 expression was instead not affected by 100 μM of compound (**2**) (1.7 ± 0.12 ng/μg protein vs 1.53 ± 0.3 ng/μg protein in LPS stimulated monocytes) and by 100 μM (**3**) (1.78 ± 0.1 ng/μg protein vs 1.53 ± 0.3 ng/μg protein in LPS stimulated monocytes).

Compounds (**5–8**) had no activity on COX-2 expression (data not shown). The use of specific anti COX-1 antibody showed that monocytes not exposed to LPS contained COX-1-like immunoreactivity that was not increased after 24 h of incubation with LPS. None of the drugs tested affected COX-1 expression (Fig. 3).

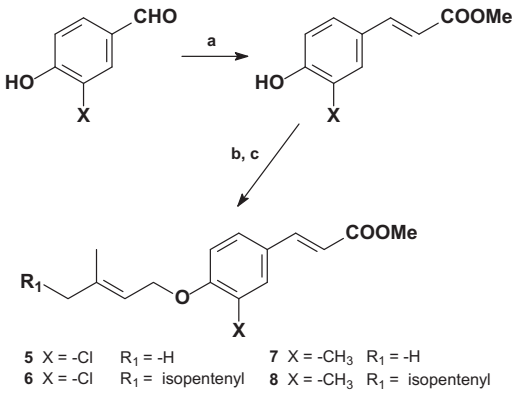
Encouraged by the results obtained in this preliminary screening, we performed further assays using the same test system in order to calculate IC_{50} values. To this aim the dose-dependent effect of compounds (**1**) and (**4**) on COX-2 expression was tested. Compounds (**1**) and (**4**) inhibit dose-dependently LPS-induced COX-2 expression: the inhibition was significant at 10 μM for compound (**1**) (20% inhibition, p <0.05 versus LPS stimulated monocytes) and at 25 μM for compound (**4**) (13% inhibition, p = 0.05), respectively (Fig. 4).

oCOX-1 and oCOX-2 activity assays were then carried out with purified oCOX-1 and oCOX-2 to determine and compare the effects of the compounds on oCOX-1 and oCOX-2 activity. After 15 min



1 R_1 = -OCH_3 R_2 = isopentenyl **5** R_1 = -Cl R_2 = -H
2 R_1 = -H R_2 = isopentenyl **6** R_1 = -Cl R_2 = isopentenyl
3 R_1 = -OCH_3 R_2 = -H **7** R_1 = -CH_3 R_2 = -H
4 R_1 = -H R_2 = -H **8** R_1 = -CH_3 R_2 = isopentenyl

Figure 1. Structures of synthesized and tested oxyprenylated cinnamic acids.



5 X = -Cl R_1 = -H **7** X = -CH_3 R_1 = -H
6 X = -Cl R_1 = isopentenyl **8** X = -CH_3 R_1 = isopentenyl

Scheme 1. Reagents and conditions: (a) $(MeO)_3POCH_2COOMe$ (3 equiv), Na (3 equiv) MeOH, 70 °C, 24 h; (b) isopentenyl bromide (1 equiv) or geranyl bromide (1 equiv) K_2CO_3 (1 equiv), acetone , 80 °C, 2 h; (c) NaOH 2 N (aq), reflux, 30 min, acid/base workup.

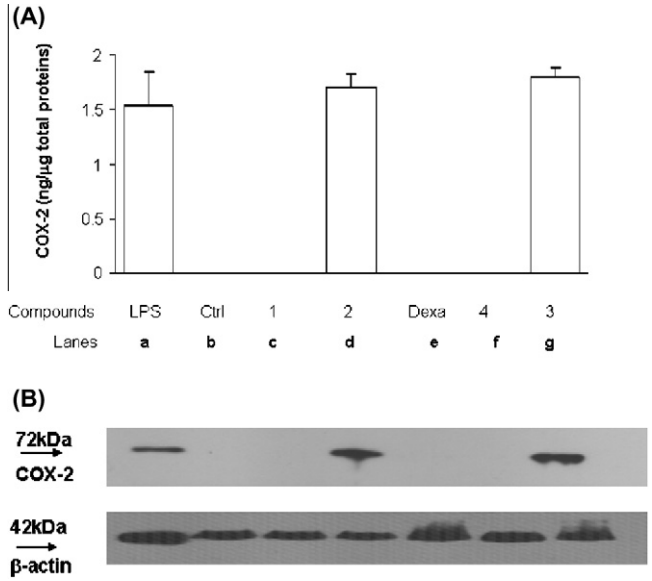


Figure 2. (A) Graphic representation of western blot analysis of COX-2 in human monocytes. Isolated human monocytes (3×10^6 cells/ml) were treated for 20 h with 100 μM of compounds **1**–**4**, vehicle of the compounds (Ctrl) and with 5 μM dexamethasone (a selective COX-2 expression inhibitor) or LPS (10 μg/ml). Cells were lysed and equal amounts of protein (20 μg) were analyzed by SDS–polyacrylamide gel and immunoblotting techniques using specific rabbit polyclonal antibody directed against COX-2. This figure is representative of three experiments. (B) Images of Western blot. The upper pannel reports COX-2 expression, while in the lower pannel beta-actin was shown as equal amounts of protein loading.

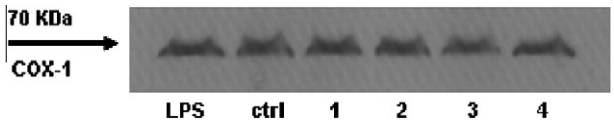


Figure 3. Western blot analysis of COX-1 in human monocytes. Isolated human monocytes (3×10^6 cells/ml) were treated for 20 h with LPS (10 μg/ml), vehicle of the compounds (Ctrl), or 100 μM of compounds **1**–**4**. Cells were lysed and equal amounts of protein (20 μg) were analyzed by SDS–polyacrylamide gel and immunoblotting techniques using specific rabbit polyclonal antibody directed against COX-1.

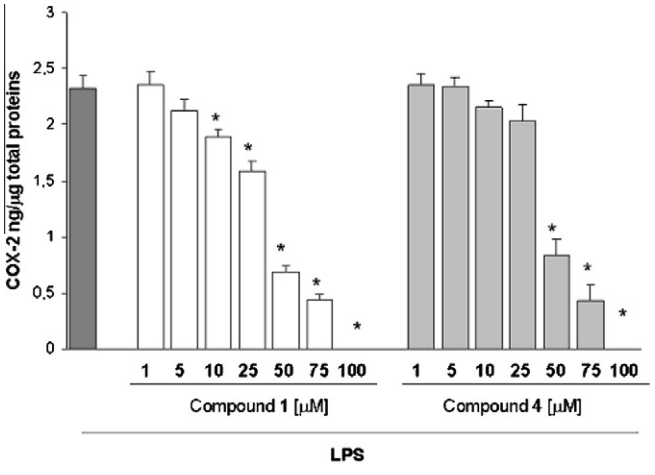


Figure 4. Dose-dependent inhibition of LPS-induced COX-2 expression. Graphic representation of western blot analysis of COX-2 in human monocytes incubated with increasing concentrations of the compounds **1** and **4**. Cells were lysed and 20 μg of protein were analyzed by western blotting techniques using specific rabbit polyclonal antibody directed against COX-2. This figure is representative of three experiments (*p <0.05 vs LPS).

preincubation, compounds (**1**) and (**4**) inhibited oCOX-1 with IC_{50} values of 54.2 ± 4.5 μM and 35.1 ± 0.5 μM, respectively. The other six compounds (**2**, **3** and **5–8**) did not affect oCOX-1 activity (data not shown).

The effects of these compounds were tested also on oCOX-2 activity. We found that 3-(4′-geranyl-3′-methoxy)phenyl *trans* propenoic acid (**1**) and 3-(4′-isopentenyloxy)phenyl-2-*trans* propenoic acid (**4**) (300 μM, respectively) inhibited oCOX-2 (97.5% and 74%, respectively), while compounds (**2**, **3** and **5–8**) did not affect oCOX-2 activity (data not shown).

From these data we can conclude that 4′-geranyloxyferulic acid (**1**) and 3-(4′-isopentenyloxy)phenyl-2-*trans* propenoic acid (**4**) inhibit COX-2 expression and the activity of oCOX-1 and oCOX-2. Trying to explain the mechanism of action underlying the observed effects on these two pro-inflammatory enzymes, it could be noticed that expression and activity of COXs can be modulated by several factors,[20] among which activation of peroxisome proliferator receptors (PPARs) play a pivotal role.[21] To this aim we have recently demonstrated that 4′-geranyloxyferulic acid is a selective inhibitor of PPARs β/δ.[22] In terms of structure–activity relationship, the presence of a geranyloxy side chain coupled to a sterically hindered group, like OCH_3, in position 3 of the aromatic ring seems to represent the best combination for COX inhibitory activities. When such a group is not present in position 3 shortening the *O*-side chain to isopentenyl provided a better activity compared to compounds having a geranyloxy moiety in the para position. The presence of medium sterical hindrance group like CH_3 or Cl abolished the activity either in the presence of a C_{10} or C_5 *O*-side chain.

In conclusion, our findings indicate that some natural and semi-synthetic prenyloxycinnamic acids, structurally derived from the natural product (**1**), can be regarded as potential novel and effective anti-inflammatory agents, the potency of which is not dissimilar from that of the well known anti-inflammatory drug dexamethasone. All tested compounds were easily synthesized starting from widely available starting materials, with good yield and cheap synthetic routes. Two of these derivatives, namely compounds (**1**) and (**4**), showed a very interesting and promising anti-inflammatory activity in the inhibition of COX-2 expression. The inhibition of both COX-1 activity and of COX-2 expression pointed out by compounds (**1**) and (**4**) should underline the innovative mechanism of action of this class of compounds. For these reasons the present findings could be considered as a basis for further studies aiming at better defining the pharmacological profile of semi-synthetic prenyloxycinnamic acids. Studies to get further insights into their mechanism of action as well as to investigate the biological activity of compounds substituted in positions other than 4′ and 3′ on the aromatic ring, as well as the effects of functionalized *O*-side chains and α,β-unsaturated double bonds and isosters of the carboxylic groups, are now ongoing in our laboratories.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.07.040.

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