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Original article

Discovery and biological evaluation of novel 1,4-benzoquinone and related resorcinol derivatives that inhibit 5-lipoxygenase

Rosanna Filosa [a,**], Antonella Peduto [a,1], Polamarasetty Aparoy [b,1], Anja M. Schaible [c,1], Susann Luderer [d], Verena Krauth [c], Carmen Petronzi [e], Antonio Massa [e], Mario de Rosa [f], Pallu Reddanna [g,h], Oliver Werz [c,*]

[a] Department of Pharmaceutical and Biomedical Sciences, University of Salerno, Via Ponte don Melillo, 84084 Fisciano, SA, Italy
[b] Centre for Computational Biology and Bioinformatics, School of Life Sciences, Central University of Himachal Pradesh, Dharamshala, India
[c] Chair of Pharmaceutical/Medicinal Chemistry, Institute of Pharmacy, Friedrich-Schiller-University Jena, Philosophenweg 14, D-07743 Jena, Germany
[d] Department of Pharmaceutical Analytics, Pharmaceutical Institute, Eberhard-Karls-University Tuebingen, Auf der Morgenstelle 8, D-72076 Tuebingen, Germany
[e] Department of Chemistry, University of Salerno, Via Ponte don Melillo, 84084 Fisciano, SA, Italy
[f] Department of Experimental Medicine, University of Naples, Via Costantinopoli, 16, 80138 Naples, Italy
[g] School of Life Sciences, University of Hyderabad, Hyderabad 500 046, India
[h] National Institute of Animal Biotechnology, Hyderabad 500046, India

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ABSTRACT

5-Lipoxygenase (5-LO), an enzyme that catalyzes the initial steps in the biosynthesis of pro-inflammatory leukotrienes, is an attractive drug target for the pharmacotherapy of inflammatory and allergic diseases. Here, we present the discovery and biological evaluation of novel series of 1,4-benzoquinones and respective resorcinol derivatives that efficiently inhibit human 5-LO, with little effects on other human lipoxygenases. SAR analysis revealed that the potency of the compounds strongly depends on structural features of the lipophilic residues, where bulky naphthyl or dibenzofuran moieties favor 5-LO inhibition. Among the 1,4-benzoquinones, compound **Ig** 5-[(2-naphthyl)methyl]-2-hydroxy-2,5-cyclohexadiene-1,4-dione potently blocked 5-LO activity in cell-free assays with IC_{50} = 0.78 μM, and suppressed 5-LO product synthesis in polymorphonuclear leukocytes with IC_{50} = 2.3 μM. Molecular docking studies suggest a concrete binding site for **Ig** in 5-LO where select π–π interactions along with hydrogen bond interactions accomplish binding to the active site of the enzyme. Together, our study reveals novel valuable 5-LO inhibitors with potential for further preclinical assessment as anti-inflammatory compounds.

1. Introduction

Leukotrienes (LTs) represent potent lipid mediators that play key roles in allergic and inflammatory processes and contribute to cardiovascular diseases (CVD) and various types of cancer [1,2]. Upon release of arachidonic acid (AA) from phospholipids of cellular membranes by cytosolic phospholipase A_2 ($cPLA_2$), 5-lipoxygenase (5-LO) converts AA into LTA_4 by the aid of the 5-LO-activating protein (FLAP) that facilitates the transfer of AA toward 5-LO [3]. LTA_4 is then metabolized by LTA_4 hydrolase into the chemotactic LTB_4 or by LTC_4 synthase into the cysteinyl (cys)-LTs C_4, D_4 and E_4 which evoke bronchoconstriction and increase vascular permeability [1]. Both, LTB_4 and the cys-LTs mediate their biological effects essentially via selective G protein-coupled receptors (at least two for LTB_4 and more than three for cys-LTs) [4]. Because of the crucial roles of LTs in pathophysiology, pharmacological strategies have been developed in order to intervene with LTs. While 5-LO inhibitors and FLAP antagonists are used to inhibit the synthesis of LTs, the so-called cys-LT receptor antagonists prevent the biological actions of cys-LTs [5]. The 5-LO inhibitors are traditionally classified according to their molecular mode of action as redox-type, iron ligand-type, and non redox-type inhibitors. However, there are currently more and more compounds described that potently inhibit cellular LT synthesis by different modes of actions such as interference with the regulatory

* Corresponding author. Tel.: +49 03641 949801; fax: +49 03641 949802.
** Corresponding author. Tel.: +39 089969398; fax: +39 089969602.
E-mail addresses: rfilosa@unisa.it (R. Filosa), oliver.werz@uni-jena.de (O. Werz).
[1] These authors contributed equally to this work.

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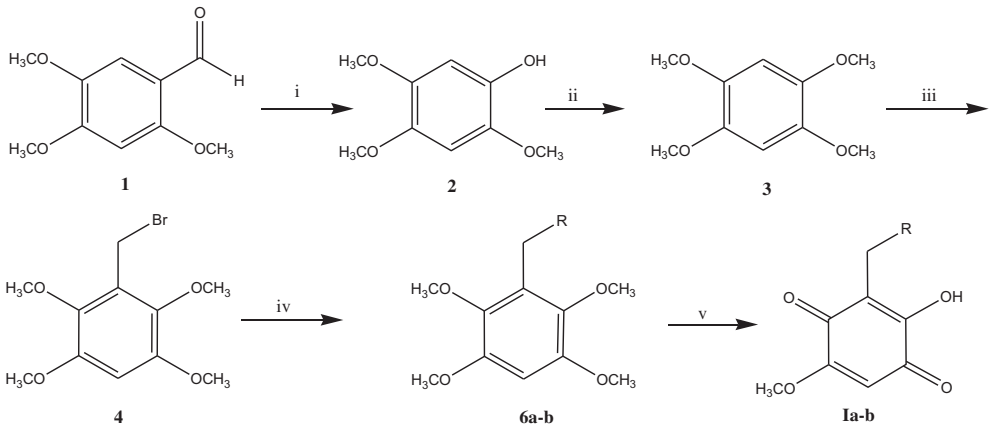
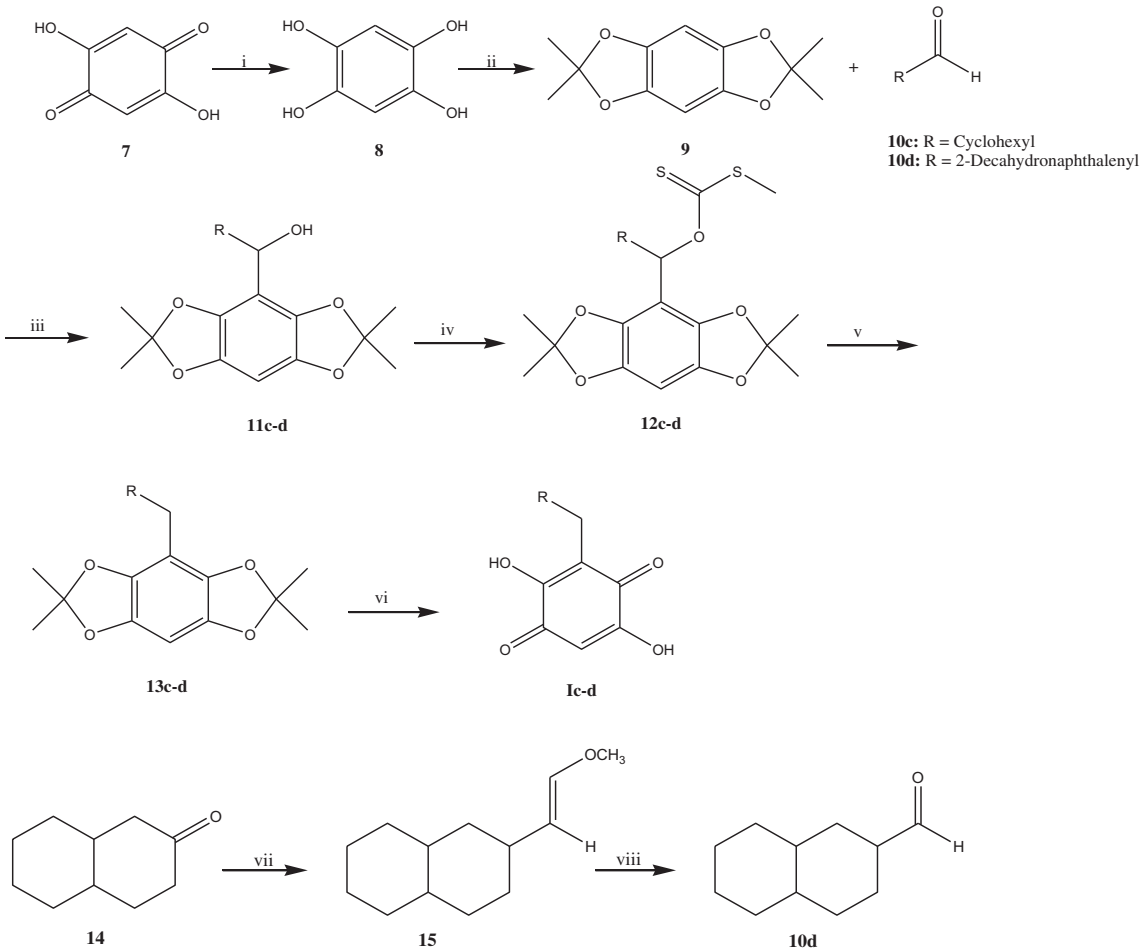


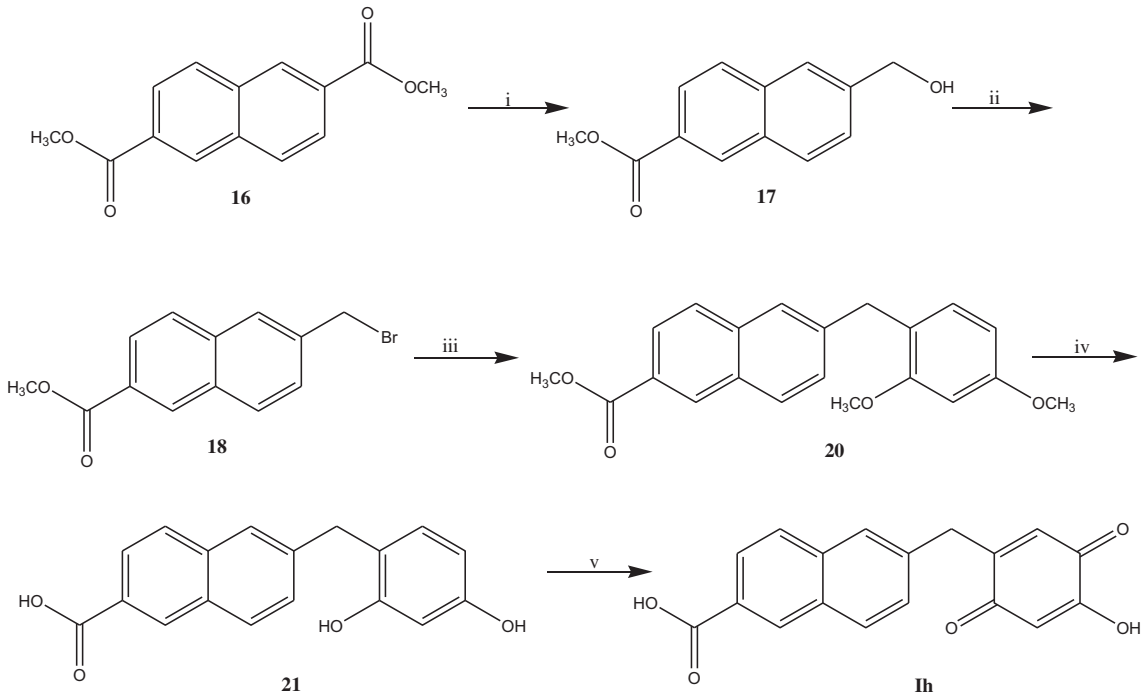
Scheme 1. Synthesis of 2-hydroxy-5-methoxy-1,4-benzoquinones **Ia**–**b**. Reagents and conditions: (i) H_2O_2 30%, H_2SO_4, MeOH, rt, 2 h; (ii) CH_3I, K_2CO_3, CH_3COCH_3, 65 °C, 18 h; (iii) H_2CO, HBr 30%, CH_3COOH, 55 °C, 6 h; (iv) phenylboronic acid (**5a**) or naphthalen-2-yl-2-boronic acid (**5b**), $Pd(OAc)_2$, PPh_3, tBuOK, toluene, 110 °C, 18 h; (v) CAN, H_2O, CH_3CN, rt, 3 h.

C2-like domain of 5-LO or by interference with 5-LO-stimulating factors or mechanisms in the cell other than FLAP [6,7]. Although much more efforts have been made in order to develop LT synthesis inhibitors, the LT antagonists dominate on the market and thus far only zileuton [8], an iron ligand-type inhibitor, has been approved for anti-LT therapy.

Polyphenols are widely distributed in nature, and many studies revealed that they constitute a rich source of inhibitors of 5-LO product synthesis [9]. Mechanistically, they may act as antioxidants thereby keeping the active site iron of 5-LO in the inactive ferrous state, even though the hydroxy groups might also contribute to 5-LO inhibition by iron-chelating properties



Scheme 2. Synthesis of 2,5-dihydroxy-1,4-benzoquinones **Ic**–**d**. Reagents and conditions: (i) Sn, HCl (37%), THF, 100 °C, 1 h; (ii) C_4H_8O, PPTS, C_6H_6, 80 °C, 24 h; (iii) n-BuLi, THF, from −10 °C to rt, 16 h; (iv) NaHDMS, CS_2, CH_3I, THF, before −78 °C, after −55 °C, then 0 °C, 3.5 h; (v) n-Bu_3SnH, AIBN, toluene, from rt to 110 °C, 2.5 h; (vi) HCl 4 M, dioxane, air, 110 °C, 7 h; (vii) MMTPPC, n-BuLi, diethyl ether, from −40 °C to rt, 30 h; (viii) $HClO_4$ 70%, H_2O, diethyl ether, reflux, 1 h.



Scheme 3. Synthesis of 2-hydroxy-1,4-benzoquinone **Ih**. Reagents and conditions: (i) DIBALH 1 M, THF, 0 °C, 30 min; (ii) PBr_3, C_6H_6, from 0 °C to rt, 1 h; (iii) 2,4-dimethoxybenzen boronic acid (**19**), $Pd(OAc)_2$, PPh_3, K_3PO_4, C_7H_8, 110 °C, 18 h; (iv) BBr_3, DCM, from −78 °C to rt, 1.5 h; (v) $K_2NO(SO_3)_2$, Na_2CO_3 (15%), THF, rt, 3 h.

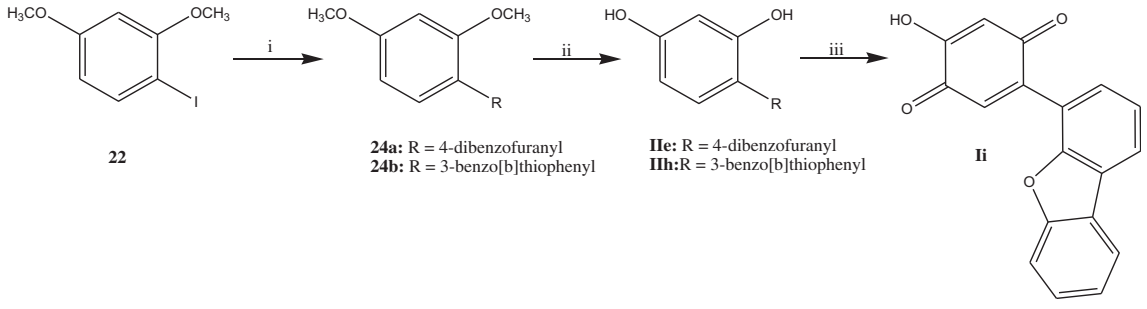
[10]. Natural compounds and synthetic derivatives possessing a 1,4-benzoquinone moiety are associated with a wide range of biological properties, including antioxidant, anti-inflammatory and anti-cancer activities [11]. The anti-inflammatory activity of 1,4-benzoquinones has been associated with suppression of LT formation [9]. In the cell, the 1,4-benzoquinone moiety can be reduced to a 1,4-diphenol structure (=1,4-hydroquinone) due to the reducing intracellular milieu (e.g. presence of glutathione in the millimolar range). Such 1,4-diphenols as well as other polyphenols have been assumed to reduce the active site iron of 5-LO, thereby inhibiting 5-LO activity primarily due to antioxidant properties [12,13]. However, it was also shown that the potency of 1,4-diphenols and polyphenols does not solely depend on the reducing properties but instead the structural features and the degree of lipophilicity are determinants [9,14]. Only few reports are available that addressed this issue in detail and respective structure–activity relationship (SAR) studies are rare. Therefore, the rational of our study was to provide insights into SARs with focus on the lipophilic residue at the phenol core.

Here we present the design and synthesis of novel 1,4-benzoquinones and corresponding resorcinol derivatives that depending on the overall structure, proved to be potent inhibitors of human 5-LO in intact cells as well as in cell-free assays, whereas others inhibited 5-LO surprisingly only in the cell-free test system. Our data shed light on the SARs related to the lipophilic moieties connected to the 1,4-benzoquinone or resorcinol backbone, respectively that determine inhibition of 5-LO without significant interference with human 12- and 15-LOs. Results from molecular docking studies that were carried out to investigate their binding interactions with 5-LO, revealed a correlation between the capacity to bind and to inhibit 5-LO.

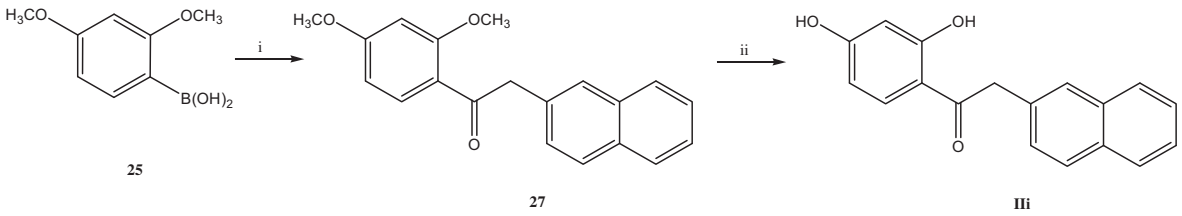
2. Results and discussion

2.1. Chemistry

Compounds **Ia–b** were synthesized starting from 2,4,5-trimethoxyphenol (**2**) [15] (Scheme 1). The phenol **2** was methylated to provide 1,2,4,5-tetramethoxybenzene **3**, which was converted into 3-(bromomethyl)-1,2,4,5-tetramethoxybenzene (**4**) [16]. Suzuki coupling with commercially available phenylboronic acid (**5a**) and naphthalen-2-yl-2-boronic acid (**5b**) yielded **6a–b**. Oxidation with ammonium cerium nitrate gave desired **Ia–b** (structures identified by ^1H NMR and confirmed by gCOSY analysis). Compound



Scheme 4. Synthesis of **IIe**, **IIh** and **Ii**. Reagents and conditions: (i) dibenzofuran-4-boronic acid (**23a**) or benzo[b]thiophen-3-yl-3-boronic acid (**23b**), K_2CO_3, $PdCl_2(dppf)$, Ethanol, MW, 25 min; (ii) BBr_3, CH_2Cl_2, −15 °C to rt, 18 h; (iii) $NO(KSO_3)_2$, EtOAc–H_2O, Na_2HPO_4, 0 °C to rt, 20 h.



Scheme 5. Synthesis of **IIi**. Reagents and conditions: (i) 2-(naphthalen-6-yl)acetic acid (**26**), $ZnCl_2$, $POCl_3$, 80 °C, 2h; (ii) BBr_3, CH_2Cl_2, −15 °C to rt, 18 h.

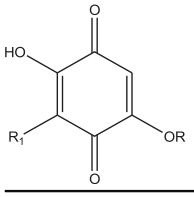
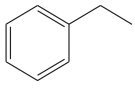
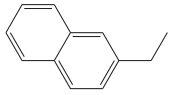
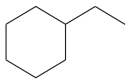
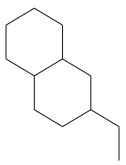
Id was synthesized according to Kim et al. [17] (Scheme 2). Briefly, diacetonide **9**, obtained from treatment of 1,2,4,5-tetrahydroxybenzene **8** [18] with 2-methoxypropene, was reacted with appropriate aldehydes **10c**–**d** to provide the corresponding alcohols **11c**–**d**. They were first converted into xanthate derivatives **12c**–**d**, according to the Barton–McCombie reaction [19]; then treatment with tributyltin hydride and 2,2′-azobisisobutyronitrile afforded compounds **13c**–**d**. Final oxidation provided the 2,5-dihydroxy-1,4-benzoquinones **Ic**–**d**. Decahydronaphthalene-2-carbaldehyde (**10d**), prepared from octahydronaphthalen-2(1H)-one (**14**) as mixture of stereoisomers, was subjected to Wittig reaction yielding decahydro-2-(methoxymethylene) naphthalene (**15**). Treatment with perchloric acid 70% (v/v) in diethyl ether provided **10d**. The 2-hydroxy-1,4-benzoquinones **Ie**–**g** and **Il**–**m** were synthesized according to Ref. [20].

The synthesis of compound **Ih** (Scheme 3) started from dimethyl naphthalene-2,6-dicarboxylate (**16**), which was mono reduced to provide the corresponding alcohol **17** [21] that, after treatment with PBr_3, furnished methyl 6-(bromomethyl)-2-naphthoate (**18**). Suzuki coupling with 2,4-dimethoxybenzen boronic acid (**19**) led to intermediate **20**, which was demethylated and hydrolyzed simultaneously with BBr_3 and finally oxidized to **Ih** using Fremy's salt. Compounds **Ii**, **IIe** and **IIh** were obtained by Suzuki coupling between 1-iodo-2,4-dimethoxybenzene (**22**) and appropriate boronic acids (**23a** and **23b**), under microwave irradiation. Intermediates **24a** and **24b** were *O*-demethylated to provide resorcinol derivatives (**IIe** and **IIh**). **IIe** was finally oxidized with Fremy's salt yielding **Ii** (Scheme 4). The resorcinol derivatives **IIb**–**d** and **IIf**–**g** were synthesized as previously reported [20]. Compound **IIa** was synthesized based on a modified procedure [22] using microwave irradiation for reaction of Suzuki coupling. Compound **IIi** was obtained via Friedel–Crafts acylation of 1,3-dimethoxybenzene boronic acid (**25**) with 2-(naphthalen-6-yl)acetic acid (**26**) and subsequent demethylation with BBr_3 (Scheme 5).

2.2. Evaluation of 5-LO activity and structure–activity relationships

In order to assess the effects of the test compounds on 5-LO product synthesis, a cell-free assay using isolated human recombinant 5-LO and a cell-based test system using intact human polymorphonuclear leukocytes (PMNL) were applied. While the cell-free assay allows the identification of compounds that directly interfere with 5-LO catalytic activity (without the need of bioactivation), the cell-based test system involves various aspects and steps that are required for 5-LO product synthesis, and as such offers several targets within the 5-LO activation and reaction cascade (e.g., $cPLA_2$, FLAP or coactosine-like protein (CLP), 5-LO-activating lipid hydroperoxides, protein kinases or Ca^{2+} mobilization, and 5-LO

Table 1A
2,3,5-Trisubstituted 1,4-benzoquinone derivatives: chemical structures and effects on the activity of 5-LO. Data are given as mean ± S.E.M., $n = 3–4$.

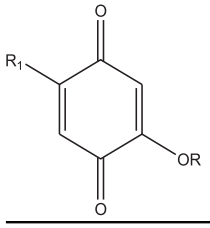
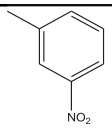
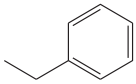
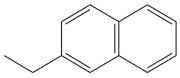
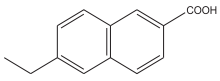
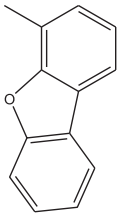
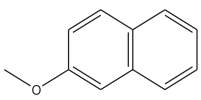
Cpd			5-LO activity; cell-free		5-LO activity; cell-based	
	R	R_1	Remaining activity at 10 μM (%)	IC_{50} (μM)	Remaining activity at 10 μM (%)	IC_{50} (μM)
Ia	CH_3		109.6 ± 5.1	> 10	103.3 ± 12.2	>10
Ib	CH_3		92.3 ± 16.3	> 10	56.1 ± 7.4	>10
Ic	H		95.2 ± 15.8	> 10	90.4 ± 12.5	>10
Id	H		51.0 ± 8.9	11 ± 5.4	5.4 ± 2.6	0.58 ± 0.2

translocation/membrane association) for a given compound in order to suppress 5-LO product generation [23]. Moreover, compounds that may require bioactivation (as in the case of quinones) in order to be able to inhibit 5-LO can be identified using the combination of these two test systems. The reference 5-LO inhibitors **29** ((*E*)-*N*-hydroxy-*N*-(3-(3-phenoxyphenyl)-allyl)acetamide; BWA4C [24]) and **30** (N-[1-(1-benzothien-2-yl)ethyl]-N-hydroxyurea; zileuton [8]) were used to control the 5-LO activity assays that blocked 5-LO activity with IC_{50} of 0.22 and 0.56 μM in the cell-free and 0.3 and 1.1 μM in the cell-based assay, respectively.

As shown in Table 1A, no remarkable inhibition of 5-LO by the 2-hydroxy-5-methoxy-1,4-benzoquinones (**Ia** and **Ib**) in either assay type was observed ($IC_{50} > 10$ μM). Also the 2,5-dihydroxy derivative **Ic**, bearing a lipophilic cyclohexyl ring, remained inactive. However, replacement of this cyclohexyl by the more hindered bicyclic decahydronaphthyl moiety led to compound **Id** with a moderate potency in the cell-free assay ($IC_{50} = 11$ μM), but this compound potently suppressed 5-LO product synthesis in intact cells with $IC_{50} = 0.58$ μM. Comparison of **Id** with inactive **Ic** demonstrates that structural features and/or size of the lipophilic residue at the 1,4-benzoquinone core strongly determine the effectiveness for inhibition of 5-LO.

By removing one hydroxyl group at the 1,4-benzoquinone core and shifting the substituent R_1 from position 3 to position 2 (**Ie**–**m**), we obtained compounds that (except **If**) directly inhibited 5-LO in the cell-free assay with IC_{50} values in the low micromolar or even submicromolar range (Table 1B). Under the assay conditions of the cell-free test system, a reduction of the quinone moiety to the hydroquinone is rather unlikely. Thus, the unexpected finding that the quinone form of **Ie**, **g**–**m** inhibits 5-LO directly in this cell-free assay is interesting and might be related to other mechanisms than simple reduction of the active site iron by the diphenol core as generally proposed for quinone-type 5-LO inhibitors with antioxidant activity [12–14]. In fact, as shown below, molecular docking and SAR experiments reveal a correlation between the number of favorable interactions with 5-LO and inhibitory potency. Compounds **Ig** and **Im**, decorated with a naphthylmethyl or a naphthoxy residue, turned out to be the most potent inhibitors of 5-LO of this particular series with IC_{50} value of 0.78 and 0.28 μM, respectively. Moreover, compound **Ig** was the most active compound in intact PMNL ($IC_{50} = 2.3$ μM) within this series. Note that **Im** carries a methoxy moiety instead of a free hydroxyl group present in the other derivatives **Ie**–**Il**, indicating that a free hydroxyl moiety at the quinone core is actually not a prerequisite for bioactivity.

Table 1B
2,5-Disubstituted 1,4-benzoquinone derivatives: chemical structures and effects on the activity of 5-LO. Data are given as mean ± S.E.M., $n = 3–4$.

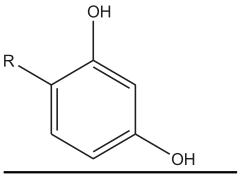
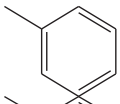
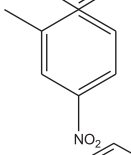
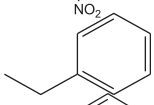
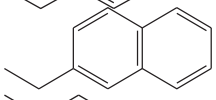
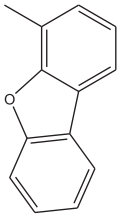
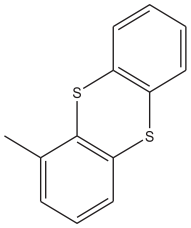
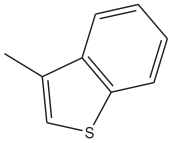
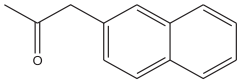
Cpd			5-LO activity; cell-free		5-LO activity; cell-based	
	R	R_1	Remaining activity at 10 μM (%)	IC_{50} (μM)	Remaining activity at 10 μM (%)	IC_{50} (μM)
Ie	H		8.0 ± 2.5	4.6 ± 1.2	61.3 ± 7.8	>10
If	H		74.3 ± 6.1	>10	93.3 ± 16.6	>10
Ig	H		8.7 ± 3.7	0.78 ± 0.1	5.3 ± 2.4	2.3 ± 0.8
Ih	H		45.2 ± 4.0	9.0 ± 1.0	74.0 ± 5.6	>10
Ii	H		30.8 ± 14.9	0.80 ± 0.1	74.1 ± 2.2	>10
Il	H		14.2 ± 8.6	1.4 ± 0.2	15.9 ± 2.7	3.0 ± 0.7
Im	CH_3		7.1 ± 3.2	0.28 ± 0.1	21.2 ± 8.6	6.7 ± 1.0
Zileuton (cmpd **30**)			6.6 ± 1.5	0.56 ± 0.1	13.4 ± 5.9	1.1 ± 0.4

Replacement of the naphthylmethyl residue by a benzyl moiety (**If**) abrogated 5-LO inhibition, suggesting again that the size and/or certain structural features of this lipophilic residue are determinants for the interference with 5-LO. Along these lines, introduction of a 3-nitro-phenyl ring, directly connected to the 5-hydroxy-1,4-benzoquinone moiety (**Ie**), restored bioactivity, at least for inhibition of cell-free 5-LO activity, and also replacement of the aromatic substituent by an *n*-hexyl chain (**Il**) retained the efficiency versus **Ig** in the cell-free assay with only a small loss of potency in intact cells. The insertion of a polar carboxyl group at the naphthalene ring (**Ih**) was clearly detrimental versus the parental **Ig**, in both read outs, whereas replacement of the naphthylmethyl group by more hindered heterocycles, like a dibenzofuran ring (**Ii**), was tolerated, at least in the cell-free assay (IC_{50} = 0.80 μM). Together, for direct interference with 5-LO, the naphthylmethyl or naphthoxy residues are most favorable.

Unexpectedly, the potency of the active compounds **Ie**–**m** was always higher in the cell-free versus cell-based assay, and in particular the potent direct 5-LO inhibitors **Ii** and **Im** hardly reduced 5-LO activity in intact cells, which is not readily understood. Possibly, the cellular uptake of these compounds might be hampered due to the sterically hindered dibenzofuran and naphthoxy ring, respectively.

To get further insights into the SARs, we next addressed the role of the 1,4-benzoquinone core and replaced it by resorcinol where the alkyl and aryl residues from above as well as other related lipophilic substituents were incorporated in position 4. As can be

Table 2
Resorcinol derivatives: chemical structures and effects on the activity of 5-LO. Data are given as mean ± S.E.M., n = 3–4.

Cpd		5-LO activity; cell-free		5-LO activity; cell-based	
	R	Remaining activity at 10 μM (%)	IC_{50} (μM)	Remaining activity at 10 μM (%)	IC_{50} (μM)
IIa		9.4 ± 2.4	2.8 ± 0.4	22.7 ± 5.3	4.2 ± 1.4
IIb		84.3 ± 8.2	>10	64.1 ± 7.4	>10
IIc		47.6 ± 4.4	9.4 ± 1.0	60.0 ± 2.2	>10
IId		37.2 ± 4.9	7.4 ± 1.5	8.9 ± 1.0	2.3 ± 0.5
IIe		30.7 ± 8.8	6.3 ± 0.9	84.1 ± 12.6	>10
IIf		25.6 ± 7.1	4.6 ± 1.8	18.6 ± 6.1	2.8 ± 2.2
IIg		15.4 ± 2.5	3.2 ± 0.4	10.5 ± 2.5	2.1 ± 0.1
IIh		100.2 ± 2.1	>10	92.8 ± 5.8	>10
IIi		57.4 ± 3.6	>10	42.4 ± 1.8	6.8 ± 0.02

seen from Table 2, compounds out of the resorcinol series except **IIb**, **IIc**, **IIh** and **IIi** showed good efficiencies for inhibition of 5-LO with IC_{50} values in the low micromolar range but they are less potent than the 1,4-benzoquinones with lowest IC_{50} values of 2.8 μM (compd. **IIa**) and 2.3 μM (compd. **IId**) in the cell-free and cell-based assay, respectively. In contrast to the 1,4-benzoquinones, the potency of the resorcinol derivatives in the two assays were rather similar, except for compound **IIe** that was inactive in intact cells. While the phenyl-substituted compound **IIa** exhibits significant 5-LO inhibitory activity (IC_{50} = 2.8 and 4.2 μM in cell-free and cell-based assays, respectively), insertion of a NO_2 group in the phenyl ring (**IIb**) or the introduction of a methylene spacer (**IIc**) was clearly detrimental. Replacement of the phenyl in **IIa** by a naphthylmethyl moiety led to compound **IId** that was still active in the cell-free but at least 2.5-fold more potent in intact cells. The elongation of the chain and the insertion of an oxo moiety led to compound **IIi** which retained 5-LO inhibitory in intact cells (IC_{50} = 6.8 μM), but was inactive against purified 5-LO. Moreover, compound **IIe**, carrying a dibenzofuranyl residue, was about equipotent to **IId** in the cell-free assay, but markedly lost its potency in the cell-based system. Again, as observed for **Ii** within the 1,4-benzoquinone series, the dibenzofuranyl moiety seemingly hampers inhibition of 5-LO in intact cells. When dibenzofuran was replaced by another hindered heterocycle like thianthrene (**IIg**) a significant improvement of potency both in the cell-based and in the cell-free assay was observed, whereas the smaller benzothiophene moiety in **IIh** did not cause 5-LO inhibition. Finally, exchange of the phenyl ring of **IIa** by an *n*-hexyl (**IIf**) was tolerated and did not significantly alter the efficiency in intact cells or cell-free assays. Together, as observed for the 1,4-benzoquinone series the ability of resorcinol derivatives to inhibit 5-LO product synthesis strongly depends on the nature of the lipophilic substituent.

2.3. Evaluation of the effects of the test compounds on other human LOs and analysis of competition with the substrate of 5-LO

Besides 5-LO, other related human LOs such as 12-LO or 15-LO that also abstract a hydrogen bond from AA via a nonheme active site iron during catalysis were investigated as potential targets of the test compounds. The activities of 12-LO and 15-LO were analyzed in cell-based assays in order to assure the availability of "bioactive" reduced 1,4-diphenol or resorcinol form, using PMNL preparations that contain 15-LO in eosinophils and 12-LO in adhering platelets. Of interest, all compounds that efficiently inhibited 5-LO product synthesis in the cell-based model with IC_{50} values = 0.58–6.8 μM (i.e., **Id**, **Ig**, **Il**, **IIa**, **IId**, **IIf**, **IIg**, and **IIi**) failed to markedly affect the activity of 12- and 15-LO (IC_{50} > 10 μM), and only compound **Ii** impaired the activity of 12-LO and compound **Im** of 15-LO at 10 μM by somewhat more than 50% (Table 3). These data suggest that the 5-LO inhibitory substances in this study exhibit a certain preference for 5-LO and are not just unspecific LO inhibitors due to reducing properties that may simply interfere with the iron-based redox cycle of any LO.

It appeared reasonable to speculate that the active compounds (e.g. **Ig**) inhibit 5-LO activity by competing with AA as substrate for the enzyme. Therefore, we assessed inhibition of 5-LO product synthesis by **Ig** in the cell-free assay, at varying AA concentrations, that is, at 2.5, 5, 10, 20 and 40 μM. In agreement with previous findings [25], 5-LO product formation continuously raised with increasing the substrate concentration, was maximal at 20 μM AA, but declined due to substrate inhibition at 40 μM AA. As shown in Fig. 1, variation of the AA concentration caused no clear pattern of competitive inhibition by **Ig** and the IC_{50} were in the close range of 0.31 (at 40 μM AA) and 0.97 μM (at 5 μM AA).

Table 3
Effects of 1,4-benzoquinone and resorcinol derivatives on the activity of 12-LO and 15-LO in PMNL. Data are given as mean ± S.E.M., n = 3–4.

Cpd	12-LO activity	15-LO activity
	Remaining activity at 10 μM (%)	Remaining activity at 10 μM (%)
Ia	84.0 ± 14.0	96.4 ± 12.8
Ib	76.4 ± 26.8	89.5 ± 25.9
Ic	85.2 ± 10.7	87.3 ± 5.7
Id	60.9 ± 1.6	87.9 ± 11.4
Ie	60.9 ± 6.7	116.3 ± 41.8
If	95.4 ± 20.4	132.3 ± 32.4
Ig	71.9 ± 13.3	147.2 ± 28.1
Ih	81.1 ± 7.4	80.9 ± 4.34
Ii	46.2 ± 6.1	79.8 ± 10.9
Il	89.8 ± 5.8	184.6 ± 20.5
Im	73.1 ± 16.0	47.9 ± 5.3
IIa	72.3 ± 3.8	106.5 ± 11.5
IIb	90.1 ± 13.5	107.5 ± 8.1
IIc	77.0 ± 1.3	115.9 ± 10.9
IId	79.2 ± 8.3	191.1 ± 20.6
IIe	93.3 ± 21.8	181.5 ± 0.7
IIf	69.3 ± 8.7	110.0 ± 15.4
IIg	91.4 ± 7.9	58.3 ± 1.6
IIh	115.4 ± 6.5	115.1 ± 15.8
IIi	96.0 ± 10.4	108.2 ± 13.3

2.4. Molecular docking studies

To understand the differences in the binding modes of various types of compounds we performed molecular docking studies. Individual binding poses of each compound were assessed and their interactions with potential amino acids in the active site of the 5-LO protein were analyzed. The best and most energetically favorable conformation of each compound was selected. The binding modes of **Ib**, **Ig** and **IId** were obtained by carefully aligning docked structures of the compounds into the active site of 5-LO and studied in detail. As shown in Fig. 2, compound **Ig** that efficiently inhibits 5-LO is stabilized in the 5-LO active site by strong hydrogen bond and π–π interactions. The bulky naphthyl moiety forms strong π–π interactions with Phe177 and His372. The hydroxyl group forms hydrogen bond interactions with Tyr181, and one of the carbonyl oxygen groups of the benzoquinone core forms strong interactions with Gln363 and His367. Similar interactions were found for the 5-LO inactive **Ib**, however,

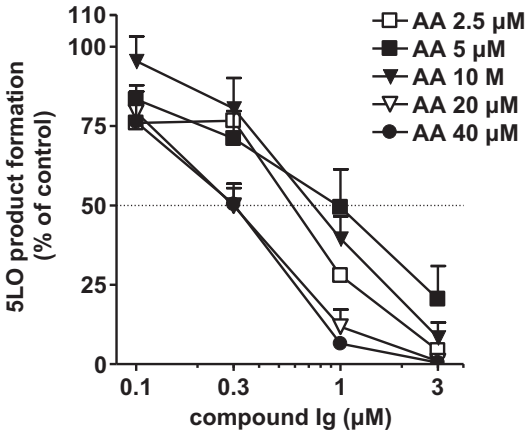


Fig. 1. Inhibition of 5-LO activity by Ig in the cell-free assay at various substrate concentrations. Purified 5-LO was pre-incubated with **Ig** or vehicle (DMSO, 0.1%) for 15 min at 4 °C. Samples were warmed up for 30 s at 37 °C and 2 mM $CaCl_2$ plus the indicated concentrations of AA were added. After 10 min at 37 °C, 5-LO activity was determined. Data are expressed as percentage of control (100%, uninhibited 5-LO), means ± S.E.M., n = 3.

it has been observed that none of the carbonyl oxygen groups of the benzoquinone is involved in interactions with 5-LO. In general, the presence of non-bonded hydrogen bond acceptor or donor in the ligand adds a lot of energy to the system, i.e., in the form of desolvation penalty [26], which may explain the low affinity of **Ib** ($IC_{50} > 10$ μM) in comparison to **Ig** ($IC_{50} = 0.78$ μM). In **Ib** the hydroxyl and the methoxy groups form hydrogen bond interactions, and one of the carbonyl oxygen in **Ib** occupies a hydrophobic point in the active site lined by Phe421 and Leu414 (Fig. 2), which is detrimental for the affinity to 5-LO. The strong π interactions observed in **Ig** correlate well with the pharmacophore model and QSAR models of 5-LO reported earlier [27,28]. For the resorcinol derivative **IId**, the two hydroxyl groups are involved in hydrogen bond interactions with Tyr181 and Gln557, and with His367, respectively. The hydrogen bond donor groups together, i.e., the two strong hydroxyl moieties, form only three hydrogen bonds. A stronger hydrogen bond group often implies higher penalty of desolvation, unless it is involved in prominent hydrogen bonds with the protein. As the 4-hydroxy moiety is involved in only one hydrogen bond it does not contribute much to the free energy, explaining why **IId** is less active on 5-LO in comparison to **Ig**. Previous reports on 5-LO inhibitors clearly suggest that the presence of hydrogen bond donors is more favorable than hydrogen bond acceptors for 5-LO inhibition [27,29]. Together, an obvious correlation between favorable/unfavorable interactions of the compounds and inhibition of 5-LO activity in the cell-free assay is evident. In general, the 1,4-benzoquinone series formed more stable interactions than the resorcinol derivatives which again correlates to the 5-LO inhibitory potencies.

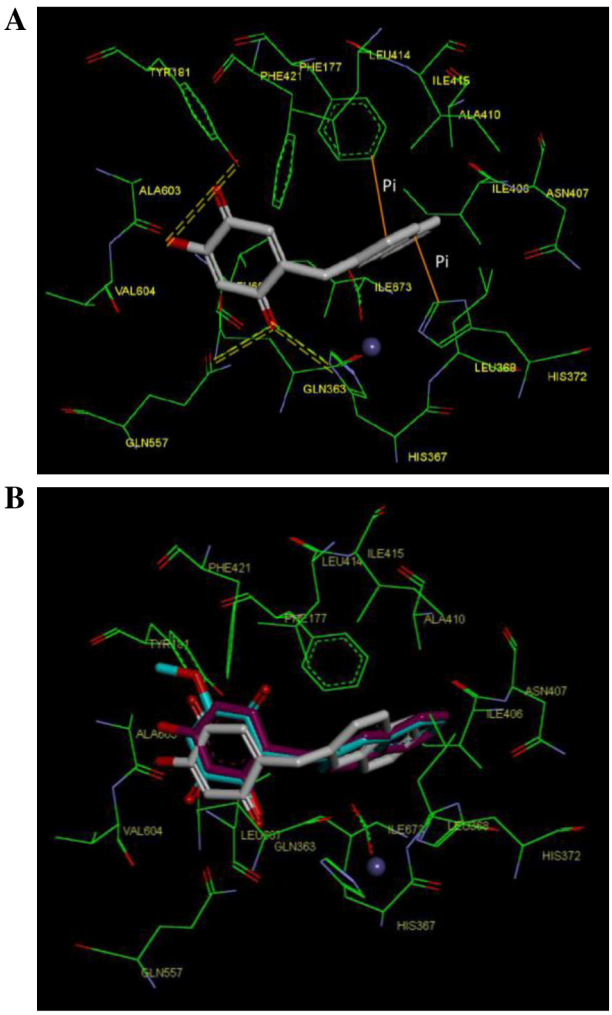


Fig. 2. Interactions of test compounds with 5-LO. (A) Interactions of **Ig** with 5-LO. The hydrogen bonds are given as double dotted lines, π interactions are shown as single solid lines. (B) Overlay of the conformations of **Ib**, **Ig** and **IId** in the 5-LO active site. The best and energetically favorable conformations of **Ib** (blue), **Ig** (white) and **IId** (purple) are superimposed. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3. Conclusions

5-LO that catalyzes the first two steps in LT biosynthesis is considered as drug target for intervention with asthma, allergic rhinitis, various autoimmune and inflammatory disorders, atherosclerosis and various types of cancer [1,5]. Therefore, the development of potent and safe 5-LO inhibitors is a major challenge that has been intensively pursued [7]. We present here novel series of 1,4-benzoquinones and related resorcinol derivatives as potent LT synthesis inhibitors, with partially submicromolar IC_{50} values. Our data provide insights related to SARs for 1,4-benzoquinone (and for resorcinol) derivatives as 5-LO inhibitors. Thus, the potency of the 1,4-benzoquinones depends on structural features of the lipophilic residues as demonstrated for instance by comparison of inactive **If** with active **Ig** in cell-free assays or of inactive **Ic** with active **Id** in intact cells. It appears that in particular bulky naphthyl or dibenzofuran moieties connected to the 1,4-benzoquinone favor 5-LO inhibition. A similar pattern was found for the resorcinol derivatives. As the results from the molecular docking simulations reflecting binding to 5-LO correlated with the 5-LO inhibitory potency, we conclude that the compounds mediate their inhibitory action on the 5-LO enzyme via discrete physical interactions, supported also by the lack of marked 12- and 15-LO interference. Together, novel 1,4-benzoquinone derivatives were revealed in this study as selective and potent inhibitors of 5-LO in cell-free and cell-based assays. Our results also stimulate for further preclinical investigations in order to assess the pharmacological properties and the anti-inflammatory efficacy of selected compounds.

4. Experimental section

4.1. Chemistry

All reagents were analytical grade and purchased from Sigma–Aldrich (Milano-Italy). Flash chromatography was performed on Carlo Erba silica gel 60 (230–400 mesh; CarloErba, Milan, Italy). TLC was carried out using plates coated with silica gel 60 F254 purchased from Merck (Darmstadt, Germany). Microwave reactions were performed on a CEM Discover® single mode platform using 10 mL pressurized vials. Melting points were determined in open capillary tubes on an Electrothermal 9100 apparatus and are uncorrected. Reaction yields refer to chromatographically and spectroscopically pure products. ^{1}H and ^{13}C NMR spectra were registered on a Bruker AC 300. gCOSY experiments were registered on Bruker AC 500. Chemical shifts are reported in ppm. All target compounds were assessed for purity by combustion analysis. All target compounds were found to be >95% purity. MS spectrometry analysis ESI–MS was carried out on a Finnigan LCQ Deca ion trap instrument. Microanalyses were carried out on Carlo Erba 1106 elemental analyzer.

4.2. General procedure for the synthesis of 2-hydroxy-5-methoxy-2,5-cyclohexadiene-1,4-dione 3-substituted derivatives (*Ia–b*)

A water solution (8 mL) of ammonium cerium (IV) nitrate (CAN) (0.486 g, 2.5 equiv.) was added rapidly to the appropriate 1,2,4,5-tetramethoxybenzene-3-substituted **6a–b** (1 equiv.) in acetonitrile

(8 mL) at room temperature. After 2 h the solvent was evaporated. The mixture was diluted with water, extracted with ethyl acetate, providing a crude product purified by column chromatography.

*4.2.1. 3-Benzyl-2-hydroxy-5-methoxy-2,5-cyclohexadiene-1,4-dione (**Ia**)*

Elution with hexane/EtOAc (50:50) afforded **Ia** (0.046 g, 55%) as yellow powder. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.81 (s, 2H), 3.90 (s, 3H), 5.92 (s, 1H), 7.20 (d, J = 8.7 Hz, 1H), 7.25–7.30 (m, 2H), 7.38–7.40 (m, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 26.2, 54.5, 101.2, 124.5, 1268, 128.1, 138.2, 150.2, 159.6, 179.3, 181.8. ESI–MS: m/z 244.9 [M^+]. Anal. Calcd for $C_{14}H_{12}O_4$: C 68.85, H 4.95, O 26.20. Found: C 68.72, H 4.83O 26.32.

*4.2.2. 3-[(2-Naphthyl)methyl]-2-hydroxy-5-methoxy-2,5-cyclohexadiene-1,4-dione (**Ib**)*

Elution with $CHCl_3$ afforded **Ib** (0.065 g, 63%) as dark yellow solid. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.85 (s, 3H), 3.97 (s, 2H), 5.86 (s, 1H), 7.41 (m, 1H), 7.44 (m, 2H), 7.48 (dd, J = 6.6 e 1.5 Hz, 1H), 7.74 (s, 1H), 7.76–7.80 (m, 3H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 27.9, 54.5, 100.1, 123.8, 124.3, 125.8, 126.2, 127.1, 150.3, 158.8, 179.8, 180.7. ESI–MS: m/z 295.60 [M^+]. Anal. Calcd for $C_{18}H_{14}O_4$: C 73.46, H 4.79, O 21.75. Found: C 73.68, H 4.96,O 21.95.

*4.3. General procedure for the synthesis of 2,5-dihydroxy-2,5-cyclohexadiene-1,4-dione 5-substituted derivatives (**Ic–d**)*

To a solution of bisacetonide **13c–d** (1.0 equiv.) in 1,4-dioxane (3.0 mL) was added an aqueous solution of HCl 4 N (4.58 mL) at room temperature. The reaction mixture was stirred for 7 h under reflux conditions and an aqueous solution of NaOH 2 N (5 mL) was added. The resulting mixture was extracted with $CHCl_3$. The organic layers were combined, dried over Na_2SO_4, filtered and concentrated *in vacuo* yielding a crude product, purified by flash chromatography.

*4.3.1. 3-(Cyclohexylmethyl)-2,5-dihydroxycyclohexa-2,5-diene-1,4-dione (**Ic**)*

Elution with EtOAc/hexane (70:30) afforded **Ic** (0.047 g, 69%) as yellow solid. Spectroscopical data are reported by Kim et al. [17].

*4.3.2. 3-((Decahydronaphthalen-3-yl)methyl)-2,5-dihydroxy-2,5-cyclohexadiene-1,4-dione (**Id**)*

Elution with EtOAc/hexane (70:30) afforded **Id** as an orange solid (0.055 g, 57%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.72–1.08 (m, 3H), 1.27–1.39 (m, 4H), 1.32–1.42 (m, 2H), 1.56–1.72 (m, 8H), 2.25–2.42 (m, 2H), 6.03 (s, 1H), 7.70 (br, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 21.2, 26.3, 28.3, 29.2 31.5, 33.3, 38.4, 42.5, 42.8, 112.3, 119.8, 150.3, 152.4, 174.6, 179.8. ESI–MS: m/z 292.33 [M^+]. Anal. Calcd for $C_{17}H_{22}O_4$: C 70.32, H 7.64, O 22.04. Found: C 70.21, H 7.75O 22.54.

*4.4. General procedures for synthesis of resorcinol derivatives (**IIe**, **IIh**, **IIi**)*

The appropriate 2,4-dimethoxybenzene aryl derivatives **24a**, **24b** and **27** (1.0 equiv.) in dry dichloromethane (12 mL), cooled to −15 °C, were reacted in the presence of boron tribromide (8.8 equiv.). The mixture was stirred for 18 h then deionized water was added, followed by dichloromethane. The combined organic layer was dried over Na_2SO_4, filtered, and purified to obtain desired compounds.

*4.4.1. 1-(4-Dibenzofuranyl)-2,4-benzendiol (**IIe**)*

Elution with hexane/EtOAc (90:10–60:40) afforded **IIe** (82%) as white solid. ^{1}H NMR (CD_3OD, 300 MHz) δ 6.85 (s, 1H), 6.87 (d, J = 8.1 Hz, 1H), 7.22–7.30 (m, 3H), 7.68 (t, J = 7.2 Hz, 1H), 7.79 (d, J = 8.5 Hz, 1H), 7.96 (m, 2H), 8.13 (d, J = 7.2 Hz, 1H). ^{13}C NMR (CD_3OD, 75 MHz) δ 104.1, 109.0, 111.6, 112.3, 113.0, 119.8, 121.0, 121.5, 123.3, 124.7, 130.7, 132.9, 137.8, 145.8, 156.8, 157.1, 158.8. ESI–MS: m/z 276.87 [M^-]. Anal. Calcd for $C_{18}H_{12}O_3$: C 78.25, H 4.38, O 17.37. Found: C 78.42, H 4.41, O 17.45.

4.4.2. 4-(Benzo[b]thiophen-3-yl)benzene-1,3-diol (IIh)

Elution with $CHCl_3/CH_3OH$ (99:1) afforded **IIh** (48%) as white solid. ^{1}H NMR (CD_3OD, 300 MHz) δ 6.84 (dd, J = 8.0 e 1.9 Hz, 1H), 6.91 (d, J = 1.9 Hz, 1H), 7.49–7.59 (m, 3H), 8.04 (d, J = 6.8 Hz, 1H), 8.45 (d, J = 6.8 Hz, 1H), 8.70 (d, J = 6.8 Hz, 1H). ^{13}C NMR (CD_3OD, 75 MHz) δ 103.7, 110.2, 118.5, 122.8, 123.2, 124.7, 141.7, 143.8, 158.5, 159.7. ESI–MS: m/z 241.13 [M^-]. Anal. Calcd. For $C_{14}H_{10}O_2S$: C, 69.40; H, 4.16; O, 13.21; S, 13.23. Found: C 68.36, H 4.95, O 12.88, S, 12.98.

*4.4.3. 1-(2,4-Dihydroxyphenyl)-2-(naphthalen-3-yl)ethanone (**IIi**)*

Elution with $CHCl_3$ afforded **IIi** (68%) as beige solid. ^{1}H NMR (CD_3OD, 300 MHz) δ 4.70 (s, 2H), 6.29 (d, J = 2.4 Hz, 1H), 6.39 (dd, J = 6.0 and 2.4 Hz, 1H), 7.33–7.48 (m, 4H), 7.78 (d, J = 9.0 Hz, 1H), 7.83–7.88 (m, 2H), 7.93 (d, J = 9.0 Hz, 1H). ^{13}C NMR (CD_3OD, 75 MHz) δ 43.6, 103.2, 107.9, 115.2, 125.1, 125.9, 126.8, 128.2, 131.5, 132.8, 136.7, 158.7, 160.2, 205.1. ESI–MS: m/z 277.54 [M^-]. Anal. Calcd. For $C_{18}H_{14}O_3$: C, 77.68; H, 5.07; O, 17.25. Found: C 77.82, H 5.12, O 17.42.

*4.5. General procedure for the synthesis of p-benzoquinones **Ih** and **Ii***

To a solution of the dihydroxyl derivatives **21** and **IIe** (1.0 equiv.) in tetrahydrofuran (10 mL) at room temperature, a solution of Fremy's salt (2.5 equiv.) in 5.7 mL of 15% Na_2CO_3, (1.6 equiv.) was added. The solution was stirred vigorously for 8 h, until reaction was completed and reaction mixture, after acidification with H_2SO_4 2 N, was extracted with EtOAc (3 × 25 mL). Desired compounds were obtained after purification by flash chromatography.

*4.5.1. 6-((4-Hydroxy-3,6-dioxocyclohexa-1,4-dienyl)methyl) naphthalene-2-carboxylic acid (**Ih**)*

Elution with a mixture of CH_2Cl_2/MEOH/CH_3COOH (80:15:05) afforded quinone **Ih** as brown powder (0.040 g, 45%). ^{1}H NMR (CD3OD, 300 MHz) δ 4.77 (dd, J = 21.8 e 8.1 Hz, 2H), 5.36 (d, J = 8.1 Hz, 1H), 5.96(d, J = 8.1 Hz, 1H), 6.17 (s, 1H), 6.36–6.28 (m, 3H), 6.46 (d, J = 8.1 Hz, 1H), 7.03 (s, 1H). ^{13}C NMR (CD3OD, 75 MHz) δ 35.2, 106.0, 109.4, 118.6, 120.2, 126.8, 127.6, 128.1, 128.4, 131.1, 132.1, 134.3, 150.4, 159.8, 160.0, 181.9, 182.1. ESI–MS: m/z 309.10 [M^+]. Anal. Calcd. For $C_{18}H_{12}O_5$: C 70.13, H 3.92, O 25.95. Found: C 70.50, H 4.10, O 26.30.

*4.5.2. 5-(4-Dibenzofuranyl)-2-hydroxy-2,5-cyclohexadiene-1,4-dione (**Ii**)*

Elution with hexane/EtOAc (60:40) afforded **Ii** (0.056 g, 54%) as light brown solid. ^{1}H NMR (DMSO-D6, 300 MHz) δ 6.11 (s, 1H), 6.99 (s, 1H), 7.30–7.36 (m, 3H), 7.46 (d, J = 7.4 Hz, 1H), 7.8–8.0 (m, 3H), 11.0 (br; 1H). ^{13}C NMR (DMSO-D6, 75 MHz) δ 110.1, 111.5, 111.6, 113.0, 119.1, 121.0, 121.7, 123.3, 124.7, 129.6, 132.7, 134.0, 145.7, 145.8, 156.3, 169.0, 181.3, 187.0. ESI–MS: m/z 291.01[M^+]. Anal. Calcd for $C_{18}H_{10}O_4$: C 74.48, H 3.47, O 22.05. Found: C 74.56, H 3.53, O 22.16.

4.6. Docking protocol

Molecular docking was performed to predict the binding mode of the ligands in the binding site of 5-LO; the recently reported crystal structure of 5-LO (PDB: 3O8Y) was used [30]. The structures of all compounds in this study were sketched and minimized using Cerius2. GOLD (Genetic Optimization of Ligand Docking), a docking program based on genetic algorithm was used to dock the inhibitors [31]. During docking, the default algorithm speed was selected. The number of poses for each inhibitor was set to 100, and early termination was allowed if the top three bound

conformations of a ligand were within 1.5 Å RMSD. As it is well known, in LOs the substrate/inhibitor binding site is near to the conserved iron binding site [29]. Thus, the active site was centered on the ligand's binding site oxyterminal of Ile. The interactions and binding modes were analyzed in Accelrys Discover Visualizer 2.5. SAR and prominent differences in the interactions of various derivatives with 5-LO have been illustrated.

4.7. Biological evaluation and assay systems

4.7.1. Cells and isolation

PMNL were isolated from buffy coats obtained from the Blood Centre, University Hospital Tuebingen (Tuebingen, Germany) as described [32]. In brief, human fresh blood was collected in heparinized tubes (16 I.E. heparin/mL blood) by venipuncture from fasted (12 h) adult healthy volunteers, with consent, and leukocyte concentrates were prepared by centrifugation (4000 × *g*, 20 min, 20 °C). The subjects had no apparent inflammatory conditions and had not taken anti-inflammatory drugs for at least ten days prior to blood collection. Cells were immediately isolated by dextran sedimentation and centrifugation on Nycoprep cushions (PAA Laboratories, Linz, Austria) and hypotonic lysis of erythrocytes was performed as described [32]. Cells were finally resuspended in PBS pH 7.4 containing 1 mg/mL glucose and 1 mM $CaCl_2$ (PGC buffer) (purity > 96–97%).

4.7.2. Determination of 5-LO, 12-LO, and 15-LO product formation in cell-based assays

For assays of 5-LO, 12-LO and 15-LO, 5 × 10^6 freshly isolated PMNL were resuspended in 1 mL PGC buffer. After pre-incubation with the compounds for 15 min at 37 °C, 5-LO product formation was started by addition of 2.5 μM A23187 and 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 PGB_1 and 500 μL of PBS were added. Formed 5-LO, 12-LO and 15-LO metabolites were extracted and analyzed by HPLC as described [33]. 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)-dihydroxy-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 C_4, D_4 and E_4 were not detected, and oxidation products of LTB_4 were not determined. The formation of the 12-LO product 12(S)-hydro(pero)xy-5,8-cis-10-trans-14-cis-eicosatetraenoic acid (12-H(P)ETE) and of the 15-LO product 15(S)-hydro(pero)xy-5,8,11-cis-13-trans-eicosatetraenoic acid (15-H(P)ETE) is expressed as ng per 10^6 cells.

4.7.3. Expression and purification of human recombinant 5-LO from Escherichia coli, and determination of 5-LO activity in cell-free systems

E. coli BL21 was transformed with pT3-5LO plasmid, human recombinant 5-LO protein was expressed at 37 °C and purified as described [34]. Purified 5-LO was immediately used for 5-LO activity assays. 0.5 μg purified 5-LO were diluted with PBS/EDTA and pre-incubated with the test compounds. After 5–10 min at 4 °C, samples were pre-warmed for 30 s at 37 °C, and 2 mM $CaCl_2$ plus 20 μM AA (or the indicated concentrations of 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 analyzed by HPLC as described for intact cells.

4.8. Statistics

Data are expressed as mean ± S.E.M. IC_{50} values were calculated by nonlinear regression using SigmaPlot 9.0 (Systat Software Inc., San Jose, USA) one site binding competition.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.06.039.

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