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Original article

Novel series of benzoquinones with high potency against 5-lipoxygenase in human polymorphonuclear leukocytes

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ABSTRACT

5-Lipoxygenase (5-LO) is a potential target for pharmacological intervention with various inflammatory and allergic diseases. Starting from the natural dual 5-LO/microsomal prostaglandin E_2 synthase (mPGES)-1 inhibitor embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone, **2**) that suppresses 5-LO activity in human primary leukocytes with IC_{50} = 0.8–2 μM, we synthesized 48 systematically modified derivatives of **2**. We modified the 1,4-quinone to 1,2-quinone, mono- or bimethylated the hydroxyl groups, and varied the C11-*n*-alkyl residue (C4- to C16-*n*-alkyl or prenyl) of **2**. Biological evaluation yields potent analogues being superior over **2** and obvious structure-activity relationships (SAR) for inhibition of 5-LO. Interestingly, conversion to 1,2-benzoquinone and bimethylation of the hydroxyl moieties strongly improves 5-LO inhibition in polymorphonuclear leukocytes versus **2** up to 60-fold, exemplified by the C12-*n*-alkyl derivative **22c** (4,5-dimethoxy-3-dodecyl-1,2-benzoquinone) with IC_{50} = 29 nM. Regarding inhibition of mPGES-1, none of the novel benzoquinones could outperform the parental compound **2** (IC_{50} = 0.21 μM), and only modest suppressive effects on 12- and 15-LOs were evident. Together, our detailed SAR study reveals **22c** as highly potent 5-LO-selective lead compound in intact cells that warrants further preclinical evaluation as anti-inflammatory agent.

1. Introduction

Leukotrienes (LTs) are bioactive lipid mediators produced from arachidonic acid (AA) with pivotal roles in immune reactions, inflammation, and allergy [1,2]. 5-Lipoxygenase (5-LO), a non-heme iron-containing dioxygenase, is the key enzyme in the biosynthesis of LTs that catalyzes the incorporation of molecular oxygen into AA yielding the intermediate 5(S)-hydroperoxyeicosatetraenoic acid (5-HPETE), and subsequently dehydrates 5-HPETE to LTA_4 [3]. The latter is either converted by LTA_4 hydrolase to LTB_4 or by LTC_4 synthases to the glutathione conjugate LTC_4 that is further degraded to LTD_4 and LTE_4. The biological effects that are evoked by LTs are mediated by different G protein-coupled receptors, specific for LTB_4 ($BLT_{1/2}$) or LTC_4, D_4 and E_4 ($cysLT_{1/2}$), as well as by additional receptors [4]. While LTB_4 causes chemotaxis towards various types of leukocytes and activates phagocytes, LTC_4, D_4 and E_4 are potent bronchoconstrictors, cause mucus secretion in the lung, and increase vasopermeability of postcapillary venules [1]. Accordingly, LTs are implicated as mediators in a variety of inflammatory and allergic diseases including asthma, allergic rhinitis,

Abbreviations: AA, arachidonic acid; CAN, cerium ammonium nitrate; COX, cyclooxygenase; $cPLA_2$, cytosolic phospholipase A_2; FLAP, 5-lipoxygenase-activating protein; H(P)ETE, hydro(pero)xyeicosatetraenoic acid; 5-LO, 5-lipoxygenase; LT, leukotriene; mPGES-1, microsomal prostaglandin E_2 synthase-1; PBS, phosphate-buffered saline; PG, prostaglandin; PGC buffer, PBS containing 1 mg/ml glucose plus 1 mM $CaCl_2$; PMNL, polymorphonuclear leukocyte; SAR, structure-activity relationship.

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and autoimmune disorders, as well as cardiovascular diseases [2]. Moreover, due to stimulation of survival, proliferation and migration of cancer cells by LTs, a role in cancer became apparent [5].

Based on its key function in the initiation of LT-related diseases, 5-LO has long been considered as a promising target for therapeutic intervention with those disorders [2]. In fact, numerous series of synthetic agents as well as natural products have been identified as 5-LO inhibitors but most of these compounds exhibited only limited potency, were rather unspecific for 5-LO with potential side effects, and/or lacked efficiency *in vivo*. [6] Moreover, the characterization of the cellular and molecular mechanism of 5-LO inhibition by the inhibitor was often neglected and detailed follow-up studies were not consequently performed or pursued [7]. For example, the 1,4-benzoquinone **1** (2,3,5-trimethyl-6-(12-hydroxy-5,10-dodecadiynyl)-1,4-benzoquinone, AA-861, Fig. 1) was initially presented in 1982 as one of the first 5-LO inhibitors [8] and many subsequent pharmacological studies confirmed the efficiency and anti-inflammatory efficacy *in vivo,* and **1** even succeeded in a clinical trial for prevention of seasonal allergic rhinitis [9]. Nevertheless, the precise mechanisms of inhibition of 5-LO has remained elusive until recently [10].

In the course of our previous biological investigations of simplified derivatives of the marine hydroxyquinone bolinaquinone with antitumour activity [11,12], we identified compound **2** (2,5-dihydroxy-3-undecyl-1,4-benzoquinone, embelin, Fig. 1), a naturally occurring 1,4-benzoquinone from *Embelia ribes*, that dually inhibits 5-LO as well as microsomal prostaglandin E_2 synthase (mPGES)-1 but does not interfere with 12/15-LOs, cytosolic phospholipase (PL)A_2 or cyclooxygenase (COX) enzymes [13]. In a parallel study we synthesized and characterized a series of related 2,5-dihydroxylated 1,4-benzoquinones with various lipophilic and bulky alkyl- or aryl-substituents in 3-position [14], exemplified by 3-((decahydronaphthalen-6-yl)methyl)-2,5-dihydroxycyclohexa-2,5-diene-1,4-dione (compound **2a**, Fig. 1), as 5-LO inhibitors with anti-inflammatory activity *in vivo* [15]. The simple structure and good potency of **2** and **2a** with IC_{50} values in the submicromolar range stimulated us to systematically modify the structure of **2** and thus, to improve the inhibitory potential as well as to investigate SARs. In particular, we aimed at improving the 5-LO inhibitory potential in intact cells, because the potency of **2** in cell-free assays (IC_{50} = 0.06 μM) was 13- to 33-fold higher than in cell-based test systems (0.8–2 μM) [13].

Here we report the synthesis and the biological evaluation of 48 different 3-mono-alkyl-substituted 1,4-benzoquinones (i.e., **17a**-**28a**, **17b**-**28b**, **17d**-**20d**, **22d**-**28d)** or 1,2-benzoquinones (i.e., **17c**-**28c)**. Whereas the potency of some representatives was clearly superior over **2** regarding inhibition of 5-LO, inhibition of mPGES-1 was not improved. Our results suggest concrete SARs for 5-LO inhibition, that is, (I) methylation of hydroxyl groups at the quinone core increases the potency especially in intact cells, (II) 1,2-quinones are more potent than 1,4-quinones, and (III) *n*-alkyl residues with 10–14 carbon atoms confer highest potency. In fact, the doubly *O*-methylated 1,2-benzoquinone **22c** with C12-*n*-alkyl chain (4,5-dimethoxy-3-dodecyl-1,2-benzoquinone) reached IC_{50} value = 29 nM in intact human polymorphonuclear leukocytes (PMNL), which is an about 60-fold improvement over **2**.

2. Results and discussion

2.1. Chemistry

The desired compounds were synthesized starting from 1,2,4,5-tetramethoxybenzene (**3**) [16] which was subjected to an ortho–metalation reaction in the presence of *n*-BuLi and hexamethylphosphoramide (HMPA) (Scheme 1) [11]. The lithium derivative

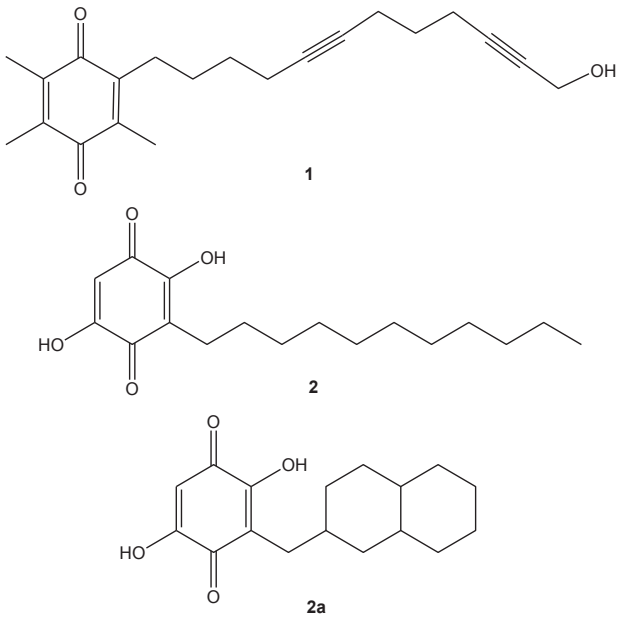


Fig. 1. Chemical structures of compound **1**, **2** and **2a** that inhibit 5-LO.

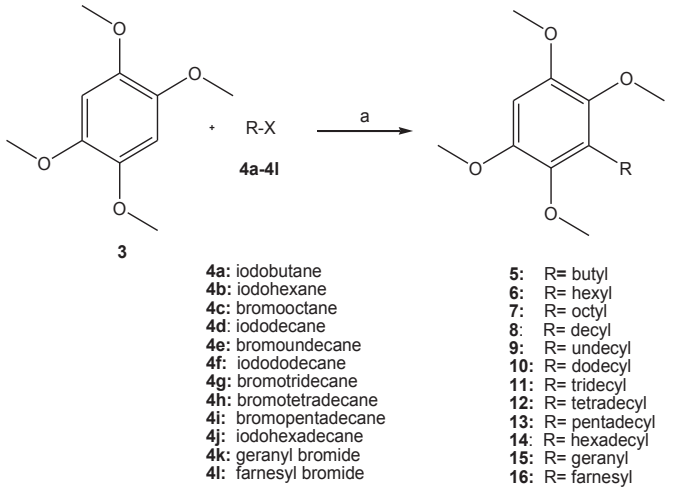
was reacted with different alkyl halides giving intermediates **5**–**16** in good yields. Cerium ammonium nitrate (CAN)-mediated oxidative reaction provided a mixture of 2,5-dimethoxy (**17a**-**28a**) and 2-hydroxy-5-methoxy-1,4-benzoquinones (**17b**-**28b**) as described for literature-known compounds **20a**, **21a** and **20b**, **21b** (Method A [17]). A mixture of 2,5-dimethoxy-1,4-benzoquinones (**17a**-**28a**) and 4,5-dimethoxy-1,2-benzoquinones (**17c**-**28c**) was achieved following a modified procedure of the reported method (Method B; Scheme 2). Treatment of **17a**-**20a** and **22a**-**28a** with 2 M NaOH allowed to obtain 2,5-dihydroxy-1,4-benzoquinones **17d**-**20d** and **22d**-**28d** in quantitative yields (Scheme 3) [18].

2.2. Biological assays

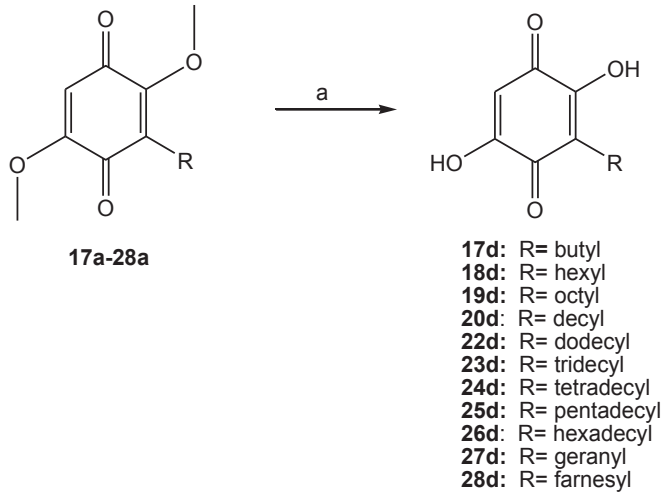
To study the ability of the test compounds for direct inhibition of 5-LO, we applied a cell-free assay using purified human recombinant 5-LO enzyme [7,19]. To study the inhibitory potency on 5-LO product formation in intact cells, we used human PMNL that are a major source for LT biosynthesis [20,21]. PMNL were either stimulated with the Ca^{2+}-ionophore A23187 alone or together with 20 μM exogenous AA. Co-addition of AA allows circumventing the absolute need for endogenous substrate release by $cPLA_2$ and the AA transfer via the 5-LO-activating protein (FLAP) [7,22]. The 5-LO inhibitor **29** (N-(1-benzo[b]thien-2-ylethyl)-N-hydroxyurea; zileuton) served as reference compound [23].

2.3. Design of compounds and structure-activity relationship studies regarding 5-LO

The focus of the compound design was placed on three structural variations of lead compound **2**: (I) the free hydroxyl groups at the benzoquinone core in comparison to *O*-methylated analogues, (II) the length of the *n*-alkyl residue in 3-position as well as modification towards prenyl moieties, and (III) alteration of the 1,4-quinone to the 1,2-quinone core. Starting with the compound **2**-derived 2,5-dihydroxy-1,4-benzoquinone core, we first varied the length of the *n*-alkyl residue in position 3 (i.e., C11 in **2**). To this aim, we introduced linear C4-, C6-, C8-, C10-, C12-, C13-, C14-, C15- and C16-*n*-alkyl residues or isoprenoid side chains with two or three



Scheme 1. Ortho-metalation of 1, 2, 4, 5-tetramethoxybenzene **3**. Reagents and conditions: a) n-BuLi, HMPA, THF, from −40 °C to room temperature, 12 h.

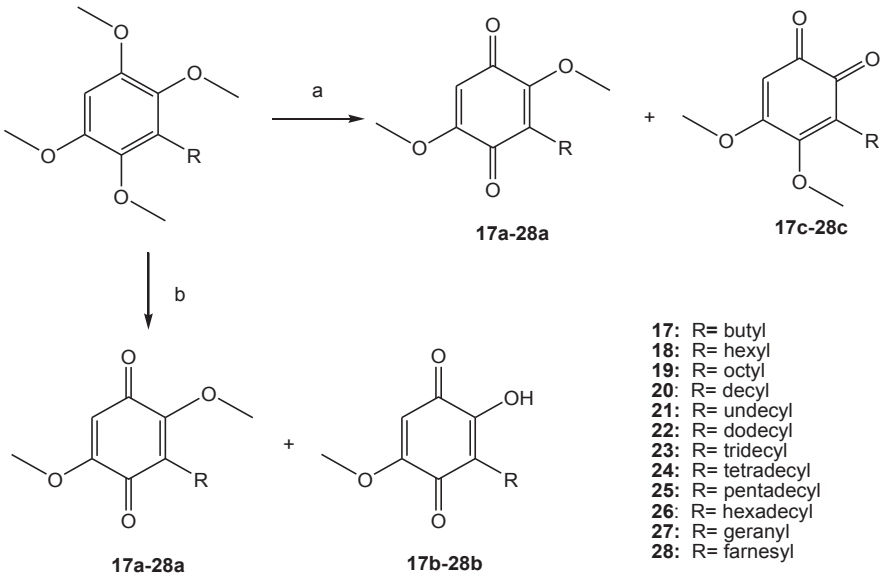


Scheme 3. Synthesis of 5-dihydroxy-1,4-benzoquinones **17d**-**20d** and **22d**-**28d**. Reagents and conditions: a) NaOH 2 M, EtOH, 80 °C, 2 h.

prenyl moieties, respectively, into 3-position, retaining the 2,5-dihydroxy-1,4-benzoquinone core ("**d**-series", Table 1).

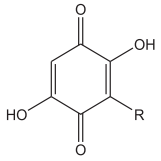
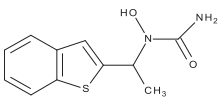
Except for the C4-*n*-alkyl-substituted compound **17d**, all derivatives inhibited the activity of isolated 5-LO in the cell-free assay with IC_{50} values = 0.17–4 μM. Compound **17d**, at a concentration of 10 μM, inhibited 5-LO by only 43.9%. Note that none of the compounds was superior over parental **2** (IC_{50} = 0.06 μM) [13], in this respect. Therefore, a relationship between the length of the *n*-alkyl chain and the 5-LO inhibitory potential of 3-substituted 2,5-dihydroxy-1,4-benzoquinones is apparent, where the C11-*n*-alkyl residue is seemingly optimal. Compounds **20d**, **22d**, **24d**, and **26d** with C10-, C12-, C14-, and C16-*n*-alkyl chains, respectively inhibited 5-LO with low IC_{50} values between 0.17 and 0.19 μM, whereas the C6-substituted compound **18d** is considerably less active (IC_{50} = 4 μM). The prenyl derivatives showed lower potency with IC_{50} values of 1.8 and 2.5 μM for compound **27d** and **28d**, respectively. Note that two (geranyl) or three (farnesyl) prenyl residues connected to 1,4-hydroquinone were found before to be superior in suppression of LTB_4 synthesis in PMNL as compared to one or four prenyl moieties [24]. Apparently, an extended lipophilic alkyl residue favours 5-LO inhibition which is in agreement with general findings by others showing that the potency of (poly)phenol-based 5-LO inhibitors is often enhanced due to increasing the lipophility [7].

Next, we evaluated the inhibitory potential of the test compounds in the 5-LO cell-based assay. In general, all compounds of this group significantly lost their potency in PMNL. In fact, compounds with C4-, C6-, C8- (i.e., **17d**, **18d**, **19d**) or C16-*n*-alkyl and prenyl residues (i.e., **26d**, **27d**, **28d**) only moderately suppressed 5-LO product synthesis (IC_{50} > 10 μM), regardless of the absence or presence of exogenous AA. However, the C10-, C12-, C13-, C-14- and C-15-*n*-alkyl derivatives **20d**, **22d**, **23d**, **24d**, and **25d**, respectively, were able to inhibit 5-LO (IC_{50} = 2 and 4.6 μM) in PMNL challenged with A23187; along these lines **2** (with a C11-*n*-alkyl residue) was active in PMNL but lost potency (IC_{50} = 1.7 μM) [13] versus cell-free 5-LO activity as well.



Scheme 2. Synthesis of 2,5-dimethoxy-1,4-benzoquinones (**17a**-**28a**), 2-hydroxy-5-methoxy-1,4-benzoquinones (**17b**-**28b**), and 4,5-dimethoxy-1,2-benzoquinones (**17c**-**28c**). Reagents and conditions: a) Method B. CAN, CH_3CN:H_2O 7:3, -7 °C, 10′; b) Method A. CAN, CH_3CN:H_2O 7:3, from −7 °C to room temperature, 2 h.

Table 1
Effects of 2,5-hydroxy-1,4-benzoquinones ("**d**-series") on 5-LO activity. Data are expressed as means ± S.E. of single determinations obtained in three to four independent experiments.

Cmpd		5-LO activity IC_{50} [μM] (remaining activity (%) at 10 μM)		
		Cell-based		Cell-free
		A23187	A23187 + AA	AA
17d	R = *n*-butyl	>10 (93.5 ± 4.4%)	>10 (92.8 ± 4.2%)	>10 (65.4 ± 14.2%)
18d	R = *n*-hexyl	>10 (133.5 ± 19.0%)	>10 (79.9 ± 6.6%)	4.0 ± 1.1
19d	R = *n*-octyl	>10 (53.5 ± 4.8%)	>10 (80.07 ± 8%)	0.38 ± 0.04
20d	R = *n*-decyl	2.0 ± 0.3	7.1 ± 1.2	0.18 ± 0.01
2 (embelin)	R = *n*-undecyl	1.7 ± 0.4	3.5 ± 0.5	0.06 ± 0.01
22d	R = *n*-dodecyl	4.6 ± 1.3	3.9 ± 1	0.17 ± 0.03
23d	R = *n*-tridecyl	2.5 ± 0.4	4.7 ± 0.2	0.22 ± 0.09
24d	R = *n*-tetradecyl	3.1 ± 0.5	5.1 ± 0.6	0.17 ± 0.01
25d	R = *n*-pentadecyl	4.3 ± 0.5	>10 (61.7 ± 2.1%)	0.23 ± 0.08
26d	R = *n*-hexadecyl	>10 (88.0 ± 16.3%)	>10 (80.5 ± 5.7%)	0.19 ± 0.04
27d	R = geranyl	>10 (114.3 ± 4.5%)	>10 (81.9 ± 10.8%)	1.8 ± 0.2
28d	R = farnesyl	>10 (85.8 ± 3.9%)	>10 (66.6 ± 2.8%)	2.5 ± 1.4
29 (zileuton)		0.9 ± 0.3	2.5 ± 1.2	0.59 ± 0.10

We assumed that the two polar hydroxyl moieties in 2- and 5-position of **2** and related compounds of the "**d**-series", which actually are part of two vinylogue acids with dissociable protons, may confer the compounds high hydrophilicity and thus, hamper membrane penetration into PMNL to access 5-LO. In fact, **2** was reported to exist in the mono-deprotonated form at pH ranges 6.0–8.0 with anionic charge [25]. Accordingly, we methylated either the hydroxyl in 5-position ("**b**-series") or both hydroxyl residues in 2- and 5-position ("**a**-series"), respectively, which may improve cellular uptake and thus potency against 5-LO. Compared to the 2,5-dihydroxy "**d**"-series, the 5-*O*-monomethylated analogues ("**b**-series") inhibited 5-LO less efficiently in the cell-free assay (Table 2). For example, compounds **18b**, **26b** and **27b** (Table 2) did not inhibit isolated 5-LO by more than 50% at 10 μM and the IC_{50} values of the most active compounds **20d** and **2** (0.18 and 0.06 μM) shifted to about 22- to 24-fold higher concentrations (i.e., 4.3 and 3.8 μM) for the corresponding 5-methoxy derivatives **20b** and **21b**, respectively. Interestingly, bimethylation of both hydroxyl moieties in 2- and 5-position ("**a**-series", Table 3) improved the efficiency in the cell-free assay, visualized by IC_{50} values of 0.21–3.3 μM for all derivatives, which are in the range of those for the unmethylated "**d**-series", and even the *n*-butyl-derivative **17a** was active (IC_{50} = 3.3 μM), in contrast to the unmethylated analogue **17d**.

Of interest, in the cell-based assay the 5-LO inhibitory capacity of the *O*-methylated derivatives was increased, in particular for the doubly methylated ("**a**-series") analogues. The *O*-monomethylated compounds carrying C10- to C15-*n*-alkyl chains were highly active with IC_{50} values in the range of 0.3–0.7 μM in PMNL stimulated with A23187 and 0.5–2.7 μM for cells stimulated with A23187 plus AA. Monomethylation conferred the prenyl derivatives **27b** and **28b** efficiency versus the inactive unmethylated counterparts **27d** and **28d**.

The 5-LO inhibitory potency in intact cells was further improved for all *O*,*O*′-bimethylated analogues ("**a**-series", except **26a)** over the monomethylated "**b**-series". In particular, bimethylated compounds carrying *n*-alkyl residues of 6–15 carbons inhibited A23187-induced 5-LO product synthesis with IC_{50} = 0.2–1.7 μM, with a tendency for improved activity in the absence of exogenous AA (Table 3). Again, also the prenylated derivatives **27a** and **28a** exhibited improved potency with IC_{50} = 0.3–1.6 μM. Taken together, *O*-mono- and even more strikingly *O*,*O*′-bimethylation of 2,5-dihydroxy-1,4-benzoquinones carrying a C10- to C15-*n*-alkyl chain in 3-position yields highly potent 5-LO inhibitors that efficiently suppress 5-LO product formation in intact cells, with up to 9-fold superiority over parental **2**.

Next, we replaced the 2,5-dimethoxy-1,4-benzoquinone core present in compounds **17a**-**28a** by a 4,5-dimethoxy-1,2-benzoquinone moiety ("**c**-series", Table 4). This modification towards 1,2-quinones further increased the potency against 5-LO in particular in intact PMNL. In fact, except for the C4-*n*-alkyl-substituted **17c**, for all other (C6- to C14-*n*-alkyl and prenyl) derivatives the IC_{50} values were in the submicromolar range. The C16-*n*-alkyl-substituted compound **26c** was poorly soluble in the aqueous assay buffer and thus, could not be tested for 5-LO inhibition. Of particular interest are the C10- to C14-*n*-alkyl derivatives **20c**, **21c**, **22c**, **23c** and **24c** which reached IC_{50} values between 29 and 150 nM in PMNL stimulated with A23187 with or without AA. Similar as the 2,5-dimethoxylated 1,4-benzoquinones most of the 4,5-dimethoxy-1,2-benzoquinones were about equipotent 5-LO inhibitors in intact cells and in the cell-free assay. Thus, compounds **20c**, **21c**, **22c**, **23c** and **24c** repressed 5-LO activity with IC_{50} values in the range of 40–130 nM, being about equipotent to **2** (IC_{50} = 60 nM). Conclusively, our SAR studies revealed 3-alkyl-substituted 4,5-dimethoxy-1,2-benzoquinones when equipped with simple C10- to C14-*n*-alkyl residues as potent 5-LO inhibitors with IC_{50} values in the range of 29–150 nM in PMNL and 40–130 nM for isolated 5-LO.

Table 2
Effects of 2-hydroxy-5-methoxy-1,4-benzoquinones ("**b**-series") on 5-LO activity. Data are expressed as means ± S.E. of single determinations obtained in three to four independent experiments.

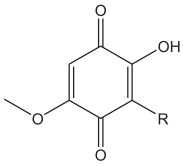
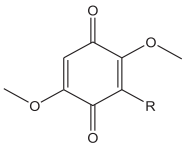
No.		5-LO activity IC$_{50}$ [μM] (remaining activity (%) at 10 μM)		
		Cell-based		Cell-free
		A23187	A23187 + AA	AA
17b	R = *n*-butyl	>10 (86.0 ± 6.0%)	>10 (95.7 ± 7.3%)	>10 (70.5 ± 17.1%)
18b	R = *n*-hexyl	>10 (68.9 ± 16.3%)	>10 (64.1 ± 8.0%)	>10 (71.1 ± 5.3%)
19b	R = *n*-octyl	>10 (73.5 ± 8.6%)	>10 (72.9 ± 11.7%)	3.0 ± 0.4
20b	R = *n*-decyl	0.59 ± 0.1	2.7 ± 0.7	4.3 ± 0.3
21b	R = *n*-undecyl	0.68 ± 0.1	1.3 ± 0.4	3.8 ± 0.5
22b	R = *n*-dodecyl	0.56 ± 0.04	1.1 ± 0.2	0.74 ± 0.08
23b	R = *n*-tridecyl	0.77 ± 0.26	2.3 ± 1.1	0.92 ± 0.47
24b	R = *n*-tetradecyl	0.27 ± 0.04	0.49 ± 0.1	0.42 ± 0.01
25b	R = *n*-pentadecyl	0.39 ± 0.14	0.86 ± 0.27	0.27 ± 0.10
26b	R = *n*-hexadecyl	1.0 ± 0.6	1.1 ± 0.8	>10 (60.1 ± 8.7%)
27b	R = geranyl	4.1 ± 1.1	>10 (65.9 ± 6.0%)	>10 (73.6 ± 8.0%)
28b	R = farnesyl	1.9 ± 0.1	2.4 ± 0.8	5.6 ± 0.1

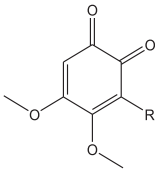
Table 3
Effects of 2,5-methoxy-1,4-benzoquinones ("**a**-series") on 5-LO activity. Data are expressed as means ±S.E. of single determinations obtained in three to four independent experiments.

Cmpd		5-LO activity IC$_{50}$ [μM] (Remaining activity (%) at 10 μM)		
		Cell-based		Cell-free
		A23187	A23187 + AA	AA
17a	R = *n*-butyl	3.4 ± 1.4	7.9 ± 1.0	3.3 ± 0.9
18a	R = *n*-hexyl	0.32 ± 0.21	1.4 ± 0.4	2.6 ± 1.2
19a	R = *n*-octyl	0.32 ± 0.01	0.57 ± 0.17	1.2 ± 0.3
20a	R = *n*-decyl	0.60 ± 0.38	1.7 ± 0.9	1.8 ± 0.7
21a	R = *n*-undecyl	0.20 ± 0.02	0.59 ± 0.11	0.93 ± 0.13
22a	R = *n*-dodecyl	0.63 ± 0.41	0.49 ± 0.10	0.61 ± 0.08
23a	R = *n*-tridecyl	0.23 ± 0.04	0.31 ± 0.05	0.26 ± 0.01
24a	R = *n*-tetradecyl	0.29 ± 0.09	1.4 ± 0.8	0.21 ± 0.03
25a	R = *n*-pentadecyl	0.39 ± 0.14	0.86 ± 0.27	0.27 ± 0.08
26a	R = *n*-hexadecyl	>10 (80.1 ± 9.1%)	3.5 ± 0.9	1.6 ± 0.2
27a	R = geranyl	0.45 ± 0.2	1.6 ± 0.2	3.3 ± 0.7
28a	R = farnesyl	0.31 ± 0.1	1.2 ± 0.2	1.7 ± 0.7

2.4. Effects of benzoquinones on the 5-LO-related enzymes 12-LO, 15-LO-1 and on mPGES-1

Besides 5-LO, other human LOs such as 12-LO and 12/15-LO (also termed 15-LO-1) catalyze the incorporation of molecular oxygen into AA (at C12 and C15, respectively) as substrate [26]. In order to determine the selectivity of the investigated benzoquinones, we analyzed their effects on the formation of 12(S)-hydroxy-6-trans-8,11,14-cis-eicosatetraenoic acid (12-HETE) and 15(S)-hydroxy-5,8,11-cis-,13-trans-eicosatetraenoic acid (15-HETE) that are produced by 12- and 12/15-LOs in PMNL incubations as well. Except for compounds **19c** and **23a** as well as for the C6-*n*-alkyl derivatives **18b** and **18c**, none of the test compounds at a concentration of 10 μM significantly suppressed the synthesis of 12-HETE or of 15-HETE in PMNL incubations (Table S1), indicating that in particular the most active compounds (e.g., **20c**, **21c**, **22c**, and **23c**) are highly

Table 4
Effects of 4,5-methoxy-1,2-benzoquinones ("**c**-series") on 5-LO activity. Data are expressed as means ± S.E. of single determinations obtained in three to four independent experiments.

Cmpd		5-LO activity IC$_{50}$ [μM] (Remaining activity (%) at 10 μM)		
		Cell-based		Cell-free
		A23187	A23187 + AA	AA
17c	R = *n*-butyl	2.2 ± 0.8	5.1 ± 0.7	>10 (82.9 ± 2.7%)
18c	R = *n*-hexyl	0.33 ± 0.10	0.99 ± 0.42	2.6 ± 0.3
19c	R = *n*-octyl	0.38 ± 0.07	0.82 ± 0.38	0.33 ± 0.05
20c	R = *n*-decyl	0.04 ± 0.01	0.07 ± 0.01	0.13 ± 0.01
21c	R = *n*-undecyl	0.06 ± 0.01	0.10 ± 0.03	0.09 ± 0.04
22c	R = *n*-dodecyl	0.029 ± 0.01	0.15 ± 0.03	0.13 ± 0.12
23c	R = *n*-tridecyl	0.12 ± 0.01	0.05 ± 0.03	0.08 ± 0.01
24c	R = *n*-tetradecyl	0.12 ± 0.03	0.09 ± 0.02	0.04 ± 0.02
25c	R = *n*-pentadecyl	>10 (57.6 ± 3.2%)	>10 (66.8 ± 3.5%)	0.62 ± 0.14
26c	R = *n*-hexadecyl	[a]n.d.	[a]n.d.	[a]n.d.
27c	R = geranyl	0.38 ± 0.08	0.39 ± 0.10	0.60 ± 0.08
28c	R = farnesyl	0.15 ± 0.07	0.10 ± 0.01	0.30 ± 0.07

[a] n.d., not determined.

selective for 5-LO (at least > 66- to 340-fold). The *n*-hexyl-substituted compounds **18b** and **18c** (at 10 μM) repressed 12-HETE formation in intact cells by 55.5 and 81.4%, respectively, and **18b** inhibited 15-HETE synthesis by 34.2%. The unspecific LO inhibitor **30** (cinnamyl-3,4-dihydroxy-alpha-cyanocinnamate, CDC) [27] inhibited 12- and 15-HETE formation under these conditions, as expected (Table S1). Moreover, analysis of **22c** for inhibition of 12/15-LO in a cell-free assay (PMNL homogenates) revealed no significant inhibition up to 10 μM, while 5-LO activity was abolished in the same incubations (not shown).

Since **2** was also active on human mPGES-1 (IC$_{50}$ = 0.21 μM) [13] we tested the novel compounds for their ability to inhibit mPGES-1 activity. For analysis of mPGES-1 inhibition, the well-recognized cell-free assay using microsomes from interleukin-1β-stimulated A549 cells and PGH$_2$ (20 μM) as substrate was utilized [28]. The mPGES-1 inhibitor **31** (3-[3-(tert-butylsulfanyl)-1-[(4-chlorophenyl)methyl]-5-(propan-2-yl)-1*H*-indol-2-yl]-2,2-dimethylpropanoic acid, MK886) served as reference compound [28]. Inhibition of mPGES-1 clearly depended on both, the (unmethylated) free hydroxyl groups and the length of the 3-substituted alkyl chain, with active compounds (i.e., IC$_{50}$ < 10 μM) carrying 8 to 15 carbons. Of interest, none of the synthetic derivatives could outperform the parental compound **2**. Mono- or bimethylation of the hydroxyl groups ("**a**-, **b**- and **c**-series") clearly decreased mPGES-1-inhibitory potency (Table S1) and only the 2,5-dihydroxy derivatives **19d**, **20d**, and **23d** with C8-, C10- and C12-*n*-alkyl chains reached IC$_{50}$ values ≤1 μM. The most potent derivative **19d** (IC$_{50}$ = 0.8 μM), carrying a C8-*n*-alkyl residue, was still about 4-fold less potent than **2**. Thus, **2** with unmethylated 2,5-dihydroxy moieties and a C11-*n*-alkyl residue and IC$_{50}$ value = 0.21 μM [13] exhibits optimal structural properties as mPGES-1 inhibitor out of all these alkyl-substituted benzoquinone derivatives.

3. Conclusions

Among the multitude of 5-LO inhibitors discovered thus far, especially from natural sources, many compounds exhibit disadvantageous structural features that impair the 5-LO inhibitory

effectiveness inside the cell, apparently due to inadequate uptake or intracellular inactivation. This is the case also for the natural product **2** that potently inhibits 5-LO in cell-free assays with an IC_{50} = 0.06 μM but showed reduced potency in leukocytes (up to 33-fold; IC_{50} = 0.8–2 μM) [13], seemingly related to the polar acidic hydroxyl groups that may hamper membrane permeability and/or might be subject to rapid oxidation in the cell. From the systematic structural modifications of **2**, aiming to obtain analogues with improved efficiency in intact cells, bimethylation of the hydroxyl moieties was revealed as successful mean to significantly improve cellular 5-LO inhibition. Conversion of the 1,4- to a 1,2-benzoquinone core further governs 5-LO inhibitory potency, and when such 4,5-dimethoxy-1,2-benzoquinones are equipped with simple C10- to C14-*n*-alkyl residues highly potent 5-LO inhibitors with IC_{50} values in the range of 29–150 nM in leukocytes and 40–130 nM for isolated 5-LO are obtained. From our SAR analysis of the 48 novel derivatives, compound **22c** turned out to be of particular pharmacological value, as it most potently inhibited cellular 5-LO product synthesis. Regarding inhibition of mPGES-1, none of the novel benzoquinones could outperform the parental compound **2** (IC_{50} = 0.21 μM). Importantly, **22c** failed to strongly inhibit mPGES-1 (IC_{50} > 10 μM) and did not affect the related 12- and 15-LO, thus implying specific interference with 5-LO rather than unspecific formation of radical intermediates. Only very few other selective 5-LO inhibitors have been reported with higher potency than **22c** in intact cells. Together, our systematic natural product-based synthesis of novel benzoquinones identified privileged, simple structures that are selective and highly potent 5-LO inhibitors with excellent cellular activity that warrant further pharmacological characterization and preclinical analysis.

4. Experimental section

4.1. Compounds and 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; Carlo Erba, Milan, Italy). TLC was carried out using plates coated with silica gel 60 F254 nm purchased from Merck (Darmstadt, Germany). ^1H and ^{13}C NMR spectra were registered on a Bruker AC 300. Chemical shifts are reported in ppm. The purity (95% or higher) of all final products that were evaluated for bioactivity was assessed by combustion analysis (using a Carlo Erba 1106 elemental analyser) and is reported in the Supporting Information. The test compounds were dissolved in DMSO and stored in the dark at −20 °C; freezing/thawing cycles were kept to a minimum.

4.1.1. General procedure for synthesis of compounds 17a-28a, 17b-28b, 17c-28c

To a solution of compounds **5**–**16** (1 mmol) in CH_3CN (10 ml), was added a solution of CAN (2.5 mmol) in CH_3CN–H_2O (10 mL, 7:3) dropwise at −7 °C (salt-ice bath).

Method A. The reaction was stirred for 10 min at −7 °C and diluted with diethyl ether (50 ml). The organic layer was washed two times with distilled water (20 ml), brine (20 ml), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified over silica gel using hexane:EtOAc (8:2) as eluent to give compounds **17a**-**28a** and **17c**-**28c**.

Method B. The reaction was allowed to stir at rt for 2 h, and diluted with ether (50 ml). The organic layer was washed with distilled water (20 ml), brine (20 ml), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified over silica gel using hexane:EtOAc (8:2–7:3) as eluent to give compounds **17a**-**28a** and **17b**-**28b**.

4.1.2. General procedure for the synthesis of compounds 17d-20d, 22d-28d

Aqueous sodium hydroxide (2 M; 1 ml) was added to a solution of **17a**-**20a** or **22a**-**28a** (0.045 mmol) in ethanol (2 ml). The mixture was heated to 70 °C for a period of 2 h and allowed to cool to room temperature. The reaction mixture was diluted with hydrochloric acid (2 M; 5 ml) then extracted with ethyl acetate (3 × 5 ml). The combined organic phases were dried over $MgSO_4$ and the solvent was evaporated to give the title compounds as orange solids.

4.2. Biological evaluation and assay systems

4.2.1. Materials

The 5-LO inhibitor **29** (zileuton) was from Sequoia Research Products (Oxford, UK), and the mPGES-1 inhibitor **30** (MK886) and the LO inhibitor **31** (CDC) were from Cayman Chemical (Ann Arbor, MI). PGH_2 was from Larodan (Malmö, Sweden). DMEM/High Glucose (4.5 g/l) medium, Nycoprep, penicillin, streptomycin, and trypsin/EDTA solution were from PAA Laboratories (Linz, Austria). HPLC solvents were from VWR (Darmstadt, Germany). All other chemicals were purchased from Sigma–Aldrich (Deisenhofen, Germany), unless stated otherwise.

4.2.2. Cells and cell isolation

PMNL were isolated from human blood as reported before [13]. In brief, human peripheral blood was obtained from fastened (12 h) healthy donors with consent that had not taken any anti-inflammatory drugs during the last 10 days, with venipuncture in heparinized tubes (16 IE heparin/ml blood) (University Hospital Jena, Germany). The blood was centrifuged at 4000× g for 20 min at 20 °C for preparation of leukocyte concentrates. Leukocyte concentrates were then subjected to dextran sedimentation and centrifugation on Nycoprep cushions. Contaminating erythrocytes of pelleted polymorphonuclear leukocytes (PMNL) were lysed by hypotonic lysis. PMNL were washed twice in ice-cold PBS (purity > 96–97%) and finally resuspended in PBS pH 7.4 containing 1 mg/ml glucose and 1 mM $CaCl_2$ (PGC buffer) as indicated.

For analysis of acute cytotoxicity of the compounds during pre-incubation periods (30 min at 37 °C), cellular integrity of PMNL was analyzed by trypan blue exclusion with a Vi-cell counter (Beckmann Coulter GmbH, Krefeld). None of the compounds caused significant loss of PMNL viability within 30 min (data not shown).

4.2.3. Expression and purification of human recombinant 5-LO

E.coli BL21 was transformed with pT3-5-LO plasmid, and recombinant 5-LO protein was expressed at 27 °C as described [29]. Cells were lysed in 50 mM triethanolamine/HCl pH 8.0, 5 mM EDTA, soybean trypsin inhibitor (60 μg/ml), 1 mM phenylmethanesulphonyl fluoride, and lysozyme (500 μg/ml), homogenized by sonication (3 × 15 s), and centrifuged at 40,000× g for 20 min at 4 °C. The 40,000× g supernatant (S40) was applied to an ATP-agarose column to partially purify 5-LO as described previously [29]. Semi-purified 5-LO was immediately used for activity assays.

4.2.4. Determination of 5-LO activity in the cell-free assay

Aliquots of semi-purified 5-LO were diluted with ice-cold PBS containing 1 mM EDTA, and 1 mM ATP was added. Samples were pre-incubated with the test compounds or vehicle (0.1% DMSO) as indicated. After 10 min at 4 °C, samples were pre-warmed for 30 s at 37 °C, and 2 mM $CaCl_2$ plus 20 μM AA was 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 RP-HPLC as described [30]. 5-LO products include the all-trans isomers of LTB_4 and 5(S)-hydro(pero)xy-

6-trans-8,11,14-cis-eicosatetraenoic acid.

4.2.5. Determination of lipoxygenase products in intact cells

For determination of LO products in intact cells, PMNL (5×10^6) were resuspended in 1 ml PGC buffer, preincubated for 15 min at 37 °C with test compounds or vehicle (0.1% DMSO), and incubated for 10 min at 37 °C with the indicated stimuli. Ca^{2+} ionophore A23187 (2.5 μM) was added with or without 20 μM AA. 10 min later the reaction was stopped on ice by addition of 1 ml of methanol. 30 μl 1 N HCL and 500 μl PBS, and 200 ng prostaglandin (PG)B_1 were added and the samples were subjected to solid phase extraction on C18-columns (100 mg, UCT, Bristol, PA, USA). 5-LO products (LTB_4, trans-isomers, 5-H(P)ETE), and the 12- and 15-LO products 12-HETE and 15-HETE, respectively, were analyzed by RP-HPLC and quantities calculated on the basis of the internal standard PGB_1. Cys-$LTsC_4$, D_4 and E_4 were not detected (amounts were below detection limit), and oxidation products of LTB_4 were not determined.

4.2.6. Preparation of crude mPGES-1 in microsomes of A549 cells and determination of PGE_2 synthase activity

Preparations of A549 cells and determination of mPGES-1 activity was performed as described previously [28]. In brief, A549 cells were treated with 1 ng/ml Il-1β for 48 h at 37 °C and 5% CO_2. Cells were harvested, sonicated and the homogenate was subjected to differential centrifugation at 10,000× g for 10 min and 174,000× g for 1 h at 4 °C. The pellet (microsomal fraction) was resuspended in 1 ml homogenization buffer (0.1 M potassium phosphate buffer, pH 7.4, 1 mM phenylmethanesulfonyl fluoride, 60 μg/ml soybean trypsin inhibitor, 1 μg/ml leupeptin, 2.5 mM glutathione, and 250 mM sucrose). Microsomal membranes were diluted in potassium phosphate buffer (0.1 M, pH 7.4) containing 2.5 mM glutathione. Test compounds or vehicle were added, and after 15 min at 4 °C reaction was initiated by addition of PGH_2 at the indicated concentration. After 1 min at 4 °C, the reaction was terminated using stop solution (40 mM $FeCl_2$, 80 mM citric acid, and 10 μM 11β-PGE_2 as internal standard. PGE_2 was separated by solid-phase extraction and analyzed by RP-HPLC as described, previously [28].

4.2.7. Statistics

Data obtained are expressed as mean ± S.E. of single determinations performed in three or four independent experiments at different days. IC_{50} values were graphically calculated from averaged measurements at 4-5 different concentrations of the compounds using SigmaPlot 12.0 (Systat Software Inc., San Jose, USA). Statistical evaluation of the data was performed by one-way ANOVA followed by a Bonferroni or Tukey–Kramer post-hoc test for multiple comparisons respectively. A p value <0.05 (*) was considered significant.

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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.2015.02.042.

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