Inhibition of Human 5-Lipoxygenase and Anti-Neoplastic Effects by 2-Amino-1,4-Benzoquinones

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Abstract: We have recently presented the synthesis of 2-amino-1,4-benzoquinones by nuclear amination of p-hydroquinones with primary aromatic amines using fungal laccases as catalysts. In the present report, a series of selected 2-amino-1,4-benzoquinones was tested for biological activities, such as inhibition of human 5-lipoxygenase and anti-proliferative/anti-neoplastic effects. Compound **9** (2-[4'-(iso-propylphenyl)-amino]-5,6-dimethyl-1,4-benzoquinone) was identified as the most potent aminoquinone derivative, suppressing 5-lipoxygenase in intact human polymorphonuclear leukocytes as well as in crude enzyme preparations in the low micromolar range (IC_{50} = 6 μM). Structure-activity relationships are discussed. Of interest, the 5-lipoxygenase inhibitory properties of 2-amino-1,4-benzoquinones in intact cells correlated to the anti-neoplastic activities of the compounds in breast and urinary bladder cancer cell lines. Based on these features, bioactive 2-amino-1,4-benzoquinones may possess potential for the pharmacological treatment of diseases associated with elevated 5-lipoxygenase activity, in particular certain types of cancer.

Key Words: Aminoquinones, antineoplastic activity, lipoxygenase, cancer.

INTRODUCTION

Aminoquinones and aminonaphtoquinones are well-known chemical compounds (see [1] and references cited therein) exerting a number of pharmacological effects including radiosensitizing [2], anti-neoplastic [3-5] and anti-microbial [6] activities, as well as anti-allergic and anti-inflammatory actions [7]. The antibacterial and anti-tumor activities of many quinone derivatives have been mainly attributed to protein binding [8] and to mechanisms involving detrimental protein modifications by free radicals and reactive oxygen species [9, 10]. A number of anti-neoblastic drugs in use or under development like mitomycin, nakijiquinone-derivatives or herbimycin-A derivatives [11, 12], contain an aminoquinone moiety, which has been suggested to be responsible for anti-cancer activity [13].

5-Lipoxygenase (5-LO) initiates the synthesis of the bioactive leukotrienes (LTs) from arachidonic acid (AA) (for review, see [14]). LTs have long been recognized as powerful mediators in allergic and inflammatory reactions [15], but have recently been implicated also in cardiovascular diseases [16], osteoporosis [17] and cancer [18]. Accordingly, an anti-LT therapy proposes benefit for the therapy of LT-related diseases, and a huge number of different types of compounds that potently suppress LT synthesis in various *in vitro* test systems (i.e. redox-, iron ligand-, and nonredox-type 5-LO inhibitors) have been produced and identified.

Several studies addressed the relation between the 5-LO pathway and cancer. Thus, various cancer cell lines as well as diseased tissues from patients over-express a functional 5-LO pathway as compared to their untransformed counterparts, and pharmacological or genetic inhibition of the 5-LO pathway inhibits cell growth and induces apoptosis, whereas supplementation of 5-LO products stimulates cell proliferation and survival [18]. Of interest, the LTB_4 antagonist LY293111 is currently undergoing phase II clinical evaluation of non-small cell lung cancer (NSCLC) and pancreatic cancer [19]. Hence, anti-LT therapy may possess potential for the treatment of cancer.

Recently, we described a synthetic route leading to aminoquinones using fungal laccases as catalysts [20]. Thus, various aniline-substituted 1,4-benzoquinones were synthesised starting from *p*-dihydroxylated benzoic acid derivatives or alkylated hydroquinones and primary aromatic amines. On one hand, aminoquinones are known to evoke anti-neoplastic effects [3-5], while on the other hand the 1,4-benzoquinone structure possesses LO inhibitory action [14, 21]. In this report, selected 2-amino-1,4-benzoquinones were evaluated for inhibition of 5-LO as well as for anti-proliferative/anti-neoplastic effects against fibroblast-like (FL) cells, an urinary bladder, and a breast cancer cell line. We found that several of the compounds tested suppressed 5-LO product formation in intact human polymorphonuclear leukocytes (PMNL) as well as in crude enzyme preparations in the low micromolar range. Intriguingly, 5-LO inhibition correlated to the anti-neoplastic activities of the compounds. Structure-activity relationships are discussed.

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RESULTS

Anti-Neoplastic Activity of Aminoquinones

The compounds shown in Table **1** were analyzed for anti-neoplastic activities in the cancer cell lines 5637 (urinary bladder carcinoma) and MCF-7 (breast cancer) as well as in non-transformed FL cells. Among these compounds, **5**, **6**, **7**, **8**, **9**, and **10** showed anti-neoplastic activity in MCF-7 and 5637 cells (Table **2**), whereas the efficacy was much reduced for compounds **1**, **2**, **3**, **4**, **11**, **12**, and **13** (IC_{50} values > 500 μM, not shown). The cytotoxic activities of the compounds do not seem to be specific for transformed cell lines, since also the non-transformed FL cell line was sensitive against the compounds, although in some cases (**5**, **7**, **8**) at significant higher concentrations (Table **2**).

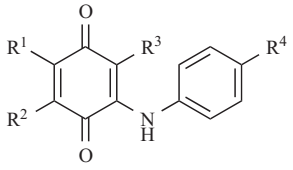
Inhibition of 5-LO Activity by Aminoquinones

First, all compounds (10 μM, each) were added to PMNL, and cells were stimulated with 2.5 μM ionophore plus 20 μM AA to stimulate 5-LO product synthesis. As shown in Fig. (**1A**), compound **9** potently blocked cellular 5-LO product formation (residual activity at 10 μM = 11 +/- 3 %), followed by **6** and **10**. Slight but significant ($p < .05$) inhibition was observed for **7**. In contrast, none of the other compounds suppressed cellular 5-LO activity. More detailed concentration-response studies with compound **9** revealed an IC_{50} of 6 μM (not shown).

Next, 5-LO inhibition was investigated in a cell-free assay using homogenates of PMNL (Fig. (**1B**)), in order to determine whether the test compounds may directly interfere

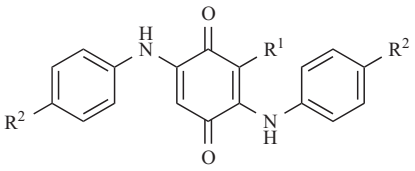
Table 1. Chemical Structures of the Aminoquinones Investigated

(A) monoaminated quinones



No	R^1	R^2	R^3	R^4
1	H	H	$CONHCH_2CH_2OH$	COOH
2	H	H	$COOCH_2CH_3$	$CONHCH_2COOH$
3	H	H	$CONHCH_2CH_2OH$	CH_2CH_2OH
4	H	H	$COOCH_3$	CH_2COOH
5	CH_3	CH_3	H	COOH
6	H	$C(CH_3)_3$	H	$COOCH_3$
7	CH_3	CH_3	H	CH_2CH_2OH
8	CH_3	CH_3	H	CH_2COOH
9	CH_3	CH_3	H	$CH(CH_3)_2$
10	H	$C(CH_3)_3$	H	$N(CH_2CH_2)_2O$

(B) diaminated quinines



No	R^1	R^2
11	$CONHCH_2CH_2OH$	COOH
12	$COOCH_2CH_3$	$CONHCH_2COOH$
13	$COOCH_3$	CH_2COOH

Table 2. Cytotoxic Activities of Aminoquinones in MCF7, 5637 and FL Cells. The Compounds Were Tested at Increasing Concentrations for Anti-Neoplastic Activity and the IC_{50} Values (μM) Were Determined. Values are Given as Mean ± S.D.

No	MCF7	5637	FL
5	31.2 ± 8.0	57.7 ± 6.5	98.1 ± 7.8
6	49.1 ± 5.1	52.2 ± 5.6	44.6 ± 5.8
7	57.4 ± 0.7	61.5 ± 9.7	115.2 ± 5.5
8	52.6 ± 4.6	67.8 ± 10.9	98.5 ± 6.5
9	32.6 ± 2.7	49.7 ± 2.0	47.9 ± 14.2
10	30.3 ± 5.6	38.0 ± 0.8	27.7 ± 6.4

with 5-LO enzyme activity. Selected compounds (that reduced 5-LO activity in homogenates more than 30 %) were also tested in the corresponding 100,000×g supernatants (Fig. (**1C**)). Again, **9** was most efficient and reduced 5-LO activity in homogenates at 10 μM by 61 +/- 7 % and in S100 by 77 +/- 6 %. Also **1**, **2**, **3**, **4**, **6**, **7**, **8** and **10**, (10 μM each) significantly inhibited 5-LO in homogenates (Fig. (**1B**)). Along these lines, selected compounds, active (>30 % inhibition) in homogenates (**3**, **4**, **6**, **7**, **8**, **10**), reduced 5-LO activity in 100,000×g supernatants at 10 μM (Fig. (**1C**)), which was even more pronounced as compared to homogenates, possibly related to depletion of membrane components (i.e. phospholipids) which may hamper the availability or the action of the compounds.

DISCUSSION

Here we present that selected representatives of the aniline-substituted 1,4-benzoquinone series, but not from the aminobenzoate-substituted 1,4-benzoquinones, act as direct 5-LO inhibitors that suppress the activity of the enzyme in cell free assays as well as in intact cells. Moreover, we present that aniline-substituted 1,4-benzoquinones possess anti-neoplastic activities, which in part correlated to the 5-LO inhibitory efficacy.

Compounds containing the 1,4-benzoquinone moiety have been reported as potent 5-LO inhibitors before [21-23]. In the cell, the quinone moiety is reduced to a hydroquinone which then reduces the active site iron in 5-LO, keeping it in the inactive ferrous form, suggesting that the reducing character confers 5-LO enzyme inhibition. However, the potency of redox-type 5-LO inhibitors not only depends on the reducing properties but also parallels their lipophilicity [24]. This is in line with our finding that **9**, a rather lipophilic structure, is most potent, whereas introduction of hydrophilic residues (R^4) such as carboxy, carboxymethylene-aminocarbonyl, hydroxyethyl or carboxymethyl groups, present in **1 - 4**, **5**, **7**, and **8**, have clearly detrimental effects. In particular compound **5**, differing from **9** solely in the R^4 substituent (carboxy group in **5**, isopropyl residue in **9**), is not active at all, supporting the hypothesis that a lipophilic (uncharged isopropyl) R^4 residue governs 5-LO inhibition. Diaminated quinones (**11**, **12**, **13**) were ineffective in the 5-LO activity assays as well as in the cytotoxicity assay.

The substitution of the aniline strongly determines the potency for inhibition of 5-LO in intact cells. Among all compounds, only **6**, **9**, and **10** possessing rather lipophilic methoxycarbonyl, isopropyl or uncharged morpholino moieties in para-position of the aniline, respectively, substantially blocked cellular 5-LO activity. In contrast, the more hydrophilic derivatives **1**, **2**, **3**, **4**, **7**, and **8** inhibit 5-LO in cell-free assays, but not (**1**, **2**, **3**, **4**, **8**) or only modest (**7**) in intact cells, presumably related to poor penetration through the plasma membrane.

Interestingly, from our data, a correlation between cytotoxicity and 5-LO inhibition is evident. Anti-tumor effects of 2,3,5-trimethyl-6-(3-pyridylmethyl)-1,4-benzoquinone (CV-6504) have been related to inhibition of the 5-LO pathway also by others [25]. In fact, 5-LO is expressed in urinary bladder carcinoma [26] as well as in breast cancer cells [27], and inhibitors of 5-LO induced apoptosis in both cell types. For the compounds presented here, **6**, **7**, **9**, and **10** were efficient in both cellular test systems. However, there is no strict correlation between 5-LO inhibition and anti-neoplastic activity, since all monoaminated quinone lacking an R^3 substituent (compound **5** - **10**) caused cytotoxicity, whereas **5** and **8** lack 5-LO inhibiting properties in intact cells. It is interesting that **5** and **9** are almost equally potent in the cytotoxicity assay, but only **9** inhibited 5-LO. Hence, while the lipophilicity of the compounds may apply for the effectiveness in suppression of 5-LO activity it seems to be dispensable for the cytotoxic efficacy.

Taken together, the bioactive 2-amino-1,4-benzoquinones presented in this study may possess potential as leads for the pharmacological treatment of diseases associated with elevated 5-lipoxygenase activity, in particular certain types of cancer.

MATERIALS AND METHODS

Materials

All aminoquinones were synthesized as described before [20]. Materials and sources: Isocove's Modified Dulbecco's Medium IMDM, Invitrogen (Karlsruhe, Germany); fetal bovine serum (FBS), Biochrome AG (Berlin, Germany); AA and ionophore A23187, Sigma (Deisenhofen, Germany); HPLC solvents, Merck (Darmstadt, Germany).

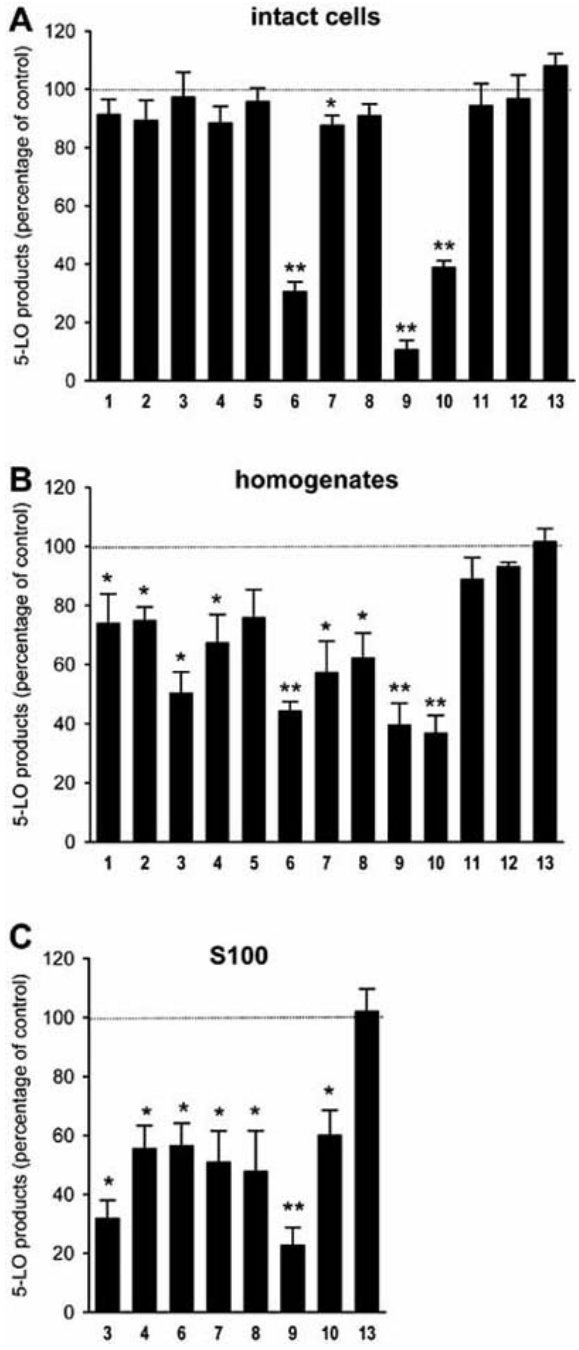


Fig. (1). Effects of 1,4-benzoquinones on 5-LO activity in intact cells and cell-free systems.

(**A**) Intact PMNL were preincubated with 10 µM of the compounds, cells were activated by 2.5 µM ionophore plus 20 µM AA and 5-LO activity was determined. (**B**) Homogenates or (**C**) 100,000g supernatants (S100) were preincubated with 10 µM of the compounds at 4 °C, prewarmed at 37 °C for 30 sec, 2 mM $CaCl_2$ plus 20 µM AA were added and 5-LO activity was determined. Values are expressed as mean + S.E., n = 3-4.

Cells

Human PMNL were freshly isolated from leukocyte concentrates obtained at St Markus Hospital (Frankfurt, Germany). In brief, venous blood was taken from healthy adult donors and subjected to centrifugation at 4,000 × g for 20 min at 20 °C for preparation of leukocyte concentrates. PMNL were promptly isolated by dextran sedimentation, centrifugation on Nycoprep cushions (PAA Laboratories, Linz, Austria), and hypotonic lysis of erythrocytes as described previously [28]. PMNL (7.5×10^6 cells/ml; purity > 96-97%) were finally resuspended in phosphate-buffered saline pH 7.4 (PBS) plus 1 mg/ml glucose (PG buffer).

MCF-7 (breast adenocarcinoma) and 5637 cells (urinary bladder carcinoma) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ Braunschweig, Germany). Cells were maintained in IMDM with 10 % FBS. FL-cells, a human amniotic epithelial cell line, were received from the American Type Culture Collection (ATCC, CCL 62, RIE 81, USA) and evaluated by the ZBV (Riems, Germany, 2005). Cells were cultivated in Eagle MEM (Sigma, St. Louis, USA), supplemented with L-glutamine (0.10 g/l), HEPES (2.38 g/l) and 8% FBS (Biochrome AG). Cells were incubated at 37°C in a 5 % CO_2 atmosphere and splitted weekly.

Determination of 5-LO Product Formation in Intact Cells

For assays of intact cells, 10^7 freshly isolated PMNL were finally resuspended in 1 ml PGC buffer. After preincubation with the indicated compounds at 37 °C, 5-LO product formation was started by addition of 2.5 µM ionophore A23187 plus 20 µM AA. After 10 min at 37 °C, the reaction was stopped with 1 ml of methanol and 30 µl of 1 N HCl, 200 ng prostaglandin B_1 and 500 µl of PBS were added. Formed 5-LO metabolites were extracted and analyzed by HPLC as described [29]. 5-LO product formation is expressed as ng of 5-LO products per 10^6 cells which includes LTB_4 and its all-trans isomers, 5(S),12(S)-di-hydroxy-6,10-trans-8,14-cis-eicosatetraenoic acid (5(S),12(S)-DiHETE), and 5(S)-hydro(pero)xy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-H(p)ETE). Cysteinyl LTs (LTC_4, D_4 and E_4) were not detected and oxidation products of LTB_4 were not determined.

Determination of 5-LO Product Formation in Cell-Free Systems

For determination of 5-LO activity in cell homogenates or 100,000g supernatants, 10^7 freshly isolated PMNL were resuspended in PG buffer containing 1 mM EDTA and a protease inhibitor cocktail, sonicated (3 × 10 s) at 4 °C, and 1 mM ATP was added. For preparation of 100,000g supernatants, homogenates were centrifuged at 100,000g for 70 min at 4 °C. Samples of either homogenates or 100,000g supernatants were supplemented with the test compounds and after 5 min at 4 °C, pre-warmed for 30 s at 37 °C and 2 mM $CaCl_2$ and 20 µM AA were added to start 5-LO product formation. The reaction was stopped after 10 min at 37 °C by addition of 1 ml ice-cold methanol and the formed metabolites were analyzed by HPLC as described for intact cells.

Assay for Antineoplastic Activity

MCF-7 (1.6×10^4/well), 5637 (0.8×10^4/well), and FL cells (10^4 /well) were seeded in 96-well plates and grown for

48 hours at 37 °C in a 5 % CO_2 atmosphere. The test compounds were added at increasing concentrations and after 48 hours incubation, cells were stained with 0.02 % crystal violet as described [30]. The viable cells were determined *via* OD measurements at 550 nm.

Statistics

All values are given as mean +/- S.E.. Statistical comparisons were determined using the Student's *t*-test for correlated samples (inhibitor versus control), and significance was accepted at $p<0.05$ (*) or $p<0.01$ (**).

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ABBREVIATIONS

AA	=	Arachidonic acid
FL	=	Fibroblast-like
5-LO	=	5-lipoxygenase
LT	=	Leukotriene
PMNL	=	Polymorphonuclear leukocytes
PBS	=	Phosphate-buffered saline
PG buffer	=	PBS plus 1 g/l glucose
PGC buffer	=	PBS plus 1 g/ml glucose and 1 mM $CaCl_2$.

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