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Characterization of scavengers of γ -ketoaldehydes that do not inhibit prostaglandin biosynthesis

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Abstract

Expression of cyclooxygenase-2 (COX-2) is associated with the development of many pathologic conditions. The product of COX-2, prostaglandin H₂ (PGH₂) can spontaneously rearrange to form reactive γ -ketoaldehydes called levuglandins (LGs). This γ -ketoaldehyde structure confers a high degree of reactivity on the LGs, which rapidly form covalent adducts with primary amines of protein residues. Formation of LG adducts of proteins has been demonstrated in pathologic conditions (e.g. increased levels in the hippocampus in Alzheimer's disease) and during physiologic function (platelet activation). Based on knowledge that lipid modification of proteins is known to cause their translocation and to alter their function, we hypothesize that modification of proteins by LG could have functional consequences. Testing this hypothesis requires an experimental approach that discriminates between the effects of protein modification by LG and the effects of cyclooxygenase-derived prostanoids acting through their G-protein coupled receptors. To achieve this goal, we have synthesized and evaluated a series of scavengers that react with LG with a potency more than two orders of magnitude greater than with the ϵ -amine of lysine. A subset of these scavengers are shown to block formation of LG adducts of proteins in cells without inhibiting the catalytic activity of the cyclooxygenases. Ten of these selective scavengers did not produce cytotoxicity. These results demonstrate that small molecules can scavenge LGs in cells without interfering with the formation of prostaglandins. They also provide a working hypothesis for the development of pharmacologic agents that could be used in experimental animals *in vivo* to assess the pathophysiological contribution of levuglandins in diseases associated with cyclooxygenase up-regulation.

Introduction

Chronic over-expression of cyclooxygenases (COX) is associated with the development of a number of diseases, including cancer and Alzheimer's disease (for review see (1–4)). Chronic inflammation in the gastrointestinal tract precedes carcinogenesis. Consistent evidence indicates that the inducible COX isoform (COX-2) can promote the multi-step sequence of events that lead to colon cancer, and it has been demonstrated that COX-2 is also over-expressed in gastric cancer as well as in esophageal carcinoma, pancreatic carcinoma, prostate carcinoma, lung cancer, and mammary cancer (5–10). In addition, substantial epidemiologic evidence suggests that long-term use of non-steroid anti-inflammatory drugs (NSAID) prevents the development of Alzheimer's disease (11,12).

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Both COX isoforms catalyze the oxygenation of arachidonic acid to the endoperoxide prostaglandin H₂ (PGH₂). PGH₂ is further metabolized to the prostanoids PGD₂, PGE₂, PGF_{2α}, thromboxane A₂, and prostacyclin by specific enzymes. In addition, prostaglandin H₂ can rapidly undergo non-enzymatic rearrangement to PGE₂ and PGD₂, and for 20% to the γ-ketoaldehydes levuglandin (LG) E₂ and D₂ (13).

Levuglandins are highly reactive molecules. They react with free amines to form covalent adducts on lysine residues in proteins (14–17) and their reactivity with proteins is at least one order of magnitude greater than 4-hydroxynonenal (15). Free levuglandins have eluded detection in biological fluid due to their high reactivity with amine groups, including those on proteins. Several isomers of levuglandins are also formed by non-enzymatic free radical-mediated peroxidation of lipids (also known as isolevuglandins or isoketals) and these γ-ketoaldehydes share the same properties as levuglandins (18). Using liquid chromatography-tandem mass spectrometry, we have characterized the LG adducts of proteins that are formed by the reaction of lysine with LGE₂ or PGH₂ (15,16), and knowledge of their structures has provided a method to detect the adducts in protein digests. We have shown that the LG-lysine adduct is formed in activated cells (19) and in brain of transgenic mice (20) in a cyclooxygenase-dependent manner. We have found that the average amount of LG-lysine lactam in the hippocampus of patients with Alzheimer's disease is 12-fold higher than in age-matched control brains; the levels of LG-lysine lactam demonstrate a significant positive relationship with Braak stages and CERAD neuritic plaque scores (21). We showed that formation of LG adducts on amyloid β (Aβ) accelerates its aggregation (22) into oligomers that are toxic to cortical neurons in primary culture (23). This concerted evidence suggests that LGs could be involved in the development of Alzheimer's disease.

The fact that other types of lipid modification of proteins can alter their cellular location and function also suggests that formation of LG adducts of proteins could have functional consequences.

In order to determine whether formation of LG adducts can alter protein function, it would be optimal to employ an experimental intervention that will block formation of LG-protein adducts without inhibiting the biosynthesis of cyclooxygenases-derived prostanoids and thromboxane A₂ that act through G-protein coupled receptors to exert numerous pathologic and physiologic functions. Such an approach would consist in using small molecules that react with LGs much faster than lysine and would not inhibit COX activity. Pyridoxamine (PM, **13**), an inhibitor of advanced glycation end-products (AGEs), has been shown to react with LGs at a rate 2300-fold higher than with lysine (24). We have recently shown that salicylamine (SA, **1**) and the PM (**13**) analog pentyl pyridoxamine (PPM, **15**) prevent formation of LG adducts on proteins in cells without interfering with the activity of COXs (25). In this manuscript, we describe the synthesis and characterization of a series of SA (**1**) and PM (**13**) analogs with the goal of selecting compounds that have enhanced effectiveness in inhibiting formation of covalent LG adducts on proteins in cells, are not inhibitors of either COX isoform, and are not cytotoxic.

Experimental Details

Materials

Dazoxiben was a generous gift from Pfizer Ltd. (Sandwich, UK). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), lipopolysaccharide (LPS), INF-γ, IL-1β, arachidonic acid, pyridoxamine dihydrochloride (**13**), pyridoxine, sodium citrate, and citric acid were purchased from Sigma-Aldrich (St Louis, MO). [¹⁴C]-arachidonic acid (AA) was obtained from Perkin Elmer Life Sciences (Boston, MA). Sepharose 2B was purchased from Amersham Biosciences (Uppsala, Sweden). Sep-Pak tC18 cartridges were obtained from

Waters Corp. (Milford, MA). Pronase and aminopeptidase M from porcine kidney were purchased from Calbiochem (Gibbstown, NJ). Silica gel 60A thin layer chromatography plates were purchased from Whatman (Clifton, NJ). Cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2) were a generous gift from Dr. Larry Marnett. COX-1 was purified from ram seminal vesicle as described (26) and wild-type murine COX-2 was expressed in SF-9 cells (Novagen, Madison WI) and purified as described (27). A549 (human alveolar basal epithelial) cells were a generous gift from Dr. Robert Coffey (Vanderbilt University). HCA-7 (human colonic adenocarcinoma), HepG2 (human hepatocellular carcinoma), and RAW 264.7 (murine leukemia virus-induced monocyte/macrophage) cells were obtained from American Type Tissue Collection (Rockville, MD). Cellular ATP content was measured using Cytolux L001-1001 assay kits with modifications (EG&G Wallac, Finland).

Characterization of structures

The identity of each compound was confirmed by mass spectrometry (MS) and nuclear magnetic resonance (NMR).

For mass spectrometry, the ThermoFinnigan (San Jose, CA) TSQ Quantum triple quadrupole mass spectrometer equipped with a standard electrospray ionization source was used. Nitrogen was used for both the sheath and auxiliary gas. The mass spectrometer was operated in the positive ion mode and the electrospray needle was maintained at 4000 V. The compounds were chromatographed on a Prodigy 5u ODS (3), 150×1.00 mm column (Phenomenex, Torrance CA) with a flow rate of 0.2 ml/min. The mobile phase consisted of solvent A (10% ACN/90 % water/0.1% acetic acid) and solvent B (ACN/0.1% acetic acid). The gradient was as follows: 0–3 min, 100% A; 3–7 min, 100 to 10% A; 7–10 min, 10% A; 10–12 min, 10 to 100% A. The ion transfer tube was operated at 35 V and 300°C. The tube lens voltage varied depending on the compound being analyzed. The ions were subjected to collision-induced dissociation at –28 eV, and daughter ions were scanned between m/z 50 and 500. Xcalibur software (version 1.3; ThermoFinnigan) was used to operate the instrument and to process the data.

Nuclear magnetic resonance spectra were acquired by a 9.4 Bruker Spectrospin magnet equipped with a Bruker AV spectrometer operating at the proton Larmor frequency of 400.13 MHz. Proton chemical shifts are reported in parts per million (ppm) on the δ scale relative to [$^2\text{H}_6$] DMSO (δ 2.5 ppm) or H_2O deuterium (δ 4.6 ppm), which also served as the ^2H lock solvent, and the coupling constants (J) are reported in Hz. ^1H spectra were acquired in 5 mm NMR tubes using a Bruker 5 mm broadband NMR probe. Typical experimental conditions included 32K data points, a 13-ppm sweep width, a recycle delay of 1.5 seconds, and 32–256 scans, depending on sample concentration.

Chemical Syntheses

Experimental details for the syntheses, analyses by ^1H NMR, and mass spectroscopic characterization of pyridoxamine (**13**), salicylamine (**1**), and their analogs are provided in Scheme 1 and 2, Fig. 1.

Synthesis of analogs of pyridoxamine (**13**)

Pyridoxine was converted to 3,4'-*O*-isopropylidenepyridoxine (**28**) and was treated with NaH in THF followed by 1-iodopentane. The pentyl derivative was hydrolyzed with formic acid to 5'-*O*-pentylpyridoxine (**29**). The side chain alcohol at the 4-position was oxidized with MnO_2 to aldehyde in chloroform. The aldehyde was converted to oxime with a slight excess of hydroxylamine hydrochloride and sodium acetate. The oxime was reduced to pentylpyridoxamine (**15**) with zinc dust in acetic acid at 10–15 °C. After filtration of zinc and removal of acetic acid, pH was raised to 8.5 with 1 M NH_4OH . The crude product was purified

by flash chromatography (10–30% methanol in ethyl acetate); m.p. 118–120 °C (23% overall yield). ^1H NMR (DMSO- d_6), δ 8.61 (brd s, 2H, NH_2), 8.21 (s, 1H, 6-H), 4.78 (s, 2H, 5- CH_2), 4.15 (s, 2H, 4- CH_2), 3.47 (t, 2H, $J = 6.60$ Hz, 5'- $\text{O}-\text{CH}_2$), 2.69 (s, 3H, 2- CH_3), 1.55 and 1.27 (dd, 6H, $\text{CH}_2\text{CH}_2\text{CH}_2$), 0.85 (t, 3H, $J = 6.76$ Hz, CH_3). MS m/z 239 ($M + 1$), 222 ($M - \text{NH}_2$), 151 ($222 - \text{C}_5\text{H}_{11}$), 136 ($151 - \text{CH}_3$).

In the second step of the sequence, instead of 1-iodopentane benzyl bromide or iodoethane was used to obtain benzylpyridoxamine (BzPM, **16**) or ethylpyridoxamine (EtPM, **14**) respectively in similar yields.

Benzylpyridoxamine (BzPM, 16)— ^1H NMR δ 8.30 (brd s, 2H, NH_2), 8.26 (s, 1H, 6-H), 7.36 and 7.31 (m, 5H, C_6H_5), 4.78 (s, 2H, 5- CH_2), 4.61 (s, 2H, 4- CH_2), 4.18 (s, 2H, $\text{C}_6\text{H}_5\text{CH}_2$), 2.63 (s, 3H, 2- CH_3). MS m/z 259 ($M + 1$), 242 ($M - \text{NH}_2$), 151 ($222 - \text{C}_5\text{H}_{11}$), 91 ($\text{C}_6\text{H}_5\text{CH}_2$).

Ethylpyridoxamine (EtPM, 14): ^1H NMR δ 8.61 (brd s, 2H, NH_2), 8.22 (s, 1H, 6-H), 4.78 (s, 2H, 5- CH_2), 4.15 (s, 2H, 4- CH_2), 3.55 (q, 2H, $J = 6.96$ Hz, CH_2CH_3), 2.69 (s, 3H, 2- CH_3), 1.16 (t, 3H, $J = 6.96$ Hz, CH_2CH_3). MS m/z 197 ($M + 1$), 180 ($M - \text{NH}_2$), 151 ($180 - \text{C}_2\text{H}_5$), 136 ($151 - \text{CH}_3$).

Synthesis of Salicylamine (1) and Its Analogs—The reduction of appropriately substituted 2-hydroxybenzaldehyde oxime to the amine, using zinc in acetic acid was the final step for preparing salicylamine (SA, **1**) and its analogs. In the case of salicylaldoxime (Acros, 2.9 g, 20 mmol), it was dissolved in acetic acid (20 mL), cooled in ice-water bath (10 °C), followed by addition of zinc dust (5 g) with stirring. The stirring was continued at 10–15 °C for 1 h and at 20 °C for 2 h. The reaction mixture was filtered and the filtrate was evaporated. The solid was crystallized from ethanol to give salicylamine acetic acid salt (SA.AcOH, **2**); 2.7 g (74%), mp 187–188 °C. It was completely converted to hydrochloride salt (SA.HCl, **1**) by refluxing in 1 M HCl for 1 h. Excess acid was removed and the residue was co-evaporated with ethanol and ether was added to get a white solid; mp 150–152 °C. 5-chlorosalicylaldehyde, 2-hydroxy-3-methoxybenzaldehyde, and 2-hydroxy-5-methoxybenzaldehyde (Alfa Aesar) were converted to the oxime with a slight excess of hydroxylamine hydrochloride and sodium acetate. Reduction of these oximes led to 5-chlorosalicylamine (CISA, **7**), 3-methoxysalicylamine (3-MoSA, **6**), and 5-methoxysalicylamine (5-MoSA, **5**), respectively. 4-Cresol, 3-ethylphenol, 4-pentylphenol, 4-hexylphenol, 4-benzylphenol, and 4-benzyloxyphenol were formylated (30) to obtain substituted salicylaldehydes that were converted to oximes and then to 5-methylsalicylamine (MeSA, **3**), 4-ethylsalicylamine (EtSA, **8**), 5-pentylsalicylamine (PSA, **9**), 5-hexylsalicylamine (HxSA, **10**), 5-benzylsalicylamine (BzSA, **11**), and 5-benzyloxysalicylamine (BoSA, **12**).

Salicylamine (2-SA, 1)— ^1H NMR δ 10.22 (s, 1H, OH), 8.28 (s, 2H, NH_2), 7.30 (dd, 1H, $J = 1.5$ Hz and 7.4 Hz, 6-H), 7.17 (dt, 1H, $J = 1.5$ and 8.0 Hz, 4-H), 6.94 (d, 1H, $J = 8.0$ Hz, 3-H), 6.79 (dt, 1H, $J = 6.80$ and 7.4 Hz, 5-H), 3.37 (s, 2H, CH_2). MS m/z 124 ($M + 1$), 107 ($124 - \text{NH}_2$), 77 (C_6H_5).

Salicylamine (SA.AcOH, 2)— ^1H NMR δ 7.04 (dt, 2H, $J = 1.44$ Hz and 5.68 Hz, 2,4-H), 6.69 (dt, 2H, $J = 1.92$ and 6.85 Hz, 4-H), 3.87 (s, 1H, CH_2), 1.62 (s, 3H, acetyl). MS of SA.AcOH (**2**) m/z 165 ($M - \text{H}_2\text{O}$), 124 ($165 - \text{acetyl}$), 107 ($124 - \text{NH}_2$), 77 (C_6H_5). SA.AcOH (**2**) has been dissolved in deuterium oxide. Hydrogen bonding between the solvent and the molecule decreases the electron density around the proton, and may undergo rapid intermediate, or slow exchange. This explains why we do not see the peaks from NH_2 and OH in the spectrum.

Salicylamine (3-SA)— ^1H NMR δ 9.63 (s, 1H, OH), 8.28 (s, 2H, NH_2), 7.18 (t, 1H, 7.8 Hz, 3-H), 6.85 (dt, 2H, $J = 8.72$ and 11.01 Hz, 2,4-H), 6.78 (dt, 1H, $J = 1.64$ Hz and 8.17 Hz, 8-H), 3.38 (s, 2H, CH_2). MS m/z 124 ($M+1$), 107 ($124-\text{NH}_2$), 77 (C_6H_5).

Salicylamine (4-SA)— ^1H NMR δ 10.22 (s, 1H, OH), 8.21 (s, 2H, NH_2), 7.28 (dd, 2H, $J = 8.53$ Hz and 14.73 Hz, 2,5-H), 6.77 (d, 2H, $J = 11.25$, 3,4-H), 3.86 (s, 2H, CH_2). MS m/z 124 ($M+1$), 107 ($124-\text{NH}_2$), 77 (C_6H_5).

5-Chlorosalicylamine (CISA, 7)— ^1H NMR δ 10.4 (s, 1H, OH), 8.10 (s, 2H, NH_2), 7.38 (d, 1H, $J = 2.6$ Hz, 6-H), 7.23 (dd, 1H, $J = 2.6$ and 8.6 Hz, 3-H), 6.92 (d, 1H, $J = 8.6$ Hz, 4-H), 3.34 (s, 2H, CH_2). MS m/z 158 ($M+1$), 141 ($158 - \text{NH}_2$), 105 ($141 - \text{Cl}$), 107 ($124 - \text{NH}_2$) and 77 (C_6H_5).

5-Methylsalicylamine (MeSA, 3)— ^1H NMR δ 9.96 (s, 1H, OH), 8.25 (s, 2H, NH_2), 7.11 (d, $J = 1.5$ Hz, 1H, 6-H), 6.97 (dd, 1H, $J = 1.5$ and 8.20 Hz, 3-H), 6.85 (d, 1H, $J = 8.2$ Hz, 4-H), 3.85 (s, 2H, CH_2), 2.17 (s, 3H, CH_3). MS m/z 138 ($M+1$), 121 ($138 - \text{NH}_2$), and the acetic acid salt gave m/z 180 ($M - \text{H}_2\text{O}$).

5-Methylsalicylamine.AcOH (MeSA.AcOH, 4)— ^1H NMR δ 6.89 (t, $J = 2.56$ Hz, 2H, CH), 6.63 (d, $J = 8.08$ Hz, 1H, CH), 3.87 (s, 2H, 1-H), 2.01 (s, 3H, 3-H), 1.67 (s, 3H, Acetyl). MS of acetic acid salt gave m/z 180 ($M - \text{H}_2\text{O}$), 138 ($M+1$), 121 ($138 - \text{NH}_2$). MeSA.AcOH (4) has been dissolved in deuterium oxide. The peak from NH_2 and OH can not be seen in the spectrum.

3-Methoxysalicylamine (3-MoSA, 6)— ^1H NMR δ 9.21 (s, 1H, OH), 8.38 (s, 2H, NH_2), 6.96 (d, 1H, $J = 7.9$ Hz, 5-H), 6.93 (s, 1H, 6-H), 6.78 (d, 1H, $J = 7.9$ Hz, 4-H), 3.79 (s, 2H, CH_2), 3.35 (s, 3H, CH_3). MS m/z 154 ($M+1$), 137 ($154 - \text{NH}_2$), 107 ($137 - \text{OCH}_3$).

5-Methoxysalicylamine (5-MoSA, 5)— ^1H NMR δ 9.68 (s, 1H, OH), 8.30 (s, 2H, NH_2), 6.9 (d, $J = 3.0$ Hz, 1H, 6-H), 6.86 (d, 1H, $J = 8.80$ Hz, 4-H), 6.76 (dd, $J = 1$ Hz, 3.0 and 8.80 Hz, 3-H), 3.88 (d, 2H, $J = 3.58$ Hz, CH_2), 3.66 (s, 3H, CH_3). MS same as 3-MOSA (6).

4-Ethylsalicylamine (EtSA, 8)— ^1H NMR δ 10.00 (s, 1H, OH), 8.11 (s, 2H, NH_2), 7.12 (d, 1H, $J = 7.6$ Hz, 6-H), 6.72 (s, 1H, 3-H), 6.58 (d, 1H, $J = 7.6$ Hz, 5-H), 3.78 (d, 2H, $J = 4.88$ Hz, CH_2N), 2.42 (q, 2H, $J = 7.65$ Hz, CH_2), 1.04 (t, $J = 7.65$ Hz, 3H, CH_3). MS m/z 152 ($M+1$) 135 ($M - \text{NH}_2$).

5-Pentylsalicylamine (PSA, 9)— ^1H NMR δ 9.23 (brd s, 2H, NH_2), 8.11 (s, 1H, 6-H), 7.9 (d, 1H, $J = 8.16$ Hz, 3-H), 7.84 (d, 1H, $J = 8.16$ Hz, 4-H), 4.85 (s, 2H, CH_2N), 3.43 and 2.28 (m, 8H, $(\text{CH}_2)_4$), 1.82 (t, 3H, $J = 6.68$ Hz, CH_3). MS m/z 194 ($M+1$), 177 ($M - \text{NH}_2$), 107.

5-Hexylsalicylamine (HxSA, 10)— ^1H NMR δ 10.94 (s, 1H, OH), 9.19 (brd s, 2H, NH_2), 8.11 (d, 1H, $J = 1.64$ Hz, 6-H), 7.97 (dd, 1H, $J = 1.64$ and 8.15 Hz, 3-H), 7.83 (d, 1H, $J = 8.15$ Hz, 4-H), 4.85 (s, 2H, CH_2N), 4.33 (s, 2H, 5- CH_2), 3.3 and 2.23 (m, 8H $(\text{CH}_2)_4$), 1.82 (t, 3H, $J = 6.3$ Hz, CH_3). MS m/z 208 ($M+1$), 191 ($M - \text{NH}_2$).

5-Benzylsalicylamine (BzSA, 11)— ^1H NMR δ 10.05 (s, 1H, OH), 8.17 (s, 2H, NH_2), 7.25 and 7.18 (5H, C_6H_5), 7.04 (dd, $J = 1$ Hz, 1.7 Hz, 8.20 Hz, 6-H), 6.86 (d, 2H, $J = 8.20$ Hz, 3-H and 4-H), 3.34 (s, 2H, CH_2N), 3.80 (m, 2H, CH_2O). MS m/z 214 ($M+1$), 197 ($M - \text{NH}_2$).

5-Benzoyloxysalicylamine (BoSA, 12)— ^1H NMR δ 9.73 (s, 1H, OH), 8.29 (s, 2H, NH_2), 7.40 and 7.34 (m, 5H, C_6H_5), 7.04 (s, 1H, 6-H), 6.85 (s, 2H, 3-H and 4-H), 4.99 (s, 2H, CH_2), 3.36 (s, 2H, CH_2N). MS m/z 230 ($M + 1$), 213 ($M - \text{NH}_2$).

Reaction rate of compounds *in vitro*

Compounds (1 mM) were reacted with 4-oxopentanal (OPA, 1 mM) in 1:1 acetonitrile-100 mM phosphate buffer, pH 7.4, at 25 °C. Aliquots were taken to measure the concentration of pyrrole, and second-order rate constants were calculated as previously described (24).

Analysis of COX activity using [^{14}C]-arachidonic Acid

Because the analogs may inhibit the COXs, they were incubated with pure COX-1 and -2 enzymes. Ovine COX-1 (3.3 nM final concentration; specific activity: oxygenation of 377 nmol of AA/min/mg enzyme) or wild-type murine COX-2 (7.7 nM final concentration; specific activity: oxygenation of 154 nmol of AA/min/mg enzyme) was preincubated on ice for 30 min with 2 molar equivalents of hematin in Tris-HCl buffer, pH 8.0, with 500 μM phenol. The enzyme solution was incubated in the absence (control) or presence of compounds (final concentrations, 0.1 or 1 mM) for 30 min at 37°C. Then, 20 μl [^{14}C]-AA (4.8 nCi, 0.5 μM final concentration) in Tris-HCl buffer, pH 8.0, was added and the activity of COXs was assayed as previously described (31). Activity of COXs is expressed as percent of control, in which no compound was added.

Preparation of washed Platelets

Human blood was obtained following a protocol approved by the Institutional Review Board of Vanderbilt University. Washed human platelets were isolated following a previously described protocol (31). Briefly, 45 ml blood was drawn into a syringe containing 5 ml of 3.8% sodium citrate and centrifuged in plastic tubes at 300 g for 10 min at room temperature. The supernatant (platelet-rich plasma) was acidified to pH 6.4 with 0.15 M citric acid and then centrifuged at 1,000 g for 10 min at room temperature. The pellet was resuspended in wash buffer (24.4 mM sodium phosphate, pH 6.5, 0.113 M NaCl, 5.5 mM glucose). After 15 min at room temperature, the platelets were purified on a Sepharose 2B column equilibrated with wash buffer. The eluted platelets were counted with a Coulter counter and diluted with resuspension buffer (8.3 mM sodium phosphate, pH 7.5, 0.109 M NaCl, 5.5 mM glucose) to a final concentration of 600,000 platelets/ μl .

Cell Culture

A549, HCA-7, and RAW 264.7 cells were cultured in 6-well plates at 37°C in a humidified atmosphere containing 5% CO_2 . HepG2 cells were cultured in T75 Falcon flasks. Cells were grown in DMEM supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 2.5 $\mu\text{g}/\text{ml}$ amphotericin B.

Cytotoxicity of compounds in HepG2 Cells

Toxicity of the compounds on HepG2 cells was assessed by measuring total cellular levels of ATP according to the previously described protocol (25). To summarize, confluent HepG2 cells were seeded in multiple CulturPlate TC-96 plates (Perkin-Elmer, Boston, MA) at 10,000 cells per well, and the cells were allowed to adhere for 4 h. The experimental compounds were dissolved in water to create 70 mM stock solutions. The compounds were serially diluted in growth medium and were added to the wells in triplicate. The cells were then incubated under normal growth conditions for 24 h. Six wells were left untreated as controls. After incubation, 100 μl of culture medium was removed and assayed according to the manufacturer's instructions. The viability of cells was quantified by measuring ATP levels with the ATPlite

luminescence ATP detection assay system (Perkin-Elmer) using a Packard Lumicount luminescent microplate reader (Global Medical Instrumentation, Ramsey, MN). Cytotoxicity was quantified based on the level of ATP in compound-treated wells compared to controls (defined as 100% viability).

Effect of the compounds on the level of LG-lysine adducts in human platelets

Washed platelets were preincubated with the thromboxane synthase inhibitor dazoxiben (10 μ M, final concentration), and then with either vehicle or varying concentrations of the different compounds (0.1 or 1 mM, final concentration) for 30 min at room temperature. Next, arachidonic acid (20 μ M, final concentration) was added and the platelets were incubated at room temperature for 2 h. After incubation, the platelets were pelleted at 2,000 g for 10 min at room temperature. The medium was removed for analysis of prostaglandins by mass spectrometry. The pelleted cells were collected and the LG-lysine lactam adduct was isolated from proteins by proteolytic digest and was analyzed by LC/MS/MS as previously described (15).

Quantification of LG-Lysine Adducts in A-549, HCA-7 and RAW 267.7 Cells Line

To assess the efficiency of compounds to scavenge LGs in different cell lines expressing COX-2, we selected four compounds that greatly inhibited the formation of adducts without inhibiting COX activity. At 90% confluence, A549 cells were activated with 1 ng/ml IL-1 β and RAW 264.7 cells were activated with 10 μ g/ml LPS and 10 units/ml INF- γ overnight, in serum-free DMEM containing penicillin and streptomycin. Overconfluent HCA-7 cells in serum-free DMEM were used without need for activation. After the addition of fresh medium to the cells, compounds were added in desired concentrations (0.1 or 1 mM, final concentration) and incubated at 37°C for 30 min. Then, arachidonic acid (20 μ M, final concentration) was added and the cells were incubated at 37°C for 1 hour. The medium was removed for analysis of prostaglandins by mass spectrometry. Adherent cells were scraped; protein concentration was estimated and was used to quantify the LG-lysine lactam adduct. Experiments were repeated twice in triplicate.

Quantification of prostaglandins by GC/MS

The effect of compounds on COX activity was determined by measuring PGE₂ and PGD₂ production. [²H₄]-PGE₂ (2 ng) was added as an internal standard to 30 μ l of the platelet supernatant or 200 μ l of conditioned cell culture medium. Prostaglandins were isolated and derivatized for GC/NICI/MS analysis, monitoring selected ions as described previously (16). The signals for the different molecules are *m/z* 524 for PGD₂/E₂, and *m/z* 528 for the [²H₄]-PGD₂/E₂ internal standard.

Statistical Methods

Statistical analysis was performed using GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA). The significance of difference between control and compound-treated experiments, in different cells lines, using COX-1/2 enzymes or for different isomers of salicylamine (**1**) was analyzed by one-way analysis of variance (Tukey's Multiple Comparison Test).

Results

Reaction of the compounds with 4-oxopentanal

SA (**1**) and PM (**13**) react extremely fast with LG when compared with N-acetyl lysine (~1000 and ~2300 times greater, respectively) (24). We synthesized a series of analogues containing the salicylamine structure and investigated their reactivity with a γ -ketoaldehyde. The

scavenging ability of the different analogs was tested using 4-oxopentanal (OPA) because the reaction forms a pyrrole, which is stable to oxidation, unlike the reaction with LGs. The absorption spectra of the reaction between the 2-methylpyrroles and 4-dimethylaminobenzaldehyde (Ehrlich's reagent) exhibited two maxima at 528 and 568 nm, and the sum of the absorbance at the two wavelengths was used as a measure of the pyrrole concentration (24). The second-order rate constants for amines are compared in Table 1. Our results show that the addition of an aliphatic substituent increases the reactivity of salicylamine by up to 1.5 fold. Increasing hydrophobicity with methoxy and benzyloxy groups increases reactivity of pyridoxamine.

Effect of the hydroxyl position on the inhibition of LG lactam adduct formation

We have previously published data suggesting that the relative position of the aminomethyl group adjacent to the hydroxyl group on the aromatic ring was critical for the increased reactivity of the scavengers for LG (24,25). We further investigated the effects of the different positions of the hydroxyl group relative to the aminomethyl group using human platelets. We found that 1 mM (final concentration) 2-HoBA (SA, **1**), 3-HoBA, and 4-HoBA did not inhibit COX-1 activity, but the amount of LG-lysine lactam measured by LC/MS varied with the position of the hydroxyl. As shown in Figure 2, formation of the LG-lysine lactam adduct was reduced by 85% and 75% respectively for 2-HoBA (**1**) and 3-HoBA. However, 4-HoBA reduced the level of LG adduct by only 30%, confirming the importance of the position of the hydroxyl group for the reactivity of the scavengers.

Cytotoxicity of compounds in hepatoma cell line HepG2

The possible toxicity of the analogs was tested in a cellular system using the hepatocarcinoma cell line HepG2. Cytotoxicity was assessed by measuring intracellular ATP levels using the luciferin-luciferase reaction (32). This assay is based on the knowledge that reduction in cellular ATP is indicative of a cytotoxic effect. Pilot experiments demonstrated that none of the tested compounds interferes with the enzymatic reaction of the assay (data not shown). The compounds' effects after 24 h of exposure were determined. Seven analogs of the salicylamine series and three analogs of the pyridoxamine series presented no cytotoxicity up to a concentration of 1 mM (Table 2). Cytotoxic effects were observed for several compounds containing hydrophobic substitutions or chloride (Table 3). Although 5-benzylsalicylamine (BzSA, **11**) and benzylpyridoxamine (BzPM, **16**), which contain 6-membered rings, caused some cytotoxicity at higher concentrations ($\geq 500 \mu\text{M}$), the strongest effects were seen with 5-benzyloxysalicylamine (BoSA, **12**), 5-hexylsalicylamine (HxSA, **10**), and 5-pentylsalicylamine (PSA, **9**) at lower concentration ($100 \mu\text{M}$).

Effect of the compounds on activity of the two isoforms of COX

To assess whether the compounds directly inhibit cyclooxygenase activity, they were incubated with purified ovine COX-1 or murine recombinant COX-2. The amounts of the two COX isoforms were selected to yield equal catalytic activity in control reactions that did not include experimental compounds. The analogs were tested at the highest concentration used for the cytotoxicity experiment (1 mM final concentration). The non-toxic compounds didn't inhibit COX-1 or COX-2 activity, suggesting that these analogs could be used to inhibit formation of levuglandin adducts in cells without blocking COX activity (Fig. 3). The most toxic two compounds inhibited both of the COX isoforms.

Effects of scavengers on LG-lysine lactam adduct formation and on COX-1 activity in platelets

We have shown previously that PM (**13**), PPM (**15**), and SA (**1**) inhibit formation of levuglandins adducts on proteins in cells (25). We used the same cellular system to assess the

effects of the ring substitutions on both the ability to inhibit the formation of LG-adducts on proteins and the catalytic activity of COX-1 in human platelets. We have previously demonstrated that inhibition of the thromboxane synthase with dazoxiben for 30 min leads to a 2.1-fold increase in the levels of LG-lysine lactam adduct (19). We used the same method to increase the sensitivity of our assay in these studies. Accordingly, we measured the levels of LG-lysine lactam as the marker of protein modification and PGE₂ as the marker of COX-1 activity. As shown in figure 4A, the formation of the LG-lysine lactam was reduced in a dose-dependent manner by all the compounds tested with maximal inhibition up to 80% by 1 mM SA (1), SA.AcOH (2), 3-MoSA (6), EtSA (8), and PPM (15); 70% by 5-MoSA (5), MeSA (3), MeSA.AcOH (4), and PM (13), and 60% by EtPM (14) compared to control. Surprisingly, the least potent compound in platelets (EtPM, 14) is one of the most reactive analogs *in vitro*, suggesting that cellular efficacy is not merely predicted by reactivity, but that other parameters are involved. It is interesting to note that the acetate salts of SA (2) and MeSA (4) (SA.AcOH and MeSA.AcOH) had activities similar to their free amines in platelets, whereas they had slightly decreased reactivity *in vitro* (table 1). The acetate forms a bond between the amine group and hydroxyl group and is easily hydrolysable in physiological or aqueous solution. Their physical properties change but their reactivity in aqueous solution is the same as the free amine. This indicates that the acetate readily dissociates in presence of cells, releasing the free compound.

In order to exclude the possibility that the analogs reduce LG adducts by inhibiting COX-1, we measured PGE₂, the major product of PGH₂ metabolism, in dazoxiben-treated platelets. None of the compounds decreased the level of PGE₂ (Fig. 4B), demonstrating that the reduced formation of protein adducts by these compounds is due to direct scavenging of levuglandins and not by inhibiting cyclooxygenase activity.

Inhibition of LG-lysine lactam adduct formation in cells that express COX-2

Based on the results with purified COXs and human platelets, we selected the two most potent analogs from each series: SA (1), 3-MoSA (6), PM (13), and PPM (15), and used them to investigate their ability to inhibit formation of LG lactam adducts in three different cell types expressing COX-2. We used the human epithelial lung carcinoma cell line A549 activated with IL-1 β and the murine monocyte-macrophage cell line RAW 264.7 activated with LPS- and INF- γ as COX-2-inducible cells, and the human colon adenocarcinoma cell line HCA-7 as a cell that expresses COX-2 constitutively. We found that SA (1), 3-MoSA (6), PM (13), and PPM (15) inhibited LG-lysine adduct formation in a dose-dependent manner (Fig 5). Treating with compounds at a concentration of 1 mM reduced the formation of the LG-lysine lactam by 75 to 85% in RAW 264.7 cells, 60 to 80% in A549 cells and 50 to 60% in HCA-7 cells, when compared to control. PGE₂ production was analyzed as a measure of COX-2 activity in all three cells lines, and no inhibition of COX-2 by the compounds was detected (data not shown).

Discussion

Evaluation of the pathophysiologic consequences of LG adducts of cellular proteins and other molecules require an experimental intervention that will block LG adduct formation without altering the formation of other cyclooxygenase products. We here investigate an approach that employs molecules that react with LGs much faster than the amino groups found on proteins, e.g. the ϵ -amine of lysine. Previously we had found that pyridoxamine (13) and its analogs, salicylamine (1) and pentyl pyridoxamine (15), react rapidly with γ -ketoaldehydes and are potent scavengers of LG *in vitro* and in cells (25). Based on the observations with these compounds, we now report on the synthesis and evaluation of a series of analogs of salicylamine and pyridoxamine. From this group, a subset was found to inhibit LG adduct

formation in cells without blocking biosynthesis of prostanoids, and to be free of toxicity to cells in culture.

The reactivity of the amino group of the scavengers is influenced by two factors: 1) the presence of the phenolic group increases the electron density in the aromatic ring. This effect maximal when the hydroxyl group is in ortho or para to the aminomethyl group. 2) The hydrogen atom of the hydroxyl group can interact with the oxygen atom of the ketone moiety in LG, holding it in position, thereby facilitating the attack of the hemiaminal amine intermediate (24). This would predict that the scavengers would become less reactive as the hydroxyl is moved farther from the aminomethyl group. We investigated the effects of the position of the phenolic hydroxyl group on the reactivity of salicylamine (**1**) by using analogs with the hydroxyl in ortho (2-HoBA, SA **1**) meta (3-HoBA) and para (4-HoBA) positions. We found that the most reactive analog is 2-HoBA (**1**) followed by 3-HoBA, and that 4-HoBA only decreased formation of LG-lysine lactam adduct by 30%. Because the effect of the hydroxyl on the reactivity of the amine should be the same for 2- (**1**) and 4-HoBA, the observed difference of reactivity suggests that the major determinant for the reactivity of the scavengers is not the electron density of the ring but the distance between the hydroxyl and the amine group that favors formation of the hydrogen bond with LG.

The acetate salts of SA (**2**) and MeSA (**4**) inhibit formation of LG-lysine adducts to an extent similar to that of their respective free amines in cellular systems whereas their reactivity *in vitro* is decreased by 24% and 22%, respectively. This observation is important with regard to cellular and *in vivo* experiments as it suggests that the water-soluble acetate salts can be used without loss of efficacy instead of the free amine compounds that require use of organic solvents.

In addition to the levuglandins, the primary amine group of these scavengers would react with other aldehydes, such as malondialdehyde (MDA), methylglyoxal (MGO), HNE, and 4-oxo-2-nonenal (ONE). MDA and MGO target amino groups, and react rather slowly with the SA group of scavengers. In these reactions, the advantage of SA (**1**) analogs seems to be their lipophilicity (33). The primary reaction of HNE and ONE is Michael addition and Cys and His residues are much more reactive than lysyl groups (33). Again, pyridoxamine (**13**) did not exhibit particularly high reactivity towards HNE (33). ONE, that is ~100 times more reactive than HNE, may react with lysyl residues in two separate pathways (34). Michael addition products of ONE are capable of condensing with amino groups to form pyrroles and ONE may react with lysyl residues in two sequential steps to form pyrrolinone. In both of these reactions it is possible to visualize SA (**1**) analogs with phenolic group at the 2-position being much more reactive than lysyl residues.

Utilization of LG scavengers to evaluate the biological consequences of LG adducts requires that the observed actions of the compounds on cells are not due to cytotoxicity. Thus, as screen to eliminate compounds with obvious cytotoxicity from further evaluation, we measured ATP depletion in HepG2 cells as a surrogate marker with which to screen for cellular toxicity (32, 35). Several analogs of SA (**1**) and of PM (**13**) were found to be cytotoxic in this assay. Among the SA (**1**) series of analogs, those with the most lipophilic substituents were toxic to these cells. Interestingly, the toxic analogs were also inhibitors of both COX isoforms *in vitro*. The pharmacological effects of the compounds that did not cause ATP depletion were then further evaluated.

The objective was to synthesize scavengers that prevent formation of LG-lysine adducts without inhibiting COX activity. Determination whether the new compounds had effects on COX-1 activity was carried out in platelets. Induction and chronic up-regulation of COX-2 is associated with inflammatory conditions and pathogenesis of many diseases. We investigated

whether the compounds could scavenge LGs produced by cells in response to COX-2 activity. Three cancer cell lines with different levels of COX-2 expression and activity were treated with the analogs, and exogenous arachidonic acid was added to achieve maximum prostanoid biosynthesis (36). Our results indicate that several analogs inhibit formation of COX-1-derived LG-lysine lactam on platelet proteins without affecting the enzyme activity. As with platelets, the same analogs inhibited formation of LG-adducts on proteins without inhibiting the release of PGE₂ in cells expressing COX-2 although with slight variations in efficacy: HCA-7 cells are less sensitive to scavengers than are A-549 or RAW264.7 cells.

Conclusion

In summary, we have identified a series of compounds that inhibit formation of LG-lysine adducts in cells without interfering with the activity of either COX isoform. These compounds do not exhibit cytotoxicity when assessed in HepG2 cells up to millimolar concentrations. Also, we provide more evidence in support of the role of the *o*-hydroxyl group of benzylamine in the mechanism responsible for the high reactivity of these molecules with γ -ketoaldehydes. These results provide the rationale and lead structures for future studies examining the biological consequence of formation of levuglandin adducts on proteins that can result from both lipid peroxidation and catalytic activity of the COXs in cells as well as *in vivo*.

Abbreviations

COX	cyclooxygenase
COX	prostaglandin H2 synthase
PG	prostaglandin
DMEM	Dulbecco's modified eagle's medium
AA	arachidonic acid
LC/ESI/MS/MS	liquid chromatography/electrospray ionization/tandem mass spectrometry
LG	levuglandin
Iso-LG	iso-levuglandins
HoBA	hydroxybenzylamine
EtPM	ethylpyridoxamine
PPM	Pentylpyridoxamine
BzPM	Benzylpyridoxamine
SA	salicylamine
CISA	5-chlorosalicylamine
MeSA	methylsalicylamine
5/3 MoSA	5/3-methoxysalicylamine
EtSA	5-ethylsalicylamine
PSA	5-pentylsalicylamine
HxSA	5-hexylsalicylamine
BzSA	5-benzylsalicylamine
BoSA	5-benzyloxysalicylamine

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Figure 1. Chemical structures of aldehyde-scavenging amine compounds discussed in the text

Figure 2. Inhibition of LG-lysine adducts and of PGE₂ production by salicylamine (1) isomers in human platelets

A) Human platelets were pre-incubated with 10 μ M dazoxiben and 1 mM of the specified compound or vehicle (control) for 30 min. After incubation, 20 μ M arachidonic acid was added for 2 h, and then the proteins were precipitated and digested to single amino acids by step-digestion with proteases. The daughter ions at m/z 84.1 for the lactam and m/z 89.1 for the internal standard were monitored. Values are means $\pm$ SD ($n = 4$). * $P < 0.01$ vs. control). + $P < 0.05$ vs. 4-HoBA. **B)** COX activity was measured by the formation of PGE₂ after exposure to exogenous 20 μ M arachidonic acid for 2 h. Values are means $\pm$ SD ($n = 9$). **C.** Structure of salicylamine analogs.

Figure 3. Inhibition of COX-1 and COX-2 by selected compounds

Ovine COX-1 (1.5 μ M) and murine COX-2 (2.7 μ M) were reconstituted in 100 mM Tris-HCl, pH 8.0 with 500 μ M phenol, two molar equivalents of hematin, and 1 mM final concentration of the selected compounds at 4°C for 30 min, as described in the text. Values are means $\pm$ SD ($n = 4$). * $P < 0.001$ vs. control.

Figure 4A

Figure 4B

Figure 4. Effect of the analogs on formation of LG-lysine adducts and COX-1 activity in human platelets

A) Inhibition of formation of LG adducts in platelets. Human platelets were pre-incubated with 10 μM dazoxiben, a thromboxane synthase inhibitor, and 1 mM of the specified compound or vehicle (control) for 30 min. After incubation, 20 μM arachidonic acid was added for 2 h, and then the proteins were precipitated and digested to single amino acids by step-digestion with proteases. The daughter ions at m/z 332.1 and 84.1 for the lactam were monitored. Values are means $\pm$ SD ($n = 9$) * $P < 0.001$ vs. control, + $P < 0.001$ vs. 100 μM concentration. **B)** Production of PGE₂ in human platelet. COX activity was measured by the formation of PGE₂ after exposure to exogenous 20 μM arachidonic acid for 2 h. Prostanoids were analyzed by GC/NICI/MS as previously described (16). The amount of PGE₂ present in the medium is represented as a percentage of vehicle-treated control. Values are means $\pm$ SD ($n = 9$).

Figure 5. Inhibition of LG-lysine adducts in cell lines that express COX-2

Cells were pre-incubated with 100 or 1000 μ M of compounds or vehicle (control) for 30 min. After incubation with 20 μ M arachidonic acid for 1 h, the proteins were scraped and digested to single amino acids by step-digestion with proteases. The daughter ion at m/z 84.1 for the lactam was monitored. **A)** RAW 264.7 cells. Values are means $\pm$ SD ($n \leq 6$). * $P < 0.001$ vs. control). + $P < 0.001$ vs. 100 μ M concentration. **B)** HCA-7 cells. Values are means $\pm$ SD ($n \leq 6$). * $P < 0.001$ vs. control). + $P < 0.01$ vs. 100 μ M concentration. **C)** A549 cells. Values are means $\pm$ SD ($n \leq 6$). * $P < 0.001$ vs. control). + $P < 0.001$ vs. 100 μ M concentration.

A.

B.

X_1	X_2	Compound
Cl	H	CISA (7)
H	$\text{CH}_3\text{O}-$	3-MoSA (6)
$\text{CH}_3\text{O}-$	H	5-MoSA (5)

Y_1	Y_2	Compound
CH_3^-	H	MeSA (3)
H	CH_3CH_2^-	EtSA (8)
$\text{CH}_3(\text{CH}_2)_4^-$	H	PSA (9)
$\text{CH}_3(\text{CH}_2)_5^-$	H	HxSA (10)
$\text{C}_6\text{H}_5\text{CH}_2^-$	H	BzSA (11)
$\text{C}_6\text{H}_5\text{CH}_2\text{O}^-$	H	BoSA (12)

Scheme 1. Synthesis of pyridoxamine (13) and its analogs

1-RX	R	Compound
Iodopentane	$\text{CH}_3(\text{CH}_2)_4-$	PPM (15)
Iodoethane	CH_3CH_2-	EtPM (14)
Benzyl bromide	$\text{C}_6\text{H}_5\text{CH}_2-$	BzPM (16)

Scheme 2. Synthesis of salicylamine (1) and its analogs

Table 1
Second-order rate constants for pyrrole formation with oxopentanal

A reaction mixture (1 ml) containing 2 mM each of compounds and OPA in 100 mM phosphate buffer (pH 7.4) was taken in a 1.5 ml eppendorf tube that was mixed at 25 °C in an Eppendorf ThermoMix. An aliquot of 40 µl was diluted with 460 µL of water and heated with 0.5 ml of Ehrlich's reagent (80 mM 4-dimethylaminobenzaldehyde in 1:1 methanol - 0.6 M HCl) for 2 min at 67–69 °C. After the mixture was cooled, the absorbances at 528 and 568 nm were measured (Shimadzu UV-1601 spectrophotometer) and used for calculating the concentration of 2-methylpyrrole. The sum of extinction coefficients for Ehrlich adducts of pyrroles in this study was $68\,000 \pm 500$.

Amine	$k^* \times 10^3 \text{M}^{-1}\text{s}^{-1}$
Ehtylsalicylamine (8)	1190±53
5-Chlorosalicylamine (7)	1209±34
Salicylammonium acetate (2)	1248±58
Salicylamine (1)	1631±139
Methylsalicylammonium acetate (4)	1863±27
Benzylsalicylamine (11)	2225±220
Pentylsalicylamine (9)	2236±94
Hexylsalicylamine (10)	2238±1.6
Methylsalicylamine (3)	2387±193
3-Methoxysalicylamine (6)	2402±186
5-Methoxysalicylamine (5)	2801±245
Benzoylsalicylamine (12)	3767±220
Benzylpyridoxamine (16)	1730±76
Pyridoxamine (13)	1835±169
Pentylpyridoxamine (15)	1868±78
Ethylpyridoxamine (14)	2419±172

* k: second order rate constant for the amine

Table 2
List of compounds devoid of cytotoxicity up to 1mM

HepG2 cells were exposed to various concentrations of compounds for 24 h at 37°C, after which total cellular ATP concentration was measured. Non-toxicity was determined as more than 75 % of the initial ATP concentration remaining in presence of a concentration of analog of 1,000 µM.

Compounds without cytotoxicity
Ethylsalicylamine (8)
Salicylammonium acetate (2)
Salicylamine (1)
Methylsalicylammonium acetate (4)
Methylsalicylamine (3)
3-Methoxysalicylamine (6)
5-Methoxysalicylamine (5)
Pyridoxamine (13)
Pentylpyridoxamine (15)
Ethylpyridoxamine (14)

Table 3
Induction of cytotoxicity by candidate compounds

HepG2 cells were exposed to various concentrations of compounds for 24 h at 37 °C, after which total cellular ATP concentration was measured. Each bar represents the mean $\pm$ S.D of six values, expressed as the percent of control (vehicle-treated cells).

Concentration (μ M)	ATP levels (% of control)			
	10	100	500	1000
BoSA (12)	90.89 $\pm$ 20.40	24.85 $\pm$ 8.29	0.25 $\pm$ 0.06	0.50 $\pm$ 0.18
BzSA (11)	98.25 $\pm$ 19.12	95.37 $\pm$ 18.27	0.475 $\pm$ 0.09	0.37 $\pm$ 0.03
HxSA (10)	107.36 $\pm$ 10.13	0.52 $\pm$ 0.06	0.33 $\pm$ 0.08	0.26 $\pm$ 0.04
PSA (9)	109.44 $\pm$ 12.87	20.14 $\pm$ 7.84	0.34 $\pm$ 0.02	0.31 $\pm$ 0.02
CISA (7)	97.86 $\pm$ 3.04	101.44 $\pm$ 5.73	98.36 $\pm$ 1.64	42.22 $\pm$ 2.43
BzPM (16)	106.01 $\pm$ 14.19	100.02 $\pm$ 13.67	59.65 $\pm$ 7.67	0.56 $\pm$ 0.15