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Synthesis and evaluation of the anti-inflammatory properties of selenium-derivatives of celecoxib

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Abstract

Celecoxib is a selective cyclooxygenase (COX)-2 inhibitor used to treat inflammation, while selenium is known to down-regulate the transcription of COX-2 and other pro-inflammatory genes. To expand the anti-inflammatory property, wherein celecoxib could inhibit pro-inflammatory gene expression at extremely low doses, we incorporated selenium (Se) into two Se-derivatives of celecoxib, namely; selenocoxib-2 and selenocoxib-3. *In vitro* kinetic assays of the inhibition of purified human COX-2 activity by these compounds indicated that celecoxib and selenocoxib-3 had identical K_I values of 2.3 and 2.4 μ M; while selenocoxib-2 had a lower K_I of 0.72 μ M. Furthermore, selenocoxib-2 inhibited lipopolysaccharide-induced activation of NF- κ B leading to the down-regulation of expression of COX-2, iNOS, and TNF α more effectively than selenocoxib-3 and celecoxib in RAW264.7 macrophages and murine bone marrow-derived macrophages. Studies with rat liver microsomes followed by UPLC-MS-MS analysis indicated the formation of selenenylsulfide conjugates of selenocoxib-2 with N-acetylcysteine. Selenocoxib-2 was found to release minor amounts of Se that was effectively inhibited by the CYP inhibitor, sulphaphenazole. While these studies suggest that selenocoxib-2, but not celecoxib and selenocoxib-3, targets upstream events in the NF- κ B signaling axis, the ability to effectively suppress NF- κ B activation independent of cellular selenoprotein synthesis opens possibilities for a new generation of COX-2 inhibitors with significant and broader anti-inflammatory potential.

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Keywords

cyclooxygenase-2; PGE₂; nuclear factor- κ B; transcription; proinflammatory genes

1. INTRODUCTION

Classical non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, naproxen, and ibuprofen are known to reduce inflammation by blocking the formation of prostaglandins (PG) and thromboxanes through inhibition of cyclooxygenase (COX)-1 and COX-2 [1]. Part of their effectiveness and/or side effects stem from their ability to block the actions of COX-1 or COX-2 or both [2]. Reduced inflammation and enhanced therapeutic value of these inhibitors are thought to arise mainly from the inhibition of COX-2, while the side-effects of gastric bleeding and ulceration arise due to the inhibition of COX-1 [3]. An increase in the expression of COX-2 in inflamed tissues is accompanied by an increase in its downstream product, PGE₂, which sensitizes peripheral nociceptor terminals causing pain [4].

Highly COX-2 selective inhibitors, such as coxibs, possess anti-cancer and anti-inflammatory activities [5]. Amongst these, celecoxib has gained considerable popularity for its dual role of selectively inhibiting COX-2 and effectively inhibiting the growth of adenomatous polyps in the colon [6]. A recent five-year efficacy and safety analysis of the adenoma prevention by celecoxib indicated that high-dose celecoxib (400 mg twice daily) was more effective than low dose celecoxib (200 mg twice daily) in reducing adenomas of the colon, but associated with an elevated risk for cardiovascular and thrombotic adverse events, particularly in patients with preexisting atherosclerotic heart disease [7]. Therefore, the ability to enhance the effect of celecoxib to promote its anti-proliferative and anti-inflammatory properties at concentrations with minimal or essentially no cardiovascular adversities would be highly desirable. We reasoned that enhancing the ability of celecoxib to inhibit COX-2 activity in addition to the inhibition of expression of COX-2 and other pro-inflammatory genes would potentially expand the spectrum of health benefits of celecoxib, particularly as an anti-cancer drug.

Emerging evidence from epidemiological studies and clinical trials show the beneficial anti-inflammatory effects of selenium (Se), an essential micronutrient. We have previously demonstrated that Se-supplementation of macrophages increased the expression of selenoproteins that effectively down-regulated lipopolysaccharide (LPS)-induced COX-2 expression [8,9]. The beneficial effects of Se, in the form of selenoproteins and novel organo-Se compounds, have been studied for their role as antioxidants, cytokine inducers, enzyme inhibitors, and antitumor agents [10–16]. Along these lines, 1,4-phenylenebis(methylene)selenocyanate (*p*-XSC), a Se-derivative of benzylthiocyanate, displayed enhanced chemopreventive activity in rodents when compared to its precursor [17]. *p*-XSC effectively inhibited COX-2 expression via the inactivation of NF- κ B [18], a redox-sensitive transcription factor that plays an important role in inflammatory process by regulating number of target genes such as COX-2, tumor necrosis factor (TNF)- α , and inducible nitric oxide synthase (iNOS). Along the same lines, recent studies by Desai *et al* (2010) [19], demonstrated that substitution of sulphur in PBIT (S,S'-(1,4-phenylenebis[1,2-ethanediyl])bisisothiurea), a well known iNOS inhibitor, with Se increased the pro-apoptotic ability of the isosteric analog towards many cancer cell lines by inhibiting PI3-kinase and Akt pathway.

The concept of synthesis of Se-derivatives of celecoxib with anti-inflammatory and chemopreventive properties could, thus, represent an effective method to treat inflammatory

processes, a hallmark of tumorigenesis. Based on our work with *p*-XSC and Se,Se'-(1,4-phenylenebis[1,2-ethanediyl])bisoselenourea (PBSe), we hypothesized that inclusion of Se into celecoxib enhances the anti-inflammatory properties by inhibiting the enzymatic activity of COX-2 in addition to targeting cellular signaling pathways in immune cells. Although, clinical trials are in progress using celecoxib and Se yeast for the prevention of colon cancer [20], there are no biochemical studies that have characterized these Se-derivatives of celecoxib. Here we report the synthesis of two Se-derivatives of celecoxib, namely, 4-(3-Selenocyanatomethyl-5-*p*-tolyl-1-yl)-benzenesulfonamide (selenocoxib-2) and 4-[5-(4-Methylselenanylmethyl-phenyl)-3-trifluoromethyl-pyrazol-1-yl]-benzenesulfonamide (selenocoxib-3) and their characterization of the inhibition of COX-2 activity and modulation of NF- κ B signaling axis in an *in vitro* macrophage model.

2. MATERIALS AND METHODS

2.1. Materials

Murine macrophage-like RAW264.7 cells were obtained from American Type Culture Collection (Manassas, VA). Bone marrow derived macrophages (BMDMs) were prepared from femoral bone marrow plugs of C57/BL6 mice as described earlier [9]. Antibodies for COX-2 and iNOS were obtained from Cayman Chemicals (Ann Arbor, MI); while anti-GPX-1 and anti-GAPDH were from Abcam and Fitzgerald Industries (Cambridge, MA), respectively. Purified ovine COX-1 and recombinant human COX-2 were obtained from Cayman Chemicals and were used without further purification. Validated taqman probes for real-time PCR analysis of COX-2 and TNF α expression were purchased from Applied Biosystems (Foster City, CA).

2.2. Synthesis of celecoxib

Celecoxib was synthesized using previously reported procedure [21]. Melting points were recorded on a Fisher-Johnson melting point apparatus. Unless stated otherwise, ^1H -NMR spectra were recorded in using a Bruker 500 MHz instrument. The chemical shifts (δ) are reported in ppm, referenced externally to tetramethyl silane at 0 ppm. All coupling constants (*J*) are given in Hertz. The signals are quoted as s (singlet), d (doublet), t (triplet), m (multiplet), and dt (doublet of triplet). Low resolution electron impact (EI) MS scans were carried out on a 4000 Q trap hybrid triple quadrupole/linear ion trap instrument (Applied Biosystems/MDS Sciex) at the Proteomic Facility within the Penn State Cancer Institute at Penn State Hershey College of Medicine, Hershey, PA. High resolution (ESI) MS were carried out at the Chemistry Instrumentation Center, State University of New York at Buffalo, NY. Thin-layer chromatography (TLC) was performed on aluminum-supported, pre-coated silica gel plates (EM Industries, Gibbstown, NJ). Celecoxib ^1H -NMR (DMSO-*d*₆) δ : 2.33 (s, 3H, CH₃), 7.20 (s, 1H, CH), 7.21–7.23 (m, 4H), 7.51 (s, 2H, NH₂), 7.55 (d, 2H, *J* = 8.5 Hz), 7.88 (d, 2H, *J* = 6.5 Hz). Methyl 2,4-dioxo-4-(4-methylphenyl)butanoate (1; Fig 2) was prepared as reported in the literature [21]. All starting materials and reagents were obtained from Sigma-Aldrich Chemical Co. (Milwaukee, WI) and used without further purification.

2.2.1. Preparation of 1-(4-Sulfamoyl-phenyl)-5-*p*-tolyl-1*H*-pyrazole-3-carboxylic acid methyl ester (2)—A solution of the diketone **1** (5.0 g, 22.7 mmol) and (4-sulfamoylphenyl)hydrazine hydrochloride (5.2 g, 23.2 mmol) (with two drops concentrated HCl) in 100 mL of methanol was stirred at room temperature for 15 min, warmed (45 °C) for 3 h and then allowed to stand overnight at room temperature. Addition of dilute HCl formed an off white colored solid that was filtered, washed with water, and dried. The residue was recrystallized with EtOAc/hexane to give pure **2** (6.54g, 77.6%) as major isomer. Mp 118–120 °C; ^1H NMR (CDCl₃) δ : 2.40 (s, 3H, CH₃), 4.00 (s, 3H, OCH₃), 4.89

(s, 2H, NH₂), 7.05 (s, 1H, H₄), 7.13 (d, 2H, aromatic, *J* = 8.0 Hz), 7.19 (d, 2H, aromatic, *J* = 8.0 Hz), 7.52 (d, 2H, aromatic, *J* = 8.5 Hz), 7.92 (d, 2H, aromatic, *J* = 8.5 Hz); MS (*m/z*, intensity): 371.0 (M⁺+1, 100), 340.2 (35), 232.1 (60).

2.2.2. Preparation of 4-(3-Hydroxymethyl-5-*p*-tolyl-pyrazol-1-yl)-benzenesulfonamide (3)

—Under nitrogen atmosphere, to a chilled solution of above cyclic pyrazole **2** (7.2 g, 19.4 mmol) in dry THF (100 mL), LiAlH₄ (0.8 g, 21.08 mmol) was added in small portions over 20 min. Subsequently, the ice-bath was removed and the resultant suspension was stirred overnight at room temperature. The mixture was poured into crushed ice containing 1N Na₂SO₄ solution that was allowed to stand for 30 min followed by extraction with EtOAc. The EtOAc extracts were dried over anhydrous MgSO₄, filtered, and concentrated to produce **3** (6.0 g, 90.1%). MP 96–98 °C; ¹H NMR (DMSO-*d*₆) δ: 2.33 (s, 3H, CH₃), 4.52 (s, 2H, CH₂OH), 5.24 (t, partially exchanged proton, OH, *J* = 5.0 Hz), 6.59 (s, 1H, H₄), 7.15 (d, 2H, aromatic, *J* = 8.0 Hz), 7.22 (d, 2H, aromatic, *J* = 8.0 Hz), 7.41 (d, 2H, aromatic, *J* = 8.5 Hz), 7.44 (s, 2H, NH₂), 7.81 (d, 2H, aromatic, *J* = 8.5 Hz); MS (*m/z*, intensity) (EMS): 344.1 (M⁺+1, 100), 326.1 (20).

2.2.3. Preparation of 4-(3-Chloromethyl-5-*p*-tolyl-pyrazol-1-yl)-benzenesulfonamide(4)

—A mixture of alcohol **3** (2.0 g, 5.8 mmol), triethylamine (0.6 g, 5.9 mmol), *p*-toluenesulfonyl chloride (1.1 g, 5.8 mmol), and anhydrous lithium chloride (0.25 g, 5.9 mmol) in 100 mL of dry THF, were refluxed for 16 h. The reaction mixture was diluted with EtOAc and then washed with 1N HCl, saturated NaHCO₃, and water; dried over MgSO₄, filtered and evaporated to give the crude products. These products were loaded onto a silica column that was developed with hexane:EtOAc (70:30) to yield the desired product **4** as white crystals (1.05 g, 49.8%). ¹H NMR (CDCl₃) δ: 2.38 (s, 3H, CH₃), 4.68 (s, 2H, CH₂Cl), 4.81 (s, 2H, NH₂), 6.58 (s, 1H, H₄), 7.11 (d, 2H, aromatic, *J* = 8.0 Hz), 7.16 (d, 2H, aromatic, *J* = 8.0 Hz), 7.44 (d, 2H, aromatic, *J* = 8.5 Hz), 7.87 (d, 2H, aromatic, *J* = 8.5 Hz); MS (*m/z*, intensity) (EMS): 362.0 (M⁺+1, 80), 326.1 (100, M⁺-Cl), 280 (5), 245.1 (20).

2.2.4. Preparation of 4-(3-Selenocyanatomethyl-5-*p*-tolyl-1-yl)-benzenesulfonamide (selenocoxib-2)

—The chloro compound **4** (0.45 g, 1.25 mmol) was added to a solution of KSeCN (0.19 g, 1.32 mmol) in acetonitrile (10 mL) under nitrogen. The solution was gently warmed for 3 h. The solvent was removed *in vacuo* and the residue was partitioned between EtOAc and water. The organic phase was separated, washed with brine and water, dried over MgSO₄, filtered and the solvent evaporated to yield selenocoxib-2 (0.40g, 74.1%) as a pale yellow compound. MP: 182–184 °C; ¹H NMR (DMSO-*d*₆) δ: 2.33 (s, 3H, CH₃), 4.38 (s, 2H, CH₂SeCN), 6.65 (s, 1H, CH), 7.16 (d, 2H, aromatic, *J* = 8.5 Hz), 7.23 (d, 2H, aromatic, *J* = 8.5 Hz), 7.44 (d, 2H, aromatic, *J* = 8.5 Hz), 7.45 (s, 2H, NH₂), 7.84 (d, 2H, aromatic, *J* = 8.5 Hz); MS (*m/z*, intensity) (EMS): 433.0 (M⁺+1, 43), 360.3 (100), 338.4 (60), 326.2 (50), 303.3 (10), 250.2 (10), Characterization of C₁₈H₁₆N₄O₂SSe by high-resolution mass spectrometry (using an ESI probe) revealed a *m/z* of 433.0230 versus the calculated *m/z* of 433.0232..

2.2.5. Preparation of 4-[5-(4-Bromomethyl-phenyl)-3-trifluoromethyl-pyrazol-1-yl]-benzenesulfonamide (5)

—A solution of celecoxib (5.0 g, 13.12 mmol) and N-bromosuccinimide (NBS) (2.4 g, 13.48 mmol) in 25 mL of CCl₄ (2 crystals of benzoyl peroxide) was irradiated for 3 h with a sun lamp. Mixture was cooled and the precipitated succinimide was filtered off and organic layer was concentrated to yield **5** (5.82g, 96.4 %) as white solid. MP: 153–154 °C; ¹H NMR (CDCl₃) δ: 4.46 (s, 2H, CH₂Br), 4.91 (s, 2H, NH₂), 6.76 (s, 1H, H₄), 7.19 (d, 2H, aromatic, *J* = 7.2 Hz), 7.39 (d, 2H, aromatic, *J* = 7.2 Hz), 7.46 (d, 2H, aromatic, *J* = 9.0 Hz), 7.90 (d, 2H, aromatic, *J* = 9.0 Hz); MS (*m/z*, intensity) (EMS): 459.9 and 461.9 (M⁺, M⁺+2, 5), 440.0 and 441.9 (20), 381.0 (100), 361.0 (25), 301.1 (45).

2.2.6. Preparation of 4-[5-(4-Methylselenanylmethyl-phenyl)-3-trifluoromethyl-pyrazol-1-yl]-benzenesulfonamide (selenocoxib-3)—Under nitrogen atmosphere, sodium borohydride was added in portions to a chilled (-78°C) solution of dimethyl diselenide (0.35 mL, 3.7 mmol) in absolute ethanol (40 mL) until the characteristic yellow color of the diselenide disappeared. At this stage, bromocelcoxib compound **5** (1.75 g, 3.8 mmol) was added to the resultant suspension and the reaction was stirred overnight at room temperature. The solvent was removed *in vacuo* and the residue was partitioned between EtOAc and water. The resultant crude residue in the organic phase was purified on a silica gel column eluting with hexane:EtOAc (70:30) to yield desired product, which on recrystallization with hexane/EtOAc yielded selenocoxib-3 (1.16 g, 64.4%) as white solid. MP: $195\text{--}197^{\circ}\text{C}$; ^1H NMR ($\text{DMSO-}d_6$) δ : 1.88 (s, 3H, SeCH_3), 3.79 (s, 2H, CH_2Se), 5.76 (s, 1H, H4), 7.25 (d, 2H, aromatic, $J = 8.5$ Hz), 7.32 (d, 2H, aromatic, $J = 8.5$ Hz), 7.52 (s, 2H, NH_2), 7.55 (d, 2H, aromatic, $J = 8.5$ Hz), 7.88 (d, 2H, aromatic, $J = 8.5$ Hz); MS (m/z , intensity) (EMS): 476.0 ($\text{M}^+ + 1$, 31), 430.9 (26), 455.0 (35), 380.2 (35), 326.2 (65). Characterization of $\text{C}_{18}\text{H}_{16}\text{F}_3\text{N}_3\text{O}_2\text{SSe}$ by high-resolution mass spectrometry (using ESI probe) revealed the m/z of 476.0166 versus the calculated m/z of 476.0165.

2.3. Inhibition of COX-2 enzymatic activity by selenocoxib derivatives using in vitro assays

Inhibition of COX-2 activity was evaluated using the oxygraph assay. For oxygraph method, oxygen consumption was measured at 37°C in the oxygraph chamber equipped with a Clark's oxygen electrode (Hansatech, Norfolk, UK). The reaction mixture contained 0.1 M Tris-Cl (pH 8.0), 1 μM hematin, 5 mM EDTA, 0.5 mM L-tryptophan, 5 μg enzyme, and 100 μM arachidonic acid. Inhibitors (100 μM stock solution) were dissolved in tissue culture-certified DMSO (Sigma). The dissociation kinetic constant (K_I) was calculated graphically from double reciprocal plots. The slopes obtained from the plots (data not shown) were used to calculate the time-dependent rate of inactivation (k_{inact}) using the formula [$\text{slope} = K_I/k_{\text{inact}}$] as reported earlier [22]. The enzyme was incubated with various concentrations of inhibitors up to 10 min. All experiments were repeated three times and the averages were computed.

2.4. Modulation of COX-2, iNOS, and GPX1 expression by selenocoxib derivatives in a LPS stimulated macrophage model

Expression studies were carried out in RAW264.7 macrophages treated with DMSO, celecoxib, selenocoxib-2, selenocoxib-3 (0.1 and 1.0 μM) for 12 h followed by *E. coli* LPS (1 $\mu\text{g}/\text{ml}$; Serotype: 0111:B4; Sigma-Aldrich) stimulation for 2–8 h. Protein estimation in the lysates was performed using the biocinchoninic acid assay (Thermo Pierce) and equal amounts of protein were used for SDS-PAGE and Western blotting with antibodies specific to GPX1, COX-2, or iNOS. The density of protein bands were quantified and normalized to GAPDH. All experiments were repeated at least four times and a representative Western blot in each case is shown. Suppression of pro-inflammatory genes by coxibs was also compared in the primary macrophage cultures (BMDMs)-derived from the bone marrow of mice. BMDMs were isolated from C57/BL6 mice and cultured with 0.1 or 1 μM of celecoxib, selenocoxib-2, or selenocoxib-3 for 12 h followed by stimulation with LPS as indicated above. Macrophages were cultured in DMEM media containing 5 % defined fetal bovine serum (7 nM Se), 2 mM L-glutamine, and 1X penicillin-streptomycin (Invitrogen). BMDMs were cultured in the above media containing L929 fibroblast conditioned media (20 %) as a source of M-CSF.

2.5. Effect of selenocoxibs on PGE_2 , TXB_2 , and $\text{TNF}\alpha$

RAW264.7 cells were pretreated with 0.1 or 1 μM of celecoxib, selenocoxib-2, or selenocoxib-3 in DMSO (0.5 % v/v) for 12 h prior to LPS stimulation for 12 h. SC-560 (50

nM; Cayman Chemicals, MI) treated cells were used to inhibit any COX-1 mediated PG production. The release of PGE₂ and TXA₂ (as TXB₂) in the cell culture media supernatant were quantitated using PGE₂ (Assay Designs, MI) and, TXB₂ (GE-Amersham, NJ) ELISA kits. To examine the anti-inflammatory effect of these compounds on TNF α and COX-2, we used real-time PCR. Pretreated RAW264.7 cells were stimulated with LPS for 2 h and the total RNA was isolated using Trizol reagent. The first strand cDNA was synthesized using the cDNA archive kit (Applied Biosystems-Invitrogen) and used in real-time PCR assays with pre-validated Taqman probes for murine TNF α , COX-2, and GAPDH. Delta C_T values were computed.

2.6. Preparation of nuclear extracts

For electrophoretic mobility shift assays, nuclear proteins were isolated as described previously [9]. The DNA sequence of the sense-strand of double stranded oligonucleotide specific for NF- κ B was 5'-GATCCAGTTGAGGGGACTT TCCAGGC-3'. Preparations of oligonucleotide probe by end-labeling and conditions for the oligonucleotide binding to nuclear proteins were as described earlier [9]. NF- κ B bands were confirmed by competition with a 100-fold excess of the respective unlabeled probe.

2.7. *In vitro* I κ B kinase assay

To assay the kinase activity of I κ B kinase (IKK) subunits, total cell lysates of RAW264.7 cells from control, DMSO with LPS stimulation, and coxibs (1 μ M) with LPS, were prepared in 50 mM Tris-HCl (pH 8.0), 100 μ M NaCl, 10 mM MgCl₂, 1 mM DTT, 10 mM NaF, 1 mM Na₃VO₄, 0.25 μ M cantharidic acid (Calbiochem). 100 μ g of the fresh cell lysate was incubated with 10 μ M ATP, 1 μ Ci [γ -³²P] ATP (Perkin Elmer), and 1 μ g of GST-I κ B α substrate (expressed in *E.coli* and affinity purified) for 30 min at 30 °C. Glutathione-sepharose beads (GE-Amersham) were added to the reaction mixture, incubated at room temperature for 1 h on an end-over-end shaker and then washed three times with PBS. The beads were boiled with 2 % SDS solution, centrifuged at 14000 g for 10 min and the radioactivity in the supernatant was determined in a Beckman LS6000LL counter. Unstimulated cells were used to calculate the fold increase in LPS treated cells in the presence or absence of coxibs.

2.8 Identification of metabolites of selenocoxibs

Two milligrams of celecoxib, selenocoxib-2, or selenocoxib-3 dissolved in 100 μ L of DMSO was added to 2 mg/ml of rat liver microsomes containing an NADPH-generating system (2 mM NADP⁺, 10 mM glucose 6-phosphate, 0.8 U of glucose 6-phosphate dehydrogenase, 5 mM MgCl₂) in a final volume of 500 μ L of 0.05 M Tris-HCl buffer, pH 7.4. The reaction mixtures were preincubated for 3 minutes at 37 °C and then the reaction was initiated by the addition of compounds. The control incubation was performed in the absence of coxibs. The incubations were carried out in a shaking water bath for 2 hr at 37 °C and terminated with 100 μ L of 15 % trichloroacetic acid. The reaction mixture was centrifuged at 15,000 g for 15 min and supernatants were analyzed by UPLC MS-MS as described below.

2.8.1. UPLC-MS-MS—Samples prepared as described above were analyzed using an Acquity LC-MS-MS system (Waters Corporation, Milford, MA, USA), consisting of an Acquity UPLC pump, an auto sampler, an ACQUITY UPLC BEH HSS T3 column (2.1 mm $\times$ 100 mm, 1.7 mm particle size; Waters Corporation, Milford, MA) at 45 °C, and with a UV-Diode Array Detector (Waters; Scan range= 200–400 nm; monitoring wavelength= 254 nm) connected to Acquity TQ tandem mass spectrometer (Waters) in serial mode. UPLC was performed at a flow rate of 0.5 ml/min using the following conditions: Solvent A was 5 mM ammonium acetate (pH 5.0), and solvent B was acetonitrile. Gradient program was

performed from 100% solvent A in 0.5 min to 95% solvent A and 5% solvent B, followed by a linear gradient for 2.5 min to 80% solvent B, and held for 1 min at 80% solvent B. The injection volume of each sample was 5 μ L. The Waters Acquity TQ tandem mass spectrometer was equipped with electrospray ionization (ESI) probe operated in both positive- and negative-ion mode, with capillary voltage at 2.5 kV. Nitrogen was used as both the cone and desolvation gases with flow rates maintained at 20 and 760 L/h, respectively. The source and desolvation gas temperatures were 140 °C and 450 °C, respectively. Single ion scan range was from 100 to 800 (m/z) for both positive and negative mode. Scan duration was 0.2 s with a 0.02 s inter-scan delay.

2.9 Effect of sulphaphenazole on Se release from selenocoxib-2

To examine the role of cytochrome P450s (CYPs) on the metabolism of selenocoxib-2, RAW264.7 cells were treated with sulphaphenazole or ketoconazole (Sigma Aldrich) at 2.5 μ M for 30 min following which celecoxib or selenocoxib-2 was added at 1 μ M for 12 h. Expression of GPX1 in such cells was analyzed by Western immunoblotting. DMSO was used as a vehicle in these studies.

2.10. Statistical analysis

The data is expressed as mean $\pm$ s.e.m. and compared to various treatment groups with Student's t test using Graph Pad Prism software program. The criterion for statistical significance was $P < 0.05$.

3. RESULTS

3.1. Synthesis of selenocoxibs

Given that the sulfonamide moiety and the pyrazole ring are essential for the activity of the coxibs, we decided to use celecoxib (Fig. 1) as a molecular platform and made modifications only at the 3- and 5-positions. Celecoxib (Fig. 1) was synthesized using reported procedure [21]. The synthesis of selenocoxib-2 is illustrated in Fig. 2A. The key intermediate in this synthesis, methyl ester of cyclic pyrazole, **2** (Fig. 2A) was prepared by reacting 2, 4-diketone, **1** with (4-sulfamoylphenyl) hydrazine hydrochloride in ethanol with a 77 % yield. Ethanol was the solvent of choice that exclusively gave desired 1,5-isomer as reported earlier [21]. Reduction of ester group in compound **2** (Fig 2A) was accomplished by using LiAlH_4 to yield hydroxymethyl derivative, **3** (Fig 2A), in quantitative yield. Chloro compound, **4** (Fig. 2A) was prepared in one-pot synthesis by reacting compound **3** with p -tosylchloride and LiCl. Above chloro compound **4** was converted to the desired compound selenocoxib-2 (Fig. 2A) by reacting with KSeCN in CH_3CN . The synthesis of selenocoxib-3 is shown in Fig. 2B. Celecoxib when reacted with NBS in CCl_4 yielded bromo compound **5** (Fig. 2B) in quantitative yield. The bromocelecoxib compound **5** was converted to selenocoxib-3 by treatment with $(\text{CH}_3)_2\text{Se}_2$ and NaBH_4 using ethanol as a solvent with a 64 % yield.

3.2. Inhibition of COX-2 enzyme activity by selenocoxibs

Since celecoxib is a well established COX-2 inhibitor, we examined if inclusion of Se within celecoxib had any effect on its inhibitory property. To characterize the kinetic mechanism of inhibition of COX-2 by celecoxib and selenocoxibs, concentration and time-dependent kinetic parameters were determined. A time-dependent inactivation of COX-2 was observed with all three compounds (Supplementary Fig. 1A). The k_{inact} was calculated to be $12.2 (\pm 2.1) \text{ sec}^{-1}$, $27.02 (\pm 1.5) \text{ sec}^{-1}$, and $24.4 (\pm 2.2) \text{ sec}^{-1}$ for celecoxib, selenocoxib-2, and selenocoxib-3, respectively. The K_I was calculated to be $2.3 (\pm 0.15)$, $0.73 (\pm 0.05)$ and $2.4 (\pm 1.2) \mu\text{M}$ for celecoxib, selenocoxib-2 and selenocoxib-3, respectively, which indicated

that selenocoxib-2 was more potent than celecoxib and selenocoxib-3 in inhibiting the cyclooxygenase activity of COX-2. Assays with ovine COX-1 did not demonstrate any time-dependent inhibition with these compounds (Supplementary Fig. 1B).

3.3. Inhibition of pro-inflammatory gene expression by selenocoxibs in LPS-stimulated RAW264.7 macrophages and BMDMs

The upregulated expression of COX-2, TNF α , and iNOS is regarded as a classical biomarker of inflammation. The effect of pretreatment of coxibs was examined on the expression of COX-2, iNOS, and TNF α by RAW264.7 cells upon stimulation with bacterial endotoxin LPS (1 μ g/ml). A statistically significant decrease in COX-2 protein expression was observed only in selenocoxib-2 treated cells at 0.1 μ M concentration for 12 h followed by 2 h LPS stimulation, when compared to the DMSO+LPS treated cells (Fig 3A). A similar trend in the downregulation of COX-2 expression by selenocoxib-2 was also seen at early time points, 30 min and 1 h, as well at later time points (8 h) post LPS-treatment (Supplementary Fig. 2). Celecoxib and selenocoxib-3 had no significant effect on LPS-induced COX-2 expression at 0.1 μ M. Further increase in inhibitor concentration to 1 μ M resulted in significant inhibition of COX-2 expression with celecoxib and selenocoxib-2, while selenocoxib-3 appeared less effective. We also tested the effect of these compounds to abrogate LPS induced iNOS expression. The dose-dependency and inhibition of iNOS were similar to that observed with COX-2. Results shown in Fig. 3A clearly indicated that selenocoxib-2 decreased the expression of iNOS in a dose dependent manner and more effectively than celecoxib and selenocoxib-3, particularly at 0.1 μ M (Fig. 3A). A similar experiment was performed in primary macrophages, derived from the mouse bone marrow (BMDMs), which also complemented the results with RAW264.7 cells. As shown in Fig. 3B, selenocoxib-2 significantly inhibited LPS-induced COX-2 expression at 0.1 μ M, when compared to LPS-treated DMSO control and celecoxib treated groups; while celecoxib and selenocoxib-3 were largely ineffective (Fig. 3B). However, at 1 μ M, celecoxib and selenocoxib-2 treatment resulted in significant inhibition of LPS-induced COX-2 expression; while selenocoxib-3 appeared to be less effective.

We further examined the modulation of COX-2 and TNF α , at the transcript level. A statistically significant decrease in COX-2 and TNF α transcript levels were observed with all three inhibitors when compared to the LPS-treated DMSO control group (Fig 4A, B). Selenocoxib-2 inhibited expression of TNF α and COX-2 more effectively than selenocoxib-3 and the parent celecoxib. Furthermore, analysis of culture media supernatant from RAW264.7 cells treated with 0.1 and 1 μ M of celecoxib, selenocoxib-2, or selenocoxib-3, showed that all three inhibitors significantly reduced LPS-induced production of PGE₂ which was the primary PG formed by the cells under these culture conditions (Fig. 4C). However, selenocoxib-2 brought about the most significant decrease in PGE₂ compared to LPS-treated celecoxib or selenocoxib-3 groups. Similarly, treatment of macrophages with all three compounds decreased LPS-induced production of TXB₂, an additional pro-inflammatory metabolite of PGH₂, with selenocoxib-2 being more potent than celecoxib and selenocoxib-3 (Fig. 4D). Taken together, these studies suggest that selenocoxib-2 likely targeted upstream events leading to the downregulation of transcription of COX-2, iNOS, and TNF α in LPS-stimulated cells.

3.4. Inhibition of LPS-induced activation of NF- κ B in macrophages

Given that NF- κ B primarily drives the expression of COX-2, TNF α , and iNOS, we examined if each of these compounds affected the activation of this redox-sensitive transcription factor by assessing the nuclear translocation and DNA-binding activity of NF- κ B. The activation of NF- κ B in LPS-stimulated RAW264.7 macrophages treated with celecoxib, selenocoxib-2, and selenocoxib-3 was followed by EMSA. We observed a down-

regulation of NF- κ B in the LPS-stimulated cells treated with selenocoxib-2 at both 0.1 and 1.0 μ M, when compared to those treated with either celecoxib or selenocoxib-3 (Fig. 5A). At 1.0 μ M, celecoxib also brought about a slight decrease in NF- κ B activation, but not to the extent as seen with selenocoxib-2. Furthermore, *in vitro* kinase activity assay with GST-I κ B α substrate also showed a similar pattern with regard to the activity of IKK subunits (predominantly IKK2 when stimulated by LPS, a toll-like receptor-4 ligand), with selenocoxib-2 being more potent than the other two coxibs (Fig. 5B).

3.5. Modulation of GPX1 expression by selenocoxibs

Based on the fact that selenocoxib-2 was more effective in inhibiting the LPS-induced expression of COX-2 in addition to its enzymatic activity, we hypothesized that the release of Se from selenocoxib-2, and not selenocoxib-3, perhaps contributed to the down-regulation of NF- κ B activation pathway. To test this hypothesis, we utilized the expression of GPX1, a selenoprotein whose expression is increased in response to bioavailable Se, to examine the release of Se from selenocoxibs. When compared to the celecoxib-treated group, an up-regulation of GPX1 protein expression was seen exclusively in selenocoxib-2 treated cells, when compared to those treated with celecoxib or selenocoxib-3 at 0.1 and 1 μ M in the presence or absence of LPS (Fig. 6A, B). In particular, at 1 μ M, a statistically significant increase in GPX1 levels were seen in LPS-stimulated cells treated with selenocoxib-2, when compared to DMSO +LPS treated cells or celecoxib + LPS treated groups. Even in unstimulated cells (Fig 6B), while celecoxib alone increased the expression of GPX1, increase in GPX1 levels with selenocoxib-2 was found to be much higher at both 0.1 and 1.0 μ M concentrations when compared to the celecoxib-treated control group. To further derive some estimate of the release of Se from selenocoxib-2, we used a semi-quantitative Western blot analysis with graded amounts of highly bioavailable sodium selenite (7–100 nM) in the presence of parent celecoxib (1 μ M). As shown in Fig 7, we estimated that the release of Se from selenocoxib-2 to be $\leq 2\%$ (Fig. 7). Treatment of macrophages with sulphaphenazole (selective inhibitor for CYP2C9) decreased the release of Se from selenocoxib-2; while ketoconazole (selective inhibitor for CYP3A4) at 2.5 μ M had no effect on the release (Fig 8A). Higher concentration of ketoconazole could not be used due to toxicity in RAW264.7 cells. Furthermore, we studied the metabolism of all three compounds by rat liver microsomes using LC-MS (Supplementary Figs. S3–S13). As shown in Fig 8B, MS/MS analysis of the metabolites of selenocoxib-2 revealed the presence of parent selenocoxib-2 along with carboxyl- (m/z 462.3), selenoic acid derivatives (m/z 438.2, data not shown), as well as N-acetylcysteine conjugates of selenocoxib-2 (m/z 568.1, data not shown) and N-acetylcysteine conjugate of 4-[3-Selenocyanatomethyl-5-(4-hydroxymethylphenyl)-pyrazole-1-yl]-benzenesulfonamide (m/z 583.2) as the major and minor LC peaks (Supplementary Figs. S7–S9). Surprisingly, in all these metabolites Se was intact suggesting that the release of Se from selenocoxib-2 comprised only a minor proportion that is in agreement with the results shown in Fig. 7.

4. DISCUSSION

Based on the previous studies that have indicated an increased chemopreventive potential of compounds with Se substitution [17], we hypothesized that inclusion of Se into celecoxib would increase the effectiveness of COX-2 inhibitory activity, by affecting the expression of COX-2, in addition to inhibiting its enzymatic activity. This is particularly relevant given that higher (more than 100–200 mg twice daily) doses of celecoxib is also associated with an increase risk of myocardial infarction and stroke, beside other side effects [23]. Moreover, such a concept would provide a new dimension to anti-cancer treatment approaches with coxibs that can impact the activation of NF- κ B, a transcription factor known to impact all

stages of carcinogenesis. To test our hypothesis, two selenocoxib derivatives were synthesized that differed in the site of insertion of Se into celecoxib.

Cyclooxygenase-2, TNF α , and iNOS are inducible gene products considered to be bonafide markers of inflammation. In addition, COX-2 has also been implicated in a variety of carcinogenic processes such as cellular invasion, angiogenesis, anti-apoptotic pathways, and augmentation of immunological resistance through PGE₂ [24]. Thus, inhibition of expression and/or activity, of COX-2 has major health implications. The two selenocoxibs were capable of inhibiting the enzymatic activity of COX-2 much like the parent celecoxib, with subtle differences. As with celecoxib, selenocoxib-2 and selenocoxib-3 also displayed characteristics of a tight-binding inhibitor with time-dependent interaction leading to potent inhibition of human COX-2. However, based on the K_I and k_{inact} values, we speculate that the two selenocoxibs possibly differ in their mode of binding to COX-2 compared to celecoxib. X-ray crystallographic and molecular modeling analyses of these complexes may shed more light on their interaction within the active site of COX-2. Although the K_i for celecoxib was in the vicinity of that reported earlier [22], k_{inact} for celecoxib was much higher. Thus all of the results with selenocoxibs have been compared to the parent celecoxib.

In addition to their inhibitory effect of LPS-induced COX-2 activity in macrophages, as seen by a decrease in PGE₂ and TXB₂, selenocoxibs also inhibited the expression of COX-2 in both primary and immortalized macrophages stimulated with LPS. In general, selenocoxib-2 clearly stood out as an effective inhibitor of LPS-induced COX-2, TNF α , and iNOS expression at 0.1 μ M compared to LPS-treated DMSO control, celecoxib, or selenocoxib-3 groups. Although less potent than selenocoxib-2, selenocoxib-3 was also found inhibit iNOS to some extent at 1 μ M, but not at 0.1 μ M. Such an effect was not seen in the case of COX-2 expression. While the reason for the differential effect is not clear, we speculate that the selenocoxib-3 may likely impact upstream signal transduction pathways to modulate the expression of iNOS at high concentrations.

The fact that NF- κ B regulates expression of COX-2, TNF α , and iNOS in a macrophage model of inflammation by LPS [25] prompted us to study the modulation of NF- κ B activation by these Se-derivatives of celecoxib. We found that selenocoxib-2 inhibited NF- κ B; whereas selenocoxib-3 did not show any discernable inhibition in LPS-induced NF- κ B binding. Thus, it is very likely that the mechanism of down-regulation of COX-2 and iNOS expression by the two selenocoxibs is most likely mediated through diverse mechanisms. Recent studies have shown that in addition to inhibiting I κ B kinase, celecoxib (>100 μ M) also affected the activity of upstream kinases such as Akt [25]. While these concentrations are unattainable even with high-doses of celecoxib, it is particularly interesting to note that Akt inhibitors display anti-metastatic potential of tumor cells, partly through the down-regulation of NF- κ B-dependent gene expression [26]. Similarly, studies by Desai *et al* (2010) [19] with the Se-analog of PBIT increased the potency of this iNOS inhibitor in addition to inhibiting PI3-kinase and Akt pathway to cause apoptosis of many cancer cell lines. Thus, the decreased phosphorylation of IKK substrate, GST-tagged-I κ B α , in macrophages treated with selenocoxib-2 could be likely due to the modulation of upstream signaling components of the NF- κ B signaling axis leading to decreased expression of downstream target genes.

To explain why only selenocoxib-2 was more effective, we hypothesized that the release of Se from this molecule was the likely to cause the down-regulation of NF- κ B. Previous studies in our laboratory have demonstrated an inverse causal relationship between Se status in macrophages and NF- κ B dependent pro-inflammatory gene expression to be dependent on the synthesis of selenoproteins [27]. GPX1 reduces reactive oxygen species in cells and,

thus mitigates oxidative stress-induced upregulation of pro-inflammatory genes [28]. Unlike *p*-XSC, where hydrogen selenide is formed during metabolism in rodents [29], we failed to see stoichiometric amounts of Se released from selenocoxib-2 by cytochrome P450 enzyme systems, such as CYP2C9, which are known to metabolize celecoxib [30]. Based on the semi-quantitative Western blot analysis, we estimated about ~ 2% of Se was available for incorporation into GPX1, which is typically not sufficient to down-regulate the NF- κ B pathway. Alternatively, it is also possible that coxibs could mediate Se-independent down-regulation of GPX1. Although there are contradictory reports regarding the role of celecoxib on the expression and activity of GPX1 *per se*, recent studies on human dermal fibroblasts suggest that celecoxib does not affect GPX1 [31], which corroborates with our observations in LPS-stimulated macrophages. Thus, it is conceivable that the effect of selenocoxib-2 on NF- κ B-dependent expression of pro-inflammatory genes is, in part, derived not from its ability to increase the levels of selenoproteins, but by other mechanisms, which are presently unclear. Based on the ability of selenocoxib-2 to form conjugates with N-acetylcysteine and GSH, we believe that the parent selenocoxib-2 may also interact with Cys thiols in proteins to modulate signal transduction pathways in a redox-dependent manner. Needless to say, identification of key metabolites of selenocoxib-2 and the impact on key signal transduction pathways leading to NF- κ B activation will be required to further understand the molecular mechanism of action of this anti-inflammatory molecule. In contrast to the idea that N-acetylcysteine conjugation of drugs is primarily a cellular detoxification mechanism, studies with N-acetylcysteine conjugates phenethylisothiocyanate and sulforaphane have shown that such conjugates serve as effective chemopreventive agents, much like their precursors [32]. In that light, it remains to be seen if the N-acetylcysteine derivative of selenocoxib-2 has all the anti-inflammatory properties of the parent selenocoxib-2, which will be addressed in the future.

In conclusion, the current study demonstrates that selenocoxib-2 displays greater anti-inflammatory property in macrophages than celecoxib in terms of the inhibition of NF- κ B activation and consequent downregulation of expression of a few downstream target genes. Taken together, our results support the idea that introduction of Se into celecoxib increases the anti-inflammatory potential of selenocoxib-2 by impacting the expression of pro-inflammatory genes at the transcription level. However, it remains to be seen if introduction of Se into celecoxib would alleviate COX-2 inhibition-dependent toxicity *in vivo*, as seen in the case of celecoxib.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

COX-2	cyclooxygenase-2
GPX1	glutathione peroxidase 1
NF-κB	nuclear factor kappa B

iNOS inducible nitric oxide synthase

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Figure 1.
Structures of celecoxib, selenocoxib-2, and selenocoxib-3.

Figure 3.

Effect of celecoxib, selenocoxib-2, and selenocoxib-3 on the LPS induced expression of COX-2 and iNOS in RAW264.7 cells. **A.** Macrophages were pretreated with indicated concentrations of coxibs followed by stimulation with LPS (1 μ g/ml). The cell lysates were used for Western immunoblot analysis. Lanes 1–4 represent DMSO + LPS, 0.1 μ M celecoxib + LPS, selenocoxib-2 + LPS, and selenocoxib-3 + LPS, respectively. Lanes 5–7 represent 1.0 μ M celecoxib + LPS, selenocoxib-2 + LPS, and selenocoxib-3 + LPS, respectively. The blots were reprobed with anti-GAPDH and densitometrically evaluated. **B.** Effect of celecoxib, selenocoxib-2, and selenocoxib-3 on the LPS induced expression of COX-2 in BMDM cells. Lanes 1–4 represent DMSO + LPS, 0.1 μ M celecoxib + LPS, selenocoxib-2 + LPS, and selenocoxib-3 + LPS, respectively. Lanes 5–7 represent 1.0 μ M celecoxib + LPS, selenocoxib-2 + LPS, and selenocoxib-3 + LPS, respectively. US represents unstimulated cells. The blots were reprobed with anti-GAPDH and densitometrically evaluated. Representative of n=3 shown. *, **, # represent $p < 0.05$ when compared to DMSO+LPS, celecoxib+LPS, and selenocoxib-3+LPS groups, respectively.

Figure 4.

Effect of celecoxib, selenocoxib-2, and selenocoxib-3 on the LPS-induced levels of TNF α (A) and COX-2 (B) mRNA, PGE₂ (C) and TXB₂ (D). A, B. RAW264.7 macrophages were treated with indicated concentrations of coxibs for 12 h followed by 2 h of LPS stimulation. RNA was isolated from cells and real-time PCR was performed to analyze changes in the transcript levels of COX-2 and TNF α and GAPDH. Representative of n= 3 shown. *, # represent p<0.05 when compared to control and celecoxib respectively. C. Effect of celecoxib, selenocoxib-2, and selenocoxib-3 on the LPS-induced production of PGE₂ in RAW264.7 cells. Macrophages (1.5×10^6 cells/well) were incubated with coxibs at indicated concentrations for 12 h followed by an additional 12 h of LPS stimulation. PGE₂ levels were measured in the culture media supernatants. Bars 1–7 represent DMSO+LPS, celecoxib, selenocoxib-2, selenocoxib-3, celecoxib+LPS, selenocoxib-2+LPS, selenocoxib-3+LPS, respectively. D. Effect of coxibs (1 μ M) on TXB₂ production in macrophages following LPS stimulation. Bars 1–5 represent DMSO, DMSO+LPS, celecoxib+LPS, selenocoxib-2+LPS, selenocoxib-3+LPS, respectively. *, # represent p<0.05 when compared to DMSO+LPS and celecoxib+LPS, respectively.

Figure 5.

Effect of celecoxib, selenocoxib-2, and selenocoxib-3 on the LPS induced NF-κB activation in RAW264.7 cells. **A.** Macrophages were treated with indicated concentrations of coxibs for 12 h followed by 2 h of LPS stimulation. Five micrograms of nuclear extract was used in the EMSA. Lanes 1 and 2 represent LPS and DMSO+LPS, respectively. Lanes 3–5 represent 0.1 μM of celecoxib+LPS, selenocoxib-2+LPS, and selenocoxib-3+LPS, respectively. Lanes 6–8 represent 1.0 μM of celecoxib+LPS, selenocoxib-2+LPS, and selenocoxib-3+LPS, respectively. CC: cold competitor control using sample from lane 1. “US” represents unstimulated cells. Representative of n=3 shown. Autoradiograms from each experiment showed a similar trend the binding of NF-κB. **B.** Total cell lysates (100 μg) from the 0.1 μM coxib treated samples were incubated with 10 μM ATP, 1 μCi [γ - 32 P] ATP, and 1 μg of GST-IκBα substrate for 30 min at 30 °C. Radioactivity associated with the glutathione-sepharose beads added to the reaction mixture, after three wash cycles, was counted. All counts are relative to that in the unstimulated cells (negative control). Mean $\pm$ s.e.m. of assays performed in triplicate shown. *, # represent $p < 0.05$ when compared to DMSO+LPS and celecoxib+LPS, respectively.

Figure 6.

Effect of celecoxib, selenocoxib-2, and selenocoxib-3 on the expression of GPX-1 in RAW264.7 cells. **A.** Macrophages were pretreated with coxibs and stimulated with LPS. Lane 1 represents DMSO + LPS, Lanes 2–4 represent 0.1 μ M of celecoxib, selenocoxib-2, and selenocoxib-3, respectively. Lanes 5–7 represent 1.0 μ M of celecoxib, selenocoxib-2, and selenocoxib-3, respectively. The blot was reprobed with anti-GAPDH and densitometrically evaluated. Representative of n=4 shown *, # represent p<0.05 when compared to vehicle control and celecoxib, respectively. **B.** Effect of treatment of celecoxib, selenocoxib-2, and selenocoxib-3 on the basal levels of GPX1 expression in unstimulated RAW264.7 cells. Macrophages were treated with indicated concentrations of coxibs for 12 h. Cell lysates were analyzed by Western immunoblotting with anti-GPX1 and anti-GAPDH antibodies. Lane 1 represents untreated cells; lanes 2–4 represent 0.1 μ M of celecoxib, selenocoxib-2, and selenocoxib-3, respectively. Lanes 5–7 represent 1.0 μ M of celecoxib, selenocoxib-2, and selenocoxib-3, respectively. Representative of n=3 shown *, # represent p<0.05 when compared to vehicle control and celecoxib, respectively.

Figure 7.

Extent of release of Se from selenocoxib-2. RAW264.7 cells were treated with selenocoxibs or or celecoxib at 1 μ M. For comparison, cells were cultured with 1 μ M of celecoxib and graded amounts of Se (7–100 nM) in the form of sodium selenite for 12 h. Concentration of Se in the basal medium was 7 nM. GPX1 expression in the cell lysates was analyzed by Western immunoblotting analysis and normalized to that of GAPDH. Based on the amount of GPX1 expression in selenocoxib-2-treated cells, the approximate concentration of Se released, shown as a dotted line, was calculated. Lanes 1–9 represent untreated, DMSO, celecoxib, selenocoxib-2, selenocoxib-3, celecoxib (1 μ M) +25 nM Se, celecoxib (1 μ M) +50 nM, celecoxib (1 μ M) +75 nM, and celecoxib (1 μ M) + 100 nM, respectively. Representative of $n = 3$ shown.

Figure 8.

Metabolism of selenocoxib-2. **A.** RAW264.7 cells were incubated with sulphaphenazole (SP) or ketoconazole (KT) at 2.5 μ M for 30 min prior to the addition to 1 μ M of celecoxib or selenocoxib-2 for 12 h. GPX1 expression was analyzed by Western immunoblot analysis. Lanes 1–7 represent vehicle (DMSO) control, celecoxib, selenocoxib-2, celecoxib with KT, selenocoxib-2 with KT, celecoxib with SP, and selenocoxib-2 with SP, respectively. **B.** HPLC UV-trace of the metabolism of selenocoxib-2 by rat liver microsomes using an NADPH generating system for 2 h at 37 $^{\circ}$ C as described in the “Methods”. The eluates were monitored at 254 nm and each peak was further analyzed by UPLC-MS/MS. Chemical structures with corresponding molecular mass of major metabolites are shown. Representative of n= 3 shown.