Simultaneous Targeting of COX-2 and AKT Using Selenocoxib-1-GSH to Inhibit Melanoma

Raghavendra Gowda, Subba Rao V. Madhunapantula, Dhimant Desai, Shantu Amin, and Gavin P. Robertson

Abstract
Melanoma is a highly metastatic and deadly disease. An agent simultaneously targeting the COX-2, PI3K/Akt, and mitogen-activated protein kinase (MAPK) signaling pathways that are deregulated in up to 70% of sporadic melanomas might be an effective treatment, but no agent of this type exists. To develop a single drug inhibiting COX-2 and PI3K/Akt signaling (and increasing MAPK pathway activity to inhibitory levels as a result of Akt inhibition), a selenium-containing glutathione (GSH) analogue of celecoxib, called selenocoxib-1-GSH was synthesized. It killed melanoma cells with an average IC₅₀ of 7.66 μmol/L compared with control celecoxib at 55.6 μmol/L. The IC₅₀ range for normal cells was 36.3 to 41.2 μmol/L compared with 7.66 μmol/L for cancer cells. Selenocoxib-1-GSH reduced development of xenografted tumor by approximately 70% with negligible toxicity by targeting COX-2, like celecoxib, and having novel inhibitory properties by acting as a PI3K/Akt inhibitor (and MAPK pathway activator to inhibitory levels due to Akt inhibition). The consequence of this inhibitory activity was an approximately 80% decrease in cultured cell proliferation and an approximately 200% increase in apoptosis following 24-hour treatment with 15.5 μmol/L of drug. Thus, this study details the development of selenocoxib-1-GSH, which is a nontoxic agent that targets the COX-2 and PI3K/Akt signaling pathways in melanomas to inhibit tumor development.

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Introduction
Melanoma remains one of the most invasive and drug-resistant cancers, making the development of clinically effective therapies a major obstacle (1). Recent U.S. Food and Drug Administration approval of vemurafenib (PLX-4032) illustrates the drug resistance hurdle faced by melanoma drugs inhibiting single targets. Vemurafenib targets mutant V600E-Raf present in 50% to 60% of sporadic melanomas, and has response rates of up to 80% (2, 3). However, almost all initially responding patients developed recurrent resistant disease within a year (4, 5). Therefore, drugs are needed that can be added to the current arsenal of compounds for use alone or in combination with agents such as vemurafenib (6). One approach is to improve the therapeutic efficacy of existing agents through chemical modification, enabling them to target multiple key pathways regulating cancer development (7, 8).

Celecoxib is a drug that inhibits COX-2 activity (9). COX-2 is a ubiquitously expressed inducible enzyme that plays an important role in the production of prostaglandin E₂ (PGE₂; refs. 10, 11). Celecoxib inhibits COX-2, thereby reducing the production of PGE₂ (9). PGE₂ affects cellular proliferation, motility, invasiveness, angiogenesis, and promotes survival by inhibiting apoptosis (10, 11). Furthermore, PGE₂ is a tumor-inducing eicosanoid that promotes tumor development and progression, leading to more invasive disease (12). COX-2 is overexpressed in carcinomas of the colon, breast, lung, prostate, cervix, stomach, and melanocyte suggesting it could be an important therapeutic target (13, 14). Initial studies in this report confirm that COX-2 expression is elevated in melanoma cell lines and in tumor biopsies compared with normal human melanocytes and that targeting COX-2 but not COX-1 in melanoma cell lines using siRNA inhibited xenografted melanoma tumor development.

Concentrations of celecoxib required to induce apoptosis of cultured cells are high and ranged from 25 to 100 μmol/L and clinical use is associated with cardiovascular side effects at doses of 200 mg per day (15, 16). Therefore, scientists are developing a variety of celecoxib analogues that are effective at lower concentrations or have altered properties (17, 18). Interestingly, some analogues maintain COX-2 inhibitory potency, whereas others do not, but all seem to decrease the viability of cancer cells in culture to varying degrees depending on the new properties of the...
agents (19–21). Several reports document that selenium incorporation into the structural backbone of certain compounds can enhance the therapeutic potential of the drug by providing it with new inhibitory properties, which involve inhibition of the Akt signaling pathway (22, 23). The Akt pathway is important in melanoma development and is activated in up to 70% of sporadic melanomas (24). Therefore, selenium incorporation into celecoxib would have potential to enhance its therapeutic efficacy by providing additional inhibitory properties unrelated to its initial COX-2 targeting efficacy. An analogue of celecoxib has been developed that contains selenium, called selenocoxib-1, but the drug is toxic to animals, limiting its use as a therapeutic agent (19). Therefore, additional modifications of seleno-oxib-1 were needed to maintain cancer cell killing efficacy, with decreased toxicity of normal cells.

The novel discovery detailed in this report is that a form of seleno-oxib-1 called seleno-oxib-1-GSH was developed, which does not exhibit the same toxicity on normal cells as seleno-oxib-1, making it a potentially useful therapeutic agent. Seleno-oxib-1-GSH more effectively killed melanoma cell lines than celecoxib. The new agent retained COX-2 targeting activity and, as predicted, had new Akt signaling inhibitory properties. Mechanistically, seleno-oxib-1-GSH inhibited melanoma cell survival by targeting the COX-2 and PI3K/Akt pathways (and increased pErk1/2 to inhibitory levels due to targeting of the Akt pathway), which decreased cellular proliferation and triggered apoptosis mediated through a G0–G1 block, resulting in fewer cells in S and G2–M phases of the cell cycle. Intraperitoneal administration of seleno-oxib-1-GSH retarded the growth of xenografted melanoma tumors up to 70% without affecting animal body weight or major organ functions. Thus, a more effective agent has been developed from a toxic agent that can decrease melanoma development by targeting key signaling pathways without causing major organ-related toxicity.

Materials and Methods

Cell lines and culture conditions

Human primary melanocytes containing wild-type B-Raf-FOM103 and NHEM 558, and human melanoma cell lines harboring mutants V600EB-Raf - WM35, WM115, WM278.1, A375M, and 1205 Lu (provided by Dr. Herlyn; Wistar Institute, Philadelphia, PA) were cultured as described (25). Human fibroblast FF2441 cells were provided by Dr. Craig Myers (Penn State College of Medicine, University of Arizona, Tucson, AZ). Wild-type B-Raf-containing C8161.C9 (provided by Dr. Danny Welch, University of Kansas, Kansas City, MO) and MelJuSo (provided by Dr. Judith Johnson, Institute for Immunology, Heidelberg, Germany) cell lines were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS. Cell lines were authenticated and maintained in a 37°C humidified 5% CO2 atmosphere incubator and periodically monitored for genotypic characteristics and tumorigenic potential to confirm cell line identity and phenotypic behavior.

Analysis of human melanoma patient tumors

Tumors were pulverized using a mortar and pestle chilled in liquid nitrogen and protein lysates extracted as reported previously (26). Western blotting was used to measure levels of COX-2 protein, normalized to 6-α-enolase using ImageJ software.

siRNA efficacy and knockdown studies

To determine efficacy of siRNA-mediated knockdown, 200 pmol of siCOX-2 #1 or siCOX-2 #2, was compared with scrambled siRNA or reconstitution buffer following nucleofection into 1 × 106 of 1205 Lu or A375M cells using an Amaxa nucleofector with solution R/program K-17 (1205 Lu) or solution R/program A-23 (A375M). Transfection efficiency of viable cells was more than 90%. Following siRNA transfection, cells were reseeded and left to recover for 2 days followed by replating in 96-well plates to measure cell viability using the MTS assay (Promega). To show siRNA-mediated protein knockdown in vitro, 1 × 106 of 1205 Lu, UACC 903, and A375M cells were similarly nucleofected with 200 pmol of siCOX-2 #1, siCOX-2 #2, and 100 pmol/L of V600EB-Raf, MEK1, MEK2, ERK1, and ERK2, scrambled siRNA, reconstitution buffer, and protein lysates were harvested at day 4 or 6, and analyzed by Western blot analysis. Duplexed Stealth siRNA (Invitrogen) was used for these studies. The following siRNA sequences were used: COX-2 #1: UCC AGA CAA GCA GGC AUA UAC UGA U; COX-2 #2: GAG UUA UGU CUU GAC AUC CAG AUC A. siRNA sequences for scrambled V600EB-Raf, MEK1, MEK2, ERK1, and ERK2 were used as previously reported (27).

COX inhibition studies

Human recombinant COX-2 activity was assayed using a commercial COX-inhibitor screening assay kit (Cayman Chemical) according to the manufacturer’s protocol. The concentrations of celecoxib and seleno-oxib-1-GSH tested were 0.2, 2.0, and 20 nmol/L. SC-560 and DuP-697, standard inhibitors for COX-1 and COX-2, respectively, were used as positive controls. Dimethyl sulfoxide (DMSO) served as a negative control for 100% activity. The assay was conducted in duplicate and repeated twice.

Synthesis of celecoxib, seleno-oxib-1, and seleno-oxib-1-GSH

Celecoxib was synthesized as described previously (28). Seleno-oxib-1 was prepared as reported (19). Seleno-oxib-1-GSH conjugate was prepared by reacting molar equivalent seleno-oxib-1 with glutathione (GSH) in tetrahydrofur- an: H2O (2:1) mixture. pH was adjusted to slightly basic conditions to generate seleno-oxib-1-GSH conjugate in a quantitative yield as a yellow powder [mp:196–198°C; 1H NMR (DMSO-d6, 500 MHz) d 1.72–1.83 (m, 3H), 1.88–1.98 (m, 1H), 2.23–2.36 (m, 2H), 2.91 and 2.94 (dd, 1H, J = 10 Hz, 10 Hz)].
3.16 and 3.18 (dd, 1H, J = 4.5 Hz), 3.57-3.72 (m, 3H). 4.18 and 4.23 (dd, 2H, J = 12.5 Hz and 22 Hz), 4.51 (td, 1H, J = 4.0 Hz), 6.62 (s, 1H, CH), 7.25 (d, 1H, aromatic, J = 2.5 Hz), 7.26 (d, 1H, aromatic, J = 3.5 Hz), 7.35-7.39 (m, 3H, aromatic), 7.41 (dt, 2H, aromatic, J = 8.5 Hz and 2.0 Hz), 7.79 (dt, 2H, aromatic, J = 8.5 Hz and 2.0 Hz), 8.44 (d, 1H, J = 7Hz), 8.75 (ds, 1H); MS (M/Z, Intensity): 681 (M+, 100%). Identity of compound was confirmed by nuclear magnetic resonance (NMR) as well as mass spectrometry (MS), and purity more than 99% was validated by high-performance liquid chromatography.

**Western blot analysis**

Cell lysates were harvested and processed as described previously (27). A total of 1.5 × 10⁶ melanoma cells were plated in 100-mm culture dishes and were treated 48 hours later with celecoxib, selenocoaxib-1-GSH (5-20 μmol/L), PLX-4032 (0.2-20 μmol/L), or U0126 (2.5-50 μmol/L) for 6 to 72 hours. Protein lysates were collected for Western blotting. Blots were probed with total and pAkt (Ser473), pPRAS40 (Thr246), pErk1/2 (Thr202/Tyr204), total and pMEK1/2 (Ser217), and cleaved PARP from Cell Signaling Technology. Total PRAS40 was obtained from Invitrogen. Erk2, cyclin D1, p27, α-enolase, and secondary antibodies conjugated with horseradish peroxidase were purchased from Santa Cruz Biotechnology. COX-1 and COX-2 antibodies were obtained from Cayman Chemical Company. Immunoblots were developed using the enhanced chemiluminescence detection system from Amersham Pharmacia Biotech.

**Cell viability, proliferation, apoptosis, and cell-cycle analysis**

Viability and IC₅₀ (μmol/L) of normal human melanocytes, fibroblast, and melanoma cells following treatment with inhibitors were measured using the MTS assay (22, 23). In brief, 5 × 10⁵ cells per well in 100 μL of media were plated and grown in a 96-well plate for 36 to 72 hours for melanoma (WM35, WM115, 1205 Lu, and UACC 903) and normal cell lines (FOM103 and FF2441). Cells were treated with 0.312 to 100 μmol/L of celecoxib, selenocoaxib-1, and selenocoaxib-1-GSH for 24, 48, or 72 hours with DMSO as vehicle control. IC₅₀ values for each inhibitor (in μmol/L) for respective cell lines were measured from 3 independent experiments using GraphPad Prism version 4.01 from GraphPad Software.

Cellular proliferation and apoptosis rates were measured by seeding 5 × 10⁵ cells in 96-well plates, followed by treatment for 72 hours with celecoxib or selenocoaxib-1-GSH. Percentage of proliferating or apoptotic cells was measured at the time of drug treatment (22, 23). The samples were processed as described previously (22, 23). Stained cells were analyzed using the FACSscan analyzer from Becton Dickinson and the data processed using ModFit software from Verity Software House (22, 23). Experiments were replicated twice.

**Reactive oxygen species assay**

The intracellular reactive oxygen species (ROS) was monitored according to a published protocol (29). A total of 1.5 × 10⁶ melanoma cells were plated in 100-mm culture dishes and treated 48 hours later with 5 to 20 μmol/L concentration of celecoxib, selenocoaxib-1, or selenocoaxib-1-GSH. After 24-hour treatment, total cells (floating and adherent) were collected in ice-cold PBS and 5 × 10⁵ cells per well placed in 100 μL of culture media in a 96-well plate containing 10 μmol/L 2′,7′-dichlorofluorescein-diacetate and incubated at 37°C for 30 minutes. Amount of fluorescent 2′,7′-dichlorofluorescein was measured using a SpectraMax-M2 plate reader. Amount of ROS present compared with DMSO vehicle-treated cells was represented in arbitrary units. The assay was conducted twice with 4 replicates each time.

**Tumorigenicity assessments following targeting of COX-2 using siRNA**

Tumor kinetic studies were undertaken in athymic-Foxn1nu nude mice (Harlan Sprague Dawley). Then, 200 pmol/L of siRNA COX-2 #1 or COX-2 #2 were nucleofected into 2 × 10⁶ of 1205 Lu cells and, after 48 hours of recovery, 1 × 10⁶ cells were collected in 0.2 mL of 10% FBS–DMEM and injected subcutaneously above both the left and right rib cages of 4- to 6-week-old female mice (5 mice/group; experiments were replicated twice). Dimensions of developing tumors were measured on alternate days up to day 21.5, using calipers by L × W × D (mm³; ref. 22).

**Animal studies using selenocoaxib-1-GSH for tumorigenicity assessments**

Six days after subcutaneous injection of 1 × 10⁶ 1205 Lu or UACC 903 cells in 0.2 mL of DMEM supplemented with 10% FBS into 4- to 6-week-old nude mice, when a fully vascularized tumor (50–75 mm³) had formed (5 mice/group; 2 tumors/mouse). Mice were treated intraperitoneally with selenocoaxib-1-GSH (0.127 μmol/L, equivalent to 10 ppm selenium) or celecoxib (0.127 μmol/L) in DMSO on alternate days for 4 weeks. Body weight (grams) and dimensions of the developing tumors (mm³) were measured at the time of drug treatment (22, 23).

**Toxicity assessments**

Four- to 6-week-old athymic-Foxn1nu nude mice were treated with either vehicle control or selenocoaxib-1-GSH (n = 5) as described in tumor kinetics studies. At the end of treatment, blood was collected from each sacrificed animal in a plasma separator tube with lithium heparin (Microtainer; Becton Dickinson) following cardiac puncture and analyzed for alkaline phosphatase, alanine...
aminotransferase, aspartate aminotransferase, total albumin, total bilirubin, creatinine, blood urea nitrogen, total cholesterol, total triglyceride, and glucose levels to ascertain possible liver, heart, kidney, and pancreas-related toxicity. A portion of vital organs, liver, heart, kidney, pancreas, and spleen, from each animal was formalin-fixed and paraffin-embedded to assess toxicity-associated changes in cell morphology and tissue organization following hematoxylin and eosin (H&E) staining. In addition, the effect of celecoxib, selenocoxib-1, and selenocoxib-1-GSH on the survival of mice was determined by intraperitoneally injecting celecoxib (0.127 μmol/L), selenocoxib-1 (0.032–0.064 μmol/L), or selenocoxib-1-GSH (0.127–0.254 μmol/L) daily for 7 days (n = 3). Number of surviving animals or changes in body weight was recorded.

Statistical analysis

Statistical analysis was carried out using Prism 4.01 GraphPad Software. One-way or 2-way ANOVA was used for group-wise comparisons, followed by the Tukey or Bonferroni post hoc tests. For comparison between 2 groups, the t test was used. Results represent at least 2 to 3 independent experiments and are shown as averages ± SEM. Results with a P value less than 0.05 [95% confidence interval (CI)] were considered significant.

Results

COX-2 expression is elevated in advanced-stage melanoma patient tumors and melanoma cell lines

Elevated expression and activity of COX-2 has been reported in cancers of the prostate, breast, colon, kidney, liver, and skin (13, 30). To confirm the initial report in melanomas (31), the expression of COX-2 was measured by Western blotting in a panel of melanoma patient tumors and cell lines representing radial (WM35), vertical (WM115 and WM278.1), and metastatic (A375M, UACC 903, and 1205 Lu) stages of development (Fig. 1A). Seventy-six percent (19/25) of melanoma patient tumors had elevated COX-2 expression when compared with normal human melanocyte (NHEM) control cells (Fig. 1A, left). Similarly, all melanoma cell lines examined had higher COX-2 protein content than that observed in melanocytes, albeit, in varying amounts (Fig. 1A, right). Expression of COX-2 in UACC 903, A375M, and 1205 Lu cell lines was 43, 76, and 329-fold higher than that observed in melanocytes, respectively.

Reduction of COX-2 protein levels using siRNA targeting V600E-B-Raf or COX-2—decreased melanoma cell viability

To determine whether targeting COX-2 would reduce viability of melanomas, metastatic 1205 Lu and A375M cells that express relatively high levels of protein were transfected with 2 different siRNAs targeting different regions of the mRNA and cell viability compared with controls nucleofected with a scrambled siRNA, buffer control, or siRNA targeting V600E-B-Raf (Fig. 1B). In both cell lines, targeting COX-2 reduced melanoma viability by 32% to 63%. Targeting mutant V600E-B-Raf using siRNA reduced COX-2 expression, suggesting protein expression was regulated through this pathway (Fig. 1B).

siRNA and pharmacologic agents targeting the MAPK pathway confirm that COX-2 expression is regulated through V600E-B-Raf signaling in melanomas

To examine whether siRNA-mediated targeting of Mek1/2 or Erk1/2 downstream of V600E-B-Raf would decrease COX-2 expression, siRNA or pharmacologic agents were used to decrease protein expression or activity. The 1205 Lu and UACC 903 cells were nucleofected with siRNAs inhibiting mutant V600E-B-Raf, Mek1/2, or Erk1/2. A significant decrease in COX-2, but not COX-1, was observed when each member of the V600E-B-Raf signaling pathway was targeted (Fig. 1C).

Next, vemurafenib (PLX-4032), a V600E-B-Raf inhibitor, was used to inhibit activity of this pathway. Cells treated with PLX-4032 showed decreased COX-2 protein expression, beginning after 12 hours of treatment for 1205 Lu cells. In the case of UACC 903, a significant decrease was seen from 24 hours of treatment (Fig. 1D, top). Similar to siRNA studies (Fig. 1C), no changes were observed in COX-1 expression in either cell line following treatment. A decrease in phosphorylation of Mek1/2 and Erk1/2 proteins showed the inhibitory activity of PLX-4032 on the V600E-B-Raf pathway. Like PLX-4032, the Mek1/2 inhibitor U0126 also reduced levels of COX-2 protein without affecting COX-1 in 1205 Lu and UACC 903 cell lines (Fig. 1D, bottom). Therefore, targeting V600E-B-Raf or downstream proteins in the signaling cascade reduced expression of COX-2 in melanomas to decrease the proliferative potential of the cells. Thus, COX-2 lies downstream of V600E-B-Raf, Mek-1/2, and Erk-1/2 in this important signaling pathway.

Development of selenocoxib-1-GSH retaining COX-2 inhibitory efficacy

Concentrations of celecoxib required to trigger apoptosis in cultured cells range from 25 to 100 μmol/L and clinical use is associated with cardiovascular side-effects at doses of 200 mg per day (32). To circumvent these concerns, an analogue of celecoxib has been created containing selenium and is called selenocoxib-1 (Fig. 2A). While selenocoxib-1 inhibited the viability of melanoma cells, it was toxic and reduced normal cell growth with similar IC50s to that observed for melanoma cells (Table 1 & Fig. 2B). To lessen the toxicity of selenocoxib-1 on normal, but not melanoma cells, the compound was further modified incorporating GSH, generating selenocoxib-1-GSH (Fig. 2A), which significantly decreased the toxicity on normal cells but maintained its melanoma cell-killing efficacy (Fig. 2B). Furthermore, selenocoxib-1-GSH inhibited the growth of melanoma cell lines irrespective of...
Figure 1. COX-2 expression increased in melanomas, regulated through the MAPK pathway. A, left, elevated levels of COX-2 expression in melanoma patient tumors and cell lines; right, COX-2 expression increased in a cell line-based melanoma tumor progression model. Lysates collected from normal human melanocytes, radial growth phase (WM35), vertical growth phase (WM115, WM278.1), and metastatic (A375M, UACC, and 1205 Lu) stage cell lines were subjected to Western blot analysis and probed for COX-2. α-Enolase served as a control for equal protein loading. B, targeting COX-2 using siRNAs decreased melanoma cell viability. The 1205 Lu and A375M melanoma cells were nucleofected with 2 nonoverlapping siRNAs against COX #1 and #2 using Amaxa nucleofector, program K17. Targeting COX-2 reduced melanoma viability by 32 to 63%. siRNA targeting mutant V600E-Raf served as a positive control. α-Enolase served as a control for equal protein loading. C, siRNA-mediated inhibition of the MAPK pathway decreased COX-2 expression in melanomas. The 1205 Lu and UACC 903 cells were nucleofected with siRNAs inhibiting mutant V600E-Raf, Mek1 or Mek2, and Erk1 or Erk2. Compared to cells treated with scrambled siRNA, decreasing protein levels of each member of the MAP kinase pathway led to a decrease in COX-2 but not COX-1 levels. α-Enolase served as a control for equal protein loading. D, top, PLX-4032 targeting of V600E-Raf decreased COX-2 expression. The 1205 Lu or UACC 903 cells were treated with 0.2–20 μmol/L PLX-4032 for 6, 12, 24, and 48 h. Levels of pMek1/2, pErk1/2, and COX-2 decreased after 12 h of drug treatment. No changes were seen in COX-1 expression. α-Enolase served as a control for equal protein loading. Bottom, U0126 targeting of Mek1/2 decreased COX-2 expression. The 1205 Lu or UACC 903 cells were treated with 2.5–50 μmol/L U0126 for 48 h. Levels of pErk1/2 and COX-2 similarly decreased following drug treatment, with no changes in COX-1 expression. α-Enolase served as a control for equal protein loading.
B-Raf mutation status (data not shown). Toxicity limiting the potential clinical utility of selenocoxib-1, but not selenocoxib-1-GSH, was corroborated in animal studies (Supplementary Table S1). Celecoxib at 0.127 mmol/L led to death of all animals following 7 days of treatment, whereas selenocoxib-1 at concentrations of 0.032 to 0.064 mmol/L, led to weight losses resulting in 14% or 100% animal mortality after 7 days of treatment. In contrast, animals receiving 0.127 to 0.254 mmol/L (equivalent to 5–10 ppm of selenium) of selenocoxib-1-GSH exhibited negligible weight loss of approximately 2% and no mortality (Supplementary Table S1). Due to the toxicity...
associated with selenocoxib-1, subsequent studies focused on selenocoxib-1-GSH. Selenocoxib-1-GSH was not predicted to have altered COX-2 inhibitory activity by incorporating selenium in the place of sulfur but rather to have Akt inhibitory properties and, as predicted, it retained COX-2 inhibitory activity similar to that of celecoxib (Fig. 2C).

**Selenocoxib-1-GSH inhibited melanoma cellular proliferation and increased apoptosis by arresting cells in the G0–G1 phase of the cell cycle**

Selenocoxib-1-GSH, but not celecoxib, inhibited melanoma cell viability in a dose-responsive manner (Fig. 3A). At 12.5 μmol/L, selenocoxib-1-GSH led to a 40% to 60% decrease in cell viability compared with control DMSO–vehicle-treated cells. Next, mechanisms leading to cell growth inhibition after treatment with selenocoxib-1-GSH were examined by measuring the level of cellular proliferation, apoptosis, and the percentage of cells in the various phases of the cell cycle. Selenocoxib-1-GSH reduced proliferation of 1205 Lu and UACC 903 melanoma cells (Fig. 3B) and increased caspase-3/7 activity, which is an indicator of apoptosis (Fig. 3C). A significant decrease in caspase-3/7 activity was observed when UACC 903 cells were treated with 50 μmol/L selenocoxib-1-GSH, which can be attributed to massive cell death (Fig. 3C). The effect of selenocoxib-1-GSH on cell-cycle distribution was measured by analyzing propidium iodide–stained 1205 Lu and UACC 903 cells using a Becton Dickinson FACScan. Selenocoxib-1-GSH treatment increased the sub-G0–G1 cell population, which is indicative of cellular apoptosis. The sub-G0–G1 cell population increased by 6.2- and 4.7-fold, respectively, when 1205 Lu and UACC 903 cells were treated with 25 μmol/L selenocoxib-1-GSH (Fig. 3D). In addition, an increase in the G0–G1 cell population was also observed at 12.5 and/or 25 μmol/L selenocoxib-1-GSH (Fig. 3D). Thus, selenocoxib-1-GSH inhibited cellular proliferation and triggered apoptosis mediated through a G0–G1 block, resulting in fewer cells in the S and G2–M phases of the cell cycle.

Selenocoxib-1-GSH inhibited Akt signaling, which activates MAPK activity to reduce melanoma cellular proliferation and promote apoptosis

Selenium incorporation into the structural backbone of certain agents can enhance the therapeutic potential of the agent by providing the compound with new inhibitory properties (19, 22, 23, 33). Selenocoxib-1-GSH retained COX-2 inhibitory activity as predicted (Fig. 2C). To determine whether selenium incorporation into selenocoxib-1-GSH provided the compound with new Akt pathway inhibitory properties, pAkt levels were examined in melanoma cells following treatment. Compared with celecoxib, selenocoxib-1-GSH treatment inhibited Akt phosphorylation in a dose-dependent manner (Fig. 4A). Furthermore, phosphorylation of the downstream Akt3 substrate PRAS40 was significantly inhibited.

The 1205 Lu and UACC 903 melanoma cells have elevated MAPK activities due to the presence of constitutively active V600EB-Raf; however, the levels are modulated into a range that promotes rather than inhibits cellular proliferation (34). Akt3 has been shown to phosphorylate V600EB-Raf to lower MAPK pathway activity to promote cellular proliferation (34). Treatment of melanoma cells with selenocoxib-1-GSH, which decreased Akt activity, led to a significant increase in pERK1/2 levels (the indicator of MAPK pathway activity; Fig. 4B), to a point where it no longer promoted proliferation but led to cell senescence. This was due to decreased phosphorylation and regulation of V600EB-Raf by Akt (34). In addition, selenocoxib-1-GSH inhibited expression of cyclin D1 and increased levels of p27 (Fig. 4C). Finally, increased caspase-3/7 and cleaved PARP levels were observed indicating higher levels of apoptosis in selenocoxib-1-GSH compared with celecoxib-treated cells (Fig. 4D).

Selenocoxib-1-GSH inhibited melanoma tumor development in mice without significant toxicity

Initially, siRNA-targeting COX-2 was used to reduce protein expression in melanoma cells to measure the effect on melanoma tumor development to serve as a control.

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**Table 1. Selenocoxib-1-GSH kills melanoma cells more effectively than normal cells**

<table>
<thead>
<tr>
<th></th>
<th>FOM103</th>
<th>FF2441</th>
<th>WM35</th>
<th>WM115</th>
<th>UACC 903</th>
<th>1205 Lu</th>
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<tr>
<td>Celecoxib</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>51.3 ± 2.3</td>
<td>54.4 ± 3.6</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>Selenocoxib-1-GSH</td>
<td>66.3 ± 3.0</td>
<td>&gt;100</td>
<td>52.6 ± 3.8</td>
<td>30.1 ± 3.3</td>
<td>30.9 ± 2.8</td>
<td>24.6 ± 2.2</td>
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<tr>
<td>Celecoxib</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>42.3 ± 1.8</td>
<td>45.3 ± 2.7</td>
<td>&gt;100</td>
<td>83.6 ± 3.3</td>
</tr>
<tr>
<td>Selenocoxib-1-GSH</td>
<td>53.4 ± 4.3</td>
<td>75.5 ± 5.6</td>
<td>4.1 ± 0.8</td>
<td>5.8 ± 0.9</td>
<td>20.6 ± 1.7</td>
<td>17.2 ± 1.6</td>
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<tr>
<td>Celecoxib</td>
<td>68.0 ± 1.2</td>
<td>65.3 ± 3.3</td>
<td>37.9 ± 3.3</td>
<td>41.8 ± 2.9</td>
<td>76.4 ± 4.4</td>
<td>66.1 ± 3.2</td>
</tr>
<tr>
<td>Selenocoxib-1-GSH</td>
<td>41.2 ± 3.2</td>
<td>36.3 ± 5.6</td>
<td>2.7 ± 0.4</td>
<td>3.1 ± 0.4</td>
<td>14.2 ± 1.2</td>
<td>10.6 ± 2.6</td>
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</table>

NOTE: Normal and melanoma cells were seeded in a 96-well plate and, after 36 to 72 hours, treated with increasing concentrations of celecoxib, selenocoxib-1, or selenocoxib-1-GSH for the indicated time period. Number of viable cells was measured using MTS and percentage decrease in viability calculated. IC50 values for each inhibitor in μmol/L for respective cell lines were measured from 3 independent experiments using GraphPad Prism version 4.01 (GraphPad Software, La Jolla, CA).
Inhibition of COX-2 protein expression using siRNAs reduced xenografted melanoma tumor development by an average of 71% after 21 days compared with controls, suggesting COX-2 was a good therapeutic target in melanomas (Fig. 5A). Next, the effect of intraperitoneal administration of selenocoxib-1-GSH on xenografted melanoma tumor development was examined (Fig. 5B and C). Decreased xenografted tumor development compared with control-treated mice was observed from day 16 in 1205 Lu tumors (Fig. 5B). Similarly, a significant decrease was observed in UACC 903 tumors from day 22 (Fig. 5C). For both cell lines at the end of treatment, up to a 70% decrease in tumor volume was observed following selenocoxib-1-GSH treatment compared with controls (Fig. 5B and C).
Figure 4. Selenocoxib-1-GSH inhibited Akt signaling to reduce the proliferative potential and promote apoptotic signaling in melanoma cells. A, selenocoxib-1-GSH inhibits the PI3K/Akt signaling pathway. B, selenocoxib-1-GSH activates the MAPK signaling pathway. C, selenocoxib-1-GSH decreased cyclin D1 protein levels, indicating a reduction in cellular proliferation. D, selenocoxib-1-GSH increased levels of cellular apoptosis.
and C). No noticeable changes in animal body weight were observed (Fig. 5B and C; insets). The levels of blood markers for major–organ-related toxicity and analysis of H&E-stained tissue sections showed negligible differences compared with controls at the concentrations examined (Fig. 5D and Supplementary Fig. S1). These data suggest that selenocoxib-1-GSH can inhibit melanoma tumor development without significant organ-related toxicity.

Discussion

Incidence and mortality rates of malignant melanoma continue to increase annually (35). Although efforts have been made to design structurally well-defined small-molecular inhibitors that interact with protein targets in melanoma cells, these efforts have failed due to development of resistant disease (36). Therefore, the realization now is that multiple important targets driving the development of this disease will need to be simultaneously targeted to most effectively manage melanoma and reduce the probability of resistant disease development. This may be achievable through the use of drug cocktails or a single drug that simultaneously inhibits multiple key signaling pathways implicated in melanoma development (37). In addition, selection of patients expressing proteins targeted by the drug would be a key...
factor that could lead to better results in the clinic. In this study, COX-2 protein levels are shown to be elevated in 76% of melanoma patient tumors and cell lines. Targeting COX-2 using siRNA, inhibited the growth of metastatic melanoma cells in culture and retarded the development of xenografted melanoma tumors in mice, indicating that COX-2 would be a good therapeutic target.

Because siRNA-mediated inhibition of COX-2 expression decreased melanoma tumor growth, pharmacological agents inhibiting COX-2 activity with high selectivity may have therapeutic potential for inhibiting melanomas. However, the COX-2 selective inhibitor celecoxib has an effect on melanoma cell proliferation but only at very high concentrations, necessitating the development of analogues better able to kill these cells at lower concentrations (38, 39). Initially, an analogue of celecoxib was developed that contained selenium, called selenocoxib-1, but the drug was toxic to normal cells and lethal to animals, which limited its use as a therapeutic agent (18). To manage this concern, a GSH derivative called selenocoxib-1-GSH was developed. It killed cultured cancer cells at doses 5-fold lower than those required to kill normal cells.

Addition of GSH to a compound can be used to increase bioavailability and reduce cytotoxicity as seen with the GSH conjugate of benzyl selenocyanate for inhibiting of colonic preneoplastic lesions and aberrant crypt foci development (40). One mechanism by which GSH conjugates inhibit cancer involves the reduction of GSH reductase activity, which depletes intracellular reduced GSH, thereby enhancing ROS-mediated cell death (41, 42). Others have reported that GSH-depleting agents can selectively sensitize cancer cells to high ROS levels (43). Another mechanism by which GSH-conjugated anticancer agents inhibit cancer cell growth involves reduction of elevated ROS levels. High ROS levels mediate cancer development and reduction reverses this process (29). Our data suggest that selenocoxib-1-GSH reduces ROS levels in melanoma cells more effectively than celecoxib or selenocoxib-1 to kill these cells (Supplementary Fig. S2).

Enhanced growth-inhibitory properties of selenocoxib-1-GSH could also be attributed to the incorporation of selenium into the structure of celecoxib. Selenium is an antioxidant nutrient reported to inhibit oncogenic Akt and NFκB pathways as well as inducing the expression of tumor suppressors PTEN, p53, and KLF-4 to mediate apoptotic cell death (19, 44). Incorporation of selenium into the structure of drugs has been shown to increase the agent’s potency by inhibiting Akt signaling (22, 23, 45). The selenium-containing analogues of the PBIT and PBITC called PBISe and ISC-4, respectively, inhibited melanoma cell growth and suppressed tumor development in animals more effectively than the sulfur-containing parental compound (22, 23). In addition, because very low levels of selenium occur in the majority of patients with melanoma, incorporating selenium may not only provide this micronutrient but also increase tumor cell-killing efficacy (33). While several reports document loss of COX-2 inhibitory activity when celecoxib was derivatized or analogues were synthesized, selenocoxib-1-GSH retained COX-2 inhibitory activity and had new Akt-targeting capabilities. In contrast, 2,5-dimethyl-celecoxib lacked the ability to inhibit COX-2, but still had antitumor activity (46).

The PI3K/Akt and MAPK signaling pathways are constitutively activated in melanoma and play a prominent role in the development of recurrent resistant disease (22, 23, 47). Selenocoxib-1-GSH while retaining COX-2 inhibitory activity at levels seen with celecoxib, also blocked Akt signaling. Decreasing levels of active pAkt3 increased V600EB-Raf activity and downstream MAPK-signaling activity to levels that are inhibitory, inducing cellular senescence (34, 48). This phenomenon occurs following selenocoxib-1-GSH treatment of melanoma cells. Selenium-containing PBISe treatment acts in a similar manner to decrease Akt activity, consequently increasing activity of MAPK pathways to inhibitory levels (23). Other studies and this one found that increased pErk-1/2 in turn upregulated COX-2 protein expression, consistent with COX-2 lying downstream in the MAPK pathway (49). High MAPK pathway activity mediated by selenocoxib-1-GSH or PBISe induced cell senescence arresting cells in G0–G1 phase of the cell cycle. Combined targeting of these pathways inhibited cell proliferation by lowering cyclin D1 and increasing p27 levels, which enhanced rates of cellular apoptosis.

In conclusion, selenium-containing selenocoxib-1-GSH retains COX-2 inhibitory activity and has new PI3K/Akt inhibitory activity to decrease melanoma cell growth by arresting cells in the G0–G1 phase of the cell cycle to promote melanoma cell apoptosis and inhibit cellular proliferation. Thus, a potentially clinically viable drug has been developed from a toxic agent that can decrease melanoma development by targeting the COX-2 and PI3K/Akt signaling pathways without causing major-organ-related toxicity.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions

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