Novel Dithiolethione-Modified Nonsteroidal Anti-Inflammatory Drugs in Human Hepatoma HepG2 and Colon LS180 Cells

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Abstract Purpose: Nonsteroidal anti-inflammatory drugs (NSAID) are promising chemopreventive agents against colon and other cancers. However, the molecular basis mediated by NSAIDs for chemoprevention has not been fully elucidated. Environmental carcinogens induce DNA mutation and cellular transformation; therefore, we examined the effect of NSAIDs on carcinogenesis mediated by the aryl hydrocarbon receptor signaling pathway. In this study, we investigated the activities of a new class of NSAIDs containing dithiolethione moieties (S-NSAID) on both arms of carcinogenesis.

Experimental Design: We investigated the effects of the S-NSAIDs, S-diclofenac and S-sulindac, on carcinogen activation and detoxification mechanisms in human hepatoma HepG2 and human colonic adenocarcinoma LS180 cells. Results: We found that S-diclofenac and S-sulindac inhibited the activity and expression of the carcinogen activating enzymes, cytochromes P-450 (CYP) CYP1A1, CYP1B1, and CYP1A2. Inhibition was mediated by transcriptional regulation of the aryl hydrocarbon receptor (AhR) pathway. The S-NSAIDs down-regulated carcinogen-induced expression of CYP1A1 heterogeneous nuclear RNA, a measure of transcription rate. Both compounds blocked carcinogen-activated AhR from binding to the xenobiotic responsive element as shown by chromatin immunoprecipitation. S-diclofenac and S-sulindac inhibited carcinogen-induced CYP enzyme activity through direct inhibition as well as through decreased transcriptional activation of the AhR. S-sulindac induced expression of several carcinogen detoxification enzymes of the glutathione cycle including glutathione S-transferase A2, glutamate cysteine ligase catalytic subunit, glutamate cysteine ligase modifier subunit, and glutathione reductase.

Conclusions: These results indicate that S-diclofenac and S-sulindac may serve as effective chemoprevention agents by favorably balancing the equation of carcinogen activation and detoxification mechanisms.

The use of nonsteroidal anti-inflammatory drugs (NSAID) has been associated with a reduced incidence of colorectal, breast, esophageal, stomach, prostate, bladder, ovary, and lung cancers (1). However, chronic treatment with conventional nonselective NSAIDs, such as aspirin, diclofenac, ibuprofen, and naproxen, is frequently associated with gastrointestinal toxicity due to the chronic suppression of cyclooxygenase-1 and subsequent inhibition of mucin secretion in the gut (2). The new generation of NSAIDs that target cyclooxygenase-2, such as rofecoxib and celecoxib, although effective as chemopreventive agents, have been recently shown to cause an increased incidence of stroke and myocardial infarctions (3).

A new series of novel redox-modified NSAIDs has been synthesized that may reduce these side effects. These compounds are conventional NSAIDs linked to a gaseous transmitter. The most extensively studied of these compounds are the nitric oxide–releasing NSAIDs, which were first described in 1994 (4) and have because been evaluated in several clinical trials in which their gastric sparing properties have been well-established (5, 6). Additional studies have shown nitric oxide–NSAIDs to be promising chemopreventive agents (7). Although nitric oxide is the best characterized of the gaseous transmitters, carbon monoxide and hydrogen sulfide (H2S) have also been recognized for their ability to affect key physiologic functions (8). In particular, H2S has been shown to exhibit complex but poorly understood roles in inflammation with evidence for proinflammatory as well as anti-inflammatory activities. H2S has also been reported to reduce NSAID-induced gastric mucosal injury.
Modulation of Phase I and II Enzymes by S-NSAIDs

Translational Relevance

Occupational and environmental exposure to polycyclic aromatic hydrocarbons (PAH) has been suggested to provoke inflammatory disorders and cancer. The molecular pathway/mechanisms invoked by PAH-mediated inflammation remain to be clarified. Our article contributed to the identification of both the molecular pathway and regulation of PAH metabolism in both human hepatoma cells, which is the active site for carcigenesis, and colon adenocarcinoma cells, which is the first exposure site for environmental xenobiotics.

The dithiolethione-NSAIDs in our study are newly synthesized drugs, which conjugate the dithiolethione moiety with NSAIDs. We found the novel compounds not only inhibited the PAH activation of phase 1 enzymes induced by PAHs but also increased the detoxification of phase 2 enzymes. We are the first to identify the dual function property of S-NSAIDs, which modulated carcinogen toxicity mediated by the aryl hydrocarbon receptor as a molecular mechanism/pathway toward chemoprotection against environmental xenobiotics.

There is additional evidence that S-NSAIDs may also show a potential usefulness in cancer treatment. Parent compounds, diclofenac and sulindac, have been investigated for their potential chemopreventive properties and have been found particularly useful for prevention and treatment in in vitro and in vivo models of colon cancer in addition to other cancer types (16, 17). Recent work on ACS 15 and 18 has also shown antiangiogenic activities of the compounds in models of tumor-driven angiogenesis (14). Our laboratory has shown the ability of sulindac as well as the dithiolethione 5-(p-methoxyphenyl)-1,2-dithiole-3-thione (ADT)8 to regulate the aryl hydrocarbon receptor (AhR) pathway and phase I enzymes (18). The AhR is the key transcriptional controller of carcinogen metabolizing pathways involving the genes that encode phase I and phase II enzymes. Inhibiting the activation of procarcinogens by phase I enzymes through suppression of the AhR signaling pathway has been suggested as an important mechanism for cancer prevention (19, 20). Based on the above data, we hypothesized that the novel compounds, ACS 15 and ACS 18, could have potential usefulness as chemoprotective compounds through regulation of the AhR pathway and associated enzymes. We show that both ACS 15 and 18 blocked carcinogen-induced phase I enzyme activity in vitro, and that this activity was regulated through inhibition of AhR-mediated transcription. In addition, ACS 18 increased the expression of several phase II genes. This dual action of reduced carcinogen activation simultaneous with increased carcinogen detoxification supports the view that the S-NSAID model may be a valuable tool for chemoprotection against environmental carcinogens.

Materials and Methods

Materials. HepG2 human hepatocellular carcinoma and LS180 human colorectal adenocarcinoma cell lines were from American Type Culture Collection. RPMI 1640, Eagle’s MEM with Earle’s balanced salt solution, glutamine, fetal bovine serum, PBS, Tris-HCl buffer, trypsin/EDTA, and TRizol were from Invitrogen. Protease inhibitor tablets were from Roche. 2,3,5,7-Tetrachlorodibenzo-p-dioxin (TCDD) was from the Midwest Research Institute. The Omniscript kit was from Qiagen. Recombinant CYP enzymes were from BD Biosciences. ACS 15 and ACS 18 were from Sulfidris. All other chemicals except where noted were from Sigma.

Cell culture. HepG2 cells were grown in RPMI 1640, and LS180 cells were grown in Eagle’s MEM with Earle’s balanced salt solution. Both media were supplemented with 10% fetal bovine serum and 2 mmol/L-l-glutamine. Cells were passed weekly with 0.05% trypsin/0.53 mmol/L EDTA. All experiments were carried out on confluent cells at 37°C in humidified atmosphere with 5% CO2 except where mentioned. ACS 15 and 18 were dissolved in DMSO; final DMSO concentration was ≤0.1% in all experiments.

Reverse transcription-PCR. Confluent cells in six-well plates were treated with DMSO (control) or the test drugs dissolved in DMSO. Total RNA was isolated using TRizol as directed. cDNA was synthesized from 4 μg of total RNA using random primers and the Omniscript kit according to manufacturer’s instructions. Real-time PCR was done in a master mix containing 12.5 μL Applied Biosystem’s TaqMan Universal PCR Master Mix, 8.75 μL DEPC water, 0.1 μg cDNA, and 1.25 μL FAM-labeled TaqMan Gene Expression Assay primer/probe sets for CYP1A1 (Hs00153120), CYP1A2 (Hs00167927), CYP1B1

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(Has00164383), GSTA2 (Has00155249), glutamate cysteine ligase catalytic (Has00157694), GR (Has00167317), and glucose-6-phosphate dehydrogenase (Has00166169) on a Bio-Rad iCycler Real Time Detection System. Amplification conditions were as follows: 1 cycle of 50°C for 2 min, 95°C for 10 min, then 50 cycles of 95°C for 15 s, 60°C for 1 min, and ended with 1 cycle of 60°C for 1 min. Threshold cycles (Ct) were determined using Bio-Rad’s iCycler 3.0a version software, allowing baseline to be set automatically by the software. Relative changes in gene expression were calculated using the 2-ΔΔCt equation. Results were normalized to 18S RNA levels.

**Transcription of CYP1A1.** The effect of ACS 15 and 18 on the transcription of the CYP1A1 gene was evaluated by measuring the level of heterogeneous nuclear RNA by real-time PCR, as described by Elferink and Reiners (21) and modified by Guigal et al. (22). This assay has been well-characterized as a valid substitute for nuclear run-on experiments as a measure of transcription rates. Sequences for hncYP1A1 forward and reverse primers were CTTGGAACCTTG-GAGCTG and TGACTGTGTCAAAACCTGGA, respectively. Amplification conditions were 15 min at 95°C, followed by 45 cycles of 15 s at 94°C, 30 s at 60°C, and 30 s at 72°C. The level of hncYP1A1 was normalized to the level of 18S RNA expression.

**Chromatin immunoprecipitation assay.** Cells were incubated with DMSO (0.06%), 10 nmol/L TCDD alone, 50 nmol/L ACS 15 or 18 alone, or a combination of TCDD and the ACS compounds as indicated for 90 min at 37°C. The chromatin immunoprecipitation (ChIP) assay was conducted as previously described (23). ChIP DNA was purified for PCR by using QIAquick PCR Purification kit from Qiagen according to the manufacturer’s protocol. Real-time PCR for the xenobiotic responsive element (XRE) of CYP1A1 was conducted with primers and conditions described by Hestermann and Brown (24) on a Bio-Rad iCycler Real Time Detection System. Results were calculated as described above and normalized to input sample DNA.

**Assay of CYP enzyme activity.** The ability of ACS 15 and 18 to affect CYP enzyme activity was evaluated in intact cells by measurement of ethoxyresorufin-O-deethylase (EROD) activity as previously described (18).

**Recombinant CYP1A1, CYP1A2, and CYP1B1 activity.** Recombinant CYP1A1 Supersomes (0.75 μmol/L), CYP1A2 Supersomes (2.5 μmol/L), or CYP1B1 Supersomes (3.5 μmol/L) were incubated with 0.4 μmol/L ethoxyresorufin and indicated concentrations of ACS 15 and 18 in a final volume of 100 μL of PBS (pH 7.2). The reaction was initiated by the addition of 500 μmol/L NADPH. The reaction mixture was transferred to a 96-well plate, and EROD activity was determined as described above. Enzyme kinetics were analyzed using the Michaelis-Menten model. Maximal velocities (Vmax) and dissociation constant parameters (Km) were calculated (Table 1) using the Michaelis-Menten equation: V = Vmax (S)/(Km + (S)).

**Statistical analysis.** Statistical analyses were done with StatView and JMP Statistical Analysis Software (SAS Institute). Differences between group mean values were determined by a one-factor ANOVA, followed by Fisher protected least significant difference post hoc analysis for pairwise comparison of means. Statistical analyses for the recombinant enzyme activity were done using the nile function in S-PLUS version 7 for Windows (InsightfulCorporation, 2005).

**Results**

For the concentration ranges used in these studies, 0 to 100 μmol/L ACS 15 and 18, we found the compounds to be nontoxic in both cell lines as measured by the cell viability assay measuring reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (data not shown).

**ACS 15 and 18 inhibit carcinogen-induced CYP mRNA expression.** TCDD (250 pmol) caused a 388-, 33.5-, and 52-fold increase in CYP1A1, CYP1A2, and CYP1B1 mRNA expression in HepG2 cells and a 99.3-, 92.6-, and 9-fold increase in LS180 cells compared with DMSO controls. Dimethylbenzanthracene (DMBA; 1 μmol/L) induced CYP1A1, CYP1A2, and CYP1B1 mRNA expression in HepG2 cells by 5-, 2- and 8.4-fold, and by 6.6-, 1.3-, and 2.5-fold in LS180 cells. These inductions were inhibited in a concentration-dependent manner.

**Table 1. Maximal velocities (Vmax) and dissociation constant parameters (Km) for the Michaelis-Menten curves analyzed for ACS 15 and ACS 18**

<table>
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<tr>
<th>Experiment</th>
<th>Vmax</th>
<th>Km</th>
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<tr>
<td>0 μmol/L ACS15 + recCYP1A1</td>
<td>4.4</td>
<td>324.4</td>
</tr>
<tr>
<td>0.05 μmol/L ACS15 + recCYP1A1</td>
<td>3.5</td>
<td>463.2</td>
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<tr>
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<td>1,085.9</td>
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<tr>
<td>50 μmol/L ACS15 + recCYP1A1</td>
<td>6.3</td>
<td>3,716.3</td>
</tr>
<tr>
<td>0 μmol/L ACS18 + recCYP1A1</td>
<td>6.6</td>
<td>416.2</td>
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<tr>
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<td>419.8</td>
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<td>50 μmol/L ACS18 + recCYP1A1</td>
<td>6.3</td>
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manner by cotreatment with ACS 15 (Fig. 2A-F). Results were similar for ACS 18 (data not shown).

**ACS 15 and 18 decrease the rate of CYP1A1 gene transcription.** The transcription rate of the CYP1A1 gene was measured by quantifying the level of heterogeneous nuclear RNA, or newly transcribed RNA, by reverse transcription-PCR. Incubation of HepG2 cells with TCDD (250 pmol; Fig. 3A) or DMBA (1 μmol/L; Fig. 3B) caused a 67.6- and 12-fold increase in hnCYP1A1 expression over DMSO control levels, respectively. Cotreatment with ACS 15 abrogated the stimulatory effects of both TCDD and DMBA. Results for ACS 18 were similar (data not shown).

**ACS 15 and 18 inhibit AhR activation.** The transcription of CYP1A1, CYP1A2, and CYP1B1 is primarily regulated by the AhR. The AhR is a cytosolic protein that, when activated by a ligand, i.e., polycyclic aromatic hydrocarbons [PAH; TCDD, DMBA, and benzo(a)pyrene (BP)], translocates to the nucleus and binds to its protein partner, the aryl hydrocarbon nuclear translocator (25). Together, this heterodimer forms a transcription factor that binds to a specific sequence of nucleotides, the XRE, located in the promoter region of a number of genes involved in xenobiotic metabolism, including CYPs. Because ACS 15 and 18 inhibit carcinogen-induced CYP1A1 transcription, we investigated whether this activity was mediated by AhR activation. To test this, the ChIP assay was used, in which binding of the activated AhR to the CYP1A1 XRE enhancer sequence was measured. We used primers and conditions as designed and tested by Hestermann and Brown (25). These primers amplify a region of the XRE 784 to 1156 bp upstream of the CYP1A1 transcriptional start site. It is known that both the TCDD-responsive CYP1B1 and CYP1A2 also contain XRE binding sites upstream of their transcriptional start sites. The CYP1A1 XRE binds to the CYP1B1 gene in gel shift assays and AhR binding to this region is confirmed by supershift (26).

Because the CYP1A1 and CYP1A2 genes are positioned in a head-to-head orientation, they share a common 5' upstream region and thus may share regulatory components. Multiple studies have shown the TCDD-responsive CYP1A1 enhancer also controls CYP1A2 expression (27, 28). The induction of CYP1A1 expression is therefore widely studied as a model for AhR activity.

Treatment of HepG2 cells with TCDD (10 nmol) caused a 6.2-fold induction of activated AhR (Fig. 3C). Cotreatment with ACS 15 and 18 inhibited the level of activated AhR to 2.6- and 0.32-fold, respectively, indicating that ACS 15 and 18 directly affect AhR activation. The ChIP assay is somewhat less sensitive than other assays done in this study, and therefore, a higher concentration of TCDD (10 nmol versus 250 pmol) was required to produce statistically significant
results. DMBA and BP are far less potent ligands of the AhR than TCDD and, therefore, could not be examined in this assay.

**ACS 15 and 18 inhibit PAH-induced EROD activities.** AhR-mediated enzymes are critical to the detoxification of PAHs, a widespread class of environmental pollutants. EROD activity is a measure of the cellular capacity for enzymatic activation of PAHs with regard to the carcinogen activating enzymes, CYP1A1, CYP1A2, and CYP1B1. Although EROD measures the combined activity of the three CYP isozymes, the 1A1 form has a much higher specific EROD activity than 1A2 or 1B1 (29). We investigated the effects of ACS 15 and 18, as well as their parent compounds, on EROD activity and PAH-induced EROD activity in both HepG2 and LS180 cell lines. Sulindac induced EROD activity by 4.45 \pm 0.09 pmoles/minute/well (Fig. 4A), as we have previously reported (23), and ACS 18 caused a slight induction of EROD activity (0.46 \pm 0.01 pmoles/minute/well). TCDD-induced EROD activity was not inhibited by 50 \mu M of either parent compound, sulindac, diclofenac; nor by NaHS, an H2S releasing compound. These results were duplicated for DMBA and BP-induced EROD activity (data not shown). Only the dithiolethione-modified compounds, ACS 15 and ACS 18, inhibited PAH-induced EROD activity. Further investigation of the ACS compounds on EROD activity show both compounds suppressed TCDD, DMBA, and BP-induced EROD activity in both cell lines (Fig. 4B-E). Inhibition by ACS 15 and 18 was concentration-dependent, and averaged, interpolated IC50s against TCDD, DMBA, and BP-induced EROD for ACS 15 were 38, 16, and 50 \mu M, respectively, and for ACS 18 were 22, 8.5, and 100 \mu M.

**ACS 15 and 18 directly inhibit recombinant CYP enzyme activity.** We investigated CYP enzyme inhibition by ACS 15 and 18 using recombinant CYP1A1, CYP1B1, and CYP1A2 supersomes to test whether the inhibition was due to a direct effect on the CYP enzymes. At low concentrations, ACS 15 caused a concentration-dependent decrease in CYP activity with interpolated IC50 values for CYP1A1, CYP1B1, and CYP1A2 of 0.05, 2.4, and 1.75 \mu M, respectively (Fig. 5A). ACS 18 also caused concentration-dependent decreases in CYP activity (Fig. 5B) with interpolated IC50 values for CYP1A1, CYP1B1, and CYP1A2 of 0.04, 0.23, and 3.1 \mu M, respectively. Kinetic analysis using the Michaelis-Menten model revealed an increase in \( K_m \) with increasing ACS 15 and 18 concentrations, whereas \( V_{max} \) remained unchanged (Table 1). This indicated CYP1A1 inhibition by both ACS 15 and 18 was competitive in nature. Residual plot analysis revealed a good fit by the data to the models. For ease in visual representation, results are displayed by the Lineweaver Burk plots (Fig. 5C and D).

**ACS 18 induces phase II enzyme mRNA expression.** The induction of phase II enzymes has been emphasized as being a critical step for the inactivation of chemical carcinogens (30). Other dithiolethiones such as ADT, 3H-1,2-dithiole-3-thione, and 5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione (oltipraz) have shown chemoprotective effects through the induction of phase II enzymes such as NAD(P)H:quinone oxidoreductase, glutathione S-transferase, and glutathione reductase (GR; refs. 31, 32). ADT-OH is released from S-diclofenac or S-sulindac after hydrolytic breakdown of the parent molecule in vivo (12). We further investigated the effect of the dithiolethione derivatives, ACS 15 and 18, on phase II gene expression. No significant increases were observed by ACS 15. We identified several phase II conjugation enzymes whose mRNA expression was up-regulated by ACS 18. ACS 18 (50 \mu M) increased the mRNA expression of GSTA2, glutamate cysteine ligase catalytic subunit, glutamate cysteine ligase modifier subunit, GR, and glucose-6 phosphate dehydrogenase by 3.6-, 1.9-, 1.6-, 1.6-, and 1.5-fold respectively over 18S RNA control as measured by reverse transcription-PCR (Fig. 6).

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**Fig. 3.** The effect of ACS15 on the transcription of CYP1A1 was measured by quantifying the expression of hnrCYP1A1 RNA by reverse transcription-PCR in HepG2 cells. Cells were treated with 250 pmol TCDD (A) or 1 \mu M DMBA (B) for 24 h, and levels of heterogeneous nuclear RNA were measured by reverse transcription-PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA. Columns, mean (n = 3); bars, SE. C: effect of ACS15 and 18 on the XRE-binding activity of the AhR. HepG2 cells were incubated for 90 min with DMSO or 10 \mu M TCDD with or without 50 \mu M ACS 15 or ACS 18. Cellular proteins were cross-linked and isolated by ChIP, and XRE mRNA was measured by real-time PCR and normalized to 18S RNA. Columns, mean (n = 6); bars, SE.
Discussion

Beginning in the early 1800s, with the isolation of salicin from willow bark, NSAIDs have become important therapeutic agents for the treatment of pain and inflammation. Since the 1970s, research has increasingly suggested that NSAIDs may be effective chemopreventive agents against colorectal and other gastrointestinal tumors. NSAIDs including sulindac, celecoxib, and aspirin have been shown to be among the most effective types of preventive agents against colorectal cancer; however, the mechanism of action has not been fully understood (1). Our laboratory has identified NSAIDs that modulate the activity of the AhR and affect CYP gene transcription and enzyme activity, e.g., salicylamide (33). We have also shown that sulindac and its metabolites modulate both phase I and phase II gene expression (18, 23).

In this study, we used three PAH: DMBA, TCDD, and BP. DMBA is a well-established model compound that induces mammary gland tumors in rodents (34). TCDD is a major environmental pollutant, generated by a variety of industrial processes that accumulates in fatty tissues of exposed animals. Humans’ primary source of exposure is through food intake, specifically the consumption of fish, meat, and dairy products. BP is an environmental contaminant found in coal tar, auto exhaust, tobacco smoke, and charbroiled foods. PAHs bind to the ligand-dependent AhR, initiating its activation and the
transcription of genes under its control. TCDD is the most potent ligand of the AhR. The PAHs are procarcinogens that are activated to highly reactive metabolites by the phase I enzymes, CYP1A1, CYP1A2, and CYP1B1. The reactive metabolites can enter the nucleus of cells and form adducts with DNA causing damage and mutations that may initiate carcinogenesis. Alternatively, the epoxide products generated by GTP metabolism may be further detoxified by pathways involving phase II enzymes including GSTA2, glutamate cysteine ligase catalytic subunit, glutamate cysteine ligase modifier, GR, and glucose-6 phosphate dehydrogenase. These enzymes mediate the biotransformation of xenobiotics into more hydrophilic and easily excretable products via the conjugation of glutathione to the xenobiotic.

Sulindac and diclofenac have been shown to inhibit PAH-induced tumorigenesis in animal models, including DMBA-induced mammary cancer and azoxymethane-induced colon cancer (17, 35). In response to the previously documented chemopreventive potential of sulindac and diclofenac, which is complicated by undesirable gastrointestinal side effects, the novel compounds ACS 15 and ACS 18 were generated. These ACS compounds have shown the ability to modify angiogenesis and to decrease gastrointestinal and cardiovascular toxicity (12–14). We undertook the current study to evaluate the effects of ACS 15 and 18 on the AhR signaling pathway and carcinogen-metabolizing enzyme gene expression in vitro. We investigated the effects of ACS 15 and 18 on phase I and phase II enzymes in HepG2 human hepatoma and LS180 human colon adenocarcinoma cells. HepG2 cells represent an aspect of liver metabolism for carcinogens, and both HepG2 and LS180 cells have been extensively used in studies of PAH metabolism and have a well-characterized AhR pathway (36, 37).

We examined the effects of ACS 15 and 18 on PAH-induced mRNA expression of CYP1A1, CYP1A2, and CYP1B1 in HepG2 and LS180 cells. We showed that both ACS 15 and 18 inhibited TCDD-induced CYP mRNA expression in a concentration-dependent manner (Fig. 2). We showed the inhibitory effect on CYP mRNA was mediated at the transcriptional level. The observed decrease in CYP1A1 heterogeneous nuclear RNA levels confirmed the decreased transcription rate, as heterogeneous nuclear RNA levels are not affected by posttranscriptional processing, transport, or stability. Cotreatment of ACS 15 or 18 with TCDD also blocked the binding of TCDD-activated AhR to the CYP1A1 enhancer sequence as shown by ChIP assay (Fig. 3C).

In agreement with our previous study that showed that sulindac is likely a weak agonist of the AhR (18), Fig. 4A

![Figure 5](cancertherapy-preclinical.org/15/6/1970/Fig5.png)
showed induction of EROD activity by both sulindac as well as ACS 18 in HepG2 cells. In contrast, ACS 15 and 18 also showed the ability to directly inhibit CYP enzymes with the strongest inhibitory effect shown against CYP1A1 enzyme activity (Fig. 5A and B). Analysis of the kinetics of recombinant CYP1A1 enzyme inhibition by Hanes-Woolf plot showed that the inhibition was competitive, i.e., $K_{in}$ values for the substrate were increased in the presence of ACS 15 and 18, whereas the $V_{max}$ remained constant. Both ACS 15 and 18 also inhibited DMBA and TCDD-induced EROD activity in intact cells, in a concentration-dependent manner, indicating the ability of the novel compounds to enter the intact cell and interact with the enzyme (Fig. 4B-E). The ability to inhibit PAH-induced CYP enzyme activity was unique to ACS 15 and 18; neither parent compound (sulindac nor diclofenac), nor other H$_2$S-releasing compounds, e.g., NaHS, could mimic the inhibitory effect (Fig. 4A). Bhatia et al. (38) showed a similar effect between ACS 15 and NaHS against lung inflammation in which ACS 15 showed protective effects, whereas other H$_2$S-releasing compounds could not. The authors concluded the effects may be contributed by the slow H$_2$S-releasing property of ACS 15 (and ACS 18), which may favor an anti-inflammatory role and other unknown unique activities. In summary, we show for the first time that the dithiolethione-modified compounds, ACS 15 and ACS 18, are potent inhibitors of the AhR pathway that block signal transduction initiated by PAHs. This mechanism of action is distinct and different from their parent compounds. In particular, in contrast to our previous work on sulindac that showed its chemoprotective mechanism to be through activation of the AhR as the first step in the detoxification process, ACS 18 was shown to inhibit the AhR. Both mechanisms can result in ultimate chemoprotection, especially when coupled with an induction in phase II enzymes, which we have also shown.

Oltipraz and related dithiolethiones are an important class of chemopreventive agents. The chemopreventive effect of dithiolethiones has been well-investigated in association with their ability to induce phase II gene expression through the Kelch ECH Associating Protein 1-nuclear factor E2 p45-related factor 2 pathway. The administration of oltipraz in rats results in 3- to 4-fold increases in hepatic cytosolic glutathione S-transferase activities and mRNA levels for some $\alpha$, $\mu$, and $\pi$ isoforms. The protective efficacy of oltipraz has been shown in animal studies and is currently under investigation in human clinical trials (39, 40). ADT is a dithiolethione that is thought to exert its chemoprotective effects by increasing levels of reduced glutathione (41) as well as inducing the phase II enzymes glutathione S-transferase, QR, and GR (31). Significant levels of cytotoxic protection were achieved with 25 $\mu$mol/L of ADT from increases in the phase II enzymes catalase and GR that were -2-fold (41). Dithiolethiones as a class have been recognized as being potent inducers of enzymes related to the maintenance of glutathione sources (42). We investigated the newly synthetic drugs, the dithiolethione-conjugated NSAIDs, ACS 15 and 18, on the expression of phase II genes related to the detoxification pathway regulated by glutathione cycles. ACS 18 treatment resulted in 1.5- to 3.5-fold increases in mRNA expression of GSTA2, glutamate cysteine ligase catalytic subunit, glutamate cysteine ligase modifier, GR, and glucose-6 phosphate dehydrogenase (Fig. 6). A coordinated increase in these enzymes would increase the overall availability of glutathione for conjugation of activated carcinogens, thereby aiding in their detoxification through increased water-solubility and excretion from the body.

H$_2$S has been shown to protect cells from oxidative stress mainly by increasing the production of glutathione (43). ADT has also been shown to be a powerful antioxidant with the ability to scavenge free radicals, inhibit lipid peroxidation, and induce catalase activity (41, 44). The mutagenic effects caused by TCDD have been attributed to its capability to generate reactive oxygen species, lipid peroxidation, and DNA damage (45, 46). The activation of TCDD is mediated by the AhR signaling pathway (46). We hypothesize that the inhibitory effect of the ACS compounds on the AhR, as well as the induction of glutathione-related phase II enzymes, in conjunction with the previously documented anti-inflammatory effects (12), will contribute to protective effects of ACS 15 and 18 against TCDD-induced oxidative stress.

AhR activation has been associated with an increase in cyclooxygenase-2 and chronic inflammation leading to increased cancer risk (47, 48). The backbone of the ACS compounds is the parent NSAID whose primary mechanism of action is the inhibition of cyclooxygenases. Additionally, in this study, we have shown ACS 15 and ACS 18 to be potent inhibitors of the AhR signaling pathway that simultaneously block carcinogen-activating phase I enzyme activity and expression while enhancing carcinogen detoxifying phase II enzymes in vitro. Our results support the model of S-NSAIDs as promising chemopreventive agents and identify the molecular mechanisms mediated by ACS 15 and 18. ACS 15 and 18 serve as important agents for prevention at the initiation stage of carcinogenesis induced by environmental carcinogens. Animal studies are needed to further confirm the mechanism of action toward chemoprevention in vivo.

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**Disclosure of Potential Conflicts of Interest**

P. Del Soldato and A. Sparatore are shareholders of Sulfridis, Milan, Italy. The company has patents on reagents used in this study. The other authors disclosed no potential conflicts of interest.
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References


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