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Modulation of Carcinogen Activation and Detoxification by Novel Dithiolethione-Modified NSAIDs in Human Hepatoma HepG2 and Colon LS180 Cells

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Abstract

Purpose—Nonsteroidal anti-inflammatory drugs (NSAIDs) 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-NSAIDs) on both arms of carcinogenesis.

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Conflict of interest

Dr. Piero Del Soldato is a shareholder of CTG Pharma, Milan, Italy. This company has patents on reagents used in this study. Dr. Anna Sparatore received a grant from CTG Pharma.

Statement of 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 manuscript 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 carcinogenesis, 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.

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 (GSTA2), glutamate cysteine ligase catalytic subunit (GCLC), glutamate cysteine ligase modifier subunit (GCLM), and glutathione reductase (GR).

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.

Keywords

aryl hydrocarbon receptor; CYP1A1; dithiolethione; sulindac; diclofenac

Introduction

The use of nonsteroidal anti-inflammatory drugs (NSAIDs) has been associated with a reduced incidence of colorectal, breast, esophageal, stomach, prostate, bladder, ovary, and lung cancers (1). However, chronic treatment with conventional non-selective 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, while 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 (NO)-releasing NSAIDs which were first described in 1994 (4) and have since been evaluated in several clinical trials in which their gastric sparing properties have been well established (5,6). Additional studies have shown NO-NSAIDs to be promising chemopreventive agents (7). Although NO is the best characterized of the gaseous transmitters, carbon monoxide and hydrogen sulfide (H₂S) have also been recognized for their ability to affect key physiological functions (8). In particular, H₂S has been demonstrated to exhibit complex, but poorly understood roles in inflammation with evidence for pro- as well as anti-inflammatory activities. H₂S has also been reported to reduce NSAID-induced gastric mucosal injury without affecting the functional suppression of prostaglandins (9). Additionally, an H₂S-releasing derivative of mesalamine reduced visceral sensitivity and pain perception in a rat model of irritable bowel syndrome (10). Finally, H₂S may also play a role in cardio protection (11). These observations, as well as the fact that many NSAIDs reduce endogenous H₂S formation(9), which may contribute to their tendency to cause gastric damage, led to the hypothesis that dithiolethione-modified NSAIDs (S-NSAIDs) may serve as valuable chemoprotective drugs.

The newly synthesized, novel compounds, S-diclofenac and S-sulindac, are comprised of an H₂S-releasing dithiolethione moiety, ADT-OH covalently conjugated with the parent NSAID by an ester linkage (Figure 1). The chemical synthesis of S-diclofenac, 2-[(2,6-dichlorophenyl)amino]benzeneacetic acid 4-(3*H*-1,2-dithiol-3-thione-5-yl)phenyl ester and S-sulindac, (Z)-5-Fluoro-2-methyl-1-[[4-(methylsulfinyl)phenyl]-methylene]-1*H*-indene-3-acetic acid 4-(5-thioxo-5*H*-[1,2]dithiol-3-yl)-phenyl ester, which are designated as ACS 15 and ACS 18, respectively, has been previously reported (12,13). Recent work on ACS 15 has shown promising results. Unlike the parent NSAID, ACS 15 did not effect blood pressure or heart rate in rat models (12) and in rabbit models, has shown anti-ischemic activities for cardio protection (14). In addition, ACS 15 was much better tolerated by the GI tract of rats, and enhanced the anti-inflammatory effect of diclofenac by interfering with transcriptional mechanisms necessary for the inflammatory response (12). The same compound as ACS 15, although coded differently, ATB-337 has also demonstrated to induce >90% less gastrointestinal damage than diclofenac in rats. ATB-337 also did not induce leukocyte adherence, and was more potent at reducing paw edema (15). These observations evidence the view that the S-NSAID model, in providing the benefit of small quantities of a dithiolethione moiety released over time, as well as the benefit of the parent NSAID, will provide an overall effect of enhanced anti-inflammatory activity with reduced gastrotoxicity and adverse cardiovascular effects.

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 demonstrated anti-angiogenic activities of the compounds in models of tumor-driven angiogenesis (14). Our laboratory has demonstrated the ability of sulindac as well as the dithiolethione ADT (5-[*p*-methoxyphenyl]-1,2-dithiole-3-thione) (Yeh, unpublished data), 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 (Manassas, VA). RPMI 1640, Eagle's minimum essential medium with Earle's balanced salt solution (EMEM), glutamine, fetal bovine serum, phosphate buffered saline (PBS), tris-HCl buffer, trypsin/EDTA, and TRIzol, were from Invitrogen (Carlsbad, CA). Protease inhibitor tablets were from Roche (Indianapolis, IN). 2,3,5,7-tetrachlorodibenzo-*p*-dioxin (TCDD) was from the Midwest Research Institute (Kansas City, MO). The Omniscript kit was from Qiagen (Valencia, CA). Recombinant CYP enzymes were from BD Biosciences (Bedford, MA). ACS 15 and ACS 18

were from Sulfidris (Milan, Italy). All other chemicals except where noted were from Sigma (St. Louis, MO).

Cell Culture

HepG2 cells were grown in RPMI 1640, and LS180 cells were grown in EMEM. Both media were supplemented with 10% fetal bovine serum and 2 mM L-glutamine. Cells were passed weekly with 0.05% trypsin/ 0.53 mM 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-Polymerase Chain Reaction (RT-PCR)

Confluent cells in 6-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 performed in a master mix containing 12.5 µl Applied Biosystem's TaqMan Universal PCR Master Mix (Foster City, CA), 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 (Hs00164383), GSTA2 (Hs00747232mH), GCLC (Hs00155249), GCLM (Hs00157694), GSR (Hs00167317), and G6PD (Hs00166169) on a Bio-Rad iCycler Real Time Detection System (Hercules, CA). Amplification conditions were as follows: 1 cycle of 50°C for 2 minutes, 95°C for 10 minutes, then 50 cycles of 95°C for 15 seconds, 60°C for 1 minute, and ended with 1 cycle of 60°C for 1 minute. Threshold cycles (C_T) 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 $2^{-\Delta\Delta C_T}$ 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 (hnRNA) 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 CTTGGACCTCTTTGGAGCTG and TGACTGTGTCAAACCCTGGA, respectively. Amplification conditions were 15 minutes at 95°C, followed by 45 cycles of 15 seconds at 94°C, 30 seconds at 60°C, and 30 seconds at 72°C. The level of hnCYP1A1 was normalized to the level of 18S RNA expression.

Chromatin Immunoprecipitation Assay (ChIP)

Cells were incubated with DMSO (0.06%), 10 nM TCDD alone, 50 µM ACS 15 or 18 alone, or a combination of TCDD and the ACS compounds as indicated for 90 minutes at 37°C. The ChIP assay was conducted as previously described (23). ChIP DNA was purified for PCR by using Qiagen's QIAquick PCR Purification Kit 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 (Hercules, CA). 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, -1A2, and -1B1 activity

Recombinant CYP1A1 Supersomes (0.75 μ M), CYP1A2 Supersomes (2.5 μ M) or CYP1B1 Supersomes (3.5 μ M) were incubated with 0.4 μ M ethoxyresorufin and indicated concentrations of ACS 15 and 18 in a final volume of 100 μ l of PBS, pH 7.2. The reaction was initiated by the addition of 500 μ M 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 (V_{max}) and dissociation constant parameters (K_m) were calculated using the Michaelis-Menten equation: $V = V_{max}[S] / (K_m + [S])$.

Statistical analysis

Statistical analyses were performed with StatView and JMP Statistical Analysis Software (SAS Institute, San Francisco, CA). Differences between group mean values were determined by a one-factor analysis of variance (ANOVA), followed by Fisher protected least significant difference post-hoc analysis for pairwise comparison of means. Statistical analyses for the recombinant enzyme activity were performed using the nlme function in S-PLUS version 7 for Windows. Seattle, Washington: Insightful Corporation (2005).

Results

For the concentration ranges utilized in these studies, 0–100 μ M ACS 15 and 18, we found the compounds to be non-toxic 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 (MTT) (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 to DMSO controls. Dimethylbenzanthracene (DMBA) (1 μ M) 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 by co-treatment with ACS 15 (Figure 2 A-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 hnRNA, or newly transcribed RNA, by RT-PCR. Incubation of HepG2 cells with TCDD (250 pmol) (Figure 3A) or DMBA (1 μ M) (Figure 3B) caused a 67.6- and 12-fold increase in hnCYP1A1 expression over DMSO control levels, respectively. Co-treatment 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 aryl hydrocarbon receptor (AhR). The AhR is a cytosolic protein that, when activated by a ligand, i.e. polycyclic aromatic hydrocarbons (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 which 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*. Since 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 employed, in which binding of the activated AhR to the *CYP1A1* XRE enhancer sequence was measured. We utilized 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 demonstrated 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 (Figure 3C). Co-treatment 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 performed 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 polycyclic aromatic hydrocarbon (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 ± 0.09 pmoles/min/well (Figure 4A), as we have previously reported (30), and ACS 18 caused a slight induction of EROD activity (0.46 ± 0.01 pmoles/min/well). TCDD-induced EROD activity was not inhibited by 50 μ 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 (Figure 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 μ M, 16 μ M, and 50 μ M respectively, and for ACS 18 were 22 μ M, 8.5 μ M, and 100 μ 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 μ M, 2.4 μ M and 1.75 μ M respectively (Figure 5A). ACS 18 also caused concentration-dependent decreases in CYP activity (Figure 5B) with interpolated IC₅₀ values for CYP1A1, CYP1B1, and CYP1A2 of 0.04 μ M, 0.23 μ M, and 3.1 μ M respectively. Kinetic analysis using the Michaelis-Menten model revealed an increase in K_m with increasing ACS 15 and 18 concentrations, while V_{max} remained unchanged (Figure 5E). 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 (Figure 5C,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 (31). Other dithiolethiones such as ADT, D3T (3*H*-1,2-dithiole-3-thione), and oltipraz (4-methyl-5-[2-pyrazinyl]-1,2-dithiole-3-thione) have shown chemoprotective effects through the induction of phase II enzymes such as NAD(P)H:quinone oxidoreductase (NQO1), glutathione S-transferase (GST), and glutathione reductase (GR) (32,33). 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 μ M) increased the mRNA expression of GSTA2, glutamate cysteine ligase catalytic subunit (GCLC), glutamate cysteine ligase modifier subunit (GCLM), GR, and glucose-6 phosphate dehydrogenase (G6PD) by 3.6-, 1.9-, 1.6-, 1.6-, and 1.5-fold respectively over 18S RNA control as measured by RT-PCR (Figure 6).

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 (34). We have also shown that sulindac and its metabolites modulate both phase I and phase II gene expression (18,23).

In this study, we utilized three polycyclic aromatic hydrocarbons (PAHs): DMBA, TCDD, and BP. DMBA is a well-established model compound that induces mammary gland tumors in rodents (35). 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 CYP metabolism may be further detoxified by pathways involving phase II enzymes including GSTA2, GCLC, GCLM, GR, and G6PD. 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 (17,36). 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 demonstrated 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 (37,38).

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 demonstrated that both ACS 15 and 18 inhibited TCDD-induced CYP mRNA expression in a concentration-dependent manner (Figure 2). We demonstrated the inhibitory effect on CYP mRNA was mediated at the transcriptional level. The observed decrease in CYP1A1 hnRNA levels confirmed the decreased transcription rate, as hnRNA levels are not affected by post-transcriptional processing, transport, or stability. Co-treatment 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 (Figure 3C).

In agreement with our previous study which demonstrated that sulindac is likely a weak agonist of the AhR (18), Figure 4A shows induction of EROD activity by both sulindac as well as ACS 18 in HepG2 cells. In contrast, ACS 15 and 18 also demonstrated the ability to directly inhibit CYP enzymes with the strongest inhibitory effect shown against CYP1A1 enzyme activity (Figure 5A,B). Analysis of the kinetics of recombinant CYP1A1 enzyme inhibition by Hanes-Woolf plot demonstrated that the inhibition was competitive, i.e. K_m values for the substrate were increased in the presence of ACS 15 and 18, while 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 (Figure 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_2S -releasing compounds, e.g., NaHS, could mimic the inhibitory effect (Figure 4A). Bhatia et. al. demonstrated a similar effect between ACS 15 and NaHS against lung inflammation in which ACS 15 showed protective effects whereas other H_2S -releasing compounds could not (39). The authors concluded the effects may be contributed by the slow H_2S -releasing property of ACS 15, (and ACS 18), which may favor an anti-inflammatory role and other unknown unique activities. In summary, we demonstrate 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 (Keap1-Nrf2) pathway. The administration of oltipraz in rats results in 3-to 4-fold increases in hepatic cytosolic GST activities and mRNA levels for some α , μ , and π isoforms. The protective efficacy of oltipraz has been demonstrated in animal studies and is currently under investigation in human clinical trials (40,41). ADT is a dithiolethione that is thought to exert its chemoprotective effects by increasing levels of reduced glutathione (42) as well as inducing the phase II enzymes GST, QR, and GR (32). Significant levels of cytotoxic protection were achieved with 25 μM of ADT from increases in the phase II enzymes catalase and glutathione reductase that were less than 2 fold (42). Dithiolethiones as a class

have been recognized as being potent inducers of enzymes related to the maintenance of glutathione sources (43). 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, GCLC, GCLM, GR, and G6PD (Figure 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.

H₂S has been shown to protect cells from oxidative stress mainly by increasing the production of glutathione (44). ADT has also been shown to be a powerful antioxidant with the ability to scavenge free radicals, inhibit lipid peroxidation, and induce catalase activity (42,45). The mutagenic effects caused by TCDD have been attributed to its capability to generate reactive oxygen species, lipid peroxidation, and DNA damage (46,47). The activation of TCDD is mediated by the AhR signaling pathway (47). 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 (48,49). 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 demonstrated 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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Abbreviations

AhR, aryl hydrocarbon receptor
 BP, benzo[*a*]pyrene
 ChIP, chromatin immunoprecipitation assay
 CYP, cytochrome P450
 DMBA, dimethylbenzanthracene
 DMSO, dimethyl sulfoxide
 EROD, ethoxyresorufin-*O*-deethylase
 G6PD, glucose-6 phosphate dehydrogenase
 GAPDH, glyceraldehyde-3-phosphate dehydrogenase
 GCLC, glutamate cysteine ligase catalytic subunit
 GCLM, glutamate cysteine ligase modifier subunit
 GR, glutathione reductase
 GST, glutathione S-transferase

H₂S, hydrogen sulfide
 NO, nitric oxide
 NSAIDs, non-steroidal anti-inflammatory drugs
 NQO1, NAD(P)H:quinone oxidoreductase
 PAH, polycyclic aromatic hydrocarbon
 RT-PCR, reverse transcription-polymerase chain reaction
 S-NSAIDs, Dithiolethione-NSAIDs
 TCDD, 2,3,5,7-tetrachlorodibenzo-*p*-dioxin
 UGT, UDP-glucuronosyltransferase
 XRE, xenobiotic responsive element

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FIGURE 1.
Chemical structures of dithiolethione, ADT-OH, ACS 15 and ACS 18.

FIGURE 2.

Effect of ACS 15 on carcinogen-induced CYP1A1, CYP1A2, and CYP1B1 mRNA expression. HepG2 (A,B,C) or LS180 (D,E,F) cells were treated with 250 pmol TCDD or 1 μM DMBA with or without increasing concentrations of ACS 15 as indicated for 24 hours. Levels of mRNA were measured by semi-quantitative PCR, and normalized to 18S RNA. n = 3 + SE. (*P<.001)

FIGURE 3.

The effect of ACS 15 on the transcription of *CYP1A1* was measured by quantifying the expression of hnCYP1A1 RNA by RT-PCR in HepG2 cells. Cells were treated with 250 pmol TCDD (**A**) or 1 μM DMBA (**B**) for 24 hours, and levels of hnRNA were measured by RT-PCR and normalized to GAPDH mRNA. N=3 + SE. (**C**) – Effect of ACS 15 and 18 on the XRE-binding activity of the AhR. HepG2 cells were incubated for 90 minutes with DMSO or 10 nmol TCDD with or without 50 μM 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. N = 6 ± SE.

FIGURE 4.

A. Confluent HepG2 cells were treated with 250 pmol TCDD, 50 μ M ACS or parent compound alone or in combination for 24 hours before enzyme activity was measured by EROD. **B-E.** Concentration dependent inhibition of carcinogen-induced EROD activity by ACS 15 and 18. Confluent HepG2 (**B,D**) or LS180 (**C,E**) were treated with 250 pmol TCDD, or 1 μ M DMBA or BP either alone or in combination with 50 μ M ACS 15 (**B,C**) or ACS 18 (**D,E**) for 24 hours before measuring EROD activity. Results are shown as EROD activity relative to control. For all, $n = 4 \pm$ standard error (SE).

FIGURE 5.

Effect of ACS 15 and 18 on recombinant CYP enzyme activity. EROD activity of 0.75 μM CYP1A1 supersomes, 2.5 μM CYP1A2 supersomes, or 3.5 μM CYP1B1 supersomes was measured in the presence of DMSO (control) or the indicated concentrations of (A) ACS 15 or (B) ACS 18. $N = 4 \pm$ standard error (SE). EROD activity of 0.75 μM CYP1A1 supersomes was measured at the indicated concentrations of ethoxyresorufin (substrate) with increasing concentrations of (C) ACS 15 and (D) ACS 18. Results are represented by Lineweaver Burk plot, $n = 4$. (E) Maximal velocities (V_{max}) and dissociation constant parameters (K_m) for the Michaelis-Menten curves analyzed for ACS 15 and ACS 18.

FIGURE 6.

Effect of ACS 15 and 18 on phase II enzyme expression. HepG2 cells were treated for 24 hours with 10 or 50 μM ACS 18 and the mRNA expression levels for GSTA2, GCLC, GCLM, GR, and G6PD were measured by real time PCR and normalized to 18S RNA. $n = 3 \pm SE$.