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Studies on the Metabolism and Biological Activity of the Epimers of Sulindac

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ABSTRACT:

Sulindac is a nonsteroidal, anti-inflammatory drug (NSAID) that has also been studied for its anticancer activity. Recent studies suggest that sulindac and its metabolites act by sensitizing cancer cells to oxidizing agents and drugs that affect mitochondrial function, resulting in the production of reactive oxygen species and death by apoptosis. In contrast, normal cells are not killed under these conditions and, in some instances, are protected against oxidative stress. Sulindac has a methyl sulfoxide moiety with a chiral center and was used in all of the previous studies as a mixture of the *R*- and *S*-epimers. Because epimers of a compound can have very different chemical and biological properties, we have separated the *R*- and *S*-epimers of sulindac, studied their individual metabolism, and performed preliminary experiments on their effect on normal and lung cancer cells exposed to oxidative stress. Previous results had indicated that the reduction of (*S*)-sulindac to sulindac sulfide, the active NSAID, was catalyzed by methionine sulfoxide reductase (Msr) A. In the present study, we purified an enzyme that reduces (*R*)-sulindac and resembles MsrB in its substrate specificity. The oxidation of both epimers to sulindac sulfone is catalyzed primarily by the microsomal cytochrome P450 (P450) system, and the individual enzymes responsible have been identified. (*S*)-Sulindac increases the activity of the P450 system better than (*R*)-sulindac, but both epimers increase primarily the enzymes that oxidize (*R*)-sulindac. Both epimers can protect normal lung cells against oxidative damage and enhance the killing of lung cancer cells exposed to oxidative stress.

Introduction

Sulindac was originally developed as a nonsteroidal, anti-inflammatory drug (NSAID) (Van Arman et al., 1976; Huskisson and Scott, 1978). Sulindac is a prodrug with a sulfoxide moiety that requires in vivo reduction to sulindac sulfide, the pharmacologically active metabolite (Duggan et al., 1977). Because sulindac has a chiral sulfur center, it can exist as either the *R*- or *S*-epimer. Earlier in vivo studies on the metabolism of sulindac, containing a mixture of both epimers, showed that sulindac could be reduced to sulindac sulfide and oxidized to sulindac sulfone (Fig. 1) (Duggan et al., 1977; Ratnayake et al., 1981).

Little is known about the enzymes that are involved in the reduction of sulindac to sulindac sulfide or the oxidation of sulindac to sulindac sulfone. Our initial interest in sulindac arose from the finding that this drug, which contains a methyl sulfoxide moiety, is a substrate for methionine sulfoxide reductase (Msr) A (Moskovitz et al., 1996; Etienne et al., 2003). The Msr family of enzymes are primarily responsible for the reduction of protein-bound methionine sulfoxide to methionine and appear to play an important role in protecting cells against oxidative damage and aging (for review, see Weissbach et al., 2005). This enzyme family includes two major classes, MsrA and MsrB, that specifically reduce the *S*- and *R*-epimers of methionine sulfoxide, Met-*S*-(o) and Met-*R*-(o), respectively (Sharov et al., 1999; Grimaud et al., 2001). In mammalian cells there is one *MsrA* gene and three *MsrB* genes, referred to as *MsrB1*, *MsrB2*, and *MsrB3* (Kim and Gladyshev, 2004). In addition to protein-bound Met-*S*-(o), MsrA can efficiently reduce the *S*-epimer of free methionine sulfoxide and other methyl sulfoxide compounds (Moskovitz et al., 1996). That the *S*-epimer of sulindac was specifically reduced by MsrA was verified in later studies (Etienne et al., 2003). In contrast, MsrB from *E. coli* and PilB from *Neisseria gonorrhoeae* appear to be relatively specific for Met-*R*-(o) in proteins with very weak activity toward free Met-*R*-(o) (Grimaud et al., 2001; Lowther et al., 2002). However, a partially purified preparation of MsrB1 from mouse liver has been reported to reduce free Met-*R*-(o) (Moskovitz et al., 2002).

With regard to the oxidation of sulindac to sulindac sulfone, sulindac oxidation in liver microsomes was reported (Kitamura and Tatsumi, 1982) and found to be enhanced by 3-methylcholanthrene treatment. It has been reported (Ciolino et al., 2006, 2008) that sulindac induces some members of the microsomal P450 system via a mechanism involving the aryl hydrocarbon receptor (AHR), but the specific enzymes responsible for sulindac oxidation were not identified. No studies on the oxidation of the individual epimers of sulindac have been reported.

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ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory; Msr, methionine sulfoxide reductase; Met-*S*-(o), *S*-epimer of methionine sulfoxide; Met-*R*-(o), *R*-epimer of methionine sulfoxide; AHR, aryl hydrocarbon receptor; TBHP, tert-butyl hydroperoxide; DABS, 4-*N,N*-dimethylaminoazobenzene-4-sulfonyl; DTT, dithiothreitol; TrxA, thioredoxin; TrxB, thioredoxin reductase; HPLC, high-performance liquid chromatography.

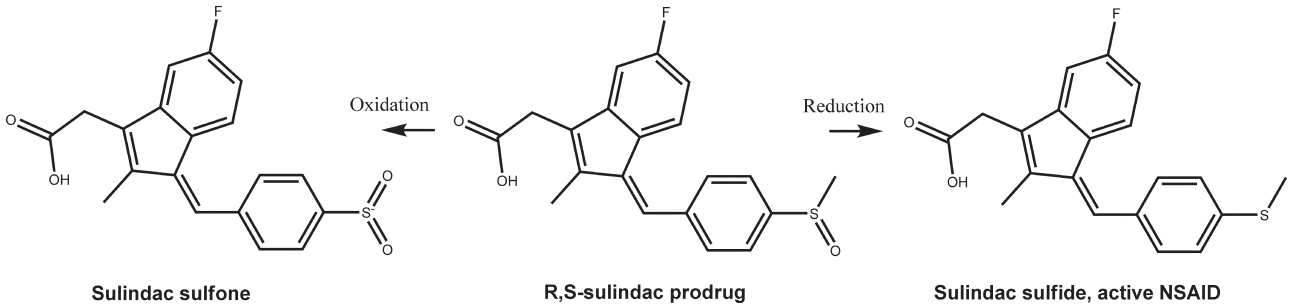

FIG. 1. Structure of sulindac and its reduced and oxidized metabolites. The methyl sulfoxide moiety of sulindac can be reduced to sulindac sulfide or oxidized to sulindac sulfone.

In addition to its anti-inflammatory effects, sulindac and its metabolites have been shown to have anticancer activity in a number of cancer cell lines and have also been tested clinically (Hixson et al., 1994; Taketo, 1998a,b; Gwyn and Sinicrope, 2002). There has been renewed interest in sulindac because of several reports that sulindac can selectively kill cancer cells in culture when combined with compounds that affect mitochondrial respiration (Park et al., 2008; Seo et al., 2008; Marchetti et al., 2009). As an example, recent results from our laboratory, using sulindac with either hydrogen peroxide or *tert*-butyl hydroperoxide (TBHP), showed that mitochondrial dysfunction, leading to ROS production, was responsible for the selective, enhanced killing of colon, lung, and skin cancer cells in culture, In addition, there have been two promising clinical studies using sulindac drug combinations. A limited, proof-of-concept clinical study, using sulindac and hydrogen peroxide for the treatment of actinic keratoses, showed a 50% cure rate (Resnick et al., 2009). In a second, larger, 3-year clinical study, sulindac was used in combination with difluoromethylornithine, an inhibitor of ornithine decarboxylase, to determine its effect on the recurrence of colon polyps. The patients receiving the drug combination showed an 80% decrease in recurrence of colon polyps and a 90% drop in the appearance of adenocarcinomas, compared with the control group of patients (Meyskens et al., 2008). However, there is no information on the activity of the individual sulindac epimers in any of the anticancer studies.

In contrast to the results in studies with cancer cells, pretreatment of normal lung cells with sulindac followed by exposure to TBHP protected these cells against oxidative stress (Marchetti et al., 2009). These studies have recently been extended to cardiac tissue using a Langendorf model, under conditions of ischemia and reoxygenation known to cause oxidative damage to cardiac tissue. It was shown that under these conditions, sulindac protected cardiac tissue from oxidative damage by a chemical preconditioning mechanism (Moench et al., 2009).

Because it is well established that epimers of a given compound can have distinct biological properties, we were interested in studying the *R*- and *S*-epimers of sulindac because of the diverse biological activities of sulindac. In the present report we have separated the *R*- and *S*-epimers of sulindac with two goals. The first was to elucidate the enzymatic systems involved in their metabolism, and the second was to examine their effect on normal lung and lung cancer cells exposed to oxidative stress.

Materials and Methods

Materials. Methionine sulfoxide, dabsyl chloride (4-*N*,*N*-dimethylaminoazobenzene-4-sulfonyl chloride) (DABS), sulindac [(*Z*)-5-fluoro-2-methyl-1-[*p*-(methylsulfinyl)benzylidene]indene-3-acetic acid], NADPH, glucose 6-phosphate, and dithiothreitol (DTT) were purchased from Sigma-Aldrich (St. Louis, MO), unless specified otherwise. Sulindac *R*- and *S*-epimers were purchased from Custom Synthesis, Inc. (Delray Beach, FL), and we also prepared them by separation of the epimers using a modification of a procedure described previously (Hamman et al., 2000). The sulindac epimers were dissolved in 1.0 M Tris buffer, pH 8.0. DABS-*S*-Met-(o) and DABS-*R*-Met-(o) were prepared as described previously (Minetti et al., 1994). Clones containing the genes for *E. coli TrxA* and *TrxB*, *E. coli* and bovine *MsrA*, and human *MsrB2* and *MsrB3* were overexpressed in *E. coli*, and the respective proteins were purified as described previously (Rahman et al., 1992; Moskovitz et al., 1996; Sagher et al., 2006a,b). Human and rat recombinant cytochrome P450 enzymes (Supersomes) were obtained from BD Gentest (Woburn, MA). The individual P450 enzymes contained various amounts of NADPH cytochrome P450 reductase, and rat 2B1 and 3A2 and human 3A4 and 2C8 enzymes also contained cytochrome b_5. Sprague-Dawley rats were supplied by Charles River Laboratories, Inc. (Wilmington, MA). A GST fusion protein (36.5 kDa) containing the first 95 amino acids of mammalian MsrB1 (10.5 kDa) and the anti-SepX1 antibody were purchased from Abnova (Taipei City, Taiwan). The GST tag was removed with PreScission protease (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

In Vivo Experiments. The sulindac epimers (2 mg/animal) were administered by intraperitoneal injection to 20 rats, 10 receiving the *R*-epimer and 10 receiving the *S*-epimer. Animals were euthanized 0.5 to 4 h postinjection. Heparinized blood was then collected, and the plasma was separated by centrifugation at 18,000*g* for 20 min. Liver, skin, and brain tissues were minced and then homogenized using 5 strokes in a Potter-Elvehjem homogenizer with 3 volumes of 50 mM Tris-Cl, pH 7.4 (buffer A). The homogenates were centrifuged for 20 min at 16,000*g*, and the supernatant was assayed.

HPLC Assay for Msr Activity and Sulindac Reduction. The Msr assay was based on the reduction of DABS-Met(o) to DABS-Met, adapted to HPLC analysis (Minetti et al., 1994; Moskovitz et al., 1997). Reaction mixtures contained 100 mM Tris, pH 7.4, 20 nmol of the indicated DABS-Met(o) epimer, and partially purified rat liver extract in a final volume of 100 μl. The reducing system consisted of either 15 mM DTT or the Trx system [1 mM NADPH, 1.2 μg of TrxB, and 5 μg of TrxA]. Incubations were performed at 37°C for the time specified. Under these conditions, the enzymatic reduction was proportional to the enzyme concentration and time until 75% (15 nmol) of the substrate was reduced. The reactions were terminated by adding 300 μl of acetonitrile. After centrifugation, 20 μl of the supernatant was injected onto a 4.6 × 75 mm C18 column (3.5-μm particle size; Waters, Milford, MA) and eluted with a buffer consisting of 55% 50 mM sodium acetate buffer, pH 4.73, and 45% acetonitrile. DABS-Met(o) and DABS-Met eluted at 0.98 and 1.7 min, respectively. Detection was by absorbance at 436 nm.

The enzymatic reduction of sulindac was assayed under the same reaction conditions but using 20 nmol of sulindac or its epimers as substrate. The amount of sulindac sulfide formed was assayed by HPLC using the same C18 column, and detection was at 330 nm. In these experiments, the eluting solution was 25% 50 mM sodium acetate buffer, pH 4.73, and 75% acetonitrile, and sulindac and sulindac sulfide eluted at 0.88 and 1.37 min, respectively. For sulindac sulfone detection, the eluting solution contained 50% 50 mM sodium acetate buffer, pH 4.73, and 50% acetonitrile, and the elution times for sulindac, sulindac sulfone, and sulindac sulfide, were 1.1, 1.5, and 7.1 min, respectively.

Isolation of Hepatic Microsomes and Oxidation of the Sulindac Epimers. Fresh rat liver was minced and then homogenized using 5 strokes in a Potter-Elvehjem homogenizer with 3 volumes of 50 mM Tris-Cl, pH 7.4. The

homogenate was centrifuged for 15 min at 10,000*g*, the pellet was discarded, and the supernatant was spun at 100,000*g* for 2 h to obtain crude microsomes. The microsomes were washed by resuspension in 3 volumes of the above buffer and centrifugation at 100,000*g* for 1 h. The microsomal pellet was finally resuspended in 3 volumes of the above buffer, aliquoted, and frozen at −80°C. The protein concentration was 25 mg/ml. In a total volume of 100 μl, 20 nmol of each sulindac epimer were incubated with 20 to 80 μg of microsomes and a NADPH-regenerating system (1.5 mM NADPH, 5 mM glucose 6-phosphate, 150 ng of glucose-6-phosphate dehydrogenase, and 5 mM $MgCl_2$) in potassium phosphate buffer (pH 7.4, 100 mM) for 60 min at 37°C. The reaction was then stopped with 3 volumes of acetonitrile and centrifuged. The supernatant was fractionated by HPLC as described above.

Oxidation of the Sulindac Epimers by Recombinant P450 Enzymes. Recombinant rat or human P450 enzymes (Supersomes) were incubated with each sulindac epimer and a NADPH-regenerating system and analyzed via HPLC as described above. Protein concentration curves and kinetic experiments were initially performed with the individual isoforms that showed the highest activities with the sulindac *R*- and *S*-epimers to ensure that the enzymatic reactions were linear with respect to protein concentration and for time periods up to 90 min. Once the enzymatic parameters were established, each P450 enzyme was assayed at least three times. In addition, it was determined that the concentration of substrate (sulindac epimer) was saturating between 200 and 500 μM and routinely either 200 or 500 μM substrate was used. The lower concentration of substrate was routinely used for the oxidation of the sulindac *S*-epimer with the P450 enzymes because the activity was quite low. The lower substrate concentration made it possible to detect the low levels of sulindac sulfone in the presence of the large excess of sulindac coming off the HPLC column. Positive controls for enzyme activity were performed with various substrates according to the manufacturer's (BD Gentest) recommendations. Two separate batches of the most-active P450 enzymes were purchased from the manufacturer, and they gave similar results.

HepG2 Cells Exposed to Sulindac Show Increased Activity of P450 Enzymes. HepG2 human hepatocarcinoma cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in modified Eagle's medium containing 10% fetal bovine serum. Cells were grown in 24-well plates to 80% confluence. For induction, 1 ml of fresh medium containing a 125 μM concentration of either the *R*- or *S*-epimer of sulindac was added per well, and the cells were incubated in 5% CO_2 at 37°C for the times indicated. To make certain that the number of cells was similar in each experiment, all wells were seeded simultaneously, and the sulindac epimers were added at the appropriate times relative to the end of the induction period. The cells were washed with phosphate-buffered saline and 100 μl/well of fresh medium containing 200 μM (*R*)-sulindac or (*S*)-sulindac were added for the assay. This second incubation was carried out for 1 h at 37°C. After centrifugation, the medium was analyzed by HPLC for sulindac and its metabolites after addition of 3 volumes of acetonitrile as described above. Control experiments showed that the cell pellets contained only trace amounts of sulindac sulfone; therefore, only the medium was analyzed in these studies.

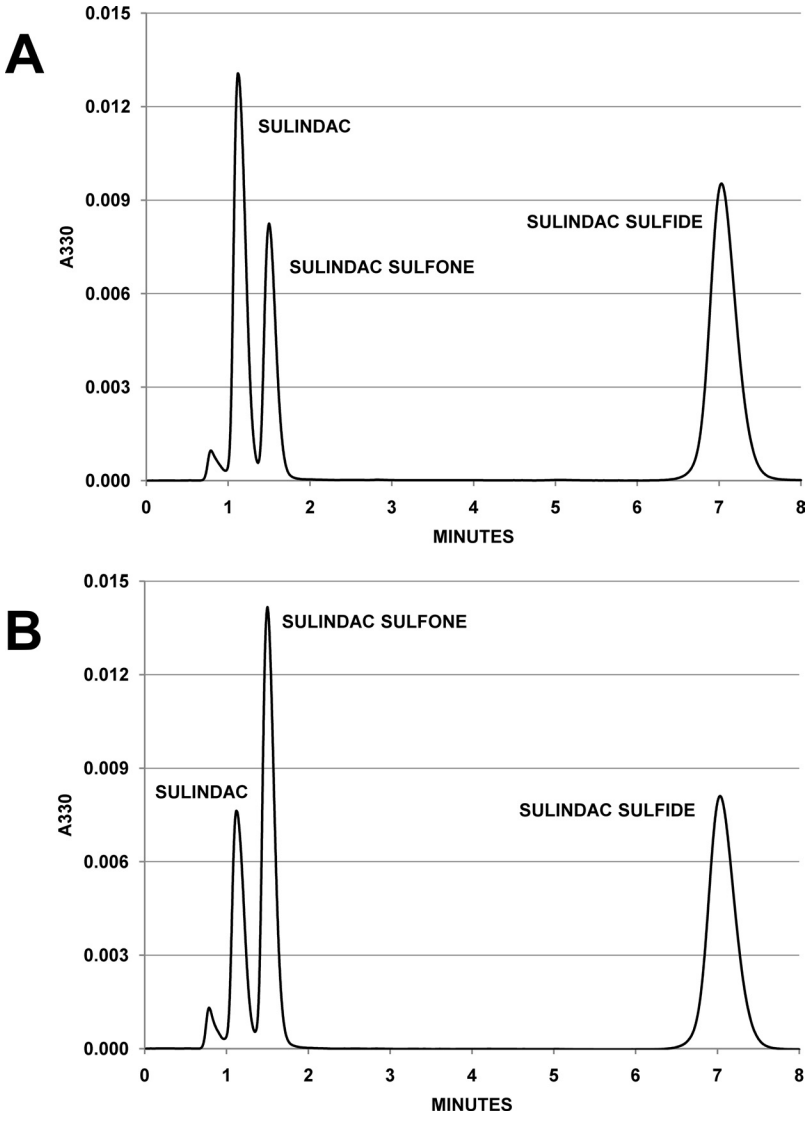

FIG. 2. Separation of sulindac and its metabolites from the plasma of rats 4 h after intraperitoneal injection of the *R*- and *S*-epimers of sulindac (see *Materials and Methods*). Plasma samples were run on a C18 column and separated as described under *Materials and Methods*, using conditions suitable for detection of sulindac sulfone. Sulindac appears at 1.1 min, sulindac sulfone at 1.5 min, and sulindac sulfide at 7.1 min. A, (*S*)-sulindac metabolites. B, (*R*)-sulindac metabolites. The ratio of sulindac sulfone to sulindac sulfide was 2.35 ± 0.19 (mean ± S.E.M.) in the animals treated with the *R*-epimer and 1.25 ± 0.49 in the animals treated with the *S*-epimer ($n = 10$, $p < 0.05$).

Purification of (*R*)-Sulindac Reductase from Rat Liver. Rat livers were homogenized in a Waring blender at high speed for 60 s in 3 volumes of 50 mM Tris buffer, pH 7.4, containing 1 mM DTT and 1 mM EDTA. The homogenate was centrifuged for 20 min at 15,000*g*, and the supernatant was further centrifuged for 2 h at 100,000*g* (S-100). The supernatant was subjected to ammonium sulfate precipitation, and the proteins precipitating between 30 and 70% saturation were resuspended in 20 mM Tris-Cl, pH 8, 1 mM DTT, and 1 mM EDTA and dialyzed against this buffer. Three grams of protein were applied to a 100-ml DEAE column and eluted using 20 mM Tris-Cl, pH 8, 1 mM DTT, and 1 mM EDTA with a salt gradient from 0 to 200 mM KCl. The major activity peak eluted at a salt concentration of approximately 100 mM. The active fractions from several runs were pooled, concentrated, and applied to a G50 superfine gel filtration column equilibrated with 50 mM Tris, pH 7.4, containing 1 mM DTT, 1 mM EDTA, and 0.15 M NaCl. The active fractions (21–26) were used for gel analyses, Western blots, and other assays.

SDS-Polyacrylamide Gel Electrophoresis and Western Blot of Purification Fractions. Proteins from various purification steps were separated on 4 to 12% NuPAGE gels (Invitrogen, Carlsbad, CA), with 2 μg of protein being loaded into each lane. After vertical electrophoretic separation, the proteins were blotted to a polyvinylidene difluoride membrane and probed with rabbit polyclonal antibody (Abnova) against SepX1 (MsrB1). A truncated, recombinant MsrB1 protein was used as a positive control, with and without cleavage of the GST moiety by PreScission protease (see *Materials*).

Results

Sulindac Metabolites Detected in Normal Rat Tissues. It is known that sulindac, which is an equal mixture of the *R*- and *S*-epimers, can be reduced to sulindac sulfide and oxidized to sulindac sulfone in vivo (Duggan et al., 1977). As a first step in establishing the in vivo metabolic pathways for the sulindac epimers, rats received intraperitoneal injections of the individual epimers (see *Materials and Methods*). The sulindac metabolites found in plasma, liver, skin, and brain were then analyzed by HPLC, and, regardless of which epimer was given, sulindac and its two metabolites, sulindac sulfone and sulindac sulfide, were found at detectable levels. A typical HPLC result from plasma is shown in Fig. 2. The ratio of sulindac sulfone to sulindac sulfide in plasma was greater by a factor of more than 2 when rats were treated with the *R*-epimer compared with the *S*-epimer (see legend to Fig. 2). Sulindac, sulindac sulfone, and sulindac sulfide were also found in liver, skin, and brain tissues but at lower concentrations than that in plasma (data not shown).

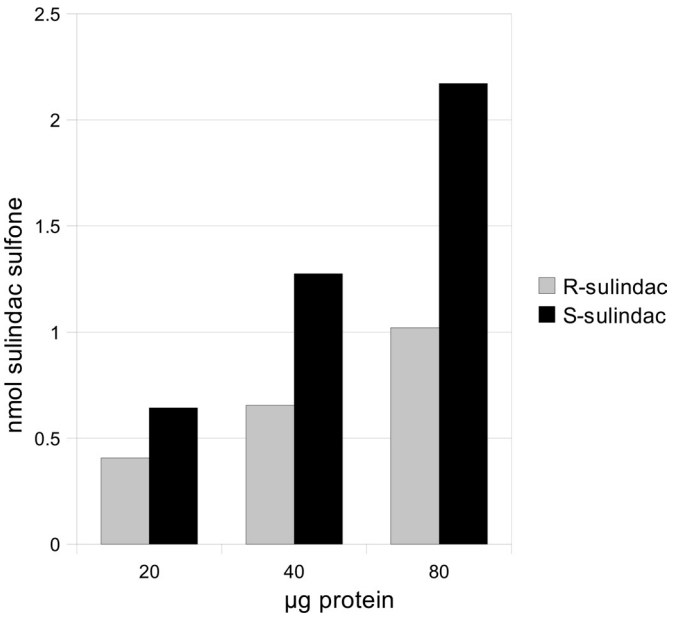

FIG. 3. Effect of rat liver microsome concentration on sulindac sulfone formation. Reaction mixtures and HPLC analysis are as described under *Materials and Methods*. The results shown, representing a microsome preparation from one of six rats, demonstrate a concentration dependence on the amount of microsomes added and greater amount of sulfone formation from the *S*-epimer than from the *R*-epimer. For the six microsome preparations that were tested, the ratio of the amount of sulindac sulfone formed from the *S*-epimer to that formed from the *R*-epimer was 2.1 ± 0.31 (mean ± S.E.M.) when 40 μg of microsomal protein was used.

TABLE 1

Sulindac oxidation by cytochrome P450 enzymes

Each enzyme was tested at least three times with each epimer. The substrate concentrations used were 500 μM for the *R*-epimer and 200 μM for the *S*-epimer; further details are described under *Materials and Methods*. Not included in the table are enzymes with very low activity for both epimers, including rat 3A2 and 2B1 and human 2C8, 2C9, 2C19, and 2D6. Values in the table are given as mean ± S.E.

P450 Enzyme	(*R*)-Sulindac	(*S*)-Sulindac
	pmol sulindac/pmol enzyme/h	
Rat 1A1	380 ± 22	22 ± 1.0
Rat 1A2	460 ± 15	24 ± 2.8
Human 1A2	130 ± 5.1	34 ± 3.5
Human 1B1	73 ± 0.8	0 ± 0
Human 3A4	47 ± 1.2	29 ± 3.5

Oxidation of Sulindac Epimers by Rat Liver Microsomes and Purified P450 Enzymes. Because many drugs are metabolized by the P450 system, the ability of rat liver microsomes to oxidize both the *R*- and *S*-epimers was examined. As seen in Fig. 3, rat liver microsomes, under uninduced conditions, catalyzed the oxidation of both *R*- and *S*-epimers. The reaction was dependent on microsome concentration, and no activity was seen in the absence of the NADPH-regenerating system (data not shown). Furthermore, no activity was detected in the liver cytosolic fraction (data not shown). Under these in vitro conditions with crude microsomes, the *S*-epimer is oxidized to the sulfone at a rate roughly twice that of the *R*-epimer. This is in contrast to what was seen in the plasma of rats injected with the sulindac *R*- and *S*-epimers (Fig. 2), in which the *R*-epimer was converted to the sulfone more efficiently than the *S*-epimer. The lower production of sulfone from the *R*-epimer in the isolated microsomes is probably due to the fact that the microsomes in these experiments were from animals not exposed to sulindac, so there was no induction of the P450 system (see below).

To determine which specific P450 enzymes might be responsible for metabolism of the sulindac epimers, 11 rat or human recombinant P450 enzymes were incubated with each epimer and a NADPH-regenerating system (see *Materials and Methods*), and the metabolites were analyzed by HPLC. Table 1 lists the activity of the five most active enzymes. The other P450 enzymes having low activities with both epimers are listed in the explanation to the table. The primary P450 enzymes responsible for *R*-epimer oxidation were rat and human 1A2, rat 1A1, and human 1B1, which are under control of the AHR. For oxidation of the *S*-epimer, the enzymes with the highest activity are human 1A2 and 3A4. It should be stressed that because the P450 Supersomes used are from two different species and are supplied by the manufacturer with varying but saturating amounts of NADPH cytochrome P450 reductase and some contain cytochrome b_5 (see *Materials and Methods*), the activity values obtained are only meant to provide an overview of which purified enzymes showed activity with each sulindac epimer under the conditions used and may not correspond to what occurs in vivo.

Induction of P450 Enzyme Activity by the Sulindac Epimers. Previous studies showed that sulindac could induce several of the P450 enzymes that were regulated by the AHR (Ciolino et al., 2006, 2008). These authors used ethoxyresorufin as a substrate to measure induction of the P450 system but did not measure the induction of the P450 enzymes that oxidize sulindac or the effect of the sulindac

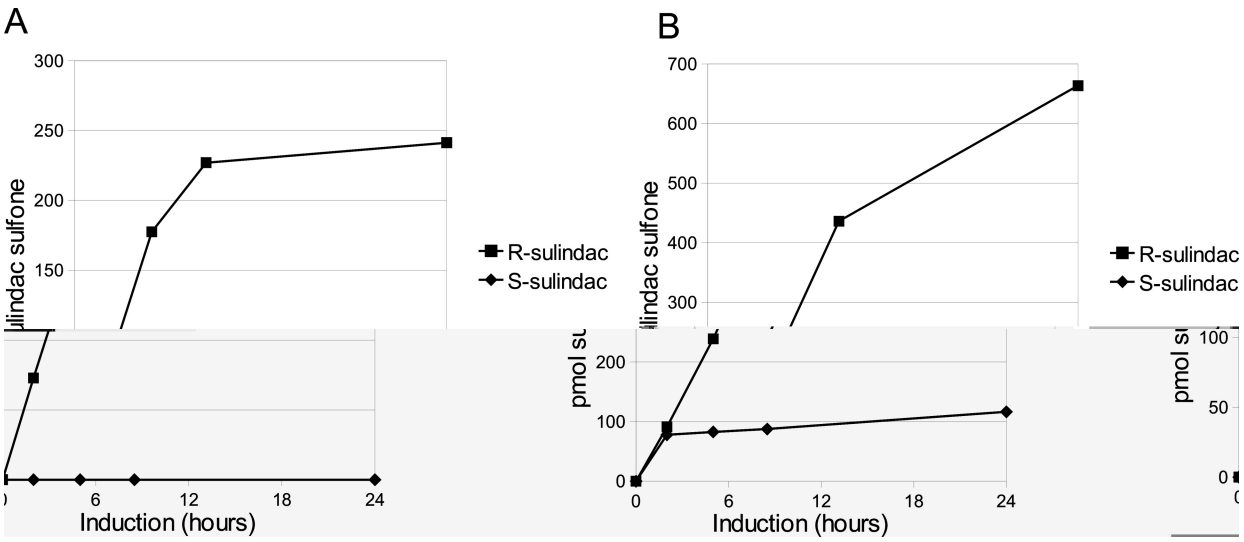

FIG. 4. Induction of sulindac oxidation in HepG2 human hepatoma cells. A, induction by pretreatment of cells with (*R*)-sulindac. B, induction by pretreatment of cells with (*S*)-sulindac. Cells in 24-well plates were incubated for the indicated time with a 125 μM concentration of the inducing epimer, followed by a 200 μM concentration of either (*R*)-sulindac or (*S*)-sulindac, as described under *Materials and Methods*. The results are expressed as picomoles per 100-μl incubation reaction. This experiment was repeated twice with a similar pattern seen in both experiments.

epimers in their P450 induction experiments. To see whether sulindac increased the activity of P450 enzymes involved in its own metabolism, human HepG2 cells were treated for periods ranging from 2 to 24 h with either (*R*)- or (*S*)-sulindac. After the pretreatment period, the medium containing sulindac was removed and replaced with fresh medium containing either the *R*- or *S*-epimer for a 1-h incubation period (see *Materials and Methods*). After this second incubation, metabolites in the medium (see *Materials and Methods*) were analyzed by HPLC. As shown in Fig. 4A, pretreatment with the *R*-epimer increases the activity of enzymes that can oxidize the *R*-epimer but not the *S*-epimer to the sulfone. Pretreatment of the cells with the *S*-epimer results in a much higher level of increase in the activity of enzymes that can oxidize both the *R*- and *S*-epimers (Fig. 4B). It should be noted that the *R*-epimer is oxidized at a faster rate than the *S*-epimer, regardless of which epimer is used during the preincubation (induction) period. We assume that the sulindac epimers are inducing the P450 enzymes in these experiments because Ciolino et al. (2006, 2008) have clearly shown that sulindac and its metabolites can induce several P450 enzymes. However, we should stress that we have not directly shown that the P450 enzymes are induced, only that there is an increase in P450 enzymatic activity after the cells are exposed to sulindac over a 24-h period.

Partial Purification of an (*R*)-Sulindac Reductase with MsrB-Like Activity. The reduction of (*S*)-sulindac has been shown to be catalyzed by MsrA (Moskovitz et al., 1996; Etienne et al., 2003). The reductase for (*R*)-sulindac was of particular interest because there is presently no known enzyme that catalyzes this reaction. Several different MsrB enzymes, including recombinant *E. coli* MsrB and human MsrB2 and B3, which are known to catalyze the reduction of protein-bound methionine-(*R*)-sulfoxide, were tested and found to have no activity in reducing the (*R*)-sulindac epimer (data not shown). Recombinant mammalian MsrB1 was not available, but its partial purification from mouse liver has been described previously (Moskovitz et al., 2002), and it was shown that the purified enzyme reduced both free Met-*R*-(o) as well as DABS-*R*-Met(o), a substrate that mimics Met(o) in peptide linkage. Thus, it was possible that MsrB1 might also reduce the *R*-epimer of sulindac. We therefore set out to purify both the (*R*)-sulindac reductase activity and DABS-*R*-Met(o) activity from a rat liver S-100 fraction to see whether the two activities copurified (see details of purification under *Materials and Methods*). A summary of a typical purification of (*R*)-sulindac reducing activity is shown in Table 2. A 270-fold purification was obtained in this run, and in several other runs the purification varied between 250- and 350-fold. (*S*)-Sulindac reducing activity was also tested on these fractions (data not shown). This activity, presumably mostly due to MsrA, was twice the (*R*)-sulindac reductase activity in the original S-100, but the final G50 fraction had only a trace of (*S*)-sulindac reductase activity (<6%), which was not further identified. In Table 3, the purification of both (*R*)-sulindac and DABS-*R*-Met(o) reductase activities from a typical purification are compared. It can be seen that the purification of both activities is very similar at each step, suggesting that the same enzyme may be responsible for both activities. In addition, the reducing requirement for both activities at the final purification stage was similar, because DTT, but not reduced Trx, could serve as the reducing agent (data not shown). Mammalian MsrB1 has a molecular mass of 12.8 kDa, and, as shown in Fig. 5A,

TABLE 2

Purification of (R)-sulindac reductase from rat liver

Aliquots from each step of purification were incubated with 200 μM (*R*)-sulindac as described under *Materials and Methods*.

Step	Specific Activity	Purification Factor	Recovery
	nmol product formed/mg protein/h		%
S-100	4.2	1	100
$(NH4)_2SO_4$, 30–70%	5.6	1.3	98
DEAE	32.6	7.8	43
G50	1160	276	40

TABLE 3

Copurification of (R)-sulindac reductase and DABS-R-Met(o) reductase

The purification factor at the different steps of purification was measured using either 200 μM (*R*)-sulindac or 200 μM DABS-*R*-Met(o), as described under *Materials and Methods*.

Step	Purification Factor	
	(*R*)-Sulindac Reductase	DABS-*R*-Met(o) Reductase
S-100	1	1
$(NH4)_2SO_4$, 30–70%	1.3	1.5
DEAE	7.7	7.5
G50 peak fraction	340	256

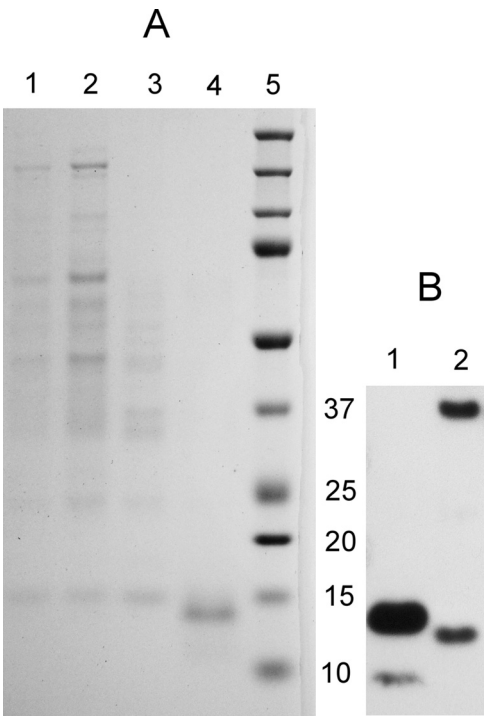

FIG. 5. Gel analysis of purified (*R*)-sulindac reductase and Western blot analysis for MsrB1. Proteins from the indicated purification steps (see *Materials and Methods*) were separated on 4 to 12% NuPAGE gels. Two micrograms of protein were applied in each lane. A, Coomassie-stained fractions: lane 1, S-100; lane 2, $(NH_4)_2SO_4$, 30 to 70%; lane 3, DEAE fraction; lane 4, G50 peak activity fraction (no. 25). B, Western blot analysis of the G50 fraction 25 as in A, along with a standard for MsrB1. Lane 1, G50 (no. 25); lane 2, incomplete digest of MsrB1-GST fusion protein standard (36.5 kDa), with the truncated MsrB1 10.5 kDa fragment clearly visible (see *Materials and Methods*).

there is a prominent Coomassie-stained band of this size in the most-purified fraction. Western blot analysis, using the SepX1 antibody (see *Materials*) against a GST fusion protein containing the N-terminal 95 amino acids of MsrB1, also provided evidence of MsrB1 at this position (Fig. 5B, lane 1). Figure 5B, lane 2, is a positive antibody control using the GST-MsrB1 fusion protein. This protein (36.5 kDa) was partially digested with PreScission protease, resulting in a second, lower band corresponding to the truncated MsrB1 (10.5 kDa). Taken together, these results strongly indicate that MsrB1 is present in the most-active fractions and may be responsible for the reduction of (*R*)-sulindac in the rat liver extracts.

Effect of Sulindac on Normal Lung and Lung Cancer Cells Exposed to Oxidative Stress. Our previous results (Marchetti et al., 2009) showed that normal lung cells, when pretreated with sulindac, were protected against oxidative stress. In Fig. 6A, we tested the individual sulindac epimers to determine whether they both had a protective effect using normal lung cells exposed to TBHP (see *Materials and Methods*). Under the conditions used, normal lung cells are quite sensitive to TBHP as indicated by the loss of viability in the absence of any drug. It can be seen that both epimers are able to protect the normal lung cells against TBHP oxidation. There is an indication that the (*R*)-sulindac epimer is slightly more protective than the *S*-epimer, but only at higher concentrations of TBHP, and we do not think that this difference has physiological or therapeutic significance.

The lung cancer cells are more resistant to TBHP, as shown in Fig. 6B. In contrast to what is seen in normal cells, pretreatment of lung cancer cells with sulindac followed by exposure to oxidative stress has been shown to selectively kill these cells (Marchetti et al., 2009). As shown in Fig. 6B, the two sulindac epimers showed similar enhanced killing of lung cancer cells under the conditions used. In all these experiments, the cells were preincubated with sulindac for 48 h before being exposed to TBHP (see *Materials and Methods* and Marchetti et al., 2009). It is concluded that the *S*- and *R*-epimers of sulindac have similar activities toward both normal lung cells, which sulindac protects against oxidative stress, and lung cancer cells, which sulindac enhances the killing of when exposed to oxidative stress. Studies on the protection of normal cells against oxidative stress by sulindac (mixture of *R*- and *S*-epimers) have been shown to involve a preconditioning mechanism (Moench et al., 2009). The selective, enhanced killing of cancer cells exposed to sulindac and oxidative stress has also been described elsewhere in detail and shown to involve mitochondrial dysfunction, leading to loss of mitochondrial membrane potential, excess production of reactive oxygen species, and apoptosis (Marchetti et al., 2009). There is no reason to believe that the individual sulindac epimers are not functioning in a similar way.

Discussion

In prior studies on sulindac, the metabolism and biological effects were investigated primarily with sulindac preparations that contained equal amounts of both epimers. Because individual epimers of a compound often have different metabolic pathways and biological effects, a complete understanding of a drug's metabolism requires study of the individual epimers. This may be especially true for sulindac, which has been shown recently to have unique anticancer

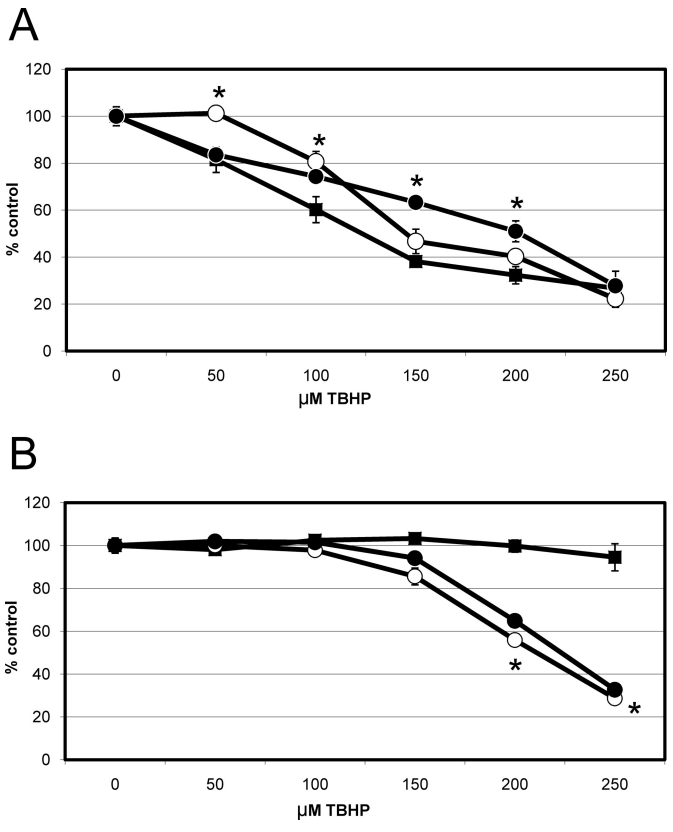

FIG. 6. Effect of sulindac epimers on the viability of lung cells exposed to oxidative stress. Cells were incubated in the presence of 500 μM (*R*)-sulindac (●), 500 μM (*S*)-sulindac (○), or no drug (■) for 48 h before exposure to TBHP, as described under *Materials and Methods* and in Marchetti et al. (2009). Viability is expressed as percentage viable cells not exposed to TBHP. A, normal lung cells. B, lung cancer cells. Data points are graphed as mean and S.E. bars from a set of five repeats. *, treatment conditions under which sulindac-treated cells showed a statistically significant ($p < 0.05$) departure from controls (no drug).

activity in combination with other agents (Meyskens et al., 2008; Park et al., 2008; Seo et al., 2008; Marchetti et al., 2009; Resnick et al., 2009; Pangburn et al., 2010) and to protect normal cells against oxidative stress (Marchetti et al., 2009). Both of these activities of sulindac are unrelated to its NSAID activity.

In the present studies using rats, we have demonstrated that both the sulfone and sulfide metabolites are present in plasma liver, skin, and brain after injection with either sulindac epimer. The oxidation of each epimer with microsomes and commercially obtained, purified cytochrome P450 enzymes has allowed us to identify some of the P450 enzymes that may be involved in the oxidation of the two epimers in vivo. Of those tested, the major purified P450 enzymes responsible for oxidation of the *R*-epimer include rat and human 1A2, rat 1A1, and human 1B1, whereas human 1A2 appeared to be most active with the *S*-epimer. From previously published rat feeding experiments, it seems that sulindac (a mixture of both epimers) did induce hepatic mRNA expression of CYP1A1, CYP1A2, and CYP1B1 (Ciolino et al., 2008), although sulindac oxidation was not tested in these studies. In fact, CYP1A1 showed the highest level of mRNA induction by the mixed epimers in these previous studies and showed high activity for oxidation of the *R*-epimer in our studies. It should also be mentioned that there is one other report showing that sulindac sulfide can be oxidized to sulindac in vitro by a flavin-containing monooxygenase, although the physiological significance of this reaction in vivo is not known (Hamman et al., 2000). Induction of P450 enzyme activity in HepG2 cells using the individual epimers showed more induced activity toward the *R*-epimer than toward the *S*-epimer. It was surprising that the induction of enzyme activity with the *R*-epimer showed little or no increase in P450 activity toward the *S*-epimer. We have not found any other report using chiral drugs for which this is the case. When the *S*-epimer was used to induce the P450 activity, once again the induced activity was primarily toward the *R*-epimer, although in these experiments there was a low level of induction of P450 activity toward the *S*-epimer. It was also of interest that the *S*-epimer has a greater capacity to induce the P450 enzyme system than the *R*-epimer. It is possible that the *S*-epimer has a higher affinity for the AHR or other P450-inducing receptors, but that the induced P450 enzyme activity oxidizes the *R*-epimer at a greater rate than the *S*-epimer in whole cells. This finding is in agreement with the in vivo plasma levels of the metabolites. In summary, our results indicate that after induction of P450 enzyme activity with either epimer, the *R*-epimer of sulindac is more readily converted to the sulfone than the *S*-epimer in cells in culture and in vivo. We have assumed that the induction of enzyme activity in the cell culture experiments is due primarily to sulindac and not to any of its metabolites, because only a small fraction of the sulindac was metabolized in these experiments. However, preliminary experiments have shown that sulindac sulfide can induce P450 enzyme activity similar to what was seen with the sulindac *R*-epimer, and thus we cannot rule out the possibility that some of the increase in P450 activity seen with the sulindac epimers is due to the sulindac metabolites.

Regarding the reduction of sulindac to sulindac sulfide, our earlier studies showed that MsrA reduced the *S*-epimer of sulindac (Etienne et al., 2003). However, MsrB2 and MsrB3, which are capable of reducing the *R*-epimer of peptide-bound methionine sulfoxide, cannot reduce (*R*)-sulindac to sulindac sulfide (data not shown). This finding prompted us to search for the enzyme(s) that could reduce the *R*-epimer of sulindac, especially because sulindac sulfide was present in the plasma of rats fed the *R*-epimer. A 250- to 350-fold purified enzyme fraction that can reduce both the *R*-epimer of sulindac and DABS-Met-*R*-(o), the latter being a known substrate for the MsrB enzymes, has been obtained. Preliminary data suggest that the enzyme may be MsrB1, a selenoprotein, because the activities that reduce both substrates copurify, and gel analyses of the most-purified preparation show a band, recognized by the anti-MsrB1 antibody, at the expected molecular weight. Matrix-assisted laser desorption ionization analysis showed that this purified preparation was grossly contaminated with fatty acid binding protein (data not shown), which prevented us from obtaining selenium measurements that could support the presence of MsrB1 in the preparation. However, we must be cautious at this time in concluding that MsrB1 is the active factor that reduces (*R*)-sulindac, because a recombinant MsrB1 (a generous gift from V. N. Gladyshev, Harvard Medical School, Boston, MA), with cysteine at position 95 instead of selenocysteine, did not have detectable activity with (*R*)-sulindac even at high enzyme concentrations, under conditions in which DABS-*R*-Met(o), a known substrate for MsrB, was readily reduced by this enzyme. This recombinant enzyme also could use thioredoxin as a reducing system, whereas the rat liver fraction that we purified could use DTT, but not thioredoxin. More studies are needed to clarify this issue. One possibility is to look at (*R*)-sulindac reductase activity in *MsrB1* knockout mice or use *MsrB1* small interfering RNA. Such studies will not provide a clear answer because the bulk of the (*R*)-sulindac reductase activity in rat liver was not in the protein fraction we purified. The DEAE fraction that contained the highest (*R*)-sulindac reductase specific activity as well as the MsrB1 activity represented approximately 30% of the total (*R*)-sulindac re-

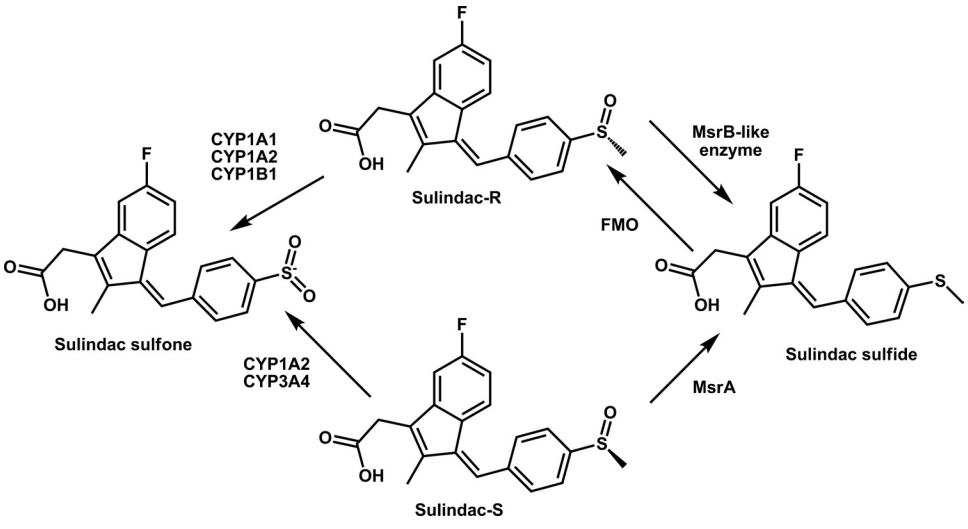

FIG. 7. Summary of P450 enzymes involved in the metabolism of the sulindac epimers. See text for details.

ductase activity in the DEAE fractions. Thus, knocking out *MsrB1* would give only a partial loss of (*R*)-sulindac reductase activity in the crude cell extracts and would require more extensive purification. Figure 7 summarizes our present knowledge of the metabolism of the *R*- and *S*-epimers of sulindac.

Finally, because of the recent interest in the role of sulindac in protecting normal cells against oxidative stress while sensitizing cancer cells to agents that affect mitochondrial function, we have checked the effect of both epimers on the protection of normal lung cells against oxidative stress as well as the ability of the sulindac epimers to selectively enhance the killing of lung cancer cells exposed to oxidative stress. Our purpose here was not to investigate specific mechanisms of normal cell protection or cancer killing by the sulindac epimers and oxidative stress, because mechanism studies with sulindac (mixture of epimers) have been described elsewhere (Marchetti et al., 2009; Moench et al., 2009). As mentioned, sulindac can protect cells against oxidative damage by a preconditioning mechanism (Moench et al., 2009), and the sulindac-enhanced killing of cancer cells exposed to oxidative stress involves mitochondrial dysfunction and death as a result of reactive oxygen species production (Marchetti et al., 2009). The goal of the present studies was to compare the relative efficacy of the two epimers. Both epimers showed similar protection of lung normal cells to oxidative stress and enhanced killing of cancer cells exposed to oxidative stress. However, differences in the metabolism of the sulindac epimers could have therapeutic significance. The *R*-epimer may have a better safety profile owing to its more efficient conversion to the sulindac sulfone, which is not a cyclooxygenase inhibitor.

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Authorship Contributions

Participated in research design: Brunell, Brot, and Weissbach.

Conducted experiments: Brunell, Sagher, and Kesaraju.

Performed data analysis: Brunell and Sagher.

Wrote or contributed to the writing of the manuscript: Brunell, Sagher, Brot, and Weissbach.

Other: Weissbach initiated the project.

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