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The *in vitro* metabolism of phospho-sulindac amide, a novel potential anticancer agent

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ABSTRACT

Phospho-sulindac amide (PSA) is a novel potential anti-cancer and anti-inflammatory agent. Here we report the metabolism of PSA *in vitro*. PSA was rapidly hydroxylated at its butane-phosphate moiety to form two di-hydroxyl-PSA and four mono-hydroxyl-PSA metabolites in mouse and human liver microsomes. PSA also can be oxidized or reduced at its sulindac moiety to form PSA sulfone and PSA sulfide, respectively. PSA was mono-hydroxylated and cleared more rapidly in mouse liver microsomes than in human liver microsomes. Of eight major human cytochrome P450s (CYPs), CYP3A4 and CYP2D6 exclusively catalyzed the hydroxylation and sulfoxidation reactions of PSA, respectively. We also examined the metabolism of PSA by three major human flavin monooxygenases (FMOs). FMO1, FMO3 and FMO5 were all capable of catalyzing the sulfoxidation (but not hydroxylation) of PSA, with FMO1 being by far the most active isoform. PSA was predominantly sulfoxidized in human kidney microsomes because FMO1 is the dominant isoform in human kidney. PSA (versus sulindac) is a preferred substrate of both CYPs and FMOs, likely because of its greater lipophilicity and masked–COOH group. Ketoconazole (a CYP3A4 inhibitor) and alkaline pH strongly inhibited the hydroxylation of PSA, but moderately suppressed its sulfoxidation in liver microsomes. Together, our results establish the metabolic pathways of PSA, identify the major enzymes mediating its biotransformations and reveal significant inter-species and inter-tissue differences in its metabolism.

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1. Introduction

Inflammation plays critical roles at various stages of tumor development, including tumor initiation, promotion, malignant conversion, invasion, and metastasis [1]. These findings provide a foundation and rationale for the use of anti-inflammatory drugs in cancer prevention and therapy. For example, sulindac, a widely-used nonsteroidal anti-inflammatory drug, prevents colon cancer in patients with sporadic adenomas or familial adenomatous polyposis [2].

Sulindac, however, has a broad range of side effects, including gastrointestinal, central nervous system, skin rash and pruritus, and transient elevations of hepatic enzymes in plasma [3] (Roberts and Morrow, 2001). This toxicity results from the inhibition of cyclooxygenase (COX) and the subsequent prostaglandin synthesis; the –COOH group of sulindac is critical for its binding to COX [4]. Therefore, we recently modified sulindac at its–COOH group to develop a sulindac derivative, phospho-sulindac (PS) (Fig. 1). PS exhibited more potent anti-cancer efficacy and markedly reduced toxicity than conventional sulindac in animal models [5]. However, the carboxylester bond of PS is labile, resulting in the extensive hydrolysis of PS to sulindac *in vitro* and *in vivo* [6]. We previously demonstrated that protecting PS against its hydrolysis enhanced its anti-cancer efficacy [7]. Thus, the extensive hydrolysis of PS *in vivo* may be detrimental to its efficacy and safety. This consideration prompted us to modify PS at its ester bond to generate phospho-sulindac amide (PSA; Fig. 1) to abrogate its undesired hydrolysis.

Drug metabolism plays a key role in defining the efficacy and toxicity of drugs. While the assessment of PSA as a novel anticancer/anti-inflammatory agent is still ongoing, herein, we report the major metabolic pathways of PSA, showing that PSA is extensively hydroxylated and sulfoxidized but not hydrolyzed by microsomes from various species and tissues. These results are

Abbreviations: CYP, cytochrome P450; FMO, flavin monooxygenase; HL, Mhuman liver microsomes; ML, Mmouse liver microsomes; PS, phospho-sulindac; PSA, phospho-sulindac amide.

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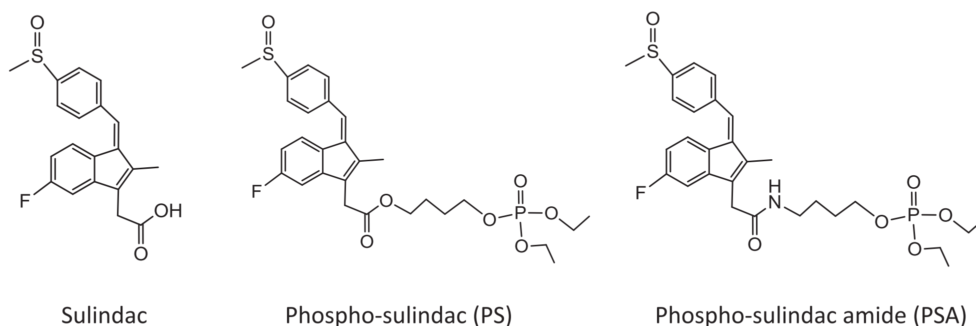

Fig. 1. Structures of sulindac, phospho-sulindac and phospho-sulindac amide.

essential for a better understanding of the pharmacology of PSA in animal and human studies.

2. Materials and methods

2.1. Reagents

PSA was provided by Medicon Pharmaceuticals, Inc. (Stony Brook, NY). Sulindac, sulindac sulfone, sulindac sulfide, dithiothreitol, methimazole, and CH_3CN of HPLC grade were purchased from Sigma-Aldrich (St. Louis, MO). Quinidine and ketoconazole were purchased from Toronto Research Chemicals (North York, ON, Canada). Mouse and human liver microsomes, rat liver cytosol, recombinant human CYPs (CYP1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1 and 3A4), FMOs (FMO1, FMO3 and FMO5), NADPH regenerating solution, and cryopreserved rat hepatocytes were purchased from BD Biosciences (San Jose, CA). Human intestine, kidney and lung microsomes were purchased from XenoTech LLC (Lenexa, KS).

2.2. HPLC-UV analysis

The HPLC system consisted of a Waters Alliance 2695 Separations Module equipped with a Waters 2998 photodiode array detector (328 nm) and a Thermo Hypersil BDS C18 column (150 × 4.6 mm, particle size 3 μm). The mobile phase consisted of a gradient between aqueous phase [Trifluoroacetic acid, CH_3CN, H_2O (0.1:4.9:95, v/v/v)] and CH_3CN at a flow rate of 1 ml/min at 30 °C. We applied gradient elution from 0% to 100% CH_3CN in 15 min, and it was maintained at 100% CH_3CN for 5 min.

2.3. LC-MS/MS analysis

The LC-MS/MS system consisted of a Thermo TSQ Quantum Access (Thermo-Fisher) electrospray ionization triple quadrupole mass spectrometer coupled to an Ultimate 3000 HPLC system (Dionex Corporation, Sunnyvale, CA). Chromatographic separations were achieved using a Luna C18 column (150 × 2 mm) and a mobile phase consisting of a gradient from 10% to 95% CH_3CN.

2.4. The metabolism of PSA in mouse and human microsomes

PSA was pre-incubated at 37 °C for 5 min with an NADPH-regenerating solution (1.3 mM NADP, 3.3 mM D-glucose 6-phosphate, 3.3 mM $MgCl_2$, and 0.4 U/ml glucose-6-phosphate dehydrogenase) in 0.1 M potassium phosphate buffer (pH 7.4). The reaction was initiated by the addition of mouse or human liver microsomes (protein concentration 0.5 mg/ml) or human intestine, kidney, liver or lung microsomes (protein concentration 0.25 mg/ml) and samples were maintained at 37 °C for various time periods. At each of the designated time-points, 0.1-ml aliquots were mixed with 0.2 ml of CH_3CN, vortexed, and then centrifuged for 10 min at 13,000 × g. The supernatants were subjected to HPLC analyses. The HPLC fractions corresponding to each metabolite of PSA were collected and analyzed by mass spectrometry.

2.5. The metabolism of PA by rat hepatocytes

Cryopreserved rat hepatocytes were thawed and incubated following the manufacturer's protocol. Briefly, hepatocytes were incubated with PSA in 24-well tissue culture plates at a density of 2.5×10^5 cells/well in 5% CO_2 at 37 °C. At the designated time-points, the cells were mixed with 2-fold volume of acetonitrile to stop the reaction. After centrifugation, the supernatants were analyzed by HPLC.

2.6. The metabolism of PSA by rat liver cytosol

PSA (50 μM) was pre-incubated at 37 °C for 5 min with 10 mM dithiothreitol in 0.1 M Tris buffer (pH 7.4). The reaction was initiated by the addition of liver cytosol (protein concentration 2 mg/ml) and samples were maintained at 37 °C for various time periods. At the end of each of the incubations, 0.1 ml aliquots were mixed with 0.2 ml of CH_3CN, vortexed and then centrifuged for 10 min at 13,000 × g. The supernatants were analyzed by HPLC.

2.7. The metabolism of PSA by human CYP and FMO isoforms

PSA were pre-incubated at 37 °C for 5 min with an NADPH-regenerating solution (1.3 mM NADP, 3.3 mM D-glucose 6-phosphate, 3.3 mM $MgCl_2$, and 0.4 U/ml glucose-6-phosphate dehydrogenase) in 0.1 M potassium phosphate buffer (pH 7.4). The reaction was initiated by the addition of individual recombinant human CYP isoforms (25 pmol/ml) or human FMO isoforms (0.125 mg protein/ml) in a total volume of 1 ml and samples were maintained at 37 °C for various time periods. At each designated time point, an aliquot was mixed with 2-fold volume of CH_3CN, vortexed, and then centrifuged for 10 min at 13,000 × g. The supernatants were subjected to HPLC analysis.

3. Results

3.1. The metabolism of PSA in mouse and human liver microsomes

We examined the metabolism of PSA in mouse liver microsomes (MLM) and human liver microsomes (HLM). We identified four mono-hydroxyl metabolites (henceforce referred to as mono-hydroxyl-PSA) and two di-hydroxyl metabolites using LC-MS/MS. The sodium adduct ions of the four mono-hydroxyl-PSA were observed at *m/z* 602.3, 602.4, 602.5 and 602.3, respectively. The sodium adduct ions of the two di-hydroxyl-PSA were observed at *m/z* 618.2 and 618.3, respectively. To determine the position of the hydroxyl groups of the metabolites, we treated these metabolites at pH 13 and 70 °C for 3 h. These metabolites were hydrolyzed to

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