Aspirin-like Molecules that Covalently Inactivate Cyclooxygenase-2

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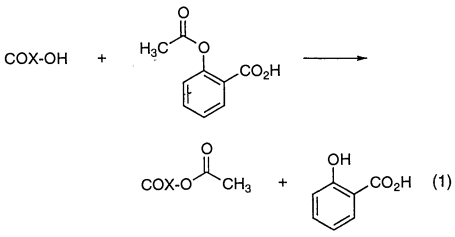
Many of aspirin's therapeutic effects arise from its acetylation of cyclooxygenase-2 (COX-2), whereas its antithrombotic and ulcerogenic effects result from its acetylation of COX-1. Here, aspirin-like molecules were designed that preferentially acetylate and irreversibly inactivate COX-2. The most potent of these compounds was *o*-(acetoxyphenyl)hept-2-ynyl sulfide (APHS). Relative to aspirin, APHS was 60 times as reactive against COX-2 and 100 times as selective for its inhibition; it also inhibited COX-2 in cultured macrophages and colon cancer cells and in the rat air pouch in vivo. Such compounds may lead to the development of aspirin-like drugs for the treatment or prevention of immunological and proliferative diseases without gastrointestinal or hematologic side effects.

Anti-inflammatory agents date to ancient times, when a variety of plant extracts containing salicylates were used for the treatment of inflammation, fever, and pain (*1*). The acetylated derivative of salicylic acid (aspirin) was introduced in 1897 (*2*). The unique properties of aspirin derive from its ability to acetylate and irreversibly inactivate COX-1 and COX-2, the in vivo targets for its action:



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(*3*, *4*). Aspirin is the only nonsteroidal anti-inflammatory drug that covalently modifies cyclooxygenases. It acetylates a serine residue positioned in the arachidonic acid binding channel (Ser^{530} of COX-1 and Ser^{516} of COX-2), thereby blocking the approach of the fatty acid substrate to the active site for its oxygenation (*5*, *6*). Although aspirin acetylates both isoforms of cyclooxygenase, it is 10 to 100 times as potent against COX-1 as against COX-2 (*7*, *8*). Attempts have been made to alter the selectivity of aspirin for the two different cyclooxygenases by varying the length of the acyl group attached to the salicylate moiety, but the compounds retain COX-1 selectivity (*9*).

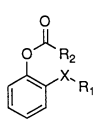
The potential utility of a selective, covalent inactivator of COX-2 prompted our current investigations. We synthesized a series of acetoxybenzenes substituted in the ortho position with alkylsulfides. *o*-(Acetoxyphenyl)methyl sulfide was identified as a compound that exhibited moderate inhibitory potency and selectivity for COX-2 (Table 1). Systematic variation of acyl group, alkyl group, aryl substitution pattern, and heteroatom identity led to the synthesis of *o*-(acetoxyphenyl)hept-2-ynyl sulfide (APHS), the most potent inhibitor in the series (Table 1).

APHS was a time- and concentration-dependent inactivator of COX-2 ($k_{inact}/K_i \sim 0.18\ min^{-1}\ \mu M^{-1}$) and was about 60 times as potent as aspirin in enzyme inhibition ($k_{inact}/K_i \sim 0.003\ min^{-1}\ \mu M^{-1}$) (*10*). The hydrolysis product of APHS was inactive. COX-2 treated with APHS produced no prostaglandin endoperoxide-derived products, but it generated 15-hydroxyeicosatetraenoic acid (15-HETE) in a fashion similar to the aspirin-inhibited enzyme (*11*, *12*).

When [^{14}C]acetyl-APHS was tested, the degree of incorporation of the [^{14}C]acetyl moiety into COX-2 and COX-1 correlated well with the relative inhibitory activity against the two enzymes (ratio of ^{14}C incorporated into COX-2 vs. COX-1 = 15.4) (*13*). Tryptic digestion and peptide mapping of acetylated COX-2 indicated that the radioactivity was incorporated into a single major peptide that included the serine acetylated by aspirin. The electrospray mass spectrum of this peptide revealed a pair of high-mass ions at mass/charge ratios (m/z) of 389 and 391, corresponding to the molecular ions of a tripeptide containing [^{12}C]acetate and [^{14}C]acetate (*14*). Collision-induced dissociation of the ion at m/z 389 allowed its mass spectrum to be determined (Fig. 1). Sequence ions were detected at m/z 260, 147, 129, 102, and 86, corresponding to the acetylated tripeptide Ser-Leu-Lys (S-L-K). The presence of an ion at m/z 86 identified the peptide as *N*-acetyl-S-L-K by comparison to authentic standards of *N*- and *O*-acetyl-S-L-K. This peptide is present in the COX-2 sequence at positions 516 to 518 and contains the Ser residue acetylated by aspirin (*15*).

Site-directed mutagenesis provided an opportunity to probe the molecular basis for the interaction of APHS with COX-2. Three different site-directed mutants were constructed that represent regions of the arachidonic acid binding site that are important for the binding of various cyclooxygenase inhibitors (Fig. 2A). Foremost among these is Arg^{106}, which is the only positively charged residue in the substrate access channel and is important for directing the salicylic acid portion of aspirin to the vicinity of Ser^{516} (*16*). Murine COX-2 containing the mutation Arg^{106}→ Gln was expressed in SF-9 insect cells from baculovirus vectors (*17*), and membrane preparations were used for enzyme assay. APHS was more active against Arg^{106}→ Gln COX-2 than against the wild-type enzyme (Fig. 2B), which is opposite to the effect of this

Table 1. Inhibition of cyclooxygenases by *o*-(acetoxyphenyl)alkyl sulfides (*32*). Each IC_{50} value corresponds to an average of at least two independent determinations. Incubations of inhibitors with human COX-2 (88 nM) or ovine COX-1 (22 nM) were conducted at 25°C for 3 hours. See (*10*) for details.



R_1	R_2	X	IC_{50} (μM) COX-2	IC_{50} (μM) COX-1	IC_{50} (COX-1)/ IC_{50} (COX-2)
CH_3	CH_3	S	250	>5000	>20
$(CH_2)_6CH_3$	CH_3	S	2.0	6.0	3.0
$(CH_2)_6CH_3$	CH_3	$N(CH_3)$	>40	>40	–
$(CH_2)_6CH_3$	CH_3	CH_2	>40	>40	–
$(CH_2)_6CH_3$	CH_3	Se	12	12	1.0
$(CH_2)_6CH_3$	CH_3	SO	>40	>40	–
$(CH_2)_6CH_3$	CH_3	SO_2	>40	>40	–
$CH_2C{\equiv}C(CH_2)_3CH_3$ (APHS)	CH_3	S	0.8	17	21.0
$CH_2C{\equiv}C(CH_2)_3CH_3$	CH_3	O	>40	>40	–
$CH_2C{\equiv}C(CH_2)_3CH_3$	CH_2CH_3	S	>40	>40	–

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mutation on the inhibitory potency of aspirin and other carboxylic acid–containing inhibitors (*16*). Similarly, the Tyr^{341}→ Ala mutant, which is juxtaposed to Arg^{106} at the mouth of the substrate access channel and appears to be important in the binding of carboxylic acid–containing inhibitors, was more sensitive than wild-type murine COX-2 to inhibition by APHS.

Comparison of the crystal structures of murine COX-2 and ovine COX-1 indicates that the major difference in the substrate access channel between the two isoforms is a side pocket off the channel in the vicinity of Val^{509} (*18–20*). Previous mutagenesis experiments have verified that differences in this region account for the selectivity of certain compounds for inhibition of COX-2 (*19–22*). Therefore, we constructed a triple mutant, Val^{509}→ Ile:Arg^{499}→ His:Val^{420}→ Ile, that incorporates the major amino acid changes between COX-2 and COX-1 in this side pocket region. Unlike other COX-2–selective inhibitors that show reduced potency when this side pocket is mutated (*21*, *22*), APHS was more potent against the triple mutant than against wild-type COX-2. Thus, the selectivity of inhibition of COX-2 by APHS appears to result from novel protein-inhibitor interactions.

The ability of APHS to inhibit COX-2 in intact cells was assayed in two systems, one in which COX-2 activity is induced by pathologic stimuli and a second in which COX-2 is constitutively overexpressed. RAW264.7 macrophages were exposed to lipopolysaccharide and γ-interferon to induce COX-2 and were then treated with APHS (*23*, *24*). The concentration of APHS necessary to inhibit prostaglandin D_2 (PGD_2) synthesis by 50% (IC_{50}) was 0.12 μM, indicating that this agent is an effective inhibitor of COX-2 activity in cultured inflammatory cells (Fig. 3A). In parallel experiments, aspirin inhibited PGD_2 synthesis at an IC_{50} of 100 μM.

We also examined the relative effects of APHS on the growth in soft agar of two types of colon cancer cells: HCA-7 cells, which express large amounts of COX-2 and are sensitive to COX-2 inhibitors, and HCT-15 cells, which do not express COX-2 and are resistant to the effects of COX-2 inhibitors (*25*, *26*). HCA-7 cells were sensitive to growth inhibition by APHS, whereas HCT-15 cells were insensitive (Fig. 3B). The IC_{50} for growth inhibition of HCA-7 cells was 2 μM, which is lower than the published IC_{50} for inhibition of the growth of HCA-7 cells by the COX-2–selective inhibitor SC-58125 (*25*). These experiments indicate that the (acetoxyphenyl)alkylsulfide pharmacophore is comparable or superior to the previously described diarylheterocycle pharmacophore for COX-2 inhibition in cellular systems. The results also confirm that COX-2 is important for the growth of colon cancer cells that express the enzyme (*25*, *27*, *28*).

The in vivo activity of APHS was assessed using the rat air pouch model (*29*). Subcutaneous air cavities were produced in

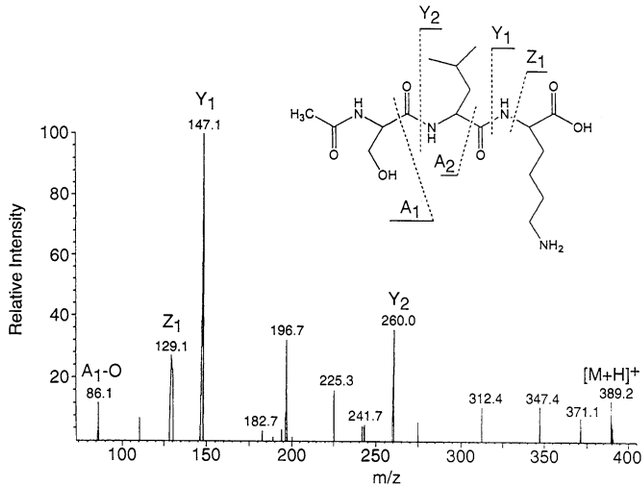
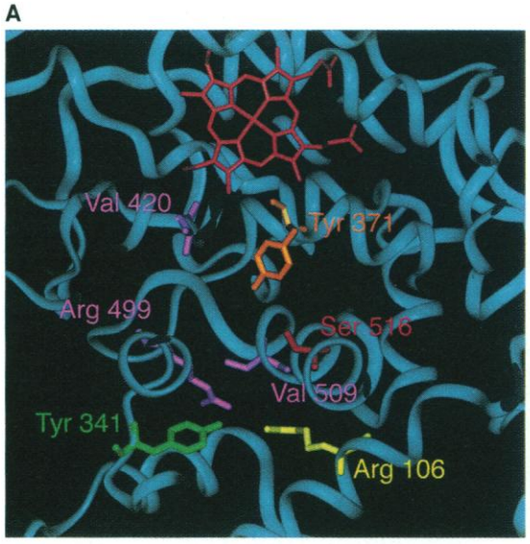


Fig. 1. Mass spectrum of the molecular ion of the acetylated peptide isolated from COX-2 treated with [^{14}C]APHS (*13*, *14*). Chromatographic and spectroscopic comparison to chemically synthesized standards indicates that the isolated peptide is *N*-acetyl-S-L-K, which results from *O*-to-*N* acetyl migration from the initial product *O*-acetyl-S-L-K.



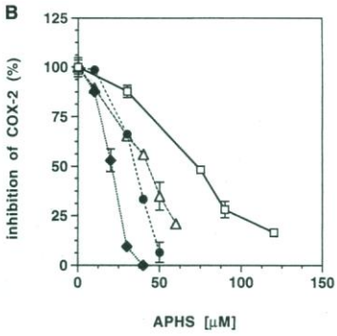


Fig. 2. (**A**) Active-site residues in COX-2 mutated to the corresponding residues in COX-1 (pink). For illustrative purposes, substitutions of COX-2 residues were made at the appropriate positions of COX-1 using the COX-1 coordinates. (**B**) Inhibition of wild-type and site-directed mutants of murine COX-2 by APHS. Membranes from SF-9 insect cells expressing wild-type (□), Arg^{106}→ Gln (◆), Tyr^{341}→ Ala (●), or Val^{420}→ Ile:Arg^{499}→ His:Val^{509}→ Ile (△) murine COX-2 were treated with APHS for 1 hour at 37°C and then assayed for COX-2 activity (*17*).

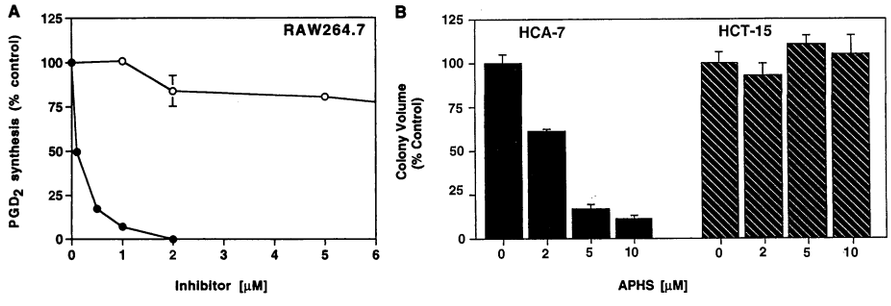


Fig. 3. (**A**) Effect of APHS on PGD_2 biosynthesis in RAW264.7 macrophages (*24*) (●, APHS; ○, aspirin). (**B**) Effect of APHS on the growth of human colon cancer cell lines in soft agar. Colony diameters were measured and volumes calculated for triplicate wells in duplicate experiments (*26*).

Table 2. Effect of APHS and indomethacin on PGE_2 and TxB_2 synthesis in rat air pouch and blood platelets. Protocols are described in (*29*). Each group contained six animals.

Treatment	Dose (mg/kg)	Pouch PGE_2 (ng/ml)	Platelet TxB_2 (ng/ml)
Vehicle	–	82 ± 6	108 ± 12
APHS	5	4 ± 1	118 ± 19
APHS	50	0	96 ± 14
Indomethacin	2	0	11.3 ± 0.6

Lewis rats, and carrageenan was injected into the cavities to induce COX-2 expression and prostaglandin biosynthesis (*29, 30*). APHS or indomethacin was administered 3 hours after carrageenan administration, the animals were killed, and the concentration of prostaglandin E_2 (PGE_2) in the pouch exudate was determined by enzyme-linked immunosorbent assay (ELISA). Blood samples were removed and the concentration of thromboxane B_2 (TxB_2) was determined by ELISA to assess the effect of APHS or indomethacin on platelet COX-1. A dose of APHS (5 mg/kg) lowered PGE_2 concentrations in the pouch exudate by 95 ± 1% but did not affect serum TxB_2 concentrations (Table 2). Increasing the dose to 50 mg/kg completely inhibited PGE_2 in the air pouch but decreased TxB_2 concentrations by only 11%. In contrast, a dose of indomethacin (2 mg/kg) inhibited PGE_2 synthesis in the air pouch by 100% and TxB_2 synthesis in blood platelets by 90%. Thus, APHS exhibits selective inhibition of COX-2 in vivo.

Our results show that potent, irreversible inhibitors of COX-2 can be designed that may provide a therapeutic equivalent for aspirin in inflammatory and proliferative diseases without the deleterious effects on stomach mucosa, which limit aspirin's use in long-term therapy. The efficacy of APHS in lipopolysaccharide- and γ-interferon–treated inflammatory cells, its selectivity in attenuating the growth of COX-2–expressing colon cancer cells, and its selectivity for inhibition of COX-2 over COX-1 in vivo indicate that this class of covalent binding inhibitor may serve as a paradigm for novel therapeutic interventions in inflammatory and proliferative disorders.

Note added in proof: The COX-2 inhibitor, celecoxib, was recently shown to be a powerful inhibitor of colon carcinogenesis induced by azoxymethane in Fischer rats (*31*).

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14. [1-^{14}C]APHS-labeled COX-2 (1.5 mg) was reduced in 300 mM tris-HCl (pH 8.0) buffer containing 4 M guanidine hydrochloride, 2 mM EDTA, and 10 mM dithiothreitol for 1.5 hours at 37°C. Iodoacetamide (25 mM) was added to the reduced protein for 1.3 hours at 25°C while covered in foil. The protein was extensively dialyzed against 100 mM NH_4HCO_3 (pH 7.4) and 0.4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, and then digested with 44:1 TPCK-trypsin (Sigma) for 21 hours at 37°C. The digestion was terminated with acid and analyzed by rpHPLC (Beckman ODS C18 column). A single radioactive peak eluted at 21.5 min in a 75-min gradient of 0% to 50% CH_3CN in 0.1% TFA in water. Further purification of the tryptic digest was effected by rechromatography on a Zorbax SB-C-18 high-resolution column. Analysis of the radiolabeled peptide was conducted by liquid chromatography–mass spectrometry.
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