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DISCOVERY OF A NEW CYCLOOXYGENASE-2 LEAD COMPOUND THROUGH 3-D DATABASE SEARCHING AND COMBINATORIAL CHEMISTRY

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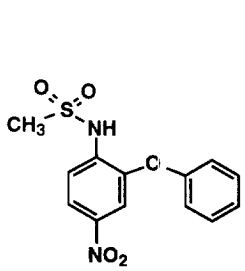
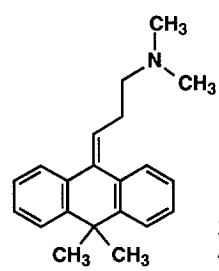
Abstract: Using a combination of computational and combinatorial chemistry methodologies, a phenothiazine compound was discovered that is a selective inhibitor of cyclooxygenase-2 and serves as a lead compound for a potentially novel series of anti-inflammatory compounds.

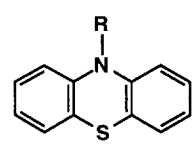
Recent efforts to discover COX-2 (cyclooxygenase-2) selective inhibitors have focused on compounds of the arylsulfonamide (e.g., nimesulide, **1**) or triaryl (e.g., DUP-697) structural types.[1] In this report, we describe a strategy to discover COX-2 selective inhibitors with novel structure. Our strategy involves a combination of computational three-dimensional database searching and combinatorial chemical production of a library of compounds. In addition to the specific disclosure of compound **12** as a COX-2 lead structure in inhibitor research, we feel that the strategy employed in the discovery of compound **12** will be of general interest to medicinal chemists seeking novel lead structures where knowledge of the three-dimensional structure of the target protein exists.



1, Nimesulide



2, Melitracene



3, R = CH_2CH_2OH
4, R = CH_2CH_2COOH
5, R = $CH_2CH(CH_3)OH$
6, R = $CH_2CH(CH_3)SH$

Results

Sequence analysis suggested that the cyclooxygenase active sites for the human COX-1 and COX-2 enzymes are likely to be very similar, when viewed in the context of the sheep COX-1 active site.[2] The human COX-1/2 residue differences that are closest to the cyclooxygenase active site are located at residue 523 (COX-1 Ile, COX-2 Val) and within the active site entry channel at residues 89, 112, 115, and 119 (COX-1 Val, Ile, Tyr, Ser; COX-2 Thr, Leu, Leu, Val, respectively). The subtle differences between the two enzymes suggested that *de novo*

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discovery of a COX-2 selective inhibitor by designing differential binding to these few residues would be extremely difficult. Therefore, we began with a more limited goal of discovering a new core template structure that would bind, but not likely discriminate between, COX-1 and COX-2 enzymes.

A DOCK search[3] of the COX-1 structure yielded structure **2**, the anti-depressant drug melitracene.[4] A striking feature of this compound's structure was the nonplanar tricyclic system that projected two benzene rings into different and nonparallel hydrophobic sections of the active site. In this model, one benzene ring projects toward Tyr 385 and Tyr 348. The other benzene ring projects toward Ile 535 and Phe 518. The *N,N*-dimethylaminoethyl group projects toward Arg 120. The orientation is illustrated in Figure 1. While we do not have experimental

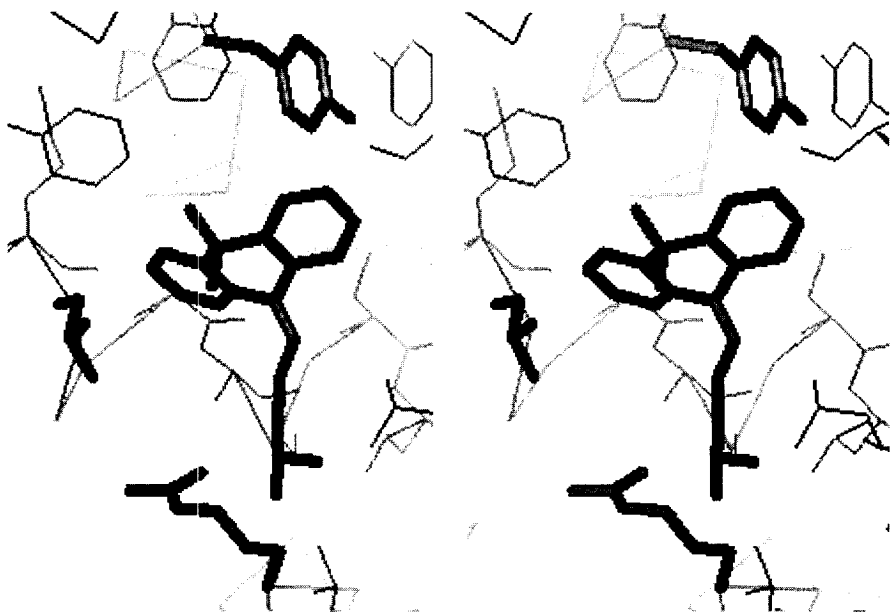


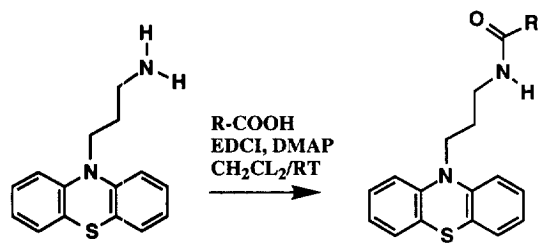
Figure 1. Stereoview of Melitracene, **2**, docked into COX-1 active site as calculated by DOCK program. Arg 120, Tyr 385, and Ile 523 are in thick bonds.

validation of this binding geometry, the orientation seemed plausible and agreed with the empirical requirements for inhibitor potency advanced in 1974 based on an active analog study.[5] Melitracene and its analogs were not available to us for biochemical screening, but we reasoned that other tricyclic ring systems that adopt nonplanar arrangements, such as phenothiazines might provide a similar binding orientation.

Four *N*-substituted phenothiazines, compounds **3–6**, were selected for testing and the results are given in table 1.[6] Compounds **5** and **6** were found to possess COX inhibitory activity in the low micromolar range. The COX 1/2 enzyme selectivity is low, in agreement with the proposed binding mode which shows a lack of contact with

residues that differ between the two enzymes. Given the binding mode shown in Figure 1, substituents extending from the phenothiazine ring nitrogen further than in compounds **3–6** might be able to project into the entry channel near Arg 120 and generate isozyme-specific specific contacts. A library of phenothiazine-based inhibitors was prepared to test this hypothesis.

Given the ready availability[7] of 10-(3-aminopropyl)phenothiazine, **7**, we envisioned a parallel synthetic method using one synthetic step of a solution-phase amide bond formation. A library containing 48 compounds was prepared, and the general synthetic procedure using EDCI coupling and DMAP catalyst is depicted below.[8]



7

8, R = -CH_2CH_3
9, R = -CH_2CH_2-cycloC_6H_{11}
10, R = -CH_2-O-(p-Br)Ph
11, R = -$C(CH_3)_2$-CH_2-O-(p-Br)Ph
12, R = -$C(CH_3)_2$-O-(p-Cl)Ph

The organic acids selected by visual inspection to provide a diverse array of structural features.[9] The library was screened against COX-2 enzyme, and inhibitory potency ranged between 0–44% inhibition at 0.3 μM inhibitor concentration. Compounds exhibiting greater than 30% inhibition were re-tested against COX-2 with IC_{50} values determined and also tested for COX-1 inhibition. Five illustrative analogs are shown in Table 1.[10]

Compd	COX-1	COX-2
	IC_{50} or % inhib. at conc. in μM	IC_{50} or % inhib. at conc. in μM
3	12% at 10	14% at 10
4	25% at 10	14% at 10
5	IC_{50} = 1	IC_{50} = 5
6	IC_{50} = 0.5	IC_{50} = 4
8	ND	18% at 0.3
9	ND	15% at 0.3
10	ND	15% at 0.3
11	12% at 0.3	34% at 0.3
	19 % at 3*	IC_{50} = 21
12	13% at 0.3	44% at 0.3
	17% at 3*	IC_{50} = 1.3
1	IC_{50} = 59	IC_{50} = 0.015

Table 1. ND = not done. *The IC_{50} values for COX-1 inhibition by **11** and **12** were not possible to determine due to solubility limits, but are estimated as > 50 μM. All IC_{50} determinations were carried out in triplicate and have less than 10% error.

While most of the compounds were inactive or only weakly active, two compounds gave measurable and reproducible IC_{50} values in the low micromolar range, compounds **11** and **12**. While the structure–activity relationship data is incomplete, both compounds were the only library entries to possess a gem-dimethyl substitution. Both **11** and **12** possess a *p*-halo substituent on the phenyl ring and are similar in structure, but **11** possesses an additional methylene linker atom. If the binding mode illustrated in Figure 1 for melitracene may be extended to apply to compounds **11** and **12**, the gem-dimethyl substitution and the halobenzene ring would be expected to project into the narrow neck of the entryway into the COX active site, near Arg 120, and into the four amphiphilic helix region which binds within the membrane. Recent X-ray crystallographic studies with COX-2 suggest that this region is indeed subtly different from COX-1.[11] Interactions between this region of the active site and compounds **11** and **12** may provide a rationale for the observed selectivity.

Discussion

We report here the discovery of compound **12** as a 1.3 μM inhibitor of COX-2 with diminished activity against COX-1 (IC_{50} > 50 μM, Table 1). While this enzyme selectivity is much less than that exhibited by established inhibitors, for example nimesulide (compound **1** in Table 1), we feel it is a useful starting point for a series with novel structure. In the first stage of this research we used a computational shape-matching algorithm which matches ligand shape with receptor shape. This work resulted in the discovery of the nonselective phenothiazine inhibitor nucleus. In the second stage of this research we utilized a combinatorial chemical strategy to produce a library of 48 analogs based on a phenothiazine core. The synthetic procedure described here for amide bond formation gave good yields and is likely to be amenable to larger libraries of compounds. Evaluation of this library resulted in discovery of one phenothiazine analog, compound **12**, with an interesting biochemical and chemical profile. This compound possessed >50-fold enzyme selectivity, low micromolar potency, a facile synthetic route suitable for further analog studies, and no complicating asymmetric centers and, therefore, would satisfy the needs of a project team desiring a lead compound. The useful interplay of computational and combinatorial methodologies has been noted by others.[12] In our studies reported here, we feel that computational chemistry was useful in finding a starting place for inhibitor design, while combinatorial chemistry was useful in building upon that starting place to provide a useful lead compound suitable for a more protracted evaluation. The computational and combinatorial effort described here was neither elaborate nor time-consuming, the entire effort being completed in less than one month.

After we completed the work described here, a more comprehensive literature review[13] revealed that the phenothiazine ring structure is present in Flutiazin, Metiazinic Acid, and Protizinic acid, three exploratory antiinflammatory compounds researched in the 1960s and 1970s. These three compounds are members of the classical benzoate, acetate, and propionate NSAIDs, respectively. These three compounds require their carboxylates for activity and therefore differ from the neutral phenothiazines reported here. We suspect that our series represents a novel series of COX inhibitors because of this charge differential, but further experimentation will be required to fully address this question.

We can envision two routes for further investigation into phenothiazine-based COX-2 selective inhibitors: A crystal structure of compound **12** bound to COX-1 and COX-2 enzymes might suggest specific analogs for targeted synthesis. Alternatively, a focused library of compounds which explore the nature of the gem-dimethyl group and/or aromatic ring substitutions might provide more active analogs. We look forward to future reports along these lines.

Acknowledgment: We thank Ms. P. Pavlik, and Drs. W. Hawe, D. Kalvin, and W. Wade for assistance and helpful discussion.

References and Notes

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4. Protein Data Base structure 1PRH was used directly in DOCK 3.0 searches with the shape-fitting algorithm. A database of a 210 entries from the Cambridge Crystallographic Database, CSD, ranging from entries AACFAZ10 to BBETIM10 was searched allowing 3 close contacts per scoring orientation. Close contacts were permitted because of the uncertainty in the protein structure due to the low resolution (3.5 Å) data that was available. CSD entry APYANB, melitracene, was ranked 1 in the hit list and filled the void volume remarkably well.

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6. Compound (3.3% DMSO final concentration) was preincubated for 60 min with aliquots of solubilized microsomes prepared from baculovirus infected Sf9 insect cells expressing recombinant human cyclooxygenase 1 or 2 in a reaction mixture containing hematin and phenol cofactors. Arachidonic acid (10 μM final concentration; Nu-Chek Prep Inc., Elysian, MN) was added to start the reactions. Following an incubation time of 2.5 min at 25 °C, the reactions were quenched with HCl and neutralized with NaOH. PGE_2 production by the reaction mixtures was measured by EIA (Cayman Chemicals, Ann Arbor, MI).

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8. A solution of 4.67 g 1-ethyl-3[3-(dimethylamino)propyl]-carbodiimide hydrochloride (24.37 mmol) and 0.975 g of 4-dimethylaminopyridine in 150 mL dichloromethane was partitioned among 48 various carboxylic acids in disposable 16 × 125 mm screw capped culture tubes (approx. twofold excess to the amount of compound **7**). A solution of 2.50 g of 1-(3-aminopropyl)phenothiazine, **7**, (9.75 mmol) in 100 mL dichloromethane was distributed equally among the tubes by a repeater pipette (2 mL each). The

reactions were shaken at ambient temperature for 48 h. The reactions were washed with 5 mL × 2 portions of 1 M citric acid, 5 mL × 2 portions of 1/2 saturated sodium carbonate solution, and 5 mL × 2 portions of distilled water. After each wash, the tubes were shaken followed by centrifugation. The solvent was then removed by a Savant speedvac overnight. The residues were purified by column chromatography utilizing sep-packs filled with silica gel. The eluant used was solutions of ethyl acetate/dichloromethane of varying proportions. Purities obtained were determined by HPLC (88%–98%). Verification of the product was determined by MS. The yields ranged from 55–98%.

9. All 48 carboxylic acids used in this library were commercially available, except the acid used in the synthesis of compound **11**. In this case, 3-(*p*-bromophenoxy)-2,2-dimethylproprionic acid was prepared by *p*-bromophenoxide reaction with the mesylate of 2,2-dimethyl-3 hydroxyproprionic acid.

10. The remaining library members are listed as follows: R = 3,5-di-CF_3-phenyl; 3,5-dicholorophenyl; 2,4-dimethoxyphenyl; 3-(2-thiophenyl)propyl; dicyclohexylmethyl; 2,2-dimethyl-3-acetyl-cyclobutylmethyl; 2,2,3,3-tetramethylcyclopropyl; 1-adamantylmethyl; 3-cyclohexylpropyl; cyclohexylmethyl; cyclopentylmethyl; cyclobutyl, 3-oxo-butyl, 3-allyl; vinyl; 4-heptyl; 3-pentyl; 3-methylbutyl; isobutyl; 4-(4-bromophenoxy)-butyl; methyl; 3-(4-(4-cyanophenyl)-phenoxy)propyl; 4-(3-bromophenoxy)-butyl, α-methoxybenzyl; 4-sulfonamide-phenyl; 4-CF_3-phenyl; 3-phenoxypropyl; styryl; 2-fluorophenyl; 2,4,6-trimethylphenylmethyl; 2-(3-methoxyphenyl)ethyl; 4-methoxyphenylmethyl; 4-phenylbutyl; 4-pentynyl; 2-nitrophenylmethyl; 5-(methoxy)-5-oxo-pentyl; 4-bromophenylmethyl; 3-(methoxy)-3-oxo-propyl; 4-*N*,*N*-(dimethyl)-aminobenzyl; 3,4,5-trimethoxybenzyl. Three additional compounds proved too insoluble to assay.

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