Novel Cyclooxygenase-1 Inhibitors Discovered Using Affinity Fingerprints

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We used protein affinity fingerprints to discover structurally novel inhibitors of cyclooxygenase-1 (COX-1) by screening a selected number of compounds, thus providing an alternative to extensive screening. From the affinity fingerprints of 19 known COX-1 inhibitors, a computational model for COX-1 inhibition was constructed and used to select candidate inhibitors from our compound library to be tested in the COX-1 assay. Subsequent refinement of the model by including affinity fingerprints of inactive compounds identified three molecules that were more potent than ibuprofen, a commonly used COX-1 inhibitor. These compounds are structurally distinct from those used to build the model and were discovered by testing only 62 library compounds. The discovery of these leads demonstrates the efficiency with which affinity fingerprints can identify novel bioactive chemotypes from known drugs.

Introduction

Molecules known to have activity against a therapeutic protein target are often the starting point for the discovery of new active molecules. These lead molecules can be commercially available drugs, compounds in clinical trials, or preclinical development candidates. Known drugs are logical starting points for developing therapeutics that will be competitive with existing therapies (i.e., "me too" drugs) or helpful in developing second-generation drugs. Molecules in preclinical development or clinical trials often fail for reasons other than lack of potency against their biological target.[1,2] Poor toxicity profiles, bioavailability, and pharmacokinetics can stop drug development. These initial compounds, however, can serve as starting structures for backup candidates. The challenge is how to use the information from known active molecules to identify a new class of molecules that is active, structurally distinct from the known active molecules, and without the undesirable properties of the original leads.

Computer models that distinguish active from inactive molecules have been used to identify promising molecules that have not been evaluated. These new molecules are evaluated in the laboratory to determine whether they display activity and fulfill the criteria for alternative leads as described above. One drawback of these methods is that they are almost entirely based on the chemical structure of molecules under consideration.[3−5] Atoms and bonds are represented in computer memory and analyzed by algorithms to construct computational models that are applied to the computer representation of the structures of untested molecules.[6] Methods based on topological similarity (i.e., patterns of atoms and bonds) are effective at finding active molecules that have the same chemotypes as the known active molecules,[7−9] while methods that consist of pharmacophores or molecular shapes are better at identifying structurally novel active molecules.[10−12] Computational docking experiments can also be effective in identifying active molecules with novel chemotypes if the three-dimensional structure of the target protein is known.[5,13,14]

An alternative approach that uses descriptors based on biological measurements rather than descriptors derived from chemical structure may offer certain advantages in identifying alternative lead molecules. This is the basis of target related affinity profiling (TRAP).[15] TRAP technology characterizes small molecules by their affinities to a panel of proteins. A compound's set of binding affinities to this panel, its *affinity fingerprint*, is the descriptor used to construct computational models for activity against a particular therapeutic target.[15−19]

In this study, we constructed an affinity fingerprint model for cyclooxygenase-1 (COX-1) inhibition using known nonsteroidal antiinflammatory drugs (NSAIDs). Our model was used to select compounds from our chemical library for assay against COX-1. As the report of a prospective study, this paper supplements previous retrospective proof-of-concept computational studies for TRAP.[16−18] In addition, the use of affinity fingerprints of known drugs as a starting point for compound selection is novel.

Target Related Affinity Profiling

TRAP technology is based on the principles that most drugs produce their biological effects by interacting with proteins and that small molecules can be classified by these interactions. By extensively surveying small-molecule interactions with several hundred proteins, we have identified a reference panel of proteins that collectively simulate the significant interactions between

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small-molecule ligands and proteins.[15,17] The binding of each small molecule in our chemical library is measured for each of the proteins in the reference panel, and the vector of each compound's binding affinities constitutes its *affinity fingerprint*. The set of affinity fingerprints collectively constitutes a database of molecular descriptors used for drug lead discovery.

Affinity fingerprints are not constructed from chemical structure; thus, they are naturally suited for finding novel chemical entities. Several earlier studies of chemical structures and affinity fingerprints have demonstrated that the relationship between small structural changes in a molecule and the resultant changes in protein binding affinities can be unpredictable.[17,20] This is consistent with the experience of medicinal chemists in lead optimization, who have found that small structural changes can produce drastic effects on potency while more structurally significant changes may have little effect on a molecule's activity.[21]

Affinity fingerprints are routinely used at Telik, Inc. for the identification of small-molecule leads in the absence of any a priori activity data.[17] For example, a training set of ~70 compounds exhibiting the greatest diversity in their affinity fingerprints is chosen and assayed against a new therapeutic target to identify a lead compound. By means of a set of proprietary algorithms, a computational model is constructed from these assay results and the affinity fingerprints of active and inactive compounds. A second set of ~70 compounds is chosen from our compound library on the basis of their fit to the fingerprint-derived computational model. The process is repeated iteratively, resulting in the identification of compounds with an increasingly better affinity to the target. Usually the model is constructed to improve potency, although we have also had success in applying models for target selectivity. Typically, biologically active molecules with affinity for the target in the low micromolar to nanomolar range emerge from three rounds of biological screening, or about 200 bioassays total.[17]

In principle, affinity fingerprints can also be used for the identification of novel chemical scaffolds exhibiting activity against a therapeutic target for which known drugs already exist. In this approach, the known bioactive molecules are fingerprinted by assaying them against the panel of TRAP proteins, and a computational model for bioactivity is constructed. The compound library is then searched to identify compounds that have fingerprints consistent with the computational model constructed from the fingerprints of the known drugs. These compounds are then tested in the assay of interest. Because the search is based on affinity fingerprint similarity rather than structural similarity to the known drugs, bioactive molecules are discovered with novel chemical scaffolds. This is the approach that we have used in this study, and it provides a novel, alternative use of affinity fingerprints to identify drug leads.

NSAIDs and Cyclooxygenases

NSAIDs inhibit cyclooxygenase (prostaglandin G/H synthase, COX), a membrane-bound enzyme responsible for the oxidation of arachidonic acid to prostaglandins that was first identified in the early 1970s.[22] The COX enzyme, a 71 kDa hemo- and glycoprotein, is expressed in two isoforms. COX-2 is believed to be the therapeutic molecular target for NSAIDs because its expression is induced in inflammation. COX-1, on the other hand, is constitutively expressed, and its inhibition by NSAIDs is believed to be responsible for their gastric and renal toxicity.

There are several structural classes of NSAIDs: (1) salicylic acid derivatives (e.g., aspirin), (2) *p*-aminophenol derivatives (e.g., acetaminophen), (3) indole and indene acetic acids (e.g., indomethacin), (4) heteroarylacetic acids (e.g., diclofenac), (5) arylpropionic acids (e.g., ibuprofen), (6) anthranilic acids (e.g., mefenamic acid), (7) enolic acids (e.g., piroxicam), and (8) alkanones (e.g., nabumetone).[23] While most NSAIDs are reversible competitive inhibitors of both cyclooxygenase isoforms, aspirin irreversibly inhibits cyclooxygenase by acetylating a serine residue in the active site.[24] Because of aspirin's different mechanism of action, it was not used in this study.

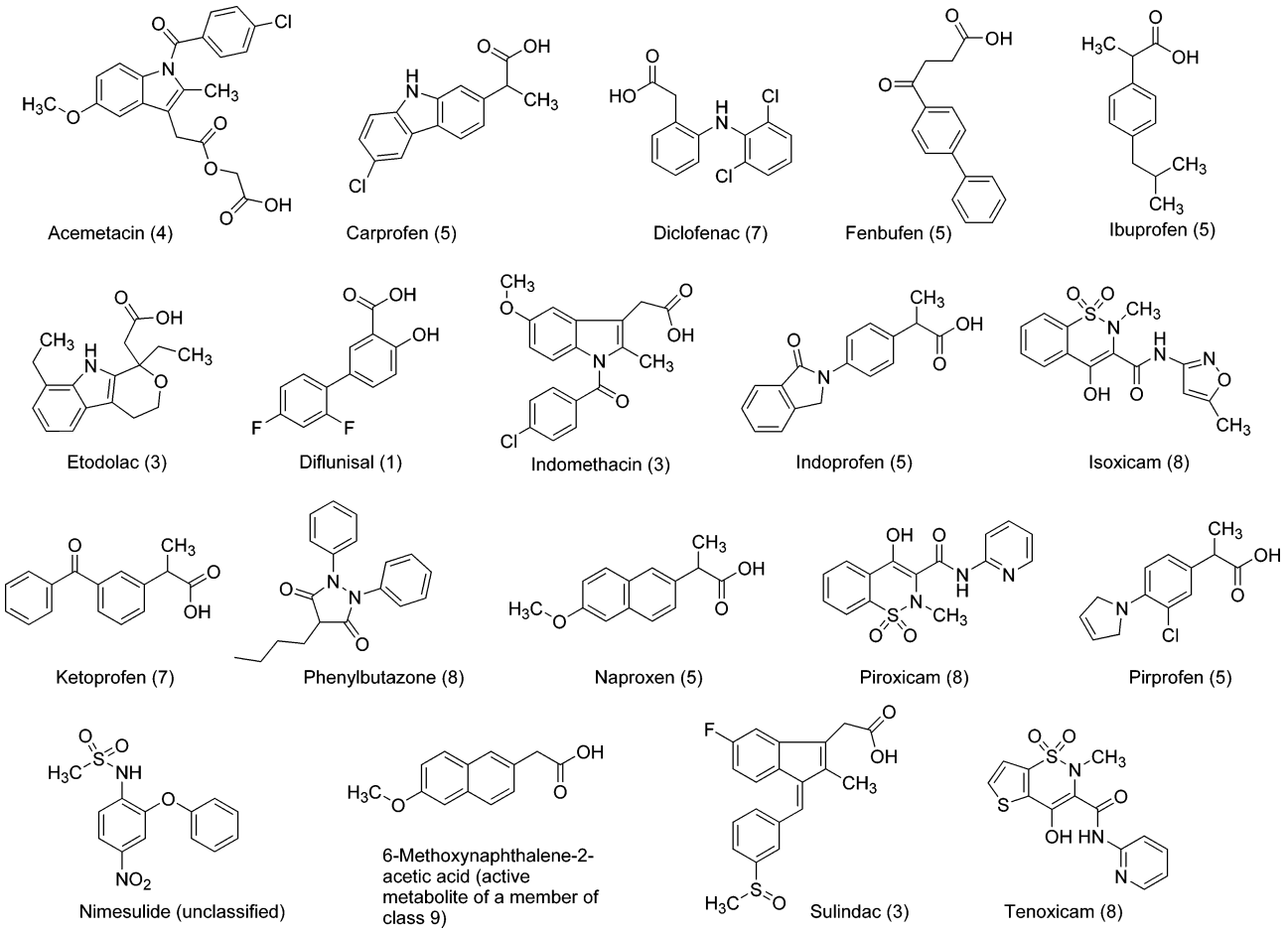
Results and Discussion

Compounds were selected to test their COX-1 inhibition in two batches. The selection of the initial batch of compounds was based on a model constructed from the fingerprints of 19 NSAIDs (Table 1). The 16 compounds selected by using this method were inactive (<50% inhibition at a compound concentration of 200 μM). The second batch of compounds was chosen with a refined computational model that incorporated the affinity fingerprints of the inactive molecules from the first batch of compounds. This refined model for COX-1 inhibition yielded an additional 46 compounds for assay. Five of these compounds showed >50% inhibition at a screening concentration of 200 μM. The concentration response curves demonstrated that three of the five active compounds had IC_{50} values in the range 1−100 μM. IC_{50} curves are shown in Figure 1. The IC_{50} value determined for ibuprofen under these assay conditions (10 μM arachidonic acid) was 76 μM, which is consistent with IC_{50} values obtained by others at lower substrate concentrations.[25,26] Note that all three active molecules identified have lower IC_{50} values than ibuprofen.

The chemical structures and IC_{50} values for the three active molecules (**1**−**3**) are shown in Table 2. Each of the molecules satisfies the general criteria associated with a good drug lead: the molecular weight is relatively low, the number of hydrogen bond donors and acceptors is small, the number of rotatable bonds is limited, and there are several regions on the molecule that could be modified in a lead optimization effort. A search of the literature revealed that none of these molecules have been reported to have antiinflammatory activity, although it is interesting to note that **3** was patented as an intermediate to antiinflammatory phenoxyalkanoic acids.[27]

The structurally most similar compounds from the 19 known NSAIDs that we used to build the model for COX-1 inhibition are shown in Table 2. The active molecules identified appear to be structurally distinct from those used to build the model for bioactivity. Each of the known NSAIDs in Table 2 has a carboxylate group, while this is not present in any of the newly identified active molecules. The diaryl ketone moiety in

Table 1. Known Cyclooxygenase-1 Inhibitors Used To Build the Fingerprint Model for COX-1 Inhibition (Structural Classes in Parentheses)



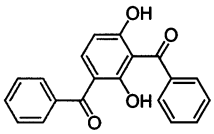
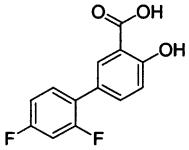
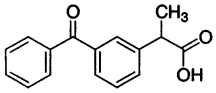
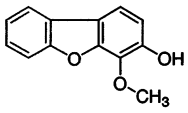
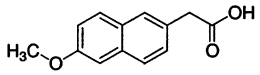
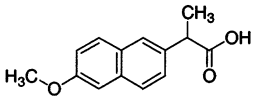
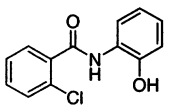
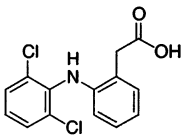
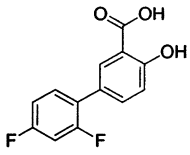
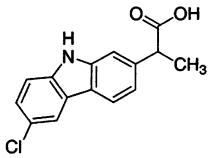
1 is interesting in that it is also present in the NSAID ketoprofen. Ketoprofen is typically classified as a propionic acid derivative, but the activity of **1** suggests that the acidic group is not required for activity. The related drug flurbiprofen contains a carboxylic acid group, and it has recently been shown that the drug and its ethyl ester bind to COX-1 in nearly identical fashion, as demonstrated by X-ray crystallography of their cocrystals with the enzyme.[28] Moreover, the diaryl ketone group of **1** is seen in a number of ketoprofen analogues[29] and has been reported in other compounds that are classified under different chemotypes. For example, chapter 6 in ref 30, which discusses sulfonamides with antiinflammatory activity, includes several active molecules with a diaryl ketone moiety. Compound **1** suggests that this group may be more important than the chemotypes that are used to classify the antiinflammatory agents in which it is present.

Affinity fingerprints of inactive compounds played a critical role in the discovery of active molecules in this study. The model constructed solely from the known NSAIDs was unsuccessful for selecting active compounds because all 16 compounds selected on the basis of this model were inactive. When the affinity fingerprint model for COX-1 inhibition was refined by including the fingerprints from these 16 inactive molecules, an improved model resulted. A total of 5 of the 46 compounds selected by the refined model showed some activity at high concentrations, and 3 were confirmed to be more potent than ibuprofen. Clearly, the fingerprints of inactive molecules were very important in constructing an effective model for COX-1 inhibition.

The influence of the affinity fingerprints of inactive molecules is best illustrated by the changes in the scaling coefficients shown in Figure 2. As described in Computational Methods, each element of a compound's affinity fingerprint is scaled by the correlation coefficient of that coordinate with activity. The initial scaling coefficients, which were determined from the affinity fingerprints of the 19 NSAIDs, differ significantly from the refined coefficients that were calculated using the fingerprints of active and inactive compounds. Of the four panel proteins whose NSAID binding affinities were most strongly correlated with activity in the first round of compound selections, none play a significant role in the second round. In the refined model, affinities to panel proteins 3, 12, and 2 are the most strongly correlated with NSAID activity. A principal components analysis of this model substantiated that these panel proteins were influential in determining the spatial relationships among the compounds. In particular, the relationships among the actives and inactives in the two-dimensional subspace defined by proteins 3 and 12, the two most influential proteins, are shown in Figure 3.

Affinity fingerprints have been shown to be efficient molecular descriptors for drug lead discovery.[17] Perhaps more importantly, these molecular descriptors are not

Table 2. Chemical Structures for Active Compounds Identified by Affinity Fingerprints and the Structures of the Most Similar NSAIDs As Measured by Tanimoto Distance (D_T) Using ISIS Structural Keys[a]

Active Compound	Most Structurally Similar NSAIDs in this study*		
			
1 (IC_{50} = 6 μM)	Diflunisal (D_T = 0.34)	Ketoprofen (D_T = 0.48)	
			
2 (IC_{50} = 29 μM)	6-Methoxynaphthalene-2-acetic acid (D_T = 0.47)	Naproxen (D_T = 0.50)	
			
3 (IC_{50} = 69 μM)	Diclofenac (D_T = 0.39)	Diflunisal (D_T = 0.49)	Carprofen (D_T = 0.49)

[a] Tanimoto distance of <50% using ISIS structural keys.

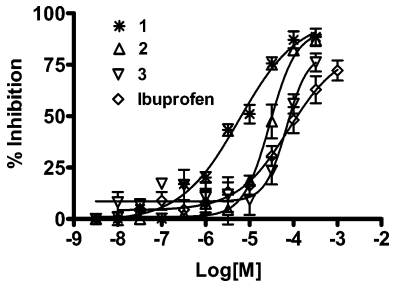


Figure 1. Concentration response for the three most potent hits identified. Concentration response for ibuprofen is shown for comparison. Data are expressed as the mean ± SEM from two to five determinations from at least two separate experimental days.

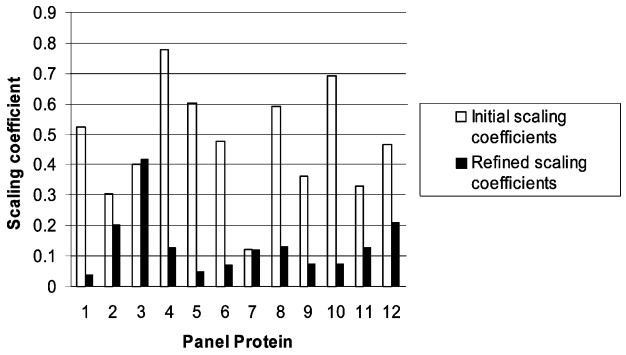


Figure 2. Activity-biased scaling coefficients for the two rounds of compound selections. The initial scaling coefficients were calculated in the absence of any data for inactive compounds. Inclusion of inactive data yields significantly different values for the refined scaling coefficients. Proteins 3, 12, and 2 correlate most strongly with activity. A principal components analysis of the activity data in the 12-dimensional scaled space confirmed this and indicated that proteins 3 and 12 were the most influential in determination of the spatial relationships among the compounds (see Figure 3).

directly related to structure. Active molecules can therefore be identified that have novel chemotypes after testing ~60 compounds, as demonstrated in the current study. Our procedure provides a distinct advantage over methods that rely on chemical structure alone for modeling bioactivity. These structural approaches may be well suited for the identification of potent molecules that are structurally related to those known to be active but may be less useful in exploring regions of chemical space that fall outside existing intellectual property claims or are known to have pharmacological liabilities.

The fact that protein affinity fingerprints provide a useful descriptor for constructing models for activity against a therapeutic target is akin to the success of in vitro "descriptors" in predicting a molecule's absorption and metabolism. In contrast to structural profiles, biological profiles of a molecule, such as measurements of CACO-2 cell transport or hepatocyte metabolism, are more closely related to what one is trying to model (i.e., absorption or metabolism). Similarly, protein affinity fingerprints are in vitro measurements of molecule−protein interactions, so it is not surprising that they are useful in predicting protein binding affinities.

Future directions for work with protein affinity fingerprints include developing models for target selectivity. Drugs have been approved that are selective for COX-2 over COX-1, and these are claimed to show the antiinflammatory behavior of traditional NSAIDs without the typical side effect profile associated with COX-1 inhibition.[31,32] Preliminary work with affinity fingerprints on other targets has shown that selectivity can

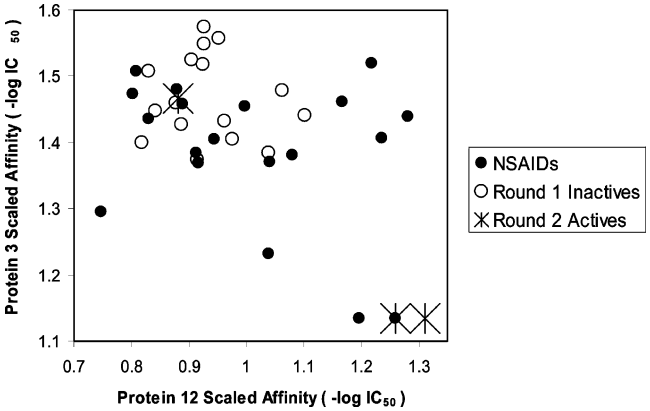


Figure 3. Spatial relationships among the 19 NSAIDs, the 16 inactive compounds identified in the first round of compound selections, and the 3 active molecules identified in the second round of compound selections, in the two dimensions most important in selecting the compounds in the second round (as determined by principal components analysis). The two active molecules in the lower-right-hand corner were selected by the interpolation model, while the active molecule in the upper left was selected by the nearest-neighbor algorithm.

be built into affinity fingerprint models for bioactivity; hence, COX-1/COX-2 selectivity is a logical next step in this work.

Conclusions

Starting from the protein affinity fingerprints of 19 known NSAIDs, three structurally novel compounds with good bioactivities toward COX-1 have been discovered by assaying only 62 compounds. This demonstrates a powerful new technique for using affinity fingerprints of known drugs to find novel drug leads for validated therapeutic targets or to find alternative chemotypes for drug candidates that display undesirable properties.

Experimental Section

Computational Methods. The computational models used to select compounds for COX-1 inhibition were based on the affinity fingerprints of 19 known COX-1 inhibitors (Table 1). Several of the structural classes of NSAIDs are represented by these compounds. The arylpropionic acid NSAIDs (e.g., ibuprofen) comprise the largest class in the set, but this is the result of the NSAIDs that happen to be present in our compound collection and not of any particular design for this experiment. The molecular weight for the 19 NSAIDs ranges from 206 to 416, with an average value of 293. To assess the structural diversity among these compounds quantitatively, we used the structural keys from the MDL's ISIS/Base software (version 2.4, MDL Information Systems, Inc., San Leandro, CA). The structural keys are a set of 166 bits that represent the presence or absence of structural elements in a molecule.

Using these descriptors, we calculated the Tanimoto distance (1 minus the ratio of the number of bits in common between the pair of molecules to the total number of bits set in either molecule) between all pairs of NSAIDs. The average Tanimoto distance was 0.65 (where 0 is most similar and 1 is most different). For comparison to typical structural differences between biologically active molecules, we repeated the distance calculation for compounds chosen from the Comprehensive Medicinal Chemistry database (version 2003.1, MDL Information Systems, Inc., San Leandro, CA). These compounds were chosen randomly with the constraint that the molecular weight distribution approximates that of the NSAIDs. The compounds had an average pairwise Tanimoto distance of 0.69. The NSAIDs that were used to build the activity model can therefore be considered structurally diverse.

To obtain their affinity fingerprints, the 19 NSAIDs were assayed against a reference panel of proteins.[15] Once an affinity fingerprint for each of the NSAIDs has been measured, it can be used to represent the NSAID as a point in 12-dimensional space, one dimension for each panel protein affinity. The compounds selected for assay against COX-1 in this study were chosen from a 20 000 compound subset of Telik's corporate collection, a small-molecule screening library that consists of internally synthesized compounds and compounds purchased from chemical vendors. These molecules had already been fingerprinted against the 12-member protein panel. They therefore populate the 12-dimensional space with 20 000 affinity fingerprint points whose COX-1 activities have not been determined. The TRAP algorithms select compounds for testing on the basis of the spatial relationships among active, inactive, and untested molecules in this 12-dimensional space.

The goal of these algorithms was to find candidate molecules (points) in this space that are structurally novel inhibitors of COX-1. To do this requires a metric. We used the weighted Euclidean distance, where the weight for each coordinate was the correlation of that coordinate with NSAID activity. For example, if all 19 NSAIDs had fingerprints that showed strong binding with panel protein 1 and none of the others, there would be a strong correlation between the value of the first dimension in the 12 dimensions in the space and weak correlation with the others. As a result, the weights of the 11 dimensions that did not correlate well would be reduced, and their influence on compound selection would be minimized. Further details are provided in ref 17.

Once the coordinates of all compounds (tested, as in the case of the NSAIDs, or untested, as in the case of the small-molecule screening library) had been rescaled, compounds were selected for screening against COX-1. The selections were made using two methods. The first method was an interpolation procedure that produces a function that predicts activity for all unscreened compounds based on their weighted Euclidean distances from compounds whose activities have already been measured. Each untested compound is assigned a predicted activity based on the value of the function at the location of that compound in the 12-dimensional space. Compounds with the highest predicted activities are submitted for assay. In the case of the activities of NSAIDs, it is difficult to compare activity values from different citations, so we assigned each an arbitrary activity of 1.0. In addition, because interpolation requires inactive data, we added a "null fingerprint" to the origin of the space and assigned it an activity of 0. In addition to the compound selections based on high predicted activity from the interpolation function, we selected compounds that were nearest neighbors to the known actives in the affinity fingerprint space.

The compound selections were carried out in two rounds. In the first, 16 new compounds were selected from our library by the methods described above. None of the compounds selected had significant activity in the initial screening assays. In the second round, the null fingerprint was discarded, and the fingerprints of the actual inactive molecules were used instead. The space was rescaled (the correlations between coordinates and activity change because of the presence of the inactive fingerprints), the interpolation function was recalculated, and an additional 46 library compounds were selected for screening. As described in the results section, five of these compounds showed activity.

In Vitro Assay. The colorimetric COX (ovine) inhibitor screening assay kit was purchased from Cayman Chemical (Ann Arbor, MI). The assay kit included the following components: 10X assay buffer (1 M Tris-HCl, pH 8), 500 μM heme, 11 unit/μL COX-1 (ovine), 22 mM arachidonic acid, 0.1 M KOH, and a solution of colorimetric substrate *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD). To make a working stock of arachidonic acid, the 22 mM solution provided was first diluted 1:1 with 0.1 M KOH in deionized distilled H_2O to 2 mL,

followed by further dilution. Ibuprofen was obtained from Assay Designs (Ann Arbor, MI), and pirprofen and phenylbutazone were provided by CIBA Geigy (now Novartis). Other NSAIDs were purchased from Sigma (St. Louis, MO) and Cayman Chemical (Ann Arbor, MI).

Enzyme assays were run in 220 μL volumes. The 1X assay buffer, heme (1 μM final), and enzyme (0.17 unit/μL final) were prepared according to the instructions provided in the kit, and this mixture was preincubated with compound for 10 min at room temperature. The reaction was initiated by the addition of TMPD solution (at a saturating concentration), followed by cold arachidonic acid to 10 μM. The assay mixture was placed on a shaker, shaken for 10 s, and incubated at room temperature for 5 min. The enzyme activity was measured as an increase in absorbance at 590 nm on a Spectra Max 250 reader (Molecular Devices, Sunnyvale, CA).

Except for ibuprofen, which was supplied in aqueous solution and diluted with deionized distilled H_2O, all compounds were dissolved in dimethyl sulfoxide (DMSO) to a final DMSO concentration of 3% (the assay was insensitive to DMSO concentration up to 4.5%). Compounds were assayed for COX-1 inhibitor activity at 200 μM in duplicate. Inhibition of enzyme activity, measured by a change in units of optical density (OD), was normalized to the maximum possible change in OD and expressed as percent inhibition.

For compounds that produced >50% inhibition under these conditions, a concentration response was measured to enable IC_{50} determination. Percent inhibition was measured in 0.5 log intervals over the concentration range $-\log [M] = 9.0-3.5$. An iterative, nonlinear curve-fitting routine (GraphPad Prism) was used to generate IC_{50} curves that obeyed

$$Y = 100/[1 + 10^{(\log IC_{50} - X)(Hill)}]$$

where Y is the percent inhibition, IC_{50} is the concentration at which the percent inhibition is 50% of its maximum value, X is the log of the molar concentration, and Hill is the Hill slope.

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