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Molecular Modeling in the Design of Phospholipase A2 Inhibitors

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The X-ray structures of pancreatic bovine and porcine phospholipases A2 have been used along with interactive computer graphics to design conformationally rigid, novel compounds (1-meta-hydroxybenzyl-2-substituted acenaphthenes) directed at the active sites of these enzymes. In vitro testing confirmed that the designed compounds are potent inhibitors of the porcine pancreatic phospholipase A2 and exhibit both stereoselectivity and structure-activity relationships that are consistent with the proposed mode of binding. These compounds take advantage of a hydrophobic "slot" positioned between residues Leu-2 and Tyr-69 while positioning hydrogen-bonding functionality directed at the nd1-N of His-48. Experimental evidence shows a regioselective preference for this H-bond acceptor. A second part of the strategy used a tethered amine to displace the essential calcium providing a bisubstrate analog.

Key words: inhibitor, structure-activity relationships, stereo selectivity

Phospholipases A2 (EC 3.1.1.4) (PLA2) are thought to be critical enzymes in the arachidonic acid cascade and may be responsible for much of the esterolytic action that releases arachidonic acid from phospholipids [1]. Arachidonic acid is the known precursor for a number of mediators of inflammation in the prostaglandin and thromboxane families, and a specific inhibitor of the release of this acid might be expected to show anti-inflammatory activity [2]. The PLA2's are, in general, small (MW 14,000), stable proteins requiring Ca^{+2} ions for the stereospecific hydrolysis of the 2-acyl group of 3-sn-phospholipids [1]. The X-ray structures of three PLA2's have been determined: pancreatic bovine [3] and porcine [4] and the *Crotolus atrox* dimer [5]. These structures, which have nearly identical active sites, have provided the basis for our synthetic design.

Several inhibitors of PLA2 have been previously described [1,6,7]. Because of the complexity of the substrate interaction with this enzyme the mode of action and thus the specificity of these inhibitors is not always clear [1]. Substrate analogs [1] and transition state analogs [7] have been prepared, but the lipophilic nature of these compounds and their tendency to form micelles, and mixed micelles with substrate, complicate the interpretation of their inhibitory action. Manoalides have been shown

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to inhibit snake venom PLA2, but they do so in an irreversible fashion and with a mechanism of action (modifying surface lysines) that may not be general [8]. A butyrophenone was reported to show inhibitory activity [6] as have a number of compounds, such as mepicrine, that modify the membrane properties of the substrate [1]. Because of the tendency of PLA2's to be inhibited in a variety of nonspecific ways, it is important, in any class of inhibitors suggested to be site directed, that a structure-activity relationship (SAR) be established.

This report describes a class of compounds, designed from a molecular modeling study of the active sites of known PLA2's that shows a well-defined structure-activity relationship as well as stereoselectivity in their binding to porcine pancreatic PLA2.

MATERIALS AND METHODS

Enzyme Assay

Phospholipase A_2 activity was measured using porcine pancreatic PLA2 as the enzyme and a sonicated dispersion of 1-palmitoyl-2-[14c]-arachidonyl-phosphatidyl choline (54.5 μCi/μmol, New England Nuclear) as the substrate in a manner similar to that described by Alonso et al. [9]. Inhibitors and buffer (25 mM Tris, 25 mM glycylglycine, 25 mM $CaCl_2$, and 0.75 mM EDTA, pH 8.5) were mixed and incubated with 25 ng porcine pancreatic PLA2 (Sigma) at 37°C for 2 min to allow interaction between the enzyme and the drugs. The substrate 0.04 μCi (7 μM final concentration) was added to initiate the reaction which continued for 5 min at 37°C. The total reaction volume was 0.1 ml. The reaction was stopped by freezing in a dry ice/ethanol slurry. Released arachidonic acid was separated from the unreacted substrate using 3-ml silica gel columns (Baker, Fisher, or Applied Separations) which were conditioned with 1 ml of ethylacetate:acetic acid, 99:1 (solvent A). Solvent A (0.5 ml) was triturated with reaction mixture as it thawed and placed on the silica column. A further 2 ml of solvent A was used to elute the arachidonic acid from the column. The unreacted phosphatidyl choline was removed using 2 × 2 ml washes of ethyl acetate:methanol:water (1:1:1). The radioactivity in the column fractions was quantitated using a scintillation counter that converted CPM to DPM to correct for solvent quenching.

Routinely, 80–85% of the radioactivity was recovered. Inhibitors were dissolved in buffer and tested in duplicate within each experiment, and each inhibitor was tested in at least two experiments. When necessary, inhibitors were dissolved in pure ethanol or DMSO and then diluted with buffer to give final concentrations of organic solvent that did not influence the enzymatic reaction. The percent inhibition at a given concentration was combined for several experiments, and the IC_{50} determined from a semilog plot of % inhibition vs. concentration. Under the conditions described the rate of hydrolysis was 5–7 pmol/min with 15–20% of the substrate being hydrolyzed in the absence of inhibitors. The substrate concentration was purposefully kept low to minimize the effects the inhibitors might have on the vesicle organization of the substrate.

A number of the inhibitors studied are very hydrophobic. To assess their solubility under the conditions of the assay the UV absorbance of compound **1** was studied as a function of its concentration in the buffer above (first dissolved in ethanol and then diluted with buffer). Absorbance at 336 and 314 nm showed a maximum solubility of 20 μM, tenfold above the micromolar range of activity.

Chemistry

The acenaphthenes were prepared by condensation of acenaphthanone with an appropriate aryl aldehyde. Catalytic reduction of the double bond and alkylation with methyl iodide at the 1-position gave 1-arylmethyl-1-methyl-2-acenaphthanones. The 2-carbonyl was converted by a Wittig reaction to the 2-methylene. 1-m-hydroxybenzyl-1-methyl-2-methylene-acenaphthanone was resolved by preparing the diasteriomers from the esterification of 1-m-methoxybenzyl-1-methyl-2-beta-hydroxy acenaphthene with both (+)- and (−)-camphanic acid. The diasteriomers were separated by preparative HPLC. Cleavage of the esters, conversion of the resultant alcohols by oxidation to the 2-ketones, and reaction of the ketones as above gave the 2-methylene compounds which in turn yielded the (+)- and (−)-enantiomers of 1-m-methoxybenzyl-1-methyl-2-methylene-acenaphthene. Details of the syntheses of these compounds will be reported elsewhere [10].

RESULTS

Several analogs of the acenaphthenes were synthesized and tested to determine their effectiveness in inhibiting the hydrolysis of 1-palmotyl-2-arachidonyl-phosphatidylcholine by porcine pancreatic PLA2. We were particularly interested in determining whether these compounds would demonstrate a structure-activity relationship that would be consistent with their proposed mode of binding. Figures 1–5 summarize these results. The relative activities shown in these figures refer to the most active compound of the series, i.e., relative activity 100 = IC_{50} of 2×10^{-7} M. Compounds **1** and **2** (Fig. 1) show the importance of the most deeply buried aromatic ring of the naphthalene part of the acenaphthene with **1** being 380× more active than **2**. In contrast, when ring B is excised from **1** to yield **3** the loss in activity is modest. Incorporation of a chain extending from the 2- or 2-alpha position (compounds **4,5**) suggests the

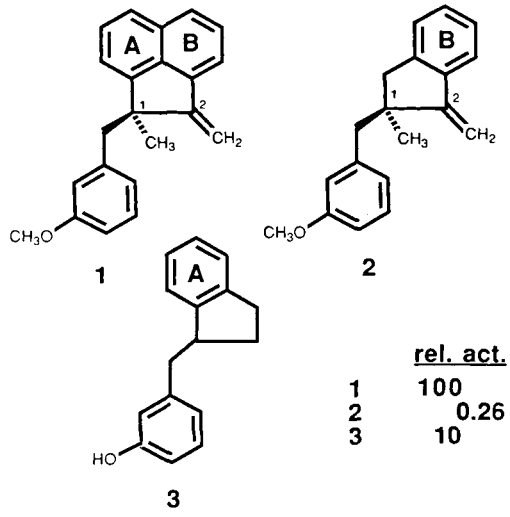
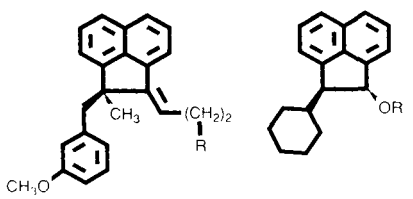
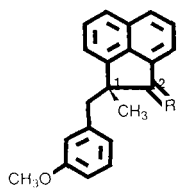


Fig. 1. Importance of ring **A** of the acenaphthene—note the loss of inhibitory activity when ring A is removed (**2**), while little is lost in the absence of ring **B** (**3**).

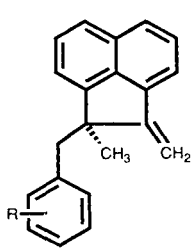


	R	rel. act.		R	rel. act.
4	NH_2	30	6	$(CH_2)_3NH_2$	14
5	CH_3	6	7	$CH_2CH{=}CH_2$	2.6



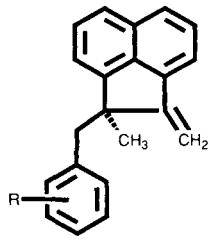
	R	rel. act.
8	$=CH_2$	100
9	$=CHCH_3$	21.4
10	$=CH(CH_2)_2CH_3$	6

Fig. 2. Effect of tethered amine—note **4,6** improve activity over the corresponding hydrocarbon. Projecting a hydrophobic chain into the hydrophilic environment of the calcium reduces activity.



	R	rel. act.
11	o-OCH_3	1.7
12	m-OCH_3	100
13	p-OCH_3	0.08
14	o-OH	1.1
15	m-OH	100
16	p-OH	17.
17	m-$NHSO_2C_6H_5$	1
18	p-$NHSO_2C_6H_5$	1.6
19	m-$NHCOCH_3$	1
20	m-CF_3	inact.
21	p-CF_3	inact.

Fig. 3. Regioselectivity for hydrogen-bond donating groups in the meta position of the pendant aryl ring – note **12,15** vs. **11,13,14,16.** Large (**17,18**) and neutral groups (**20,21**) are inactive.



	R	rel. act.
12	m-OCH_3	100
22	p-Br,m-OCH_3	.06
23	p-H	8
24	p-$CH(CH_3)_2$	inact.
25	p-CF_3	inact.
26	p-NO_2	inact.
27	p-F	.08

Fig. 4. Effects of para substitution of pendant aryl group—compound **12** is inactivated by para substitution (**22**).

importance of the amine group (Fig. 2). Thus, amine (**4**) is a fivefold better inhibitor than **5**. A similar trend is seen for **6** and **7**. Compounds **8–10** show that as the length of the alkyl chain is increased the compounds bind less well. Substitution in the aryl ring of the pendant benzyl group shows a preference for a hydrogen bond acceptor in the meta position (Fig. 3). This regioselectivity is shown by the activity of the m-methoxy

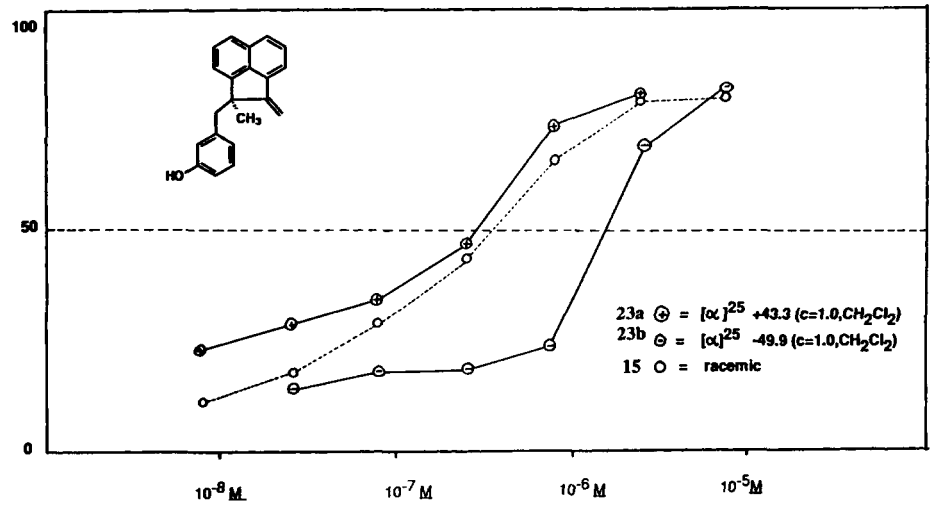


Fig. 5. Stereoselectivity of binding of acenaphthenes. The y-axis is the % inhibition and the x-axis concentration of inhibitor. The (+) isomer is consistently more active than the (−) at all but the highest concentrations.

and m-hydroxy compounds (**12,15**) compared to the relatively inactive ortho and para isomers (**11,13,14,16**). Larger groups in the meta position reduce activity (**17–19**), while neutral substituents (**20,21**) render the compounds inactive. Para substitution, in general, decreases activity (Fig. 4). In particular, when an active compound, **12,** is modified with a para substituent (**22**), it loses activity. For the case of the enantiomeric pair, **23a** and **23b,** the (+) isomer is about 5–10× more active than the (−) (Fig. 5).

DISCUSSION

The x-ray structure of three known PLA2's, pancreatic porcine, bovine, and the *Crotolus atrox* dimer, have been used to design novel PLA2 inhibitors directed at the active sites of these enzymes. In part, this strategy involved an analysis of how the substrate phospholipid was bound at this site, and this in turn suggested the starting acenaphthene framework which could be located in a preferred packing orientation [11] in a "slot" between Leu-2 and the aryl ring of Tyr-69. Details of this strategy are included in a preliminary report of this work [12]. The acenaphthene structure provided an anchoring framework which possessed two stereochemical centers from which substituents could be directed stereospecifically into each part of the active site (Fig. 6). The benzyl group was selected as an ideal substituent to be projected from the 1-position since it could be easily substituted in a position (meta) that was predicted [12] to be able to hydrogen bond to His-48 nd1-N. It also located the aryl ring of the benzyl group in the preferred perpendicular arrangement to Phe-5, although with a somewhat greater separation (5.8 Å) than is considered ideal (4.6 Å) [11]. A second design target was the carboxyl group of Asp-49 or the essential calcium near the bottom of the site. Our strategy called for extending an amine group into this part of the site to either interact with the Asp-49 carboxyl group or to displace the calcium with a protonated amine to provide a bisubstrate analog. Bisubstrate analogues, which in this case would combine a ligand and cofactor in a single molecule, can have binding constants lower than the *product* of the binding constants of the two independent ligands

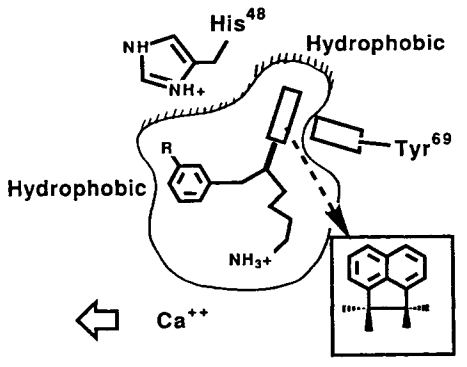


Fig. 6. PLA2 inhibitor design strategy. The acenaphthene is seen edge on (note box view) in the active site "slot" formed by Leu-2 and Tyr-69. See reference 12 for details of the rationale behind this design.

[13]. Finally, the proposed mode of binding suggests stereoselectivity should be observed with one of the enantiomers binding better than the other.

The acenaphthenes synthesized appear to be consistent with their predicted structure-activity relationships. The more buried of the two acenaphthene aromatic rings is clearly more important for activity. The small improvement in binding of amines **4,6** is consistent with the idea that the free-energy difference between free, solvated charged ligands in aqueous solution and the buried complexed ligand may be small because the substantial energy achieved by pairing charged groups in the complex is partially offset by the energy lost in desolvating the free ligand. Compounds **8–10** show that as the hydrophobic alkyl chain is extended into a hydrophilic part of the enzyme site binding is reduced as expected. This is particularly significant for inhibitors of PLA2 since *in general* increasing hydrophobicity of nonspecific inhibitors increases binding. A very important feature of this series of compounds is the predicted regioselectivity of a hydrogen-bond donor or acceptor (depending on the ionization state of His-48 in the complex) in the meta-position of the pendant benzyl group. Compounds **11–21** show a clear preference for meta substitution of an H-bond acceptor and a value for this interaction of about 1.5 kcal/mol [cf. (**12,15**)(Fig. 3) vs. (**23**)(Fig. 4)]. This is consistent with experimental determinations of an uncharged hydrogen bond in protein-ligand complexes (0.5–1.5 kcal/mol) [14]. Finally, stereoselectivity is demonstrated by the preferred binding of the (+) isomer of **23ab** over the (−) (Fig. 5). Taken together, this SAR strongly suggests a specific binding of these compounds to the active site of PLA2 and confirms the predictions of the modeling study. Unequivocal evidence for our proposed binding would come from an x-ray or two-dimensional nuclear magnetic resonance analysis of an enzyme-inhibitor complex and these studies are underway.

Comparison of the potency of these compounds with other published PLA2 inhibitors is difficult because of the substantially different conditions employed in the various reported assays. Although these compounds appear quite potent in our assays (IC_{50} of 10^{-7} M), it should be recognized they are competing against low substrate concentrations (ca. 10^{-5} M). This concentration is just below the concentration (for this particular substrate and enzyme) which shows the well-known rate acceleration due to substrate aggregation and the enzymes preference for these aggregates [1]. It was chosen to give a reproducible assay. Preliminary studies, however, have also shown these compounds inhibit the *Crotalus adamanteus* (snake venom) with *Escherichia coli* as substrate although at a much lower level (IC_{50}'s in the mM range) [15]. More important, however, than absolute potency is the fact these compounds represent a class that shows well-defined structure-activity relationships as well as stereoselectivity against these important enzymes.

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