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1,3-DIOXANE-4,6-DIONE-5-CARBOXAMIDE-BASED INHIBITORS OF HUMAN GROUP IIA PHOSPHOLIPASE A_2: X-RAY STRUCTURE OF THE COMPLEX AND INTERFACIAL SELECTION OF INHIBITORS FROM A STRUCTURAL LIBRARY.

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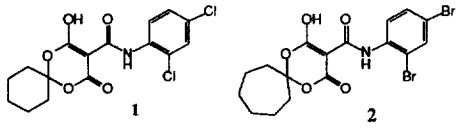
Abstract: A library of 109 1,3-dioxane-4,6-dione-5-carboxamides was prepared by solution-phase methods as potential inhibitors of human group IIa phospholipase A_2. Tight binding inhibitors were found by an interfacial affinity selection method. The crystal structure of the secreted phospholipase A_2 containing one of the inhibitors was determined, and it reveals the inhibitor-calcium bidendate coordination.

Inhibitors of phospholipases A_2 (PLA_2s) are of interest as potential antiinflammatory agents since these enzymes catalyze the liberation of arachidonic acid from the *sn*-2 position of cellular phospholipids for the biosynthesis of eicosanoids. Based on amino acid sequence patterns, ten groups of PLA_2s have been identified.[1,2] Of these, it appears that both the 87 kDa cytosolic PLA_2 (group IV) and at least 4 groups of 14–18 kDa secreted PLA_2s (groups IIa, IIc, V, and X) are responsible for arachidonic acid release in mammalian cells stimulated with pro-inflammatory agonists.[3–6]

Breitenstein et al. have found that enolized 1,3-dioxane-4,6-dione-5-carboxamides are potent reversible inhibitors of human group IIa PLA_2 (hIIa-PLA_2).[7] Compounds **1** and **2** were among the most potent in the series (Scheme 1). These authors proposed that both the ionized form of the enol hydroxyl group and the carbonyl oxygen of the carboxamide of **1** and **2** are directly bonded to Ca^{2+} at the active site of the enzyme. The cycloalkyl and disubstituted aromatic rings presumably engage in van der Waals contact with hydrophobic residues that line the active site of secreted PLA_2s.

Scheme 1



These 1,3-dioxane-4,6-dione-5-carboxamides are readily assembled in a two-step sequence by reacting a ketone with malonic acid to form the 1,3-dioxane-4,6-dione intermediate, which reacts with an isocyanate to form the desired compounds (Scheme 2). We have made two libraries of 1,3-dioxane-4,6-dione-5-carboxamides by solution-phase methods. One library was made from a mixture of 54 ketones and a single isocyanate, and the other from a single ketone and a mixture of 59 isocyanates. We were able to use an interfacial-based selection strategy employing a detergent micelle embedded inhibitor mixture and solid-phase bound hIIa-PLA_2 to identify

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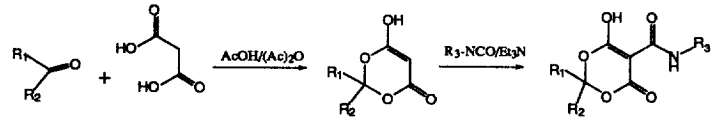
Scheme 2



those compounds that bind most tightly to the enzyme. We have also determined the molecular basis for binding of **1** to the active site of hIIa-PLA_2 by determining the X-ray crystal structure of the enzyme-inhibitor complex.

Results and Discussion

Preparation of 1,3-dioxane-4,6-dione-5-carboxamide libraries. We took advantage of the ease of assembly of 1,3-dioxane-4,6-dione-5-carboxamides from a ketone, malonic acid, and an isocyanate (Scheme 2) to construct libraries of potential hIIa-PLA_2 inhibitors that could be screened by an enzyme affinity approach. The ketone library was made by condensing a equimole mixture of 54 ketones[8] (0.1 mmol each ketone) with excess malonic acid (10 g, 96 mmol) in 10 mL of acetic anhydride containing ~ 0.5 g of 4 Å molecular sieves and 0.2 mL of concentrated sulfuric acid. After stirring for 2–3 days at room temperature, most of the solvent was removed *in vacuo*, and the residue was loaded on to a pad of silica gel (~ 30 mL). The silica was washed with 100 mL hexane, and the mixture of 1,3-dioxane-4,6-diones was eluted with 200–300 mL of ethyl acetate (until no more UV absorbing material was seen by spotting a small aliquot of eluant on a silica TLC plate with fluorescent indicator). The eluant was concentrated to dryness *in vacuo* and the residue taken up in 20 mL acetone (freshly distilled under N_2) containing 1 mL triethylamine. 2,4-Dichlorophenylisocyanate (2.0 g) was added, and the mixture was stirred overnight under nitrogen. The library was stored in basic acetone at -20°C for several weeks.

Prior to making this ketone library we showed that a mixture of acetone and phenyl cyclohexyl ketone underwent high yield conversion to the 1,3-dioxane-4,6-dione-5-carboxamides in the presence of either 2,4-dichlorophenylisocyanate or ethyl isocyanate. Yields were obtained by separating the mixture on a C18 reverse phase HPLC column using a water-methanol gradient and comparing the product peak integrals to a standard curve made with all four authentic and pure 1,3-dioxane-4,6-dione-5-carboxamides. The overall yields ranged from 63% for the compound containing phenyl cyclohexyl/ethyl to 96% for the dimethyl/2,4-dichlorophenyl compound.

We also made a library of 1,3-dioxane-4,6-dione-5-carboxamides in which the single ketone cyclohexanone was used together with 59 different isocyanates.[9] To 10 mL acetone (freshly distilled under N_2) was added 1 mL of triethylamine and 0.05 mmol of each isocyanate. The 1,3-dioxane-4,6-diones made from malonic acid and cyclohexanone[10] (2.4 g, ~threefold excess) was added, and the mixture was stirred overnight at room temperature under N_2. The library was stored at -20 °C for up to several weeks.

Interfacial-based selection of hIIa-PLA_2 inhibitors. Secreted PLA_2s bound to phospholipid interfaces bind ligands in their active sites more tightly than do enzymes in the aqueous phase.[11,12] Thus, extraction of tight binding PLA_2 inhibitors from a library of compounds should be carried out in the presence of an interface, and we choose micelles of hexadecylphosphorylcholine as the matrix to which enzyme and inhibitor bind. Micelles are much smaller than vesicles and can penetrate the solid support gel used for affinity selection. hIIa-PLA_2 binds tightly to heparin, and the interaction is disrupted by high salt.[13] Thus, we used heparin-Sepharose as a solid support for non-covalent attachment of enzyme.

To a solution of 20 mM HEPES, pH 8.0, 100 mM NaCl, 1 mM $CaCl_2$, 5 mM hexadecylphosphorylcholine (Sigma) was added 1.4 mg of hIIa-PLA_2 (prepared as described[14]) and 1 mol of total library components per mole of enzyme (added from a methanol stock). Heparin-Sepharose (110 mg of dry powder, Pharmacia) was added. Insoluble material was removed by centrifugation. The sample was mixed by inversion for 3 h at room temperature. The Sepharose was pelleted in a microfuge and the supernatant removed. The pellet was washed five times with 1

mL portions of buffer without detergent. The pellet was treated with 20 mM HEPES, pH 8.0, 2 M NaCl, 1 mM $CaCl_2$ to release enzyme from heparin-Sepharose. The mixture was extracted with 5 volumes of $CHCl_3$, and the organic phase was concentrated to dryness *in vacuo*. As a control, this procedure was repeated as above except that hIIa-PLA_2 was omitted.

The residue from the $CHCl_3$ extract was taken up in 20 μL of $CHCl_3$, and the sample was injected on to the GC column (Supelco SP-1) installed on a Hewlett Packard HP5090A/HP5871A benchtop GC/MS (50 °C for 5 min, 3 °/min to 300 °C). The minus enzyme control was also analyzed in the same way. Prior to this analysis, it was shown that 1,3-dioxane-4,6-dione-5-carboxamides decompose in the GC to yield the corresponding ketones,[15] and the latter were detected by GC/MS. The GC trace obtained with the enzyme-based selection of the ketone library is shown in Figure 1. Peaks marked by an asterisk were also seen in the minus enzyme control. The large peak at ~33 min is 2,4-dichloro-phenylisocyanate. Peaks 1, 2, and 3 were identified from the electron impact fragmentation pattern as cyclohexanone, methylcyclohexane, and 2,2-di-methylcyclopentanone, respectively. Peaks 4-6 are sharp and may be due to instrument noise (ketone identification could not be made from MS data).

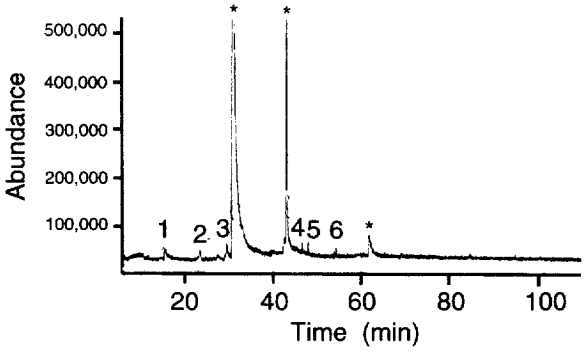


Figure 1. Total ion current trace of ketones formed by thermal decomposition of 1,3-dioxane-4,6-dione-5-carboxamides obtained by enzyme affinity purification of the ketone library (see text for additional information).

Based on these results, we synthesized individual 1,3-dioxane-4,6-dione-5-carboxamides derived from cyclohexanone, 2-methylcyclohexane, 3-methylcyclohexane, 4-methylcyclohexane and 2,2-dimethylcyclohexanone. These compounds were tested as inhibitors of hIIa-PLA_2 using the scooting mode assay with anionic dimyristoyl phosphatidylmethanol vesicles to avoid artifacts associated with analyzing PLA_2 inhibitors.[16] Table 1 gives the mole fraction of inhibitor in the vesicle interface that produces a twofold reduction in the initial enzymatic velocity, $X_i(50)$. The most potent compounds, derived from 3- and 4-methylcyclohexanone and 2,2-dimethylcyclopentanone are twofold better inhibitors than the original lead compound (derived from cyclohexanone). Although this represents a modest improvement in potency, the library approach has generated a large amount of semi-quantitative structure–activity data.

The isocyanate library was also analyzed using enzyme-based affinity selection exactly as described above. The residue from the $CHCl_3$ extract (see above) was taken up in 1 mL of water:methanol (1:1), and 1 mL of concentrated HCl was added. After stirring overnight at room temperature, solvent was removed *in vacuo*, and the residue was taken up in 4 mL of saturated aqueous $NaHCO_3$, and 1 mL of benzyl bromide was added. The emulsion was stirred vigorously at room temperature overnight to benzylate the amines liberated from acid hydrolysis of the 1,3-dioxane-4,6-dione-5-carboxamides. Solvent was removed *in vacuo*, and the residue was taken up in a small volume of $CHCl_3$ and analyzed by GC/MS as above. Peaks not seen in the minus enzyme control were analyzed by electron impact mass spectrometry. Three benzylated amines, derived from 2-chloro-6-methylaniline, 2,6-dimethylaniline, and 2,4-dichloroaniline, were observed. The 1,3-dioxane-4,6-dione-5-

carboxamides derived from the these substituted phenylisocyanates were individually synthesized, and all these compounds display similar inhibition potencies (Table 1).

Table 1. Vesicle assay of the potency of 1,3-dioxane-4,6-dione-5-carboxamides as hIIa-PLA_2 inhibitors.

Ketone Component	Isocyanate Component	$X_I(50)$ (mole fraction)[a]
Cyclohexanone	2,4-Dichlorophenylisocyanate	0.022
2-Methylcyclohexanone	2,4-Dichlorophenylisocyanate	0.022
3-Methylcyclohexanone	2,4-Dichlorophenylisocyanate	0.012
4-Methylcyclohexanone	2,4-Dichlorophenylisocyanate	0.011
2,2-Dimethylcyclopentanone	2,4-Dichlorophenylisocyanate	0.010
Cyclohexanone	2-Chloro-6-methylphenylisocyanate	0.03
Cyclohexanone	2,6-Dimethylphenylisocyanate	0.025

[a]Estimated errors are ≤ 15% based on triplicate analysis.

Crystal structure of hIIa-PLA_2 with bound 1. In order to determine the molecular details of the interaction with **1** with hIIa-PLA_2 and to attempt to interpret the structure–activity data from the library screening, we co-crystallized enzyme with **1**[17] and determined the X-ray structure of the complex (coordinates are being deposited in the PDB). After data collection,[18] the structure was solved by molecular replacement[19] and refined to a resolution of 2.5 Å with a final R factor of 23.1%.[20] As shown in Figure 2, the enolate and carboxamide carbonyl oxygens of **1** bind directly to the active site Ca^{2+} with a bond length of 2.2 Å.

Figure 2. Stereoview of the complex of hIIa-PLA_2 with **1**. Atom colorings are: C, green; O, red; N, blue; Cl, magenta. Ca^{2+} is shown as a yellow ball. H48, Y52, F106, K53 are colored orange. A portion of the complex of hIIa-PLA_2 with phospholipid analog is superimposed on the enzyme-**1** structure with the analog shown in cyan, the Ca^{2+} binding loop in yellow, and Ca^{2+}as a cyan cross.

Interestingly, the *ortho* chlorine of the carboxamide side chain of **1** makes a polar van der Waals contact with the delta NH of H48 (Cl-to-N distance of 2.7 Å) (residue numbering is based on the common scheme for secreted PLA_2s[21]). The *para* Cl is packed against the edges of the phenyl rings of F5 and F106 and also the NH, carbonyl, and CH_2 of G22. The cyclohexyl ring of **1** engages in limited interaction with the enzyme, contacting only the α, β, and γ CH_2s of K53 and the β CH_2 of Y52. The carboxamide NH and malonic acid-derived carbonyl oxygen of **1** form an intramolecular hydrogen bond (N-to-O distance of 2.4 Å).

The Ca^{2+} coordination geometry of bound **1** may be compared to the structure of the same enzyme containing a bound *sn*-2 phosphonate phospholipid analog[22] (Figure 2). In both structures, the enzyme donates 5 oxygen ligands to Ca^{2+}, and the inhibitor donates two oxygens. In both cases the backbone carbonyls of G29 and H38 and both oxygens of the side chain carboxylate of D49 form pseudo-equatorial ligands to Ca^{2+}, and the backbone carbonyl of G31 donates a pseudo-axial ligand. Nonbridging oxygens of the *sn*-2 phosphonate and *sn*-3

phosphate groups of the bound phospholipid analog engage in pseudo-axial and pseudo-equatorial interactions with Ca^{2+}, respectively. These latter two ligand positions are not occupied by enzyme-bound **1**. Rather the carboxamide oxygen of **1** that binds to Ca^{2+} sits at a position between those occupied by the *sn*-2 phosphonate and *sn*-3 phosphate oxygens of bound phospholipid analog, and the enolate oxygen of **1** sits at a position between those occupied by the *sn*-3 phosphonate oxygen and the G31 carbonyl oxygen of the enzyme. Interestingly, part of the Ca^{2+} binding loop in the complex of enzyme with **1** is shifted away from the metal compared to the structure of hIIa-PLA_2 containing bound phospholipid analog. In the complex with **1** the α-carbons of G29 and V30 are 1.7 and 2 Å, respectively, further away from Ca^{2+} than in the complex with phospholipid analog. Although this movement changes the ligand geometry somewhat, all Ca^{2+}-O distances are 2.2–2.6 Å in both complexes. In the structure of hIIa-PLA_2 containing bound phospholipid analog, the side chain of K53 is rotated away from its position occupied in the complex of enzyme with **1**.

The structure of **1** bound to hIIa-PLA_2 provides a platform for understanding the structure–activity data obtained from the library screening. The fact that the cyclohexyl group of **1** is largely exposed to solvent explains why methylated cyclohexanes are tolerated. Replacement of the cyclohexyl group with an alkyl chain would probably not allow for the interaction with the side chain of K53. Inhibitors derived from bulkier ketones such as cyclohexyl phenyl ketone would not be tolerated because one of the large substituents would be forced against enzyme residues that lie on one side of the cyclohexyl ring. The 2,4-dichlorophenyl group of **1** makes a snug fit with the enzyme. Inspection of the X-ray structure suggests that a methyl substituent at the 6 position of the phenyl ring would be tolerated, as is the case (Table 1). Replacement of the 2-chloro group by alkyl and alkoxy groups would lead to unfavorable clashing with H48. Finally, replacement of the substituted phenyl substitutent of **1** with smaller alkyl groups would lead to significant gaps between the substituent and the enzyme.

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8. Ketones R_1COR_2 are listed as R_1/R_2: Me/Me, Me/Pr, Me/Bu, Et/Et, Et/Pr, Pr/Pr, Bu/Bu, Et/CH_2Ph, Me/CH_2OPh, Et/CH_2OH, Me/CH(OH)Me, Me/CH(Cl)Me, Et/3-Cl-Pr, Me/2-F-phenyl (and 2-Cl, 2-Br, 2,4-$(OH)_2$, 2,6-$(OH)_2$, 3,4-$(OMe)_2$, 3,4-Cl_2), Ph/Heptyl, Et/3-Cl-Ph (and 4-Cl-Ph), Ph/3-Cl-Pr, Ph/4-Cl-Bu, cyclohexyl/Ph, Ph/CH_2NO_2. Other ketones: cyclohexanone (and 2-Me, 3-Me, 4-Me, 2-OMe, 2-Cl, 2-NO_2, 3-NO_2, and 2,4,4-Me_3), 2,2-Me_2-cyclopentanone, tetrahydro-4H-pyran-4-one, 2-acetylfuran, 2-acetylpyridine (and 3-acetyl, 4-acetyl), acetylpyrazine, 1-indanone, phorone, tropinone, 3-nonen-2-one, 3-chloro-2-norbornanone, α-tetralone, *trans*-1-decalone, 5-methoxy-1-indanone, *cis*-jasmone, 4-Ph-cyclohexanone, 6-OMe-1-tetralone, 1-Br-pinacolone, geranylacetone, 1-phenethyl-4-piperidone.
9. Isocyanates: Et, Bu, *t*-Bu, 3-Cl-Pr, hexyl, cyclohexyl, benzyl, 2-Me-Ph (and 2-F, 3-F, 4-F, 3-CN, 2,6-Me_2, 2-Et, 2-OMe, 3-OMe, 4-OMe, 2-Cl, 3-Cl, 4-Cl, 2,4-F_2, 2,5-F_2, 2,6-F_2, 2-OEt, 4-OEt, 4-*i*-Pr, 2-NO_2, 3-NO_2, 4-NO_2, 4-CH_2Cl, 2-Cl-6-Me, 4-Me-3-NO_2, 2,4-$(OMe)_2$, 2-CF_3, 3-CF_3, 4-CF_3, 2,3-Cl_2, 3,4-Cl_2, 2,6-Cl_2, 3,5-Cl_2, 2-Br, 3-Br, 4-Br, 2-OCF_3, 2,6-$(i\text{-Pr})_2$, 4-Cl-2-CF_3, 4-Cl-3-CF_3, 2-Cl- 5-CF_3), $C_{16}H_{33}$, 1,1,3,3-Me_4-Bu.

Other isocyanates: (*R*)-α-methylbenzyl, methyl (*S*)-2-isocyanato-3-methylbutyrate, methyl-2-isocyanato benzoate, ethyl-3-isocyanato benzoate, 3-isopropenyl-α,α-dimethylbenzyl, (*S*)-(-)-2-isocyanato-3-phenylpropionate.

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15. The 1,3-dioxane-4,6-dione-5-carboxamides made from 4-methylcyclohexanone and 1-phenyl-2-butanone and 2,4-dichlorophenylisocyanate decomposed to the ketones in 87% and 82% yield, respectively.
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17. Crystallization experiments were carried out using the method of vapor diffusion in hanging drops. Protein concentrations ranged from 4-12 mg/mL. The crystallization solution in the reservoir had a volume of 300 μL and was composed of: 25-35% (w/v) PEG 8000, 100 mM sodium cacodylate, 200 mM ammonium sulfate, pH 6.5. Five μL of protein solution containing 10 mM **1** and 10 mM $CaCl_2$ and 5 μL of reservoir solution were mixed and equilibrated against reservoir at 15 °C for 2 weeks before crystals appeared. The dimensions of the crystals used were 0.2 mm x 0.2 mm x 0.1 mm. Crystals grew in space group $P4_12_12$ with cell dimensions a = b = 47.6 Å, c = 108.7 Å, with a single enzyme monomer/inhibitor forming the asymmetric unit.
18. Diffraction data were collected on an RAXIS II area detector (Molecular Structure Corporation) with a CuK_α x-ray source from a Rigaku RU-200 rotating anode operated at 50 kV and 100 mA (graphite monochromator). Crystals were flash-cooled at 100 K after they were equilibrated with a cryo-solvent containing 30% glucose and 10% glycerol in the reservoir buffer described above. Data were processed using DENZO and SCALEPACK (Otwinowski, Z. *DENZO*; Sawyer, L., Issacs, N., Bailey, S., Eds.; SERC Daresbury Laboratory: Warrington, UK., 1993, pp 56–62).
Additional crystallographic data. Resolution, 2.5 Å; Number of reflections measured, 59,092; Number of unique reflections, 4557; Redundancy, 13.0; Completeness (outer resolution bin 2.54–2.50), 96.4% (63.0%); Average $I/\sigma(I)$, 15.7; R_{sym} (outer resolution bin 2.54-2.50), 4.9% (17.8%).
19. The structure of hIIa-PLA_2-**1** was solved by molecular replacement using the AMORE software package (Navaza, J. *Acta Cryst.* 1994, *A50*, 157) using coordinates of the apo enzyme as the search model. For the rotation function only Patterson densities inside a spherical shell of inner and outer radii of 3 Å and 35 Å were used in Patterson map superpositions. The best peak from a rotation search (with a correlation coefficient 50% higher than the next best solution) was used to determine the location of the molecule using the translation function calculation. The correlation coefficient and R-factor calculated for the best resulting peaks in the translation function were 43.8 and 43.2, respectively. The next best solution yielded values of 17.3 and 52.5, so that the correct solution was unambiguous. A translation search conducted in the (incorrect) enantiomorphic space group $P4_32_12$ yielded a best peak with a much lower correlation coefficient. Rigid body refinement of the orientation and position of the two molecules using data between 10.0 Å and 3.0 Å resulted in a crystallographic R-factor of 41.7%.
20. Prior to refinement, 10% of the data was set aside for cross validation with R_{free}. Refinement of the complex was performed with X-PLOR (Brunger, A. T. *Science* 1987, *235*, 458). Following an initial positional refinement the inhibitor and calcium were built into the active site using a F_O-F_C map. Iterative rounds of simulated annealing, positional refinement, and rebuilding were conducted until the value of R_{free} had converged. $2F_O$-F_C and F_O-F_C maps were used to manually rebuild side chains and adjust the model. After final refinement the R-factor was 23.1% and R_{free} was 31.2%. The final model for the asymmetric unit of the complex contains one protein subunit consisting of 127 residues, 64 water molecules, a single bound calcium, and a single bound **1**.
Additional refinement data. Resolution Range, 100-2.5 Å; R-factor, 23.1%; R-free, 31.2%; Number of protein atoms, 4948; Number of solvent molecules, 64; Ramachandran plot distribution (# core, allowed, disallowed), 88, 19, 2; r.m.s. on bonds, 0.011 Å; r.m.s. on angles, 1.922°; Average B (Protein, Ligand) 25.7, 33.4 $Å^2$; Inhibitor /calcium (B-factor, occupancy), 43.1, 1.0 / 33.2, 1.0.
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