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Biochemical Characterization of Selective Inhibitors of Human GIIA Secreted Phospholipase A₂ and Hyaluronic Acid-Linked Inhibitor Conjugates[†]

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Abstract

We explored the inhibition mode of group IIA secreted phospholipase A₂ (GIIA sPLA₂) selective inhibitors and tested their ability to inhibit GIIA sPLA₂ activity as chemical conjugates with hyaluronic acid (HA). Analogs of a benzo-fused indole sPLA₂ inhibitor were developed where the carboxylate group on the inhibitor scaffold, that has been shown to coordinate to a Ca²⁺ ligand in the enzyme active site, was replaced with other functionality. Replacing the carboxylate group with amine, amide, or hydroxyl groups had no effect on human GIIA (hGIIA) sPLA₂ inhibition potency but dramatically lowered inhibition potency against hGV and hGX sPLA₂s. An alkylation protection assay was used to probe active site binding of carboxylate and non-carboxylate inhibitors in the presence and absence of Ca²⁺ and/or lipid vesicles. We observed that carboxylate-containing inhibitors bind the hGIIA sPLA₂ active site with low nanomolar affinity, but only when Ca²⁺ is present. Non-carboxylate, GIIA sPLA₂ selective inhibitors also bind the hGIIA sPLA₂ active site in the nanomolar range. However, binding for GIIA sPLA₂ selective inhibitors was dependent on the presence of a lipid membrane and not Ca²⁺. These results indicate that GIIA sPLA₂ selective inhibitors exert their inhibitory effects by binding to the hGIIA sPLA₂ active site. An HA-linked GIIA inhibitor conjugate was developed using peptide coupling conditions and found to be less potent and selective against hGIIA sPLA₂ compared to the unconjugated inhibitor. Compounds reported in this study are some of the most potent and selective GIIA sPLA₂ active site binding inhibitors reported to date.

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Supporting Information Available

Semilogarithmic inactivation plots and Scrutton and Utter plots used to compute K_d values in Table 3 (Figures S1-S5), Semilogarithmic inactivation plots of compound **1** in the absence of calcium (Figure S6), Semilogarithmic inactivation plots of compounds **2a** and **2c** in the absence of DTPM (Figure S7), ITC binding curves for **4c** and **4i** (Figure S8) and ITC experimental procedure, semilogarithmic inactivation plots of N-methyl compounds (Figure S9), molecular modeling studies of **HA-4d** (Figure S10), Inhibition of hGV and hGX sPLA₂ activity by **HA-4d** (Figure S11), Semilogarithmic inactivation plots of hGIIA sPLA₂ activity in the presence of **HA-4d**, **HA-4d-Nme**, or **4d** (Figure S12), Percent Loading determination of **HA-4d** and **HA-4d-Nme** (Table S1), Inhibition data of N-methyl oxalamide control inhibitors (Table S2), synthetic schemes and details of synthetic methods for all compounds and pyrPG, including, NMR and MS data. This information is available free of charge via the Internet at <http://pubs.acs.org>.

Keywords

eicosanoids; lipid mediators; inflammation; sPLA₂ inhibitors; phospholipases; hyaluronic acid bioconjugates

INTRODUCTION

Secreted phospholipases A₂ (sPLA₂s) are a family of Ca²⁺-dependent, disulfide-rich enzymes that catalyze the hydrolysis of glycerophospholipids at the *sn*-2 position to liberate lysophospholipid and fatty acid products.¹ Ten sPLA₂s have been identified in mammals (groups IB, IIA, IIC, IID, IIE, IIF, III, V, X, XIIA) and, in humans, all of these enzymes are present except group IIC which exists as a pseudogene.^{2,3} The lipolytic activity of these enzymes has important implications in inflammation since hydrolysis products such as arachidonic acid (AA) can be further processed into important proinflammatory mediating eicosanoids such as prostaglandins and leukotrienes.^{1,3} In fact, sPLA₂ activity has been connected to a number of inflammatory diseases that include atherosclerosis, asthma, and arthritis.^{1,3} Not surprisingly, this has led to increased efforts to develop small molecule inhibitors that target sPLA₂s.^{4,5}

Some of the earliest evidence for a proinflammatory function of sPLA₂s came over two decades ago with the discovery of large amounts of human GIIA (hGIIA) sPLA₂ in rheumatoid arthritic and osteoarthritic synovial fluid.^{6,7} The discovery of increased levels of GIIA sPLA₂ in arthritic synovial fluid has raised the possibility that this and other sPLA₂ enzymes may be involved in arthritis development through generation of eicosanoids and that inhibition of sPLA₂ activity may alleviate joint inflammation. Current evidence suggests that treatment of arthritis through sPLA₂ inhibition may require selective targeting of GIIA over other sPLA₂s.⁸ Using a mouse arthritis model, the Lee lab showed that GIIA and GV sPLA₂s play opposing roles in arthritis disease progression where the GIIA enzyme contributes to arthritis development but GV plays an anti-inflammatory role.⁸ This is among the first evidence showing that sPLA₂s can perform opposing roles in an inflammatory disease, and it may explain why earlier attempts to treat rheumatoid arthritis with an sPLA₂ indole-based inhibitor that potentially inhibits both GIIA and GV failed to show efficacy.⁹ Furthermore, this suggests an important need for developing GIIA sPLA₂ selective inhibitors that can target the proinflammatory functions of GIIA, but not the protective functions of GV in arthritis development.

We recently reported on the development of an indole-based inhibitor that was selective for GIIA over GV and other sPLA₂s.¹⁰ In this study, we showed that compound **1** (Figure 1A) was a generally potent sPLA₂ inhibitor with nanomolar inhibition potency against all sPLA₂s except GIII and GXIIA.¹⁰ A key binding feature of compound **1** is the carboxylate group that has been shown, using a similar structural analog, to contact a calcium ion in the enzyme active site (Figure 1B).¹¹ However, removal of this Ca²⁺-binding, carboxylate moiety on compound **1** to give **2a** (Figure 1A) severely diminished inhibition potency against most sPLA₂s, but had no effect on human and mouse GIIA sPLA₂ inhibition potency.¹⁰ From these studies it was unclear whether the GIIA sPLA₂ inhibition caused by **2a** still required calcium and/or whether the inhibitory effects were due to active site or allosteric site binding. Given the recent interest in developing GIIA sPLA₂ selective inhibitors as described above, we performed further studies investigating the mode of GIIA sPLA₂ inhibition for this compound.

We first investigated the range of functional groups that could replace the 4-position carboxylate on compound **1** without disrupting hGIIA sPLA₂ inhibition potency and

selectivity. We then explored the ability of GIIA sPLA₂ selective compounds to bind the enzyme active site using an alkylation protection assay that specifically probes active site binding. These active site studies were carried out in the presence and absence of Ca²⁺ and/or membrane to test the importance of these two factors on inhibitor binding. Since the carboxylate group of indole-based compounds does not appear to be important for GIIA sPLA₂ inhibition selectivity, we investigated the novel use of GIIA sPLA₂ selective inhibitors as chemical conjugates with hyaluronic acid (HA) for the potential treatment of osteoarthritis (OA). Intra-articular injection of HA is a current treatment option that can provide symptomatic relief from OA by temporarily restoring viscoelasticity and other normal properties to the diseased joint.^{12,13} We reasoned that HA conjugated with a GIIA sPLA₂ selective inhibitor could combine the anti-inflammatory properties of GIIA sPLA₂ inhibition with the lubrication and viscosupplementation features provided by the HA polymer. As a proof-of-principle, we also set out to synthesize HA-inhibitor conjugates and then test their ability to inhibit hGIIA sPLA₂ activity in vitro.

MATERIALS AND METHODS

Materials

hGIIA sPLA₂ was prepared and purified as described previously¹⁴, and hGV and hGX sPLA₂s were prepared and purified as described previously¹⁵, DMPM was from Alexis Corp., DTPM was synthesized as described¹⁶, pyrPG was purchased from Molecular Probes or prepared as described in the Supporting Information, compounds **1**, **2a**, and **3a** were synthesized as described¹⁰, phenacyl bromide was from Sigma, fatty acid free Bovine Serum Albumin (BSA) was from Sigma (cat. no. A6003), 20-kDa HA was from Lifecore Biomedical, hydroxybenzotriazole (HOBt) was from Pierce, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was from TCI America, purified water was from a Milli-Q system (Millipore Corp, Billerica, MA).

Synthesis of sPLA₂ Inhibitors

Inhibitors were prepared as described in Supporting Information.

Determination of IC₅₀ values

IC₅₀ values were obtained from each of the enzyme assays reported below using five inhibitor concentrations ranging from 10% to 90% inhibition of sPLA₂ activity. IC₅₀ values were determined by nonlinear regression analysis of a semilog plot of percent inhibition versus log of inhibitor concentration. The inhibition curves were generated using the Kaleidagraph software.

Fluorometric Enzyme Assay

This assay was performed as previously described.^{17,18}

Radiometric Enzyme Assay

This assay was performed as previously described.¹⁰ hGIIA sPLA₂ was used at 15 pg/reaction tube.

pH-Stat Titration Enzyme assay

This assay has been described previously¹⁹ but was slightly modified for our inhibition studies. hGIIA sPLA₂ activity was monitored by continuous-titration of hydrolyzed fatty acid at a constant pH (8.0) in a pH-stat instrument (Radiometer, Copenhagen, Denmark). The pH-stat consisted of a pH meter (PHM 82), titrator (TTT 80), autoburette (ABU 80), a thermostat-controlled titration assembly unit (TTA 80) and pH electrode (Radiometer

Analytical, PHC 4006-9). All assays were carried out at room temperature under N₂. Five milliliters of assay solution (1 mM NaCl, 0.6 mM CaCl₂) was placed into the reaction vessel, and the pH was adjusted to 8.0 using an autoburette with 2.8 mM NaOH as titrant. Once the baseline had stabilized with zero drift, 62 μL of DMPM vesicles from a 16 mM stock solution in purified water were added to the reaction vessel to give a final concentration of 200 μM. Preparation of DMPM vesicles was carried out exactly as described.¹⁹ The reaction was adjusted to pH 8.0 with the autoburette, and the baseline was allowed to stabilize over a 3-5 minutes. hGIIA sPLA₂ in 5 μL of purified water was added to the reaction to give a final concentration of 14 nM, and the enzyme activity was monitored by autotitration using 2.8 mM NaOH at a maintained pH of 8.0. Under these conditions, we generally observed a linear initial velocity (v_o) over the first 6-8 minutes of the reaction progress curve. For inhibitor studies, enzyme was first added to the reaction vessel, and the v_o was measured over the first 2-3 minutes. Inhibitor in 2 μL DMSO was then added, and the reaction velocity in the presence of inhibitor (v_i) was measured over 2-3 minutes. Percent inhibition was calculated as $100 - ((v_i/v_o) \times 100)$.

Alkylation Protection Studies

Inactivation of hGIIA sPLA₂ activity in the presence of phenacyl bromide was carried out as reported previously but with a few modifications.²⁰ Binding assay buffer (200 μL) consisting of 50 mM sodium cacodylate (pH 7.3), 50 mM NaCl, 0.1% BSA and either 200 μM CaCl₂ or 100 μM EGTA was added to a 600 μL microcentrifuge tube (Neptune Plastics). Inhibitor (1 μL in DMSO) or DMSO vehicle control was added to the reaction mixture. hGIIA sPLA₂ in 1 μL 10 mM Tris pH 8.0 was then added to the reaction mixture to give a final concentration of 250 nM and briefly vortexed. For inactivation studies involving membrane, 2.5 μL of DTPM vesicles from a 17 mM stock solution in purified water were added to the assay buffer at 200 μM prior to addition of the inhibitor. DTPM vesicles were prepared following the exact procedure outlined for preparation of DMPM vesicles.¹⁹ Phenacyl bromide was dissolved in acetonitrile at 350 mM and added to the assay mixture at 3.5 mM to start the assay. Aliquots (2 μL) of the assay mixture were removed at appropriate time points and diluted 400-fold in solution A consisting of 50 mM Tris (pH 8.0), 50 mM KCl, 100 μM EGTA, 0.1% BSA. 50-100 μL of the diluted assay mixture was then added to a well of a 96-well microtiter plate and diluted to 200 μL with solution A. This was followed by addition of 100 μL solution B consisting of 4.2 μM pyrPG in 50 mM KCl, 100 μM EGTA, and 50 mM Tris-HCl (pH 8.0). Enzyme activity was initiated by addition of 20 μL 50 mM CaCl₂. The initial velocity of pyrPG hydrolysis was monitored over 2-3 minutes by measuring the increase in pyrene monomer fluorescence (ex. = 342 nm, em. = 395 nm) with a Victor3V microtiter plate spectrophotometer (Perkin-Elmer). In all alkylation assays, the first time point was taken seconds after addition of phenacyl bromide ($t = 0$ minutes) followed by three other time points taken over the course of the inactivation assay until $\approx 10\%$ of enzyme activity remained. Percent enzyme activity over the course of the reaction was determined by dividing the initial velocity of enzyme activity at a given time point by the initial velocity of enzyme activity at $t = 0$ minutes. Semilog plots of log (% activity) vs. time gave a straight line from which the inactivation half-times were determined.

Values of K_d were calculated from a modified form of the Scrutton and Utter equation (1):

$$\frac{1}{[1 - (t_o/t_i)]} = \frac{[(K_d/[I]) + 1]}{[1 - (k_i/k_o)]} \quad (1)$$

where t_o and t_i are the respective inactivation half-times in the absence and presence of inhibitor, $[I]$ is the inhibitor concentration, k_i and k_o are the inactivation rate constants in the presence of saturating inhibitor concentration or absence of inhibitor, respectively.²¹ Note that $[I]$ is used in place of X_L from the original equation since we do not accurately know

the mole fraction of inhibitor that partitions onto the lipid surface when membrane is used in the assay. A plot of $(1/(1-(t_o/t_i)))$ vs. $(1/[I])$ yields a straight line in which the x-intercept equals $(-1/K_d)$. Scrutton and Utter plots were generated from five different inhibitor concentrations, and the K_d value was determined from the x-intercept.

Synthesis of HA-Inhibitor Conjugates

Hyaluronic acid (20-kDa) (5 mg, 0.012 mmol) was dissolved in 1.25 mL of a 1:1 mixture of THF:0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) pH 5.1. The free amine compound **4d** or **4d-Nme** (2.5 mg, 0.006 mmol) was dissolved in 1 mL of THF:0.1 M MES pH 5.1 (1:1) and added to the reaction mixture and stirred for 2-3 minutes (see supporting information for preparation of **4d** or **4d-Nme** free amines). HOBt (3.6 mg, 0.024 mmol) was added to the reaction mixture and stirred until completely dissolved. EDC (35 mg, 0.18 mmol) was dissolved in 250 μ L of THF:0.1 M MES pH 5.1 (1:1) and added dropwise to the reaction mixture under constant stirring. The reaction mixture was then stirred for two hours at room temperature. Purified water (1.25 mL) was then added to the reaction mixture, and the sample was placed in a Speed-Vac to remove the THF ($\approx$ 1.25 mL). The remaining reaction mixture was diluted to a final volume of 2.5 mL with purified water and loaded onto a PD-10 disposable size exclusion column (bed volume, 8.3 mL) (GE Healthcare) that was pre equilibrated with 100 mM NaCl and then eluted off the column with 3.5 mL 100 mM NaCl. The eluted product was concentrated to final volume of 2.5 mL in a Speed-Vac. This reaction mixture was loaded onto a second PD-10 column (equilibrated with purified water) and eluted off of the column with 3.5 mL purified water. The product was then flash frozen and lyophilized to dryness to give a white fibrous material. The dried product was first dissolved in 100 mM NaOH at 4 mg/mL and stirred for 1-2 minutes and then diluted to 0.5 mg/mL with buffer consisting of 50 mM Tris pH 7.3, 50 mM KCl, 1 mM CaCl_2 . The product was then tested for sPLA₂ enzyme inhibition using the fluorometric enzyme assay or for alkylation protection of hGIIA sPLA₂.

Percent loading for the HA-inhibitor conjugation reaction was determined from the molar ratio of inhibitor to HA carboxylate (HA-COOH) groups and is defined as: $((\text{moles inhibitor}/\text{moles of HA-COOH}) \times 100)$. Molar ratios were obtained from two different methods (fluorescence-based or NMR-based). In the fluorescence based method, molar ratios were calculated by obtaining the fluorescence of a known milligram amount of HA-inhibitor conjugate and comparing it to a standard curve of fluorescence vs. known nmole amount of free inhibitor (fluorescence of conjugated inhibitor and free inhibitor were assumed to be identical) (see Table S1). The fluorescence value of the HA-inhibitor conjugate was converted to nmoles of inhibitor per milligram of HA and then to nmoles of inhibitor to nmoles of HA disaccharide unit. This ratio was then converted to nmoles of inhibitor to nmoles of HA-COOH. Fluorescence measurements were carried out on a Victor3V microtiter plate spectrophotometer (Perkin Elmer) with an excitation filter of 355 nm and emission filter of 460 nm. In the NMR-based method, percent loading of inhibitor onto total available HA-COOH groups was obtained by comparing the integrated methyl peak of the N-acetyl group of HA to the integrated methylene peak of the inhibitor N-benzyl group. For ^1H -NMR experiments, dried HA-inhibitor conjugates were suspended in D_2O followed by addition of 5-10 μ L of NaOD to solubilize the product. Typically, the dried HA-Inhibitor conjugates could not be dissolved in acidic or neutral solutions and required basic conditions for solubilization.

RESULTS

Structural Features of GIIA sPLA₂ Selective Inhibitors

To further investigate the GIIA sPLA₂ selectivity effect observed for compound **2a**, we generated analogs of compound **1** as well as similar indolizine (**3a-e**) and carbazole (**4a-h**) analogs where the carboxylate group responsible for the general sPLA₂ potency was replaced with other functionality (Table 1). We found that replacing the carboxylate with hydroxy (**2b**, **3b**), amino (**2c**, **3c**, **4c**), or amide (**3e**) groups did not alter hGIIA sPLA₂ inhibition potency, but led to 70-fold increase in the IC₅₀ values against hGV and hGX sPLA₂s (Table 1). Slightly larger groups such as methyl ether (**3d**) or dimethylamino (**4g**) substituents resulted in a seven- to ten-fold decrease in hGIIA sPLA₂ inhibition potency, and even larger groups such as a piperidine (**4h**) completely abolished the inhibitory effect. The length of the substituent also influenced hGIIA sPLA₂ inhibition potency. Carbazole-inhibitors **4c-f**, where the chain length was increased in increments of ethylene glycol units, experienced a corresponding decrease in hGIIA sPLA₂ inhibition potency, but not selectivity (Table 1). Also, the type of scaffold (benzo-fused indole, indolizine, or carbazole) had no influence on the hGIIA sPLA₂ inhibition potency or selectivity.

In addition to replacing the carboxylate moiety with other functional groups, we also investigated the effects of lengthening the distance between the carboxylate group and inhibitor scaffold. Increasing the distance from one methylene (compound **1**) to three methylene groups (compound **4b**) had a minimal effect on hGIIA sPLA₂ inhibition potency, but severely diminished inhibition potency on hGV and hGX sPLA₂s (Table 1).

Benzo-fused indole compounds **1**, **2a** and **2c** were selected for further hGIIA sPLA₂ inhibition studies in radiometric and pH-stat enzyme assays. Results from these studies are listed in Table 2 along with results from the fluorometric assay. IC₅₀ values for compound **1** and **2a** were all 100 nM among the three assays (Table 2). Some variation in IC₅₀ values was observed for **2c** where the IC₅₀ ranged from 35 nM in the fluorometric assay, 520 nM in the radiometric assay, and 95 nM in the pH-stat assay. We have shown previously with this class of inhibitors that replacing one of the oxalamide hydrogens with a methyl group results in a dramatic decrease in inhibition of GIIA, GV and GX sPLA₂ activity.¹⁰ This is most likely because addition of the methyl group to the oxalamide would introduce steric clash and disrupt a key hydrogen bond interaction that is depicted in the crystal structure of Me-Indoxam bound in the active site of hGX sPLA₂ (Figure 1B). We thus synthesized N-methyl oxalamide analogs of compounds **1**, **2a**, and **2c** (referred to as **1-Nme**, **2a-Nme**, and **2c-Nme** respectively) to test for the importance of the oxalamide group on inhibition potency. As expected, compounds **1-Nme**, **2a-Nme**, and **2c-Nme** showed no observable inhibition potency in the fluorometric assay (Table S2). We also tested **2a-Nme** in the radiometric and pH-stat assays and observed <15% inhibition at concentrations that gave >90% inhibition for **2a** (Table S2).

Investigation of Active Site Binding

Attempts to obtain a crystal structure of **2a** or **2c** bound to hGIIA sPLA₂ were unsuccessful, so we turned to an enzyme alkylation assay to probe hGIIA sPLA₂ binding. In this assay, hGIIA sPLA₂ is incubated in the presence of an alkylating agent that irreversibly alkylates the active site histidine to shutdown enzyme activity. However, in the presence of an active site binding ligand, the rate of enzyme alkylation decreases depending on the ligand type and/or concentration. We can therefore use this assay to probe whether GIIA sPLA₂ selective compounds bind the hGIIA sPLA₂ active site, as well as determine equilibrium dissociation constants of an inhibitor from the active site. This assay has been used previously to study ligand active site binders of hGIIA and other sPLA s.²⁰⁻²² We tested

compounds **1**, **2a**, and **2c** for their ability to protect against active site alkylation in the presence or absence of calcium or a membrane interface. Since all three compounds inhibited hydrolysis of DMPM vesicles at nanomolar concentrations in the pH stat assay (Table 2) we selected the non hydrolysable ether analog of DMPM, known as DTPM, to act as the membrane surface in the alkylation protection studies. We also chose DTPM because it has been previously shown that hGIIA sPLA₂ binds tightly to the surface of DTPM vesicles but has very low binding affinity for DTPM monomers in the enzyme active site.²⁰

The effects of DTPM vesicles on hGIIA sPLA₂ inactivation half-times in the presence or absence of calcium were tested in our assay conditions. We found the half times for hGIIA sPLA₂ inactivation in the absence of CaCl₂ (100 μ M EGTA) and the absence or presence of DTPM vesicles to be 11 $\pm$ 1 and 12 $\pm$ 2 minutes, respectively (average of at least three experiments). Similarly, we found the half times for hGIIA sPLA₂ inactivation in the presence of 200 μ M CaCl₂ and the absence or presence of DTPM vesicles to be 20 $\pm$ 3 and 24 $\pm$ 4 minutes, respectively (average of at least six experiments). The similar inactivation rates of enzyme activity in the presence or absence of DTPM indicates that very little DTPM monomer occupies the hGIIA sPLA₂ active site to protect against alkylation. Also, under our assay conditions of 200 μ M DTPM and 250 nM hGIIA sPLA₂, the enzyme is expected to be >90% bound to the membrane surface.²⁰ Thus we can test inhibitors using a scenario in which the hGIIA sPLA₂ enzyme is essentially all bound to a membrane surface but the active site is mostly occupied by solvent molecules.

Representative results from the alkylation protection assay are shown in Figure 2A with semilogarithmic plots of enzyme inactivation in the presence of calcium, DTPM and five different **2a** concentrations. As depicted in Figure 2A, increasing inhibitor concentration has a corresponding decrease in the rate of enzyme alkylation that ranges from mostly complete protection at 5 μ M **2a** to almost no protection at 0.1 μ M **2a**. A Scrutton and Utter plot was generated from the half-times of inactivation in the presence and absence of inhibitor and then used to calculate the K_d value of **2a** for hGIIA sPLA₂ in the presence of calcium and membrane (Figure 2B and Table 3). Similar plots were obtained for **1**, **2a**, and **2c** in the presence and absence of calcium and/or membrane (Figures S1-S5). The K_d values obtained from these plots are reported in Table 3.

As reported in Table 3, compound **1** showed tight binding to hGIIA sPLA₂ in the presence of 200 μ M CaCl₂ and the presence or absence of DTPM. Unfortunately, limitations in the alkylation protection assay prevented us from using lower enzyme concentrations to obtain more accurate values of K_d for compound **1**. In the presence of 200 μ M CaCl₂ and 200 μ M DTPM we observed near stoichiometric binding of **1** to the hGIIA sPLA₂ active site whereas in the absence of 200 μ M DTPM, a ten-fold higher concentration of compound **1** over enzyme was required to fully protect against alkylation (Figures S4 and S5). Compound **1** showed no measurable affinity for the hGIIA sPLA₂ active site in the absence of calcium (Table 3 and Figure S6). For compounds **2a** and **2c**, protection against alkylation was only observed in the presence of DTPM, and the K_d values for **2a** and **2c** were all 400 nM (Table 3 and Figure S7). Interestingly, binding of **2a** and **2c** in the presence of DTPM membranes was Ca²⁺-independent. This lack of hGIIA sPLA₂ binding in the absence of membrane was further supported by isothermal titration calorimetry involving compound **4c**. No heat of binding was detected when **4c** was added to hGIIA sPLA₂ in the absence of membrane (Figure S8). However, titration of a carbazole analog of compound **1** (**4i**) into a solution of hGIIA sPLA₂ and no membrane resulted in a binding curve that could be fit to the standard single site binding model with a K_d $\approx$ 270 nM (Figure S8).

We also tested the N-methyl control compounds **1-Nme**, **2a-Nme**, and **2c-Nme** in the alkylation protection assay and mostly observed no measurable binding affinity for the

hGIIA sPLA₂ enzyme (Figure S9). The one exception was **2c-Nme** which showed an 18 fold reduction in K_d when tested in the presence of 200 μM DTPM and no calcium (Figure S9).

Development of Hyaluronic Acid-Inhibitor Conjugates

Our approach to developing an HA-inhibitor conjugate was to covalently attach a GIIA sPLA₂ selective inhibitor with a free amine to the carboxylate group of HA using peptide coupling chemistry. Methods to conjugate free amine containing small molecules to HA have been reported previously.^{23, 24} For HA conjugation, we selected compound **4d** because our docking studies suggested that the linker distance between the free amine and inhibitor scaffold of **4d** was sufficient for the inhibitor to attach to HA and extend into the enzyme active site (Figure S10). Also, we chose the carbazole scaffold because its highly fluorescent properties provide an analytical advantage over the benzo fused indole and indolizine scaffolds. The HA conjugate, **HA-4d**, was prepared from peptide coupling conditions using EDC/HOBt in THF:0.1 M MES pH 5.1 (1:1) (Scheme 1). The percent loading of **4d** onto HA was calculated from the molar ratio of inhibitor to HA-COOH using two different methods (fluorescence-based and NMR-based, see experimental section). Both methods gave similar values of 9% loading (fluorescence-based) and 8% loading (NMR-based) of inhibitor onto the total possible HA-COOH groups (Table S1). For these initial studies we selected 20-kDa HA because of its lower viscosity and ease of handling compared to higher molecular weight HA preparations.

HA-4d was tested for in vitro potency against hGIIA sPLA₂ (Figure 3). We observed that **HA-4d** displayed significant inhibition potency with an IC₅₀ of 7 ng/JL **HA-4d** (Figure 3). At 8% loading, this corresponds to an IC₅₀ of 1.5 μM conjugated **4d**. As controls, we tested untreated HA, HA conjugated with linker only (**HA-NH₂CH₂CH₂OCH₂CH₂OH**), or HA mixed with **4d** but no coupling reagents and then purified from the reaction mixture (**HA+4d**). We observed little to no inhibition from these control mixtures (Figure 3). As another control, we prepared HA conjugated with the N-methyl control of **4d** (**4d-Nme**). Like all of our other N-methyl compounds, **4d-Nme** is devoid of inhibition potency against hGIIA, hGV, and hGX sPLA₂s (IC₅₀ >3300 nM for all three enzymes). We expected the **HA-4d-Nme** conjugate to poorly inhibit hGIIA sPLA₂ activity, but instead we found that it inhibited hGIIA sPLA₂ activity with an IC₅₀ of 33 ng/JL which corresponds to 5.6 μM of conjugated **4d-Nme** (Figure 3). We also tested **HA-4d** and the other controls for inhibition of hGV and hGX sPLA₂s (Figure S11). We observed that **HA-4d** inhibited hGV activity with an IC₅₀ of 33 ng/JL and showed ≈20% inhibition against hGX activity at 33 ng/JL (Figure S11). **HA-4d-Nme** inhibited hGV and hGX activity with nearly the same potency as **HA-4d** (Figure S11).

To investigate whether **HA-4d** exerts its inhibitory effect by binding the hGIIA sPLA₂ active site, we tested this conjugate in the alkylation protection assay. At concentrations of 100 ng/JL **HA-4d** (14.2 μM of conjugated inhibitor), we did not observe any measurable protection against active site alkylation in the presence or absence of membrane (Figure S12). In the presence of membrane, the free inhibitor **4d** showed nearly complete protection against alkylation at 4 μM concentrations (Figure S12).

DISCUSSION

The inhibition data in Table 1 show that a number of different functional groups can replace the carboxylate of compound **1** without altering the inhibition potency against hGIIA sPLA₂. Perhaps the most surprising observation is that a complete charge reversal from carboxylate to amine (**2c**, **3c**, **4c**) has no impact on hGIIA sPLA₂ inhibition potency.

However, there appears to be a limit to the length and size of the group that replaces the carboxylate. We found that large increases in chain length in compounds **4c-f** resulted in a corresponding decrease in inhibition potency (Table 1). Increasing the chain length probably interferes with inhibitor membrane partitioning (see below) and/or ability of the inhibitor to access the active site due to the increasing floppiness afforded by the ethylene glycol chain. Also, going from free amine in compound **4c** to a larger piperidine group in **4h** completely abrogated inhibition potency on hGIIA sPLA₂ (Table 1). This size limitation suggests that this portion of the inhibitor is not completely solvent exposed and is likely contacting a region of the hGIIA sPLA₂ enzyme. Another important result was the lack of hGIIA sPLA₂ inhibition potency displayed by the N-methyl amide compounds (**1-Nme**, **2a-Nme**, and **2c-Nme**) (Table S2). This suggests that the oxalamide hydrogens make important contributions to hGIIA sPLA₂ inhibition potency. Interestingly, this effect is identical for both the carboxylate inhibitor (**1**) and the hGIIA sPLA₂ selective inhibitors (**2a** and **2c**) suggesting that the oxalamide-GIIA sPLA₂ interactions are similar for these compounds.

It remains perplexing that the carboxylate side chain is not required for tight binding of these compounds to hGIIA sPLA₂ given that the carboxylate directly coordinates to the active site Ca²⁺ of sPLA₂s in general including hGIIA.²⁵ We therefore considered the possibility that the inhibition of hGIIA sPLA₂ is due to some sort of artifact in the fluorometric assay involving pyrPG. This seemed unlikely given that other sPLA₂s are not inhibited by these compounds lacking the carboxylate side chain. We tested the inhibition potency of compounds **1**, **2a**, and **2c** in a radiometric assay and a pH-stat assay (Table 2), and observed that potent inhibition of hGIIA sPLA₂ persisted in these assays. All three compounds inhibited hGIIA sPLA₂ activity at nanomolar concentrations strongly suggesting that they bind tightly and directly to hGIIA sPLA₂ and do not result in false positive inhibition in the fluorometric assay. Interestingly, we observed a more dramatic variation in IC₅₀ values for the GIIA sPLA₂ inhibitors **2a** and **2c** compared to compound **1** in these three different assays suggesting that the GIIA sPLA₂ inhibitors are more sensitive to their membrane environment compared to carboxylate inhibitors. The membrane environment is known to have important effects on the inhibition potency of sPLA₂ inhibitors. This is because the inhibition of sPLA₂s has additional complexity in that the degree of inhibition depends on the concentration of inhibitor in the membrane interface in relationship to the interfacial equilibrium dissociation constant (enzyme-inhibitor complex in the membrane giving free enzyme and free inhibitor both in the membrane).²⁶ The concentration of inhibitor in the membrane depends on the concentration of membrane in the assay and the equilibrium constant for partitioning of inhibitor between aqueous and membrane phases. Furthermore, favorable interactions between inhibitor and membrane components would tend to pull the inhibitor off of the enzyme, whereas unfavorable membrane inhibitor interactions would tend to promote enzyme inhibitor binding. Thus, the degree of inhibition of sPLA₂s depends also on the structure of the lipids that make up the membrane interface.²⁷ All of these factors contribute in a complex way to the extent of sPLA₂ inhibition as the concentration and structure of the membrane in the different assays change.

Given that the GIIA sPLA inhibitors lack the Ca²⁺-binding carboxylate, we considered the possibility that they bind remote to the active site and cause inhibition either by some sort of allosteric effect on enzyme structure or on the way in which the enzyme sits on the membrane surface. Proper positioning of enzyme at the membrane surface presumably allows a single phospholipid molecule to efficiently diffuse from the plane of the membrane into the catalytic site to reach the catalytic residues.^{28,29} Starting first with the carboxylate inhibitors, which likely bind to the active site based on several x-ray structures of related inhibitors bound to various sPLA s^{11, 25}, we showed that compound **1** protects the active site histidine from alkylation by phenacyl bromide both in the absence and presence of a non-hydrolyzable membrane interface (vesicles of DTPM) (Table 3). This was confirmed

with isothermal calorimetry studies on the carboxylate inhibitor **4i** showing that it binds hGIIA sPLA₂ in the absence of membrane (Figure S8). In contrast, the GIIA sPLA₂ specific inhibitors **2a** and **2c** provided no protection to alkylation in the absence of DTPM vesicles (no binding isotherm was detected for GIIA sPLA₂ specific inhibitor **4c** by isothermal calorimetry as well) (Table 3 and Figure S8). However, in the presence of DTPM membranes, **2a** and **2c** provide protection from alkylation (Table 3). Thus, inhibition by these inhibitors lacking the carboxylate is due to occupancy of the active site of hGIIA sPLA₂ on the membrane surface.

Ca²⁺ binds to the active site of GIIA sPLA₂ yet Ca²⁺ is not required for the binding of the GIIA specific inhibitors to the active site. This argues that the inhibitor is not ligated to the active site Ca²⁺, which in turn suggests that water ligates directly to Ca²⁺ in the enzyme inhibitor complex. In the absence of inhibitors, the x ray structures of several sPLA₂s show two water molecules directly bound to Ca²⁺ with the remaining ligands coming from the protein. Both waters are displaced when phospholipid analogs and Ca²⁺-requiring indole sPLA₂ inhibitors bind to the active site. If a water remains bound to Ca²⁺ in the GIIA sPLA₂-inhibitor complex, the inhibitor is likely to be pushed away from the Ca²⁺ site toward the membrane. Thus, while the inhibitor protects the active site histidine from the alkylating agent, it may not sit as deeply in the active site slot as inhibitors that do coordinate to Ca²⁺. In this model, more of the GIIA sPLA₂-selective inhibitor sits in the membrane where a portion of it would directly contact phospholipids in the membrane. This would explain why binding of GIIA sPLA₂-selective inhibitors requires the presence of membrane phospholipids, and it would also explain why the energetics of inhibitor binding depends on the structure of the phospholipids that make up the membranes (recall that that IC₅₀s and inhibitor concentration required for 50% protection from alkylation depend on the structure of the phospholipid used in the assays). In the case of inhibitors that require Ca²⁺ for sPLA₂ binding, they are pulled more deeply into the active site slot and may not interact significantly with phospholipids left in the membrane. Jain and Berg have shown that for inhibitors that fully leave the membrane to dock into the active site slot of the membrane bound sPLA₂, partitioning of the inhibitor into the membrane does not enhance inhibitor enzyme binding.²¹ This is because there is essentially no local concentration advantage to confining the inhibitor and enzyme to the same smaller volume (aqueous phase versus surface of the vesicles) if the inhibitor has to fully give up its interaction energy with the membrane to bind to the enzyme's active site. In other words, the favorable binding energy of inhibitor with membrane which favors partitioning of inhibitor from the aqueous phase to the membrane has to be given up when inhibitor leaves the membrane to bind to the enzyme's active site. On the other hand, if a portion of the inhibitor remains in the membrane when it is docked into the active site (inhibitor is not fully extracted from the membrane), then binding of inhibitor to the membrane may enhance enzyme inhibitor binding. We cannot rule out the possibility that binding of enzyme to the vesicles leads to a conformational change in the active site that is required for binding of inhibitors that lack the carboxylate but not those that contain the carboxylate; however, this seems unlikely.

Since the carboxylate is not required for high affinity binding of these compounds to GIIA sPLA₂, we thought it would be possible for GIIA sPLA₂ inhibitors, bound through a linker at the 4-position of the indole scaffold, to act as potent GIIA sPLA₂ inhibitors. Thus, we explored GIIA sPLA₂ inhibitors as conjugates with HA. As stated in the introduction, these conjugates may be useful therapeutics for the treatment of joint disorders. In comparison to free **4d**, the IC₅₀ for **HA-4d** was ten fold less potent against hGIIA sPLA₂ (1500 nM for conjugated **HA-4d** vs. 130 nM for free **4d**). However, **HA-4d** demonstrated much higher GIIA sPLA₂ inhibition potency compared to free HA, **HA-NH₂CH₂CH₂OCH₂CH₂OH**, and **HA-4d** controls (Figure 3). The fact that both HA and **HA-NH₂CH₂CH₂OCH₂CH₂OH** failed to inhibit hGIIA sPLA₂ activity rules out any possible

non specific inhibitory effects from HA, the linker, or from the conditions or reagents associated with synthesis or purification of these conjugates. The small inhibition observed for **HA-4d** is likely due to trace **4d** that remains after purification. We also observed that the N-methyl control **HA-4d-Nme** was four fold less potent than **HA-4d** against hGIIA sPLA₂ activity. This result indicates that some of the inhibitory effect of **HA-4d** may be nonspecific.

To gage hGIIA sPLA₂ inhibition selectivity, we tested the inhibition potency of **HA-4d** against hGV and hGX sPLA₂. The inhibition potency of **HA-4d** against hGV sPLA₂ was five fold lower than the inhibition potency against hGIIA. Compared to free **4d** where the difference in IC₅₀s for hGIIA and hGV sPLA₂ is >12-fold, conjugating the compound to HA lowers the selectivity of the GIIA sPLA₂ inhibitor. In addition, inhibition of hGV sPLA₂ activity by **HA-4d-Nme** approached the inhibition levels observed for **HA-4d** (Figure S11). Since free **4d** and **4d-Nme** display no inhibition potency against hGV, this suggests that hGV inhibition by the conjugated forms of these inhibitors (**HA-4d** and **HA-4d-Nme**) probably occurs through a non specific mechanism. Interestingly, the two heparin-binding sPLA₂s, GIIA and GV, show inhibition by **HA-4d** and **HA-4d-Nme** (Figures 3 and S11). It is possible that the affinity of hGIIA and hGV sPLA₂s for polyanionic surfaces, such as HA, may contribute to the inhibition. This was not the case for hGX sPLA₂, a poor binder of heparin, which was essentially unaffected by **HA-4d** or **HA-4d-Nme** (Figure S11). This result rules out **HA-4d** inhibition through general membrane disruption or some other general mechanism.

To investigate whether **HA-4d** was able to bind the enzyme active site, we tested **HA-4d** in the alkylation protection assay at 100 ng/Jl. This concentration was three fold higher than the concentration of **HA-4d** that inhibited 90% of hGIIA sPLA₂ activity in the fluorescence based assay. Results from the alkylation protection assay show that **HA-4d** has no measurable affinity for the active site of hGIIA sPLA₂ in the presence or absence of DTPM (Figure S12). It appears that linking **4d** to HA prevents active site binding since free **4d** is able to protect against alkylation (Figure S12). These data suggest that the hGIIA sPLA₂ inhibition observed from **HA-4d** is probably not occurring through binding the active site. Thus, while **HA-4d** shows inhibition of hGIIA sPLA₂ activity, the decreased inhibition potency and selectivity compared to free **4d** makes it a poor candidate for further study in arthritis disease models. To get around these problems, HA and the inhibitor as two separate molecules or an HA inhibitor conjugate containing a hydrolyzable linker could be developed for injection into the synovial space of the arthritic joint.

In summary, we explored the mode of inhibition of an important class of sPLA₂ inhibitors that are selective for GIIA sPLA₂.¹⁰ Previously it was shown that removal of the carboxylate moiety on compound **1** significantly decreased inhibition potency against hGV and hGX sPLA₂s, but not against hGIIA. We found that the carboxylate moiety could be replaced with a number of different functional groups that are similar in size to the carboxylate without affecting hGIIA sPLA₂ inhibition potency and selectivity. Using inhibition data and an alkylation protection assay, we provide evidence that hGIIA sPLA₂ selective inhibitors are able to bind the hGIIA enzyme active site in a similar orientation as carboxylate containing compounds, and that this binding does not require Ca²⁺. We also conjugated the hGIIA sPLA₂ selective inhibitor **4d** to HA (**HA-4d**) and observed that **HA-4d** is able to inhibit hGIIA sPLA₂ activity but not through active site binding. These results are part of a growing initiative to design and develop inhibitors that selectively target proinflammatory sPLA₂s.

Supplementary Material

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ABBREVIATIONS

DMPM	1,2-dimyristoyl- <i>sn</i> -glycero-3-phosphomethanol
DTPM	1, 2-ditetradecyl- <i>sn</i> -glycero-3-phosphomethanol
GIIA sPLA₂	group IIA secreted phospholipase A ₂ (likewise for other group names)
HA	hyaluronic acid
hGIIA sPLA₂	human group IIA secreted phospholipase A ₂ (likewise for other group names)
pyrPG	1-hexadecanoyl-2-(1-pyrenedecanoyl)- <i>sn</i> -glycero-3-phosphoglycerol

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Figure 1.
A, Structures of the generally potent (**1**) and GIIA selective (**2a**) sPLA₂ inhibitors. B, Crystal structure highlighting the key binding interactions between the structurally related inhibitor, Me Indoxam, and the hGX active site.

Figure 2.

Representative example of K_d determination using the alkylation protection assay. A, Semilogarithmic plots for kinetics of hGIIA sPLA₂ inactivation by phenacyl bromide in the presence of 200 μM CaCl₂, 200 μM DTPM, and different concentrations of compound **2a** (see figure legend). Negative control denotes conditions where phenacyl bromide is not included in the assay mixture. Each plot represents the mean of quadruplicate analysis with error bars representing the standard deviation. B, Scatchard and Utter plots were generated for each of the four individual experiments and the K_d value was determined from the $\times$ intercept ($-1/K_d$) of the linear equation fitting each plot.

Figure 3.

Inhibition of hGIIA sPLA₂ activity by **4d** or **4d-Nme** conjugated to Hyaluronic Acid (HA) (**HA-4d** and **HA-4d-Nme**, respectively) in the fluorometric enzyme assay. Also, HA mixed with **4d** but no coupling reagents (**HA+4d**), HA treated with linker only (**HA-NH₂CH₂CH₂OCH₂CH₂OH**) and free HA were also tested for inhibition of hGIIA sPLA₂. Each plot is the mean of triplicate experiments with error bars representing the standard deviation.

Scheme 1a.

^aReagents and conditions: a) EDC, HOBT in THF: 0.1 M MES pH 5.1 (1:1), 2hrs at room temperature.

Table 1IC₅₀ values of Inhibitors against human GIIA, GV, and GX sPLA₂s

<div> </div>				
IC ₅₀ (nM)				
comp	R ₁	hGIIA	hGV	hGX
1		40±2	35±7	20±3
2a		14±2	>1600	1500±300
2b		20±1	>1600	≈1600
2c		35±5	>1600	1400±150
3a		35±2	>1600	>1600
3b		20±3	>1600	>1600
3c		20±2	>1600	>1600
3d		200±40	>1600	>1600

IC ₅₀ (nM)				
comp	R ₁	hGIIA	hGV	hGX
3e		25±5	≈1600	>1600
4a		70±20	>1600	>1600
4b		100±15	>1600	≈1600
4c		40±2	>1600	≈1600
4d		130±15	>1600	>1600
4e		210±20	>1600	>1600
4f		430±30	>1600	>1600
4g		140±20	>1600	>1600
4h		>1600	>1600	>1600

IC₅₀ values were obtained from the fluorometric assay and reported as the mean of triplicate analysis with standard deviations. Values of >1600 nM or ≈1600 nM are reported for compounds with IC₅₀ values >1600 nM.

Table 2

Inhibition potency of Benzo-fused indole inhibitors in different sPLA₂ activity assays.

IC ₅₀ (nM) against hGIIA sPLA ₂			
comp	Fluorometric Assay ^a	Radiometric Assay ^b	pH-Stat Assay ^c
1	40±2	2±1	<14
2a	14±2	95±10	25
2c	35±5	520±90	95

^aIC₅₀ values reported from Table 1.

^bIC₅₀ values reported as the mean of duplicate analysis with standard deviations.

^cIC₅₀ values reported from singlet analysis.

Table 3

K_d values determined from alkylation protection assay.

comp	K_d (nM)			
	0 μ M DTPM 200 μ M CaCl_2	0 μ M DTPM 100 μ M EGTA	200 μ M DTPM 200 μ M CaCl_2	200 μ M DTPM 100 μ M EGTA
1	≈ 250	>2500	<125	>2500
2a	>5000	>5000	400 ± 50	350 ± 40
2c	>5000	>5000	330 ± 20	280 ± 30

Each K_d value is reported as the mean of at least quadruplicate analysis with standard deviations. Values of >5000 nM or >2500 nM were reported in cases aof no measurable binding.