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Letter

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Discovery of AZD2716: a novel secreted phospholipase A_2 ($sPLA_2$) inhibitor for the treatment of coronary artery disease

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Discovery Sciences

Sections of [d]Structure & Biophysics, [e]Reagents and Assay Development and [f]Screening Sciences and Sample Management.

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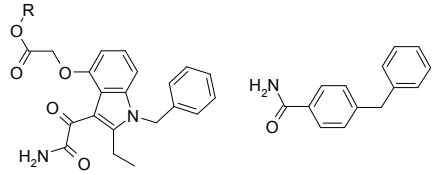
KEYWORDS. secreted phospholipase A_2; $sPLA_2$; inhibitor; fragment-based drug discovery, fragment screening, atherosclerosis, coronary artery disease

ABSTRACT: Expedited structure-based optimisation of the initial fragment hit **1** led to the design of (*R*)-**7** (AZD2716) a novel, potent secreted phospholipase A_2 ($sPLA_2$) inhibitor with excellent preclinical pharmacokinetic properties across species, clear *in vivo* efficacy and minimized safety risk. Based on accumulated profiling data, (*R*)-**7** was selected as a clinical candidate for the treatment of coronary artery disease.

Secreted phospholipase A_2 ($sPLA_2$) are enzymes that hydrolyze the acyl ester at the sn-2 position of sn-3 glycerophospholipids[1], a process characterised by complex interfacial kinetics of substrate-enzyme binding and catalysis[2]. Eleven $sPLA_2$ enzymes (group Ib-XIIb) have so far been identified in mammals[3-5], several of which have been detected in human atherosclerotic lesions[6]. Among these, group IIa, V, and X $sPLA_2$ isoforms are present in human carotid atherosclerotic lesions and have been associated with disease progression. They have been implicated in several proatherogenic actions in the arterial wall.[7-9] Due to their hydrolytic action on lipoprotein phospholipids, $sPLA_2$s promote lipid accumulation, induce significant lipoprotein remodeling, macrophage activation and foam cell formation.[10-11] Furthermore, as the rate-limiting step in eicosanoid production, $sPLA_2$-mediated release of arachidonic acid from the *sn*-2 position of phospholipids renders them highly pro-inflammatory enzymes.[10-11] In addition, epidemiological data has shown that increased levels of $sPLA_2$ protein and $sPLA_2$ activity are independently associated with risk of cardiovascular events and prevalence of atherosclerosis.[11] Owing to the pivotal role of $sPLA_2$s in regulating lipoprotein function and inflammatory mechanisms, two crucial components of atherogenesis, $sPLA_2$ inhibitors[12] could be useful for the treatment of atherosclerosis. Interestingly, the archetypal $sPLA_2$ inhibitor varespladib methyl (Figure 1), was evaluated in short duration clinical trials for the treatment of rheumatoid arthritis and acute coronary syndrome with negative results.[13-14]

We thus set out to identify novel $sPLA_2$ inhibitors that could be used in longer term coronary artery disease-based clinical studies to more properly assess the relevance of their lipoprotein-modifying effects[15-17]on cardiovascular disease, alongside their anti-inflammatory properties.



R=Me, varespladib methyl
R=H, varespladib **1**

Figure 1. $sPLA_2$ inhibitor varespladib methyl, its active metabolite varespladib and initial fragment hit **1**.

Given the competitive landscape, medicinal chemistry precedents and available structural information for $sPLA_2$ enzymes, we opted for a structure-based fragment approach to maximize the chances of novelty and developability.[18] Analysis of potency data in combination with the available ligand-bound $sPLA_2$ crystal structures[19] indicated that primary amides are extremely effective $sPLA_2$ warheads, as they establish three hydrogen bonds with $sPLA_2$ and one co-ordination bond with the catalytic calcium ion. We assembled a selection of primary aromatic carboxamide-containing fragments (heavy atom count ≥ 10 and ≤ 18) by mining in house biochemical and biophysical assay data against the $sPLA_2$-IIa and $sPLA_2$-X isoforms. The selection was based on activity against $sPLA_2$-IIa, which is the most widely expressed isoform in humans[20], but also on inspection of crystal structures and chemical evolution potential.

Table 1. Initial profile for the fragment hit 1.

entry	sPLA$_2$-IIa IC_{50} (μM)	Plasma $IC_{u,50}$ (μM)[a]	F_u (%)	LE/LLE[b]
Varespladib	0.028	0.008	12.5	0.37/7.2
1	24	0.9	1.8	0.39/2.2

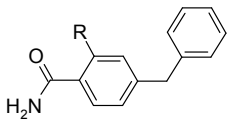
[a]Calculated as Plasma sPLA$_2$ IC_{50} (μM) × compound's unbound fraction in human plasma (F_u) /100. F_u = 100-human protein binding(%). [b]Ligand efficiency, LE (kcal/mol/HAC), calculated as -RTln(sPLA$_2$-IIa IC_{50}) / Heavy Atom Count. Ligand Lipophilicity efficiency (LLE) calculated as pIC_{50} (sPLA$_2$-IIa) – logD.

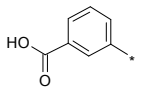
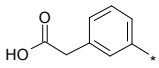
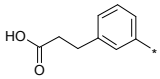
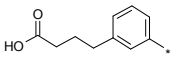
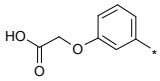
The triaging process identified compound **1** (LE: 0.39) originating from a legacy fragment/HTS campaign as the most promising fragment lead . As the translatability to a clinical setting was of special importance, we also triaged the actives for sPLA$_2$ activity inhibition in human plasma (the mode-of-action biomarker to be used in clinical trials), as measured using a previously established protocol.[21]

Compound **1** inhibited sPLA$_2$-IIa (IC_{50}: 24 μM) and human plasma sPLA$_2$ activity ($IC_{u,50}$: 0.9 μM) in a concentration dependent manner (Table 1). Going forward, as plasma sPLA$_2$ activity is the result of various sPLA$_2$ isoforms and we were interested in identifying a broad spectrum sPLA$_2$ inhibitor, we also monitored inhibition of sPLA$_2$-V and sPLA$_2$-X, given their potential role in lipoprotein modulation.[16] Lastly, to avoid the need for a prodrug strategy (eg varespladib methyl) we carefully evaluated compound lipophilicity against passive permeability, solubility and metabolic stability, prior to verifying the pharmacokinetic (PK) and pharmacodynamic (PD) profile *in vivo*.

The crystal structure of **1** bound to sPLA$_2$-X confirmed the binding mode of of the primary amide, with hydrogen-bonding to sPLA$_2$-X's G28, H46 and D47 (corresponding to sPLA2-IIa G29, H47 and D48) and coordination to the calcium ion observed, as shown in Figure 2. Additionally, the 4-benzyl substituent was located in a lipophilic pocket consisting of residues I2, L5, A6, V9, P17, I18 and M21 (corresponding to L2, F5, H6, I9, A17, A18 and G22 in sPLA$_2$-IIa, Figure 2). While the two sPLA$_2$ isoforms are identical in the amide coordinating residues, the lipophilic pocket that accommodates the benzyl group is slightly smaller in sPLA2-IIa. However, superposition of the sPLA$_2$-IIa and sPLA$_2$-X crystal structures suggested that the benzyl group of **1** could fit in the slightly smaller sPLA$_2$-IIa pocket.

Table 2. sPLA$_2$ potency and ligand efficiencies for compounds 2 – 6.[a]



Entry	R	sPLA$_2$-IIa IC_{50} (μM)	sPLA$_2$-V IC_{50} (μM)	sPLA$_2$-X IC_{50} (μM)	Plasma $IC_{u,50}$ (μM)[b]	LE/LLE[c]
varespladib		0.028	0.12	0.041	0.008	0.37/7.2
1		24	NA[d]	2.2	0.9	0.39/2.2
2		0.91	>10	NA[c]	NA[d]	0.33/4.9
3		0.07	1.4	1.1	0.04	0.38/5.9
4		0.012	0.36	0.28	0.007	0.4/6.4
5		0.11	4.1	5.4	0.03	0.34/5
6		0.19	3.7	3.7	ND[e]	0.34/5.4

[a]Results are mean of at least two experiments. Experimental errors within 20% of value. [b]Calculated as Plasma sPLA2 IC_{50} (μM) × compound's unbound fraction in human plasma (F_u) /100. F_u = 100-human protein binding(%) [c]Ligand efficiency, LE (kcal/mol/HAC), calculated as -RTln(sPLA$_2$-IIa IC_{50}) / Heavy Atom Count, Ligand Lipophilicity efficiency (LLE) calculated as pIC_{50} (sPLA$_2$-IIa) – logD. [d]Not active at maximum tested concentration (25 μM). [e]Not determined.

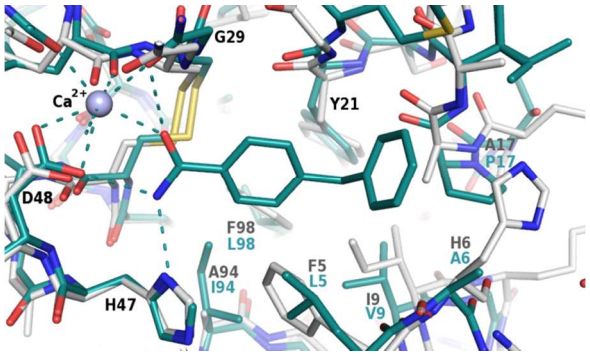
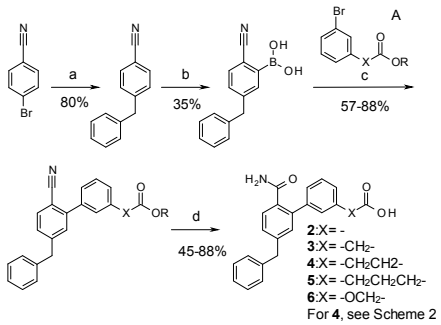


Figure 2. Superposition of the crystal structure of **1** bound to sPLA$_2$-X (cyan) and a crystal structure of sPLA$_2$-IIa (grey). Residues that differ between the two isoforms are labelled in cyan and grey, respectively. The calcium ion is depicted as a purple sphere and hydrogen bonds are displayed as dashed lines.

We therefore devised a chemical exploration strategy starting from the binding mode of **1**. Here, special effort was placed upon establishing a second coordination bond to the catalytic calcium ion. The reasoning was two-fold: a) to increase affinity and functional inhibition of the enzyme as a result of a bidentate calcium chelate and additional van der Waals contact with the enzyme, b) to allow a more balanced lipophilicity profile of the final compounds as the additional calcium interacting moiety was anticipated to be a carboxylic acid. The *ortho* position of the benzamide ring was identified as a favourable substitution vector to deploy such a strategy. Based on iterative molecular modelling and careful consideration of theoretical affinity gain and ligand efficiencyprediction, we synthesised compounds **2** – **6**, according to Scheme 1 (for compound **4** refer to Scheme 2). Key steps involved the formation of the boronic acid by ortho lithiation[22] of the 4-benzylbenzonitrile (step b) followed by a Suzuki-Miyaura coupling and a controlled hydrolysis (step d) to generate both the amide and carboxylic acid functions (Scheme 1).

Scheme 1. General synthesis of compounds 2, 3, 5, 6[a]



[a] Conditions: (a) benzylzinc bromide, Pd(Ph$_3$)$_4$, THF, 60 °C. (b) 1. nBuLi, tetramethylpiperidine 2. B(OiPr)$_3$, THF -78 °C. (c) Compound **2**, **3**, **5**: 3-bromophenyl carboxylic acid **A,** PdCl$_2$(dppf), Cs$_2$CO$_3$, DMF, 60-90 °C. Compound **6**: Pd(P(Ph$_3$)$_4$, Cs2CO3, DMF 90 (d) Compound **2**, **3**, **5**: n-propanol:H$_2$O (10:1), KOH (10 eq), 80-100 °C, or THF, H$_2$SO4. Compound **6**: KOH (10 eq), MeOH/H$_2$O, microwawe 130 °C, 20 min.

Introduction of a 3-benzoic acid moiety at the 2-position of 4-benzylbenzamide **1**, albeit notoptimal, confirmed the potential for growth at that position. (**2**, Table 2). Progressive elongation at the carboxylic acid position by introduction of methylene units (**3** – **5**) had a parabolic effect on potency, with the 3-phenylpropionic acid side chain yielding the most potent and ligand efficient derivative (**4**, Table 2). Replacement of the benzylic methylene by an ether oxygen or further elongating the hydrocarbon chain were not tolerated (cf. **5** and **6**, Table 2), hinting at a specific conformational requirement for the carboxylic acid-containing side chain. The co-crystal structure of sPLA$_2$-IIa and **4** confirmed the previously hypothesised ligand-mediated calcium chelation, as displayed in Figure 3.

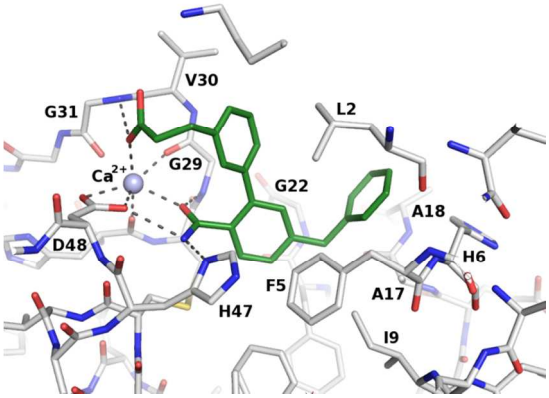


Figure 3. Cocrystal structure of **4** bound to sPLA$_2$-IIa. The calcium ion is depicted as a purple sphere, and relevant hydrogen bonds are displayed as dashed lines.

The carbonyl oxygen atom of the amide group of **4** provided the first coordination bond to calcium, analogously to **1**. The carboxylate moiety established the second coordination bond to calcium (*d*: 2.4 Å) and a hydrogen bond to the backbone amide group of G31, as shown in Figure 3, while the additional phenyl ring made significant van der Waals contacts with the side chains of L2, G29 and V30. The 4-benzylbenzamide componentof **4** displayed a similar interaction pattern as in **1**, except for the benzamide ring which was rotated by ca. 50 degrees. This rotation is induced by the introduction of the substituent at the 2-position and, in the case of sPLA$_2$-X, this comes with a penalty. This is exemplified by **2** and **3**, where the affinity gain is very limited, despite the addition of more lipophilic interactions (Table 2). In type IIa, on the other hand, this conformational lock is further stabilized by a edge-to-face pi interaction with F5, which is not available in sPLA2-X, This is reflected by the steep improvement in affinity when adding the substituents. For example, **3** despite having a linker too short to form the additional calcium interaction, still gains more than 300-fold in affinity. This is further improved by the propanoic acid side chain of **4** where the second calcium coordination bond is properly established leading to a 2000 fold increase in potency compared to **1**. The active site of sPLA2-IIa is smaller, F5 (L5 in sPLA$_2$-X) affects the benzamide moiety and I9 (V9 in sPLA$_2$-X) is located close to the hinge between the two benzyl groups of **1** (*d*:3.7Å), thereby slightly altering the angle in which the 4-benzyl enters the pocket and potentially introducing some strain in the fragment, where the larger pocket of sPLA$_2$-X offers a less restrained binding mode

The high ligand efficiency and potency of **4**, coupled with its marked plasma sPLA$_2$ inhibition ability (IC$_{u,50}$ 7 nM) triggered a broad characterisation campaign to identify potential shortcomings.

As summarised in Table 3, compound **4** proved to be soluble, highly permeable and metabolically stable;characteristics which translated well *in vivo* with high bioavailability and low systemic clearance recorded in rat and dog (Table 3). This provided a significant improvement over varespladib, which required a methyl ester prodrug approach (ie. varespladib methyl) to afford moderate oral absorption (F: 40 – 55%) in the same species. Compound **4** did not show any significant inhibition of cytochrome P450 enzymes or ion channel activity relevant to cardiac function. Nevertheless, **4** inhibited the uptake of pivastatin in HEK293 cells transfected with the human organic anionic transporter polypeptide 1B1 (OATP1B1) at an estimated IC_{50} of 2.2 μM, as shown in Table 3. The OATP1B1 transporter is necessary for statin's hypocholesterolemic action as it mediates their access to the liver compartment where they can then inhibit the function of HMG-CoA reductase. [23-25]

Table 3. Profile[a] of compound 4.

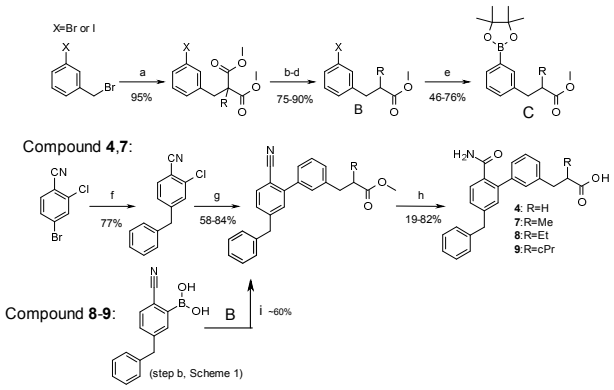
Solubility (pH=7.4) (μM)	98	
P_{app} (10^{-6} cm/s)	40.1	
HEP Cl_{int} (μL/min/10^{-6} cells)	5.2	
hERG, $Na_v1.5$, IKs, $K_v4.3$, $Ca_v3.2$, $Ca_v1.2$ IC_{50} (μM)	>33.3	
CYP450 IC_{50} (μM)	>20	
OATP1B1 IC_{50} (μM)	2.2	
PK	Rat	Dog
Dose i.v./p.o. (μmol/kg)	2/4	1/2
CL (mL/min/kg)	1	0.3
V_{ss} (L/kg)	0.22	0.26
F (%)	81	82

[a] Please see the supporting information for experimental details.

Considering that an eventual $sPLA_2$ inhibitor for the treatment of coronary artery disease will need to be co-administered with a statin, as an established standard of care, minimizing the risk for such drug-drug interaction was required. As OATP1B1 recognises anionic compounds, we reasoned that modification of the molecular environment around the carboxylic acid of **4** might alleviate its interaction with OATP1B1. More specifically, substituting the carbon atom alpha to the carboxylic acid was of special interest as a) it was postulated to provide a steric impediment to OTAP1B1, b) it seemed compatible with the binding pocket of the $sPLA_2$-IIa enzyme, and c) could enhance potency and/or selectivity through conformational "freezing" of the carboxylic acid side chain via gauche-like effects. Due to structure-based constraints and in order to minimize the impact on compound lipophilicity, we targeted small substituents and synthesised compounds **7** – **9**, following Scheme 2.

According to scheme 2, the appropriate dimethyl malonate was alkylated using 3-bromobenzylbromide. The propionic acid derivative was then obtained by hydrolysis, decarboxylation and re-esterification to yield the methyl ester. Boronylation was accomplished by standard protocols using $(Bpin)_2$ and $PdCl_2(dppf)$ to yield the pinacol borane ester which could be used in the subsequent Suzuki-Miyaura coupling using the benzylated chloro benzonitrile. Finally, racemic **7** – **9** could be obtained by hydrolysis using hydroxide in alcohol/water mixtures (careful monitoring of the reaction to avoid over hydrolysis to the corresponding di-acid is needed).

Scheme 2. General synthesis of $sPLA_2$ inhibitors 4, 7-9[a]



[a] Conditions: (a) appropriate malonate, Cs_2CO_3, DMF, 70 °C, 2h. (b) NaOH (4 eq), H_2O:MeOH 3:1 80 °C 2h. (c) HOAc (3 M), reflux. (d) HCl, MeOH 60 °C, 2.5 h. (e) $(Bpin)_2$ (1.3 eq), KOAc (2.5 eq), $PdCl_2(dppf)$ (6.5mol%), dioxane, 90 °C. (f) BnZnBr (1.5 eq, 0.5 M in THF), $Pd(PPh_3)_4$ (5 mol%), THF, 60 °C, 2h. (g) Compound **4**: 3-(3-methoxy-3-oxopropyl)phenylboronic acid (commercial), $Pd(Ph_3)_4$. Compound **7**: $PdCl_2(dbpf)$ (5 mol%), C. Cs_2CO_3, DMF, 90 °C, 1.5 h. (h) n-propanol, H_2O (10:1), KOH (10 eq), 80-100 °C. i) **B,** $PdCl_2(dppf)$, Cs_2CO_3, DMF, 90 °C.

Addition of a methyl group (**7**, Table 4) displayed an isoform-specific effect on potency: while it was neutral at $sPLA_2$-IIa, it enhanced inhibition of $sPLA_2$-V and reduced that of $sPLA_2$-X. Overall, this yielded excellent plasma $sPLA_2$ inhibition ($IC_{u,50}$: 0.1 nM). Remarkably, structural manipulation of the carboxylic acid surroundings by an alpha-methyl group demonstrated the intended effect at a transporter level and **7** was devoid of OATP1B1 inhibition at the maximum tested concentration (25 μM). Ethyl and cyclopropyl substitutions (**8, 9**) were not as efficient and had a deleterious effect on metabolic stability in human hepatocytes (cf. **8**, **9** and **7**, Table 4). We therefore proceeded to separate and characterise the two enantiomers of **7**. In line with expectations, $sPLA_2$ inhibition was affected by stereochemistry and the *R* enantiomer proved to be the most active, half-maximally inhibiting $sPLA_2$-IIa, -V and -X at 10, 40 and 400 nM, respectively (Table 4). As a result, (*R*)-**7** was the most potent plasma $sPLA_2$ inhibitor in this study ($IC_{u,50}$: 0.1 nM) and, based on its minimized risk for drug-drug interactions with statins, was progressed to further profiling.

When incubated with HepG2 cells, (*R*)-**7** effectively inhibited $sPLA_2$ activity (IC_{50} <14 nM) and suppressed production of $sPLA_2$-IIa (IC_{50} 176 ± 28 nM) via a mechanism not yet elucidated.[7] Importantly, (*R*)-**7** demonstrated significant $sPLA_2$ activity inhibition (IC_{50} 56 ± 10 nM) in atherosclerotic plaque homogenates, as obtained from carotid endarterectomy of coronary artery disease patients (N = 7).[26]

In vivo PK analysis of (*R*)-**7** showed consistent high bioavailability and low clearance across different animal species, as summarised in Table 5.

Table 4. $sPLA_2$ potency and optimisation parameters for compounds 4 – 9 .

Entry	R	$sPLA_2$-IIa IC_{50} (μM)[a]	$sPLA_2$-V IC_{50} (μM)[a]	$sPLA_2$-X IC_{50} (μM)[a]	Plasma $IC_{u,50}$ (nM)[b]	HEP Cl_{int} (μL/min/10^{-6} cells)[c]	OATP1B1 IC_{50} (μM)[d]
4	H	0.012	0.36	0.28	7	5.2	2
7	Me	0.011	0.07	0.75	1	9.6	NA[e]
8	Et	0.021	0.07	0.43	0.8	25	ND[f]
9	CyPr	0.018	0.25	0.58	0.9	21	ND[f]
(*S*)-7	(*S*)-Me	0.038	1.2	3.8	ND	9.3	ND[f]
(*R*)-7	(*R*)-Me	0.010	0.04	0.4	0.1	12	NA[e]

[a] Mean of at least two experiments. Experimental errors within 20% of value. [b]Calculated as Plasma $sPLA_2$ IC_{50} (μM) × unbound fraction in human plasma (F_u) /100. [c]Intrinsic clearance of test compounds after incubation with human hepatocytes. [d]Inhibition of pivastatin uptake to HEK293 cells transfected with human OATP1B1. [e]Not active at maximum tested concentration (25 μM). [f]Not determined.

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Table 5. PK parameters of compound (*R*)-7

	Mouse	Rat	Dog	Cyno-molgus
N i.v./p.o.	2/2	2/2	2/2	2/2
Dose i.v./p.o. (μmol/kg)	10/50	2/8	1/3	5/15.8
Oral $AUC_{0\text{-}inf}$ (μM×h)	69.7	70	284.1	71.3
Cl (mL/min/kg)	9.2	1	0.2	2.8
V_{ss} (L/kg)	2.8	0.21	0.15	1.1
F (%)	76.3	84	91	81

Building on the observed PK parameters, and the *in vitro* $sPLA_2$ inhibition measured in cynomolgus monkey plasma ($IC_{u,50}$ 1 nM), we resolved to evaluate the *in vivo* $sPLA_2$ inhibitory effect of (*R*)-**7**.

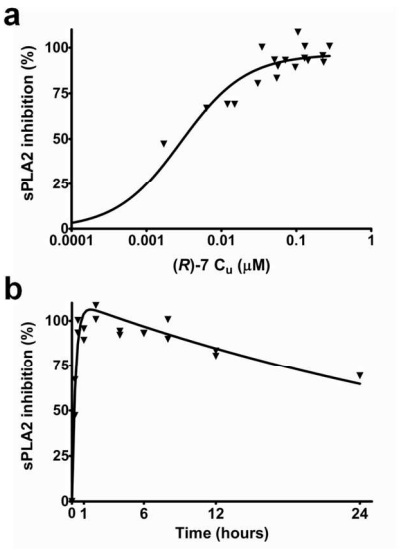

Figure 4. Vehicle-corrected plasma $sPLA_2$ activity inhibition, corresponding PK-PD relationship (a) and time course (b) following oral administration of 30 mg (*R*)-**7** to cynomolgus monkeys (N=2).

A 30 mg dose of (*R*)-**7** was orally administered to cynomolgus monkeys (N = 2), as shown in Figure 4. This generated a concentration-dependent inhibition of $sPLA_2$ activity in plasma ($IC_{u,80}$ 13 ± 3 nM) that well reflected the time course of (*R*)-**7**'s exposure profile (Figure 4).

In summary, starting from the original fragment hit **1**, two design cycles based on structural information, ligand efficiency reasoning, physicochemical property control, medicinal chemistry tactics and readily available experimental data resulted in the discovery of (*R*)-**7** (AZD2716), a novel, potent $sPLA_2$ inhibitor with excellent PK properties, *in vivo* efficacy and minimized risk for drug-drug interactions. Based on the available results and the favourable toxicological profile in rats, dogs and cynomolgus monkeys, (*R*)-**7** was selected as a clinical candidate for the treatment of coronary artery disease

SUPPORTING INFORMATION

Synthesis details for **2** - **9**, assay protocols and X-ray data are enclosed in the supporting information. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

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Notes

The authors declare no competing financial interest.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. Project leader, lead author FG; Author DP; Medicinal chemists DP, PN, MD, IS, LK, NS; Bioscientists EH-C, BR; Enzyme assays UK; DMPK L-OL; X-ray JS, ND, MC.

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Discovery of AZD2716: a novel secreted phospholipase A_2 ($sPLA_2$) inhibitor for the treatment of coronary artery disease

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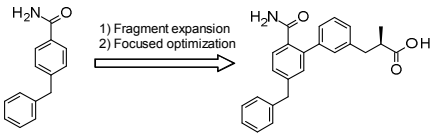

1
Plasma $sPLA_2$ $IC_{u,50}$ = 0.9 uM

AZD2716
Plasma $sPLA_2$ $IC_{u,50}$ = 0.1 nM