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Synthesis and Enzyme Inhibitory Activities of a Series of Lipidic Diamine and Aminoalcohol Derivatives on Cytosolic and Secretory Phospholipases A_2

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Abstract—We have synthesised some lipidic diamines and aminoalcohols and examined their behaviour as inhibitors of secretory and cytosolic PLA_2. Some structure–activity relationships considerations have been deduced. Compound **14** was a potent and selective inhibitor of $cPLA_2$ and compound **4** showed a dual inhibitory profile against both types of PLA_2 while no cytotoxicity at 10 μM on human neutrophils or on murine macrophage line was observed for both.

There is evidence that phospholipases A_2 (PLA_2) play an important pathophysiological role in various inflammatory diseases, such as septic shock,[1] adult respiratory distress syndrome,[2] arthritis[3] and acute pancreatitis.[4] PLA_2 catalyzes the hydrolysis of a fatty acyl ester bond at the *sn*-2 position of a glycerophospholipid, being the enzyme responsible for arachidonic acid release for the biosynthesis of the eicosanoids, including prostacyclin, thromboxanes, and other prostaglandins as well as leukotrienes.[5] The other product regulated by PLA_2 enzymes is the platelet-activating factor (PAF), which is also a potent cellular mediator.[6] It is known that inhibition of PLA_2 can modulate the production of different inflammatory mediators. However, it is not clear which forms of PLA_2 should be inhibited, because PLA_2 really comprises a rapidly growing superfamily of enzymes like other signal transduction proteins. In this regard, distinct forms of PLA_2 have been characterised.[5] Two main groups have been reported, the secretory PLA_2 ($sPLA_2$ groups I, II, III, V, IX and X with a relatively small molecular weight), and the cytosolic PLA_2 ($cPLA_2$ groups IV, VI, VII and VIII with a higher molecular weight). They also differ in Ca^{2+} requirements and molecular characteristics.

We report the synthesis and the in vitro pharmacological evaluation of a series of diamine and aminoalcohol derivatives sharing a long alkyl chain as the common feature. Some of these compounds were tested for their inhibitory effect on $sPLA_2$, belonging to the groups I (*Naja naja* venom and porcine pancreatic enzymes), II (human synovial recombinant enzyme) and III (bee venom enzyme) as well as on $cPLA_2$ from macrophage line RAW 264.7 (group IV), using manoalide[7] and palmitoyl trifluoromethyl ketone (PTK) as reference inhibitors.

Chemistry

Compounds were designed as not too close structural analogues of the phospholipidic substrate of PLAs. The aliphatic chain ($R = C_{14}H_{29}$), was included to mimic the *sn*-1 chain of the substrate and its size was selected to provide the molecules with an adequate lipophilic character, which would be modulated by substituents at positions *sn*-2 and *sn*-3. The absence of any hydrolysable function at the chain would prevent the undesirable deactivation by chemical or enzymatic factors. Main functional groups attached to positions 1 and 2 of the aliphatic long-chain were separated differently by increasing the number of ethylene groups between them in order to define the best distance for calcium coordination in the enzyme. Compounds selected for testing

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were synthesised, among others, as depicted in Scheme 1. The Boc-aminoacid **1**, prepared from diethyl 2-acetamidomalonate, as reported by Gibbons et al.,[8] was transformed into the Boc-aminoalcohol **2** by the Kokotos procedure.[9] **2**, through mesylation, followed by substitution with sodium azide and reduction with $NaBH_4$/MeOH, in presence of Pd-C,[10] led to the Boc-diamine **3**, which on alkylation/acylation followed (or not) by Boc-deprotection, yielded the diamine derivatives **4**–**6** (or **7**), respectively.

The Boc-aminoalcohol **2** was benzylated to obtain the Boc-aminoether **8**, which was Boc-deprotected to give compound **9.** The benzylic aminoether **9**, treated with 1.2 and 2.2 equivalents of EtBr, gave **10** and **11**, respectively. Acylation of **9** with succinic, maleic and glutaric anhydrides led to the amidoethers **12**, **13** and **14** respectively. Hydrogenolytic debenzylation of **10** and **14**, gave **15** and **16** respectively. Alkylation of **9** with ethyl bromoacetate, followed by debenzylation and hydrolysis with 10% KOH/MeOH, yielded the glycine derivative **17**.

The Boc-aminoalcohol **2** was treated with heptanoyl chloride and palmitoyl chloride to give the esters **18a** and **18b**, respectively. The attempted Boc-deprotection of these compounds with TMSCl/PhOH[11] in $HCCl_3$, followed by purification on silica gel, led to the intramolecularly transamidated compounds **18c** and **18d**, which were further treated with the appropriate acyl chlorides to obtain the amidoesters **19**, **20** and **21**.

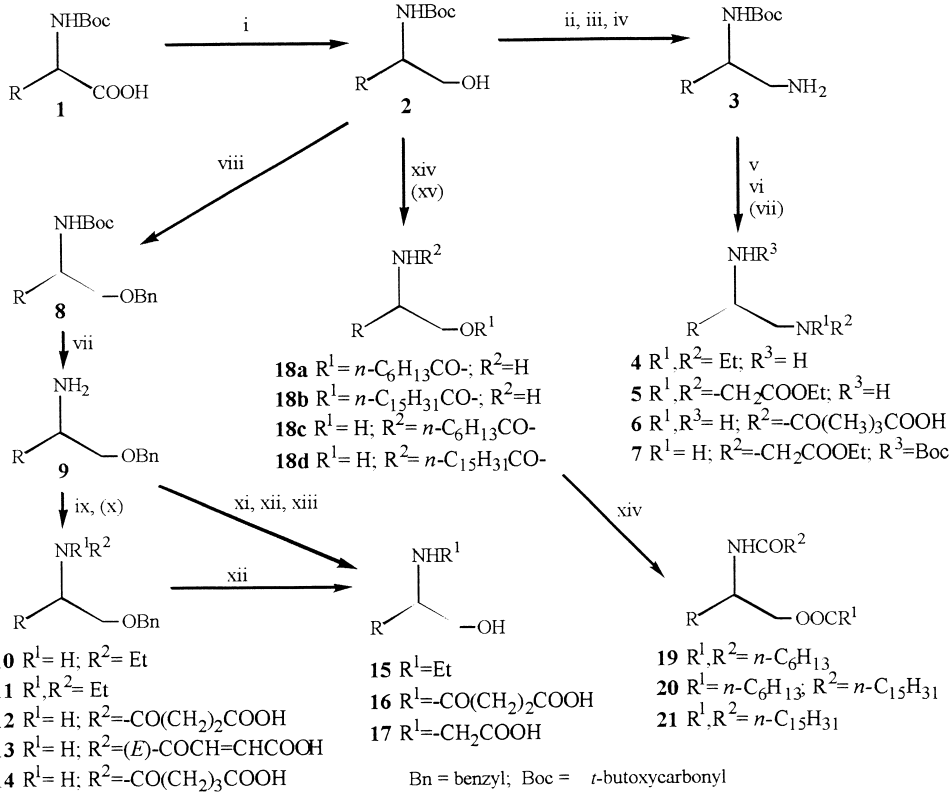
Complete details on the synthetic procedures, product characterisation and yields will be published elsewhere. New studies relating the resolution of racemic samples and/or the synthesis of enantiopure compounds will be done.

Biological Evaluation

$sPLA_2$ was assayed by using autoclaved [^{3}H]oleate-labeled membranes.[12] *Naja naja* venom enzyme, porcine pancreatic, human recombinant synovial enzyme, and bee venom enzyme were used. $cPLA_2$ activity was measured as the release of radiolabeled arachidonic acid[13] using cytosolic fractions of murine macrophage cell line RAW 264.7 as the source of enzyme**.** The mitochondrial dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan[14] was used to assess the possible cytotoxic effects of test compounds on human neutrophils as well as on murine macrophage line.

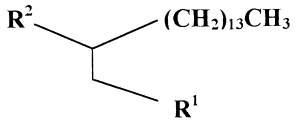
Results and Discussion

Inhibition of specific PLA_2s constitutes a potentially useful approach for treating a great variety of inflammatory



Scheme 1. Synthesis of lipidic diamine and aminoalcohol derivatives. i: a) EtOOCCl/N-methylmorpholine/THF; b) $NaBH_4$/MeOH. ii) MsCl/Et_3N/CH_2Cl_2. iii. NaN_3/DMF. iv. Pd-C/$HCCl_3$, $NaBH_4$/MeOH. v: EtBr (or $EtOOCCH_2Br$)/Et_3N/DMF. vi: glutaric anhydride/EtOAc. vii: HCl/THF, Argon. viii: BnCl/NaH/DMF. ix: EtBr (1.2 or 2.2 eq.)/Et_3N/DMF. x: succinic (maleic, glutaric) anh./EtOAc. xi: $EtOOCCH_2Br$/Et_3N. xii: H_2/Pd-C/AcOH. xiii: 10% KOH/MmeOH. xiv: *n*-$C_6H_{13}COCl$ (*n*-$C_{15}H_{31}COCl$)/Et_2O. xv: TMSCl/PhOH/$HCCl_3$.

Table 1. Effect of lipidic diamine and aminoalcohol derivatives on cytosolic PLA_2 activity[a]



Compound	R^1	R^2	$cPLA_2$%I (100 μM)[b]	IC_{50}[c] (μM)
4	$-NEt_2$	$-NH_2$	90.6 ± 5.7**[d]	11.9
5	$-N(CH_2COOEt)_2$	$-NH_2$	43.9 ± 10.9**	ND[e]
6	$-NHCO(CH_2)_3COOH$	$-NH_2$	0.0 ± 0.0	ND
7	$-NHCH_2COOEt$	-NHBoc	0.0 ± 0.0	ND
10	-OBn	-NHEt	0.0 ± 0.0	ND
11	-OBn	$-NEt_2$	1.9 ± 1.9	ND
12	-OBn	$-NHCO(CH_2)_2COOH$	72.0 ± 3.5**	62.8
13	-OBn	-NHCOCH=CHCOOH(*E*)	60.7 ± 6.1**	69.5
14	-OBn	$-NHCO(CH_2)_3COOH$	84.8 ± 9.6**	10.5
15	-OH	-NHEt	25.2 ± 3.8**	ND
16	-OH	$-NHCO(CH_2)_3COOH$	0.0 ± 0.0	ND
17	-OH	$-NHCH_2COOH$	25.0 ± 3.7**	ND
19	$-OCO(CH_2)_5CH_3$	$-NHCO(CH_2)_5CH_3$	2.3 ± 1.2	ND
20	$-OCO(CH_2)_5CH_3$	$-NHCO(CH_2)_{13}CH_3$	0.5 ± 0.5	ND
21	$-OCO(CH_2)_{13}CH_3$	$-NHCO(CH_2)_{13}CH_3$	5.1 ± 3.3	ND
PTK	$CH_3(CH_2)_{14}COCF_3$		67.7 ± 3.1**	11.7

[a]Mean ± SEM ($n = 6$).
[b]%I = % inhibitory effect of compounds at 100 μM.
[c]IC_{50} values were determined for those compounds that reached 50% of inhibition at 100 μM.
[d]** $P < 0.01$.
[e]ND = not determined.

Table 2. Effect of compounds **4** and **15** on different secretory PLA_2 activities[a]

Compound	*Naja naja* venom		Pancreas		Human synovial		Bee venom	
	%I (10 μM)[b]	IC_{50}[c]	%I (10 μM)	IC_{50}	%I (10 μM)	IC_{50}	%I (10 μM)	IC_{50}
4	51.5 ± 3.0**[e]	9.5	68.5 ± 4.9**	3.6	82.7 ± 1.6**	5.6	57.6 ± 3.9**	11.0
15	62.6 ± 3.2**	6.2	81.9 ± 5.1**	1.4	80.0 ± 1.3**	3.7	63.5 ± 3.6**	6.1
Manoalide	17.0 ± 1.7*[d]	ND[f]	32.3 ± 2.7**	ND	93.2 ± 0.2**	3.9	62.5 ± 3.8**	7.5

[a]Mean ± SEM ($n = 6$).
[b]%I = % inhibitory effect of compounds at 10 μM.
[c]IC_{50} values (μM) were determined for those compounds that reached 50% of inhibition at 10 μM.
[d]* $P < 0.05$.
[e]** $P < 0.01$.
[f]ND = not determined.

disorders. Unfortunately, no potent and absolutely type-specific PLA_2 inhibitors are widely available to investigators. Non-specific covalent-modifying PLA_2 drugs such as manoalide[7] have received much attention as $sPLA_2$ inhibitors. On the other hand, owing to the central role of $cPLA_2$ in arachidonic acid signalling, design of $cPLA_2$ inhibitors has recently been an area of great interest. In this group, palmitoyl trifluoromethyl ketone (PTK) is the most well known $cPLA_2$ inhibitor.[15] Some long-chain 3-amino-1,2-diols have been reported to exert anti-inflammatory properties[16] but they have not been evaluated against different PLA_2s. Herein we report some structure–activity relationship considerations that can be deduced from the in vitro pharmacological study of these new diamine and aminoalcohol derivatives (Tables 1 and 2). Several compounds of these series displayed potent inhibitory activities and, in some cases, even higher than those of reference compounds PTK (for $cPLA_2$) and manoalide (for $sPLA_2$). In addition, none of them presents any sign of cytotoxicity at 10 μM (data not shown).

Looking at data in Table 1, particular stucture–activity relationships observations can be made: (a) both series have representative compounds able to inhibit extensively $cPLA_2$ at the 100 μM level, the diamine **4** and the benzylated amidoacid **14** being the most potent for each series; (b) compounds in which both diamino or aminoalcohol groups were acylated/alkylated were inactive (**7**) or practically inactive (**19**, **20**, **21**), probably due to their excessive lipophilicity or low solubility; (c) compounds having both extreme functions free of any alkyl or acyl substituent and separated by eight bonds (**6** and **16**) were inactive, while if the distance was only five bonds (**17**), a 25% inhibition was observed, thus indicating a fair structure–activity dependence; (d) compounds having only one of the extreme functions blocked by alkylation or acylation are good inhibitors,

independently of the position, 1 or 2, of the blocked function in the long chain, thus informing about the minor relevance of the location of the long chain in the molecule. A relevant comparison illustrating the above statements involves the closely related compounds **14** and **16**, for which the presence or absence of the benzyl moiety is definitive for the activity. This behaviour seems parallel to that of compounds **4** or **5** in relation with the aminoacid **6** within the diamine family. (e) Simple diethylation of the amino group at C-1 (**4**), seems to be the most beneficial transformation of diamines for activity, because the introduction of larger substituents (*bis*-ethoxycarbonylmethyl, glycinyl ester residue, **5**) at this part of the molecule decreases the potency substantially.

On the other hand, the presence of a larger acyl side chain having an ionisable free carboxylic group at its end (**6**), provoked the complete disappearance of the inhibitory activity. (f) Relating to the most potent group of aminoalcohol derivatives (R^1 = -OBn), it seems clear that there exists an optimum size, of five or more carbon atoms length, for the acyl chain and that the introduction of a *trans* double bond decreases the inhibitory potency.

Evaluation of the diamine **4** and the aminoalcohol **15** in their ability to inhibit four different classes of secretory PLA_2 (Table 2) showed that the former compound displayed slightly better IC_{50} values than that found for $cPLA_2$ but did not show any appreciable selectivity. In contrast, in the case of compound **15**, the inhibitory potency for $sPLA_2$ was much stronger (IC_{50} = 1.4–6.2 μM) than for $cPLA_2$ (≅ 200 μM), thus revealing a fair selectivity, ranging between 30 to 140 times, for the preferential inhibition of the $sPLA_2$s.

Evaluations will be extended to other members of the series for defining more precisely the new series of compounds to be prepared and tested for optimising the inhibitory response. Furthermore, taking into account that all the compounds tested were racemic mixtures, stronger and/or more selective inhibitions can be expected for one of the enantiomers of each pair, and additional work in this sense will be done. In conclusion, we have developed a novel series of lipidic diamine and aminoalcohol derivatives with a PLA_2 inhibitor profile, showing potency and selectivity towards cytosolic and secretory PLA_2. Based on these results, two compounds have been selected for extensive preclinical evaluation.

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