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Small Ligands Interacting with the Phosphotyrosine Binding Pocket of the Src SH_2 Protein

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Abstract—Various small fragments bearing phosphate, phosphonate or phosphonic acid moieties have been prepared through parallel synthesis and their binding potencies evaluated on the Src SH_2 protein using a BIAcore assay. This provided us insight into the requirement of the Src SH_2 pTyr binding pocket and some promising small ligands have been characterised.

Many of the key signaling pathways are mediated by phosphorylation/dephosphorylation of tyrosine residues found in intracellular proteins.[1] Thus tyrosine kinases can bind to phosphotyrosine sequences via their Src homology 2 (SH_2) domain[2] thus affording molecular association and translocation. Tyrosine kinases are involved in many pathologies such as cancer, inflammation or osteroporosis and are consequently important targets for the pharmaceutical industry.[3]

In our research program on Src SH_2 inhibitors, we were looking for peptidomimetics of the pYEEI tetrapeptide. Many X-ray co-structures of pYEEI or other ligands within SH_2 domains have been reported,[4–6] indicating that there are two major binding pockets, one interacting with the phosphotyrosine itself, and the other with the hydrophobic iLeu residue.

Our strategy was to use a modular approach and to try to optimise each binding site independently, and we would like to disclose in this letter some of our results on the search of pTyr surrogates. A similar modular strategy with the evaluation of small organic molecules has also been used for the search of new inhibitors of protein tyrosine phosphatase CD 45[7] and PTP1B.[8] Very recently, Fesik et al. also used a small fragment approach with a NMR based screen for the discovery of novel phosphotyrosine mimetics that bind to the Lck SH_2 domain. [9]

The pTyr binding pocket is a positively charged pocket interacting with the phenyl phosphate pTyr residue and is formed in part by Arg 12, Arg 32 and Lys 60. Thus strong ionic interactions between the doubly negatively charged phosphate and the two Arg are observed. Additional polar interactions also exists with the phosphate and Ser 34, Glu 35 and Thr 36. Moreover, the hydrophobic alkyl side chain of Lys 60 interacts with the phenyl ring. Thus, it appears that the phenyl phosphate group is a tight binder of this pocket. However, to overcome the problems associated with the phosphate moiety of pTyr, many groups have searched phosphate surrogates, resulting in the identification of phosphonate, difluorophosphonate or carboxyl based mimetics amongst others.[10]

Our purpose in this study was to focus on the Src SH_2 protein and to find out whether it could be possible to improve the binding affinity of phenyl phosphate by modifying the phenyl ring (with substitution on the phenyl ring or by its replacement with another aromatic ring) and/or by replacing the phosphate with a non hydrolysable group that retained potent Src SH_2 binding affinity.

Chemistry

Phosphate compounds have been prepared using parallel synthesis, either by solid-phase or solution-phase chemistry. In both cases, the desired phosphate is obtained in two steps from the corresponding alcohol

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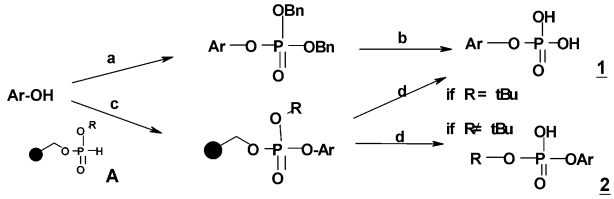
using a phosphite (bound on the resin or not) as the phosphorylating agent.

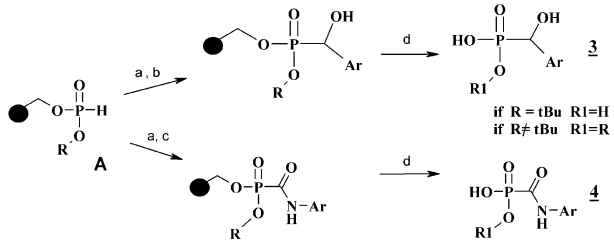
For solution-phase chemistry, we adapted the phosphorylation procedure described by Silvenberg et al.[11] to a parallel synthesis approach (Scheme 1). Thus, in our procedure, 0.95 equiv of alcohol was reacted with 1 equiv of dibenzyl phosphite in the presence of CCl_4, and the resulting dibenzyl phosphate was purified by liquid liquid extraction, followed by SPE on a silica cartridge. Debenzylation was performed in parallel either by hydrogenation over Pd/C or TFA/CH_2Cl_2 treatment depending of the compound, which led to the desired pure free phosphate **1** after an aqueous extraction and lyophilisation with an overall yield of 50–60%.

For the preparation of phosphates **1** and **2**, we have also developed[12] a solid-phase version of this synthesis using the phosphite bound on the Wang resin **A**[13] (Scheme 1). This methodology has the advantage over the solution-phase version of allowing the quick synthesis of a larger number of compounds, the final phosphates being purified with SPE on reverse-phase cartridges. Using this pathway with various R groups (different to ^{t}Bu), phosphate monoesters **2** can also be prepared easily.

α-Hydroxy phosphonates **3** and amido phosphonates **4** have also been prepared by solid phase from the same starting bound phosphite following a methodology published by Mjalli et al.[12] Activation of the phosphite



Scheme 1. Solid-phase and solution-phase phosphate syntheses: (a) ArOH (0.95 equiv), dibenzylphosphite $HPO(OBn)_2$ (1 equiv), DIEA (2 equiv), CCl_4 (5 equiv); DMAP (0.1 equiv) in CH_3CN, −10 °C, 2 h, then KH_2PO_4 treatment and AcOEt extraction followed by SPE on silica (70 cyclohexane/30 AcOEt); (b) H_2, Pd/C cat in MeOH, 2 h or TFA/CH_2Cl_2 1/1 overnight, then aqueous extraction (H_2O/toluene); (c) 1 M solution of ArOH (5 equiv), DIEA (6.5 equiv), CCl_4 (5 equiv); DMAP (0.5 equiv) in CH_3CN, 23 °C, 1 h, then filtration and washing; (d) 15% TFA/CH_2Cl_2 30 min.



Scheme 2. Solid-phase synthesis of phosphonates and phosphonic acids: (a) 1 M solution of DBU in CH_3CN, 10 min; (b) 1 M solution of the aldehyde (5 equiv) in CH_3CN, 30 min; (c) 1 M solution of the isocyanate (5 equiv) in CH_3CN, 30 min; (d) 15% TFA/CH_2Cl_2 30 min.

with DBU generated the anion which was then trapped by an aromatic aldehyde (Scheme 2). Similarly, we describe here the preparation of amido phosphonic acids using an aromatic isocyanate as the electrophilic agent. Here again, depending on the nature of R group (^{t}Bu does not withstand TFA treatment), we also can have access to amido phosphonatate mono esters or amido phosphonic acids **4**.

Results and Discussion

In the search for phenyl phosphate surrogates, around 120 compounds have been prepared and can be classified as phosphates **1**, α-hydroxy phosphonic acids **3** and α-amido phosphonic acids **C**. The corresponding monoesters of these three classes have also been synthesised and a selection of representative compounds are described in Tables 1 and 2 (phosphate and phosphonate).

Our methodology to evaluate binding affinity of these small ligands was based on BIAcore technology.[14] In this assay,[15] the compound to be evaluated is in the presence of the Src SH_2 protein and has to compete for binding with the pYEEI tetrapeptide bound on the sensor chip. This assay is sensitive enough to detect millimolar binding affinity and, as a reference, the IC_{50} of phenyl phosphate **1a** has been measured at 2.5 mM.

Substitution in the *ortho*, *meta* or *para* position of the phenyl ring (**1b–1q**, Table 1) in general gives compounds with lower binding affinity to the Src SH_2 protein. Exceptions have been observed with the introduction

Table 1. Biacore Src SH_2 binding affinity of aryl phosphate fragments

Compd	Ar–OH	Ar–X, X =	IC_{50} (mM)
1a	PhOH	OPO_3H_2	2.5
1b	3-COOMe PhOH	OPO_3H_2	4
1c	3-CHO PhOH	OPO_3H_2	2.4
1d	3-Me PhOH	OPO_3H_2	4
1e	4-Me PhOH	OPO_3H_2	2
1f	4-*t*Bu PhOH	OPO_3H_2	4
1g	4-OMe PhOH	OPO_3H_2	4
1h	4-COOMe PhOH	OPO_3H_2	4
1i	4-NH_2 PhOH	OPO_3H_2	0.6
1j	4-NO_2 PhOH	OPO_3H_2	1
1k	4-SCF_3 PhOH	OPO_3H_2	1.2
1l	2-Cl PhOH	OPO_3H_2	3.5
1m	2-COOH PhOH	OPO_3H_2	2
1n	2-CHO PhOH	OPO_3H_2	0.2
2a	2-CHO PhOH	OPO_3H(Me)	3.3
2b	2-CHO PhOH	$OPO_3H(CH_2$ c-hexyl)	2.3
2c	2-CHO PhOH	OPO3H(iPr)	2.1
1o	2-Me 6-Me PhOH	OPO3H2	>10
1p	2,6 CHO PhOH	OPO3H2	0.5
1q	2-CHO 6-OMe PhOH	OPO3H2	0.9
1r	1-Naphthol	OPO3H2	1
1s	2-Naphthol	OPO3H2	1
1t	2-$COCH_3$ 1-Naphthol	OPO3H2	3.5
1u	3-$COOMe_2$-Naphthol	OPO3H2	3.2
1v	8-OH Quinoline	OPO3H2	>10
1w	2-CN, 8-OH Quinoline	OPO3H2	>10
1x	1,2,3,4-Tetrahydro 8-OH quinoline	OPO3H2	0.3

Table 2. Biacore Src SH_2 binding affinity of aryl phosphonate fragments

Compd	Ar	Ar–X, X =	IC_{50} (mM)
3a	Ph	$CH(OH)PO_3H_2$	> 10
3b	2-OMe Ph	$CH(OH)PO_3H_2$	> 10
3c	4*i*Pr-Ph	$CH(OH)PO_3H_2$	> 10
3d	4PhPh	$CH(OH)PO_3H_2$	> 10
3e	Cyclohexyl	$CH(OH)PO_3H_2$	> 10
3f	1-Naphthyl	$CH(OH)PO_3H_2$	> 5
3g	2-Naphthyl	$CH(OH)PO_3H_2$	4.4
3h	4-OPr Ph	$CH(OH)PO_3H_2$	2.4
4a	4-Et Ph	$NHCOPO_3H_2$	4.4
4b	EtPh	$NHCOPO_3H_2$	> 5
4c	4-Et Ph	$NHCOPO_3H(Bn)$	> > 5
4d	CH_2Ph	$NHCOPO_3H_2$	4.4
4e	4-COOEt Ph	$NHCOPO_3H_2$	3.6
4f	4-OBu Ph	$NHCOPO_3H_2$	3.9
4g	2-OCF_3 Ph	$NHCOPO_3H_2$	4.0
4h	3,4,5-OMe Ph	$NHCOPO_3H_2$	2.1
4i	4-OBn Ph	$NHCOPO_3H_2$	0.8

of the NH_2, NO_2 and SCF_3 groups (**1i, 1j** and **1k**) in the *para* position which give a 2–3-fold increase in binding affinity, but without clear explanation.

More significant was the 10-fold improvement with an *ortho* aldehyde substitution (**1n**, 0.2 mM). This latter result was not a surprise to us and confirmed the 15-fold increase in potency already observed by Charifson et al.[6] with the Src SH_2 protein when they introduced an *ortho* aldehyde on their peptidic inhibitors. This improvement is explained by the presence of the cysteine 42 in the pTyr binding site which is trapped by the aldehyde group to form a covalent and reversible bond. This is clearly visible on the X-ray co-structure of this fragment within the Src SH2 protein.[19] Confirmation of the potency of the known *ortho* aldehyde fragment with this small fragment approach was important because it validated this methodology.

Conversely, introduction of the aldehyde at the *meta* position does not influence the binding (**1c**, 2.3 mM) indicating that the aldehyde is poorly placed in that case to interact with the cysteine. It was of interest to reduce the overall charge of the compound with the expectation of improvement of cellular uptake of the inhibitor. Potency of the *ortho* aldehyde phosphate monoesters **2a**–**c** (one negative charge) is similar to that of the phenyl phosphate **1a** (two negative charges), indicating that the aldehyde interaction is strong enough to compensate the loss of one charge of the phosphate. However, it is also clear that the phosphate monoesters **2a**–**c** are 10 times less potent that the corresponding free phosphate **1n**. This result is consistent with the fact that the two negative charges of the phosphate are useful for potent binding. With the synthesis of phosphate esters, we were wondering whether the loss in ionic binding could be counterbalanced by some additional interactions with the ester side chain. It appears not to be the case whatever the ester group introduced.[16] This suggests that the ester group on the phosphate does not interact with the pTyr pocket and probably lies outside the protein.

Replacement of the phenyl group by other aromatic rings (especially bicyclic rings) has also been studied (**1r**–**x**). As shown in Table 1, the naphthyl phosphates **1r** and **1s** are twice as potent as the phosphate **1a**. The naphthyl ring has already been identified as a good phenyl replacement for phosphatases and SH_2 domains.[17] More interestingly, tetrahydroquinoline **1x** appeared to be the most potent aromatic scaffold (10-fold increase in binding) despite the lack of activity of the corresponding quinoline **1v** (or **1w**).

Knowing that some hydroxy phosphonic acids have been found as inhibitors of tyrosine phosphatase CD45,[7] we were wondering whether the additional hydroxy group could serve as hydrogen bonding mediator within the Src SH_2 protein. Thus, hydroxy phosphonic acids **3** have been prepared and evaluated against Src SH_2 (Table 2). Generally speaking, these compounds **3**, if not inactive, are less active that the corresponding phosphates **1** as indicated by at least a 5-fold decrease in binding affinity between **1a** and **3a** (or **1r** and **3f**, **1s** and **3g**). This is in line with some recent results[18] on Src SH_2 indicating that the hydroxyphosphonomethyl cannot be considered as a very potent phosphate surrogate. In this series, all the corresponding monoesters prepared are inactive (data not shown).

Finally, amido phosphonic acids **4** have been evaluated (Table 2). Interestingly, many of them have similar activity to phenyl phosphate **1a**. Some substituted phenyl hydroxyphosphonates displayed promising affinity, the best compound in the series being the 4-OBn substituted analogue **4i**. To our knowledge, this is the first time that amido phosphonic acids have been identified as good surrogates of phosphate and have the advantage of not being so easily hydrolyzable.

In conclusion, different series of aryl phosphates and aryl phosphonic acids (and their corresponding mono esters) have been prepared and evaluated as potential phosphotyrosine surrogates on the Src SH_2 protein. This study has given us an insight into the ability of some fragments to be good replacements of the phenyl phosphate group present in pTyr of the pYEEI tetrapeptide. We confirmed the importance of the aldehyde in the *ortho* position of the phenyl ring for the Src SH_2, thus validating the small fragment methodology, and we identified the naphthyl and tetrahydroquinoline derivatives as possible phenyl replacements. Amido phosphonate is also a potential candidate for phosphate mimic.

To confirm how these selected fragments interacted with the pTyr binding pocket and whether they adopt a similar positioning to that for the phenyl phosphate of the pYEEI tetrapeptide, some X-ray studies have thus been undertaken. Some co-structures of fragments within the Src SH2 protein have been obtained, confirming their interaction with the pTyr binding pocket and giving essential information on the positioning of these fragments. These data, as well as the incorporation of the most promising surrogates into full Src SH2 ligand, will be reported shortly.[19]

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15. The Src-specific phosphopeptide was immobilized to a biosensor chip CM5 through injection of a 0.5 mM solution of the phosphopeptide in 0.5 M NaHCO3, across the chip surface previously activated according to the instructions of the manufacturer. Injection of anti-phosphotyrosine antibody was used to confirm that successful immobilization of the peptide was achieved. For the competition binding assay 0.5 μM of the Src SH_2 protein was premixed in HBS (10 mM Hepes/150 mM NaCl/0.05% surfactant P20, pH 7.4) at room temperature with increasing concentrations (up to 5 mM) of competing molecules and injected at a flow rate of 30 μL/mn for 4 mn across a surface to which the phosphopeptide was immobilized. Prior to each run the phosphopeptide surface of the chip was regenerated using 2 M guanidium HCl. The amount of bound Src SH_2 domain was estimated from the surface plasmon resonance signal at a fixed time just before the end of the injection and the percentage bound, relative to injection of Src SH_2 alone, calculated.
16. All phenyl phosphate monoesters prepared (>10 ester groups introduced) are inactive ($>>10$ mM in the BIAcore assay).
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