Examination of Phosphoryl-Mimicking Functionalities within a Macrocyclic Grb2 SH2 Domain-Binding Platform

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Abstract: Reported herein are the design, synthesis, and Grb2 SH2 domain-binding affinities of several phosphoryl-mimicking groups displayed within the context of a conformationally constrained macrocyclic platform. With use of surface plasmon resonance techniques, single-digit nanomolar affinities were exhibited by phosphonic acid and malonyl-containing diacidic phosphoryl mimetics (for **4h** and **4g**, K_D = 1.47 and 3.62 nM, respectively). Analogues containing monoacidic phosphoryl mimetics provided affinities of K_D = 16−67 nM. Neutral phosphoryl-mimicking groups did not show appreciable binding.

SH2 domain-binding antagonists have been developed by a number of groups as potential alternatives to kinase inhibitors.[1−4] Despite their central roles in SH2 domain recognition processes, phosphotyrosyl residues (pTyr, **1**, Figure 1) are contraindicated as components of such inhibitors because of their hydrolytic lability and the poor cellular bioavailability of the doubly ionized phosphoryl moiety. Replacement of the phosphoryl ester oxygen with either $-CH_2-$ or $-CF_2-$ has yielded hydrolytically stable analogues such as Pmp (**2**) and F_2Pmp (**3**), respectively. However the anionic charge of these phosphonate-based structures still presents problematic issues of bioavailability. Therefore, phosphoryl mimetics continue to be sought that retain high binding affinity, yet exhibit reduced net anionic charge.[5,6]

To date, monoacidic phosphoryl mimetics have tended to exhibit less affinity than diacidic mimetics.[7,8] This can be attributed in part to the presence of two highly conserved positively charged Arg residues within the SH2 domain pTyr binding pocket.[9] If one role served by pTyr binding is to promote capture of conformationally flexible peptides from solution and thereby allow secondary ordering of the peptide along the protein surface, the importance of interactions within the pTyr-binding pocket may be diminished for ligands whose solution conformations are preordered for binding. This could be reflected in a reduced requirement for diacidic phosphoryl-mimicking charge for certain conformationally constrained ligands.

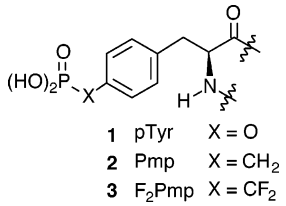

Figure 1. Structures of pTyr and pTyr mimeitcs.

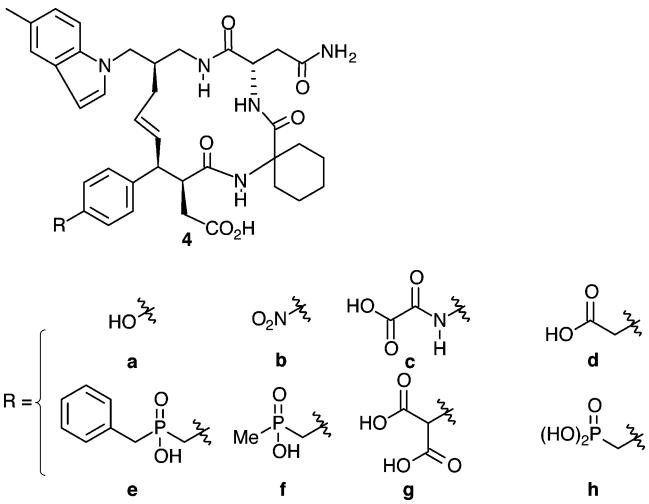

Figure 2. Structures of macrocyclic Grb2 SH2 domain-binding antagonists.

Recently, a series of macrocyclic peptide mimetics typified by **4h** have been reported that bind to Grb2 SH2 domains with extremely rapid on-rates (Figure 2).[10,11] The high k_{on} values may indicate that solution conformations are preordered for binding. Therefore, macrocycles such as **4** may provide qualitatively different platforms for investigating phosphoryl mimicking functionality than afforded by conventional nonconstrained peptides. Since the 5-methylindolyl-containing **4** has only been reported bearing a diacidic phosphonic acid-based phosphoryl mimetic (**4h**), the current study was undertaken to use this platform to examine additional diacidic (**4g**), monoacidic (**4c**−**f**), and neutral (**4a**,**b**) phosphoryl mimetics.

All analogues were prepared as shown in Scheme 1. A characteristic feature of macrocycle construction was the use of protected β-vinyl-containing pTyr mimetics **10**,[12] which was coupled to the 5-methylindolyl-containing dipeptide **11**[11] to yield metathesis precursors **12**. The synthesis of **10g** has been reported previously.[13] Ring closure of **12** using second-generation Grubbs' catalyst [((PCy$_3$)(Im(Mes)$_2$)Ru=CHPh) (**11**))][14,15] provided the protected macrocycles **14**, which were converted to the final products **4** by treatment with acid. In the case of *N*-oxalyl-containing **4c**, the nitro-containing precursor **14b** was first reduced to the corresponding amine using freshly prepared Al−Hg amalgam, then reacted directly with *tert*-butyl oxalyl chloride/^{i}Pr$_2$NEt to give the protected amide **15c**, which was converted to **4c** by treatment with TFA (Scheme 2). The carboxymethyl ana-

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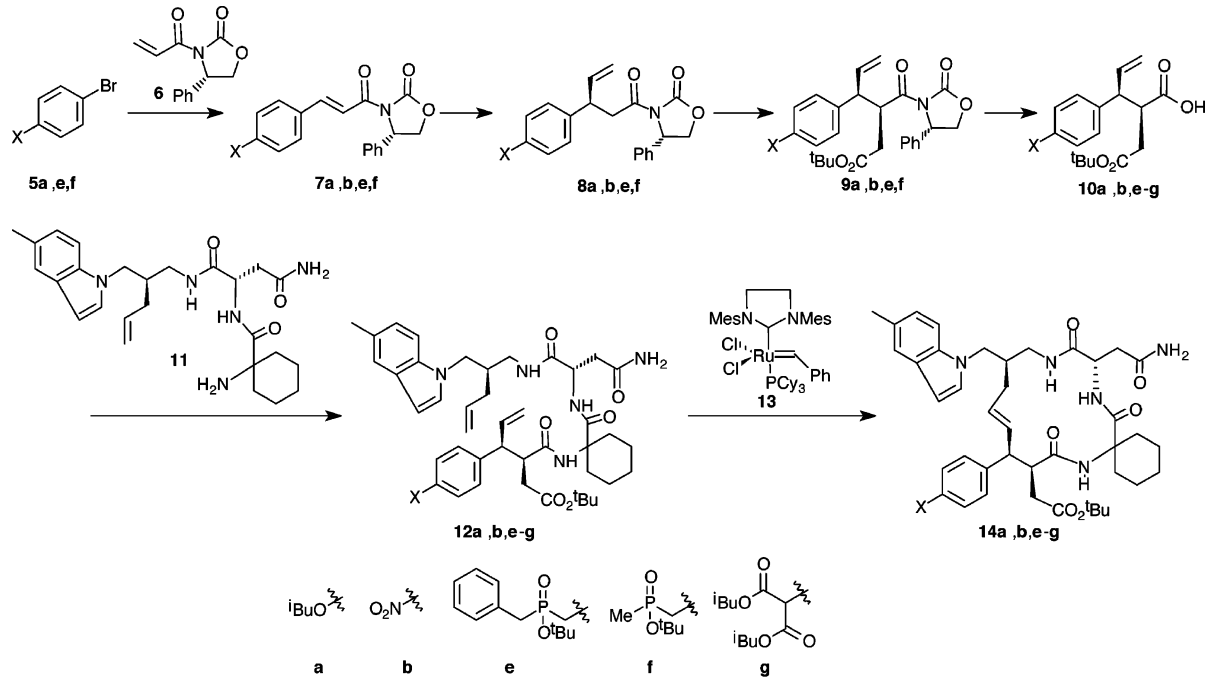
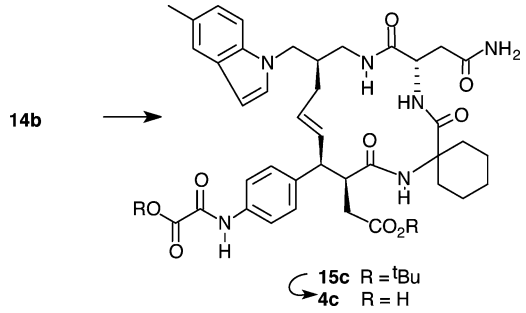
Scheme 1



Scheme 2



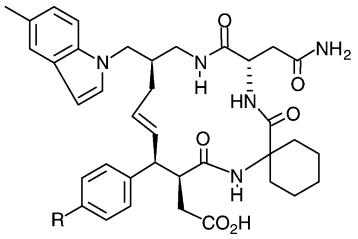
logue **4d** was obtained by lithium hydroxide mediated decarboxylation of **4g**.

Preparation of the key β-vinyl-containing pTyr mimetics **10** proceeded from the α,β-unsaturated oxazolidinone-containing **7**[16] through **8** by initial 1,4-addition of vinylmagnesium bromide[17] followed by conversion to **9** using stereoselective alkylation with α-bromo *tert*-butyl acetate.[12] With the exception of the nitro-containing **7b**, construction of intermediates **7** was by Heck reaction of 4-substituted phenylbromides (**5**) with known (S)-*N*-acroyl-4-phenyl-2-oxazolidinone (**6**).[17] The corresponding Heck reaction of 4-bromonitrobenzene failed. However the desired nitro-containing **7b** can be obtained by condensation of commercially available 4-nitrocinnamic acid with the Evans auxiliary (*S*)-4-phenyl-2-oxazolidinone.

Starting aryl bromide **5a**[18] was obtained by reaction of 4-bromophenol with *tert*-butyl 2,2,2-trichloroacetimidate/$BF_3{\cdot}Et_2O$. For the synthesis of starting *tert*-butyl phosphinate **5e**, ammonium phosphinate was first prepared by careful addition of 50% aqueous hypophosphorus acid to an equal-molar quantity of aqueous ammonia at 0 °C. This was stirred for 1 h at room temperature and then lyophilized to provide a hygroscopic white solid, which was dried over P_2O_5 under high vacuum. The freshly prepared ammonium phosphinate was converted to bis(trimethylsilyl)phosphonite by heating under argon with hexamethyl disilazane under argon. [Caution: vapors are flammable.] This was then reacted with benzyl bromide, followed by a second treatment with hexamethyl disilazane. Alkylation with 4-bromobenzyl bromide gave (4-bromobenzyl)benzylphosphinic acid, which was converted to **5e** by esterification with *tert*-butyl 2,2,2-trichloroacetimidate/$BF_3{\cdot}Et_2$.[19,20] The corresponding *tert*-butyl (4-bromobenzyl)methylphosphinate **5f** was prepared in a similar fashion by sequential treatment of bis(trimethylsilyl)phosphonite with 4-bromobenzyl bromide, then hexamethyl disilazane, then methyl iodide, and finally, *tert*-butyl 2,2,2-trichloroacetimidate/$BF_3{\cdot}Et_2$.

Evaluation of binding affinity to recombinant Grb2 SH2 domain protein was accomplished using an enzyme linked immunosorbent assay (ELISA) based competition assay[21,22] and a surface plasmon resonance (SPR) assay that provided real-time kinetic data, including k_{on} and k_{off} values (Table 1).[23,24] On the basis of SPR data, the highest affinities were exhibited by diacidic phosphoryl mimetic-containing **4g** and **4h**,[25,26] which showed enhanced binding on-rates (k_a) that were more than 5-fold greater than analogues having fewer acidic groups. The highest affinity among the monoacidic phosphoryl mimetics was shown by the methylphosphinic acid **4f**, which had twice the affinity of the benzylphosphinic acid **4e** or the carboxymethyl analogue **4d**. The oxalylamido analogue **4c** showed the poorest affinity among the monoacidic phosphoryl mimetics because of a disproportionately rapid off-rate (k_d). Compounds with uncharged phosphoryl mimetics (**4a** and **4b**) showed little affinity (up to 1000 nM). ELISA-based IC_{50} values for **4d** and **4g** were in close agreement with SPR-derived k_D values. However, **4c**,**e**,**f** gave lower IC_{50} values than would be expected on the basis of SPR data, while **4h** gave a higher IC_{50} value. The reasons for these differences are not known. However, the ELISA IC_{50} values are more dependent on experimental conditions than

Table 1. Grb2 SH2 Domain-Binding Results



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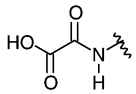
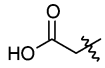
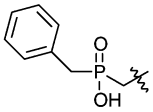
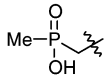
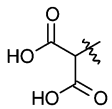
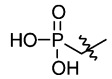
No.	R Group	k_a ($M^{-1}s^{-1}$)[a]	k_d (s^{-1})[a]	K_D +/- S.E. (nM)[a]		IC_{50} +/- S.E. (nM)[b]	
4a		—	—	**> 1000**	**(n = 2)**	—	
4b		—	—	**> 1000**	**(n = 2)**	—	
4c		8.66×10^5 +/- 6.25×10^3	5.08×10^{-2} +/- 2.14×10^{-4}	**67 +/- 0.1**	**(n = 6)**	**8.8 +/- 3.0**	**(n = 3)**
4d		3.06×10^5 +/- 1.20×10^3	1.01×10^{-2} +/- 2.25×10^{-5}	**33.0 +/- 0.1**	**(n = 4)**	**22.5 +/- 4.7**	**(n = 3)**
4e		1.45×10^5 +/- 2.45×10^2	5.12×10^{-3} +/- 8.65×10^{-6}	**35.3 +/- 0.1**	**(n = 6)**	**8.4 +/- 0.7**	**(n = 3)**
4f		6.09×10^5 +/- 1.47×10^3	9.88×10^{-3} +/- 1.11×10^{-5}	**16.2 +/- 0.01**	**(n = 4)**	**6.3 +/- 2.0**	**(n = 1)**
4g		6.27×10^6 +/- 3.09×10^4	2.27×10^{-2} +/- 3.86×10^{-5}	**3.62 +/- 0.02**	**(n = 6)**	**3.3 +/- 0.3**	**(n = 3)**
4h		5.46×10^6 +/- 3.66×10^4	8.01×10^{-3} +/- 4.40×10^{-5}	**1.47 +/- 0.01**[c]	**(n = 6)**	**15.0 +/- 8.0**	**(n = 1)**

[a] Obtained by SPR experiments as described in ref 23, where "*n*" refers to the number of surfaces fit to a global analysis of a simple Langmuir binding isotherm and where the errors in k_a and k_d reflect the error of the global fit to these paramenters. K_D and associated SE values were calculated as described in ref 24, with the SE values also being the errors of the global analysis. [b] Obtained by ELISA-based experiments as described in ref 22, wherein IC_{50} values ± the standard error of the mean (SE) were obtained as the mean of "*n*" independent experiments performed with nine doses per curve with each dose done in quadruplicate. [c] Value as reported in ref 25.

the SPR-derived K_D values, and the SPR data should be used as the more reliable indicator of binding affinity.

Significant effort has been directed toward reducing phosphoryl-mimicking charge on SH2 domain binding antagonists. For example, on the basis of a phosphate-containing $p56^{lck}$ SH2 domain-binding dipeptide mimetic ($K_D = 40$ nM), a group from Boehringer Ingelheim Pharmaceuticals obtained low micromolar K_D values using several monoacidic phosphoryl mimetics, including carboxymethyl and oxalylamido-based analogues.[8] In our own hands, open-chain analogues containing monocarboxy-based pTyr mimetics have been shown to exhibit Grb2 SH2 domain IC_{50} values in the low micromolar to submiromolar range.[7] Novartis Corporation has reported low micromolar to submicromolar affinity constants for monoacidic phosphinic acid based pTyr mimetics that include benzyl and methyl phosphinates upon which analogues **4e** and **4f** of the current study were predicated.[27] In all of these prior reports, affinities of inhibitors bearing monoacidic phosphoryl mimetics were in the submicromolar range at best. Accordingly, to our knowledge, **4f** is the most potent Grb2 SH2 domain-binding antagonist yet reported having a monoacidic phosphoryl mimetic.

In the interpretation of these results, care should be taken in equating the number of acidic groups contained in a phosphoryl mimetic with formal charge, since complete ionization may not occur at a given pH. For example, it is known that because of elevated pK_{a2} values, bis-acidic phosphonic acids are only partially ionized at neutral pH, in contrast to phosphoryl groups, which are di-ionized at pH 7.[28] Differences in ionization states between phosphoryl groups and phosphonic acids have been used to at least partially explain reduction in SH2 domain-binding affinity often observed when the pTyr phosphate group is replaced with a phosphonic acid moiety.[29] Additionally, factors other than anionic charge, such as hydrogen-bonding acceptor/donor properties, may contribute to variations in affinity exhibited by different phosphoryl mimetics.

It is of note that although large losses of affinity are observed for **4a** and **4b**, which lack any phosphoryl-mimicking acidic groups, only modest losses of affinity occurred for monoacidic phosphoryl mimetics (i.e., a

10-fold reduction for **4f** compared to **4h**). This may be attributed to the differential roles in pTyr recognition played by the ArgβB5 residue, which is situated in the rear of the binding pocket compared to the more anteriorly located ArgαA2 residue. Thermodynamic studies have shown that while the ArgβB5 residue is critical for recognition and binding of pTyr-containing peptides, the ArgαA2 residue is less important.[30] Data presented in Table 1 highlight the need for high-affinity binding of at least one anionic interaction with the ArgβB5 residue. It is of note that cyclic Grb2 SH2 domain-binding peptides have been reported that are devoid of phosphate-mimicking functionality at the Y^0 position. However, these peptides contain acidic residues at the Y-2 amino acid position that are hypothesized to bond with the ArgβB5 residue, thereby highlighting the importance of this critical interaction.[31]

In conclusion, by examination of a variety of phosphoryl mimicking functionalities within the context of a conformationally constrained Grb2 SH2 domain-binding platform, ligands have been identified that contain monoacidic phosphoryl mimetics that exhibit low nanomolar affinities. The current study may facilitate the development of therapeutically relevant Grb2 SH2 domain-binding antagonists.

Supporting Information Available: Detailed experimental procedures for the preparation of final products **4a**−**g** and results from elemental analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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