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STRUCTURE-BASED DESIGN OF A NON-PEPTIDIC ANTAGONIST OF THE SH2 DOMAIN OF GRB2

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Abstract: The structure-based design and synthesis of a completely non-peptidic, micromolar antagonist of the SH2 domain of Grb2 is presented. The compound mimics the two main pharmacophores of the natural ligand, the phenylphosphate of the phosphotyrosine residue and the β-carboxamide of the X_{+2} asparagine, which are linked by a rigid aromatic spacer.

Introduction: Tyrosine phosphorylation of intracellular proteins is an early and reversible step in the signal transduction pathways, which occurs after the stimulation of cells by a variety of external factors. Subsequent, specific binding of Src homology 2 (SH2) domain-containing proteins to these newly formed tyrosine phosphorylated sequences couples receptor activation to downstream effector molecules and leads eventually to multiple cellular responses[1-3]. The growth factor receptor bound protein 2 (Grb2) is one of more than 50 mammalian proteins carrying an SH2 domain. Our interest for Grb2 comes from the fact that it links activated receptors to the mitogenically important Ras pathway. Compounds that prevent this link by inhibiting the binding of the SH2 domain of Grb2 to upstream phosphoproteins are expected to be useful for the treatment of hyperproliferative diseases like cancer.

The design of low molecular weight compounds that mimic or inhibit the action of naturally occurring peptidic mediators or which block protein-protein interactions is a continuing challenge. Peptides often lack the appropriate physico chemical properties and metabolic stability to be useful as therapeutic agents. This has led to the well-established concept of peptidomimetics. Many strategies have been utilized which vary from a stepwise de-peptidization approach to the structure-based *de novo* design of completely non-peptidic molecules. Here we report an example of the latter, which has been used to generate a Grb2-SH2 antagonist.

Design/Modeling: The recent determination in our group of the X-ray crystal structure of the Grb2-SH2 domain in complex with a phosphopeptide sequence belonging to one of its endogenous ligands (Lys-Pro-Phe-pTyr-Val-Asn-Val-NH2) has revealed the structural basis of ligand recognition by this SH2 domain, offering opportunities for structure-based design[4]. Key elements for recognition are the phosphotyrosine and

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asparagine residues of the ligand whose side chains are seen to make multiple electrostatic and hydrogen bond interactions with the protein in the X-ray structure. The carboxamide group of the side chain of the asparagine residue makes three specific hydrogen bonds with the peptide backbone of the protein while, as observed in other SH2 domain structures, the phenyl phosphate moiety of the tyrosyl residue is involved in salt bridges and in an amino-aromatic interaction with arginines (Figure 1). The importance of these two amino acids also demonstrated by peptide studies[5] led us to envisage a minimal pharmacophore strategy where we would seek to mimic the phenyl phosphate and asparagine side chain carboxamide moieties of the natural ligand with small non peptidic molecules.

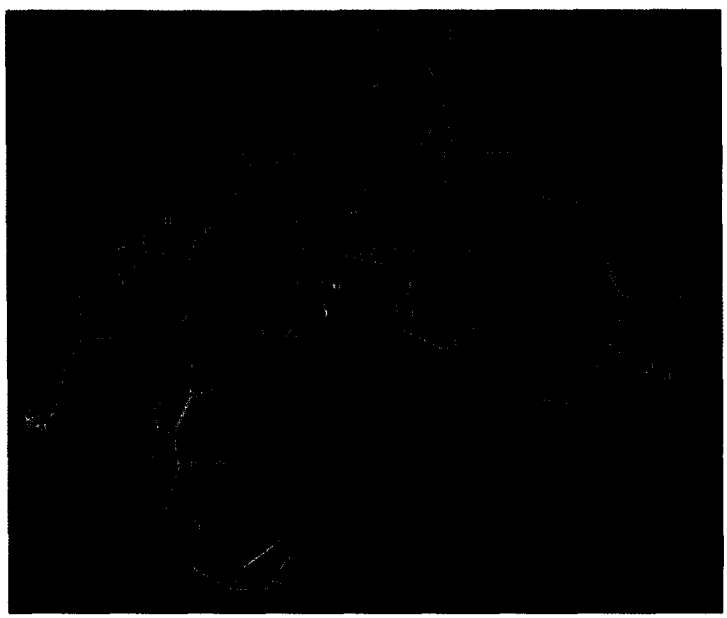
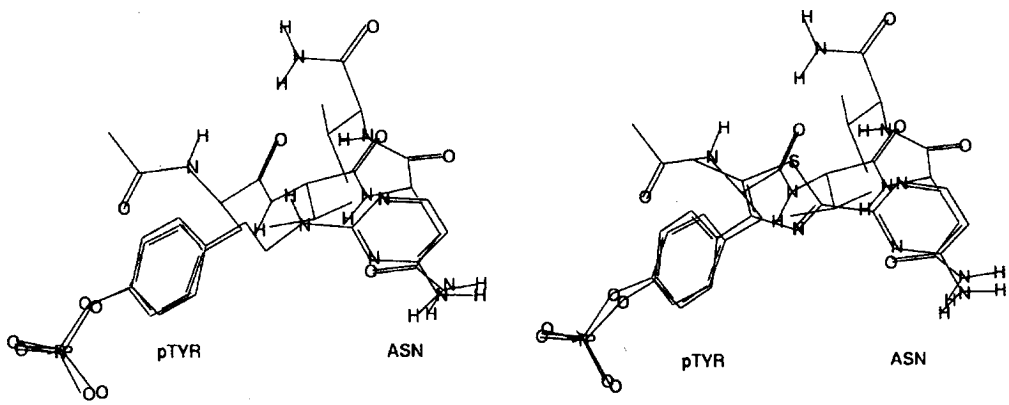


Figure 1: Main binding interactions between the Grb2-SH2 domain and its phosphopeptide ligand in the X-ray structure (residues of the ligand N-terminal to the phosphotyrosine are omitted for clarity). The three intermolecular hydrogen bonds made by the side chain carboxamide group of the ligand asparagine are represented in red while the intramolecular hydrogen bond appears in blue.

Besides making the three intermolecular hydrogen bonds above mentioned, the asparagine side chain carboxamide group is involved in an intramolecular hydrogen bond with the backbone NH of the residue resulting in the formation of a pseudo six-membered ring. This suggested that a six-membered ring moiety bearing appropriate hydrogen bonding functionalities would be a suitable mimic of this pharmacophore element. Design effort[6] along this line led to the conclusion that a 4-aminopyrimidine moiety could adequately serve this purpose without introducing unfavorable steric interactions with the SH2 domain. Further modeling work established that a spacer with three atoms was required to link the synthetically accessible 2-position of the aminopyrimidine ring to the 4-position of the phenyl ring of the aryl phosphate pharmacophore. The 4-position was the position of this ring most proximal to the aminopyrimidine moiety. In addition, not being in contact with the SH2 domain, it was sterically accessible.

Two molecules, **1** and **2**, were designed following this concept (Figure 2). In **1**, the spacer is an amino ethyl chain that must adopt an extended conformation to obtain a good superposition of the molecule with the two pharmacophore elements of the phosphopeptide as can be seen in Figure 2. Concerned by the possibility that **1** would be conformationally too flexible and its binding entropically unfavorable, we introduced in **2** an aromatic ring spacer giving rigidity to the molecule by conjugation. The 5-methyl substituent of the thiazole spacer was designed to increase the value of the twist angle between the phenyl phosphate moiety and the 5-membered ring. This is not large enough to ensure a good geometric fit of the mimetic to the pharmacophore elements in the absence of an α substituent on at least one side of the biaromatic system[7]. In addition, the thiazole spacer was designed such that its nitrogen atom points towards the protein while the larger and less electronegative sulfur atom is oriented towards the solvent because of space restriction and the requirement for negative polarity in the region of the SH2 domain proximal to this part of the ligand.



1 **2**

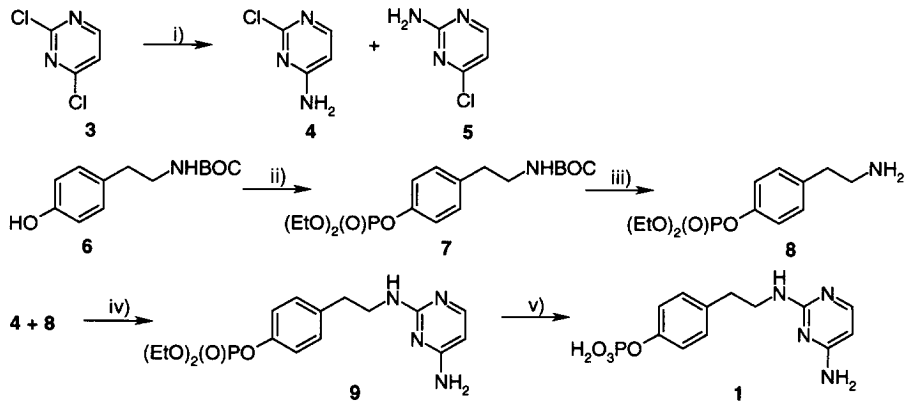
Figure 2: Designed peptidomimetics **1** and **2** (blue) superimposed on the X-ray structure phosphopeptide ligand (red). The models for **1** and **2** come from fully minimized ligand-protein complexes.

Chemistry: The synthesis of compound **1** (Scheme 1) starts with the phosphorylation of BOC-protected tyramine using triethylphosphite/iodine[8]. Removal of the BOC group using hydrogen chloride in dioxane gives the diethylphosphate of tyramine (**8**) as the hydrochloride salt. The subsequent reaction of **8** with 4-amino-2-chloropyrimidine **4** requires prolonged heating in isopropanol and gives the desired diamino pyrimidine **9** only in low yield. Cleavage of the diethylphosphate esters is accomplished with trimethylsilyl iodide[9]. The final product **1** was purified by absorption on an ion exchange resin (DOWEX-1®, OH^- form) and elution with diluted hydrochloric acid.

The synthesis of the constrained, tricyclic analog **2** involved as key step the construction of the thiazole ring in **15** from thioamide **11** and α-bromoketone **14** (Scheme 2). Building block **11** is obtained from **4** by reaction with sodium cyanide in DMF followed by conversion of the nitrile to the thioamide under standard

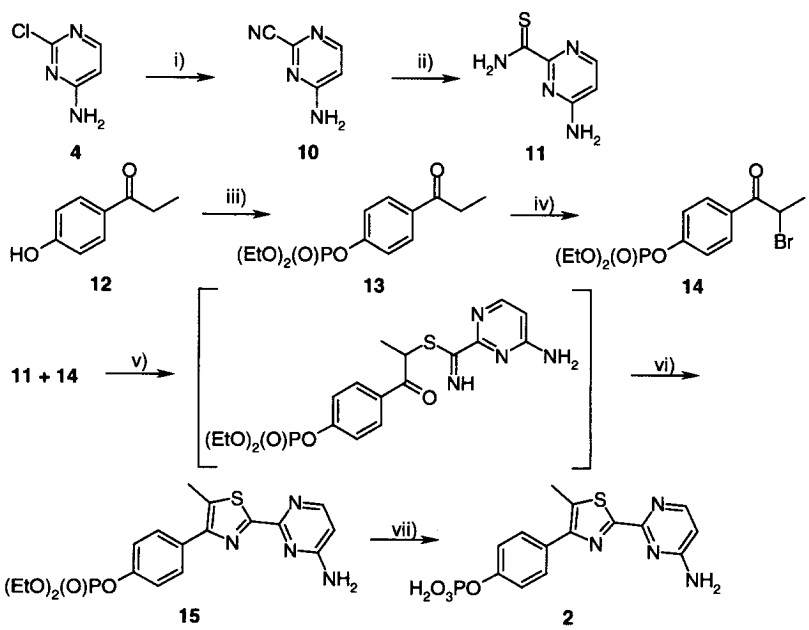
conditions[10]. The α-bromoketone **14** is synthesized by phosphorylation of 4-hydroxy-propiophenone followed by bromination. Cleavage of the phosphate esters in **15** was accomplished with trimethylsilyl iodide[9] as described for compound **1**.

Scheme 1: Synthesis of compound **1**



i) conc. NH_3, EtOH, RT, separation of 4 (23%) and 5 (10%) by sublimation; ii) $P(OEt)_3/I_2$, CH_2Cl_2, Pyridine, 0°-RT (59%); iii) 4 n HCl/dioxane, 0°-RT (100%); iv) Isopropanol, reflux, 24 hrs. (15%); v) TMSI, CH_2Cl_2, 0°-RT (27%).

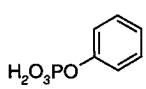
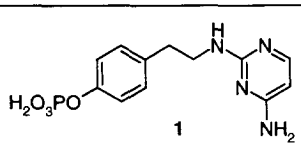
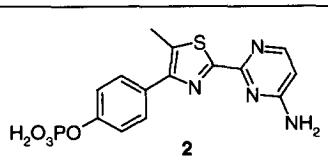
Scheme 2: Synthesis of compound **2**



i) 2 equiv. NaCN, DMF, 110°C, 65 hrs. (27%); ii) H_2S, Pyridine, Et_3N, RT, 6 hrs. (94%); iii) $P(OEt)_3/I_2$, CH_2Cl_2, Pyridine, 0°-RT (79%); iv) Br_2, CCl_4, reflux, 5 min. (48%); v) $NaHCO_3$, acetone, reflux, 19 hrs.; vi) acetic acid, reflux, 1.5 hrs. (v + vi 50%); vii) TMSI, CH_2Cl_2, 0°-RT (62%).

Results and discussion: The binding affinity for the Grb2-SH2 domain was assessed by an ELISA-type assay that measures the ability of a compound to inhibit the binding of the phosphorylated C-terminal intracellular domain of the epidermal growth factor receptor (EGFR) to this SH2 domain[11]. IC_{50} values of compounds **1** and **2** in this assay are reported in Table 1. Also included in the table are the IC_{50} values of two reference peptides (**16** and **17**) corresponding to the minimal phosphopeptide sequence retaining micromolar affinity for the Grb2-SH2 domain[12]. As can be seen, compound **1** has no detectable activity at a concentration as high as 200 μM. However, remarkably, with an IC_{50} value of 26 μM, the conformationally restricted compound **2** shows an activity in the same range as those of the reference phosphopeptides. The high rigidity of **2** conferring an entropic advantage very likely contributes to the substantial affinity of the compound for the Grb2-SH2 domain. This view is supported by the fact that the reference peptides, besides presenting the two essential pharmacophore moieties as does **2**, can form additional hydrogen bond interactions with the SH2 domain whose beneficial contribution to affinity is documented[5,13]. These hydrogen bonds, which involve the carbonyl of the acetyl N-terminal group of the peptides and the backbone NH of their X_{+1} residue, are not mimicked by **2**, still the compound displays the same level of affinity. One should also notice that the hydrophobic interactions between the SH2 domain and the X_{+1} valine side chain present in **17** that are seen in the X-ray structure, are also omitted with **2**. The fact that compound **2** is ~ 200 fold more active than phenylphosphate[14,15] proves that the aminopyrimidine moiety contributes substantially to the binding affinity and therefore is very likely to act as designed.

Table 1: Grb2-SH2 Domain Inhibitory Activity of the Peptidomimetics and the Related Peptides

Compound	IC_{50} (μM)
Ac-pTyr-Gly-Asn-NH_2 **16**	67 ± 8.42
Ac-pTyr-Val-Asn-NH_2 **17**	4.32 ± 0.86
	55% inhibition at 5 mM
 **1**	> 200
 **2**	25.9 ± 2.4

In conclusion, we have shown that it is possible to design a fully non peptidic molecule that mimics the natural ligands of the Grb2-SH2 domain in their main interactions with the protein . The affinity of the mimetic is in the micromolar range and further optimization is certainly required to obtain a drug candidate. However, this successful example of peptidomimetic design opens the way to the discovery of more potent low molecular weight compounds capable of inhibiting the cellular Ras activation pathway.

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(6) The design and molecular modeling work was carried out interactively in MacroModel (a) using the AMBER force field (dielectric constant of 4r) (b) to energy minimize the protein-ligand complexes.
(a) MacroModel v.4.0: Mohamadi, F.; Richards, N. G. J.; Guida, W. C.; Liskamp, R.; Lipton, M.; Caufield, C.; Chang, G.; Hendrickson, T.; Still, W. C. *J. Comput. Chem.* **1990**, *11*, 440.
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(7) Calculations in Macromodel using the AMBER forcefield suggests a twist angle of 25°in 4-phenyl thiazole the value of which increases to 50°when a 5-methyl substituent is introduced.
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(12) Peptide studies in our group have established that the minimal sequence retaining micromolar affinity for the Grb2-SH2 domain is the tripeptide Ac-pTyr-X_{+1}-Asn-NH2. Asparagine at X_{+2} is absolutely required while position X_{+1} is more versatile: valine, isoleucine, glutamine and glutamic acid being the preferred natural amino acids at this position (Garcia-Echeverria, C.; et al. Novartis Pharma Inc., Oncology Research Department, unpublished results). Compound **16** which has glycine in X_{+1} was included in the table to show the contribution of the side chain of the X_{+1} residue to affinity. Hydrophobic contacts between the SH2 domain and the X_{+1} valine side chain are seen in the X-ray structure.
Phosphopeptide residues are numbered relative to the position of phosphotyrosine which is denoted 0. Positive numbers are used for amino acids C-terminal to phosphotyrosine.
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(14) In our assay, phenylphosphate has approximately the same activity as phosphotyrosine[15], 55 % inhibition at 5 mM and 6.2 mM (IC_{50}), respectively.
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