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Identification of a High-Affinity Phosphopeptide Inhibitor of Stat3

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Abstract—Stat3 is a latent transcription factor that exhibits elevated activity in a variety of human cancers. To find a lead peptide for peptidomimetic drug development we synthesized and tested phosphopeptides derived from known receptor docking sites and found Y(p)LPQTV as the optimal sequence. SAR studies showed that each residue from pY to pY + 3 provided binding energy.

Stat3 (Signal Transduction and Activator of Transcription 3) is a member of the STAT family of latent, cytosolic transcription factors that directly relate signals from the plasma membrane to the nucleus.[1,2] Stat3 mediates IL-6 signaling and has been shown to be constitutively activated in multiple myeloma and cancers of the head and neck, breast, prostate, and brain (reviewed in ref 3). Downstream targets of Stat3 include bcl-x_L, a member of the bcl-2 family of anti-apoptotic proteins, cell cycle regulators such as cyclin D1 and $p21^{WAF1/CIP1}$, and other transcription factors including *c-myc* and *c-fos*.[1,3,4] Stat3 is recruited to phosphorylated receptors via its SH2 domain. It then becomes phosphorylated on Tyr^{705} by JAK kinases, Src, Abl, or the kinase activity of the receptor. Upon tyrosine phosphorylation, Stat3 forms a dimer in which the SH2 domain of one protein molecule binds to the $pTyr^{705}$ residue of the other and vice versa. The dimer migrates to the nucleus, binds to specific DNA sequences and initiates transcription. As mentioned above, aberrantly activated Stat3 has been observed in a variety of tumors and is an attractive target for therapeutic intervention. To this end, we have embarked on a program to impede Stat3 signaling by inhibiting receptor docking and/or dimer formation by the use of phosphotyrosine-based peptidomimetics targeted to the SH2 domain. To date we are aware of only one publication concerning the development of Stat3 inhibitors.[5]

As part of its role in signaling, Stat3 has been shown to be recruited to phosphotyrosine residues on gp130,[6] leukemia inhibitory factor receptor (LIFR),[6] the epidermal growth factor receptor (EGFR),[7] interleukin 10 receptor (IL-10R),[8] and granulocyte colony stimulating factor receptor (G-CSFR),[9] presumably by binding via its SH2 domain. Phosphopeptides derived from putative binding sites were able to inhibit Stat3 binding to these receptors in cell free systems.[6–9] It was noted early on that in these receptors a consensus sequence for recognition was YXXQ.[6]

Although phosphopeptides derived from Stat3 Tyr^{705} have been assayed for their ability to inhibit dimerization and DNA binding by electrophoretic mobility shift assays (EMSAs),[5,10] to date, no reliable information is available on the affinity of this protein for the amino acid sequences surrounding the phosphotyrosine docking sites of receptors. Haan et al.[11] assayed several phosphopeptides based on gp130 and LIFR as well as the sequence surrounding Stat3 Tyr^{705} for binding to the isolated SH2 domain from Stat3. At pH 7.5 no complex formation was observed whereas binding occurred at pH 5.5. It was noted that at the higher pH the SH2 domain existed in a dimerized state, which appears to have precluded phosphopeptide binding.

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Binding constants determined at pH 5.5 must be considered tenuous since the conformation of the protein may be altered by the acidic conditions. To find a lead peptide for the development of Stat3 inhibitors, we measured the ability of peptides derived from several membrane binding sites to inhibit dimerization and DNA ability. The results are reported herein.

Tyrosine-phosphorylated hexapeptides based on the known Stat3 docking sites of gp130,[6] LIFR,[6] EGFR,[7] IL-10R,[8] and GCSFR,[9] were assayed for their ability to impede DNA binding by Stat3 using EMSA's (Table 1). Included in the survey was acetyl-Y(p)LKTKF-amide (peptide **1**), derived from the phosphorylated tyrosine 705 in Stat3. This peptide serves as a standard since the sequence surrounding Tyr^{705} was shown to bind to the Stat3 SH2 domain in a crystal structure of the dimer of Stat3.[12] It was also used to interfere with the DNA binding of recombinant Stat3.[10] In the phosphopeptide series reported by Turkson et al.,[5] also derived from the Stat3 phosphorylation site, changing a proline to an alanine at the pY-1 position did not result in a significant change in inhibitory activity. We concluded that this position did not provide binding affinity so we acetylated the N-terminal phosphotyrosine in our peptides to simplify their structures.

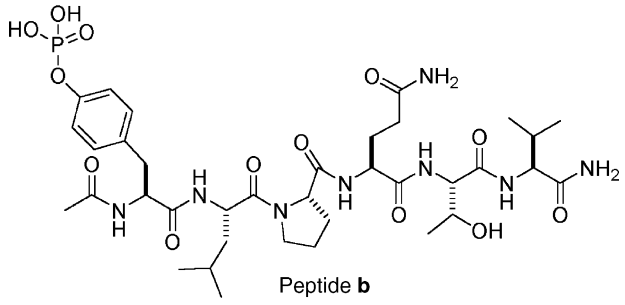
Peptides were synthesized by solid phase Fmoc techniques on Rink resin on an Advanced Chemtech 348 multiple synthesizer. Phosphotyrosine was introduced either by global phosphorylation using dibenzyl-*N,N*-diisopropylphosphoramidite/tetrazole phosphitylation followed by mCPBA oxidation,[13] or by the use of Fmoc-protected phosphotyrosine in peptide assembly.[14] Peptides were purified by HPLC and gave the correct mass by MALDI or electrospray mass spectrometry. Peptide concentration was determined by using the phosphotyrosine extinction coefficient of 695.[15] Peptides were assayed using electrophoretic mobility shift assays as described by Park et al.[10] His_{10}-Stat3 was expressed in Sf9 cells from a baculovirus encoding the recombinant protein. Jak-1 and Jak-2 were co-expressed to ensure phosphorylation of Tyr^{705}. A nuclear extract of the Sf9 cells was incubated with ^{32}P-labeled high affinity c-*fos sis* inducible element (hSIE) either alone or in the presence of inhibitor. After 20 min of incubation, samples were electrophoresed on 4% native polyacrylamide gels. The gels were dried, exposed to a phosphorimager screen and scanned. The image was quantified using Imagequant™ software and IC_{50} values were derived from plots of spot intensity versus phosphopeptide concentration. As mentioned, Stat3 dimerizes via reciprocal phosphotyrosine-SH2 domain interactions, and the dimer binds to DNA. Phosphopeptides targeted to the Stat3 SH2 domain block dimerization and therefore DNA binding. The affinity of peptides to the SH2 domain is measured by the intensity of the radioactivity of the Stat3-DNA complex band in the electrophoresis gel.

Table 1. The inhibition of Stat3 dimerization and DNA binding by receptor-derived phosphopeptides as measured by EMSAs

Peptide	Receptor/protein	Tyr position	Sequence[a]	IC_{50} (μM)[b]
1	Stat3	705	Y(p)LKTKF	20
2	gp130	673	Y(p)SDGNF	>150
3	gp130	759	Y(p)STVVH	>150
4	gp130	767	Y(p)RHQVP	6.0
5	gp130	813	Y(p)FKQNC	60
6	**gp130**	**904**	**Y(p)LPQTV**	**0.15**
7	gp130	915	Y(p)MPQ	1.5
8	LIFR	981	Y(p)QPQAK	6.0
9	LIFR	1001	Y(p)KPQMH	0.40
10	LIFR	1028	Y(p)RPQAM	1.0
11	EGFR	1068	Y(p)INQSV	30
12	EGFR	1086	Y(p)HNQPL	150
13	mIL-10R	427	Y(p)QKQTR	8.0
14	mIL-10R	477	Y(p)LKQES	6.0
15	GCSFR	708	Y(p)VLQGD	8.0
16	GCSFR	748	Y(p)LRCDS	80

[a]All peptides are acetylated on the N-terminus and are C-terminal amides.
[b]IC_{50} values were determined by EMSA and are the averages of two determinations.

In general the Y(p)XXQ peptides displayed greater activity than those with other amino acids at pY + 3 and exhibited IC_{50} values of 60 μM or less (Table 1). The exception was peptide **12** derived from position 1086 of the EGF receptor. Interestingly, peptide **1**, possessing Thr at pY + 3, had an IC_{50} value of 20 μM. Of the Y(p)XXQ peptides, those possessing a proline residue at pY + 2 (peptides **6**–**10**) were the most active, with IC_{50} values of 6 μM or less. Of these, peptide **6** was the most active (IC_{50} = 150 nM), exhibiting a 133 - fold increase in activity over the Stat3-derived peptide, **1**. This compound was derived from the sequence following Tyr^{904} of gp130. This protein forms a heterodimer with the IL-6 receptor and it is this complex that transmits the signal from this cytokine to Stat3 and to the nucleus.



Peptide **b**

Peptide **6** was chosen for further development because of its high affinity. To gain an understanding of the minimum size necessary for inhibition and the role of individual residues in peptide-protein interactions, truncation experiments, an alanine scan, and other substitutions were performed. Truncation experiments (Table 2) show that that the minimum size for potent inhibition is four amino acids and that addition of an additional amino acid at position pY + 4 provides slightly increased affinity. The alanine scan (Table 2) shows that considerable binding energy results from the side chain of leucine at pY + 1, as evidenced by the 10-fold higher IC_{50} value of peptide **20**. The proline to alanine substitution (peptide **21**) also resulted in a considerable loss of activity, again suggesting the importance of the proline at pY + 2. Glutamine appears to provide extensive binding energy as indicated by the high IC_{50} value of the Ala substitution in peptide **22**.

Substitution of the pY + 4 and pY + 5 residues with alanine did not significantly hinder activity (peptides **23** and **24**).

Deletion of the phosphoryl group from phosphotyrosine resulted in extensive loss of inhibitory activity (peptide **25**) which increases our confidence that we are indeed targeting the SH2 domain of Stat3. Substituting the phosphate group with a carboxyl group[16] or the negatively charged tetrazole group[17] also severely impaired inhibition (peptides **26** and **27**). This is no doubt a result of poor contact with the side chains of Lys591 (αA2), Arg609 (βB5), Ser611 (βB7), and Ser613 (BC2) and the main chain NH of Glu612 (BC1) that make contact with the phosphate oxygen atoms in the crystal structure of Becker et al.[12] Substitution of the non-hydrolyzable phosphotyrosine mimic, 4-phosphonomethylphenylalanine, for phosphotyrosine resulted in a 40-fold loss in inhibition. Loss of activity of phosphonomethyl-substitution has been observed in peptide ligands of the p85 PI3K C-terminal SH2 domain[18] as well as in the case of Src family SH2 domains.[19] This it attributed to a higher pKa of the acidic oxygens on the phosphono group compared to those of the corresponding phosphate. Thus, in regard to phosphotyrosine binding, the SH2 domain of Stat3 appears to be normal.

The substitutions of glutamic acid and asparagine for the glutamine at pY + 3 resulted in decreased activity (peptides **29** and **30**). Thus negative charge at this position is detrimental. The reduced inhibition of the asparagine-containing peptide indicates that the distance of the side chain carboxamide group from the backbone is important.

Table 2. Inhibition of Stat3 dimerization and DNA binding by analogues of the gp130 (904) peptide, **6**

Peptide	Sequence[a]	IC_{50} (μM)[b]
Minimum size		
17	Y(p)LPQT	0.6
18	Y(p)LPQ	1
19	Y(p)LP	>6
Alanine scan		
20	Y(p)APQTV	1.6
21	Y(p)LAQTV	4
22	Y(p)LPATV	>6
23	Y(p)LPQAV	0.8
24	Y(p)LPQTA	0.4
pTyr replacements		
25	Y LPQTV	>6
26	pCpaLPQTV[c]	>6
27	Tpa LPQTV[d]	>6
28	Pmp LPQTV[e]	8
Gln replacements		
29	Y(p)LPETV	>6
30	Y(p)LPNTV	2

[a]All peptides are acetylated on the N-terminus and are C-terminal amides.
[b]IC_{50} values were determined by EMSA and are the averages of two determinations.
[c]pCpa, 4-carboxyphenylalanine.
[d]Tpa, 4-tetrazolylphenylalanine.
[e]Pmp, 4-phosphonemethylphenylalanine.

The YXXQ motif is present in most of the peptides derived from the receptors. Glutamine was found to be optimal. Those peptides possessing amino acids other than glutamine at pY + 3 were very weak inhibitors. Substituting Ala for Gln in peptide **6** resulted in >60-fold loss in activity. Therefore it appears that the SH2 domain of Stat3 binds to phosphopeptide sequences in the 'two-pronged plug' motif comprising the pY and the pY + 3 residues of the ligand seen in a large number of other SH2 domains.[20] Typically, the pY + 3 residue is hydrophobic, such as Ile for Src-family SH2 domain phosphopeptides and Met in the p85 phosphoinositol kinase SH2 domain. In contrast, Stat3 requires the hydrophilic residue, Gln, at pY + 3.

Both the initial screen and the alanine scan indentified proline at pY + 2 as important for potency. Proline experiences no rotation about the Nα–Cα bond and this constraint may be important in pre-folding the peptide to a population of conformations resembling the bound conformation. This residue is often involved in backbone turn sub-structures in proteins and peptides. Additionally, the Xxx-Pro peptide bond can exist in both *cis* and *trans* conformations, which would alter the overall fold of the peptide. *Cis* and *trans* prolyl peptides have been observed to exhibit markedly different activities in other systems such as opioids.[21] In the case of the C-terminal SH2 domain of the p85 PI3 kinase, peptide inhibitor studies revealed that the optimum sequence is pTyr-Val-Pro-Met-Leu, also possessing a proline at pY + 2. According to the X-ray structure, this peptide binds to the SH2 domain in a extended conformation with a *trans* Val-Pro peptide bond.[22] The interaction is also a pY–pY + 3 'two pronged plug' motif and the proline serves to 'present' the side chain of Met for insertion into the pY + 3 binding pocket. In contrast, the X-ray structure of the Stat3 dimer[12] reveals that there is no pocket for the pY + 3 residue (Thr^{708}) that would fit the side chain of Gln in peptide **6.** The sequence from Tyr^{705} to Ile^{711} from one Stat3 monomer binds in a β-strand conformation across the surface of the SH2 domain of the other. Thus the role of the proline at pY + 3 in the bound conformation of peptide **6** is very important and the structure of the peptide bound to Stat3 will greatly enhance the design of peptidomimetic drugs against this target. Structural studies are currently underway and will be reported at a later time.

In summary, testing a battery of known receptor docking sites for Stat3 has resulted in the identification of a high affinity phosphopeptide inhibitor of dimerization and DNA binding. This peptide can be used as a starting point for peptidomimetic drug design to develop anti-cancer therapeutics.

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