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Isoform selective inhibition of STAT1 or STAT3 homo-dimerization via peptidomimetic probes: Structural recognition of STAT SH2 domains

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Abstract—The identification of constitutively activated STAT (Signal Transducers and Activators of Transcription) proteins in aberrant cell signaling pathways has led to investigations targeting the selective disruption of specific STAT isoforms directly associated with oncogenisis. We have identified, through the design of a library of peptidomimetic inhibitors, agents that selectively disrupt STAT1 or STAT3 homo-dimerization at low micromolar concentrations. ISS840 has 20-fold higher inhibition of STAT1 homo-dimerization (IC_{50} value of 31 μM) relative to STAT3 (IC_{50} value of 560 μM).

Selective binding to closely related protein isoforms represents a particularly challenging problem in the search for molecules that might control cell signaling. This is nowhere more true than in the disruption of constitutively activated Signal Transducer Activators of Transcription (STAT) proteins as a viable anti-cancer therapy. Constitutive activation in numerous cancers, including leukemia and lymphomas, has provided evidence of a relationship between aberrant STAT activation and oncogenesis.[1] As a result, the emerging significance of STAT signaling in the development of human cancers makes it an excellent target for new therapeutic intervention. There have been a number of recent examples of phosphopeptide-based compounds shown to display high affinity for STAT proteins.[2] In this paper, we report the design of a series of phospho-peptidomimetic probes that display selective inhibition of specific STAT isoform homo-dimerization.

The STATs are a family of cellular proteins important in cell proliferation, differentiation, apoptosis, and survival.[3] STAT proteins are triggered through extracellular cytokine and growth factor stimulation resulting in receptor activation.[4] Phosphorylation of a crucial tyrosine residue provides binding sites for the recruitment of monomeric, non-phosphorylated STAT proteins via their Src homology 2 (SH2) domain.[5] Receptor-bound STAT is then tyrosine phosphorylated by nonreceptor tyrosine kinases such as JAK and Src. Phosphorylated STAT proteins are then released from the receptor, and dimerization occurs through reciprocal phosphotyrosine–SH2 interaction.[6] STAT dimers immediately translocate to the nucleus and bind with promoter regulatory elements.[7] In normal functioning cells STAT activation is transitory and tightly regulated. However, aberrant activation of some STAT proteins such as STAT3 and STAT5 leads to the up-regulation of oncogenic pathways through dysregulated growth, angiogenesis, and survival. The alleviation of irregular STAT signaling through dimerization inhibition provides a focused target for molecular intervention. For example, suppression of homo- and hetero-dimerization through specific peptide sequences interacting with STAT3 has served to establish the structural attributes required for SH2 domain-inhibitor complementarity and further development of anti-cancer agents. Critical for their roles in cell biology, the seven individual isoforms of

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STAT exhibit different functional properties.[3] For example, STAT1 deficient mice display an impaired response to interferons, are susceptible to viral or bacterial pathogens, and spontaneously develop tumors.[3] Also, constitutively activated STAT1 signaling is present in a number of human cancers although its role in oncogenic pathways has yet to be fully elucidated. In contrast to STAT1, inhibition of persistent STAT3 activation by blocking tyrosine kinase activity has been repeatedly associated with tumor selective growth suppression and cell death.[4]

We have previously reported[10,11] the synthesis of STAT3 homo-dimerization inhibitors through the use of the peptide PY*LKTK (Y* = phosphotyrosine), which corresponds to the core of the native C-terminal STAT3 SH2 domain binding sequence GSAA-PY*LKTKFIC.[9] Further optimization through the synthesis of a focused tripeptide library identified the importance of the central Y*L residues for inhibitory activity. STAT selectivity and affinity were associated with functional group substitution of the peptide termini. Selectivity is in part explained by the difference in the STAT3 binding sequence and the known STAT1 binding sequences Y*DKPH and Y*IKTE. By testing with a range of substituents of varying polarity, size, and orientation we have shown that a phenyl nitrile substituent at the Y* N-terminus is crucial in the series for enhanced inhibition of STAT3 dimerization.[10,11]

We now report progress toward the development of peptidomimetic probes of STAT1 function that provide the means to preferentially inhibit STAT1 over STAT3 through the exploration of the Leu C-terminus. Improved activity and selectivity were achieved and explained through a targeted synthetic program and computational techniques including exploratory genetic optimization for ligand docking (GOLD)[12] and quantitive structural activity relationship (QSAR) modeling.[13] In vitro evaluation was carried out through pre-incubation of specific concentrations of peptidomimetics with nuclear extracts containing STAT1 and STAT3 for 30 min at room temperature before incubation with radiolabeled hSIE oligonucleotide probes and ESMA analysis. Standard peptide synthetic procedures using HBTU and DIPEA were employed to furnish the library of functionalized inhibitors (see Fig. 1).

Effective STAT3 isoform dimerization was achieved through previously reported ISS610 (IC_{50} value of 42 ± 23 μM), a mono-functionalized dipeptide. ISS610 was tested as a representative STAT3 selective peptidomimetic against constitutively active STAT3-dependent v-Src transformed fibroblasts and was found to suppress cell growth significantly.[11]

Modification of ISS610 via amide coupling to the free acid of the Leu residue was explored to identify more potent inhibitors of STAT3 dimerization. Theses changes were found to reduce activity toward STAT3, but they imparted unexpected selectivity for STAT1 (Table 1). For example, the addition of an isopropyl or cyclohexyl substituent results in a drop in DNA binding disruption to 760 μM (ISS834) and 605 μM (ISS836), respectively, compared to 42 μM (ISS610). GOLD docking of ISS610 and ISS840 within the SH2 domain of STAT3 (Fig. 2B) was used to confirm the limited space available for scaffold extension.

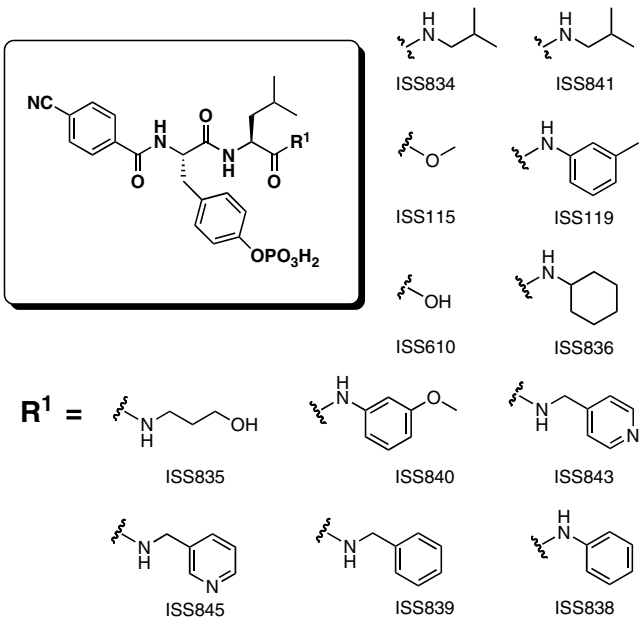

Figure 1. Peptidomimetic library; substitution of central (4-phenyl nitrile)Y*L core with a range of functional groups projected from the C-terminus.

Molecular docking of STAT3 peptidomimetic derivatives suggested that the location of the pendant phosphorylated tyrosine within a hydrophilic pocket was conserved in all the peptidomimetic binding models. Control compounds synthesized without the phosphorylated Tyr lost all dimerization inhibitory activity toward STAT isoforms. The orientation of the peptide and subsequent projection of functionality is determined by the positioning of the phosphate in relation to the hydro-

Table 1. Selective disruption of STAT 3 and STAT1-DNA binding activity by peptidomimetics

	IC_{50} values (μM) STAT-DNA binding disruption		
Inhibitor	STAT3:STAT3 IC_{50} (μM)	STAT1:STAT1 IC_{50} (μM)	STAT1v STAT3
ISS610	42 ± 23	310 ± 145	1:7.4
ISS834	760 ± 65	58 ± 29	13:1
ISS835	210 + 78	620 ± 120	1:3
ISS836	605 ± 87	58 ± 32	10:1
ISS838	380 ± 65	310 ± 78	~1:1
ISS839	760 ± 130	800	~1:1
ISS840	560 ± 100	31 ± 22	20:1
ISS841	250 ± 85	74 ± 22	3:1
ISS843	402 ± 22	123 ± 30	3:1
ISS845	na	na	—
ISS119	na	na	—
ISS115	na	na	—

Note: Nuclear extracts containing active STAT1 and STAT3 were preincubated with or without peptidomimetics for 30 min before incubation with radiolabeled hSIE probe and EMSA analysis.

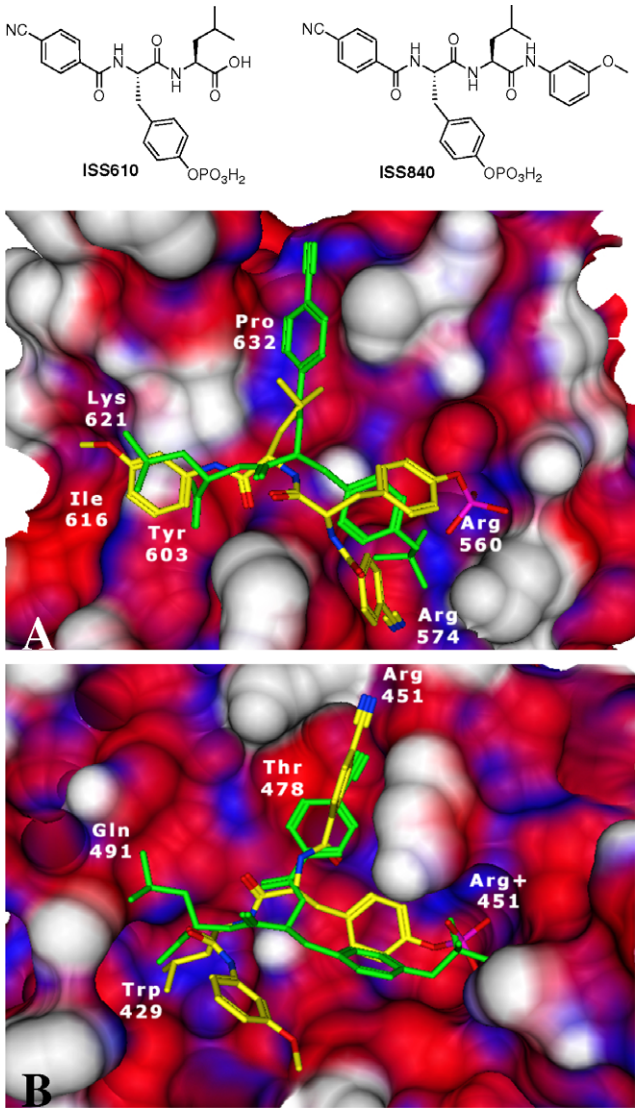

Figure 2. (A) Comparative GOLD (Genetically Optimized Ligand Docking) docking of ISS840 (yellow) and ISS610 (green) within the SH2 domain of STAT1 protein. Residue numbers refer to the corresponding residues of unphosphorylated STAT1 (*Homo sapiens*) (pdb:1YVL[8]). (B) Comparative GOLD docking of ISS840 (yellow) and ISS610 (green) within the SH2 domain of STAT3 protein. Residue numbers refer to the corresponding residues of STAT3β homodimer bound to DNA (*Mus musculus*) (pdb: 1BG1[9]) (red hydrophobic to blue hydrophilic).

philic Arg cleft. Spatial constraints upon ISS610 limited the possibility of chain elongation; experimental results confirmed these model findings.

Suitable spatial arrangement of functional groups at the Leu C-terminus was critical for STAT1 inhibition. Substitution with *m*-methoxyaniline (ISS840) gave excellent STAT1 homo-dimerization inhibition (IC_{50} value of 31 μM) (Fig. 3), with little effect on STAT3:STAT3 dimer formation (IC_{50} value of 560 μM). Selectivity of a lesser magnitude toward STAT1 was achieved with ISS834 (55 μM), 836 (58 μM), 841 (74 μM), and 843 (123 μM). STAT1 affinity appears to require projection of a hydrophobic group toward hydrophobic residues, Tyr 603 and Ile 616. STAT1 inhibitor development

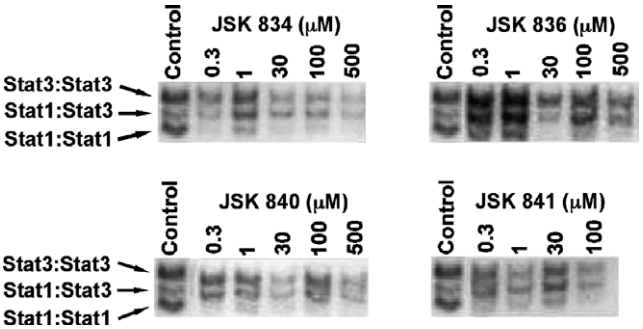

Figure 3. EMSA analyses of homo and hetero STAT1 and STAT3 dimerization showing the effects of pepdidomimetics. Nuclear extracts containing activbated STAT1 and STAT3 are treated with the indicated concentrations of peptidomimetics ISS834, 836, 840, and 841.

was achieved without the assistance of crystal structure docking models.

When developed with our library of STAT1 and STAT3 peptidomimetic inhibitors, a QSAR model showed crucial structural attributes necessary for isoform selectivity and affinity. PLS and genetic PLS (G/PLS) algorithms, as implemented within Cerius,[3,14] were used to model STAT1 and STAT3 inhibitors. We focused on specific descriptors, which were deemed pertinent to disrupters of protein–protein interactions. Our model indicated that increasing the number of hydrogen bond donors elevated STAT1 selectivity, whereas preferential STAT3 affinity was obtained through increased hydrogen bond acceptor sites. Isoform selectivity was also directly related to inhibitor size, which correlated well with the experimental findings; STAT1 inhibitors were larger in dimensions than the corresponding STAT3 agents.

Docking studies were performed on our lead STAT1 agents using coordinates from the unphosphorylated STAT1 crystal structure.[8] ISS840 had excellent complementarity with the protein surface as shown in Figure 2A. Phosphate incorporation into the Arg 560 cleft and projection of the benzyl nitrile toward Arg 574 suggested a basis for its high affinity. Additional contacts appear to include hydrogen-bonding between the terminal methoxy group and Lys 621 which presumably stabilizes the C-terminal region of the peptidomimetic within the SH2 domain.

In conclusion, we have demonstrated a peptidomimetic approach for the inhibition of STAT protein dimerization which can be extended to display selectivity toward specific STAT isoforms. Our multifaceted approach using GOLD and QSAR modeling is now being applied to a non-peptidomimetic scaffold to improve the membrane permeability of our agents while retaining the key structural attributes required for selectivity.

Acknowledgments

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