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Targeting Protein–Protein Interactions: Suppression of Stat3 Dimerization with Rationally Designed Small-Molecule, Nonpeptidic SH2 Domain Binders

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Protein–protein interactions remain a daunting target for disruption by small molecules due to their large interfacial areas and their often noncontiguous contact points. The prospect for inhibition increases when small interaction modules (such as SH2 domains) participate in the binding. SH2 domains are found in the family of signal transducers and activators of transcription (STAT) proteins,[1] which mediate the relay of extracellular signals from various cell-surface protein receptors to the nucleus, where they help to initiate and regulate specific gene expression.[2] In particular, the Stat3 protein is known to directly upregulate Bcl-x_L, c-Myc, Mcl-1, VEGF, and cyclin D1/D2, and contributes directly to compromised cellular regulation by stimulating cell proliferation and preventing apoptosis in numerous human cancers.[2, 3]

Stat3 activation occurs through phosphorylation of tyrosine 705, which promotes the formation of a Stat3 dimer through reciprocal Stat3 phosphotyrosine 705–SH2 domain interactions.[4] In general, STAT dimers then translocate to the nucleus, where they regulate unique gene expression programs through interaction with specific DNA-response elements. Stat3-targeted gene expression confers resistance to apoptosis in many tumor cells and promotes cell survival, which contributes to the resistance of these cancers to currently available chemotherapeutics.[5] Successful Stat3 inhibitors might thus be used to sensitize human cancers that harbor constitutively active Stat3 to existing chemotherapeutic agents. Further, the specificity of these inhibitors toward Stat3 might potentially reduce the side effects that are associated with conventional, aggressive chemotherapy.

Despite the difficulties in identifying protein surface-recognition agents, the promise of Stat3 modulators warrants investigation.[6] Successful peptidic[7a–f] and nonpeptidomimetic small molecules[8–11] that are capable of targeting malignant cell lines with constitutively activated Stat3 protein are limited to a few examples that include Stattic,[8] STA-21,[9] and S3I-201,[10] which were all identified through high-throughput virtual or biochemical screening approaches. Our first-generation designs were simple peptidomimetics derived from the natural sequence, of which ISS610 was the most potent (see Figure 1).[7f] More recently we have discovered S3I-M2001 (**15**, Table 1) an oxazole-based small-molecule inhibitor that shows promising inhibition of Stat3 function,[11] and we herein report a family of rationally designed small-molecule, nonpeptidic Stat3 inhibitors. These agents inhibit Stat3 protein dimerization and induce apoptosis in Stat3-transformed cells and Stat3-dependent breast oncogenic cell lines.

The crystal structure of the Stat3-SH2 domain reveals a shallow triangular pocket that is composed of two hydrophobic sites and a hydrophilic phosphate-recognition pocket. Docking studies on our initial lead, peptidomimetic ISS610, showed that only two of the hydrophobic pockets in the binding domain were effectively occupied (See Figure 1 A).[7f] To address this problem we envisaged that trisubstituted heterocyclic scaffolds, such as oxazoles and thiazoles, could effectively access all three sites (Figure 1 B). Flexible ligand-docking studies (GOLD) directed the design and assembly of oxazole and thiazole scaffolds. The oxazole inhibitors were prepared as outlined in Scheme 1 (see the Supporting Information for thiazole synthesis). GOLD docking studies predicted a focused set of substituents that might effectively occupy the SH2 domain. Both R^1 and R^2 appendages need to be predominantly hydrophobic in nature to interact with the hydrophobic surface presented by residues that are present in the upper (Phe716, Met660, Pro715) and lower right (Ser636, Arg595, Lys591) pockets of the Stat3 active site (Figures 1 A, B).

Our initial screening process sought to evaluate in vitro disruption of Stat3:Stat3–DNA complex formation through a previously published in vitro DNA-binding EMSA-based assay,[7e] which would give valuable insight into inhibitor action against the Stat3 in terms of disruption of Stat3:Stat3 homodimer. An initial series of Stat3 oxazole and thiazole inhibitors is shown in Table 1. Oxazole **7** was prepared as a control to explore the efficacy of compounds that do not project sizable appendages from the R^2 position. It closely resembles lead peptidomimetic ISS610. Stat3–DNA binding inhibition by **7** was shown to be negligible, presumably due to poor inhibitor–protein complementarity. Substitution of ISS610 at the acidic terminus had been shown previously to decrease potency in all cases studied; this suggests that the oxazole scaffold is able to project substituents in a substantially different orientation than could be achieved with a peptide-based inhibitor (Figure 1). Five compounds displayed IC_{50} values below 100 μM, with oxazole

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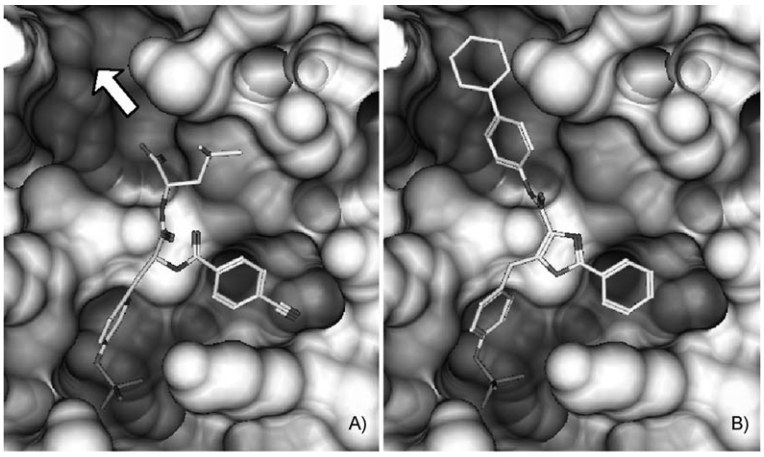
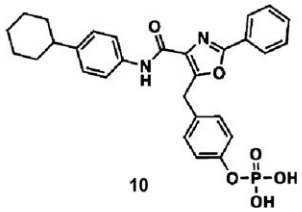
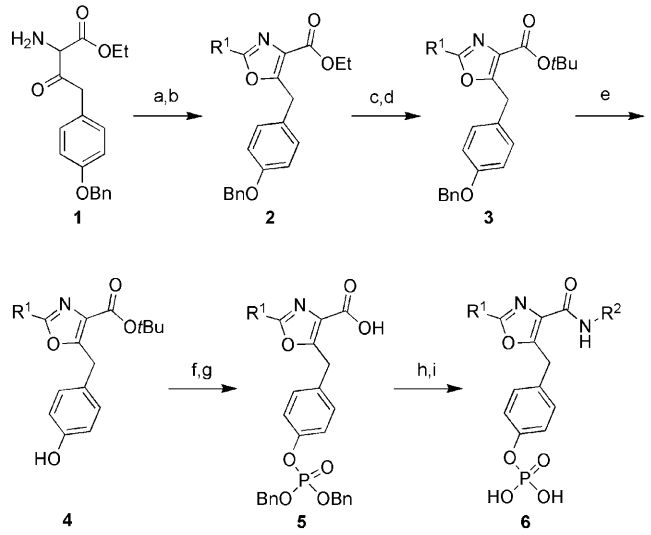


ISS610



10

Figure 1. A) Active-site conformation of ISS610, as determined by flexible ligand docking in the active site of Stat3 (PDB ID: 1BG1) by using GOLD 3.0.[12] Lead peptidomimetic ISS610[7f] does not completely occupy the active site. The site available for expansion is indicated by an arrow. B) Oxazole **10** replicates the functionality projection of **10** and accesses the previously unoccupied region of the SH2 domain.



Scheme 1. Conditions: A) R^1COOH, HBTU, DIPEA, DMF; B) PPh_3, I_2, TEA, CH_2Cl_2; C) 2 N NaOH, THF; D) *t*-butyl trichloroacetamide, $BF_3{\cdot}(OEt)_2$, CH_2Cl_2; E) H_2, Pd/C, THF; F) dibenzyldiisopropyl phosphoroamidate, tetrazole, *m*-CPBA, CH_2Cl_2; G) TFA, Et_3SiH, CH_2Cl_2; H) R^2COOH, HBTU, DIPEA, DMF; I) H_2, Pd/C, EtOAc. HBTU = 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, DIPEA = *N*,*N*-diisopropylethylamine, DMF = *N*,*N*-dimethylformamide, TEA = triethylamine, TFA = trifluoroacetic acid, *m*-CPBA = *m*-chloroperbenzoic acid.

10 ($IC_{50} = 33 \pm 16$ μM), and thiazoles **14** and **16** showing significant dimer-disruption potential.

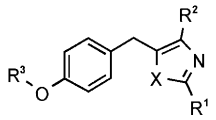
In several cases large effects were observed upon replacement of the central core. This is seen in **10** and **11**, where the high potency displayed by the oxazole analogue **10** ($IC_{50} = 33 \pm 16$ μM; Figure 2A) is severely diminished in the analogous thiazole **11** ($IC_{50} = 775$ μM). Conversely, thiazole **16** ($IC_{50} = 25$ μM) has improved activity compared to corresponding oxazole **15** ($IC_{50} = 58 \pm 10$ μM). Molecular modeling studies showed that the thiazole scaffolds facilitated improved projection, and concomitant higher complementarity of the R^1, R^2, and R^3 groups to the protein surface. In almost all examples, increased potency is derived from a thiazole core. Oxazole **12**, which contains the most potent arrangement of R^1 and R^2 elements, but lacks a phosphate group, was shown to be devoid of activity. Further nonphosphorylated scaffolds were prepared and found to be impotent Stat3 disruptors.

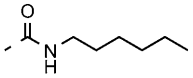
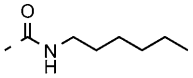
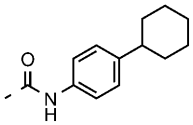
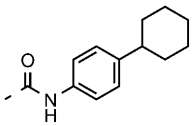
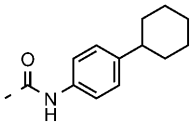
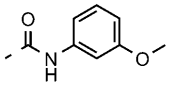
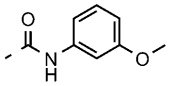
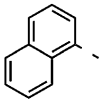
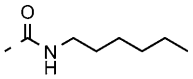
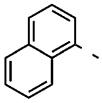
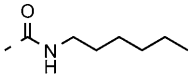
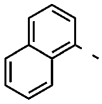
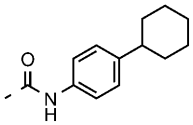
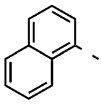
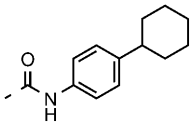
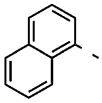
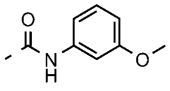
Given the extended planar aryl–oxazole construct and the propensity of such scaffolds to intercalate with DNA, experiments were conducted to discount interaction with DNA as a source of the biological activity.[13] By using fluorescence spectroscopy it was found that the amount of DNA-bound ethidium bromide did not vary upon addition of **10** (up to concentrations of 100 μM); this compound can thus be considered to be inert toward DNA (See the Supporting Information).

Work was undertaken to establish the affinity of the lead oxazole **10** for the inactive (unphosphorylated) Stat3 monomer to help determine the mode of action. By using fluorescence spectroscopy, K_i values were calculated through displacement of an SH2 binding fluorescein-labeled GpYLPQTV-NH_2 peptide.[14] The most potent inhibitors were found to have low affinity with the unphosphorylated Stat3 monomer (**10** and **15**, $K_i \sim 1$ mM; Figure 3). These results were attributed to the smaller size of the oxazole scaffolds relative to the phosphopeptide, which is predicted to make contacts with both the BG and EF loops that recognize ligand residues at the pTyr + 3 site.[15,16] Structural limitations of the inhibitors might preclude effective interactions with the SH2 domain and reduce their ability to displace the extended peptide sequence.

Despite the inherent difficulties associated with the cell permeability of phosphate derivatives, we observed promising whole-cell activities for several lead compounds. Initial in vitro whole-cell experiments were conducted against normal mouse NIH-3T3 fibroblasts and v-*src*-transformed counterparts (NIH-3T3/v-*src*) that harbor aberrant Stat3. Inhibitor effects upon cell viability, proliferation, and cytotoxicity were assessed through WST-1, a cell proliferation reagent that measures the metabolic activity of viable cells. The most potent in vitro inhibitor, **10**, displayed at least ten-fold selectivity toward malignant NIH-3T3/v-*src* fibroblasts with aberrant Stat3 ($EC_{50} = 120$ μM) and negligible effects toward normal NIH-3T3 fibroblasts, in which Stat3 pathways are tightly regulated. Conversely, the suppression by lead peptidomimetic ISS610 in whole cells required millimolar concentrations of inhibitor. It is assumed that the

Table 1. IC_{50} values (μM) for the inhibition of Stat3 DNA-binding activity in vitro via oxazole and thiazole analogues of ISS610.



Compound Number	R^1	R^2	X	R^3	IC_{50} values [μM]
7			O	PO_3H_2	>1000
8			O	PO_3H_2	>1000
9			S	PO_3H_2	150
10			O	PO_3H_2	33
11			S	PO_3H_2	775
12			O	H	>1000
13			O	PO_3H_2	90
14			S	PO_3H_2	28
15			O	PO_3H_2	58
16			S	PO_3H_2	25
17			O	PO_3H_2	255
18			S	PO_3H_2	350
19			S	PO_3H_2	125

[a] Inhibitor concentration required to decrease Stat3-DNA binding two-fold.

predominantly hydrophobic nature of our compounds facilitated successful permeation of the cell membrane despite the phosphate.

In addition, cell-based EC_{50} values were determined for potent inhibitors against human breast (MDA-MB-231) cancer cell lines (Table 2). Oxazole **10** had the best activity against breast cancer (ED_{50} = 180 μM) and NIH-3T3/v-*src* cells (ED_{50} = 120 μM) and lacked toxicity for control NIH-3T3 cells.

In summary, we have developed the first rationally designed small-molecule inhibitors of Stat3 dimerization and thereby

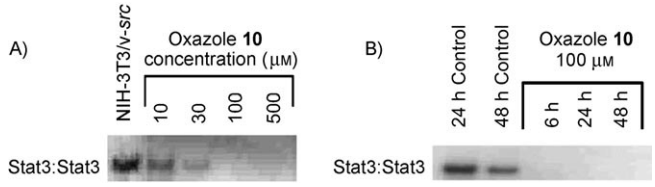


Figure 2. A) EMSA data for in vitro disruption of Stat3 dimers by **10**. B) NIH-3T3/v-*src* whole-cell in vitro disruption with **10** (100 μM) over 6, 24, and 48 h periods.

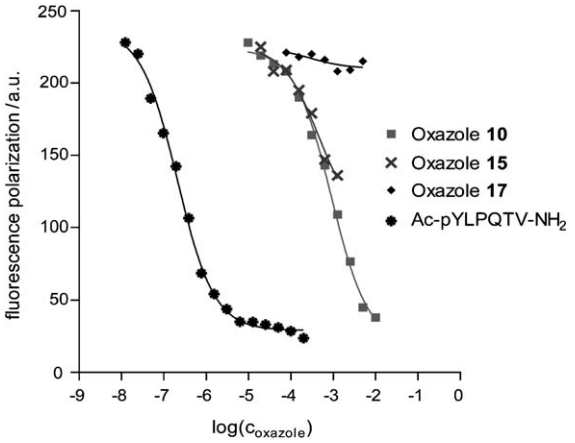


Figure 3. Competitive binding experiment between the fluorescent probe 5-carboxyfluorescein-GpYLPQTV-NH_2 and oxazoles **10**, **15**, **17** as well as unlabeled Ac-pYLPQTV-NH_2 peptide to Stat3 protein. Whereas Ac-pYLPQTV-NH_2 displays a 200 nM affinity towards Stat3, oxazoles **10**, **15**, and **17** do not interact significantly with Stat3.

Table 2. EC_{50} values for selected Inhibitors against constitutively activated Stat3 containing cell lines NIH/3T3/vSrc, MDA-MB-231 and unactivated Stat3 NIH3T3 cells.

Compound No.	EC_{50} [μM] NIH3T3	NIH3T3/vSrc	MDA-MB-231
10	>1000	120	180
14	>1000	480	>1000
16	>1000	145	300
19	>1000	700	>1000

have disrupted Stat3-mediated cell proliferation pathways. Suitably substituted oxazole and thiazole scaffolds, derived from peptidomimetic leads, disrupted Stat3:Stat3–DNA-binding activity in vitro at low micromolar concentrations, but showed low affinity to the unphosphorylated Stat3 monomer. This might suggest that our compounds preferentially bind with activated Stat3, which is further supported by the lack of activity against control cells (NIH-3T3); this still remains to be verified. Lead agents showed potency and selectivity against specific human cancer cell lines and negligible toxicity towards normal NIH-3T3 fibroblasts. Our studies highlighted the widely acknowledged belief that Stat3 is a potent target for disruption by small-molecule inhibitors for novel anticancer drug development and that targeting of Stat3 will require a higher level of investigation.

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Keywords: antitumor agents · molecular recognition · protein–protein interactions · small molecules · Stat3

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