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Design and synthesis of a new, conformationally constrained, macrocyclic small-molecule inhibitor of STAT3 via 'click chemistry'

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Abstract—STAT3 is a promising molecular target for the design of new anticancer drugs. In this paper, we report the design and synthesis of a conformationally constrained macrocyclic peptidomimetic **2** via click chemistry. Compound **2** was determined to bind to STAT3 with a K_i value of 7.3 μM in a competitive fluorescence-polarization-based binding assay, representing a promising initial lead compound for further optimization.

Constitutive activation of the signal transducers and activators of transcription 3 (STAT3) is frequently detected in human cancer specimens from patients with advanced disease and cancer cell lines, but not in normal epithelial cells.[1,2] Persistent activation of STAT3 signaling has been demonstrated to contribute directly to oncogenesis by stimulating cell proliferation and preventing apoptosis in human cancer cells.[1,2] STAT3 activation may not only provide a growth advantage, allowing accumulation of tumor cells, but also confer resistance to conventional therapies that rely on apoptotic machinery to eliminate tumor cells.[1,2] STAT3 is an important and specific molecular target for the design of an entirely new molecularly targeted therapy for human cancer with constitutively active STAT3. Such therapeutic agents should have low toxicity to the normal cells without constitutive STAT3 signaling.[1–4]

STAT3, recruited from cytosol, makes specific interactions through its SH2 domain with phosphotyrosine docking sites on different cytokine receptors. STAT3 then becomes phosphorylated on a carbonyl terminal tyrosine (Tyr705).[1,2] Tyrosine phosphorylation of STAT3 causes it to dimerize and translocate to the nucleus and bind to specific promoter sequences on its target genes.[1–6] Dimerization of STAT3 is a decisive event for its activation[1–5] and blocking this dimerization with a small-molecule antagonist is a very attractive therapeutic approach to the development of a molecularly targeted therapy for the treatment of human cancers in which STAT3 is constitutively activated.[7–10]

Two approaches are currently being pursued for the design of small-molecule antagonists to block STAT3 dimerization.[7–10] The first is to design peptide-based antagonists and peptidomimetics[7,8] and the second is to discover non-peptidic small-molecules.[9,10] While a number of peptide-based ligands can achieve quite high binding affinities to STAT3, they are generally not cell-permeable due to their peptidic nature and the negatively charged phosphotyrosine group in the ligands. For non-peptide small-molecule inhibitors, the major advantage is their good cell permeability,[7,8] but the inhibitors reported to date still have relatively poor binding affinities to STAT3. Although they may

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be considered as potential lead compounds, their binding affinity and specificity to STAT3 must be significantly improved. In this paper, we wish to present our structure-based design, synthesis, and biochemical evaluation of a conformationally constrained macrocyclic peptidomimetic as a new inhibitor of STAT3. The present study represents our initial but an important step toward our ultimate goal for the design of potent, cell-permeable, conformationally constrained peptidomimetics that block STAT3 dimerization.

The phosphopeptide, acetyl-pY*LKTKF-amide **1** (Fig. 1), has been found to inhibit STAT3 dimerization[8] and was the starting point in our design. The corresponding peptide segment (pY*LKTKF) in a complex with STAT3 (Fig. 2) in the crystal structure of the dimeric STAT3[6] showed that the phosphotyrosine has extensive electrostatic interactions with the side chains of Lys591, Arg609, Ser611, and Ser613, the hydrophobic side chains of leucine and phenylalanine residues interact with several hydrophobic residues or hydrophobic side chains of polar residues (Trp623 with leucine and Cys712, Y640, Met648, Asn647 with phenylalanine) in the protein, and the side chain of the threonine residue forms a hydrogen bond with Y640 in STAT3. However, the side chains of the two lysines are exposed to solvent (Fig. 2). This provided us with an opportunity to cyclize these two lysine residues to form a macrocyclic ring, obtaining conformationally constrained, macrocyclic, new STAT3 inhibitors. Compared to linear peptides, conformationally constrained, macrocyclic peptidomimetics can be much more resistant to protease degradation and may also achieve higher binding affinities to the target protein than its linear counterparts by reducing the entropic cost associated with loss of conformational degrees of freedom upon binding to the target protein.

Based upon these considerations, we have designed compound **2**, in which the ring cyclization was accomplished via 'click chemistry',[11] a highly efficient synthetic method for coupling. Modeling showed that compound **2** interacts with STAT3 in a similar fashion as the linear peptide **1** (Fig. 2) and predicted that compound **2** would bind to STAT3 with a good affinity.

The synthesis of compound **2** is shown in Scheme 1. Boc-L-6-hydroxynorleucine (**3**) was condensed with *O*-*t*-butyl-L-threonine methyl ester (**4**) to afford compound **5**. Compound **5** was treated with methanesulfonyl chloride to give the methanesulfonate ester, which was then converted to the azide by the treatment with sodium azide in DMF at 90 °C. The methyl ester was hydrolyzed to the carboxylic acid **6**, condensation of which with (*S*)-propargylglycine methyl ester furnished compound **7**. This was converted in 80% yield into the key intermediate **8**[12] via click chemistry[11] in the presence of $CuSO_4{\cdot}5H_2O$/sodium ascorbate. Compound **8**, whose structure was confirmed by NMR spectroscopy,[11] was hydrolyzed to the acid **9**, which was coupled with L-phenylalaninamide to afford compound **10**. Removal of the Boc group gave **11** and coupling with *N*-Fmoc-L-leucine furnished compound **12**. Removal of the Fmoc in **12** followed by coupling with Cbz-phosphotyrosine di-*t*-butyl

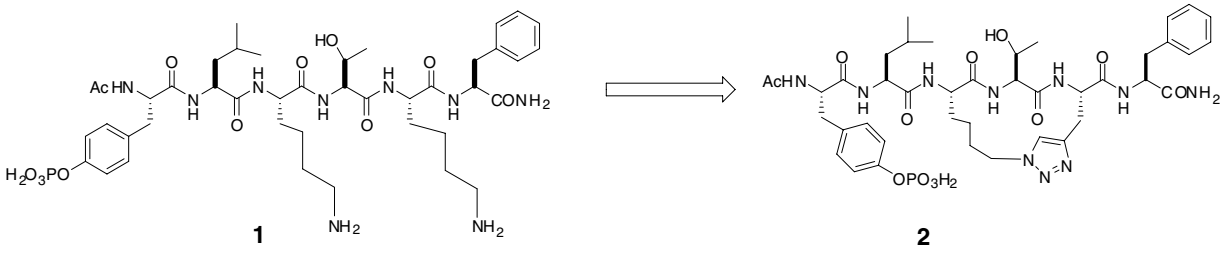


Figure 1. Design of a conformationally constrained, macrocyclic STAT3 inhibitor.

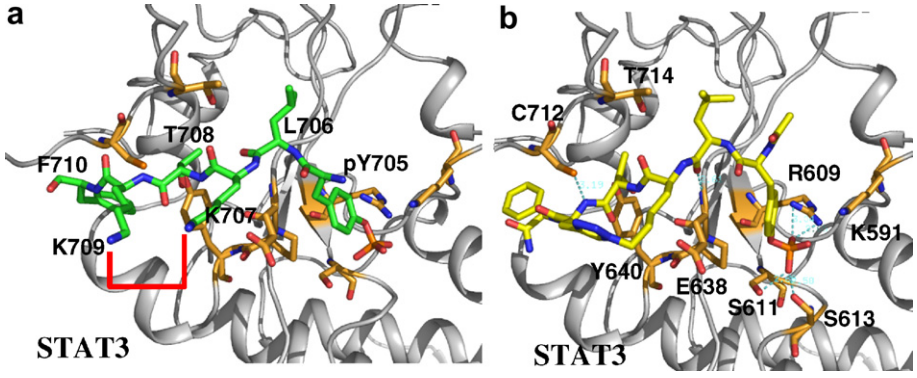
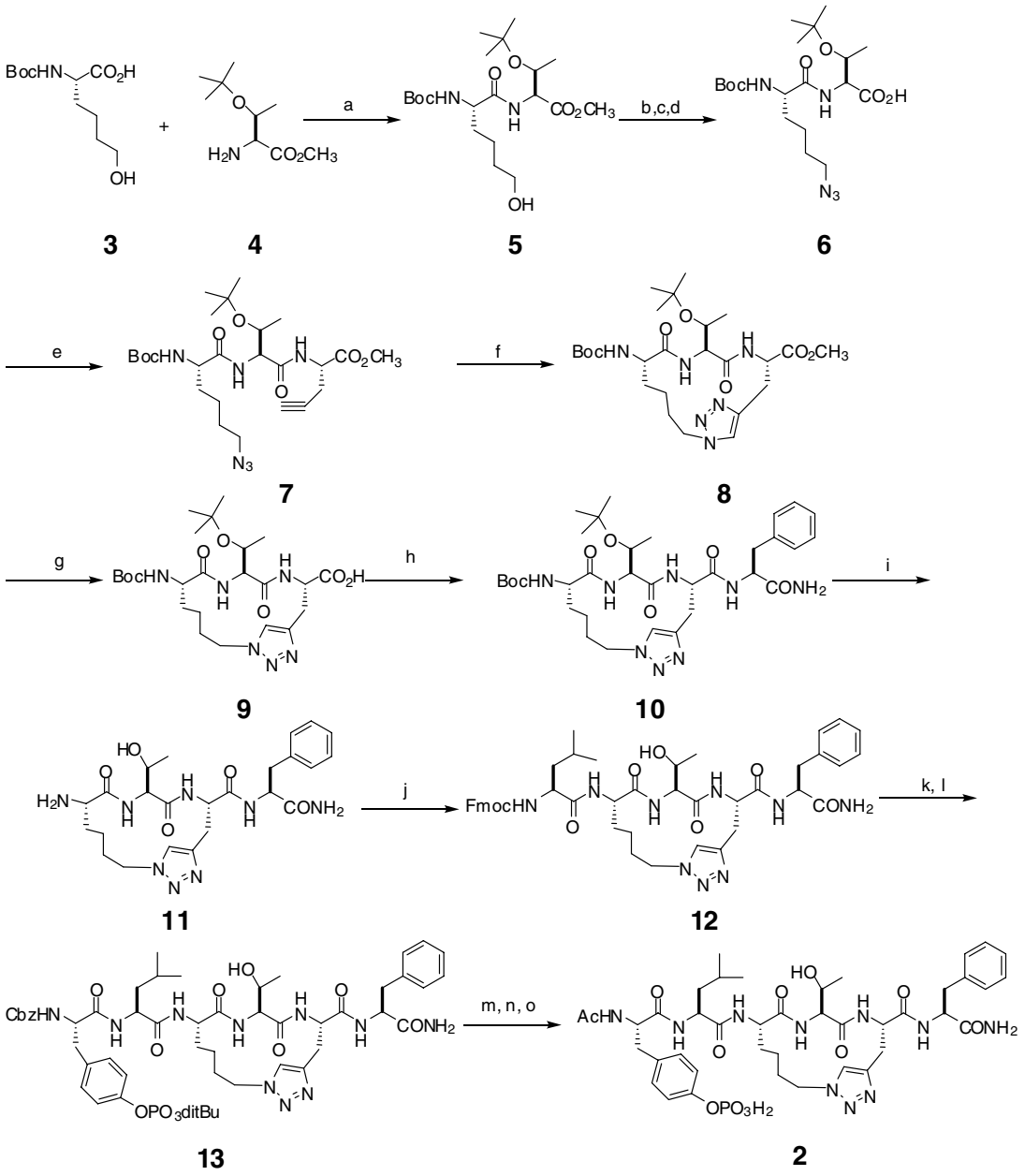


Figure 2. The STAT3 protein monomer is drawn in ribbon representation. STAT3 residues interacting with the ligand are also displayed in the stick model. The peptide segment (pY*LKTKF) is colored in green (a), while the designed compound (**2**) is colored in yellow (b). Hydrogen bond interactions are drawn as cyan dashed lines. The linkage sites are depicted as red lines.



Scheme 1. Reagents and conditions: (a) EDCI/HOBt/*i*-Pr_2NEt/DCM; (b) MsCl/*i*-Pr_2NEt/DCM; (c) NaN_3/DMF/90 °C; (d) 2 N LiOH/dioxane, then 1 M HCl; (e) (*S*)-propargylglycine methyl ester, EDCI/HOBt/*i*-Pr_2NEt/DCM; (f) $CuSO_4{\cdot}5H_2O$/Na ascorbate/H_2O/*t*-BuOH; (g) 2 N LiOH/dioxane, then 1 M HCl; (h) L-phenylalaninamide, EDCI/HOBt/*i*-Pr_2Net/DCM; (i) 4 M HCl; (j) *N*-Fmoc-L-leucine, EDCI/HOBt/*i*-Pr_2NEt/DCM; (k) diethylamine/acetonitrile; (l) Cbz-phosphotyrosine di-*t*-butyl ester, EDCI/HOBt/*i*-Pr_2NEt/DCM; (m) H_2/10% Pd–C/MeOH; (n) acetic anhydride/*i*-Pr_2NEt/DCM; (o) TFA/TES/DCM, rt, 30 min.

ester yielded compound **13**. Hydrogenation of **13** to remove the Cbz group, followed by acetylation, and removal of the two *t*-butyl groups afforded the designed target compound **2**.

In order to determine quantitatively the binding affinity of compounds **1** and **2** to STAT3, we have developed a fluorescence-polarization-based binding assay. A previous study[13] showed that a peptide with the GpY*LPQTV sequence derived from gP120 protein binds to STAT3 with a much higher affinity than the native STAT3 peptide. We have therefore synthesized a peptide with the β(Ala)-β(Ala)-G-pY*LPQTV sequence labeled with a fluorescence tag, 5-carboxyfluorescein (5-FAM) at the N-terminus and expressed and purified a recombinant STAT3 protein (residues 127–722). It was determined that this fluorescently tagged peptide binds to the recombinant STAT3 with a K_d value of 50 nM, consistent with its high affinity reported in the previous study.[13] Using this FP-based assay, it was determined that compounds **1** and **2** are capable of competing with the binding of the fluorescently tagged peptide to STAT3 in a dose-dependent manner with K_i values of 25.9 and 7.3 μM, respectively (Fig. 3). Hence, compound **2** is 3 times more potent than the lead peptide **1**.

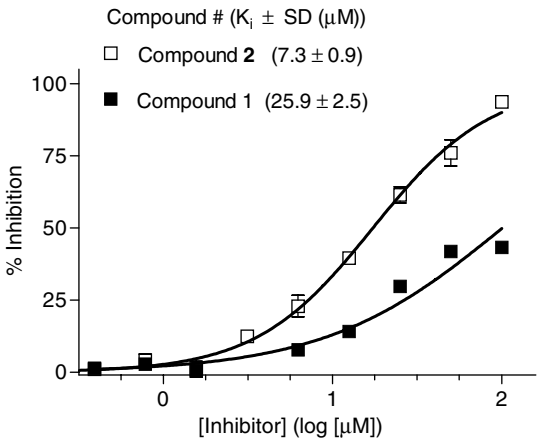


Figure 3. Competitive binding curves of compounds **1** and **2** to STAT3 as determined using a fluorescence-polarization-based binding assay.

In summary, a new, conformationally constrained macrocyclic compound **2** has been designed and synthesized using intramolecular 'click chemistry' as an inhibitor of STAT3. Compound **2** binds to STAT3 with a K_i value of 7.3 μM, and is a promising initial lead compound for further optimization for the design and development of potent, cell-permeable, small-molecule inhibitors of STAT3 as a new class of anticancer drugs.

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12. ^{1}H NMR of key intermediate **8**: ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 8.55 (d, J = 9.0 Hz, 1H), 7.89 (d, J = 9.4 Hz, 1H), 7.51 (s, 1H), 6.49 (d, J = 7.8 Hz, 1H), 4.87–4.80 (m, 1H), 4.32–4.09 (m, 4H), 3.72–3.69 (m, 1H), 3.67 (s, 3H), 3.20–3.15 (m, 1H), 3.01–2.92 (m, 1H), 1.80–1.30 (m, 4H), 1.35 (s, 9H), 1.01 (s, 9H), 1.02 (d, J = 6.0 Hz, 3H), 0.92–0.60 (m, 2H).
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