

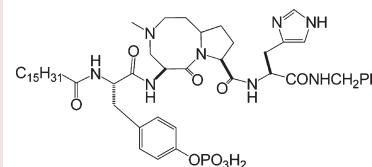
Structure-Based Design of Conformationally Constrained, Cell-Permeable STAT3 Inhibitors

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ABSTRACT We report herein the structure-based design of a class of conformationally constrained, potent, cell-permeable small-molecule inhibitors to target the SH2 domain in STAT3. Compound **11** (CJ-1383) binds to STAT3 with a K_i value of 0.95 μM , dose-dependently inhibits cellular STAT3 signaling and cancer cell growth, and induces apoptosis in the MDA-MB-468 cancer cell line with constitutively activated STAT3.

KEYWORDS STAT3 inhibitor, SH2 domain, apoptosis



STAT3, the signal transducer and activator of transcription 3, is a transcription factor that transmits signals directly from cell surface receptors to the nucleus.¹ Persistent activation of STAT3 signaling has been demonstrated to contribute directly to oncogenesis by stimulating cell proliferation and preventing apoptosis in human cancer cells.^{1–7} Activation of STAT3 not only provides a growth advantage to tumor cells, allowing their accumulation, but also confers resistance to conventional therapies that rely on apoptotic machinery to eliminate tumor cells.^{1–7} STAT3 is thus an attractive cancer therapeutic target.^{1–8}

STAT3 is recruited from the cytosol and has specific interactions through its Src homology 2 (SH2) domain with different cytokine receptors possessing phosphotyrosine docking sites. STAT3 then becomes phosphorylated on a carboxyl terminal tyrosine, and this phosphorylation causes it to dimerize, translocate to the nucleus and bind to specific promoter sequences on its target genes. This dimerization of STAT3 is decisive to its activation.^{7,8} Thus, it has been proposed that small-molecule inhibitors that bind the SH2 domain of STAT3 can block its interaction with cytokine receptors, as well as its homodimerization, leading to efficient inhibition of its activity.⁹

There have been considerable efforts in the design of both peptidic and non-peptidic inhibitors that target the SH2 domain in STAT3.^{9–27} While peptide-based inhibitors can bind to STAT3 with high affinities, they suffer from the lack of cellular permeability due to both their peptidic nature and the negative charges on the phosphotyrosine group. On the other hand, non-peptidic small-molecule inhibitors are cell-permeable, but most of the reported compounds bind to STAT3 with weak affinities and the cellular activity cannot be clearly attributed to STAT3 targeting. Recently, McMurray and colleagues have reported the successful design of cell-permeable peptidomimetics that inhibit STAT3 activity in cells using a prodrug strategy.²⁶ Despite these efforts, design

of potent and cell-permeable STAT3 inhibitors remains a very challenging task.

In this study, we report the structure-based design, synthesis and evaluation of a new class of conformationally constrained peptidomimetic inhibitors of STAT3. Our efforts have produced a set of compounds that bind to STAT3 with high affinities. Furthermore, by incorporating a long hydrocarbon chain into one STAT3 ligand to improve cell-permeability, we have successfully obtained a potent and cell-permeable STAT3 inhibitor.

We previously reported the design and synthesis of compound **1** (Figure 1), starting from the phosphorylated hexapeptide (pYLPQTV) derived from the gp130 protein sequence.²⁵ Compound **1** binds to STAT3 with a K_i value of 350 nM.²⁵ Examination of the predicted binding model for **1** in complex with STAT3 suggested that the isobutyl group of the leucine may be cyclized with the five-membered ring of the proline to form the bicyclic lactam without distorting the β -turn conformation of **1** (Figure 2). This led to the design of compound **2**.

Compound **2** was synthesized (Supporting Information) and determined to have a K_i value of 17 nM to STAT3 in our competitive, fluorescence-polarization (FP)-based assay (Figure 3 and Supporting Information). Hence, compound **2** is 20 times more potent than the initial peptide **1**, representing a potent lead compound for further structure–activity relationship studies.

Previous studies have shown that the Gln residue is of critical importance for the binding of peptide-based inhibitors to STAT3.¹⁰ Compounds **3** and **4** were thus designed to further investigate the importance of the side chain of the Gln residue in compound **2** for binding to STAT3. When the Gln side chain in **2** is replaced by a methyl group or an ethyl

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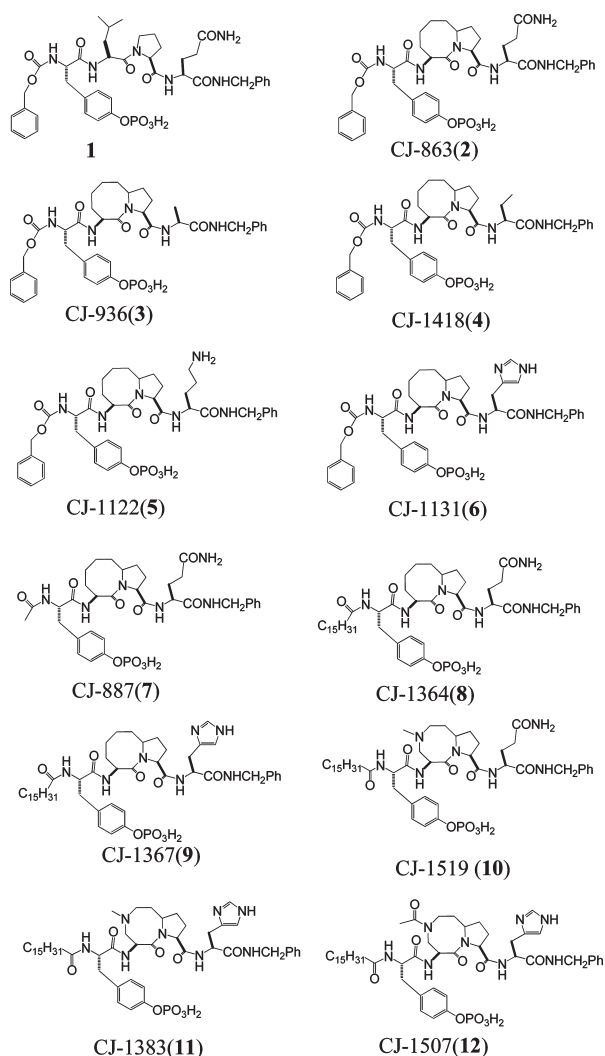


Figure 1. A peptide lead and designed conformationally constrained peptidomimetics.

group, the K_i values of resulting compounds 3 and 4 are 3.23 μM and 8.57 μM , respectively. Thus, compounds 3 and 4 are 190 and 500 times less potent than 2, respectively. When the amide carbonyl group of the Gln residue is reduced, the resulting compound 5 binds to STAT3 with a K_i value of 207 nM, being 10 times less potent than 2. When the Gln residue is replaced by a His residue, the resulting compound 6 has a K_i value of 435 nM, being 25 times less potent than 2. Taken together, these data clearly show that both the carbonyl and amino groups in the side chain of the Gln residue make significant contributions to the binding of compound 2 to STAT3.

To investigate if the Cbz group in 2 contributes to its binding to STAT3, it is replaced by an acetyl group. The resulting compound 7 has a K_i value of 15 nM and is thus equipotent with 2, indicating that the Cbz group in 2 makes no significant contribution.

We evaluated the ability of compounds 2–7 to inhibit cell growth in MDA-MB-231 and MDA-MB-468, two human breast cancer cell lines with very high levels of phosphorylated

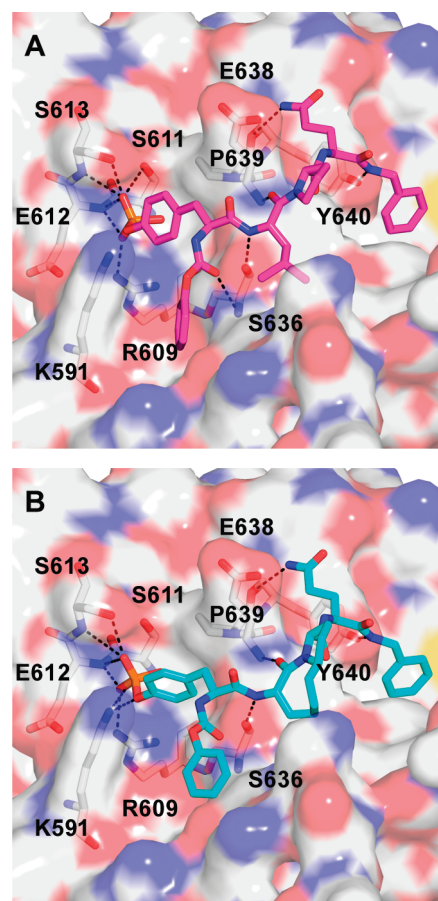


Figure 2. Predicted binding mode for compound 1 (A) and compound 2 (B). Carbon in gray, violet, and cyan for the protein, compound 1 and compound 2, respectively. Nitrogen, oxygen, sulfur and phosphorus are shown in blue, red, yellow and orange, respectively. Protein residues likely to be involved in polar interactions are shown in sticks, with interactions indicated by dashed lines. The figures were generated using Pymol.

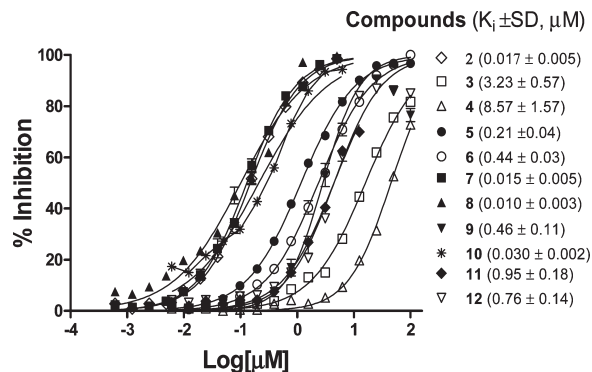


Figure 3. Competitive binding curves of designed compounds to STAT3 protein as determined in an FP-based binding assay and their K_i values and standard deviations. For each compound, 3–5 independent experiments were performed according to the optimized assay conditions, and the K_i values were calculated using a mathematical model developed for FP-based assays.

STAT3. To our disappointment, none of these compounds show any cellular activity at concentrations as high as 100 μM ,

with the exception of compound **3**, which shows weak activity.

In an effort to overcome the lack of cellular activity of compound **7**, we introduced a long lipid chain to its N-terminus. The resulting compound **8** has a K_i value of 10 nM and is as potent as compound **7**. Compound **8** dose-dependently inhibits cell growth in the MDA-MB-231 and MDA-MB-468 cell lines with IC_{50} values of 25 and 35 μ M, respectively (Supporting Information).

Although the amide group in the Gln side chain in compound **8** is clearly important for binding to STAT3 based upon the data for compounds **2–6**, the primary amide group may be detrimental for cell-permeability. We therefore designed **9** in which the Gln residue is replaced by histidine. Compound **9** binds to STAT3 with a K_i value of 458 nM, and is thus 45 times less potent than **8**. However, compound **9** has a similar potency to compound **8** in inhibition of cell growth in both the MDA-MB-231 and MDA-MB-468 cancer cell lines (Supporting Information), with IC_{50} values of 50 and 70 μ M, respectively. These data suggest that the Gln residue is indeed detrimental to the cellular permeability of our STAT3 ligands.

Both compounds **8** and **9** bear negative charges due to their phosphotyrosine group. We next investigated if replacement of a carbon atom in the 8-membered ring in these compounds with a positively charged nitrogen, which reduces the overall negative charge of the entire molecule, can further improve their cellular activity without significant detrimental effect on their binding to STAT3. The resulting compounds **10** and **11** have K_i values of 45 nM and 950 nM, respectively, to STAT3, and are thus 2–5 times less potent than their counterparts **8** and **9**. Hence, introduction of a positively charged nitrogen into the 8-membered ring has a modest negative effect on their binding to STAT3. Both **10** and **11** dose-dependently inhibit cell growth in the MDA-MB-231 and MDA-MB-468 cancer cell lines (Supporting Information). While **10** is only slightly more potent than **8**, compound **11** is 5–10 times more potent than compound **9** in both cancer cell lines. Compound **11** achieves IC_{50} values of 11.2 μ M and 3.6 μ M, respectively, in the MDA-MB-231 and MDA-MB-468 cancer cell lines (Supporting Information), respectively, in the cell growth inhibition assay.

To investigate whether the positively charged nitrogen in the 8-membered ring is important for cellular activity, we have synthesized compound **12**, in which the methyl group is replaced with an acetyl group. While compound **12** binds to STAT3 with a K_i value of 0.76 μ M, similar to that of compound **11**, it is 4–10 times less potent than **11** in the cell growth assay in the MDA-MB-231 and MDA-MB-468 cancer cell lines.

It has been hypothesized that direct inhibition of the STAT3 activity in tumor cells can lead to apoptosis induction.^{3–5} We next evaluated compound **11** for its ability to induce apoptosis in the MDA-MB-468 cancer cell line. As can be seen in Figure 4, compound **11** effectively induces apoptosis in a dose- and time-dependent manner. For example, compound **10** at 10 μ M for 24 and 48 h treatment induces 26% and 35% of the MDA-MB-468 breast cancer cells to undergo apoptosis, respectively.

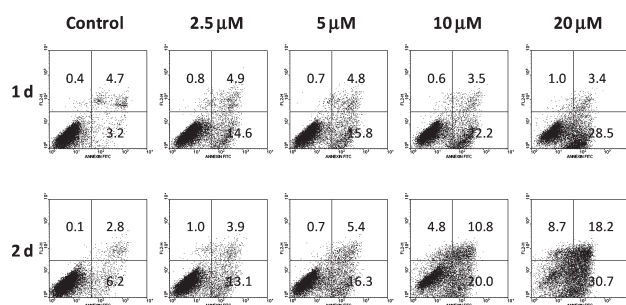


Figure 4. Analysis of apoptosis induction by compound **11** in the MDA-MB-468 cell line. Cells were treated with different concentrations of **11** for 24 or 48 h, and apoptosis was analyzed using Annexin V and propidium iodide double staining by flow cytometry. Percentage of early apoptotic (Annexin V+/PI-), late apoptotic (Annexin V+/PI+), and dead (Annexin V-/PI+) cells are shown, respectively.

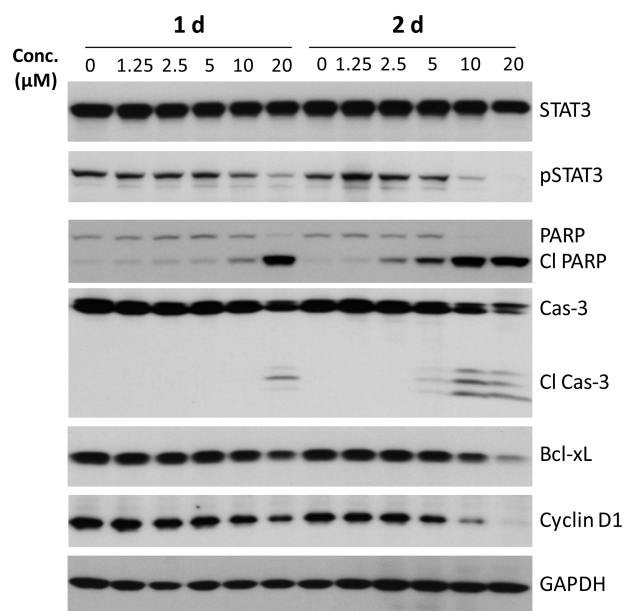


Figure 5. Western blot analysis of biochemical markers for apoptosis induction and inhibition of STAT3 activity by compound **11** in the MDA-MB-468 cell line. Cells were treated with **11** for 24 and 48 h, and levels of STAT3, pSTAT3, full-length PARP, cleaved PARP (Cl PARP), caspase-3, cleaved caspase-3 (Cl Cas-3), Bcl-xL, and cyclin D1 were probed by specific antibodies. GAPDH was used as the loading control.

To test whether **11** can effectively inhibit STAT3 activity and induce apoptosis at similar concentrations and time-points, we examined a number of molecular markers in the MDA-MB-468 cancer cells. As shown in Figure 5, compound **11** induces cleavage of caspase-3 and PARP, two critical apoptosis biochemical markers in a dose- and time-dependent manner. Compound **11** induces robust cleavage of caspase-3 and PARP at 5–10 μ M for 2-day treatment. Consistent with its mechanism of action, compound **11** also effectively reduces the levels of phospho-STAT3 protein at 5–10 μ M for 2-day treatment, without affecting the levels of total STAT3 protein. Furthermore, it significantly decreases the levels of Bcl-xL and cyclin D1 proteins, two known

transcriptional targets of STAT3 at 10 μM for 2-day treatment (Figure 5).^{1–5} These cellular data clearly show that compound **11** effectively inhibits STAT3 activity in the MDA-MB-468 cancer cell line at concentrations and time-points at which robust apoptosis of the tumor cells is induced.

In summary, we have successfully designed and synthesized a series of potent, conformationally constrained peptidomimetic inhibitors of STAT3. Several of these compounds bind to STAT3 with low nanomolar affinities. Significantly, compound **11** binds to STAT3 with a K_i value of 0.95 μM and effectively inhibits cell growth with IC_{50} values of 3–11 μM in two breast cancer cell lines with high levels of phosphor-STAT3. Our data further show that compound **11** is effective in inhibition of STAT3 activity and induction of apoptosis in the MDA-MB-468 cancer cell line in a time- and dose-dependent manner. Hence, compound **11** is a cell-permeable and a *bona fide* STAT3 inhibitor. Compound **11** therefore represents a useful pharmacological tool with which to investigate the role of STAT3 in cancer cells, and it is a promising lead compound for further optimization toward our goal of developing a new class of anticancer drugs by targeting STAT3.

SUPPORTING INFORMATION AVAILABLE The information on the synthesis and chemical data for compounds **2–12**, molecular modeling methods and results for **2–12**, the experimental procedure for the fluorescence polarization-based binding assay and details on the cellular growth inhibition assay. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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