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Discovery of the catechol structural moiety as a Stat3 SH2 domain inhibitor by virtual screening

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ABSTRACT

The Stat3 SH2 domain is essential for its activation, and development of a potent SH2 inhibitor will be therapeutically valuable in treating cancers with constant Stat3 activation. We report here the identification of the catechol (1,2-dihydroxybenzene) structural moiety by virtual screening as a Stat3 SH2 inhibitor. The catechol compound docked to the Stat3 SH2 domain in computer modeling forms hydrogen bonds with the conserved pTyr-interacting amino acids. In the biochemical assay, a catechol-containing compound, but not the hydroxyl group-acetalized analogue, was able to inhibit Stat3 DNA-binding activity. Furthermore, the catechol compound was demonstrated to compete with pTyr peptides in binding to the Stat3 SH2 domain, suggesting that the catechol moiety is a pTyr bioisostere and may potentially be used for designing cell-permeable SH2 inhibitors. In our preliminary effort, we also demonstrated that the potency of catechol compound as Stat3 SH2 inhibitors could be improved by modifying the non-catechol part of the compound structure.

Excessive activation of signal transducers and activators of transcription 3 (Stat3) has been proven to be protumorigenic by promoting cancer cell growth, survival, angiogenesis, and immune evasion.[1] Suppression of Stat3 activation inhibits growth and induces apoptosis in cultured cancer cells and cancer animal models, validating Stat3 as an attractive anticancer drug target.[1,2] Activation of Stat3 requires Stat3 Src homology 2 (SH2) domain to bind first phosphorylated tyrosine of activated growth or cytokine receptors at plasma membranes, and then reciprocally the pTyr705 from another Stat3 molecule to form transcriptionally active dimers.[3] Targeting Stat3 SH2 by small molecule inhibitors is therefore expected to effectively abrogate Stat3 activity, as being demonstrated with phosphotyrosyl peptides targeting the SH2 domain.[4,5]

The SH2 protein domain is a well-characterized small protein module of approximately 100 amino acids identified in proteins of diverse functions.[6] Since SH2 domains of signaling proteins selectively bind phosphotyrosine (pTyr) peptides to mediate cellular signaling pathways that are frequently implicated in cancer, inflammation, allergy, and many other diseases, developing SH2 inhibitors to disrupt pTyr–SH2 interactions has long been an attractive strategy for drug discovery.[7] The overall structure of the SH2 domain is conserved among proteins, in which a central β sheet plane divides the compact domain into a pTyr-binding side and a side for interacting with the 3–5 amino acids immediate C-terminal to the pTyr.[8] This detailed characterization of SH2–pTyr peptide interactions in 3D structures has greatly assisted in structure-based approaches to develop potent SH2 inhibitors for Src and Grb2.[9]

The X-ray structure of Stat3 dimer complexing with DNA[10] was previously used in designing peptidomimetic SH2 inhibitors,[11,12] and led to two successful virtual screening efforts that identified Stat3 inhibitors STA-21[13] and S3I-201[14] with in vitro and in vivo anti-tumor activity. A Stat3 SH2 inhibitor was also identified through a biochemical screening of the chemical library against a Stat3 SH2 domain-binding phosphopeptide, further supporting that the domain is a potential target for drug delivery.[15,16] Here, we report the discovery of the catechol structural moiety as a Stat3 SH2 inhibitor by virtual screening of the Wyeth's proprietary small molecule collection.

We conducted a virtual screening against the Stat3 SH2 domain using the database of 112,386 lead-like compounds with known 3D structures. The crystal structure of the dimerized Stat3 at 2.25 Å was downloaded from the RCSB Protein Data Bank (PDB code: 1BG1) for the virtual screening. Schrödinger's docking program GLIDE (grid-based *li*gand *d*ocking with *e*nergetics) was employed to dock the pre-generated 3D structures of compounds

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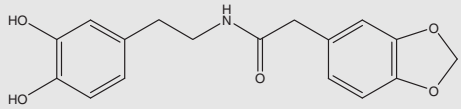
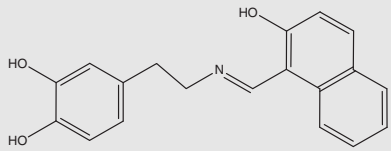
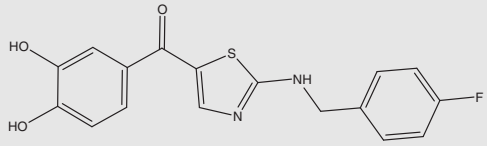
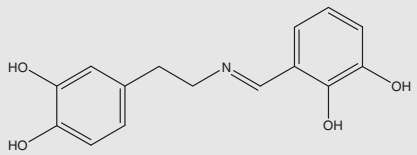
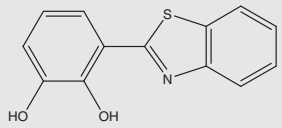
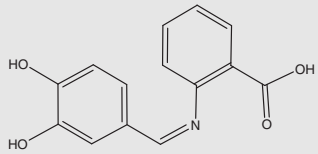
into the SH2 domain. The screening target is prepared as follows: (1) monomer A of 1BG1 was used as the docking target with water molecules removed. (2) Hydrogens and OPLS-defined atomic parameters were assigned to the protein residues, and a restrained minimization of the protein was performed by the IMPREF utility of GLIDE. The minimized geometry had an RMSD of all heavy atoms within 0.8 Å of the crystallographically determined positions. Residues were not neutralized. (3) The centroid of the pTyr705 of the monomer B was used as the center of the grid box. The size of the box was $20 \times 20 \times 20$ Å^3, which covered the entire dimerization interface. No constraints were employed in the docking.

A quick docking run with high-throughput-virtual screening mode was performed with a limit of five retained poses per molecule. The top 50,000 poses were saved and later were identified from approximate 12,000 molecules. Next, these molecules were re-docked with Standard-Precision (SP) mode and a list of top 1200 molecules was generated. These 1200 molecules were finally docked with Extra-Precision (XP) mode and visualized to generate a list of 1000 molecules for physical screen in the bioassay. During the in silico screening, the van der Waals radii of the ligands were scaled by 0.8 for non-polar atoms to introduce a modest induced fit binding of compounds to the domain.[17]

The 1000 compounds coming out of the in silico screening were tested in a Stat3 DNA-binding ELISA that, similar to an electrophoretic mobility-shift assay (EMSA), measured the binding of active Stat3 dimers to its cognate DNA-binding elements.[18] The active form of Stat3 was prepared in whole-cell lysate from human multiple myeloma U266 cells that contained constitutively active Stat3 as the result of IL-6 autocrine stimulation.[19] A Stat3 SH2 domain-binding pTyr peptide, S(pY)LPQTV from IL-6 receptor gp130, inhibited Stat3 activity in the assay with an IC_{50} of 8 μM. In contrast, a non-phosphorylated peptide tested up to 1000 μM had no effect (data not shown), demonstrating that the assay is capable of identifying SH2 inhibitors.[20] The compounds were screened at a concentration of 100 μg/mL in the assay[20] and 56 were shown to inhibit Stat3 DNA-binding by greater than 10% as compared to the DMSO-treated control. By scrutinizing of the structures of the active compounds, we identified a group of hits that shared in common a catechol (or pyrocatechol, IUPAC) structural moiety were identified. The GLIDE scores of the catechol hits ranged from −6.75 to −4.59 with the lower values generally corresponding to more potency in the biochemical assay (Table 1). Compound **6** was a catechol compound ranked close to the top 1000 with a GLIDE score of −4.59 and showed only 8% inhibition of Stat3 activity (Table 1). In comparison, a Stat3 SH2 inhibitor STA-21 previ-

Table 1
Selected catechol structures coming out of the virtual screening and the Stat3 DNA-binding ELISA screening

Compound	Structure	GLIDE score[a]	VS rank[a]	% Inhibition in ELISA[b]	Screening concentration[c] (μM)
1		−6.23	229	40	317.2
2		−6.75	101	50	325
3		−6.57	126	57	290.4
4		−5.33	701	51	365.9
5		−4.83	963	56	411
6		−4.59	1059	8	388.7

[a] GLIDE scores and virtual screening (VS) ranking of the catechol compounds among the top 1200 compounds by the Extra-Precision mode of the computer program.
[b] % Stat3 inhibition in the ELISA at a screening concentration of 100 μg/mL.
[c] Molar concentrations (μM) of compounds screened at 100 μg/mL.

ously identified through in silica screening[13] was docked with a GLIDE score of −5.48. The results validated the effectiveness of our virtual screening model in identifying hits, and particularly catechol hits, as Stat3 inhibitors. The purity and integrity of **1** were confirmed with HPLC, and its activity as a SH2 inhibitor was further studied.

The significance of the catechol moiety in contributing to Stat3 SH2 binding was revealed when **1** was docked onto the domain. The modeling positioned catechol right in the pTyr-binding pocket surrounded by the conserved pTyr-binding polar residues Lys591, Arg09, Glu612, and Ser613 (Fig. 1). The two hydroxyl groups were close to Arg609 and Glu612, respectively, and formed hydrogen bonds with the residues, a common feature also observed for other catechol hits (Fig. 1). The GLIDE XP docking result of **1** on the Stat3 SH2 domain and the enrichment of the catechol moiety in active hits suggested that this unit could bind to the SH2 domain as a pTyr mimetic.

To confirm this idea, we synthesized **7** (Fig. 2A), which had the catechol hydroxyl groups of **1** protected by acetals and lost the two hydrogen donors involved in the binding, and tested **1** and **7** in the Stat3 DNA-binding ELISA at various concentrations. Like the Stat3 SH2 domain-binding pTyr peptides, **1** could also inhibit Stat3 DNA-binding in a dose-dependent manner with an IC_{50} at 357 ± 35 μM (n = 7) (Fig. 2B and C). In contrast, **7** with the lost hydrogen donors abolished the activity of Stat3 inhibition (Fig 2C), strongly supporting the role of catechol moiety in inhibiting Stat3 activity. The **1**-treated whole-cell lysates were also analyzed by Western blot and no loss of Stat3 phosphorylation at Tyr705 was detected (Fig. 2D), which is consistent with the idea that the mechanism of action of **1** as a Stat3 inhibitor is to bind to the SH2 domain rather than dephosphorylate pTyr705. To further confirm that **1** binds to the SH2 domain, we tested the compound in a fluorescence polarization-based binding assay previously developed for assaying the binding of pTyr peptides to SH2 domains of Stat proteins.[21] We expressed and purified the flag-tagged mouse Stat3 from serum-starved Hek293 cells[22] to maximize the amounts of unphosphorylated Stat3 in the purification (Fig. 3A). Incubation of increasing concentrations of the purified Stat3 with 3 nM of carboxyfluorescin-S(pY)LPQTV probes generated a binding curve with a calculated dissociation constant K_d at 650 nM (Fig. 3B), which is in alignment with binding affinities between pTyr peptides and Stat SH2 domains.[21] As expected, an excessive amount of unlabeled pTyr peptides, but not non-phophorylated peptides could compete with the probe (data not shown). To this binding assay, 50, 100, and 200 μM of **1** were added. The increasing concentrations of the compound led to a right-shifted Stat3-peptide binding curve (Fig. 3C). At 2000 nM of Stat3, a concentration that nearly saturates the peptide binding as suggested by the binding curve, the presence of the compound up to 200 μM did not inhibit Stat3-peptide binding (Fig. 3C). Thus, the data are consistent with that **1** is a competitive Stat3 SH2 inhibitor, as proposed by the computer modeling.

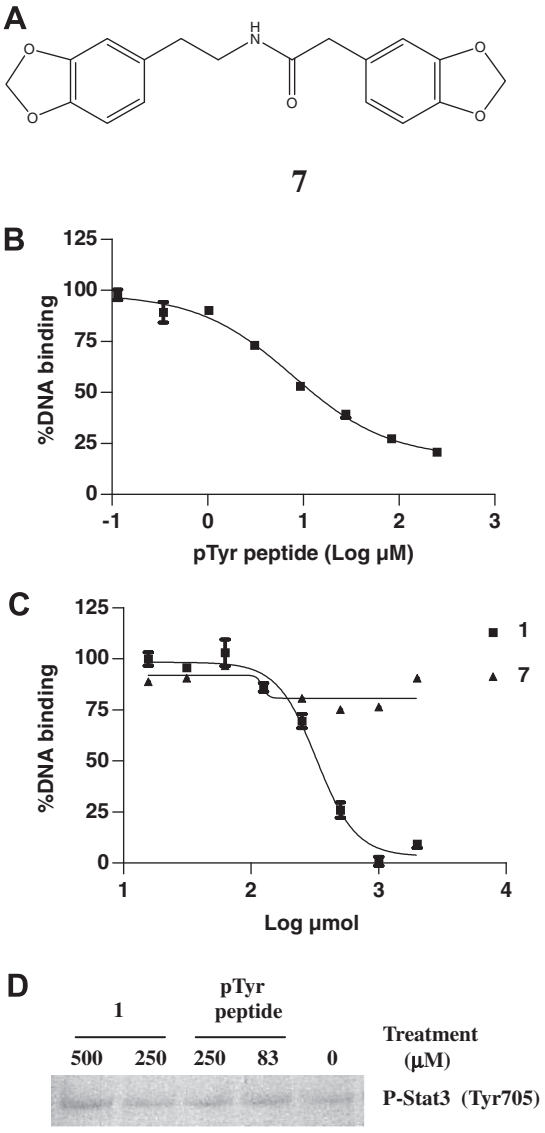


Figure 2. (A) Structure of **7**. (B) Inhibition of Stat3 DNA-binding by pTyr peptide. (C) Inhibition of Stat3 DNA-binding by **1** and **7**. (D) pTyr peptide- and **1**-treated lysates were also analyzed by Western blot to examine Stat3 phosphorylation at Tyr705.

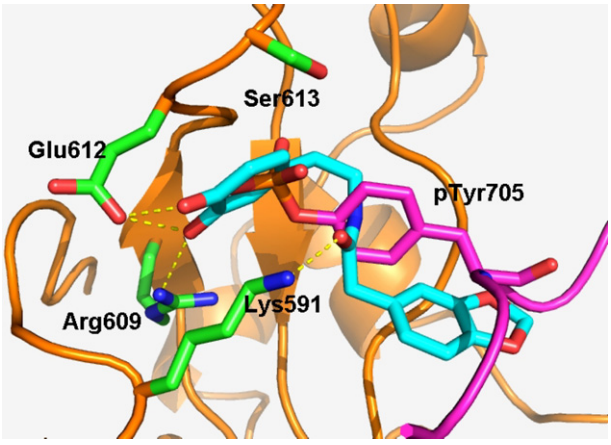


Figure 1. Proposed binding modes of 1 (cyan) and pTyr705 (magenta) in the SH2 domain of stat3. The predicted hydrogen bonds are indicated with yellow dotted lines. The pTyr705 is from the monomer B of stat3 homodimer.

With the confirmation of **1** as the Stat3 SH2 inhibitor, we searched and collected the existing catechol-containing compounds from Wyeth's compound library. We screened the compounds in Stat3 DNA-binding ELISA and identified compound **8** with an IC_{50} of 106 ± 19 μM (n = 4) that is more than 3-fold improvement from that of **1** (Fig. 4), suggesting that modification on non-catechol structure of the compound has the potential to further improve compound potency.

The well-characterized SH2 structures from many proteins have led to the successful design of highly potent SH2 inhibitors for Src and Grb2 using structure-based drug design approaches.[9] These SH2 inhibitors are bipartite in composition, including a pTyr bioisostere to mimic pTyr binding and peptidomimetic or non-peptide

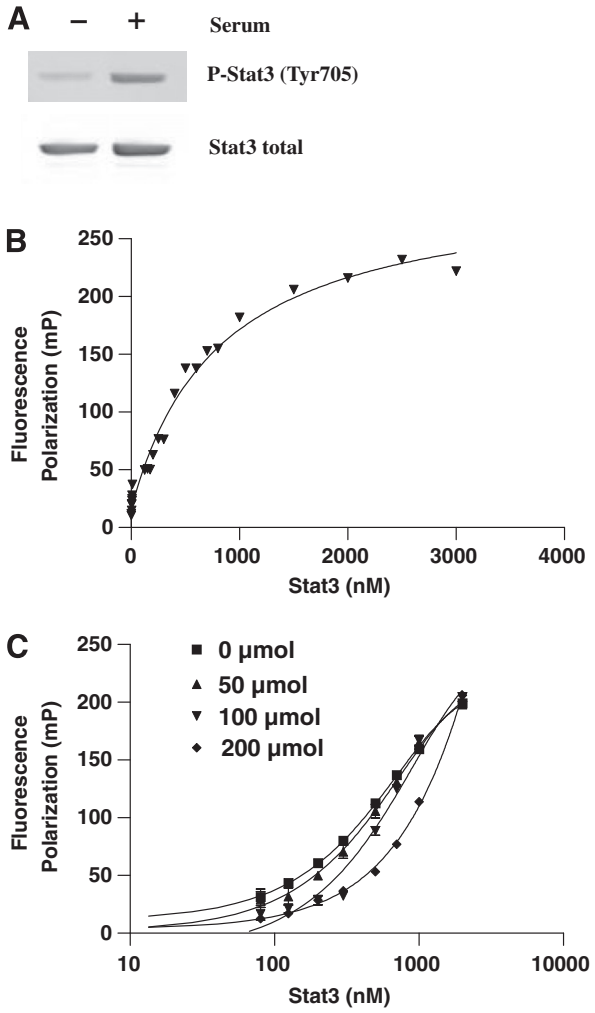


Figure 3. (A) Affinity-purified Stat3 from serum-starved or non-starved Hek293 cells was examined by Western blot with antibodies against Stat3 phosphorylation or total protein. Serum-starvation significantly lowered level of Stat3 phosphorylation. (B) Fluorescence polarization of fluorescent pTyr peptide as a function of Stat3 concentration. (C) Increasing concentration of catechol **1** was added to the mixture of fluorescent pTyr and Stat3 to test its inhibitory effect on the binding. Each data point represents the mean of three measurements.

templates to explore the interactions outside of the pTyr-binding pocket. The commonly used pTyr mimetics include phosphonate- or carboxylate-based negative-charged moieties.[9] Compared to these, catechol forms fewer hydrogen bonds and no charged interactions in the Stat3 SH2 domain in the computer modeling, which may account partly for its lower potencies in the ELISA and fluorescent binding assays. However, unlike the charged pTyr mimetics, catechol is cell-permeable and the catechol-containing compounds are active in cellular assays (data not shown). Future efforts will continue to focus on improving the potency for catechol-containing compounds. Work on pTyr peptide–SH2 interactions and current SH2 inhibitors has suggested that structures responsible for interactions outside of the SH2 pTyr-binding pocket work synergistically with phosphotyrosine to determine SH2-binding affinity. For example, it has been shown that the pTyr within a peptide of optimal amino acid sequence can have more than a 1000-fold higher affinity for the SH2 domain than pTyr within a peptide of random sequence context.[23] Following this direction, there is a great potential to further improve on catechol compounds to generate high affinity and cell-permeable SH2 inhibitors, as we have demonstrated in the preliminarily effort.

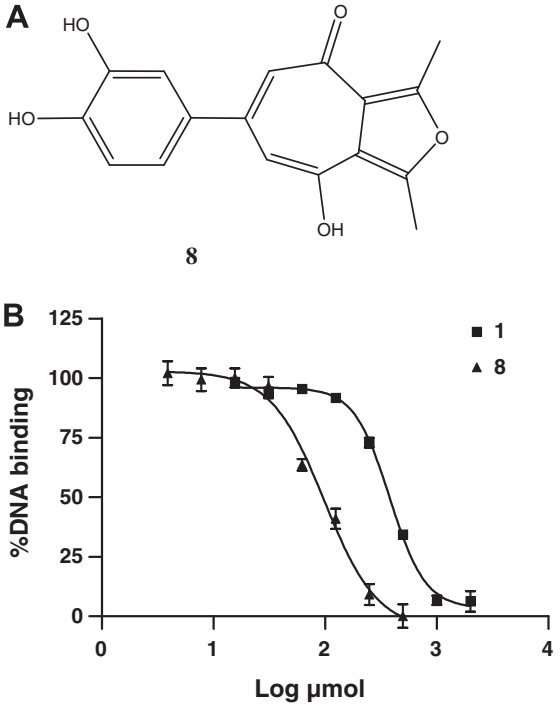


Figure 4. Compound **8** showed an improved potency of inhibiting Stat3 activity.

Acknowledgments

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18. Stat3 DNA-binding ELISA kit was purchased from ActiveMotif. To test Stat3 DNA-binding, 5 μL of the U266 whole-cell lysate was diluted with 25 μL of the complete lysis buffer from the kit. Next, 25 μL of the mixture was transferred into the wells of the assay plate (provided in the kit) pre-loaded with 25-μL complete binding buffer from the kit. The reaction mixture was incubated at room temperature for 1 h and the assay was proceeded to the completion as instructed.
19. The whole-cell lysate was made from U266 cells growing in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1 mM glutamine, 50 U/mL penicillin G (sodium salt), and 50 μg/mL streptomycin sulfate (Invitrogen). Cells at a density of 75,000 cells/mL were collected by centrifugation. The cell pellet was washed once with cold PBS containing phosphatase inhibitors from

the ActiveMotif's Nuclear Extract Kit and lysed with the buffer of 20 mM Hepes, pH 7.5, 350 mM NaCl, 20% glycerol, 1% igepal, 1 mM $MgCl_2$, 0.5 mM EDTA, and 0.5 mM EGTA. The cell pellet from 1-mL culture was solubilized in 30 μL of lysis buffer. The lysate was vortexed briefly and incubated on ice for 15 min, followed by centrifugation at 15,000*g* for 20 min at 4 °C. The supernatant was collected and stored frozen at −80 °C until use. The protein concentration was measured by Bradford dye-binding assay from Bio-Rad.

20. To test Stat3 inhibition, inhibitors were first diluted in the completed binding buffer prior to mixing with the whole-cell lysate diluted in the complete lysis buffer. Equal volumes of diluted compounds and the whole-cell lysates were mixed, and 50 μL of the mixture was then added to the wells of the assay plate.
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22. The Stat3 expression vector pCMV-Stat3-Flag was kindly provided by Dr. Jiayuh Lin, Ohio State University, Columbus, Ohio. The vector was transfected by Lipofectamine 2000 (Invitrogen) into Hek293 cells cultured in DMEM with 10% fetal bovine serum and 1 mM glutamine (Invitrogen). After 68-h protein expression, the medium was changed to serum-free DMEM to starve cells for 4 h in an attempt to increase the unphosphorylated Stat3 in the purification. The cells were then washed with cold PBS and lysed in CelLytic M buffer (Sigma). Stat3 was purified by ANTI-FLAG M2 affinity gel (Sigma) following the manufacturer's instructions. The protein was eluted from the beads with the FLAG peptides (Sigma) at 100 μg/mL in 50 mM Hepes, pH 7.5 containing 100 mM NaCl, 1 mM EDTA, 2 mM DTT, and 10% glycerol, concentrated to the desired concentration and stored frozen at −80 °C.
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