Novel peptidomimetic inhibitors of signal transducer and activator of transcription 3 dimerization and biological activity

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Abstract

The critical role of signal transducer and activator of transcription 3 (Stat3) in the growth and survival of human tumor cells identifies it as a promising target for cancer drug discovery. We previously identified a Stat3 SH2 domain-binding phosphopeptide, PY*LKTK, and its tripeptide derivatives, PY*L and AY*L (where Y* represents phosphotyrosine), which inhibit Stat3 biochemical activity and biological function. Here, we report novel peptidomimetic compounds based on PY*L (or AY*L) with substitution of the Y-1 residue by benzyl, pyridyl, or pyrazinyl derivatives that are selective and greater than 5-fold more potent in disrupting Stat3 activity *in vitro* than lead tripeptides. The biological activities of these derivatives mirror that originally observed for peptides. In this context, the representative peptidomimetic ISS 610 with 4-cyanobenzoate substitution inhibits constitutive Stat3 activity in Src-transformed mouse fibroblasts and human breast and lung carcinoma cells. This effect is not evident with the non-phosphorylated counterpart, ISS 610NP, consistent with interaction of peptidomimetics with the SH2 domain of Stat3. Moreover, ISS 610 induces cell growth inhibition and apoptosis of Src-transformed fibroblasts that contain persistently active Stat3. We present the first report of a peptidomimetic approach to design of small-molecule inhibitors of Stat3 that are also among the first examples of disruptors of transcription factor dimerization with the potential for novel cancer therapy. [Mol Cancer Ther. 2004;3(3):261–269]

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Introduction

Signal transducer and activator of transcription (STAT) proteins are latent cytoplasmic transcription factors that are activated in response to cytokines and growth factors, and consequently regulate cellular processes, including proliferation, differentiation, and survival [reviewed in Refs. (1–10)]. STAT activation is dependent on tyrosine phosphorylation, which induces dimerization via reciprocal phosphotyrosine (pTyr)-SH2 interactions between two STAT monomers and is a requirement for binding to specific DNA response elements (11).

A large number of studies on persistent activation of specific STAT family members, particularly Stat3, have shown a strong link to growth and survival of transformed and tumor cells [reviewed in Refs. (12–17)], thus establishing a causal role for abnormal Stat3 activity in malignant transformation. In many tumor cells harboring persistent Stat3 activity, inhibition of Stat3 signaling induces growth arrest and apoptosis. This critical role of Stat3 in the molecular pathogenesis of many tumors provides a validation for targeting this protein for cancer drug discovery [reviewed in Refs. (15, 18)].

We have taken a semi-rational approach to develop specific small-molecule inhibitors of Stat3. On the basis of the known importance of pTyr-SH2 interactions for STAT dimerization (11, 19, 20), we previously identified a dimerization-disrupting phosphopeptide sequence that is derived from the SH2 domain-binding region of Stat3, PY*LKTK (where Y* represents phosphotyrosine) and its tripeptide derivatives PY*L and AY*L, as inhibitors of Stat3 activation and biological function (21). As leads, the tripeptides PY*L and AY*L were modified to generate a series of synthetic compounds with reduced peptide character, some of which reproduce the activities originally observed for the tripeptides. Specific peptidomimetics selectively disrupt Stat3 DNA-binding activity *in vitro* with potencies that are 5- to 10-fold better than previously obtained for lead peptides. Moreover, representative peptidomimetic ISS 610 reproduces the biological effects observed for phosphopeptide leads and induces suppression of Stat3-dependent Src transformation and cell proliferation, and promotes apoptosis. These novel peptidomimetic derivatives of Stat3 phosphopeptides retain the potency of interaction with and disruption of Stat3 activity as compared to the original peptides, and thus demonstrate the applicability of a peptidomimetic approach to the design of Stat3 inhibitors. Our findings also provide proof-of-principle that small-molecule inhibitors of Stat3 represent potential novel cancer therapeutic agents, and together with an earlier report of a peptidomimetic inhibitor of Myc/Max (22) are among the first examples of small-molecule inhibitors of transcription factor dimerization (1).

Materials and Methods

Cells and Reagents

Src-transformed NIH3T3/v-Src, NIH3T3/v-Src/pLucTKS3, NIH3T3/v-Src/pRLSRE, and Ras-transformed NIH3T3/v-Ras fibroblasts, human breast carcinoma MDA-MB-231, MDA-MB-435, MDA-MB-453, and MDA-MB-468 cells, as well as human lung carcinoma A459 cells have been previously described (21, 23–25). Cells were grown in DMEM containing 5% iron-supplemented bovine calf serum (BCS), with or without G418 or zeocin. The Apo-BrdUrd kit was from PharMingen (San Diego, CA).

Peptides

Peptides used in studies include PY*LKTK-AAVLLPVLLAAP and PYLKTK-AAVLLPVLLAAP and peptidomimetics based on PY*L and AY*L. The underlined amino acid sequence represents the membrane translocation sequence (MTS) (26). Peptides were synthesized by the Peptide Synthesis Laboratory, Queen's University, Kingston, ON, Canada. Peptidomimetics were synthesized manually using standard Fmoc solid phase chemistry. Peptides or peptidomimetics were used at concentrations up to 1 mM as indicated.

Plasmids

The Stat3 reporter, pLucTKS3, driving expression of the firefly luciferase gene and the Stat3-independent plasmid, pRLSRE, containing two copies of the serum response element (SRE) from the c-*fos* promoter (27, 28) that drives renilla luciferase gene (Promega, Madison, WI) expression, have been previously described (21, 29).

Recombinant Baculoviruses and Infection of Sf-9 Insect Cells

Stat1, Stat3, Jak1, and c-Src recombinant baculoviruses and infection of Sf-9 insect cells have been previously described (30). For protein expression of activated Stat1 or Stat3, Sf-9 insect cells were infected with viruses expressing either Stat1 or Stat3 in combination with viruses expressing Jak1 and/or c-Src.

Cytosolic Extract Preparation and Luciferase Assays

Cytosolic extract preparation from fibroblasts and luciferase assays were previously described (27, 29). Briefly, after two washes with PBS and equilibration for 5 min with 0.5 ml PBS-0.5 mM EDTA, cells were scraped off the dishes and the cell pellet was obtained by centrifugation (4500 × *g*, 2 min, 4°C). Cells were resuspended in 0.4 ml of low-salt HEPES buffer [10 mM HEPES (pH 7.8), 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM DTT] for 15 min, lysed by the addition of 20 μl of 10% NP40, and centrifuged (10,000 × *g*, 30 s, 4°C) to obtain the cytosolic supernatant, which was used for luciferase assays (Promega) measured with a luminometer. Cytosolic lysates containing activated Stat3 or Stat1 that were used for dissociation-reassociation analysis were prepared from baculovirus-infected Sf-9 insect cells as previously described (21, 30). Briefly, cultured dishes of Sf-9 cells were washed twice with ice-cold 1× PBS and then PBS containing 1 mM sodium orthovanadate. Cells were then lysed in 1% NP40 lysis buffer [50 mM HEPES (pH 7.9), 150 mM NaCl, 1% NP40, 20 mM NaF, 1 mM sodium orthovanadate, 1 mM tetrasodium pyrophosphate, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM EGTA, 2 mM EDTA, 0.1 μM aprotinin, 1 μM leupeptin, and 1 μM antipain] on ice for 10 min, and centrifuged (13,000 × *g*, 30 s, 4°C) to obtain lysate.

Nuclear Extract Preparation and Gel Shift Assays

Nuclear extracts were prepared from cell lines and used for electrophoretic mobility shift assay (EMSA) as previously described (23, 24, 27). In some cases, cells were pretreated with peptidomimetics for the indicated times (12–48 h) before harvesting for nuclear extract preparation. In other studies, nuclear extracts were preincubated with peptidomimetics for 30 min at room temperature before incubation with radiolabeled probe. The ^{32}P-labeled oligonucleotide probes used are hSIE (high affinity *sis*-inducible element, m67 variant, 5′-AGCTTCATTTCCCGTAAATCCCTA) that binds both Stat1 and Stat3 (23, 31) and MGFe (mammary gland factor element from the bovine β-casein gene promoter, 5′-AGATTTCTAGGAATTCAA) that binds Stat1 and Stat5 (32, 33).

Dissociation-Reassociation Analysis

Two independent preparations of lysate (from baculovirus-infected Sf-9 cells) containing either active Stat1:Stat1 or Stat3:Stat3 were pooled together. Aliquots of mixed lysates of equal total protein were pretreated with or without 30–300 μM PY*LKTK or 3–1000 μM ISS 610 for 30 min before incubation with ^{32}P-labeled hSIE and subjected to EMSA (19).

Cell Proliferation, Soft-Agar Growth, and Apo-BrdUrd Labeling Studies

Proliferating fibroblasts and human tumor cells were counted by phase-contrast microscopy for viable cells (using trypan blue exclusion). Colony formation assays in six-well dishes and quantification of colonies by iodonitrotetrazolium violet have been previously described (29). Treatment of cells with inhibitors was initiated 1 day after seeding cells by adding 75 μl of medium with or without inhibitor, and repeated every 2–3 days. Apoptosis was measured by Apo-BrdUrd labeling and following the supplier's (PharMingen) instructions. Cells (NIH3T3 or NIH3T3/v-Src) were first treated with or without PY*LKTK-MTS or ISS 610 for 48 h before labeling and than analyzed by flow cytometry for detection of apoptotic cells.

Results

Design of PY*L Peptidomimetics: Substitutions of Proline by Aromatic Groups Generate Strong Disruptors of Stat3 DNA-Binding Activity *in Vitro*

We have previously reported that PY*LKTK, the putative Stat3 SH2 domain-binding sequence, and the tripeptides PY*L and AY*L, disrupt Stat3:Stat3 dimer formation and subsequent Stat3 DNA-binding activity. In this manuscript, we have used PY*L (and AY*L) as peptide leads from which to design peptidomimetics. Our initial strategy focused on substitution of the Y-1 prolyl (or alanyl) residue by aromatic groups and replacing the peptide bond that is

NH_2-terminal to the phosphotyrosine (Y*) to generate peptidomimetics for evaluation against STAT DNA-binding activities *in vitro*. The activities of STATs were measured in nuclear extracts [prepared from epidermal growth factor (EGF)-stimulated fibroblasts] as DNA-bound protein complexes by EMSA. STAT-DNA complexes detected include Stat3:Stat3 homodimers (upper band), Stat1:Stat3 heterodimers (intermediate band), and Stat1:Stat1 homodimers (lower band) (Fig. 1A) when a ^{32}P-labeled hSIE oligonucleotide probe is used, or Stat5:Stat5 (upper band) and Stat1:Stat1 dimers (lower band) (Fig. 1B) with ^{32}P-labeled MGFe as probe.

Evaluation of compounds reveals peptidomimetics that reproduce and/or enhance the activities originally observed for lead tripeptides. Preincubation of peptidomimetics with nuclear extracts results in potent and dose-dependent inhibition of DNA-binding activities of Stat3, and to a lesser extent of Stat1 or Stat5 (Fig. 1). From densitometric analysis of band intensities, we determined the IC_{50} values (concentration of peptidomimetic at which DNA-binding activity is reduced by 50%), which are reported in Tables 1 and 2. We note that replacement of proline or alanine in PY*L or AY*L with 4-cyanobenzoate (ISS 610) or 2,6-dimethoxybenzoate (ISS 637) results in a 5-fold increase in potency (compared to tripeptide leads) in inhibiting Stat3 DNA-binding activity *in vitro* [IC_{50} value decreased from 217 ± 55 μM (AY*L) or 182 ± 15 μM (PY*L) to 42 ± 23 μM (ISS 610) or 55 ± 35 μM (ISS 637)] (Tables 1 and 2; Fig. 1A). In their inhibitory activities, these peptidomimetics also show preference for Stat3 over Stat1 or Stat5. In contrast, substitutions of the Y-1 residue by other groups, such as 2-fluoro-5-nitrobenzoate (ISS 375), 2-aminobenzoate (ISS 265), or 3-aminobenzoate moiety (ISS 248), make the corresponding peptidomimetics ineffective at disrupting Stat3 activity (Tables 1 and 2), suggesting that peptidomimetics with these substitutions may not interact with Stat3. The non-tyrosine-phosphorylated form of ISS 610 (ISS 610NP) has no effect on Stat3 (and Stat1 or Stat5) DNA-binding activity *in vitro* (Fig. 1, A and B, *bottom panels*), reflecting the importance of the pTyr for disruption of Stat3 by this peptidomimetic. These findings provide support for the engagement of pTyr-SH2 interaction as being the basis for Stat3 dimer disruption by ISS 610 (see below).

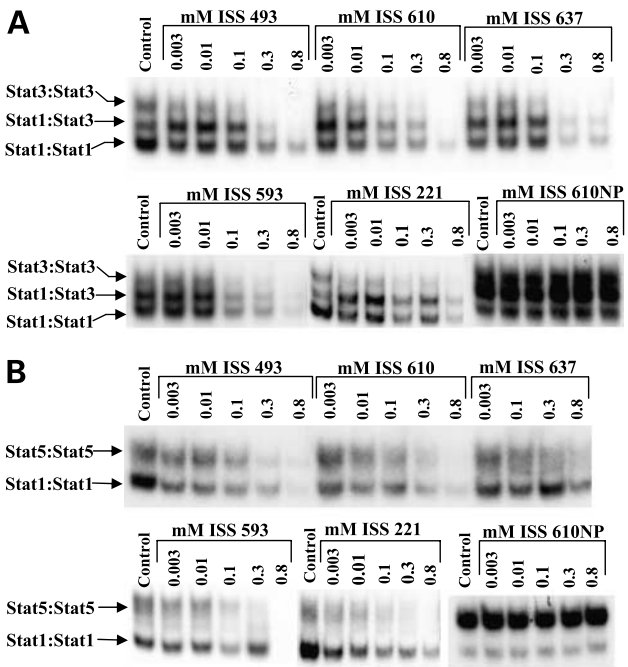


Figure 1. EMSA analyses of STAT DNA-binding activities showing effects of peptidomimetics. Nuclear extracts containing activated Stat1, Stat3, and Stat5 are treated with the indicated concentrations of peptidomimetics ISS 493, ISS 610, ISS 637, ISS 593, ISS 221, or ISS 610NP for 30 min at room temperature before incubation with radiolabeled oligonucleotide probes. Stat1 and Stat3 binding to hSIE probe (**A**) and Stat1 and Stat5 binding to MGFe probe (**B**). Positions of STAT:DNA complexes in gel are labeled. *Control lanes,* nuclear extracts from NIH3T3 cells stimulated with EGF but not treated with peptidomimetics.

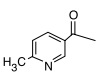
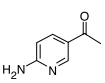
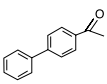
Replacement of proline or alanine by pyridine 3-carboxylic acid as in ISS 219 did not affect potency (IC_{50} = 232 ± 16 μM) compared to lead tripeptide. However, substitution by 2-methyl pyridine 3-carboxylic acid in ISS 221 but not 6-methyl pyridine 3-carboxylic acid in ISS 223 increased potency by 3-fold (IC_{50} = 75 ± 36 μM), suggesting that hydrophobic aliphatic substitutions on the pyridine ring are favored at position 2 over position 6. Furthermore, fluoro and dichloro substitutions in 2,6-dichloro, 4-fluoro pyridine-3 carboxylic acid (ISS 593) result in increased potency (IC_{50} = 48 ± 32 μM), suggesting that those substitutions are also favored. This is in contrast to amino substitution at position 6 of the pyridine ring (in ISS 249), which is not tolerated and the corresponding peptidomimetic is a weak inhibitor of Stat3 activity. Our data indicate that substitutions of the Y-1 residue by 5-methylpyrazine carboxylic acid (ISS 493) result in a gain in potency [IC_{50} value of 38 ± 16 μM (ISS 493)], while incorporation of bulky quinoline, naphthoic, or biphenyl carboxylic acid moieties (ISS 352, ISS 353, ISS 355, ISS 360, or ISS 363), or simple aliphatic chains (ISS 230, ISS 231, and ISS 234) at the Y-1 position generates peptidomimetics that have no effect on Stat3 activity.

Certain substitutions at the Y-1 position (of PY*L or AY*L) lead to preferential suppression of Stat3 activity over those of Stat1 or Stat5 (Table 2, ISS 493, ISS 610, and ISS 637), while others, such as in ISS 593, result in peptidomimetics that have enhanced potency against Stat5 over Stat3 (IC_{50} values of 10 ± 6 μM). The inhibition of Stat5 has important implications for some types of human tumors, such as chronic myelogenous leukemia, which depend on constitutively active Stat5 for growth and survival (12, 34, 35). Altogether, we have generated potent peptidomimetic disruptors of Stat3 activity *in vitro* that are also selective for Stat3 over Stat1 or Stat5.

Disruption of STAT Dimerization: Evidence for Dissociation of STAT Dimers

To define the interaction of Stat3:Stat3 dimers with PY*LKTK, PY*L, and AY*L, we have previously proposed a STAT dimerization disruption model (21). In this model, phosphopeptides engage in pTyr-SH2 interactions with

Table 1. Disruption of Stat3 DNA-binding activity by peptidomimetics

Compound	R′	$IC_{50}(\mu M)$[a]
Prolylphosphotyrosylleucine		182±15
Alanylphosphotyrosylleucine		217±55
ISS 248		ne
ISS 265		ne
ISS 375		ne
ISS 610		42±23
ISS 637		55±35
ISS 219		232±16
ISS 221		75±36
ISS 593		48±32
ISS 223		225±15
ISS 249		ne
ISS 493		38±16
ISS 352		410±15
ISS 353		650±22
ISS 355		ne
ISS 360		420±35
ISS 363		643±43

Note: Nuclear extracts containing active Stat3 were preincubated for 30 min with or without peptidomimetics before incubation with radiolabeled hSIE probe and analysis by EMSA. Results are representative peptidomimetics from over 80 that have been evaluated.

[a]Values are the means and SDs of at least three independent assays. *ne,* no effect at 1 mM. Structural formula of compounds: R′Y*L (where R′ is defined as shown).

STAT:STAT dimers, which results in STAT dimer dissociation into monomers (in complexes with phosphopeptides), some of which may in turn reassociate into STAT dimers. To test this model, we performed dissociation-reassociation analysis (19) with two independent cell lysate preparations, one containing only active Stat1:Stat1 dimers and the other only Stat3:Stat3 dimers. Lysates were mixed together with or without PY*LKTK, ISS 610, or its non-phosphorylated counterpart, ISS 610NP, and then incubated with radiolabeled hSIE probe and subjected to EMSA analysis.

Either cell lysate preparation alone shows one band corresponding to Stat1:Stat1 dimers (Fig. 2, *Stat1*) or Stat3:Stat3 dimers (Fig. 2, *Stat3*). For mixed cell lysates, two bands with migrations consistent with Stat1:Stat1 dimers (lower band) or Stat3:Stat3 dimers (upper band) (Fig. 2, *Stat1 + Stat3, 0*) are observed in the absence of the phosphopeptide, PY*LKTK, or ISS 610. However, EMSA analysis of mixed cell lysates that are preincubated with PY*LKTK or ISS 610 shows three bands: (*a*) lower and upper bands corresponding to Stat1:Stat1 and Stat3:Stat3 dimers, respectively (Fig. 2, *Stat1 + Stat3, 30–150*), which are of decreasing intensities with increasing concentrations of phosphopeptide or peptidomimetic, consistent with results in Fig. 1A; and (*b*) appearance of an additional intermediate band representing Stat1:Stat3 heterodimers (Fig. 2, *Stat1 + Stat3, 30–100*) (19) that hitherto were not present and could only have formed from random reassociation of two dissociated phosphorylated monomers. Results also show decreasing band intensities or complete disappearance of the three STAT-DNA complexes at higher concentrations of PY*LKTK or ISS 610 (Fig. 2, *Stat1 + Stat3, 150* and *200*) due primarily to total disruption of all STAT:STAT dimers and formation of only complexes of STAT protein with peptidomimetics (or phosphopeptides). The apparently stronger disruption of Stat1:Stat1 dimer might be due to a relatively lower amount of Stat1 protein in starting material (lysate) compared to Stat3 protein. The non-phosphorylated ISS 610NP has no effect (data not shown), which demonstrates the importance of pTyr for disruption of Stat3 dimers and reflects its requirement for engagement in interactions with the SH2 domain. These findings together suggest that PY*LKTK or ISS 610 physically interacts with and disrupts STAT:STAT dimers and requires pTyr for this effect.

Peptidomimetic Selectively Blocks Stat3 Signaling and Constitutively Active Stat3-Dependent v-Src Transformation

We previously showed that PY*LKTK-MTS [MTS, membrane translocation sequence, is a sequence of hydrophobic amino acids that facilitates transport of peptides across cell membranes (26)] inhibits constitutive Stat3 activation in Src-transformed fibroblasts (21). We similarly evaluated the biological activities of peptidomimetics in transformed fibroblasts and human tumor cells. Because peptidomimetics are phosphorylated on tyrosine that greatly reduces membrane permeability and do not contain MTS to enhance permeability, ISS 610 was used at 1 mM concentrations in whole-cell studies (ISS 610-MTS was not synthesized due to concern over size of MTS in relation to

Table 2. Selective disruption of STAT family members by peptidomimetics

Mimetics	IC_{50} Values (μM) against STAT Dimers[a]			
	Stat3:Stat3	Stat1:Stat3	Stat1:Stat1	Stat5:Stat5
ISS 221	75 ± 36	455 ± 97	310 ± 74	50 ± 12
ISS 493	38 ± 16	230 ± 22	273 ± 22	300 ± 23
ISS 593	48 ± 32	87 ± 15	175 ± 65	10 ± 6
ISS 610	42 ± 23	125 ± 15	310 ± 145	285 ± 32
ISS 637	55 ± 35	195 ± 12	255 ± 55	695 ± 123

Note: Nuclear extracts containing active Stat1, Stat3, and Stat5 were preincubated with or without peptidomimetics for 30 min before incubation with radiolabeled hSIE probe and EMSA analysis.

[a]Values are the means and SDs of at least three independent assays. *ne,* no effect at 1 mM. Results are representative peptidomimetics from over 80 that have been evaluated.

peptidomimetic and its potential to obstruct interaction with Stat3). Luciferase reporter assays on Src-transformed mouse cells that stably express Stat3-dependent and Stat3-independent dual luciferase reporters (21) and are treated with ISS 610 show a significant suppression of transcriptional induction of the Stat3-dependent luciferase reporter, pLucTKS3 (Fig. 3A), with no effect on the induction of the Stat3-independent luciferase reporter, pRLSRE (Fig. 3A). Similarly, DNA-binding studies and EMSA analysis reveal a time-dependent reduction of Stat3 activation in Src-transformed fibroblasts (Fig. 3C), human non-small cell lung carcinoma (A549), and breast carcinoma (MDA-MB-231, MDA-MB-435, and MDA-MB-468) cells that harbor constitutive Stat3 activation (36, 37) and are treated with ISS 610 (Fig. 3E, *second lane* for each cell line). We note that inhibition of Stat3-dependent luciferase induction and Stat3 activation in whole cells was not complete at 48 h post-treatment (Fig. 3, A and C), possibly due to low intracellular levels of peptidomimetic as a result of weak uptake, rapid degradation, or both. In other studies, we evaluated the effect of ISS 610 on ligand (EGF)-induced STAT activation in mouse fibroblasts and observed preferential inhibition of activation of Stat3, and to a lesser extent of that of Stat1 (data not shown). Moreover, the non-phosphorylated ISS 610NP has no effect on Stat3 activation in Src-transformed cells or Stat3-mediated gene expression (Fig. 3, B and D), indicating that pTyr is required for disruption of interaction between Stat3 dimers. By selectively suppressing constitutive Stat3 signaling in whole cells, ISS 610 therefore reproduces the biological activity of phosphopeptide leads.

Our previous studies with PY*LKTK-MTS (21) confirmed that inhibition of constitutive Stat3 activation blocks Src transformation (27, 29, 38). Using growth in soft agar as a measure of transformation, we sought to determine the effect of representative peptidomimetic, ISS 610. Growth of NIH3T3/v-Src in soft-agar suspension is significantly suppressed by the addition of ISS 610 (Fig. 3F). In contrast, ISS 610 has no effect on soft-agar growth of Stat3-independent Ras-transformed fibroblasts (NIH3T3/v-Ras) (Fig. 3F). These findings indicate that selective blocking of constitutive Stat3 signaling by the peptidomimetic inhibitor ISS 610 suppresses transformation of mouse fibroblasts by v-Src.

ISS 610 Induces Growth Inhibition of Malignant Cells That Contain Persistent Stat3 Activity

Stat3 has an essential role in cell proliferation, and constitutively active Stat3 is required for growth of transformed and tumor cells that harbor aberrant Stat3 signaling. We extended our studies to determine the effects of the peptidomimetic-mediated inhibition of Stat3 on cell proliferation using trypan blue exclusion for viable cell counts. Results show that treatment with ISS 610 (or PY*LKTK-MTS for comparison) of Src-transformed mouse fibroblasts (NIH3T3/v-Src), or the human breast carcinoma cells MDA-MB-231 and MDA-MB-435 that harbor constitutively active Stat3, significantly suppresses proliferation compared to control (non-treated cells) (Fig. 4). In comparison to the effect observed for PY*LKTK-MTS, the inhibition of cell proliferation by ISS 610 was only partial. Unlike the phosphopeptide, which is linked at the COOH terminus to the MTS (26) to facilitate intracellular uptake across cell membrane, ISS 610 lacks this MTS and therefore may not be efficiently taken up into cells, which will in turn be reflected in its biological activity. Treatment of cells that lack constitutive Stat3 activity [normal NIH3T3 cells, Ras-transformed counterparts (NIH3T3/v-Ras), or MDA-MB-453 cells] shows no effect on cell proliferation (Fig. 4, data not shown), suggesting that ISS 610 has no general cytostatic or cytotoxic effects. These findings show that peptidomimetics inhibit growth of transformed mouse and human tumor cells that harbor constitutive Stat3 activity.

Peptidomimetic Induces Apoptosis in Transformed Fibroblasts That Contain Persistent Stat3 Activity

Because one of the functions of Stat3 is to protect transformed or tumor cells from apoptosis (39–43), we

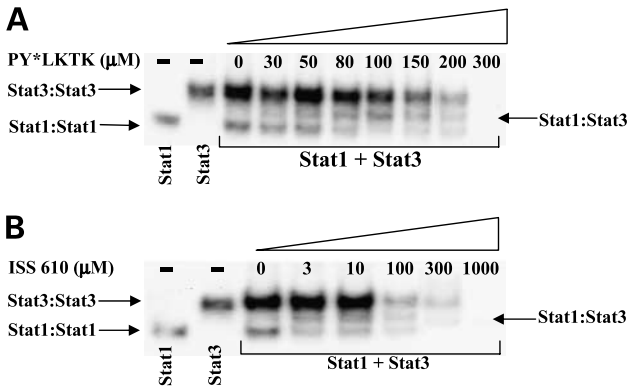


Figure 2. Evidence for dissociation of STAT dimers by phosphopeptide or peptidomimetic. Cell lysates contain either only activated Stat1 (*Stat1*), Stat3 (*Stat3*), or both [pooled lysates (*Stat1 + Stat3, 0–300*)] and are treated (*Stat1 + Stat3, 30–300*) with the indicated concentrations of PY*LKTK (**A**) or ISS 610 (**B**) for 30 min at room temperature before incubation with radiolabeled hSIE oligonucleotide probe. Positions of STAT:DNA complexes in gel are labeled. Cell lysates were prepared from recombinant baculovirus-infected Sf-9 cells as described in Materials and Methods.

evaluated the ability of ISS 610 (and PY*LKTK-MTS for comparison) to induce apoptosis in Stat3-dependent transformed cells. Viral Src-transformed NIH3T3/v-Src fibroblasts were treated with or without compounds for 48 h. Treated cells were then labeled with Apo-BrdUrd (PharMingen) for detection of DNA breaks. Results from flow cytometric analysis of cells show dramatic increases in incorporated BrdUTP in Src-transformed fibroblasts treated with PY*LKTK-MTS or ISS 610 compared to controls (nontreated cells or treated with control peptide, PYLKTK-MTS) or to normal NIH3T3 fibroblasts treated with peptides or peptidomimetic (Table 3). Together, these findings show that PY*LKTK-MTS and ISS 610 induce apoptosis in Stat3-dependent transformed cells but not in normal cells. This observation reflects the inhibition of Stat3 activity and its biological function, which together with the other data demonstrates that selective suppression of constitutive Stat3 activation and its biological function induces apoptosis in model transformed cells that harbor constitutive Stat3 activity.

Discussion

Inhibitors of STAT signaling have become increasingly important because of the role of STATs in the pathogenesis of human diseases (15, 18, 44, 45). While significant progress has been made in using antisense or decoy oligonucleotides (46) and dominant-negative mutants (27, 38, 42, 47) to elucidate STAT signaling and biological effects, these STAT inhibitory modalities do not easily lend themselves to clinical applications. Another approach has been to use small-molecule inhibitors of the upstream tyrosine kinases that phosphorylate and activate STATs (15, 36, 48). However, because other signaling pathways are also activated downstream of tyrosine kinases, non-Stat3-related biological effects might ensue from inhibition of these kinases. In an effort to develop Stat3-specific small-molecule inhibitors, we previously showed that a Stat3 SH2 domain-binding phosphopeptide, PY*LKTK, and its truncated tripeptide derivatives, PY*L and AY*L, disrupt Stat3 activity (21), thus providing leads for peptidomimetic drug design. We have now designed and investigated several tripeptide-based small molecules as inhibitors of Stat3, and show here that some of these peptidomimetics reproduce effects observed for the original phosphopeptides, thus validating the peptidomimetic approach for design of Stat3 inhibitors. On the mode of action, our data provide strong support for disruption of dimerization of STAT:STAT protein complexes (19) by phosphopeptides or synthetic peptidomimetics, and causing dissociation into functionally incompetent monomers, thereby inhibiting Stat3-mediated gene regulation and inducing apoptosis of Stat3-dependent transformed cells.

In designing tripeptide (PY*L or AY*L)-based peptidomimetics, we have modified the Y-1 residues and peptide bond. Evaluation of these peptidomimetics as small-molecule inhibitors of Stat3 activity *in vitro* reveals a broad range of efficacies and selectivities. Substitutions of proline

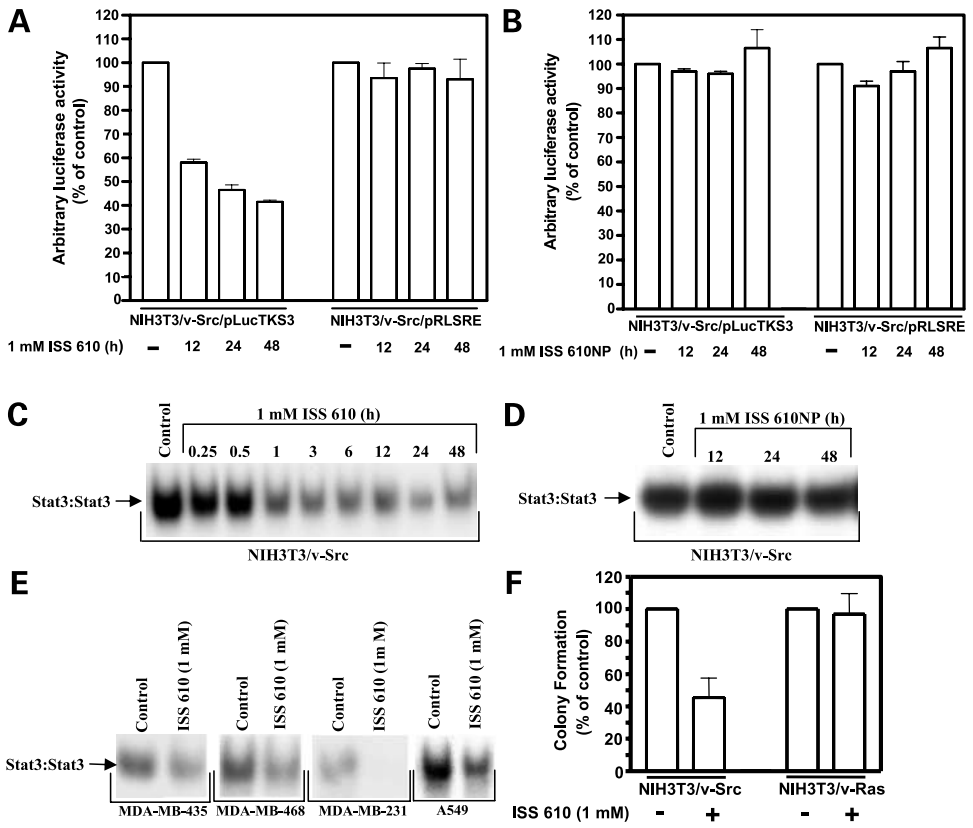


Figure 3. Evaluation of peptidomimetic effects on Stat3 activation and Stat3-mediated gene expression in intact cells, and on Src transformation. **A** and **B,** luciferase activities in extracts prepared from ISS 610- or ISS 610NP-treated v-Src-transformed mouse fibroblasts that stably express Stat3-dependent (*NIH3T3/v-Src/pLucTKS3*) and Stat3-independent (*NIH3T3/v-Src/pRLSRE*) luciferase reporters. *Columns,* means of three independent assays; *bars,* SD. EMSA analyses of Stat3 DNA-binding activities (using hSIE oligonucleotide probe) in nuclear extracts prepared from v-Src-transformed NIH3T3/v-Src (**C** and **D**), and human breast carcinoma MDA-MB-435, MDA-MB-468, MDA-MB-231, and non-small cell lung carcinoma A549 (**E**). **F,** effect of ISS 610 on soft-agar growth of v-Src-transformed fibroblasts (*NIH3T3/v-Src*) and their v-Ras-transformed counterparts (*NIH3T3/v-Ras*). Transformed cells were seeded in soft agar and treated every 2–3 days with or without ISS 610 until large colonies were evident. *Columns,* means of three independent assays; *bars,* SD.

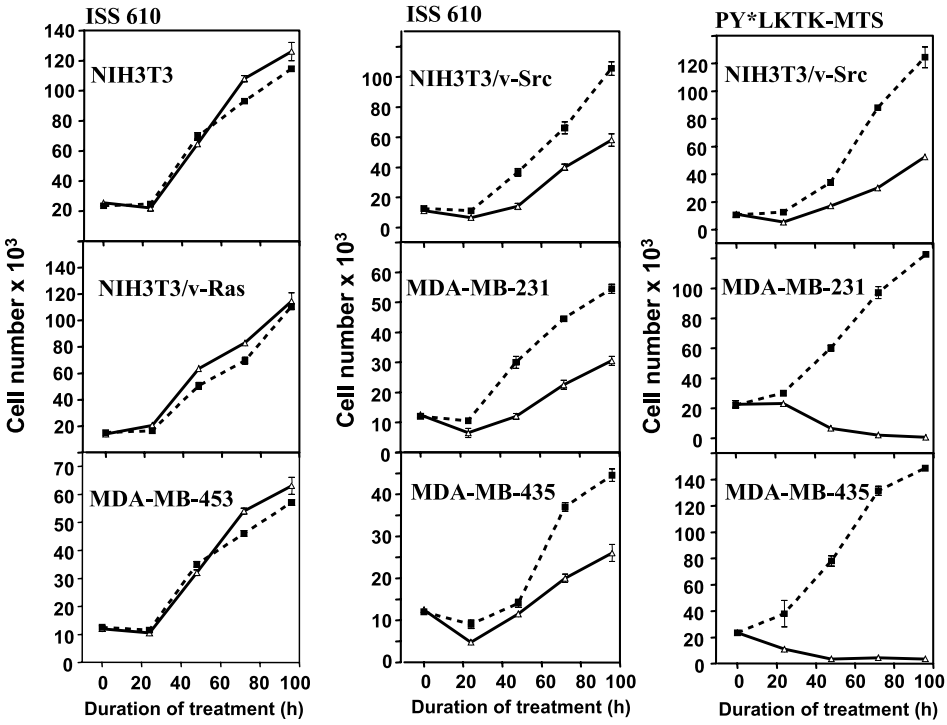


Figure 4. Evaluation of peptidomimetic effects on cell proliferation. Growth curves for transformed and tumor cells. Normal and transformed fibroblasts (*NIH3T3*, *NIH3T3/v-Src*, or *NIH3T3/v-Ras*) as well as human breast carcinoma (*MDA-MB-231*, *MDA-MB-435*, or *MDA-MB-453*) cells were treated with or without compounds and counted by trypan blue exclusion on each of 4 days. Cells were untreated (*dotted lines*) or treated with 1 mM ISS 610 or PY*LKTK-MTS (*solid lines*). *Points,* means of four independent determinations; *bars,* SD.

by benzyl, pyridyl, and pyrazinyl moieties result in increased potency, which contrast that by more bulky groups, such as naphthalene, quinoline, or biphenyl that lead to inactive peptidomimetics. Thus, the bulky groups may not be accommodated in the SH2 domain pocket that binds PY*LKTK. Although hydrophobic, these substituents may be constrained (by size) from making effective contact with the side chains of those amino acids that form the hydrophobic core of the SH2 pocket (49, 50), thus limiting the overall binding affinity. On the other hand, the small size or the limited hydrophobicity of the Y-1 group in inhibitors ISS 230, ISS 231, ISS 234, or ISS 374 may explain their weak effects on Stat3 activity. Emerging from the SAR analysis are five peptidomimetics, ISS 221, ISS 437, ISS 593, ISS 610, and ISS 637, which are potent disruptors of active Stat3 and contain derivatives of pyridine, pyrazine, or benzene at the Y-1 position of PY*L (or AY*L). We note that while peptidomimetics with pyrazine or benzene substitutions are generally more selective for Stat3, the selectivity of these compounds may be influenced by the presence, the type, as well as the positioning of functional groups on the aromatic ring.

Table 3. Flow cytometric analysis of cells treated with PY*LKTK-MTS and ISS 610

	NIH3T3	NIH3T3/v-Src
Control	2.1%	1.8%
1 mM PYLKTK-MTS	1.2%	0.4%
1 mM PY*LKTK-MTS	3.7%	27.6%
1 mM ISS 610	0.3%	21.4%

Note: Cells were treated with compounds for 48 h, labeled with Apo-BrdUrd and analyzed by flow cytometry for percentage apoptotic cells. Control represents no treatment.

The peptidomimetic inhibitors with improved potency based on *in vitro* assays presumably have enhanced affinity for Stat3 and so effectively disrupt and dissociate active Stat3:Stat3 dimers. Shown in Fig. 5 is the lowest energy GOLD (51) docked conformation of ISS 610 in the SH2 domain of Stat3β, as compared to the observed SH2 domain-bound phosphopeptide AAPY*LK of the associated monomer of Stat3β in the X-ray crystal structure (49). The modeling by GOLD (51) flexible docking program reveals that ISS 610 has access to the hydrophobic pocket and available hydrogen bonding interactions on the protein surface, which are not accessed by the native peptide sequence. Thus, the improved activity of peptidomimetics ISS 493, ISS 610, and ISS 637, with IC_{50} values that are 5- to 10-fold better than those obtained for lead peptides, likely comes from the increased hydrophobic surface area that is present relative to the Pro and Ala residues in PY*L and AY*L. In summary, we have achieved significant gain in potency against *in vitro* Stat3 activity by modifying peptides into peptidomimetics. Our findings that ISS 593 has a relatively higher preference for Stat5 over Stat3 or Stat1 is least expected, because the SH2 domain-binding peptides were originally designed from Stat3. How such pyridine substitutions at the Y-1 position enhance preference for Stat5 remains to be defined.

As seen with PY*LKTK-MTS (21), ISS 610 shows activity in intact cells that is consistent with disruption of activated Stat3 *in vitro* and suggests that peptidomimetic reproduces the biological activity of phosphopeptide. Functional

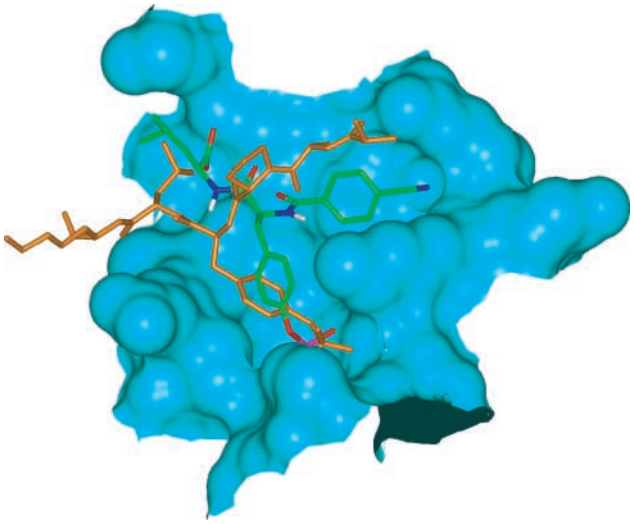


Figure 5. Computer modeling of ISS 610 bound to the SH2 pocket. Comparison of the lowest GOLD (51) docked conformation of ISS 610 (*green*), and the COOH-terminal phosphotyrosine peptide, AAPY*LK (*orange*), of the associated Stat3β monomer determined from the crystal structure (49), in the SH2 domain of Stat3β (*pale blue*).

specificity is evident in that ISS 610 selectively targets Stat3 signaling without affecting Stat3-independent induction of transcriptional events or constitutive Stat5 activation in human K562 chronic myelogenous leukemia cells (data not shown). The inhibitory effects of ISS 610 (or PY*LKTK-MTS) on Stat3 activation in whole cells also extend beyond cell type and species differences, because they are evident in murine fibroblasts as well as human breast and lung carcinoma cells. Thus, modifications in peptidomimetics have enhanced *in vitro* potency while maintaining biological activity profile comparable to that of the original membrane-permeable lead phosphopeptide. Together with the reduced peptidic character and smaller size, peptidomimetics therefore represent an improvement over lead phosphopeptides in development of small-molecule inhibitors of Stat3.

Results from the *in vitro* DNA-binding assay would suggest that possible modes of inhibition by peptidomimetics *in vivo* include disruption/dissociation of preexisting constitutively active Stat3:Stat3 dimers (21).Additionally, peptidomimetics might associate with non-phosphorylated Stat3 monomer proteins through pTyr-SH2 interactions (the peptide or mimetic contains the pTyr motif and the Stat3 monomer has an SH2 domain) to form a heterocomplex (21). This in turn would decrease the levels of free non-phosphorylated Stat3 monomers that are available for *de novo* phosphorylation and activation. While not mutually exclusive, these two modes of action likely occur concurrently *in vivo*. Other factors that regulate constitutively active Stat3 levels, including physiological dephosphorylation and degradation, might augment peptidomimetic effects on Stat3 signaling.

Our studies demonstrate significant biological effects of Stat3 inhibitor peptide or peptidomimetic ISS 610, which are consistent with inhibition of Stat3's biological functions of promoting proliferation and survival of transformed and tumor cells (12, 18). This repression of the biological consequences of persistently activated Stat3 implies inhibition of a significant portion of the downstream transcriptional events of Stat3, which are dysregulated in malignant cells (15). Because of their critical dependence on constitutive Stat3 activity and its induced dysregulation of downstream transcriptional events for growth and survival, malignant cells that harbor aberrant Stat3 signaling are preferentially more sensitive to changes in Stat3 signaling caused by the Stat3 inhibitor peptidomimetics. Thus, inhibition of Stat3 activity may not necessarily result in general growth inhibition or cytotoxicity in normal cells that do not harbor or depend on constitutively active Stat3 signaling. On these grounds, it is possible for specific inhibitors against Stat3 protein to distinguish normal cells from the malignant ones that require constitutive Stat3 activity for growth and survival. Peptidomimetic ISS 610 also shows good selectivity against Stat3 and its functions, and its overall biological effects are consistent with this. Thus, we show that a Stat3 SH2 domain-binding peptidomimetic inhibits Stat3 signaling *in vivo* and thereby induces growth inhibition and apoptosis. This provides support for the viability of peptidomimetic approaches to design of small-molecule inhibitors of Stat3, as well as proof-of-principle for the therapeutic potential of such compounds. Together with an earlier report of a peptidomimetic inhibitor of Myc/Max dimerization (22), this is among the first examples of small-molecule inhibitors of a transcription factor that functions by disruption of protein-protein interactions.

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