First-in-Human Trial of a STAT3 Decoy Oligonucleotide in Head and Neck Tumors: Implications for Cancer Therapy

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RESEARCH ARTICLE
**ABSTRACT**

Despite evidence implicating transcription factors, including STAT3, in oncogenesis, these proteins have been regarded as “undruggable.” We developed a decoy targeting STAT3 and conducted a phase 0 trial. Expression levels of STAT3 target genes were decreased in head and neck cancers following injection with the STAT3 decoy compared with tumors receiving saline control. Decoys have not been amenable to systemic administration due to instability. To overcome this barrier, we linked the oligonucleotide strands using hexaethylene glycol spacers. This cyclic STAT3 decoy bound with high affinity to STAT3 protein, reduced cellular viability, and suppressed STAT3 target gene expression in cancer cells. Intravenous injection of the cyclic STAT3 decoy inhibited xenograft growth and downregulated STAT3 target genes in the tumors. These results provide the first demonstration of a successful strategy to inhibit tumor STAT3 signaling via systemic administration of a selective STAT3 inhibitor, thereby paving the way for broad clinical development.

**SIGNIFICANCE:** This is the first study of a STAT3-selective inhibitor in humans and the first evidence that a transcription factor decoy can be modified to enable systemic delivery. These findings have therapeutic implications beyond STAT3 to other “undruggable” targets in human cancers. Cancer Discov; 2(8): 694–705. ©2012 AACR.

**INTRODUCTION**

Transcription factors are attractive as therapeutic targets due to their critical role in regulating gene expression associated with the development and progression of many diseases, including cancer (1). STATs are one such class of transcription factors that regulate various aspects of cell proliferation, survival, and differentiation (2). Among the 7 known members of the mammalian STAT family (STAT1, 2, 3, 4, 5α, 5β, and 6), STAT3 functions as a key mediator of oncogenic signaling (3). Constitutive STAT3 activation has been detected in a large number of human cancers, where increased STAT3 signaling is commonly associated with a poor clinical prognosis (4–7). In vitro studies have shown that inhibition of STAT3 expression or function attenuates the proliferation and survival of a wide variety of cancer cell lines characterized by overexpression/hyperactivation of STAT3, suggesting an addiction to the oncoprotein (8, 9). In contrast, although STAT3 gene inactivation results in embryonic lethality (10), many normal adult tissues are unaffected by loss of STAT3 (2, 11, 12). Collectively, these findings point to STAT3 as a highly attractive target in cancer therapy.

Several strategies have been developed to inactivate STAT3, including the use of aptamers and peptidomimetics to target STAT3 protein and antisense oligonucleotides to decrease STAT3 expression. However, to date, challenges in drug delivery have limited the clinical translation of these approaches (5–7, 13). Small molecules that reportedly inhibit STAT3 generally function by targeting upstream receptor and non–receptor tyrosine kinases and therefore lack specificity. In hepatocellular carcinoma, sorafenib, a multikinase inhibitor, decreased STAT3 phosphorylation in association with inhibition of phosphoinositide 3-kinase (PI3K)/Akt and MEK/ERK pathways (14). NSC74859, a chemical probe inhibitor of STAT3 activity, inhibited tumor development in a hepatocellular carcinoma model by blocking STAT3; however, its application has only been as a preclinical tool (15). WP1066, a JAK2 inhibitor, showed antitumor activity against renal cell carcinoma in conjunction with decreased STAT3 phosphorylation (16). Knocking down STAT3 by an RNA interference approach—a preclinical tool—suppressed proliferation in vitro and tumorigenicity in vivo (17). Curcumin analogues LLL12 and FLLL32 were evaluated for their ability to inhibit STAT3 activity in vitro and antitumor efficacy in vivo (18). In osteosarcoma, LLL12 and FLLL32 inhibited STAT3 activity in vitro and reduced tumor growth. However, there is no evidence of direct binding of LLL12 or FLLL32 to phosphorylated-STAT3 (pSTAT3) protein. In an effort to develop a highly specific inhibitor of STAT3, we generated a double-stranded STAT3 oligonucleotide decoy (19).

Transcription factor decoys consist of nucleotide sequences derived from conserved genomic regulatory elements that are recognized and bound by the transcription factor in question. Transcription factor decoys elicit their biologic effects by competitively inhibiting binding of the transcription factor to corresponding cis elements in genomic DNA, preventing expression of target genes. The STAT3 decoy was derived from the conserved hSIE genomic element found in the c-fos gene promoter and was composed of a 15-bp duplex oligonucleotide with free ends and phosphorothioate modifications.
of the three 5′ and 3′ nucleotides (19). This STAT3 decoy showed selective binding for STAT3 protein and inhibited the proliferation and survival of head and neck squamous cell carcinoma (HNSCC) cells in vitro (19). Intratumoral administration of STAT3 decoy inhibited the growth of HNSCC xenograft tumors in vivo (20). Subsequent investigations by others showed that this STAT3 decoy exhibits antitumor activity in a variety of preclinical models including cancers of the lung, breast, skin, and brain (21–24). Preclinical studies of the STAT3 decoy in animal models showed that it is well tolerated and lacks toxicity (25).

The U.S. Food and Drug Administration (FDA) introduced the concept of phase 0 clinical trials in 2006 to accelerate cancer drug development. One of the objectives of a phase 0 cancer clinical trial is to establish at the very earliest opportunity whether an agent is modulating its target in a tumor and consequently whether further clinical development is justified. Given the paucity of clinical studies testing the biologic effects of a STAT3-selective inhibitor in humans, we designed a trial with a primary endpoint of target gene modulation in the tumor. To ensure that we could obtain high-quality tissue specimens, we elected to directly inject the STAT3 decoy immediately before HNSCC tumor resection when the patient was under anesthesia. Although control groups are not typically included in early-phase clinical trials, we chose to also enroll subjects exposed to a saline injection, rather than the STAT3 decoy, to serve as controls for the assessment of target gene modulation in the decoy-treated tumors. We received support from the (former) NIH Rapid Access to Interventional Development (RAID) program to manufacture clinical grade material. Because of the relative ease of obtaining biopsies of HNSCC before surgical resection, cumulative evidence supporting STAT3 as a therapeutic target in this cancer, and the urgent need for more effective therapies, we conducted a phase 0 study to evaluate the biologic effects of the STAT3 decoy in patients with HNSCCs.

Because the potential for broad clinical application of the STAT3 decoy in its original formulation is limited by its sensitivity to degradation and the necessity for intratumoral administration, we also sought to develop STAT3 decoy modifications that would improve stability and facilitate effective systemic administration. These studies resulted in a chemically modified cyclic STAT3 decoy that shows antitumor activity following systemic delivery. This strategy of decoy modification should allow further clinical development and testing of the STAT3 decoy and may have important implications for the generation and therapeutic evaluation of a wide variety of decoys targeting previously considered “undruggable” transcription factors.

RESULTS

Intratumoral Administration of a STAT3 Decoy Oligonucleotide Abrogates Target Gene Expression in Human HNSCC

STAT3 is a plausible therapeutic target in cancers characterized by STAT3 hyperactivation. To date, no STAT3-selective small molecule has reached clinical testing. We developed a novel strategy to specifically target STAT3 using a decoy oligonucleotide. A phase 0 clinical trial was conducted to evaluate the pharmacodynamic effects of this STAT3 decoy, compared with saline control, in patients with HNSCC (ClinicalTrials.gov number NCT00696176). Patients undergoing surgery for HNSCC were enrolled in this phase 0 clinical trial (Fig. 1A). The STAT3 decoy dose was escalated in successive cohorts at 3 dose levels from 250 μg to 1 mg per injection (5–6 patients per dose). Patients received a single intratumoral injection of STAT3 decoy or vehicle control (saline). Biopsies of the tumors were performed before treatment and after completion of surgery. Levels of STAT3 target gene expression were assessed in the tumors before and after treatment. In anticipation of the phase 0 trial, we conducted xenograft studies to determine the kinetics of downregulation of target gene expression in the tumors and concluded that decreased protein was observed by 4 to 6 hours (data not shown). Thirty patients were enrolled (Table 1). No grade III/IV or dose-limiting toxicities were noted. No toxicities were reported, and a maximum tolerated dose (MTD) was not reached. The time between pre- and posttreatment biopsies was similar for the group that received the STAT3 decoy and the group that received saline (4.1 ± 2.7 vs. 4.5 ± 2.1 hours; P = 0.643). There was evidence of decreased expression of STAT3 target genes, including cyclin D1 and Bcl-XL, in the posttreatment tumors compared with levels in the pretreatment biopsies in the group that received STAT3 decoy, compared with expression in the tumors from the group that received saline (Fig. 1B–F). There was no evidence of a dose–response on the modulation of target gene expression levels (data not shown). There was no apparent association between baseline levels of total or phosphorylated STAT3 in the tumor and degree of modulation of target gene expression (data not shown). To ensure that global gene expression or RNA stability was not impacted, we carried out ion reverse transcription (RT)-PCR on a subset of tumors. While RNA expression levels for cyclin D1 and Bcl-XL clearly declined, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were unchanged (data not shown). These findings suggest that intratumoral administration of a transcription factor decoy targeting STAT3 is safe and may decrease target gene expression in HNSCC tumors. Future studies of STAT3 inhibitors in patients with cancer are warranted (ClinicalTrials.gov number NCT00696176).

Intravenous Injection of Parental STAT3 Decoy Fails to Abrogate Xenograft Tumor Growth

The STAT3 decoy used in the phase 0 trial consists of a 15-mer duplex oligonucleotide with phosphorothioate modifications at the 5′ and 3′ ends to enhance stability as described previously (19). To determine whether this parental STAT3 decoy could be administered systemically and retain antitumor effects, mice harboring cancer xenografts were given daily intravenous injection of the decoy (5 mg/kg). No reduction of tumor growth (Supplementary Fig. S1A) or downmodulation of STAT3 target genes in the tumors (Supplementary Fig. S1B) was observed, showing that the parental STAT3 decoy requires local/intratumoral delivery to inhibit STAT3 signaling.

Design of Modified STAT3 Decoys

A plausible explanation for the lack of antitumor activity of the systemically administered parental STAT3 decoy is
Figure 1. Intratumoral administration of a STAT3 decoy oligonucleotide abrogates target gene expression in patients with HNSCC. A, schema of phase 0 trial. Biopsies of HNSCC tumors were performed and the tumors were injected with a single dose of a STAT3 decoy oligonucleotide (or saline) followed by tumor resection and analysis of target gene expression in the paired tumor samples. OR, operating room. B, downmodulation of STAT3 target genes in representative HNSCC tumors injected with STAT3 decoy as shown by Western blot analyses. Whole-tumor lysates were prepared from 4 HNSCC tumors before and after injection with the STAT3 decoy enrolled on the first dose tier. Proteins (40 μg) were resolved on a 12.5% SDS-PAGE gel and subjected to immunoblotting with anti-Bcl-XL and cyclin D1 antibody. β-Actin was used as a loading control. C, representative images of immunohistochemical staining for cyclin D1 protein expression before and after injection of STAT3 decoy oligonucleotide or saline from 6 patients. D, cumulative quantitative determination of cyclin D1 expression in tumors of all 30 patients with HNSCC showed a significant decrease in expression in tumors injected with STAT3 decoy oligonucleotide compared with saline injection (P = 0.0431). E, representative images of immunohistochemical staining for Bcl-XL protein expression before and after injection of STAT3 decoy oligonucleotide or saline from 6 patients with HNSCC. F, cumulative quantitative determination of Bcl-XL expression in tumors of all 30 patients with HNSCC showed decreased expression in tumors injected with STAT3 decoy compared with the tumors injected with saline (P = 0.0634).
the vulnerability of this reagent to degradation and/or thermal denaturation in vivo, due to the presence of free ends. Modifications of the parental STAT3 decoy were undertaken in an effort to improve serum half-life and thermal stability and thereby facilitate systemic delivery. Because nucleolytic degradation predominantly occurs at the 3′ end of single-stranded DNA or frayed ends of duplexes, we predicted that linkage of the 2 strands, as well as complete circularization, would improve stability in serum, while also enhancing thermal stability, ensuring that the decoy remains in annealed duplex form. Three distinct, unimolecular derivatives of the parental STAT3 decoy were generated and evaluated (Fig. 2).

### Modified STAT3 Decoys Exhibit Longer Half-lives in Serum

Following incubation in mouse serum for varying lengths of time, approximations of decoy half-life of the parental and modified STAT3 decoys were determined (Fig. 3A and B). Consistent with its lack of antitumor activity when administered systemically, the parental STAT3 decoy exhibited a relatively short serum half-life of approximately 1.5 hours. In contrast, each of the modified decoys exhibited substantially longer half-lives. The half-life of DN4 was approximately

**Table 1. Clinical and pathologic features of patients with HNSCC enrolled in phase 0 STAT3 decoy trial**

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<td>Time between pre- and posttreatment biopsies, h</td>
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<td>4.5 ± 2.1</td>
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4 hours, whereas that of DS18 was about 3.5 hours. The most stable derivative was the cyclic decoy, which was detected up to 12 hours in serum. The markedly improved stability of the cyclic STAT3 decoy indicated that removal of all free ends, via circularization, was important for enhancing resistance to degradation.

Because the decoy acts to mimic double-stranded STAT3 response elements in target genes, thermal denaturation temperatures above 37°C will be important for effective systemic administration. UV denaturation determinations (Supplementary Fig. S3) revealed a melting temperature of only 30°C for the parental STAT3 decoy. However, generation of unimolecular decoy forms resulted in enhanced thermal stability, with the DN4 and DS18 STAT3 decoys yielding melting temperatures of 57°C and 54°C, respectively. Moreover, complete circularization resulted in dramatic resistance to thermal denaturation, with the cyclic STAT3 decoy showing a melting temperature of 80°C, well above body temperature.

**Modified STAT3 Decoys Bind Avidly to pSTAT3 Protein**

We next determined whether any of the chemical modifications of the parental STAT3 decoy interfered with binding to STAT3 protein. Binding assays were conducted using recombinant, tyrosine (Y705)-phosphorylated STAT3 protein (pSTAT3), representing the activated form of the transcription factor (26, 27). Parental or modified STAT3 decoys were first incubated with the pSTAT3 protein, followed by non-denaturing PAGE and SYBR-Gold staining of the nucleic acid decoys (Supplementary Fig. S4). Results from these experiments suggested that the DN4, DS18, and cyclic STAT3 decoys exhibited similar binding as the parental STAT3 decoy. These findings were subsequently confirmed by surface plasmon resonance (SPR) measurements, which additionally provided quantitative binding interaction parameters (Fig. 4). SPR analyses permitted derivation of the rates and affinities of association and dissociation between the decoys in solution and immobilized pSTAT3 protein, as monitored in real time. In general, the chemical modifications introduced in the DN4, DS18, and cyclic STAT3 decoys did not significantly perturb the kinetics of complex formation, with the $k_a$ and $k_d$ of the DN4, DS18, and cyclic decoys to immobilized pSTAT3 remaining largely unchanged compared with the parental STAT3 decoy (Fig. 4). To quantitatively evaluate the strength of interactions between the 4 STAT3 decoys and the pSTAT3 protein, their equilibrium dissociation constants ($K_D$) were determined by fitting the SPR data according to a 1:1 Langmuir binding model. The $K_D$ values were calculated as a function of their rates of dissociation relative to association, according to the following equation $K_D = 1/K_A = k_d/k_a$ ($K_A$ is the equilibrium association constant). The immobilized pSTAT3 protein bound the parental
and modified decoys (DN4, DS18, and cyclic) with similar nanomolar affinities (Supplementary Table S1). Thus, the chemical modifications introduced in the parental decoy, resulting in the enhanced serum half-lives and thermal stabilities of the DN4, DS18, and cyclic STAT3 decoys, did not adversely affect their binding to pSTAT3 protein.

Modified STAT3 Decoys Inhibit In Vitro Viability and Expression of STAT3 Target Genes in Cancer Cell Lines

To determine whether chemical modifications in the STAT3 decoy resulted in altered in vitro activities, HNSCC cells (UM-SCC1, UM-22B) and bladder cancer cells (T24) were treated with varying concentrations of parental STAT3 decoy, DN4, DS18, or cyclic STAT3 decoy to determine EC_{50} values (Supplementary Table S2). Corresponding mutant control decoys that differed from the parental or modified decoys at a single base pair (as described in Methods) were also evaluated. In the 3 cell lines tested, the parental and modified STAT3 decoys exhibited EC_{50} values in the low nanomolar range (<100 nmol/L) at the end of 24, 48, and 72 hours. In contrast, none of the mutant control decoys showed nanomolar activity.

Transcription factor decoys act by interfering with the transcription of target genes. To determine the impact of the modified STAT3 decoys on expression of key STAT3 target genes, UM-SCC1 (Supplementary Fig. S5A), UM-22B (Supplementary Fig. S5B), and T24 cells (Supplementary Fig. S5C) were treated with IC_{50} concentrations of DN4, DS18, cyclic STAT3 decoy, or corresponding mutant control decoys. Following incubation, immunoblotting was used to assess Bcl-X\textsubscript{L} and cyclin D1 expression levels. Treatment with DN4, DS18, and cyclic STAT3 decoy led to downregulation of both Bcl-X\textsubscript{L} and cyclin D1 when compared with treatment with vehicle alone or with the corresponding mutant control decoy. Thus, in cancer cell lines, the modified DN4, DS18, and cyclic STAT3 decoys retained the ability to decrease the expression of STAT3 target genes.

Cyclic STAT3 decoy does not inhibit cell viability or STAT3 target gene expression in STAT3-null cells but potently reduces cell viability and downmodulates STAT3 target genes in cells expressing wild-type STAT3. To determine the specificity of the cyclic STAT3 decoy, A4 colon cancer cells expressing human wild-type STAT3 (A4 STAT3 wild-type) or isogenic cells engineered to serve as STAT3-null cells (A4 STAT3-null cells; ref. 28) were used to determine the effect of the parental or modified decoys. The A4 STAT3-null cells when treated with the parental or cyclic STAT3 decoy did not show downmodulation of STAT3 target genes (Supplementary Fig. S6A) or inhibition of growth. In contrast, the isogenic cells that retain STAT3 expression were potently growth inhibited by treatment with the parental or cyclic STAT3 decoy (Supplementary Fig. S6B) in association with downregulation of STAT3 target gene expression (Supplementary Fig. S6C). These results suggest that STAT3 is the selective target of the STAT3 decoys and indicate that tumors that do not express STAT3 are unlikely to be responsive to treatment with the STAT3 decoy.

**Systemic Administration of Cyclic STAT3 Decoy Inhibits Tumor Growth and Expression of STAT3 Target Genes In Vivo**

Our in vitro studies revealed that the modified, unimolecular DN4, DS18, and cyclic STAT3 decoys showed enhanced serum half-lives and thermal stabilities while retaining biologic and biochemical activities. On the basis of these results, we sought to determine whether systemic intravenous administration of the modified decoys would exert effects on xenograft tumors. To evaluate the antitumor effects of systemic administration of the cyclic STAT3 decoy, mice bearing established HNSCC xenografts (10 mice per group) were given daily intravenous injections (tail vein) of the cyclic decoy or the corresponding cyclic mutant control decoy (5 mg/kg/d), and tumor growth was monitored for 19 days. Tumors treated with the cyclic STAT3 decoy exhibited significant growth inhibition relative to tumors treated with cyclic mutant control decoy \((P < 0.0001, \text{Fig. 5A})\). Moreover, 2 of 10 tumors treated with cyclic STAT3 decoy experienced complete tumor regression. To ascertain the impact of the systemically administered cyclic STAT3 decoy on the expression of STAT3 target genes, tumors were harvested after 19 days of treatment, and the levels of cyclin D1 and Bcl-X\textsubscript{L} in the tumors were determined. Relative to treatment with cyclic mutant control decoy, systemic administration of cyclic STAT3 decoy resulted in a significant decrease in cyclin D1/\beta-actin ratio \((P = 0.0015)\) and Bcl-X\textsubscript{L}/\beta-actin ratio \((P = 0.0021; \text{Fig. 5B})\). Cyclic STAT3 decoy treatment did not alter the expression of total or phosphorylated STAT3 in the tumors (Supplementary Fig. S7). These results indicate that the cyclic STAT3 decoy exerts its antitumor effects by impacting STAT3 activity and not by inhibiting the expression of either total or phosphorylated STAT3.
DISCUSSION

To date, there have been only a handful of phase 0 trials reported in patients with cancer, and none have targeted a transcription factor, included a control group, or used an intratumoral route of administration. We selected the phase 0 trial model for this first-in-human study of a STAT3-selective inhibitor in patients with cancer to determine whether the STAT3 decoy warranted further clinical development. The intraoperative setting used in our trial allowed for the collection of tumor tissue before and after administration of the STAT3 decoy. The inclusion of a control group enabled us to determine the specificity of the STAT3 decoy on target gene expression in human HNSCC tumors. Our results show significant pharmacodynamic activity of the intratumoral STAT3 decoy relative to saline control. Phase 0 trials have also proven useful in guiding subsequent studies as seen in the development of the PARP inhibitor ABT-888 where the phase 0 results showed significant inhibition of PAR levels in tumor biopsies and peripheral blood mononuclear cells at specific dose levels (29).

STAT3 is hyperactivated in the majority of human cancers, where preclinical evidence supports STAT3 as a therapeutic target. Agents amenable to clinical administration
that inhibit STAT3 generally lack specificity, target upstream receptor or non-receptor kinases, or represent natural products, which have a plethora of molecular targets (13, 30). Oligonucleotide (ODN) decoys selectively inhibit the action of transcription factors but have been limited by their bioavailability due to rapid degradation. There has only been a single decoy oligonucleotide tested clinically in the setting of vascular disease and none to date for cancer therapy (31). The results of our phase 0 trial showed downregulation of STAT3 target gene expression in HNSCC tumors after a single intratumoral inoculation. This is especially compelling because expression level changes were compared with levels in paired biopsies from a control group that received an injection of vehicle (saline) alone into their tumor. Furthermore, most preclinical studies have examined the effects of decoys on target gene expression at far later time points (e.g., days) compared with the 2- to 6-hour exposure in our intraoperative phase 0 trial. Cancer is largely a systemic disease, and therapeutic benefits are likely limited for an agent that requires intratumoral injection. These cumulative findings underscore the need for decoy formulations that are amenable to repeat systemic administration.

Transcription factor decoys are generally composed of double-stranded oligonucleotides with a high affinity for a transcription factor and compete for binding to the protein with specific cis elements present in the promoter region of target genes. Although phosphorothioate modifications represent the most common strategy to improve stability, we and others have found that decoys with a fully modified phosphorothioate backbone have reduced affinity for the specific DNA binding site and hence reduced efficacy (19, 32). Oligonucleotides modified with only terminal phosphorothioate linkages exhibit increased resistance to exonucleases but retain susceptibility to endonuclease activity (33, 34). The unmodified parent STAT3 decoy with terminal phosphorothioate modifications showed high affinity as well as efficacy both in vitro (19) and when administered intratumorally (20) but failed to show antitumor efficacy when injected intravenously, indicating degradation of the STAT3 decoy by serum nucleases as a significant limitation to systemic delivery.

To date, chemical modifications of decoy oligonucleotides to improve serum stability have been associated with reduced biologic efficacy and diminished binding to target proteins (35). Several strategies have been adopted to structurally modify transcription factor decoys in attempts to overcome some of the limitations associated with phosphorothioation. Transcription factor decoys modified with peptide nucleic acids have shown increased serum stability but often at the cost of binding specificity and affinity to target proteins (35, 36). Oligonucleotides have also been modified with locked nucleic acids (LNA), a nucleic acid analogue to improve resistance to nuclease degradation. However, substitution of nucleotides with LNA close to the transcription factor-binding region induces conformational changes of adjacent nucleotides that can interfere with binding affinity (35). Crinelli and colleagues reported that substituting nucleotides in an NF-kB decoy with LNA at different positions increased the half-life of the decoys in serum to 40 to 48 hours but led to failure of the LNA-modified decoys to bind to NF-kB protein (35). Osako and colleagues modified an NF-κB decoy into a circular oligonucleotide (RODN) and compared it with phosphorothioate-modified (PODN) and -unmodified (NODN) NF-kB decoys (37). Although RODN and PODN had serum stabilities of 6 and 24 hours, respectively, compared with less than an hour for NODN, binding assays showed that PODN had very low affinity for NF-kB protein. Another transcription factor decoy targeting activator protein 1 (AP-1) was modified to form a dumbbell-like structure (CD AP-1; ref. 38). The activity of CD AP-1 has been studied in vitro; however, serum stability data pertaining to the resistance of CD AP-1 to nuclease degradation has not been reported.

Our results suggest that altering the STAT3 decoy to create a unimolecular structure by a hairpin loop containing 4 single-stranded nucleotides (DN4) or with a hexaethylene glycol spacer (DS18) or by complete cyclization results in a more stable therapeutic compound by making it more resistant to serum nucleases while retaining potency and target specificity, in contrast to the parent decoy, which is highly susceptible to degradation and thermal denaturation. Our modified STAT3 decoys inhibited cell proliferation and reduced the expression of STAT3 target genes in vitro and in vivo and inhibited the growth of xenograft tumors.

Cumulative evidence has implicated a variety of transcription factors, including STAT3, in the development and/or maintenance of an oncogenic phenotype. To date, however, transcription factors have generally been regarded as “undruggable” target molecules. This is the first report of administration of a STAT3-selective molecular targeting agent to patients with cancer resulting in a pharmacodynamic signature of biologic activity. Cyclization of this decoy allowed for successful systemic administration and suggests that clinical development of the STAT3 decoy and other transcription factor decoys may yield effective therapeutic agents.

METHODS

Production of Clinical Grade STAT3 Decoy

The STAT3 decoy (NSC-741763), which is an annealed, double-stranded oligonucleotide that is fully complementary and partially phosphorothioated, with the sense sequence being 5′-C*A*T*TTCGCCGTTT*A*T*C-3′ and the antisense sequence 5′-G*A*T*TTACGGGA*A*T*G-3′ (“*” denotes phosphorothioated sites), was manufactured at National Cancer Institute (NCI)-Frederick, Biopharmaceutical Developmental Program (Frederick, MD). The STAT3 decoy was formulated in PBS (purchased from Amresco Solon) at 3.5 mg/ml stock concentration. The NIH RAID program assisted in the manufacture of the decoy compound under Good Manufacturing Practice conditions and oversaw the preclinical toxicology studies (25). The potency of the clinical grade decoy was confirmed by assessing its effects on STAT3 promoter activity and target gene expression as described previously (19).

Phase 0 Clinical Trial

Patients ≥18 years with primary or recurrent HNSCC that was histologically confirmed and amenable to surgical resection were eligible to enroll in this study. Other eligibility criteria included Eastern Cooperative Oncology Group scale 0–2, hemoglobin ≥10 g/dL, absolute neutrophil count (ANC) ≥1,500/μL, platelets ≥100,000/μL, creatinine ≤1.5 times upper limit of normal, bilirubin ≤1.5 times normal.
upper limit of normal, aspartate aminotransferase (AST) ≤2.5 upper limit of normal, and corrected serum calcium ≤10.5 mg/dL. Patients treated previously with radiation, chemotherapy, or targeted agents were permitted. Ineligible patients included those who were pregnant, had tumors too small to reserve a portion for research purposes, or had received neoadjuvant radiotherapy and/or chemotherap during a 4-week period before enrollment. The study was approved by the University of Pittsburgh Institutional Review Board (IRB# 08020216, UPCI# 07-022) and registered on Clinicaltrials.gov (NCT00696176). Written informed consent was obtained from patients. Patients were enrolled sequentially on 1 of 3 dose tiers (250 mg decoy in 250 μL of saline, 500 mg in 500 μL, 1,000 mg in 1 mL), which were based on extrapolation of the relative size of a xenograft in a mouse to the average volume of a human head and neck tumor. After the administration of general anesthesia for the tumor resection, the pretreatment tumor biopsy was obtained. Next, the STAT3 decoy was administered by direct inoculation of the tumor with a 25-gauge needle. After resection of the tumor, a posttreatment biopsy was obtained from the area of the tumor that had been injected with the decoy. Control patients who were injected with saline rather than the decoy were included to determine the specificity of the STAT3 decoy and to distinguish between the effects of the decoy and the effects of surgery. Patients were monitored for adverse events using the NCI Common Terminology Criteria for Adverse Events version 3.0. Patients were followed for survival until 2 years after the date of surgery.

**Tissue Acquisition, Processing, Tissue Microarray Construction, and Immunohistochemistry**

Biopsies of the tumors were performed before and after administration of the STAT3 decoy (or saline) intraoperatively. The injection site was marked to assure that the posttreatment sample represented tissue that was exposed to the decoy. Tissue was processed primarily for the construction of a tissue microarray to allow for assessment of protein expression across the entire cohort. When possible, fresh-frozen material was processed for Western blotting as described previously (39). A tissue microarray of the paired specimens from decoy- and saline-treated HNSCC tumors was constructed as described previously (40). Tissues were snap-frozen in a cryobath before being placed in a freezer at −80°C. Sections were cut at 5 μm and mounted on Superfrost Plus slides, dried overnight at room temperature, and then at 60°C for an hour. The sections were deparaffinized and hydrated before heat-induced epitope retrieval by a retrieval buffer. Blocking was conducted with Invitrogen CAS block. Slides were washed with antibodies and Dako Substrate Chromagen and incubated with TBS. Slides were then counterstained with Harris Hematoxylin, dehydrated, and cleared. The tissue microarray was stained with STAT3 (catalog number 9132, Cell Signaling Technology), pSTAT3 (catalog number 9145, Cell Signaling Technology), cyclin D1 (Clone SP4, catalog number M3635, Dako Technology), and Bcl-XL (catalog number 2762, Cell Signaling Technology). All assessments of levels of STAT3 activation or target gene expression were determined by laboratory personnel blinded to the treatment group (STAT3 decoy vs. saline), and the time of sampling (pre- vs. postinjection). Assays were conducted only after the specimens from the entire cohort were collected to minimize assay variability. The immunostaining was interpreted as the intensity of staining (0–3 scale), and the percentage of positively staining cells in the tissue section (0%–100%) reported as intensity multiplied by expression.

**Cell Line Validation**

Cell lines were validated using the AmpEFlSTR Profiler Plus Kit from PE Biosystems according to the manufacturer’s instructions. T24 bladder cancer cell line was obtained from American Type Culture Collection. UM-SCC1 and UM-22B were a kind gift from Dr. Thomas E. Carey (University of Michigan, Ann Arbor, MI).

**Generation of Modified STAT3 Decoys**

Our initial design was to convert the bimolecular parental decoy (19) into a unimolecular system by bridging the sense and antisense strand through a 4-base linker (DN4) or by a hexaethylene glycol linkage (DS18). The parental STAT3 decoy was also circularized using 2 hexaethylene glycol linkers attached to the sense and the antisense strands followed by enzymatic ligation of the 3’ and 5’ ends of the oligonucleotides. The mutant controls differed by one nucleotide at position 9 (G to T). We have previously shown that mutation of this nucleotide position abrogates decoy binding to STAT3 protein (19). The single-stranded sense and antisense oligonucleotides of the STAT3 decoy/STAT3 mutant, DN4/MN4, and DS18/MS18 were obtained from Integrated DNA Technologies. The cyclic STAT3 decoy/cyclic STAT3 mutant was obtained from Oligos, Etc.

**Serum Stability Assays**

For the serum stability assay, the parental STAT3 decoy, DN4, DS18, and cyclic STAT3 decoy were incubated in 20 μL mouse serum at a final concentration of 0.05 μg/μL according to standard protocol (41). Following separation, the gels were stained with SYBR-Gold and imaged using the Gel Logic 2200 Imaging System.

**Thermal Denaturation Assay**

Thermal denaturation studies were conducted using a Varian Cary 300 Bio spectrophotometer equipped with a thermodenatured multiecell holder, using 1.5 μmol/L strand concentration each in 10 mmol/L Tris and 1 mmol/L EDTA, pH 8.0. Thermal denaturation of STAT3 decoy, DN4, DS18, and cyclic STAT3 decoy were monitored at 260 nm. Both the heating and cooling runs were conducted at the rate of 1°C/min. Melting transitions (Tm values) were determined by taking the first derivatives of the UV melting curves.

**Therm 3-Binding Assays**

For in vitro binding assays, parental and modified decoys for pSTAT3 protein were accomplished by SPR analyses, using a BiAcore 3000 instrument (GE Healthcare) following standard protocols (42, 43). Unreacted sites on the chip surface were blocked using 1.0 mol/L ethanolamine-HCl (pH 8.5). Binding of parental STAT3 decoy, DN4, DS18, and cyclic STAT3 decoy to pSTAT3 protein was determined at several concentrations of analyte solutions, at a flow rate of 30 μL/min in a running buffer (20 mmol/L HEPES, pH 7.0, 200 mmol/L NaCl, 10 mmol/L MgCl2).

**Dose Response to Determine EC50 Value**

UM-SCC1, UM-22B, and T24 cells (30,000 cells per well) were seeded in 24-well plates in Dulbecco’s Modified Eagle Media (DMEM) containing FBS. After 24 hours, cells were transfected with varying concentrations of STAT3 decoy, DN4, DS18, and cyclic STAT3 decoy. After 4 hours, the transfection media were replaced with DMEM containing 10% serum. At the end of 24, 48, and 72 hours, MTT assays were conducted to determine the percentage of cell viability.

**Immunoblotting**

Immunoblotting was conducted as previously described (20). Antibodies used for immunoblotting included rabbit anti-human cyclin D1 polyclonal antibody, mouse anti-human Bcl-XL monoclonal antibody (Santa Cruz Biotechnology), rabbit anti-mouse pSTAT3 monoclonal antibody and rabbit anti-mouse STAT3 polyclonal...
antibody (Cell Signaling). Blots were developed using the enhanced chemiluminescence (ECL) detection system (Amersham Life Sciences Inc.). The membranes were stripped and then probed with rabbit anti-human β-tubulin polyclonal antibody (Abcam, Inc.). Densitometric analyses were conducted using ImageJ software.

**Systemic Administration of Parental STAT3 Decoy In Vivo**

Female athymic nude mice naïve (4–6 weeks old; 20 grams; Harlan Sprague-Dawley) with T24 xenograft tumors were treated with intravenous injection of STAT3 decoy or saline (vehicle; 100 μg) on a daily basis. Tumor volumes were measured 3 times per week. On day 18, the tumors were harvested, and immunoblotting of the tumor tissue was conducted to detect Bcl-XL and cyclin D1. Detection of β-tubulin was used to assess protein loading. Animal care was in strict compliance with institutional guidelines established by the University of Pittsburgh (Pittsburgh, PA), the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, 1996), and the Association for Assessment and Accreditation of Laboratory Animal Care International.

**Systemic Delivery of Cyclic STAT3 Decoy In Vivo**

Female athymic nude mice naïve (4–6 weeks old; 20 grams; Harlan Sprague-Dawley) with UM-SCC1 tumors were treated daily with intravenous injections of cyclic STAT3 decoy or cyclic mutant STAT3 decoy (100 μg). Palpable tumors were detected by day 3, and there was 100% tumor take for the cell lines used. During the treatment period, tumors were measured 3 times a week for 19 days. The size of the control tumors (treated with the mutant cyclic decoy) reached the maximum allowable tumor volume by day 19 so we elected to stop the experiment at that time point. At the end of the treatment period, the tumors were harvested and subjected to immunoblot analyses.

**Statistical Analyses**

The goal of the clinical trial was to monitor toxicity and to obtain preliminary estimates of biologic efficacy of the STAT3 decoy. As a phase 0 trial, no hypotheses about therapeutic efficacy, biologic activity, or optimum dose level were specified. A minimum of 5 patients were accrued to each dose level to provide a one-tailed signed-rank test to reject the null hypothesis of no change at α = 0.03125. In vivo tumor volumes for the systemic delivery of cyclic STAT3 decoy experiment have been estimated with linear regression using a smoothing spline to capture nonlinear tumor volume growth profiles. The spline fit is a flexible model that summarizes the overall trend and unlike a simple mean plot, borrows information from all profiles. The spline fit is a flexible model that summarizes the overall trend and unlike a simple mean plot, borrows information from all profiles. The model estimates the overall trend and is robust to outliers.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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