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DRUGGING "UNDRUGGABLE" DISEASE-CAUSING PROTEINS: FOCUS ON SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION (STAT) 3

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Abstract

Signal transducer and activator of transcription (STAT) 3 has been assigned to the group of "undruggable" disease-causing proteins, despite its containing a Src-homology (SH) 2 domain, a potential Achilles' heel that has eluded successful targeting by academic and pharmaceutical groups over the past 30 years. Based on mutational and modeling studies, our group developed a unique virtual ligand screening strategy targeting the STAT3 SH2 domain that was coupled to robust biochemical and cellular assays and structure-based medicinal chemistry and led to the identification of TTI-101. TTI-101 represents one of the most advanced, direct, small-molecule inhibitors of an SH2 domain-containing, disease-causing protein in clinical development. TTI-101 is currently being evaluated in a Phase 1 study to determine safety and tolerability in addition to pharmacodynamic effects and efficacy in patients with advanced solid tumors.

INTRODUCTION

The Undruggable Human Proteome

While the complete sequence of the human genome, including "dark DNA," only became available this year (1), the sequence of the functional portion of the human genome has been available for over two decades. One hope arising from this first major scientific accomplishment of the new millennium was that it would mark the dawn of a new era in therapeutics. While some of this hope has been realized, especially with the development of targeted therapies, notably tyrosine kinase inhibitors (TKI) and monoclonal antibodies, progress has been stymied by the finding that many disease-causing proteins are not "druggable" (i.e., not amenable to either small-molecule or antibody targeting). Of the 20,500 proteins encoded by human genome, only 3,051 (15%) have been deemed druggable by small or large molecules (e.g., monoclonal antibodies) (2).

The 20,000 or so FDA-approved drugs currently available target only 2% of human proteins (2), with these proteins falling into five major families: (1) G-protein coupled receptors (e.g., adrenergic receptors), which have been targeted to treat cardiac and pulmonary diseases; (2) kinases, in particular tyrosine kinases, starting with BCR-ABL, the underlying cause of chronic myelogenous leukemia, which was first successfully targeted with imatinib; (3) proteases critical for viral diseases caused by HIV, hepatitis C, and SARS-CoV-2, which have been effectively targeted and may, in the case of hepatitis C, lead to its eradication; (4) nuclear hormone receptors, in particular the estrogen and androgen receptors, which have successfully been targeted to treat breast and prostate cancer; and (5) cell surface receptors, including those mediating pathological cytokine signaling (TNF receptor; etanercept), B-cell lineage (CD20; rituximab), and immune checkpoint couplets, such PD-1/PD-LI and CTLA-4/B7.1/2. One or both partners of these couplets have been successfully targeted with monoclonal antibodies to treat cancer (e.g., PD-1 with pembrolizumab and CTLA-4 with ipilimumab).

In oncology, only 40 of the 700 or so cancer-causing proteins have been successfully targeted to date (2). Key features of undruggable disease-causing proteins, including those causing cancer, are their intracellular location, which limits accessibility of large molecules, particularly monoclonal antibodies, to the protein target and the lack of a small-molecule binding pocket within the protein. Some high-value protein targets for cancer treatment include mutated RAS and MYC proteins and STAT3.

STAT3 Is a Major Disease-Causing Protein Yet to Be Drugged

A substantial body of evidence summarized in recent reviews (3-5) supports a critical contribution of STAT3 to inflammation, fibrosis, and cancer. In the area of fibrosis, for which there is a particularly large unmet medical need, STAT3 activation occurs within fibroblasts following binding of both IL-6 and TGF-b, which are produced by inflammatory and fibrogenic macrophages, respectively (6). Activation of STAT3 within fibroblasts leads to their differentiation into myofibroblasts, which are the major cells responsible for mediating tissue fibrosis (6). In the field of cancer, another area with a large unmet medical need, STAT3 has been shown to contribute to 10 of the recently updated 14 hallmarks of cancer (3, 5, 7), including proliferation and survival, which are intrinsic to cancer cells, and immune suppression and angiogenesis, which arise extrinsically within the tumor microenvironment (TME).

Structural Basis for Small Molecule Targeting of STAT3

The first step in STAT3 activation is its recruitment via its SH2 domain to specific tyrosine-phosphorylated peptide motifs within cytokine receptors. Following its recruitment, STAT3 is itself phosphorylated on tyrosine (pY) 705, either by receptor-intrinsic tyrosine kinases or receptor-associated tyrosine kinases. STAT3 then undergoes homodimerization and translocation into the nucleus where it accumulates and binds to DNA within the promoter regions of genes resulting in their transcriptional activation, and in some cases, transcriptional repression.

Our group first identified a protein in human myeloid cells with characteristics of a STAT protein that transiently associated with the G-CSFR receptor, along with two tyrosine kinases, LYN and SYK, following G-CSF binding (8). We initially referred to this novel STAT protein as STATG (9) but subsequently determined that it was the newly identified β isoform of human STAT3 (10). STAT3 binds to two pY-peptide regions within the G-CSFR (11). One region contains residue Y704 that resides within the canonical STAT3 pY-peptide binding motif—YXXQ. In this canonical motif, the +3 residue C-terminal to the Y residue, glutamine (G), is conserved, while residues at the +1 and +2 positions (X) are not. The second STAT3 binding pY-peptide region within the G-CSFR is located at Y744. Interestingly, the +3 residue within this pY-peptide motif is cysteine (C). Detailed studies by our group demonstrated that when C is in a reduced state (-SH), it is able to form H-bonds critical for its binding to STAT3 in a manner similar to the -OH group within the G of YXXG-containing canonical peptide motifs (11, 12).

Additional studies by our group and others that interrogated at the atomic level the binding pocket within STAT3 that binds pY-peptides (13) led to the structural underpinnings of the virtual ligand screening (VLS) strategy we used to identify small molecules that competitively target this interaction (see in next section below). Crucial to the success of this effort was the availability of the crystal structure of the SH2 domain of GRB2 bound to a pY-peptide based on the Y1068-peptide located within the EGFR (14). This peptide also is bound by STAT3 and has a beta turn structure when bound by GRB2 rather than being a linear peptide, which is the configuration that pY-peptides assume when bound to the SH2 domain of SRC and most other SH2-domain containing proteins (14). Critically, the pY-peptide binding pocket within STAT3 was found to be structurally similar to that of GRB2 (15). Since it also bound to the pY1068-peptide with high affinity, we reasoned that when bound by STAT3, the pY1068-peptide would assume a beta turn. Furthermore, by assuming a beta turn, the residues within the STAT3 SH2 domain that interacted with the pY1068-peptide, similar to those within the SH2 domain of GRB2, are located within 10 angstroms of each other, a distance that can be bridged by a smallmolecule, including drug-like molecules able to fulfill Lipinski's rule of 5 (16).

MATERIAL, METHODS, RESULTS, AND DISCUSSION

TTI-101 Is a Potent, Direct, Oral, Small-Molecule STAT3 -Inhibitor

To date, no small molecule that directly targets STAT3 has been approved for clinical use. To begin to test the hypothesis that STAT3 can be targeted with a small, drug-like molecule, our lab used computer-based docking of 920,000 compounds from commercial chemical libraries into the pocket within the SH2 domain of STAT3 responsible for its binding to pY-peptide ligands within cytokine-activated receptors. We identified three hits [C3, C30, and C188; (17)] capable of directly blocking two steps in STAT3 activation—its recruitment to cytokine-activated receptors and its homodimerization. Hit-to-lead strategies focused on the most potent initial hit, C188 (17,18), and led to the identification of TTI-101, formerly C188-9 (3,19). TTI-101 binds directly to STAT3 to inhibit its activities, including: (1) potent inhibition of STAT3 binding to its pY-peptide ligand (Ki=12.4 nM) in surface plasmon resonance (SPR) assays (20); (2) reduction of constitutive levels of pY-STAT3, in human cancer cell lines at concentrations as low as 4 nM (21); (3) inhibition of cytokine-stimulated increases in pY-STAT3 at 3.7 ± 1.9 μ M (20); and (4) potent inhibition of proliferation driven

by either WT STAT3 or GOF STAT3 mutants (22). Pharmacokinetic (PK) and toxicology studies in mice, rats, and dogs demonstrated that TTI-101 administration provides excellent plasma exposures following oral administration [65 to 78% of IV; (20)] and revealed no toxicity detectable in gross, microscopic, or clinical laboratory evaluations at a Good Laboratory Facility (GLP) when administered in maximum doses of up to 100 mg/kg/day for 28 days in dogs and up to 200 mg/kg/day for 28 days in rats (20). In addition, tumor PK studies in mice demonstrated tumor levels 2.6 times those of simultaneously measured plasma levels (20). Thus, based on the mean IC_{90} for inhibition of growth induced by WT STAT3 (2.8 ρM) and a 2.6-fold concentration of TTI-101 in tumor, the plasma concentration of TTI-101 predicted to inhibit 90% of STAT3-driven proliferation of tumor cells is 1.1 ρM.

Binding of TTI-101 to STAT Proteins Was Selective for STAT3

To assess the selectivity of TTI-101 for STAT3 versus the other six members of the STAT protein family, we synthesized cDNAs encoding each human STAT protein optimized for expression in *E. coli*. Recombinant STAT proteins were labeled with Atto488 NHS dye and examined for binding of TTI-101 using a Monolith NT.115 MST device. Binding affinities of TTI-101 to each STAT protein revealed three affinities: (1) strong affinity (K_D = 405-1766 nM) shown for STAT3, STAT1, STAT5A, and STAT5B; (2) weak affinity (K_D ≥ 5-fold that for STAT3; $K_{D/STAT3}$ = 798 ± 197 nM) shown for STAT2 and STAT6; and (3) no affinity shown for STAT4.

TTI-101 Has No Off-Target Effects

TTI-101 did not inhibit tyrosine kinases known to activate STAT3 (19) and was specific for inhibition of STAT3 activation by IL-6 in human cells. Briefly, human myotubes were pre-incubated with or without TTI-101 (30μM) for one hour and treated with or without IL-6 plus sIL6Rα (@100 ng/ml) for 30 minutes (23). Luminex bead-based analysis revealed that pre-treatment with TTI-101 significantly inhibited STAT3 tyrosine phosphorylation ($p \leq 0.0001$). Analysis of 1,320 proteins within the Phospho Explorer Antibody Array using ANOVA-normalized p-values revealed 55 analytes that were significantly different within the four groups. Of the 55 analytes, 48 were significantly modulated by IL6/sIL6R treatment (38 upregulated and 10 downregulated). Out of these 48, TTI-101 pre-treatment significantly modulated the IL6/sIL6R-mediated effect of 16 (33.3%) analytes (15 of 38 of those upregulated and 1 of 10 of those downregulated). Several of these analytes were previously reported to be modulated by STAT3, while others were newly identified. Importantly, of the IL6-upregulated analytes that are known to be upregulated through non-STAT3 mechanisms (e.g., MAPK, PI3K, or AP-1), TTI-101 either had no effect or resulted in further upregulation. Similarly, within the group of the IL6/sIL6R-downreglated analytes known to be downregulated by non-STAT3 pathways, each was largely unaffected (8 of 9) or further downregulated by TTI-101 (1 of 9). Altogether, these results provide strong evidence that TTI-101 only affected levels of analytes or phosphorylation events dependent on STAT3 signaling in human cells.

TTI-101 Has No Adverse On-Target Effects

In addition to sparing tyrosine kinases and its specificity for STAT3 signaling downstream of IL-6, TTI-101's mechanism of action does not involve chemical modification of STAT3, which our group and others have demonstrated for other small-molecule STAT3 inhibitors, including Stattic (24) and several members of the SI3-102 family of small-molecule inhibitors (25-27). Also, unlike the Otsuka series of small-molecule STAT3 inhibitors—OPB-31121, OPB-51602, and OPB-111077 (27)—TTI-101 does not cause intracellular aggregation of STAT3 (24). Importantly, also unlike the Otsuka series of compounds and WP1066 (28), TTI-101 does not inhibit STAT3's mitochondrial function; thus, it spares oxidative phosphorylation and avoids lactic acidosis and peripheral neuropathy (24), both of which were observed in Phase I/II studies of the Otsuka compounds (27). In fact, not only did TTI-101 not cause peripheral neuropathy, but it also reversed -chemotherapy-induced peripheral neuropathy (CIPN) caused by -cisplatin, docetaxel, and paclitaxel in mouse models (29).

TTI-101 Administration to Healthy Normal Mice Reduced Th17 Cells by 50%, Had the Expected Effects on Myeloid Cells, and Had Minimal Effects on Circulating Cytokines

Two genetically engineered mouse (GEM) models revealed the impact on immunocyte development of genetic reduction of STAT3 activity in the whole animal: (1) mice bearing two copies of the dominant negative STAT3 mutation identified in patients with autosomal dominant hyper-IgE (Job's) syndrome (mut*Stat3* mice) demonstrated impaired Th17 cell differentiation (30) and (2) mice deficient in STAT3 within the bone marrow cell compartment demonstrated increased circulating mononuclear phagocytes (monocytes/macrophages), as well as neutrophils (31). We administered TTI-101 to three sets of 6- to 10-week-old female C57BL/6 mice (n=4-5/group) to assess whether TTI-101 administration phenocopied these mouse models or had other unexpected effects on immunocyte development. One set of mice (7-day set) received either TTI-101 or DMSO and were euthanized at 7 days. A second set of mice (14-day set) received TTI-101 or DMSO or were untreated and euthanized at 14 days. A third set of mice (wash-out set) received TTI-101 or DMSO for 14 days, were left untreated for 30 days, and then were euthanized at 44 days. Total leukocyte counts within PBMC, spleen, and lymph nodes were similar in all groups at 7, 14, and 44 days. Multi-parameter flow cytometry analysis performed on T cells and myeloid cells from PBMC, spleen, and lymph nodes revealed a 50% decrease in the number of IL-17-producing $CD4^+$ cells within the spleen of TTI-10-treated mice at 44 days compared to DMSO-treated mice ($p<0.05$, ANOVA plus Tukey). TTI-101 treatment, in this respect, phenocopied the mut*Stat3* mouse. This ability of TTI-101 to reduce IL-17-producing $CD4^+$cells is noteworthy given the evidence for the contribution of Th17 cells to immune checkpoint inhibitor (ICI)-induced immune-related serious adverse effects (SAE) (32). Of additional interest, the reduction of IL-17-producing $CD4^+$ cells was accompanied by a 20–30% increase in IL-2-producing $CD4^+$ cells in the spleen and lymph nodes at 44 days ($p<0.05$, ANOVA plus Tukey), suggesting that TTI-101 treatment also may skew T cell development away from Th17 cells and toward Th1 cells. Thus, the ability of TTI-101 to target STAT3 *in vivo* appears to override any inhibitory effect on STAT1, which may further promote activation of T-cell mediated anticancer immunity. In addition, at 14 days the percentage of $CD45^+$ leukocytes positive for myeloid cell markers (CD11b, Ly6C, and F4/80) in the spleen was mildly increased in TTI-101-treated mice compared to untreated mice ($p<0.05$ for each, ANOVA plus Tukey). In this respect, TTI-101 treatment phenocopied the bone marrow STAT3 knockout mouse (31). Levels of plasma IL-1α, IL-2, and IL-9 were below the detection levels for the Luminex assays. Among mea-

surable cytokines, there were no changes in plasma levels of IL-1α, IL-4, IL-6, IL-10, IL-17A, IL-21, IFN- γ, or TNF- α. In contrast, the levels of IL-5 were increased two-fold at 44 days in TTI-101-treated mice compared to all other groups ($p<0.05$, ANOVA plus Tukey). Thus, examination of the effects of TTI-101 administration to healthy adult mice on immunity revealed: (1) a reduction in Th17 cell development, as expected; (2) an increase in T helper subsets that produce IL-2, suggestive of increased development of Th1 cells; and (3) a mild increase in myeloid cells. Importantly, none of these changes would be expected to result in significant impairment of immunity; rather, they likely would promote immune activation of T cells in cancer.

TTI-101 Arrested Tumor Growth, Reversed Nonalcoholic Steatohepatitis (NASH), Reduced Fibrosis, and Markedly Reduced Hepatocellular (HCC) Tumor Development in Mouse Models of HCC

More than 90% of HCC arise in the setting of hepatic injury and inflammation (33-35), which involve production of several cytokines, notably hepatocyte growth factor (HGF) and IL-6 that activate STAT3 (36,37). STAT3 activation contributes to HCC oncogenesis by promoting tumor cell growth, tumor vascularization, and an immunosuppressive tumor microenvironment through a combination of tumor cell intrinsic signaling and tumor cell extrinsic signaling (38). STAT3 has been shown to be activated [phosphorylated on tyrosine (Y) 705; pY-STAT3] in 50–64% of HCC patient samples (39, 40) where its activation correlated closely with tumor vascularity (39) and the intensity of pY-STAT3 staining was highly correlated with the aggressiveness and undifferentiated state of the tumors (40). To determine the effect of TTI-101 on HCC, we tested TTI-101 in two mouse models of HCC—the Hep*Pten*$^{-}$ model and the Stk4$^{-/-}$Stk3$^{f/-}$ model. We treated Hep*Pten*$^{-}$ mice after HCC tumors developed (11 months old) with TTI-101 (50 mg/kg) in DMSO or with DMSO alone by intraperitoneal (IP) injection daily for four weeks (41). MRI examination showed that the average tumor volume increased by 2.5-fold in DMSO-treated mice but did not change in mice that received TTI-101 ($p=0.04$). TTI-101 treatment also reduced the tumor cell proliferation index (% cells staining by IHC for Ki67) by 50% ($p<0.001$). In addition to blocking tumor growth and proliferation, TTI-101 treatment reduced hepatic fibrosis by 75% ($p<0.001$); normalized liver injury as determined by levels of hepatocyte enzymes, ALT and AST ($p<0.001$ for each); and reduced serum levels of inflammatory chemokines, CXCL9 and CXCL10, by 60% ($p<0.01$) and 50% ($p<0.05$), respectively (41). RNA-seq analysis of livers from TTI-101- versus vehicle-treated mice identified STAT3 as the main upstream regulator of genes downregulated by TTI-101 in the liver and tumors (41). Similar results on tumor growth were obtained with TTI-101 in Stk4$^{-/-}$Stk3$^{f/-}$ mice that had dysregulation of the Hippo/YAP pathway and previously were validated for preclinical testing of new HCC therapies (42). In a separate experiment, treatment of eight-month-old Hep*Pten*$^{-}$ mice before appearance of tumors with TTI-101 (50 mg/kg/d) for four weeks markedly reduced HCC development (41).

TTI-101 Enhanced the Antitumor Effect of Anti-PD-1 in Syngeneic and Humanized Mouse Models of Hepatocellular Carcinoma (HCC)

Immune checkpoint inhibitor (ICI) therapy, either alone (nivolumab or pembrolizumab) or in combination with bevacizumab (atezolizumab plus bevacizumab), has been approved for treatment of HCC. TTI-101 recently was shown to augment responses of anti-PD-1 MoAb therapy in humanized mouse models of patient-derived xenografts (PDX) with subcutaneous and orthotopic HCC tumors. In these -humanized -models, immunodeficient mice were immune–reconstituted with human leukocyte antigen (HLA)-matched human immune cells. The addition of TTI-101 to pembrolizumab or to the combination of pembrolizumab and bevacizumab decreased the growth of the human PDX tumors by ~70% and was significantly more effective than monotherapy (pembrolizumab alone) or dual therapy (pembrolizumab and bevacizumab) (43).

The combined data from these models suggest the utility of TTI-101 in a variety of cancer indications, most notably HCC. STAT3 inhibition can be used in a monotherapy setting to reverse the intrinsic tumorigenesis (Hep*Pten*$^{-}$ and Stk4$^{-/-}$Stk3$^{f/-}$ models) as well as relieve extrinsic tumor microenvironment immunosuppression (humanized PDX HCC models). Further, STAT3 inhibition via TTI-101 provides a distinct and complementary mechanism to existing therapeutic options in HCC of angiogenesis inhibitors (e.g., bevacizumab and sorafenib) and immune checkpoint inhibition (e.g., atezolizumab and pembrolizumab).

TTI-101 Reduced Liver, Lung, Skin, and Cardiac Fibrosis in Mouse Models

In addition to reducing fibrosis in the Hep*Pten*$^{-}$ model of NASH as summarized above, we and our collaborators have demonstrated that TTI-101 administration reduced fibrosis in the bleomycin mouse model of idiopathic pulmonary fibrosis (44), in both the bleomycin and thick skin (Tsk-1) models of scleroderma (45), as well as in a mouse model of chronic Chagasic cardiomyopathy (46). Remarkably, the reduction in bleomycin-induce lung fibrosis was observed when TTI-101 treatment was delayed for two weeks and accompanied by reversal of hypoxia. The ability of TTI-101 to reverse hypoxia has not been demonstrated in preclinical studies of pirfenidone and nintedanib, the two drugs currently FDA-approved for IPD treatment.

Phase 1 Clinical Trial of TTI-101

Tvardi Therapeutics, Inc., sponsors a Phase 1 study of TTI-101 in patients with advanced cancers at multiple U.S. sites (NCT03195699). In this study, TTI-101 is administered as monotherapy and is being evaluated for safety and preliminary efficacy in a parallel dose-escalation/expansion study in patients with advanced HCC and other solid tumors. The dose-escalation phase has completed enrollment, and enrollment in the dose-expansion phase is ongoing. TTI-101 has been well tolerated and has demonstrated multiple durable radiographic objective responses in patients treated with TTI-101 monotherapy.

DISCUSSION

STAT3 is a transcription factor that is well validated as a high-value target for treatment of inflammation, fibrosis, and cancer, yet no drug targeting it has been approved for clinical use. TTI-101 was identified in a chemical genetics probe and drug-development program using a virtual ligand

screening (VLS) strategy that targeted the pY-peptide binding pocket within the SH2 domain of STAT3 that was uniquely defined by our group. This strategy also incorporated rigorous biochemical and cellular screens, as well as several rounds of structure-based medicinal chemistry.

TTI-101 has several highly favorable characteristics as a chemical genetics probe. It binds to STAT3 with high affinity and potently blocks STAT3 binding to its receptor-based pY-peptide ligand thereby interfering with all canonical STAT3 functions tested. TTI-101 is selective and lacks both off- and on-target toxicity, in particular sparing STAT3's non-canonical contribution to oxidative phosphorylation. In addition, TTI-101 demonstrated excellent oral bioavailability in mice, rats, monkeys, and humans. Preclinical studies using TTI-101 as a chemical genetics probe have established the contribution of STAT3 in aggregate across all tissues in many human and rodent models of disease, including cancer (19-21, 41, 47), most notably HCC; chronic inflammatory diseases (24, 48-50); and fibrotic diseases (41, 44-46), most notably IPF. Pharmacology and toxicology studies were performed over 28 days in rats and dogs to determine the first-in-human dose to test in cancer patients and to enable a Phase 1 study of TTI-101 in patients with solid tumors sponsored by Tvardi Therapeutics.

Based on potency, lack of adverse off-target effects, lack of adverse on-target effects, and proof-of-concept results, TTI-101 is the most promising small-molecule STAT3 inhibitor currently in clinical development for treatment of cancer and fibrotic diseases. Successful progression of TTI-101 into a new drug application (NDA) would enable similar small-molecule drug development programs aimed at targeting SH2-containing, disease-causing proteins.

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DISCUSSION

Mackowiak, Baltimore: David, fascinating, potentially very important, thank you. We had grand rounds at Maryland given by the head of hepatology at USC this past week focused primarily on fatty liver disease. She concluded her presentation with a claim to a wealth of data showing that coffee is beneficial to the liver. I believe that I couldn't start a day without coffee! But it raises two

questions relative to your presentation: (1) Is there a natural source for a TTI-101-like substance that could possibly be obtained from one's diet? and (2) Do you know anything about the relationship of coffee consumption to STAT3 or a TTI-101-like material?

Tweardy, Houston: Well, those are great questions. Actually, a lot of natural products have been demonstrated to target STAT3 and reduce its activation, along with a lot of other things. They're pretty non-selective. I have not seen anything about caffeine.

Mackowiak, Baltimore: She wasn't sure it was caffeine—it could be coffee, but it could've been anything.

Tweardy, Houston: I am not aware of what compound(s) in coffee might be beneficial to the liver. On the other hand, a number of natural products, such as curcumin, which is present in turmeric, have STAT3-targeting effects. Actually, we were very interested in following up on a compound called piperlongumine that clearly targets STAT3. Piperlongumine is a derivative of the long pepper (*Piper longum*). The problem with developing curcumin or piperlongumine into a drug is that they tend to be non-selective and not sufficiently potent with respect to the intended target, in this case STAT3.

Boyce, Boston: Fascinating presentation. As I listened to your talk, I was thinking about another aspect of STAT3, which is the loss of function mutations that cause hyper IgE syndrome that we occasionally see as immunologists. In that disease, there is sort of an ineffectual default immune response to staph and fungi that drive the IgE. It's a type 2 inflammatory response that in some cancer models is actually pretty potently anticancer. I'm wondering if you think that perhaps some of the antitumor effects of the drug may be related to the immune response and whether you have either detected or plan on looking for type 2 inflammatory signatures in the patients who are treated.

Tweardy, Houston: Great question. We are very interested in examining the immune pathways you cite, along with others, thinking that some benefit of targeting STAT3 in cancer may be extrinsic to malignant or premalignant cells and reside in the tumor microenvironment. We have just started to look at that. Frankly, I have no information to give you. We have several grants that are funded to look at just that question because it's a very important one.

Sherman, Houston: Thank you, David. You mentioned reversal of fibrosis. Do you have histological evidence that it's actually a reversal versus a prevention phenomenon? And what data do you have on optimal timing between the lung insult and development of irreversible damage?

Tweardy, Houston: Great questions too. We don't have histological data at the time of intervention with TTI-101 in the experiment I presented to prove there was reversal of fibrosis. However, we think this occurred based on the oxygen saturation measurements. TTI-101 was started after there was a substantial decline in oxygenation saturation, and its addition reversed the oxygen saturation deficit even with continued treatment using bleomycin. We have not worked further on the timing.

Sherman, Houston: Yes.

Oates, Charleston: Thank you for that wonderful talk. We have a scleroderma lung group here in Charleston and a liver and pulmonary fibrosis group who would be interested in talking to you, but I want to ask you about SH2 domains. They are important in redox regulation on signaling proteins, and I wonder whether the redox state of the host cells affects binding of the drug or if the drug affects redox regulation at all.

Tweardy, Houston: At this point, we do not have any evidence that the binding of TTI-101 to STAT3 is affected by the redox state of the cell, but we haven't looked carefully at that issue. We have indirectly examined the second issue, however, with a focus on mitochondria, which, as you know, generate reactive oxygen species (ROS). The bottom line is that TTI-101 does not target STAT3's mitochondrial function and would not be expected to increase production of ROS and affect the redox state of the cell.

Kaelin, Boston: Most epithelial cancer cell lines, including liver cancer cell lines, will tolerate complete loss of STAT3. For example, by CRISPR or at least in cell culture, the rules might be different of course *in vivo*. Have you done or are you contemplating doing the "killer" experiment that says your drug is acting on a target by doing a rescue with a drug-resistant STAT3 that you can either engineer or screen for?

Tweardy, Houston: We have not done the "killer" experiment you described, but we have knocked out STAT3 in murine embryonic fibroblasts (MEF) and showed reduced growth that returns to normal with restoration of STAT3. This restored growth is reversed by treatment with TTI-101 with an IC50 of 1.4 micromolar.

Kaelin, Boston: I think that speaks to the plausibility that STAT3 is the target, but I think the "killer" experiment is the drug-resistant mutant experiment, which would demonstrate it's on target.

Tweardy, Houston: So, when you say "drug-resistant"—resistant to what drug?

Kaelin, Boston: If you take liver cells that are expressing a point mutant of STAT3 and the point mutant renders STAT3 resistant to the drug and you don't see the phenotype, then I think this proves that the drug is acting on target. A number of nice technologies are available now if you're looking for such mutants, but you may be able to predict such a mutant based on your structural information in terms of how the drug is docking.

Tweardy, Houston: I might actually speak to you afterwards just to walk through that experiment, because that type of experiment has been proposed by others but not as clearly as you have.

Kaelin, Boston: Yes, I think it's important because STAT3, as you know, has been touted as a target for over 10 years. You may have the best molecule, but I think someone has to drive the stake through the heart and say this is all on target and not just some insidious off-target effect we didn't know about.

Tweardy, Houston: Well spoken, thanks.

Bishopric, Washington, DC: I'm interested in these undruggable targets and generally in how you decided to screen for that specific property of STAT3. I'd love to know how you decided to go after its intracellular, intranuclear partners and its ability to activate transcription. More specifically, have you looked at where it's acting on STAT3? Is it preventing translocation into the nucleus? Is it affecting specific transcription complexes? Is it affecting the STAT3 transcriptome? What kinds of validation have you done that might support the targeting?

Tweardy, Houston: We have done essentially all of those things. We have shown that TTI-101 does in fact block translocation of STAT3 into the nucleus, and we've shown that it blocks transcription of known STAT3 gene targets. We targeted the SH2 domain because I have long viewed it, as have others, as a potential Achilles' heel of any protein that contains it. I recall as a postdoctoral fellow seeing a blot of phosphorylated proteins being developed with an SH2-containing protein that was radiolabeled. The thing that impressed me was the implied high affinity of binding of the SH2 domain to blotted phosphoproteins. I viewed its affinity of binding as similar in magnitude to the affinity of binding achieved by 4G10, a monoclonal antibody that Brian Drucker helped develop that binds to the phosphotyrosine residue itself. I began to think that a small phosphopeptide or small molecule might be identified that would bind selectively to an SH2 domain with similar affinity blocking its interaction with its cognate phosphopeptide and be used as a chemical probe, or even a drug to target STAT3.

Bishopric, Washington, DC: Thank you.

Footnotes

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Potential Conflicts of Interest: David J. Tweardy co-founded and holds equity in Tvardi Therapeutics. The intellectual property used in the research supporting this publication was created by Dr. Tweardy and licensed to Tvardi Therapeutics, Inc. Dr. Tweardy also receives compensation from Tvardi as a member of its scientific advisory board.

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