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Two decades of research in discovery of anticancer drugs targeting STAT3, how close are we?

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Abstract

Signal transducer and activator of transcription 3 (STAT3) controls many biological processes including differentiation, survival, proliferation, and angiogenesis. In normal healthy cells, STAT3 is tightly regulated to maintain a momentary active state. However, aberrant or constitutively activated STAT3 has been observed in many different cancers and constitutively activated STAT3 has been shown to associate with poor prognosis and tumor progression. For this reason, STAT3 has been studied as a possible target in the treatment of many different types of cancers. However, despite decades of research, a FDA-approved STAT3 inhibitor has yet to emerge. In this review, we will analyze past studies targeting STAT3 for drug discovery, understand possible causes of failure in these studies, and provide potential insights for future efforts to overcome these roadblocks.

Keywords: STAT3 inhibitor, cancer therapeutics, drug discovery, small molecule compounds, molecular probes, clinical trials.

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Abbreviations:

DBD, DNA binding domain; HNSCC, head and neck squamous cell carcinoma; JAK, Janus kinase; SH2, Src-homology 2; STAT, Signal transducer and activator of transcription.

1. Introduction

Signal transducer and activator of transcription 3 (STAT3) belongs to a family of Janus kinase (JAK)/STAT transcription factors, which regulate responses to extracellular signals (Rawlings, Rosler, & Harrison, 2004). In normal tissues/cells, the STAT family proteins become transiently activated to relay transcriptional signals from cytokine and growth factor receptors at the plasma membrane to the nucleus (J. Bromberg & Darnell, 2000) (see **Figure 1A**). Stimulation of these receptors leads to their autophosphorylation, recruitment of, and activation of JAK. Phosphorylation of tyrosine residues on the receptor and JAK leads to the recruitment of STAT3 by binding to the SH2 domain of STAT3. JAK then phosphorylates STAT3, causing its activation, dimerization, and translocation into the nucleus, where it controls transcription of various genes important for many normal cellular processes including embryo development, cell differentiation, survival, proliferation and angiogenesis (X. Wang, Crowe, Goldstein, & Yang, 2012). Signaling by STAT3 under normal physiologic conditions is tightly regulated to maintain a transiently active state (Kortylewski & Yu, 2007). However, STAT3 is constitutively activated in many types of human cancers including but not limited to colorectal, lung, breast, prostate, liver, and pancreas cancers and STAT3 activation associates with poor prognosis of these cancers (Alvarez, Greulich, Sellers, Meyerson, & Frank, 2006; Corcoran, et al., 2011; X. Wang, et al., 2012; H. Yu & Jove, 2004).

Constitutive activation of STAT3 in cancers results from activation of several oncogenic pathways and dysregulation of negative regulators of STAT3 signaling (Leeman, Lui, & Grandis, 2006). Most frequently, gain-of-function mutations and overexpression of tyrosine kinases, such as PDGFR, EGFR, Her2/Neu, IL-6R/gp130, c-Met, and BRC-ABL, leads to the persistent activation of STAT3 (Kortylewski & Yu, 2007). The dysregulation of STAT3 downstream target

genes, including those involved in survival, proliferation, angiogenesis, and suppression of host immune surveillance, have been implicated in tumor initiation and formation (Haura, Turkson, & Jove, 2005; H. Yu & Jove, 2004). Aberrant STAT3 activation has also been shown to contribute to tumor progression by promoting invasion, metastasis and drug resistance (Al Zaid Siddiquee & Turkson, 2008; Lee, et al., 2014; Yue & Turkson, 2009; Zhao, Li, et al., 2016).

Direct evidence on the oncogenic function of STAT3 comes from the over-expression studies of a constitutively-activated STAT3 molecule, STAT3c. It was shown that STAT3c over-expression transformed human mammary epithelial cells *in vitro* (Dechow, et al., 2004) and *in vivo* (J. F. Bromberg, et al., 1999). Transgenic over-expression of STAT3c in airway epithelial cells also led to lung inflammation and consequently spontaneous lung cancer (Y. Li, et al., 2007). Furthermore, use of antisense oligonucleotides significantly impaired the growth of human and mouse nucleophosmin-anaplastic lymphoma kinase tumors in xenograft models by inhibiting STAT3 expression (Chiarle, et al., 2005). These studies along with other evidences on the multiple roles of STAT3 in tumor initiation, progression, resistance, and immunosuppression suggests that STAT3 is an attractive target for anticancer drug discovery (Costantino & Barlocco, 2008). Indeed, many approaches have been used in an attempt to discover drugs targeting STAT3 over the last 20 years. However, no STAT3-targeting therapeutics have been approved despite the fact that several drugs have entered clinical trials. In this review, we will provide an in-depth analyses of past studies on STAT3 inhibitors that have been studied to date and illustrate possible challenges ahead.

2. Structure and Activity of STAT3

In 1998, the crystal structure of STAT3-ß bound to a DNA molecule was solved, offering insight into the function and steps necessary for STAT3 to transduce signal into transcription activation (Becker, Groner, & Muller, 1998). STAT3 consists of 770 amino acids that constitute

six different functional domains (**Figure 1B**), including amino-terminal domain (NTD), coiled-coil domain (CCD), DNA-binding domain (DBD), linker domain, SH2 domain, and the carboxyl-terminal transactivation domain (TAD). The NTD is a conserved sequence, which is essential for cooperative binding of STAT proteins to multiple consensus DNA sites (X. Xu, Sun, & Hoey, 1996). The CCD is critical for recruitment of STAT3 to the receptor and subsequent phosphorylation, dimerization, and contains residues essential for nuclear translocation (Ma, Zhang, Novotny-Diermayr, Tan, & Cao, 2003; Z. Yu & Kone, 2004; T. Zhang, Kee, Seow, Fung, & Cao, 2000). The DBD governs the DNA-binding activity and specificity, allowing recognition of and binding to a specific consensus DNA sequence (Horvath, Wen, & Darnell, 1995). The SH2 domain is required for recruitment and activation as well as dimerization of the STAT3 molecule by interacting with phosphorylated tyrosine residues in the opposing subunit (Haan, et al., 1999). The linker domain connects the DBD with the SH2 domain and mutational studies have shown that it is important in transcriptional activation (Mertens, Haripal, Klinge, & Darnell, 2015). An important residue, tyrosine 705, is located in the TAD and becomes phosphorylated upon activation of STAT3. Phosphorylation of tyrosine 705 is required for STAT3 monomers to form a dimer by binding to the SH2 domain in the opposing subunit (X. Wang, et al., 2012). The STAT3 dimer can then bind to DNA at its 9-base-pair consensus sequence, TTCCGGGAA, located in the promoters of STAT3 target genes (Becker, et al., 1998). An additional phosphorylation site within the TAD, serine 727 (**Figure 1B**), maximizes transcriptional activity and phosphorylation of both tyrosine 705 and serine 727 allows the TAD to recruit transcriptional machinery to initiate transcription of STAT3 target genes (Wen, Zhong, & Darnell, 1995).

3. Targeting STAT3 for Therapeutic Development

Over the last 20 years, various approaches have been tested to target STAT3 for discovery and development of potential therapeutics. These approaches include molecular probes such as decoy oligonucleotides, peptides and small molecule inhibitors targeting different domains of STAT3 (see **Figure 1C** for representative inhibitors bound to different domains of STAT3**)**. Targeting upstream regulators of STAT3 such as JAK has also been considered. Here, we will focus on only approaches and molecules that directly target and bind to the STAT3 protein.

3.1. Molecular Probes.

The first studies that showed promise in the treatment of various cancers by inhibiting STAT3 involved molecular probes such as STAT3 dominant negative molecules, decoy oligonucleotides, and peptidomimetics (**Table 1**). In 1996, a dominant negative STAT3 was generated by mutating tyrosine 705 to a phenylalanine, which inhibited activation of wild type STAT3 (Kaptein, Paillard, & Saunders, 1996). This dominant negative STAT3 has since been tested in several different cancer models and was shown to inhibit cancer cell proliferation and induce apoptosis (C. L. Chen, et al., 2008; Corvinus, et al., 2005; Niu, et al., 1999; G. Xu, Zhang, & Zhang, 2009). Although it is possible to deliver large cDNAs encoding the dominant negative STAT3 in a vector *in vivo*, the efficacy to suppress xenograft tumors is relatively low as shown in the study using intratumoral electroinjection (Niu, et al., 1999). Viral vectors for more effective delivery of the dominant negative STAT3 into xenograft models have not yet been tested as a gene therapy.

Another molecular probe is a STAT3 decoy oligonucleotide, consisting of a 15-bp duplex (**Figure 2**) representing the genomic element found in *c-fos* gene promoter, which has been shown to inhibit STAT3 activity and proliferation of head and neck cancer cells (Leong, et al., 2003). This STAT3-specific decoy was also able to inhibit the growth of xenograft tumors of head and

neck (Xi, Gooding, & Grandis, 2005) and lung (X. Zhang, Zhang, Wang, Wei, & Tian, 2007) cancer cells via daily intratumoral injection. However, this decoy had no effect on head and neck xenograft tumor growth when applied systematically via IV injection (Sen, et al., 2012). This observation is also consistent with the previous study where intratumoral injection of this decoy into one xenograft tumor did not affect the growth of the counter lateral tumor inoculated on the different flank of the same mouse (Xi, et al., 2005). Apparently, this STAT3 decoy is unstable with a very short half-life of 1.5 hrs in mouse serum. Nevertheless, a phase 0 clinical trial was conducted on treatment of head and neck tumors using this STAT3 decoy with direct intratumoral injection (Sen, et al., 2012). While a single injection of the STAT3 decoy did not show toxicity in a dose-escalating study up to 1 mg/injection, it lowered STAT3 target gene expression in tumor biopsies. Its efficacy on the tumor growth or patient outcome of this clinical trial was not reported.

To eliminate the stability problem, a modified STAT3 decoy was created by closing the ends of the 15-bp duplex forming a cyclic structure (**Figure 2**), which was able to increase its stability with a longer half-life (4 hrs) in mouse serum and allowed for systematic delivery (Sen, et al., 2012). Although the modified decoy was effective in inhibiting xenograft tumors and STAT3 target gene expression in mice via intravenous injection, further studies are needed to move forward this modified decoy for potential clinical testing. Although STAT3 decoys may successfully inhibit xenograft tumor growth and expression of STAT3 target genes, developing these decoys into clinically useful therapeutics may face challenges including stability issues for systematic use.

Since STAT3 activation may require homo-dimerization via binding of the SH2 domain from one subunit to the phosphorylated tyrosine 705 in another subunit, it was thought that a peptide mimicking the sequence containing phosphorylated tyrosine 705 would be able to bind to

the SH2 domain of STAT3 and inhibit its activation and dimerization and, thus, its activity. Indeed, many peptidomimetics have been synthesized and tested (**Table 1**).

Firstly, a phosphopeptide with a sequence of PpYLKTK from STAT3 was used to inhibit the DNA-binding activity of STAT3 but not other STATs such as STAT1 (Turkson, et al., 2001). Addition of a membrane-permeabilization sequence (AAVLLPVLLAAP) to this peptide at the carboxyl terminus led to a peptide that was able to inhibit STAT3 activity in cells using luciferase reporter assay (Turkson, Kim, et al., 2004; Turkson, et al., 2001). However, it is unclear how stable this phosphorylated peptide is in cells or in animal models.

Using alanine-scanning mutagenesis assay, it was found that a minimum of three amino acid residues PpYL from the above peptide is sufficient to inhibit STAT3 (Turkson, et al., 2001). In a follow-up study with modification of this tripeptide, the newly synthesized peptidomimetics have improved activity shown using EMSA (Turkson, Kim, et al., 2004). For example, the peptidomimetics ISS 610 with 4-cyanobenzoate replacing the proline residue had 5-fold increase in potency for inhibiting STAT3 binding to DNA (Turkson, Kim, et al., 2004). However, despite the 5-fold increase, the IC50 is still very high at 42 μM as demonstrated using EMSA. Furthermore, although ISS 610 inhibited constitutive activation of STAT3 and cancer cell growth, very high concentrations (1 mM) are required to achieve 50% inhibition of cancer cell proliferation in a 3-day treatment. With such a high cytotoxicity IC50, ISS 610 will likely have difficulty for clinical development.

Another mimetic S3I-M2001 was developed by identifying key hydrogen bonding, hydrophobic, and electrostatic interactions between ISS 610 and the STAT3 in the X-ray crystal structure (K. A. Siddiquee, et al., 2007). While S3I-M2001 still had a high IC50 value for DNA binding (79 μM), it was used in a xenograft model of breast cancer and was shown to inhibit

xenograft tumor growth and led to tumor regression. Although these results are promising, further optimization is required to improve potency and selectivity and to eliminate potential off-target effects.

In addition to peptidomimetics derived from STAT3 sequence, which have poor affinities for STAT3 (K_i=25.9 μM), other peptidomimetics derived from receptor tyrosine kinase sequences have been designed recently with improved activity. For example, pYLPQTV, a peptidomimetic derived from the phosphorylated gp130 receptor, had a higher affinity for STAT3 than the previous peptidomimetics derived from STAT3 (Gomez, et al., 2009). This peptidomimetic was used as a starting point for optimization, which led to the development of compound 1 with a high binding affinity (K_i=350 nM). Furthermore, it was found that addition of a 7-membered Freidinger lactam, to conformationally constrain the peptide, increased the binding affinity with a Ki=190 nM (Gomez, et al., 2009). While this compound is effective in binding STAT3in *in-vitro* studies, it is unable to cross cell membranes to act on intracellular STAT3. Continued development of this peptidomimetic led to CJ-1383 with increased cell permeability and a cytotoxicity IC50 of 3.6 μM against MDA-MB-468 breast cancer cells (J. Chen, et al., 2010).

XZH-5 is another peptidomimetic generated using structure-based design focusing on the hydrogen bonds that could be formed between the tyrosine 705 and the side pocket in the SH2 domain (A. Liu, Y. Liu, Z. Xu, et al., 2011). XZH-5 is cell permeable and prevents STAT3 phosphorylation, DNA binding, and downregulation of STAT3 target gene expression in multiple cancer cells including breast, pancreatic, liver, and rhabdomyosarcoma cells (A. Liu, et al., 2012; Y. Liu, et al., 2011). Furthermore, XZH-5 treatment increased the cytotoxicity of chemotherapeutic drugs doxorubicin and gemcitabine in cancer cells (A. Liu, et al., 2012). While XZH-5 had an increased solubility and selectivity over previous peptidomimetics, it had decreased

potency with cytotoxicity IC50 between 15 and 25 μM. New analogues that have better IC50's, as low as 6.5 μM against breast cancer cells, have been identified from modification of XZH-5 as a lead (Daka, et al., 2015). While XZH-5 and its analogs show promising results in *in-vitro* and cell-based studies, no *in-vivo* testing have been conducted on these mimetics.

Peptide aptamers have also been used to inhibit STAT3 by targeting the dimerization domain. rS3-PA, for example, is a 20-amino-acid peptide attached to a protein transduction domain and a thioredoxin scaffold protein. These attachments increased stability as well as cellular uptake of the peptide (Schoneberger, et al., 2011). rS3-PA was shown to reduce STAT3 phosphorylation without any effect on STAT1 phosphorylation (Borghouts, et al., 2012). Additionally, the use of rS3-PA together with irinotecan augmented its cytotoxic effect on colon cancer cell lines (Weber, et al., 2012). While rS3-PA was successful at reducing Tu-9648 glioma xenograft tumors in mice, it had limited systemic stability and its effects were transient and declined a few hours after administration (Borghouts, et al., 2012). Nevertheless, increasing the dosing frequency to 3 times daily improved tumor growth inhibition (Borghouts, et al., 2012). Clearly, optimization to increase the stability of rS3-PA may be required to improve its therapeutic efficacy for further testing.

Using two-yeast hybrid screening system and the DNA-binding domain (DBD) of STAT3 as a bait, a peptide aptamer (DBD1, **Table 1**) was identified as a binder to STAT3 (Nagel-Wolfrum, et al., 2004). Transient transfection of DNAs encoding this peptide into NIH3T3 or mouse melanoma B16 cells inhibited STAT3-dependent luciferase reporter expression and STAT3 binding to DNA using EMSA. Interestingly, this peptide when fused with a protein transduction domain consisting of 9 Arginine residues was able to dose-dependently inhibit proliferation of B16 cells and induce apoptosis. Although this peptide shows promise as a biological drug targeting the DBD of STAT3, no studies have be conducted to test its *in-vivo* activity.

While molecular probes such as peptidomimetics and aptamers, as described above are promising strategies in inhibiting STAT3 for development of biological drugs, many challenges exist with these approaches including stability and delivery as described above. In addition, these molecular probes have low affinity to STAT3 as determined *in vitro*, which certainly hinders their clinical development. The molecular probes including peptides and oligonucleotides may cause host immunogenic reactions, which would effectively prohibit their clinical development. The peptidomimetics are the first group of inhibitors targeting STAT3 to be studied. Yet, none of these mimetics were able to move into phase I clinical testing. Clearly, this approach faces enormous challenges to be successfully developed into clinically useful biologic drugs. Novel approaches to increase their affinity and perhaps humanization are in dire need to overcome these challenges for developing STAT3-targeting biological drugs.

3.2. Small molecule inhibitors targeting the SH2 domain

While many issues have arisen in association with developing SH2-domain-targeting biologic drugs, as discussed above, other past efforts have focused on developing small molecule inhibitors targeting the SH2 domain and many such inhibitors have been identified **(Table 2)**. Below we will discuss these SH2-domain-targeting small molecules and how successful they have been.

Stattic was identified from screening a diverse chemical library, containing 17,000 small molecules, using a fluorescence polarization-based binding assay targeting the SH2 domain (McMurray, 2006; Schust, Sperl, Hollis, Mayer, & Berg, 2006). It was shown to inhibit STAT3 dimerization, nuclear translocation and activity (Adachi, Cui, Dodge, Bhayani, & Lai, 2012; Schust, et al., 2006). Stattic was also shown to inhibit proliferation and induce apoptosis of glioblastoma cells with IC50's at 1-2.5 μM (Villalva, et al., 2011). Similar results were also

observed for breast, prostate, and colon cancer cells (Chung, Giehl, Wu, & Vadgama, 2014; Han, et al., 2014; Lin, et al., 2013; Lin, Liu, et al., 2011). Additionally, combination treatments of Stattic with other chemotherapeutics, such as temozolomide, cisplatin, Herceptin, and radiation increased the efficacy of these treatments, leading to increased apoptosis in glioma, head and neck squamous cell carcinoma (HNSCC), and breast cancer cells (Adachi, et al., 2012; Chung, et al., 2014; Pan, Zhou, Zhang, & Claret, 2013; Villalva, et al., 2011). Stattic has also been tested in several animal models such as, HNSCC, esophageal squamous cell carcinoma, colon, prostate and ovarian cancer, which showed that Stattic alone reduced tumor growth and has enhanced effect when given in combination with radiation and chemotherapeutic treatments (Adachi, et al., 2012; Han, et al., 2014; Ji, et al., 2013; Spitzner, et al., 2014; Q. Zhang, et al., 2015).

In-silico screening of a chemical database targeting the SH2 domain of STAT3 led to identification of STA-21 (Song, Wang, Wang, & Lin, 2005). The initial studies showed that STA-21 inhibited DNA binding activity, phosphorylation, dimerization, and transcriptional activity of STAT3 (Song, et al., 2005). STA-21 was later used in numerous studies to determine its potential in inhibiting cancer cells. Notably, STA-21 inhibited cell growth, viability and induced apoptosis through the activation of caspases in many different cancers including breast, bladder, rhabdomyosarcoma, and osteosarcoma cell lines with cytotoxicity IC50's ranging from 12-25 μM (C. L. Chen, et al., 2008; C. L. Chen, et al., 2007; Song, et al., 2005). Additionally, mesenchymal stem cells co-cultured with glioma cells in different chambers showed a transformation to a malignant phenotype, which was abrogated by STA-21 (Cui, Liu, Bai, Tian, & Zhu, 2014).

STA-21 has also been tested in the treatment of psoriasis and rheumatoid arthritis. Psoriatic lesions are characterized by increased levels of cytokines and growth factors as well as an increase in activated STAT3 (Miyoshi, et al., 2011). Topical treatment of a mouse model of psoriasis

prevented skin lesions (Miyoshi, et al., 2011). Use of STA-21 in the treatment of psoriasis has advanced to Phase I/II clinical trials (Miyoshi, et al., 2011; Nadeem, et al., 2017) and has shown improvement in psoriatic lesions when administered topically. In rheumatoid arthritis, STAT3 activation leads to an increase in cytokines that allow the retention of inflammatory cells, which attack joints (Alam, Jantan, & Bukhari, 2017; Nowell, et al., 2009). Using mouse models of arthritis, it was found that STA-21 could decrease inflammation and inhibit downstream signaling pathways known to be involved in rheumatoid arthritis pathology (Ahmad, et al., 2017; Park, et al., 2014). While *in-vivo* models have shown STA-21's success at treating psoriasis and rheumatoid arthritis, it is still unclear if STA-21 is active *in vivo* in treating cancers.

Analyses of STA-21 analogues led to identification of LLL3 and LLL12, which have smaller molecular weight and improved potency in cancer cell lines with *in-vivo* antitumor activity (Bhasin, Etter, Chettiar, Mok, & Li, 2013; Fuh, et al., 2009; Lin, Hutzen, Li, et al., 2010). LLL12 contains a sulfonamide, which dramatically increased its potency over STA-21 and LLL3 (see **Table 2**) (Bhasin, et al., 2013). Although further modification of LLL3 improved its *in-vitro* activity, the increase is still far below that of LLL12 (Bhasin, et al., 2013). LLL12 is specific to STAT3 and prevented the phosphorylation, nuclear translocation, DNA binding activity of STAT3, and decreased expression of STAT3 target genes. LLL12 also blocked IL-6-induced STAT3 phosphorylation and led to the induction of apoptosis by increasing the cleavage of caspase-3 and PARP in multiple cancer cell lines including breast, pancreatic, colon, and liver cancer cells (Ball, Li, Li, & Lin, 2011; Lin, et al., 2012; Lin, Liu, et al., 2011; A. Liu, Liu, Li, Li, & Lin, 2011; Y. Liu, Li, Li, & Lin, 2010; Onimoe, et al., 2012; Wei, et al., 2011). LLL12 has been tested in multiple tumor models and inhibited xenografts of glioblastoma, hepatocellular carcinoma, osteosarcoma, childhood astrocytoma, multiple myeloma, and cancers of prostate,

colon, and breast (Bid, et al., 2013; Fuh, et al., 2009; Kroon, et al., 2013; Lin, et al., 2012; Lin, Hutzen, Li, et al., 2010; Lin, Liu, et al., 2011; Onimoe, et al., 2012; Zuo, Li, Lin, & Javle, 2015). LLL12 also inhibited growth of xenografts of primary multiple myeloma cells from patients who were clinically resistant to lenalidomide and bortezomib (Lin, et al., 2012).

LLL-12 also potently inhibited stem-like cell populations of breast, colon, pancreas and prostate cancers. This included a decrease in their viability, tumor sphere-forming capacity and clonogenicity (Kroon, et al., 2013; Lin, et al., 2013; Lin, et al., 2016; Lin, Liu, et al., 2011). Strikingly, pretreatment with 5 and 10 μM LLL12 of cells derived from a patient with castration resistant prostate cancer prior to implantation completely inhibited xenograft tumor growth of these cells (Kroon, et al., 2013). Thus, inhibiting STAT3 may help eliminate caner-initiating stem cells and help overcome castration resistance.

While LLL12 shows promising results in preclinical studies, it suffers from low solubility and requires high doses for *in-vivo* studies due to low bioavailability. Additionally, hypoxia-induced resistance to LLL12 has been observed (J. Xu, et al., 2017). Recently, it was shown that stimuli responsive microdroplets containing LLL12 and oxygen could be used to overcome the delivery and hypoxic dilemma by releasing oxygen and LLL12 upon stimulation using ultrasound (J. Xu, et al., 2017). While this approach could go a long way to solve the limitations of LLL12, it has not yet been tested *in vivo* and its efficacy needs to be validated.

Using *in-silico* site-directed fragment-based drug design, a new small molecule inhibitor of STAT3, LY5, was made based on known STAT3 inhibitors targeting the SH2 domain with LLL12 as the starting point to synthesize fragments (W. Yu, Xiao, Lin, & Li, 2013). LY5 successfully bound to the SH2 domain, as determined using fluorescence polarization assay, and inhibited constitutively-activated STAT3 and IL-6-induced STAT3 activation in several different

cell lines including osteosarcoma, medulloblastoma, ewing sarcoma, breast cancer, and rhabdomyosarcoma cells (Xiao, et al., 2015; W. Yu, et al., 2013; Zhao, Wang, et al., 2016). It had no effect on the phosphorylation of STAT1 induced by IFN-γ (Zhao, Wang, et al., 2016), suggesting that LY5 is STAT3 selective over STAT1. LY5 was also able to inhibit nuclear translocation, migration and expression of STAT3 downstream target genes in medulloblastoma, hepatocellular carcinoma, and colon cancer cell lines (Xiao, et al., 2015; Zhao, Wang, et al., 2016). STAT3 phosphorylation is inhibited by LY5 and LY5 suppressed breast and colon xenograft tumor growth in mouse models (W. Yu, et al., 2013; Zhao, Wang, et al., 2016). Furthermore, LY5 combination with cisplatin or radiation caused greater decrease in cell viability of medulloblastoma cells than with either single agent/treatment alone (Xiao, et al., 2015). Overall, the cytotoxicity IC50 (0.5-1.4 μM) of LY5 is lower than the previous SH2 domain inhibitors and it has shown promise at overcoming resistance to a variety of anticancer agents.

S3I-201 was also identified via virtual screening targeting the SH2 domain of STAT3 (K. Siddiquee, et al., 2007) and it represents the most studied STAT3 inhibitor. Early studies showed that S3I-201 disrupted STAT3 dimerization and decreased the DNA-binding and transcriptional activity of STAT3 (K. Siddiquee, et al., 2007). S3I-201 also inhibited proliferation, migration, and invasion of several cancer cell lines (Bu, Deng, et al., 2015; C. L. Chen, et al., 2007). It was found that S3I-201 increased the transcription of caspase genes and may lead to apoptosis of prostate cancer and osteosarcoma cell lines through the caspase pathway (Gurbuz, et al., 2014; X. Wang, Goldstein, Crowe, & Yang, 2014). S3I-201 also enhanced doxorubicin sensitivity in hepatocellular carcinoma cells (Q. D. Hu, et al., 2012). In urothelial carcinoma and bladder carcinoma, S3I-201 overcame resistance to paclitaxel and cisplatin (W. J. Wang, et al., 2016). Additionally, S3I-201 sensitized esophageal squamous cell carcinoma to radiation (C. Zhang, et al., 2014). S3I-201

enhanced the therapeutic effect of cisplatin, docetaxel, and 5-FU in head and neck squamous cell carcinoma by targeting stemloid cells (Bu, Zhao, et al., 2015). When used in a xenograft model of HNSCC, at 5 mg/kg every other day, in combination with 10 mg/kg of cisplatin or docetaxel on day 14 or combination with 15 mg/kg 5-FU administered day 14 through day 19 inhibited tumor growth more than any single agent alone (Bu, Zhao, et al., 2015). Use of other mouse models, including models of breast and gastric cancer as well as hepatocellular carcinoma, have shown that S3I-201 aids in chemotherapy sensitization and is effective at reducing tumor growth while prolonging survival (Bu, Zhao, et al., 2015; Lin, et al., 2009; K. Siddiquee, et al., 2007).

Several S3I-201 analogs have been created and tested in preclinical models. The first one is S3I-201.1066, which was generated using computer-based design and information on the key structural elements of S3I-201 involved in binding to the SH2 domain of STAT3 (X. Zhang, et al., 2010). S3I-201.1066 had improved potency in inhibiting the DNA-binding activity of STAT3 with an IC50 of 35 μM compared to 86 μM for the parent compound S3I-201 as determined using EMSA (X. Zhang, et al., 2010). These high IC50's suggest that it may be difficult to compete for the SH2 domain in active and dimerized STAT3 in the *in-vitro* EMSA assay. Nevertheless, S3I-201.1066 treatment resulted in reduced STAT3 activity, expression of STAT3 downstream target genes, and inhibited survival of cancer cells and xenograft tumor growth. Interestingly, there is a ~3-fold difference in cytotoxicity IC50 of S3I-201.1066 between cancer cells and MEF cells that lack STAT3, suggesting that it has a therapeutic window. However, despite the improvement of S3I-201.1066 in inhibiting STAT3 activity over S3I-201, the cytotoxicity IC50 of S3I-201.1066 for cancer cells is >37 μM, making it a less likely candidate for clinical development.

Additional effort has also been made to further optimize S3I-201 and S3I-201.1066 to improve potency (Urlam, et al., 2013). which led to the identification of several analogues

including S3I-1757 (X. Zhang, et al., 2013), BP-5-087 (Eiring, et al., 2015), BP-1-102 (Resetca, Haftchenary, Gunning, & Wilson, 2014; X. Zhang, et al., 2012), SH-4-54, SH-5-07 (Haftchenary, et al., 2013), and PG-S3-001 (Arpin, et al., 2016). S3I-1757 is a novel non-sulfonamide containing salicylic acid, which inhibited STAT3 dimerization and nuclear translocation. It also inhibited anchorage independent growth, invasion and migration of human breast and lung cancer cell lines.

BP-5-087 differs from S3I-201.1066 by substitution of a 2-methylbenzyl group on the sulfonamide group (Eiring, et al., 2015). This modification prevented STAT3 binding to a phosphotyrosine peptide with an IC50 of 5.6 μM. Furthermore, BP-5-087 was tested in primary chronic myeloid leukemia cells with BCR-ABL1 tyrosine kinase inhibitor resistance and it restored sensitivity to treatment and reduced colony formation ability and survival (Eiring, et al., 2014; Eiring, et al., 2015).

BP-1-102 contains a pentafluorobenzene in place of the 3-methylbenzene of S3I-201.1066. The substitution of pentafluorobenzene decreased the IC50 for DNA binding down to 6.8 μM with an improved selectivity to STAT3 over STAT1 and STAT5 (X. Zhang, et al., 2012). BP-1-102 had a significant impact on growth, survival, migration, and invasion of tumor cells with constitutively active STAT3. BP-1-102 is orally available and inhibited human breast, lung and colon tumor growth in xenograft models (De Simone, et al., 2015; X. Zhang, et al., 2012).

Increased potency was achieved by the development of SH-4-54 and SH-5-07 with cytotoxicity IC50's ranging 66-235 nM and 195-1120 nM in brain cancer stem cells, respectively (Haftchenary, et al., 2013). SH-4-54 was achieved by removing the hydroxyl group from the salicylic acid forming benzoic acid while SH-5-07 exchanged the salicylic acid for an N-hydroxylamine (Haftchenary, et al., 2013). These compounds were effective at suppressing STAT3 phosphorylation and transcriptional activities. They are permeable to the blood-brain

barrier and inhibited growth of glioblastoma cells as well as breast cancer in *in-vivo* mouse models (Haftchenary, et al., 2013). *In-vivo* PK/PD studies have shown accumulation of SH-4-54 in the brain and its ability to target STAT3 with limited side effects. Furthermore, PG-S3-001, a derivative of SH-4-54, was shown to be able to inhibit proliferation of pancreatic cancer cells using a 3D culture system with an IC50 of 15.2 μM and growth of patient-derived xenograft tumors (Arpin, et al., 2016).

STX-0119, another STAT3 inhibitor targeting the SH2 domain, was also identified using in-silico screening (Matsuno, et al., 2010). STX-0119 treatment of lymphoma cell lines led to a significant growth inhibition and induction of caspase cleavage (Ashizawa, et al., 2011). Oral administration of STX-0119 led to reduction in STAT3 target gene expression as well as induction of apoptosis in xenograft tumors from lymphoma cell line SCC3. STX-0119 has also been used to treat glioblastoma by targeting the stem cell populations (Ashizawa, et al., 2013). While STX-0119 had little effect on glioblastoma tumor growth derived from U87 cells, it was able to inhibit growth of xenograft tumors of temozolomide-resistant U87 cells (Ashizawa, et al., 2014). While the finding on STX-019 inhibition of temozolomide resistant U87 xenograft tumors is exciting, it is not clear why it had no effect on the parental U87 xenograft tumors and if the effect on temzolomide resistant U87 tumors was STAT3-dependent.

Additionally, a natural product, curcumin, has also been shown to inhibit STAT3 signaling (Zhao, Liu, & Liang, 2013). However, due to its low bioavailability and being quickly metabolized, different curcumin analogs have been synthesized to increase its bioavailability and stability (Lin, Hutzen, Zuo, et al., 2010; Wei, et al., 2011; Zhao, et al., 2013). Two such analogs, FLLL31 and FLLL32, had the 2 hydrogens in the center of curcumin replaced with methyl groups and a spiro-cyclohexyl ring, respectively, and have a 3,4-dimethoxy substitute, which helped

increase stability (Lin, Hutzen, Zuo, et al., 2010). Both FLLL31 and FLLL32 inhibited STAT3 phosphorylation, DNA-binding activity *in vitro*, colony formation and cell invasion as well as induced apoptosis of pancreatic and breast cancer cells (Lin, Hutzen, Zuo, et al., 2010). Further study of FLLL32 showed that it potently caused apoptosis of melanoma, multiple myeloma, osteosarcoma, rhabdomyosarcoma, renal cell carcinoma, glioblastoma, liver and colorectal cancer cell lines at micromolar concentrations (Bill, et al., 2010; Bill, et al., 2012; Fossey, et al., 2011; Lin, Deangelis, et al., 2010; Lin, Fuchs, et al., 2011; Onimoe, et al., 2012; Wei, et al., 2011). In colon and pancreatic cancers, FLLL32 reduced cell viability of the stem-like cell population, decreased tumorsphere formation and expression of STAT3 downstream target genes, and induced caspase dependent apoptosis in colon cancer stem-like cells (Lin, Fuchs, et al., 2011; Lin, et al., 2016). Use of FLLL32 has been shown to decrease growth of xenograft osteosarcoma and breast tumor (Onimoe, et al., 2012; Yan, et al., 2015). FLLL32 significantly reduced tumor growth and tumor vascularity in a chicken embryo xenograft model of pancreatic cancers (Lin, Hutzen, Zuo, et al., 2010). However, whether FLLL31 and FLLL32 directly bind to the SH2 domain requires further studies.

Most recently, a synthetic derivative of a natural product bisindolylmaleimide alkaloid, BMA097, was shown to directly bind to the SH2 domain and inhibit STAT3 dimerization and activation (X. Li, et al., 2018). It is effective in suppressing breast cancer cell growth with IC50's ranging 0.9-3.9 μM against different cell lines. It also inhibited growth of breast xenograft tumors and induced spontaneous apoptosis. The structure-activity relationship analysis revealed that the hydroxymethyl group in the 2,5-dihydropyrrole-2,5-dione is important for the STAT3-inhibitory activity. Although other BMAs have previously been shown to inhibit PKC, the synthetic

analogue, BMA097, does not appear to have this activity. Nevertheless, whether BMA097 has other potential targets remains to be determined.

Finally, several FDA-approved drugs have also been shown to inhibit STAT3 signaling and therefore, may be repurposed. These drugs include celecoxib (H. Li, et al., 2011), piperlongumine (Bharadwaj, et al., 2015), artesunate (Ilamathi, Santhosh, & Sivaramakrishnan, 2016; C. Kim, Lee, Kim, Sethi, & Ahn, 2015), nifuroxazide (Nelson, et al., 2008; Yang, et al., 2015), and niclosamide (Ren, et al., 2010). While celecoxib, piperlongumine, and artesunate have been shown to bind directly to STAT3, possibly at the SH2 domain, nifuroxazide was shown to inhibit the upstream JAK autophosporylation and niclosamide was shown to inhibit STAT3 phosphorylation on Tyr705 but does not bind to the SH2 domain. Although more detailed studies are required to understand if and how effective these drugs are in inhibiting STAT3 activity and signaling, these approved drugs may prove to be an interesting approach and a faster way to translate into clinical testing.

In summary, many small molecule inhibitors targeting the SH2 domain of STAT3 have been identified with some having *in-vivo* activity and the list of such inhibitors continues to grow. Although some of these inhibitors have been shown to bind directly to the SH2 domain, many others are only predicted to bind to the SH2 domain based on computational modeling and inhibition of STAT3 phosphorylation. Some studies lack evidence to eliminate the possibility that the inhibitor may inhibit JAK2, which results in reduced STAT3 phosphorylation, raising concerns on specificity. Another common issue of these inhibitors is their high cytotoxicity IC50, which would preclude them from further clinical development. Although medicinal chemistry has been conducted to optimize some of these inhibitors, the improvement appears to be limited and none has resulted in a lead with cytotoxicity IC50 in the low nanomolar range. Clearly, future studies

may need to focus on optimizing the existing inhibitors to generate more active derivatives. Only when SH2 domain-targeting STAT3 inhibitors that are more active and more selective than those described above are identified, will there be clinically testable inhibitors (see below). However, it is concerning that most of SH2 domain inhibitors have high cytotoxicity IC50, which raises a possibility that the SH2 domain may be difficult to target. It is also noteworthy that natural products may be a rich resource to discover potential STAT3 inhibitors targeting its SH2 domain. However, caution should be considered when testing some of these natural products such as curcumin, which may be promiscuous with limited selectivity.

3.3. SH2 domain inhibitors in clinical trials

As described above, many SH2 domain inhibitors have shown promise in laboratory studies. However, only a few have successfully made it into clinical trials (**Table 3**). While STA-21 has been tested for the treatment of psoriatic lesions as discussed above (Miyoshi, et al., 2011), it has yet to be tested clinically for cancer treatment in systematic use.

Two of the most promising STAT3 inhibitors in clinical trials were OPB-31121 and OPB-51602. These compounds, which were thought to target the SH2 domain, are very potent and have been shown to have antitumor effects in several different cancers in preclinical *in-vitro* and *in-vivo* studies (Brambilla, et al., 2015; Hayakawa, et al., 2013; M. J. Kim, et al., 2013). They both have completed Phase I/II clinical trials for multiple cancers including advanced solid tumors, non-Hodgkin's lymphoma, multiple myeloma, hepatocellular carcinoma, and hematologic malignancies (Bendell, et al., 2014; Ogura, et al., 2015; Oh, et al., 2015; Okusaka, et al., 2015; Wong, et al., 2015). While initial studies for OPB-31121 showed feasibility in inhibiting STAT3 and having antitumor effects, clinical trials in hepatocellular carcinoma showed minimal antitumor activity, poor pharmacokinetic properties, and peripheral nervous system toxicity (Okusaka, et al.,

2015). OPB-51602 only demonstrated some antitumor activity in non-small cell lung cancer but not in hematologic malignancies (Ogura, et al., 2015; Wong, et al., 2015). The clinical trials were terminated for both of these inhibitors due to poor pharmacokinetic properties and intolerability. These inhibitors require further optimization to improve pharmacokinetic properties and to limit observed toxicity.

Pyrimethamine, originally identified as an antimalarial drug by inhibiting dihydrofolate reductase, is currently in phase I/II clinical trial in the treatment of chronic lymphocytic leukemia and small lymphocytic leukemia (https://clinicaltrials.gov/ct2/show/NCT01066663) after being identified as a potential STAT3 inhibitor via screening a library of 1120 drugs that are known to be safe in human using cell-based STAT3-dependent luciferase reporter assay (Takakura, et al., 2011). Pyrimethamine inhibited tyrosine phosphorylation of STAT3 without inhibiting its upstream kinase JAK2 and, thus, was also thought to bind to the SH2 domain of STAT3. Recent pre-clinical studies have shown that pyrimethamine may be effective at treating glioblastoma and acute myeloid leukemia (Baritchii, et al., 2016; Sharma, et al., 2016). Very recently, a xenograft mouse model of breast cancer showed reduction in STAT3 activation, tumor growth, and in inflammation following pyrimethanmine treatments (Khan, et al., 2018). There was also indications of increased cytotoxic granule release by tumor infiltrating CD8+ cells in this model as indicated by Lamp1 expression. However, it is yet to be shown whether the anticancer activity of pyrimethamine is via inhibiting STAT3, dihydrofolate reductase or both.

Recently, another STAT3 inhibitor targeting its SH2 domain, C188-9, has entered phase I trial in treating advanced cancers (https://clinicaltrials.gov/ct2/show/NCT03195699). C188-9 was a more effective analogue of one of the hit inhibitors derived from in-silico screening of a chemical library targeting the SH2 domain of STAT3 (Redell, Ruiz, Alonzo, Gerbing, & Tweardy, 2011;

X. Xu, Kasembeli, Jiang, Tweardy, & Tweardy, 2009). C188-9 was able to bind directly to STAT3 with a K_D of 4.7 nM (Bharadwaj, et al., 2016) and the binding prevents STAT3 phosphorylation, induces apoptosis, and inhibits xenograft tumor growth (Bharadwaj, et al., 2016; Jung, et al., 2017; Lewis, et al., 2015; Redell, et al., 2011). C188-9 has a good safety profile and good pharmacokinetic properties in mice, rats and dogs (Jung, et al., 2017), providing the required basis for phase I testing.

Moving some of the SH2 domain inhibitors into clinical trials is very encouraging despite the possibility that these inhibitors may never gain FDA approval. In addition to the potential toxicity and poor pharmacokinetics in the case of OPB-31121 and OPB-51602, other possible issues may need to be considered in future clinical trials in order to ensure its success. One such consideration should be stratifying patient population for clinical trials and testing specific cancer types that are driven by STAT3 activation. For example, activating mutations in the SH2 domain have been identified that associates with large granular lymphocytic leukemia with 40% mutation rate (Koskela, et al., 2012). Similar mutations in the SH2 domain have also been identified in inflammatory hepatocellular adenomas (Pilati, et al., 2011). While it remains to be determined, these somatic mutations may affect the binding of the SH2 domain inhibitors to STAT3 and reduce the efficacy of these inhibitors. The fact that OPB-51602 had no effect on hematological malignancies (see above) is consistent with this speculation. Thus, further study of these mutations and identifying patients with these mutations will likely benefit clinical testing of the SH2 domain-targeting STAT3 inhibitors and benefit patients with precision use.

3.4. Small molecule inhibitors targeting the DNA-binding domain (DBD)

The SH2 domain has largely been the focus in drug discovery targeting STAT3. However, successes of the past studies on SH2 domain inhibitors have been limited as discussed above.

Furthermore, it has been shown that the import of STAT3 into the nucleus and binding to DNA can occur independent of its phosphorylation status (L. Liu, McBride, & Reich, 2005; Nkansah, et al., 2013). This observation indicates that SH2 domain inhibitors may not be sufficient to fully inhibit STAT3 function, which may contribute to the limited success of these inhibitors. Interestingly, few studies have been conducted to target the other domains of STAT3 (**Figure 1**). Of these other domains of STAT3, only the DBD has been tested for discovery of small molecule inhibitors (**Table 4**). In general, the DBD of transcription factors are considered "undruggable". This is due to the fact that DBDs are flat with similarities among different isoforms of the same transcription factor family and, thus, potentially limiting selectivity.

A class of platinum compounds including IS3-295, CPA-1, CPA-7, and platinum (IV) tetrachloride has been found to block the DNA-binding activity of STAT3, inhibit cell growth, and induce apoptosis while having no effect on cells that did not have persistent STAT3 activation (Turkson, et al., 2005; Turkson, Zhang, et al., 2004). These compounds had better inhibitory profiles than the peptide inhibitor as described above. Of these compounds only CPA-7 has been tested in *in-vivo* models. CPA-7 had antitumor activity in prostate, colon, and glioma mouse xenograft models (Assi, et al., 2014; Liang, et al., 2016; Turkson, Zhang, et al., 2004; L. Zhang, et al., 2009). However, its inability to cross the blood brain barrier limits its use in the treatment of tumors in the central nervous system. While cursory toxicology studies have been performed on tumor-bearing animals, a full assessment is needed before CPA-7's clinical relevance can be determined.

A natural product, Galiellalactone, was identified to inhibit the DNA-binding activity of STAT3 while screening for compounds that inhibited IL-6-induced gene expression (Weidler, Rether, Anke, & Erkel, 2000). Using biotinylated Galiellalactone as a probe in combination with

MS analysis, it was found that the DBD of STAT3 was likely the binding site for Galiellalactone (Don-Doncow, et al., 2014). The cysteine residues Cys-367, Cys-468 and Cys-542 in the linker and DBD of STAT3 were covalently modified by Galiellalactone since it is a cysteine-reactive Michael acceptor (Don-Doncow, et al., 2014; Garcia, et al., 2016). Galiellalactone has been shown to reduce tumor size and to reduce metastatic spread in prostate cancer cell lines and xenograft mouse model (Canesin, et al., 2016; Hellsten, et al., 2008). Furthermore, Galiellalactone was able to reduce the number of ALDH+ cells, or stem-like cells, in DU145 xenograft tumors (Hellsten, Johansson, Dahlman, Sterner, & Bjartell, 2011). However, the bioavailability of orally administered Galiellalactone is low and the *in-vivo* studies required IP injection. To increase the oral bioavailability of Galiellalactone, a prodrug, GPA512, was created by adding an N-Acetyl L-cysteine methyl ester to the thiol of Galiellalactone (Escobar, et al., 2016). GPA512, at an oral dose of 40 mg/kg daily, inhibited growth of prostate xenograft tumors, which was similar to the outcomes observed when Galiellalactone was used via intraperitoneal injection (Escobar, et al., 2016). Thus, GPA512 may function as an orally available prodrug of Galiellalactone. However, Galiellalactone has been shown to interrupt other signaling pathways such as NF-kB and TGF-ß and it can bind several other target proteins due to its Michael acceptor activity (Don-Doncow, et al., 2014). This lack of specificity may prove to be an issue during its future development.

While curcumin and a few of its analogues were discussed above to target the SH2 domain, HO-3867, another curcumin analog, has been suggested to bind to the DBD of STAT3 (Rath, et al., 2014). HO-3867 has been tested in several cancer cell lines including breast, colon, head and neck, pancreas, liver, lung, ovarian and prostate cancer cell lines. HO-3867 has also been tested in an ovarian xenograft mouse model (Selvendiran, et al., 2011; Selvendiran, Ahmed, Dayton, Kuppusamy, et al., 2010). In these studies, HO-3867 had preferential cytotoxicity towards cancer

cells versus non-cancerous cells (Selvendiran, Ahmed, Dayton, Kuppusamy, et al., 2010). Furthermore, STAT3 was thought to mediate the cellular effect of HO-3867 as determined by overexpressing or knocking down STAT3 (Tierney, et al., 2012). In an ovarian xenograft mouse model, it was shown that HO-3867 inhibited tumor growth, angiogenesis, and metastasis (Saini, et al., 2017; Selvendiran, et al., 2011). However, it was also found that HO-3867 decreased the phosphorylation status of STAT3, which indicates that it may not bind to the DBD of STAT3. Interestingly, HO-3867 has also been shown to increase FASN degradation, inhibit JAK activation, and produced reactive oxygen species (Y. Hu, et al., 2017; Selvendiran, Ahmed, Dayton, Ravi, et al., 2010; Selvendiran, Tong, et al., 2010). These findings further highlight the promiscuity of curcumin and its analogues and, thus, they are unlikely specific to STAT3.

Using an improved in-silico screening method, a small molecule inhibitor, inS3-54, was identified targeting the DBD of STAT3 (Huang, et al., 2014). inS3-54 selectively inhibited the DNA-binding activity of STAT3 both *in vitro* and *in situ* but had no effect on the phosphorylation of STAT3. Additionally, in-silico screening showed that inS3-54 does not bind to STAT1 and is selective for the DBD of STAT3, which was confirmed using EMSA. inS3-54 also effectively inhibited IL-6-induced activity of STAT3 and proliferation, migration, and invasion of both breast and lung cancer cell lines (Huang, et al., 2014).

Further investigation of inS3-54 analogues led to identification of a pharmacophore as shown in **Figure 3A** (Huang, et al., 2016)**.** Of the analogues tested, inS3-54A18, A26, and A69 (**Figure 3B**) were identified to be more active than the parent inS3-54. Interestingly, the cytotoxicity IC50 of all these three analogues negatively associates with the level of activated STAT3 in different cell lines tested (**Figure 3C**). InS3-54A18 (A18) was selected as a lead compound based on its enhanced properties in solubility, specificity and pharmacology. It has

IC50s ranging from 1.8 to 5.6 μM for cancer cells and from 4.0 to 12.0 μM for non-cancer cells. A18 not only inhibited the constitutive and IL-6-stimulated expression of STAT3 downstream target genes, it also effectively inhibited lung xenograft tumor growth and metastasis with little adverse effect on animals. Importantly, it was also shown, using pull-down assay of purified recombinant STAT3 and immobilized compounds, that A18 directly binds to the DBD of STAT3. Thus, A18 is a potential candidate for further development as anticancer therapeutics targeting the DBD of human STAT3.

Recently, pyrimidinetrione and its derivatives have been identified as potential STAT3 DBD inhibitor by screening 300 compounds using EMSA and Sitemap program analyses (Shan Sun, 2017). The pyrimidinetrione derivatives were shown to decrease cancer cell viability and growth but had little effect on STAT3 null MEF cells. Thus, the pyrimidinetrione compounds may be used to help design future STAT3 DBD inhibitors.

As stated above, relatively few studies have been focused on targeting domains other than the SH2 domain. Although progress in targeting the DBD of STAT3 has been made recently, developing inhibitors targeting DBD will likely face challenges as well. Similar to the SH2 domain, as discussed above, somatic mutations have also been identified in the DBD of STAT3. One such mutation is H410R, which activates STAT3 without upstream stimulation (Andersson, et al., 2016). While the histidine to arginine mutation was thought to increase hydrophilicity of the DBD and STAT3 binding to DNA, it may also prohibit binding of STAT3 inhibitors to the DBD. Several dominant negative mutations in the DBD have also been identified and associated with hyper-IgE syndrome (Minegishi, et al., 2007). Although it is unknown if these mutations could occur in human cancer cells, such dominant negative mutations will likely render ineffectiveness of any STAT3-targeting inhibitors in cancer treatment.

4. Conclusions and Perspectives

It has been clearly shown and accepted that constitutive STAT3 activation gives tumor cells a survival advantage. Targeting STAT3 within tumor cells may prove to be successful in treating cancers and sensitizing them to current chemotherapeutics. However, while many different STAT3 inhibitors have been identified, with few in clinical trials, there are currently no approved drugs targeting STAT3. Based on the above discussion and history of development, it is likely that we will face many challenges ahead in developing STAT3 targeting inhibitors.

The vast majority of past STAT3 inhibitors, as discussed above, targeted the SH2 domain with few targeting other domains of the protein. Targeting the SH2 domain has limited success due to insufficient inhibition of STAT3. STAT3 has been shown to translocate into the nucleus and bind to DNA even without Tyr705 phosphorylation. With new approaches being developed and the increasing studies of targeting other domains, such as the DBD of STAT3, novel inhibitors with higher affinity to STAT3 and lower cytotoxic IC50's may emerge and enter into clinical trials. Testing additional FDA-approved drugs for their potential in inhibiting STAT3 may result in novel inhibitors for repositioning and quick translation into clinical testing for treating human cancers by targeting STAT3.

One of the major problems with STAT3 inhibitors, as discussed above, is their high cytotoxic IC50 values, which prohibits further development. Clearly, medicinal chemistry work is needed to optimize some of these promising inhibitors. It is also noteworthy that the inhibitors with high affinity to STAT3 often show adverse effects such as fatigue, diarrhea, infection, and periphery nervous system toxicities. These adverse effects may be associated with the physiological function of STAT3 in normal tissues and, thus, result in STAT3-specific toxicity. For example, it has been shown previously that tissue-specific conditional STAT3 inactivation

leads to enterocolitis and Crohn's disease-like pathogenesis (Alonzi, et al., 2004; Kortylewski, et al., 2005; Welte, et al., 2003). Thus, consideration and use of targeted delivery for high affinity STAT3 inhibitors may help avoid or eliminate these adverse effects in future direction of research on developing STAT3 inhibitors.

Another major potential problem is somatic mutations in STAT3, which may inhibit the binding of inhibitors to STAT3 and cause ineffectiveness of these inhibitors as discussed above. Considering that these mutations occur at a rate of 40% in large granular lymphocytic leukemia, it is conceivable that the outcome of clinical studies could be affected by these mutations should they prohibit inhibitor binding to STAT3. Unfortunately, no studies have addressed if these mutations affect inhibitor binding to STAT3. Understanding the relationship between these somatic mutations and STAT3 inhibitor efficacy in future studies will be very important for developing effective STAT3-targeting drugs for precision use.

Tumor heterogeneity is another potential area of research that deserves thoughtful consideration in developing STAT3 inhibitors. Cancer is a dynamic disease and constantly changes during the development of this disease, resulting in diversity and heterogeneity (Dagogo-Jack & Shaw, 2018). Analysis of several cell lines have revealed cell-cell heterogeneity in STAT3 activation in a cell population (Gough, et al., 2014). Recently, it was discovered that oncogenically transforming normal human bronchial epithelial cells resulted in tumor cells with heterogeneity of two major mutually exclusive subpopulations (Deb, et al., 2017). One subpopulation had upregulated STAT3 activation and was sensitive to STAT3 inhibitors while the other had downregulation of SMAD2/3 signaling and were resistant to STAT3 inhibitor treatments. It is also tempting to speculate that tumor heterogeneity with cells expressing drug efflux pumps may cause resistance to STAT3 inhibitors. Thus, tumor heterogeneity will likely influence the outcome of

STAT3 inhibitor treatment. Combination of STAT3 inhibitors with other agents may help overcome these problems as demonstrated by Deb et al (Deb, et al., 2017), who showed that combining STAT3 and BCL6 inhibitors more effectively eliminated their heterogenic cancer cells than any single treatment alone. Future studies should be conducted to determine other mutually exclusive pathways from STAT3 in order to develop therapeutic combinations.

Our lack of understanding of cross-talks between signaling pathways and compensatory pathways may have also contributed to the past failure in developing STAT3 inhibitors. Indeed, combinations of STAT3 inhibitors with other targeted therapeutics have shown promises. For example, crosstalk between MEK and STAT3 activation has been observed to lead to resistance to MEK inhibitors in K-ras mutant pancreatic and colon cancer cells and combination of STAT3 inhibitor LY5 with MEK inhibitor Trametinib had synergistic effect on these cells both *in vitro* and *in vivo* (Zhao, et al., 2015). STAT3 has also been shown to contribute to EGFR inhibitor resistance and combination of STAT3 inhibitor S3I-201 with EGFR inhibitor overcame the resistance and increased the anti-proliferative effect (W. Chen, et al., 2012). Thus, combination therapies may help overcome the challenges created by signaling cross-talk and compensatory pathways. Additional research into how these pathways interact will help determine the best course of actions and the most promising combinations.

Another line of future research direction with potentials is combination of STAT3 inhibitors with immunotherapeutics. Immune surveillance plays a significant role in eliminating tumor cells. Activation of STAT3 in cancer cells increases expression of immunosuppressive factors which leads to suppression of immune cells (H. Yu, Kortylewski, & Pardoll, 2007). This immunosuppression is maintained in immune cells by STAT3 activation. Considering the function

and activity of STAT3 in immune evasion of cancer cells, STAT3 activation may be an effective way to both target tumor cells and stimulate the immune system to eliminate tumor cells.

In summary, while we have come a long way in understanding and targeting STAT3 for drug discovery and development, there is still no FDA approved STAT3 inhibitor. This may be due to a lack of full inhibition of STAT3 while targeting the SH2 domain, lack of understanding of signaling cross talk, and adverse events related to STAT3-specific activity in normal tissues. Recently, targeting other domains of STAT3 as an alternative approach began to attract attention to overcome the limitations of SH2 targeting molecules. Further understanding the relationship between tumor heterogeneity and STAT3 signaling, cross-talks between STAT3 and other signaling pathways, STAT3 dependency and specific cancer types, as well as the effect of somatic mutations on cellular response to STAT3 inhibitors will all likely help successfully develop STAT3 inhibitors. This information will likely also contribute future patient stratification and precision use of STAT3 inhibitors in the clinical setting. Finally, consideration of tumor site delivery of STAT3 inhibitors using special vehicles will likely help avoid STAT3-specific toxicity in normal tissues.

5. Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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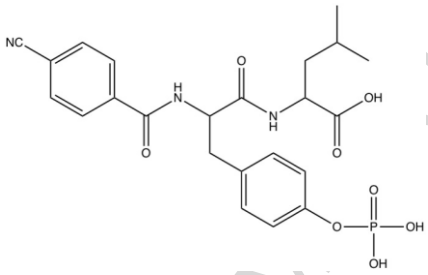
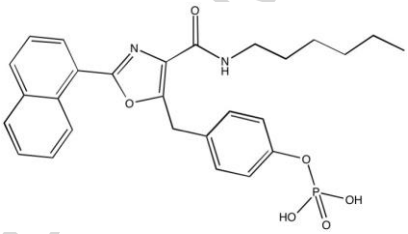
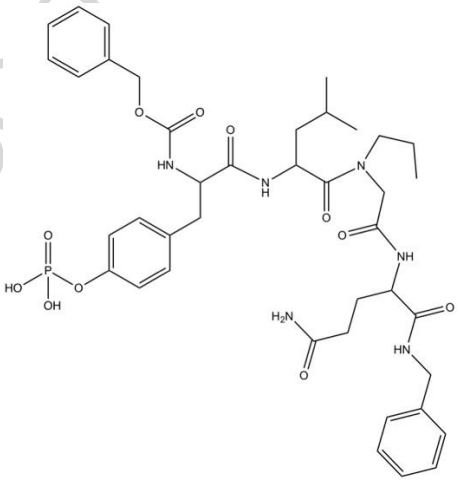
Table 1. Molecular probes targeting STAT3

Name	Structure/Sequene	Activity	Remarks	
Mimetic derived from STAT3	PpYLKTK	IC50=235 µM (DNA binding)		(Turks Tur
Minimum required mimetic sequence	PpYL	IC50=182 µM (DNA binding)		(Turks Tur
Mimetic derived from GP130	pYLPQTV	Kd=35 nM (Binding affinity)		(Go
ISS610		IC50=42 µM (DNA binding)		(Tu
S3I M2001		IC50=79 µM (STAT3 dimerization) IC50=50-100 µM (cytotoxicity)	Effective on breast xenograft tumors	(K. A
Compound 1		Ki=350 nM (Phosphopeptide Binding)		(Go

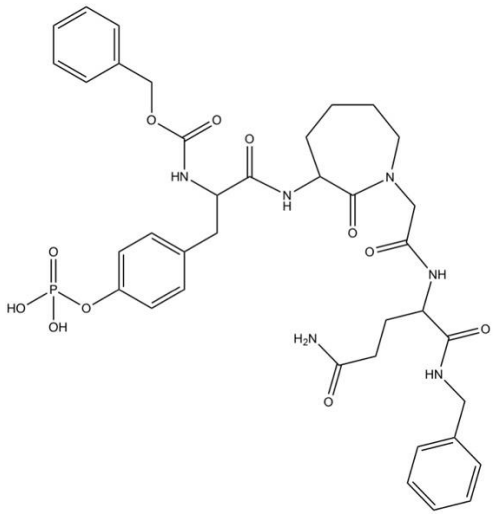
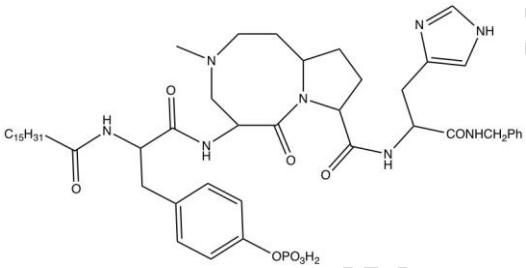
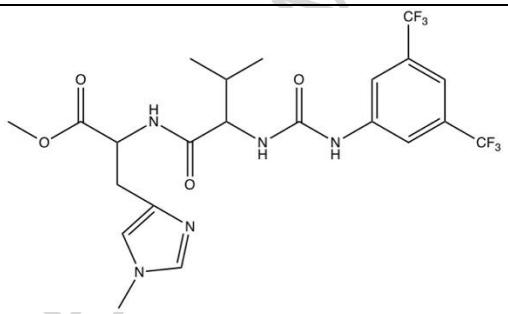
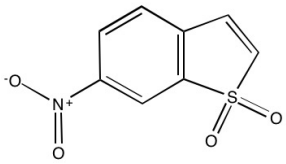
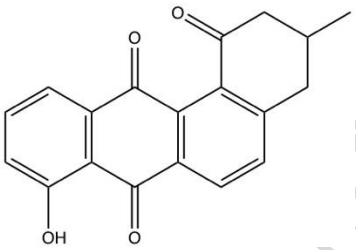
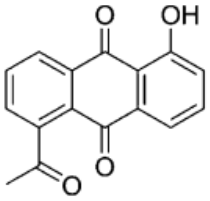
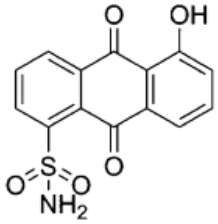
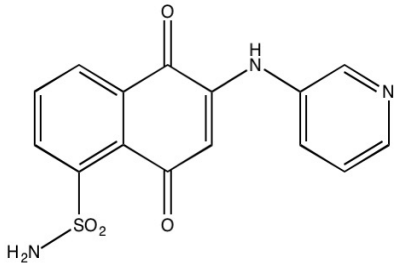
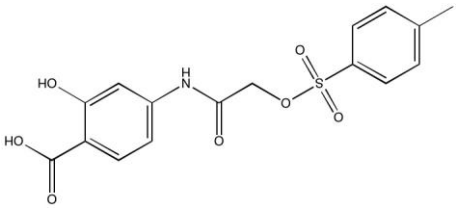
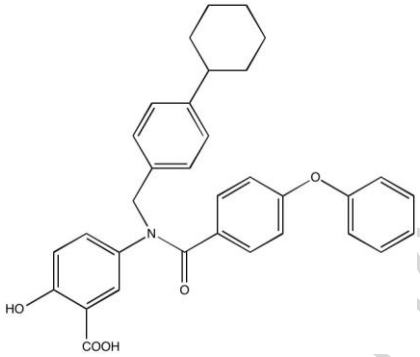
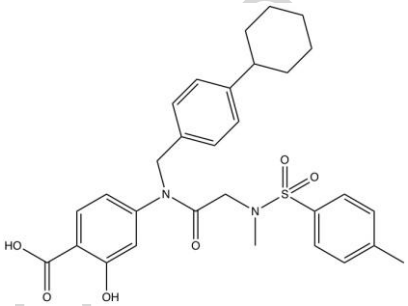
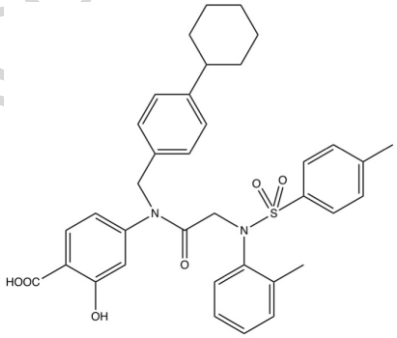
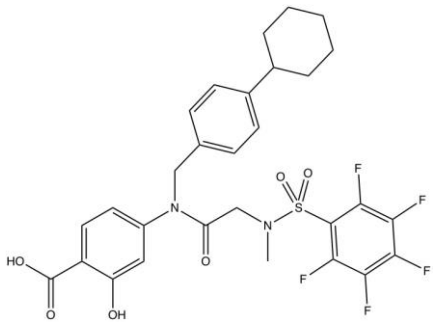
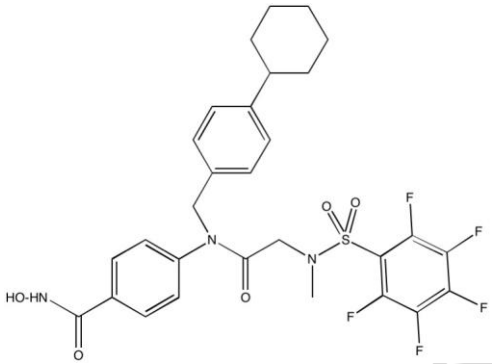
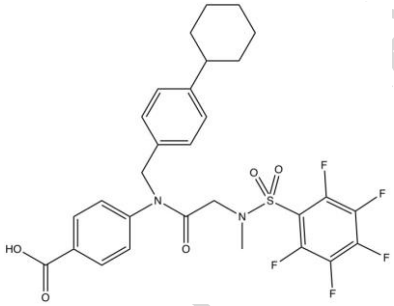
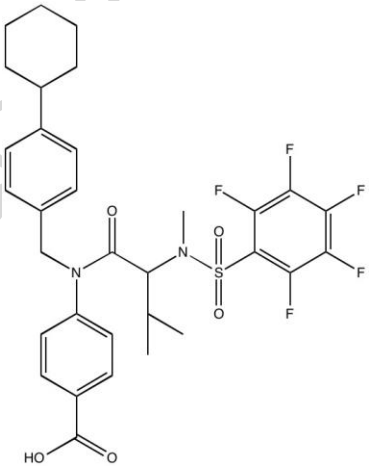
Compound 3		Ki=190 nM (Phosphopeptide Binding)		(Go
CJ1383		Ki=950 nM (Phosphopeptide binding) IC50=3.6-11.2 µM (Cytotoxicity)		(J.
XZH-5		IC50=15-25 µM (Cytotoxicity)		(Daka, et al., Z. Xu,
rS3-PA	N-VRH SAL HMA VGP LSW PAR VS-C		Effective on xenograft glioma	(Bor Schon We
DBD1	PLTAVFWLIYVLAKALVTVC	IC50=270 nM (Cytotoxicity)		(Nag

Table 2. Small molecule inhibitors targeting the SH2 domain of STAT3

Drug	Structure	IC50	Remarks	
STATTIC		IC50=5.1 µM (Phosphopeptide binding) IC50=1-4 µM (Cytotoxicity)	Effective on xenograft HNSCC, esophageal squamous cell carcinoma, ovarian, colorectal, and prostate cancers	(Agu 201 2012; Han, e 2013 Lin, e et al. 2006 Schus Gu, Sou Spit Vill
STA-21		IC50=12-20 µM (Cytotoxicity)	Effective on psoriatic lesions in mouse models	(Ahn L. Che et al. al., 20 2017 So
LLL3		IC50 = 6.8-11.5 µM (Cytotoxicity)	Effective on xenograft glioblastoma	(Bh Fosse
LLL12		IC50=0.11-6 µM (Cytotoxicity)	Effective on xenograft glioblastoma, multiple myeloma, osteosarcoma, astrocytoma, hepatocellular carcinoma, breast, and prostate cancers	(E Bhasi et al 2013; al., 20 Lin, Liu, al., 2 201 2012; Xu, e

LY5		IC50=0.5-1.4 µM (Cytotoxicity)	Effective in xenograft breast, colon, and pancreatic cancers	(W Zhao,
S3I-201		IC50=85-150 µM (Cytotoxicity)	Effective on xenograft HNSCC, hepatocellular carcinoma, breast, and gastric cancers	(Q. L Lin Siddi
S3I-1757		IC50=13.5 µM (Phosphopeptide binding)	Effective on xenograft melanoma	(X. Z
S3I-201.1066 (SF-1-1066)		IC50=35µM (DNA binding) Kd=2.74 µM (Binding affinity)	Effective on xenograft breast cancer	(Eiri Zh
BP-5-087		IC50=5.6 µM (Phosphopeptide binding)		(Ei

BP-1-102		Kd=504 nM (Binding affinity) IC50=4.1 µM (STAT3 binding to RTK) IC50=6.8 µM (DNA binding) IC50=2-19 µM (Cytotoxicity)	Effective on xenograft breast, colon, and lung cancers	(De S X. Z
SH-5-07		IC50=195-1120 nM (Cytotoxicity)		(Ha
SH-4-54		Kd=300 nM (Binding affinity) IC50=66 nM-9.2 µM (Cytotoxicity)	Effective on xenograft glioblastoma	(H
PG-S3-001		Kd=324 nM (Binding affinity) IC50=3.7 µM (Cytotoxicity)	Effective on patient-derived pancreatic cancer xenograft	(A

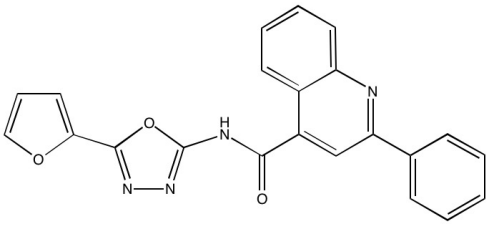
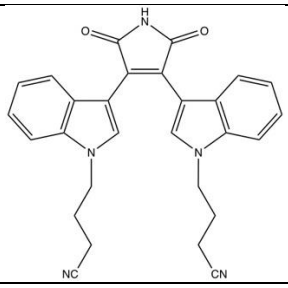
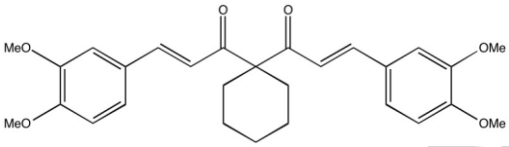
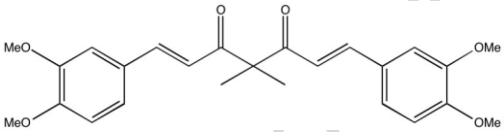
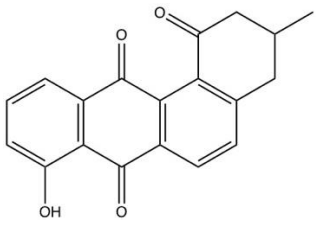
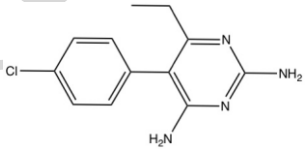
STX-0119		IC50=5-10 µM (Cytotoxicity)	Effective on xenograft lymphoma and glioblastoma	(Aki Ashi Ashi Ashi Mat Mi
BMA097		IC50=0.9-3.9 µM (Cytotoxicity)	Effective on xenograft breast cancer	(X
FLLL32		IC50=0.53-0.77 µM (Cytotoxicity)	Effective on xenograft osteosarcoma	
FLLL31				

Table 3. SH2 domain inhibitors in clinical trials

Drug	Structure	Diseases Tested	IC50	
STA-21		Psoriasis	IC50=12-20 μM (Cytotoxicity)	(Ahma L. Che et al., al., 201 2017; Son
OPB-31121	Not reported	Leukemia, advanced cancer, solid tumor, Non-Hodgkin's lymphoma, multiple myeloma, and hepatocellular carcinoma	IC50=0.3-100 nM (Cytotoxicity)	(Ben Bram Hayak M. J. Oh, et a
OPB-51602	Not reported	Nasopharyngeal carcinoma, advanced cancer, multiple myeloma, non-Hodgkin lymphoma, acute myeloid leukemia, acute lymphoid leukemia, chronic myeloid leukemia, solid tumor		(Og Wo
Pyrimethamine		Chronic lymphocytic leukemia, small lymphocytic leukemia		(J. Takal

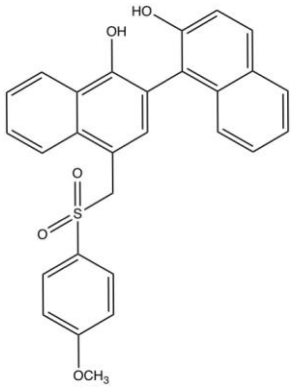
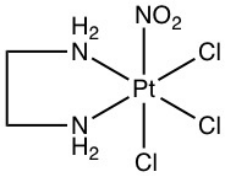
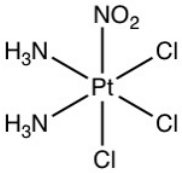
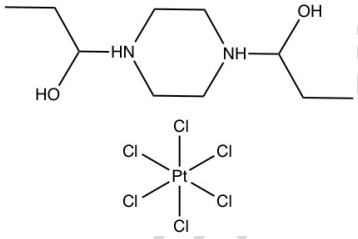
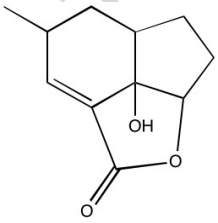
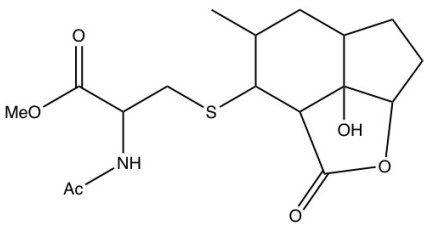
C188-9		Breast cancer, HNSCC, non-small cell lung cancer, hepatocellular cancer, colorectal cancer, gastric adenocarcinoma, melanoma, advanced cancer	IC50=0.4-18 µM (Cytotoxicity)	(Bhara Jung, e et al., 2

Table 4. Inhibitors targeting the DBD of STAT3

Drug	Structure	IC50	Remarks
CPA-1		IC50=5 μM (DNA binding)	
CPA-7		IC50=1.5 μM (DNA binding)	Effective on xenograft glioma, melanoma, prostate and colon cancers but not effective on xenograft tumors implanted in brain
IS3-295		IC50=1.4 μM (DNA binding)	
Galiellalactone		IC50=250-500 nM (SEAP expression) IC50=5 μM (Luciferase expression) IC50=3 μM (Cytotoxicity)	
GPA512		IC50=4.81 μM (Cytotoxicity)	Effective on xenograft prostate cancer

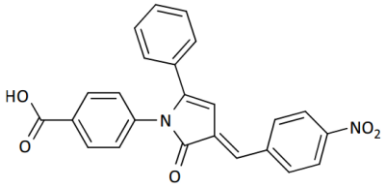
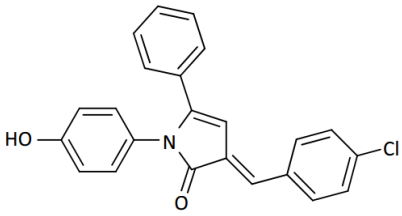
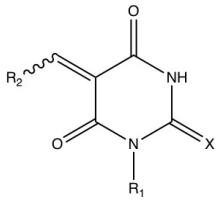
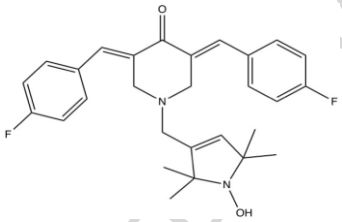
InS3-54		IC50=13.8 µM (Luciferase expression) IC50=20 µM (DNA binding) IC50=3.2-5.4 µM (Cytotoxicity)	
InS3-54A18		IC50=8.8-12.6 µM (Luciferase expression) IC50=3.2-4.7 µM (Cytotoxicity)	Effective on xenograft lung cancer
Pyrimidinetrione		IC50=2.5-3.8 µM (DNA binding)	
HO-3867			Effective on xenograft ovarian cancer.

Figure Legend

Figure 1. JAK/STAT3 pathway. (A) STAT3 becomes activated by binding of a ligand to its receptor on the cell surface. Ligand binding leads to activation of JAK, which recruits and phosphorylates STAT3. STAT3 then forms a dimer moves into the nucleus and controls transcription. (B) Schematic domain structure of STAT3. pY and pS indicate the phosphorylated tyrosine 705 and serine 727 residues. NTD, amino terminal domain; CCD, coiled coil domain; DBD, DNA-binding domain; TAD, transactivation domain. (C) Crystal structure of STAT3 with representative STAT3 inhibitors BMA097 and inS3-54 docked into the SH2 and DBD, respectively. STAT3 structure (pdb code: 1bg1) containing residues 130 to 722 was colored by their domain consistent to the color scheme of the domain structure (B) in ribbon representation. BMA097 and inS3-54 are shown by their molecular surface in green and magenta, respectively. InS3-54 binds to the DBD and replaces the double strand DNA shown in gray. BMA097 binds to the SH2 domain and replaces the phosphorylated Tyr705 shown by as ball-and-stick in pink.

Figure 2. STAT3 decoy oligonucleotides and the modified cyclic structure.

Figure 3. DBD inhibitor, inS3-54, and its derivatives. (A-B) The pharmacophore (A) and chemical structures (B) of inS3-54 and its active analogues targeting the DBD of STAT3. (C) Association of IC50 of active inS3-54 analogues with expression level of activated STAT3 in different cell lines.