Chapter 5
STAT3 Inhibitors in Cancer: A Comprehensive Update

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Abstract STAT3 is an important signaling molecule that modulates a wide range of genes by relaying extracellular signals from the plasma membrane to the nucleus in response to peptide hormone binding. It is known to play a prominent role in the initiation and progression of cancer, as it is constitutively activated in 25–100 % of more than 25 different malignancies and has been implicated in nearly all the hallmarks of cancer. In addition, STAT3 contributes to development and maintenance of cancer stem cells, as well as to cancer immune evasion and resistance to chemotherapy and radiotherapy, making it an even more attractive target for cancer therapy. In this chapter, we give an overview of strategies involved in targeting STAT3 and discuss recent advances in the development of STAT3 modulating agents.

Keywords Cancer • Oncogene • Kinase • Inhibitor • Signaling • Phosphorylation • High throughput screen • Transcriptional activation • Therapeutic • Dysregulated • SH2 • Peptidomimetics • Aptamer • Decoy • Drug design • Nuclear • Allosteric • Interference • Rational • Clinic • Clinical trial • STAT3 • Resistance

5.1 Introduction

Signal transducer and activator of transcription 3 (STAT3) is a member of a family of seven proteins that are known to play important roles in growth factor and cytokine signaling [1]. Canonical signal transduction by STAT3 is initiated by the recruitment

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of STAT3 to ligand activated membrane receptor complexes leading to a key phosphorylation event on Y705, which in turn induces a configuration change leading to tail-to-tail dimerization mediated by reciprocal SH2/pY705-peptide ligand interactions [2, 3]. The active dimer accumulates in the nucleus, where it binds to promoters and transcriptionally regulates a large number of target genes encoding proteins involved in cell survival, cell cycle progression, homeostasis, and inflammation.

Under normal physiological conditions the phosphorylation status of STAT3 in the cell is closely tied to receptor activation in response to extracellular stimuli, such that the intensity and duration of the intended signal is tightly regulated. Regulation of STAT3 is achieved by a number of elements that either act through negative feedback control on the phosphorylation of STAT3 or deactivation by dedicated nuclear phosphatases. Pathological conditions may arise in those instances where anomalies in the STAT3 signaling cascade lead to constitutive activation [1]. Hyperphosphorylation of STAT3 has been shown to occur through a variety of mechanisms, including, unregulated autocrine and paracrine secretion of cytokines and growth hormones [4], expression of intrinsically activated tyrosine kinases or receptors [5], or reduced levels of endogenous negative regulators of STAT3 signaling such as SOCS3, PIAS3, nuclear phosphatases [6, 7].

5.2 STAT3, The Oncogene

Dysregulated activation of STAT3 has been linked to the etiology and molecular pathogenesis of many diseases, most prominently cancer [4, 8], where the STAT3 signaling pathway has been implicated in nearly all features of cancer biology [7], including anti-apoptosis [9], cell transformation [8], growth and proliferation [2], angiogenesis [10], metastasis [11], and cancer stem cell maintenance [12]. Accordingly, over-expression or constitutive activation of STAT3 frequently occurs in a large number of both solid and hematological tumors (Table 5.1).

In addition to its established role in cell transformation and tumorigenesis, STAT3 oncogenic signaling has been implicated in immune regulatory mechanisms of multiple tumors [13]. For example, several studies showed that persistent activation of STAT3 leads to the suppression of anti-tumor immunity by promoting Treg recruitment within the tumor microenvironment, while negatively regulating antitumor Th1-mediated immune response [14, 15]. In addition, recent findings also revealed that STAT3 plays a crucial role in tumor immune resistance, as constitutive STAT3 activation has been shown to drive the expression of PD-L1, an immune checkpoint ligand that mediates immune inhibition within the tumor microenvironment [16]. Overall, it appears that STAT3 plays an important role in anti-tumor immune response by up regulating immune inhibitors while at the same time suppressing tumor immune activators.

From a therapeutic perspective, another significant aspect of STAT3 signaling that also merits attention is its role in chemotherapy resistance. Despite initial clinical responses to both targeted and cytotoxic cancer drugs, relapses are frequent and drug resistance remains a major obstacle in curing cancer [17, 18]. Because STAT3

Table 5.1 Constitutively activated STAT3 in various cancers

Tumor type	STAT3 activity in tumor tissue	STAT3 and clinicopathological features
Acute myelogenous leukemia (AML)	33 % pY-STAT3 + ve compared to bone marrow cells from normal donors [95]	NA
Chronic myelogenous leukemia (CML)	48.5 % CML patients pY-STAT3 + ve compared to 36.8 controls (P = 0.033) [316]	pY-STAT3 higher in advanced phase CML patients than in chronic phase patients (22.77 ± 4.41 % vs. 11.47 ± 3.14 %), P = 0.003 [316]
T cell large granular lymphocytic (T-LGL) leukemia	100 % of T-LGL pY-STAT3 + ve [317]	NA
	~40 % of T-LGL patients harbor mutations in *STAT3* gene and/or STAT3 pathway related genes and harbor increased pY-STAT3 activity [318]	
Chronic lymphocytic leukemia (CLL)	100 % of CLL patients PBMC constitutive pS-STAT3 + ve in contrast to none among normal PBMC or CD5+ B cells isolated from tonsil [319, 320]	NA
Lymphoma	87 % of Hodgkins Lymphoma (HL), 46 % of B-cell NHL, 73 % of T-cell NHL stained pY-STAT3 + ve [321]	61 % ALCL tumors constitutive STAT3 activation (84 % of ALK^+, 47 % of ALK^-). ALK correlates with STAT3 activation ($P < 0.0001$). ALK^- group: lack of STAT3 activation correlated with a favorable 5-year overall survival ($P = 0.0076$) [322]
	100 % lymphoma pY-STAT3 + ve, most intense staining in marginal sinus [210]	
Sézary syndrome (SS), type of cutaneous T-cell lymphoma (CTCL)	100 % of SS pY-STAT3 + ve compared to CD4+ T-cells from healthy controls [323]	NA
NPM-ALK + ve anaplastic large cell lymphoma (ALCL)	95 % NPM-ALK+ ALCL tumors nuclear STAT3 + ve, vs. surrounding non- neoplastic lymphocytes. ALK-ve cases primarily cytoplasmic STAT3 [324]	Survivin associate to nuclear pY-STAT3 (P = 0.007). ALK + ve group: 5-year failure-free survival (FFS): 34 % in survivin + ve vs 100 % in survivin-ve (p = 0.009). ALK-ve group: 5-year FFS: 46 % in survivin + ve vs. 89 % in survivin-ve (p = 0.03) [325]
Diffuse large B-cell lymphoma (DLBCL)	Strong pY-STAT3 (32.4 %) and nuclear STAT3 (25.7 %), more frequent in non-germinal center B cell-like (non-GCB) DLBCL than in GCB [326]	High nuclear STAT3 correlated with poor overall survival (OS, p = 0.005), and is an independent prognostic factor for DLBCL [326]. Detectable pY-STAT3 associated with improved 5-year EFS (93% vs. 47 %, p = 0.006) [327]

(continued)

Table 5.1 (continued)

Tumor type	STAT3 activity in tumor tissue	STAT3 and clinicopathological features
Non-germinal center B-cell–like (GCB-DLBCL) including activated B-cell–like (ABC-DLBCL)	61 % non-GCB-DLBCL positive for pY-STAT3 [328]	pY-STAT3 associated with shorter survival in patients (n = 185) treated with RCHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone) [329] pY-STAT3 associated with worse event free survival [329]
Breast cancer	46 % expressed high to moderate levels (3, 2+) of nuclear pY-STAT3, 23 % low levels (+1), and 31 % no detectable pY-STAT3 [330]	pY-STAT3 higher in invasive carcinoma (52 %) than in non-neoplastic tissue (27.8 %, $p < 0.001$). pY-STAT3 lower in patients showing complete pathologic response, suggesting that higher levels of activated STAT3 made tumors less responsive to the treatment [331]
Lung cancer (non-small-cell)	(64.6 %) pY-STAT3 + ve, in carcinoma tissue vs. 37.5 % in normal tissue (p = 0.001) [332]	78.8 % lymph node metastasis + ve patients pY-STAT3 + ve ($p = 0.009$) [332]. High STAT3/pY-STAT3 strong predictor of poor prognosis [333]
Endometrial cancer	64.9 % pY-STAT3 + ve (Scores 2 and 3) compared to normal endometrial tissues [334]	High pY-STAT3 (Scores 2 and 3) detected in 11.8 % of grade I, 25.8 % of grade II, and 27.3 % of grade III patients [334]
Cervical cancer	10.8 % grade II and 10.5 % grade III patients pY-STAT3 + ve [334]	56.8 % patients + ve for pY-STAT3 which also correlated with lymph node metastasis, lymph vascular space invasion, and large tumor diameter (>4 cm). pY-STAT3 + ve indicative of a poor OS (p = 0.006) and DFS (p = 0.010) [335]
Clear cell renal cell carcinoma (ccRCC)	76.3 % RCC tissues were nuclear pY-STAT3 + ve [336]	High CD44 correlates with high pY-STAT3 (r = 0.4013, p = 0.0004), high tumor grade (p < 0.001), large tumor size (p = 0.009) and advanced T stage (p = 0.004). CD44-high/pY-STAT3-high had poor survival vs. CD44-low/pY-STAT3-low (p = 0.024) [336]
Hepatocellular carcinoma (HCC)	100 % HCC nuclear pY-STAT3 + ve, intense, moderate and weak staining in 28.5, 28.5 and 43 %, vs. weak nuclear pY-STAT3 in 40 % of normal liver. Intense/moderate STAT3 in 89 % of HCC vs. normal liver samples [337]	49.3 % HCC, vs. 5.8 % of adjacent non-tumor liver (p < 0.001) + ve for pY-STAT3 which correlated with intratumour MVD (p = 0.002) and was a predictor of OS (p = 0.036) [338]

Cholangiocarcinoma (CCA)	44 % of CCA tissues STAT3 positive [339]	STAT3 and STAT5b associated with non-papillary, poorly differentiated CCA ($p=0.032$ and $p=0.001$); STAT3 associated with shorter survival ($p<0.001$) [339]
Colorectal cancer	62 % pY-STAT3 + ve; 18 % high expression (pY-STAT3 high), 34 % low-level expression (pY-STAT3-low) [340]	pY-STAT3 associated to higher colorectal cancer-specific mortality [log-rank p = 0.0020; univariate HR (pY-STAT3-high vs. pY-STAT3-ve): 1.85, 95 % confidence interval (CI) 1.30–2.63, ptrend = 0.0005; multivariate HR: 1.61, 95 % CI (1.11–2.34), ptrend = 0.015) [340]
Ovarian carcinoma (OC)	74 % nuclear pY-STAT3 + ve [341]	pY-STAT3 increased in aggressive, high-grade vs. low-grade, indolent carcinomas (p<0.005) [342]
Pancreatic adenocarcinoma (PAC)	70.4 % PAC pY-STAT3 + ve compared to none in normal pancreas [343]	pY-STAT3, a risk factor for prognosis, correlated to tumor size, TNM staging and lymphatic metastasis [343]
Head and neck squamous cell carcinoma (HNSCC)	75 % HNSCC tumors increased pY-STAT3 + vity vs. normal mucosa [344]	pY-STAT3 levels correlated to presence of lymph node metastasis (p<0.0001) [345] in HNSCC and decreased survival in oral and tongue tumors [346, 347]
Glioblastoma (GBM)	55.6 % astrocytomas (AA) and 56.4 %, GBMs were pY-STAT3 + ve [348]	40 % of Gliomas pY-STAT3 + ve with 27 %, 29 %, 57 % and 66 % + vity in Grade I, II, III and IV gliomas, respectively [350]
	50 % of AA and 51 % of GBM pY-STAT3 + ve [226, 346, 348–351]	
Extramammary Paget disease (EMPD)	91.6 % Paget cells pY-STAT3 + ve [351]	Strong nuclear pY-STAT3 staining in invasive EMPD [351]
Papillary thyroid cancer (PTC)	56.7 % of PTC vs. 10.9 % of adjacent normal thyroid tissues were pY-STAT3 + ve [352]	Nuclear pY-STAT3 positively correlated with presence of ETE and LNM, and higher TNM stage (p < 0.05) [352]

signaling drives gene expression promoting cell growth and resistance to apoptosis, persistent activation of STAT3 is thought to confer resistance to drug mediated apoptosis [19]. Numerous studies show that hyper-activated STAT3 signaling plays a significant role in chemotherapy resistance. Accordingly, the inhibition of activated STAT3 signaling appeared to sensitize resistant tumor cells to the cytotoxic agents [20]. STAT3 is also emerging as a major contributor to adaptive resistance to targeted drug therapy. Notably, it has been demonstrated that STAT3 activation via a positive feedback mechanism underpins frequently observed drug resistance in many oncogene addicted tumor cells. Similarly, inhibition of STAT3 reversed drug resistance to RTK targeting. Taken together, these findings support targeting STAT3 to overcome resistance to cancer therapy [17, 21].

There is an overwhelming amount of clinical and preclinical data in solid and hematological cancers supporting STAT3 as a pharmacological target, which has prompted substantial efforts to develop STAT3 inhibitors. Currently, there are a number of STAT3 inhibitors in clinical trials and many more in active development, as will be discussed later in this chapter. Here we provide an update on efforts to develop inhibitors of STAT3 to treat various cancers and will discuss the strategies involved in targeting STAT3 and the advantages and pitfalls of each approach.

5.3 Strategies for STAT3 Inhibition

The STAT3 signaling cascade provides many opportunities to manipulate its activity, because each step in the activation process can serve as a potential target. In order to pharmacologically modulate STAT3 activity, it is important to understand how each step contributes to the transcriptional function of STAT3, as this information forms a basis for target identification and design of specific inhibitors (Fig. 5.1).

5.3.1 Structure and Biochemical Properties of STAT3

The initial steps in STAT3 activation are triggered by tyrosine phosphorylation events that drive key protein-protein interactions, which are necessary for signal transduction from the plasma membrane to the nucleus [22]. STAT signaling initiated by peptide hormones generally occurs through 3 types of receptors—receptor kinases, receptor-linked kinases, or G–coupled receptors [23, 24]. Peptide ligand binding stimulates cytoplasmic receptor-associated kinase activity leading to phosphorylation of receptors at key tyrosine residues. Phosphorylated tyrosine residues on the receptors act as anchors that recruit STAT3 proteins via their SH2 domains [25]. STAT3 is phosphorylated at Y705 and subsequently dimerizes in a tail-tail conformation.

Migration from the cytoplasm into the nucleus is required for STATs to transduce signals and regulate gene expression in response to extracellular stimuli. It has been noted that once dimerized in a tail-to-tail configuration, STATs rapidly accumulate

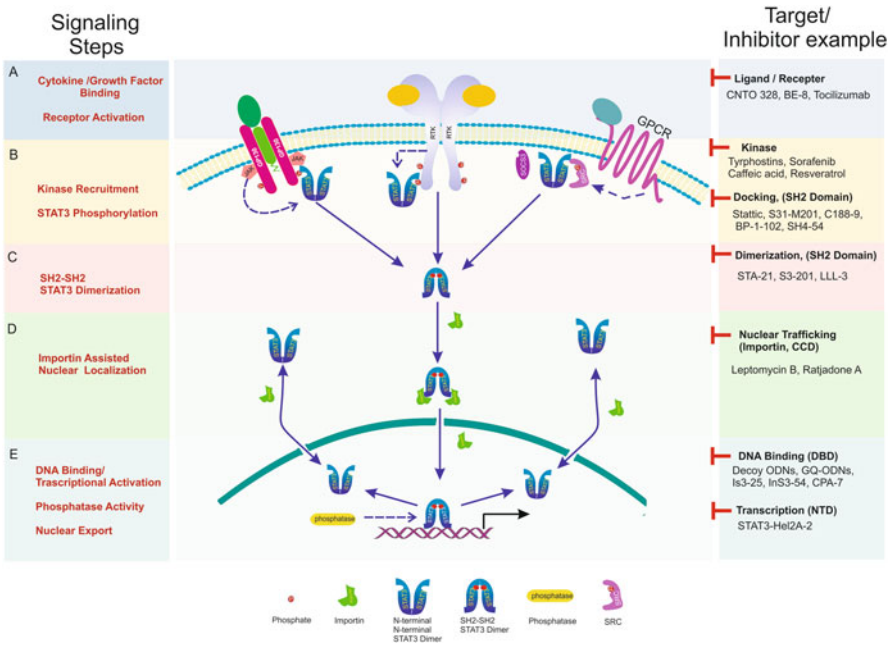

Fig. 5.1 Strategies for targeting STAT3 signaling. STAT3 signaling cascade is triggered by phosphorylation. (**a**) Upstream events including ligand binding, receptor activation or kinase activity can be blocked to prevent STAT3 phosphorylation. (**b**) Blocking STAT3 recruitment onto receptors inhibits phosphorylation of STAT3 at Y705 and consequently SH2-SH2 dimerization. (**c**) Inhibitors that disrupt the SH2-SH2 dimer block the transcriptional activity of STAT3. (**d**) Nuclear localization can be blocked by targeting importins or importin binding sites on STAT3. (**e**) The DNA binding domain can be targeted to inhibit STAT3 DNA binding, consequently transcriptional activity

in the nucleus. Though initially thought to be dependent on tail-to-tail dimerization of STAT3, subsequent studies now suggest that STAT3 is constitutively shuttled between the cytoplasm and nucleus independent of phosphorylation [26]. Studies show that rather than a passive process dependent on diffusion, nuclear translocation of STAT3 is an active process. Indeed, the nuclear import and export of STAT3 as well as other STATs is facilitated by a group of proteins belonging to the karyopherin-B family called importins [27]. Available data shows that importin α3, α5, α6, and α7 are involved in the nuclear translocation of STAT3. Importin α3 and α6 are linked to translocation of unphosphorylated STAT3 while α5 and α7 are required for pY-STAT3 nuclear import [28]. All importins involved in STAT3 trafficking appear to utilize a NLS located within the coiled-coiled domain of STAT3 [29, 30]. Once localized in the nucleus, STAT3 binds to specific DNA elements via its DNA binding domain (DBD), whereby it engages the transcriptional machinery by recruiting a number of coactivators and chromatin remodelers, such as cAMP response element binding protein/p300 (CBP/p300) complex and steroid receptor coactivator 1 [31, 32].

Fig. 5.2 Domains structure of STAT3. STAT3 has 6 domains with specific biochemical functions. NH2-terminal domain (NTD), coil coiled domain (CCD), DNA binding domain (DBD), linker domain (LD), SRC homology domain (SH2), and transactivation domain (TAD)

5.3.2 Functional Domains of STAT3

STAT3 is composed of an N-terminal domain (NTD), a coiled-coil domain (CCD), a DNA-binding domain (DBD), a linker domain (LD), an SH2 domain, and a C-terminal domain. The structure of the core fragment of STAT3, which includes the CCD, DBD, LD and SH2 showed that each domain of STAT3 has a distinct function and is essential for the signal transduction and transcriptional activity of STAT3 (Fig. 5.2).

STAT3 has no enzymatic activity that would make it amenable to small-molecule intervention; rather, its mode of action depends on protein-protein interactions (PPI) and protein-DNA interactions. Thus, strategies for targeting STAT3 mainly rely on the ability to disrupt these interactions. Although the prevailing dogma is that PPI interfaces generally lack special topological features amenable to small molecule inhibition, STAT3, nonetheless, has proven to be a compelling protein to target using small molecules. The available X-ray crystallographic data of both the monomer and dimerized STAT3 bound to DNA have been instrumental in revealing physical chemical properties of phosphotyrosyl (pY) peptide binding, as well as DNA recognition that have laid the foundation for the development of many STAT3 inhibitors by rational design.

5.3.3 Inhibitors Acting Upstream of STAT3 Activation

There is a strong correlation between the phosphorylation status of STAT3 at Y705 with tumor initiation and progression (Table 5.1), yet the reason for dysregulated STAT3 signaling is only rarely due to mutations in the signaling molecule itself. Although the reason for abnormal STAT3 signaling in cancer is not fully understood, most instances of hyper-phosphorylated STAT3 observed in cancer are mediated by receptor tyrosine kinases (RTK), for example EGFR, or non-receptor tyrosine kinases, such as JAK and SRC, more specifically, by unchecked intrinsic tyrosine kinase activity of RTK, over expression of RTK, or persistent stimulation of RTK or tyrosine kinase-associated receptors by cytokines and growth factors [33–35]. As such, intense efforts have focused on inhibiting events upstream of STAT3 that drive STAT3 phosphorylation [36, 37].

There are several therapeutic strategies used to block upstream activation of STAT3, One involves targeting the tyrosine kinase enzymatic activity of specific receptors or associated kinases using small molecule inhibitors of RTKs, JAK2 and SRC kinases. Another strategy involves disruption of protein-protein interactions necessary for receptor mediated signal transmission across the plasma membrane. The later strategy has been achieved in several ways including blocking cytokine binding to the extracellular portions of the receptors, and disruption of receptor oligomerization. These strategies primarily involve blocking cytokine or growth factor activation of cognate receptors with the use of monoclonal antibody-based inhibitors that target either the ligand or critical sites on extracellular portion of receptors. Another strategy in this category involves the use of an aptamer, a short peptide portion derived from a random peptide library integrated into the thioredoxin scaffold protein, which specifically binds to the intracellular domain of the EGF receptor blocking the recruitment of substrate to the receptor [38].

All the above approaches have shown success in targeting STAT3 activation leading to induction of cancer cell death (Table 5.2) and have demonstrated significant clinical efficacy. However, acquired resistance against tyrosine kinase inhibitors remains a significant challenge [21, 39]. Besides, there have been inhibitors (e.g. OPB-31121) that showed very low nanomolar level IC_{50}s in pre-clinical settings, but eventually failed to show efficacy in clinical trials. Moreover, due to the pleiotropic nature of cytokines such as IL-6 there are always concerns of potential toxicity due to off-target effects [40, 41]. Recent studies now provide a rationale for direct targeting of STAT3 by itself or in combination with other therapeutic approaches for combating drug resistance in cancer treatment [21, 42].

5.3.4 Inhibitors Targeting the STAT3 SH2 Domain

The SH2 domain presents a defined and well-characterized targeting site with suitable topological features amenable to small molecule intervention and has proven to be tractable for small molecule inhibition of STAT3. Additionally, the SH2 domain of STATs have a dual function where they act as receptor recruitment modules as well as dimerization domains necessary for high-affinity STAT DNA-binding. The SH2 domain has become the favored target for platforms geared towards rational design, as well as *in vitro* and cell based screens for several reasons, including: (i) the pY-peptide binding site provides a suitable druggable site for *in silico* docking screens, (ii) pY705 phosphorylation is a convenient surrogate for STAT3 activation making it amenable to very robust cell based high-throughput screening (HTS) assays, and (iii) the SH2 domain binds short cognate pY-peptide ligands and, thus, provides a platform for competitive inhibition bind assays such as SPR and fluorescence polarization that have routinely been used to directly screen for competitive inhibitors of pY-peptide binding. The greatest effort at designing STAT3 inhibitors has been directed at the SH2 domain, as summarized below (Table 5.3).

Table 5.2 STAT3 upstream inhibitors in development

Inhibitors	Type	Description	Blocks	IC50 STAT3 inhibition (assay)	IC50 cell growth inhibition, cells	Pre-clinical animal models	Ref
PD153035	SM	EGFR TK inhibitor	pY, DM, NT, DB, GT	~100 nM, EGF-stimulated pSTAT3 MDA-MB-468	0.2–2.5 μM, HER2/Neu +ve cancer cells	80 mg/kg, IP, A431 xenografts	[353–355]
Oleanolic Acid	SM	JAK2, SRC, EGFR, STAT3 inhibitor	pY, DM, NT, DB, GT	~20^{E} μM, constitutive pSTAT3, U373	~20^{E} μM, U373	NA	[356, 357]
Brevilin A	SM	JAK inhibitor	pY, DM, NT, DB, GT	10.6 μM, constitutive pSTAT3 A549R	>20 μM, A549R	NA	[358]
Tofacitinib (CP-690,550)	SM	JAK3 inhibitor, inhibits pSTAT1/3/4/5/6	pY, DM, NT, DB, GT	0.07 μM, constitutive pSTAT3, JAK2V617F/FDCP-EpoR	0.07 μM, JAK2V617F-transduced FDCP-EpoR	NA	[359–363]
Sorafenib	SM	JAK2/STAT3 inhibitor	pY, DM, NT, DB, GT	<3 μM, constitutive pSTAT3, U87	1–2 μM glioblastoma cells	100 mg/kg, IP, U87-Luc xenografts	[364]
AZD1480	SM	JAK1/2 inhibitor, inhibits pSTAT1/3/5/6	pY, DM, NT, DB, GT	0.35 μM, nuclear translocation	0.36–5.37 μM, Ewing sarcoma cells	30–50 mg/kg, OG, DU145, MDA-MB-468, MDAH2774 xenografts	[41, 269, 272, 365–370]
Atiprimod	SM	JAK2/3 inhibitor, inhibits pSTAT3/5	pY, DM, NT, DB, GT	~$4–8^{E}$ μM, constitutive pSTAT3, U266-B1	0.5–1.5 μM, HEPG2	50 mg/kg/2d, IV, OPMI xenografts	[371–375]
Auranofin	SM	JAK1/STAT3 inhibitor	pY, DM, NT, DB, GT	<1^{E} μM, IL6-stimulated pSTAT3, HepG2	0.05 μM, U266	7 mg/kg/d, IP, IM-resistant Bcr-Abl-T315I xenografts	[376–379]
Sanguinarine	SM	JAK2, Src, STAT3 inhibitor	pY, DM, NT, DB, GT	~$1–2^{E}$ μM, IL6-stimulated pSTAT3, DU145	1.3/1.6 μM, A17, MDA-MB-231	2.5/5 mg/kg/2d, 7d, IG, GTL-16 xenografts	[380–382]
Cucurbitacin I (JSI-124)	SM	JAK2/STAT3 inhibitor	pY, DM, NT, DB, GT	7.5/0.5 μM, constitutive pSTAT3 MD-MB-468, A549	2.9–10.5 μM, cells from CLL patients	1 mg/kg/d, 15d, IP, HRas/3 T3/A549/MDA-MB-468/Calu-1 xenografts	[383, 384]

Cucurbitacins B	SM	JAK2/STAT3 inhibitor	pY, DM, NT, DB, GT	Low nM–high μM, constitutive pSTAT3, PANC-1, K-562	~0.5 nM, leukemia, HCC, breast cancer cells	1 mg/kg, IP, Panc-1 xenografts	[385–392]
Cucurbitacin E	SM	JAK2/STAT3 inhibitor	pY, DM, NT, DB, GT	1.4 μM, constitutive pSTAT3, MD-MB-468	~10 nM, HUVEC, PC3)	3 mg/kg, IL, PC3 xenografts	[393–395]
Celastrol	SM	JAK2, SRC, STAT3 inhibitor	pY, DM, NT, DB, GT	~2.5[E] μM, constitutive pSTAT3, C3A	1.1–3 μM, H1650/ H1975/H2228	1–2 mg/kg, IP, PLC/PRF5 cells	[396–398]
Emodin	SM	JAK2/STAT3 inhibitor	pY, DM, NT, DB, GT	25–50[E] μM, constitutive pSTAT3, HepG2	35.8–46 μM, FaDu/ HSC-3	20–50 mg/kg, IP, HCCLM3_Luc2	[399]
Dasatinib	SM	SRC/ABL / KITSTAT3 inhibitor	DM, NT, DB, GT	Does not block pSTAT3	8 nM, MTT CME -1, cells 13 nM, MTT SYO-1 cells	10 mg/kg, IP, SYO-1 xenografts	[400, 401]
Caffeic Acid (CA) CADPE	SM	JAK2, SRC, STAT3 inhibitor	pY, DM, NT, DB, GT	CA: 70–100 μM CADPE 15–30 μM, hypoxia-induced pSTAT3, Caki-1	~50[E] μM, Huh-7	5 mg/kg, IP, Caki-I xenografts	[402–404]
AG490	SM	JAK3	pY, DM, NT, DB, GT	50–100 μM, constitutive pSTAT3, renal/colon cancer cells	40 μM HT29, cells 50 μM Caki-1 cells	5 mg/kg, IC, EGFRvIII cell intracranial xenografts	[405, 406]
WP1066	SM	JAK2/STAT3 inhibitor	pY, DM, NT, DB, GT	1–2[E] μM, constitutive pSTAT3, HEL	2.5 μM Caki-1 cells 2.3 μM, HEL cells	40 mg/kg, OG, Caki-1, GL26 xenografts	[151, 288, 407]
TG101209	SM	JAK2/STAT3 inhibitor	pY, DM, NT, DB, GT	~2–5[E] μM, constitutive pSTAT3, HEL	2–5 μM myeloma cells	100 mg/kg, PO, Ba/F3 V617F–GFP xenografts	[408, 409]
FLL32	SM	JAK2, STAT3 inhibitor, binds to STAT3 SH2 domain	pY, DM, NT, DB, GT	2.5–5 μM, constitutive pSTAT3, MDA-MB-231, PANC-1	0.11–0.64 μM Pancreatic 0.14–0.60 μM Breast cancer cells	50 mg/kg, IP MDA-MB-231 xenografts	[410, 411]

(continued)

Table 5.2 (continued)

Inhibitors	Type	Description	Blocks	IC50 STAT3 inhibition (assay)	IC50 cell growth inhibition, cells	Pre-clinical animal models	Ref
Avicin D	SM	JAK1/2/3 and STAT3 inhibitor, acts through SHP1 upregulation too	pY, DM, NT, DB, GT	~1[E] μM, pSTAT3, U266 cells	5[E] μM, U266 cells, 0.32 μM, Jurkut cells	NA	[412, 413]
E738	SM	SRC and JAK inhibitor	pY, DM, NT, DB, GT	1–5 μM, constitutive pSTAT3, PaCa cells	0.68–2.2 μM, pancreatic cancer cell	NA	[414]
MLS-2384	SM	SRC and JAK inhibitor	pY, DM, NT, DB, GT	1–2.5 μM, constitutive pSTAT3, DU145, MDA-MB-468, A2058, A549	2 μM, DU145, MDA-MB-468, A2058, A549	25 mg/kg, PO, melanoma	[415]
CYT387 (Momelotinib)	SM	JAK2 Inhibitor	pY, DM, NT, DB, GT	NA	1.5 μM, Ba/F3-JAK2V617F, HEL cells	15 mg/kg, PO, HEY cell xenografts	[416, 417]
Ergosterol peroxide (EP)	SM	JAK, SRC, STAT3 inhibitor	pY, DM, NT, DB, GT	8–12.5 μM, constitutive pSTAT3, U266, SCC4, DU145. MDA-MB-231	NA	100 mg/kg, IP, U266 cells xenografts	[418]
PP2	SM	SRC inhibitor/ STAT3		μM, LIF-activated pSTAT3	0.2–3 μM, melanoma cell		[419, 420]
Ponatinib	SM	FGFR4 inhibitor	pY, DM, NT, DB, GT	0.2–0.8 μM, constitutive pSTAT3, RH4/RH5/RH41	0.2–0.9 μM, rhabdomyosarcoma	30 mg/kg, PO, RMS722 xenograft	[421]
Benzyl isothiocyanate	SM	Inhibits SRC recruitment and hence STAT3	pY, DM, NT, DB, GT	5–10 μM, constitutive pSTAT3, PaCa cells	8–10 μM, PANC-1, BxPC3	12 μM, PO, BxPC3 xenografts	[308, 422]
CNTO-328 (Siltuximab)	Ab	MAb to IL6 JAK/STAT inhibitor	pY, DM, NT, DB, GT	0.06–0.6 μM, IL6-activated pSTAT3 HKOV3	No effect on viability, H1650 cells	10 mg/kg, 36d, IP, H1650 xenografts, NSCLC PDX	[423, 424]

Toclizimab	Ab	MAb to IL-6R JAK/STAT inhibitor	pY, DM, NT, DB, GT	NA	0.09–0.27 nM Ba/F3 cells	20 mg/kg, IP, HepG2 xenografts	[425, 426]
Cetuximab	Ab	MAb to EGFR	pY, DM, NT, DB, GT	NA	NA	0.2–0.5 mg/mice, IP, A431 xenografts	[427]
KDI1/KDI3/ KDI4	AP	EGFR intracellular domain amino acids 688–821, interacting aptamers	pY, DM, DB, NT, GT	NA	NA	NA	[38]
Xanthohumol	SM	EGFR inhibitor	Py, DM, NT, DB, GT	~5–10^{E} μM, pSTAT3, PANC-1	~10^{E} μM, pancreatic cancer cells	25 mg/kg, IP, 27d, PANC-1 xenografts	[428, 429]

Note: Information for Pre-clinical animal models consist of Dose, route of administration, duration (if available) and animal model used. Abbreviations: *E* estimated from descriptive data on inhibition, from corresponding reference, *NA* not available, *Ab* antibody, *SM* small molecule, *pY* STAT3 phosphorylation at Tyr-705, *DM* dimerization, *NT* nuclear translocation, *DB* DNA-binding, *GT* gene transcription, *IG* intra-gastrical, *TK* tyrosine kinase, *IC* intra-cranial, *PO* per Os (by mouth), *IP* intra-Peritoneal, *MAb* monoclonal Ab, *AP* aptamer

Table 5.3 Direct STAT3 inhibitors in development

Inhibitors	Type	Description	Blocks	IC50 STAT3 inhibition (assay)	Kd (STAT3 binding)	IC50 Cell growth inhibition, cells	Pre-clinical animal models	Ref
Inhibitors targeting STAT3 SH2 domain								
PY*LKTK(-mts)	P	Phosphopeptide derived from STAT3 (vicinity of Y-705)	DM, DB, GT	235 μM, STAT3-hSIE binding EMSA	NA	>500 μM, NIH 3T3/vSrc colony	NA	[45]
PY*L	P	Minimal STAT3 p-peptide required for STAT3 inhibition	DM, DB, GT	182 μM, STAT3-hSIE binding EMSA	NA	NA	NA	[45]
ISS 610	PM	Peptidomimetics developed replacing P by 4-cyanobenzoate	DM, DB, GT	42 μM, STAT3-hSIE binding EMSA	NA	>1000 μM, NIH 3T3/vSrc colony	NA	[45, 49, 430, 431]
PDP/ Phosphododeca peptide(-mts)	P	p-peptides Y1068 + p/LPVPE(pY) INQSVP & Y992 + p/TDSNF(pY) RALMDE from STAT3 binding sequence of EGFR	pY, DM, DB, GT	350–750 μM, IL6/ EGFR stimulated STAT3 binding EMSA, HepG2/ UM-SCC-23	NA	~750 μM, A431	NA	[25
Ac-Y*LPQTV	P	p-peptide from STAT3 binding sequence of gp130	DM, DB, GT	0.15 μM, STAT3-hSIE binding EMSA	NA	NA	NA	[44, 52]
Hydrocinnamoyl-Tyr(PO3H2)-L-cis-3,4-methanoPQ-NHBn	P	p-peptide from STAT binding sequence of gp130	DM, DB, GT	0.125 μM, STAT3-hSIE binding EMSA	NA	NA	NA	[44, 52]
CJ-1383	PM	PM developed from Ac-Y*LPQTV	pY, DM, DB, GT	~10 μM[E], pSTAT3, MDA-MB-468	0.95 μM, STAT3 binding, FP	3.6–11.2 μM, MDA-MB-468, MDA-MB-231	NA	[433]
PM-73G	PM	Mimetic developed from Ac-Y*LPQTV	pY, DM, DB, GT	0.1–0.5 μM, pSTAT3-inhibition, cancer cells	NA	≥30 μM, MDA-MB-468, A549	5 mM, IT, MDA-MB-468 xenografts	[53, 54]

APT_{STAT3}-9R	AP	Tryptophan zipper scaffold attached to a STAT3-binding peptide and cell-penetrating motif	pY, DM, DB, GT	NA	231 nM STAT3 binding, SPR	10–20 μM, A549	8 mg/kg, IT, A549 xenografts	[55]
Recombinant STAT3 inhibitory peptide aptamer (rS3-PA)	AP	Recognizes dimerization domain and inhibits STAT3 function	Py, DM, NT, DB, GT	NA	1–4[E] μM, ligand-stimulated pSTAT3, HepG2	1–4[E] μM, various cancer cell lines	7.5 mg/kg, IV 15d, Tu9648 xenografts	[56–59]
DD1/DD2/DD3	AP	STAT3-DD (amino acid positions 655–755) binding aptamers	Py, DM, NT, DB, GT	NA	NA	NA	NA	[163]
		DD-1: PPLVCIRSWCPLMVPHSA DLGPASQWLCHRVASIALLPRYSS						
		DD-2: VGWTWMSVLVCCDGSGLV PEGPVVVQAGGAVPISGSVALMTD						
		DD-3: SPISIPIGFVVRHCALHMAV GPLSWPARVSGYSFALEVLTNF						
Dipicolylamine copper complexes 1,2,3	PM	Phosphate binders e.g. Lewis acidic metal–picolylamine complexes acting as SH2-proteomimimetics, disrupt phosphopeptide–STAT3 complexes	DM, DB, GT	15–128 μM, F*pYLPQTV gp130p-STAT3 binding, FP	8–100 μM, pY-LKTK STAT3p binding, IGT	77/11/100 μM, DU145	NA	[60]
						73/11/100 μM, OCI-AML2		
						115/5/56 μM, MDA-MB-468		
S3I-M2001	SM	Developed from ISS-610 peptidomimetic	pY, DM, DB, GT	79 μM, STAT3-hSIE binding EMSA	NA	~100 μM	5–20 mg/kg, IV, MDA-MB-231 xenografts	[50]
STA-21	SM	Structure-based virtual screening and screening for STAT3-luciferase yielded STA-21	DM, NT, DB, GT	20–30[E] μM, STAT3-hSIE binding EMSA	NA	12.2/18.7 μM, DU145, PC3,	NA	[61–63]

(continued)

Table 5.3 (continued)

Inhibitors	Type	Description	Blocks	IC50 STAT3 inhibition (assay)	Kd (STAT3 binding)	IC50 Cell growth inhibition, cells	Pre-clinical animal models	Ref
LLL-3	SM	Structural analogue of STA-21	DM, NT, DB, GT	~40[E] μM, STAT3-hSIE binding EMSA, SJSA cell	NA	6.3 μM/K562, 10–20 μM, U87, U251, U373; 11.3 μM, DU145	50 mg/kg, IT, intra-cranial U87 xenografts	[64, 434, 435]
LLL-12	SM	Derived by replacing acetyl group of LLL-3 with sulfonamide to	pY, DM, NT, DB, GT	0.16–3.09 μM, pSTAT3, various cancer cells	NA	0.3–0.8 μM, U2Os, SAOS2, SJSA 0.97–3.1 μM, MDA-M231, SKBR3,		[65–73]
Stattic	SM	Hit from HT fluorescence polarization screen for binding to STAT3 SH2 domain	pY, DM, DB, GT	5.1 ±0.8 μM, gp130-derived p-peptide binding to Stat3 SH2 domain	NA	0.43–2.6 μM, C, MM, G cells 4.3–5.6 μM, Nasopharyngeal cancer cells	50 mg/kg, PO (UM-SCC-17B orthotopic xenografts	[74–76]
S31-201/NSC 74859	SM	Structure based virtual screening of NCI chemical libraries with computer model of SH2-pYpeptide interaction	pY, DM, NT, DB, GT	86 μM, STAT3-hSIE binding EMSA	NA	300 μM, LNCaP	5 mg/kg, once/2day, 16d, IV, MDA-MB-231 xenografts	[81, 82]
S31-201.1066/ SF-1066	SM	Resulted from molecular modeling of the pTyr–SH2 interaction combined with in silico structural analysis of S3I-201	pY, DM, NT, DB, GT	35 μM, STAT3-hSIE binding EMSA 23μM, pY-peptide STAT3 binding, SPR	2.7 μM, pY-peptide STAT3 binding, SPR	35/48/37 μM, NIH3T3/v-Src, Panc-1, MDA-MB-231	5 mg/kg, once/2day, 17d, IV, MDA-MB-231 xenografts	[83, 84]

BP-1-102/17o	SM	Screening of salicyclic acid-containing STAT3 SH2-inhibitors	pY, DM, NT, DB, GT	6.8 μM, STAT3-hSIE binding EMSA	Ki 13 μM, pY-peptide STAT3 binding, SPR	10.9–22.7 μM, MDA-MB-468, DU145, JJN3	1/3 mg/kg, once/2day, 15d, IV or PO, MDA-MB-231,A549 xenografts	[87, 88]
SH4-54	SM	Screening BP-1-102 analogues for anti-STAT3 and anti-tumor functions	pY, DM, NT, DB, GT	4.7 μM, STAT3-hSIE binding EMSA	Kd 0.3–2.4 μM, direct binding to STAT3, SPR	0.07–0.2 μM, 25EF, 67EF, 73E, 84EF, and 127EF 1.9–9.6 μM, vSrc, 231, DU145, Panc-1, U251MG, U87MG, U373MG, SF295	10 mg/kg, 15d, IP/PO, BT73 xenografts	[91, 92]
SH5-07	SM	Screening BP-1-102 analogues for anti-STAT3 and anti-tumor functions	pY, DM, NT, DB, GT	3.9 μM, STAT3-hSIE binding EMSA	Kd 2.4 μM, direct binding to STAT3, SPR	1.1–10.3 μM, growth, vSrc, 231, DU145, Panc-1, U251MG, U87MG, U373MG, SF295	5 mg/kg, 15d, IP or 3 mg/kg, OD, PO, U251/ MDA-MB-231 xenografts	[92]
S3I-V3-31/32/33/34	SM	GOLD and 3D-pharmacophore analysis with known STAT3 inhibitors and subsequent wet lab screening	pY, DM, NT, DB, GT	27–84 μM STAT3-hSIE binding EMSA	0.8–12 μM pY-peptide STAT3 binding, SPR	41–80 μM, various cancers	NA	[89]
C188	SM	Virtual ligand screening, by docking 920,000 small molecules into the pY-binding pocket of the STAT3 SH2 domain and further high-throughput screens	pY, DM, NT, DB, GT	7.5–20 μM, pY-peptide STAT3 binding, SPR 16.2 μM, G-CSF-stimulated pSTAT3, Kasumi-1, Luminex	Ki 37.3 nM, pY-peptide STAT3 binding, SPR	0.7–3.9 μM, ED50, apoptosis MDA-MB-468/ MDA-MB-231,	12.5 mg/kg, 14d, IP, chemoresistant PDX models 50 mg/kg, 14d, IP, UM-SCC-17B xenografts	[93, 94, 96]

(continued)

Table 5.3 (continued)

Inhibitors	Type	Description	Blocks	IC50 STAT3 inhibition (assay)	Kd (STAT3 binding)	IC50 Cell growth inhibition, cells	Pre-clinical animal models	Ref
C188-9	SM	2D similarity screening using scaffold of C188, and 3D pharmacophore analysis in a hit-to-lead program identified C188-9	pY, DM, NT, DB, GT	2.5 μM, pY-peptide STAT3 binding, SPR	Ki 12.4 nM, pY-peptide STAT3 binding, SPR;	0.7–14.8 μM, growth, HNSCC cells, MTT	100 mg/kg, 14d, IP, UM-SCC-17B xenografts	[95–98, 231]
				3.7 μM, G-CSF-stimulated pSTAT3, Kasumi-1, Luminex	Kd 4.7 nM, STAT3-binding, MST	0.8–25 μM, ED50, apoptosis in primary AML		
Cryptotanshinone	SM	Screen of natural compound library with HT STAT-luciferase screen	pY, DM, NT, DB, GT	4.6 μM, STAT3-luciferase	NA	7 μM, DU145; 5.8–15.1 μM, growth AML, colon cancer, breast cancer cells	NA	[100–105]
STX-0119	SM	In-silico docking and screening through biochemical methods	DM, NT, DB, GT	4.6 μM, STAT3-luciferase	NA	1.4–18.3 μM, growth, hematological cancer cells,	40 mg/kg, 5d, IP, SCC-3, GBM-SC xenografts	[110–113]
C48	SM	Hit from VLS screen using entire STAT3 SH2 domain	pY, DM, DB, GT	3–10 μM, OSM-induced STAT3-dependent luciferase activity	NA	10–20 μM, apoptosis induction MDA-MB-468	200 mg/kg, IP, MDA-MB-468 xenografts in nude mice	[79]
							100–200 mg/kg, syngeneic C3L5 mouse model	

Piperlongumine	SM	High throughput screen of a repositioning library for inhibitor of STAT3 nuclear translocation	pY, DM, NT, DB, GT	0.9–2.7 μM, IL6/ sIL6R-induced pSTAT3, luminex	Ki 68 nM, STAT3-pY-peptide binding, SPR	0.2–4.6 μM, growth, breast cancer cell, MTT	15 mg/kg, 21d, MDA-MB-468 xenografts	[115]
OPB-31121	SM	Very potent SH2 domain inhibitor with activity in low nM range	pY, DM, NT, DB, GT	3–10 μM, OSM-induced STAT3-dependent luciferase activity	Kd 10 nM STAT3 binding	Low nM range IC50, STAT-addictive oncokinases (SAO) +ve cells from various cancers	100–300 mg/kg, IP, xenografts in nude mice. Reduction of tumor by 79–95 %	[273–278]
Withacnistin	SM	Stops STAT3/5 recruitment to EGFR and gp130. Doesn't affect upstream kinases, most probably targeting SH2	pY, DM, NT, DB, GT	1.4 μM, constitutive pSTAT3 MD-MB-468	NA	1–3^{E} μM, colony MD-MB-468	20 mg/Kg, IP, FVB/N--Tg(MMTV neu)202Mul/J) mice; 0.5 mg/kg A549.v -Src/3 T3 xenografts	[395, 436]
XZH-5	SM	Peptide mimicking small molecule design from known STAT3 inhibitor knowledge	pY, DM, NT, DB, GT	~20^{E} μM, constitutive pSTAT3, HCC cells	NA	15–50 μM, growth, breast, pancreatic, HCC, rhabdom yosarcoma, MTT	NA	[119–121]
T2, T3, Celecoxib	SM	FBDD using MLSD and drug repositioning screen	pY, DM, NT, DB, GT	~20–50^{E} μM, constitutive pSTAT3, HCT-116 cells	NA	9.7/10.1/43.3 μM, HCT-116, growth, MTT	NA	[123]

(continued)

Table 5.3 (continued)

Inhibitors	Type	Description	Blocks	IC50 STAT3 inhibition (assay)	Kd (STAT3 binding)	IC50 Cell growth inhibition, cells	Pre-clinical animal models	Ref
HJC0123	SM	FBDD based on structure of niclosamide and other STAT3 inhibitors	pY, DM, NT, DB, GT	~1[E] μM, constitutive pSTAT3, MDA-MB-231 cells	NA	0.1–1.2 μM, breast and pancreatic cancer cells, growth, MTT	50 mg/kg, PO, MDA-MB-231 xenografts	[119, 125, 126]
Ly5	SM	FBDD. By inking naphthalene-5,8-dione-1-sulphoneamide fragment of LLL12 as binding moiety to pTyr705 of STAT3-SH2 domain	pY, DM, NT, DB, GT	~0.5–1.4 μM, stimulated STAT3, MDA-MB-231 cells	NA	0.5–1.39 μM U2OS/RD2, growth, MTT 0.32–0.48 μM, UW288-1, UW426, and DAOY	NA	[127, 437–439]
T40214/T40231	GQ-ODN	G-quartet oligonucleotide binds to STAT3 SH2 domain	pY, DB, GT	5 μM· STAT3 DNA-binding, prostate, breast, HNSCC cells	NA	NA	10 mg/kg, IP, NSCLC, HNSCC, prostate, breast xenografts	[144, 145, 147, 440–443]
Inhibitors targeting STAT3 DBD								
Decoy ODN	ODN	Sense: 5′ C*A*T*TTCCCGTTA*A*T*C 3′ AS: 5′ G*A*T*TTACGGGAA*A*T*G 3′, "*" denotes phosphorothioated sites)	NT, DB, GT	NA	NA	~12.5[E] μM, 1483 cell growth MTT 8 nM, HUVEC and 137 nM, HDMEC	25 μg, IT, 1483 xenografts	[133, 140, 292, 444, 445]
13410/13410A/SeqD	ODN	Oligonucleotide decoy, modification of consensus STAT3-binding sequence	NT, DB, GT	NA	NA	40–200 nM, apoptosis DU-145	NA	[137, 446]
CPA-7	SM	Platinum (IV) complexes	DB, DM, DB, GT	1.5 μM, DNA binding EMSA	NA	2.9–23.7 μM, GL26, SMA560, CNS1, IN859, U251, HF2303	5.5 mg/kg, tail-vein, GL26 xenografts	[149, 151]

IS3 295	SM	Platinum (IV) compound screened from NCI 2000 diversity set, non-competitive	DB, DM, DB, GT	1.4 μM, DNA binding EMSA	NA	<10 μM [E] (colony formation Src/ NIH3T3	NA	[149]
inS3-54	SM	Virtual screening for binding to DBD of STAT3	DB, DM, DB, GT	20 μM, DNA binding EMSA	NA	3.2–5.4 μM (MDA-MB-468, MDA-MB-231, A549, H1299	NA	[154]
inS3-54A18	SM	Activity-guided hit optimization and mechanistic characterization from inS3-54	DB, DM, DB, GT	11 μM, STAT3 dependent luciferase	NA	3.2–4.7 μM, MDA-MB-468, MDA-MB-231, A549, H1299	8 mg/kg, OG, A549 xenografts in nude mice	[150]
HO-3867	SM	Conjugation of a diarylidenyl-piperidone, DAP) backbone to N-hydroxypyrroline (-NOH) group	pY, DM, NT, DB, GT	<10 μM, constitutive pSTAT3, HO-3867	NA	3–5 μM, BRCA-1 mutated ovarian cancer cells	50/100 ppm, in feed, A2780 xenografts	[156–158, 447, 448]
Galiellalactone	SM	Fungal metabolite, co-valent modifier	DM, DB, GT	~4 μM[E], DNA binding DU145, EMSA	NA	3.4 μM, DU-145 cell growth, MTT	5 mg/kg, IP DU-145-Luc xenografts	[160–162]
DBD-1/DBD-1-9R	AP	STAT3-DBD (aa 322–483) binding aptamer, p-seq: PLTAVFWLIYVLAKALVTVC	DM, DB, GT	NA	NA	180–369 nM[E], U266	NA	[38, 163]
Inhibitors targeting STAT3 ND								
Hel2K-Pen/ ST3-HA2A	SM	Cell permeable analogs of the STAT3 second helix	DM, NT, DB, GT	NA	NA	0.7–3.5[E], Du145, LNCap, PC3	NA	[151, 288, 407]

Note: Information for Pre-clinical animal models consist of Dose, route of administration, duration (if available) and animal model used

Abbreviations: *E* estimated from descriptive data on inhibition, from corresponding reference, *NA* not available, *Ab* antibody, *SM* small molecule, *pY* STAT3 phosphorylation at Tyr-705, *DM* dimerization, *NT* nuclear translocation, *DB* DNA-binding, *GT* gene transcription, *IG* intra-gastrical, *TK* tyrosine kinase, *IC* intra-cranial, *PO* per Os (by mouth), *OG* oral gavage, *FP* fluorescence polarization. *IP* Intra-Peritoneal, *MAb* Monoclonal Ab, *P* peptide, *PM* peptidomimetic, *AP* aptamer, *GQ-ODN* G-quartet oligonucleotide, *ppm* parts per million

5.3.4.1 Peptides and Peptidomimetics

Elucidation of the crystal structure of STAT3β-STAT3β-DNA complex [43] and subsequent studies [25, 44–46] indicated that the SH2 domain facilitates binding to specific pY-peptide motifs within receptor complexes and mediates dimerization of two STAT3 monomers via reciprocal interaction between the SH2 of one monomer and pY-peptide motif, ^{702}AAPY*LKTKFI^{711}, on the other. Strategies to target STAT3 by identifying pY-peptide inhibitors of STAT3 SH2 binding to pY-peptide ligands have been pursued by several groups (Table 5.3) [47]. Turkson et al. showed that pY-peptides based on the sequence PY*LKTK surrounding Y705 within STAT3, inhibited STAT3 DNA binding (IC_{50} = 235 μM) and pulled down STAT3 from lysates of unstimulated cells [45]. Alongside the usual limitations of the peptide approaches, e.g. low cell permeability, instability, and the consequential low biological activities, the requirement for the phosphorylation on Tyr for the inhibitory activity presented another challenge to making this approach biologically useful. Covalently attaching a membrane-translocating sequence (mts) of hydrophobic amino acids (AAVLLPVLLAAP) to the C-terminus of the peptide improved membrane permeability and PY*LKTKmts inhibited STAT3-mediated gene transcription and malignant transformation, and induced apoptosis in v-Src-transformed NIH3T3 fibroblasts albeit at 1 mM concentration [45, 48], underscoring the potential difficulty of converting this approach into an effective therapeutic modality. The exploration of peptidomimetic and phosphotyrosine (pY) mimic approaches led to the identification of ISS 610, a peptidomimetic analog of the tripeptide, PY*L [49], the minimal peptide from PY*LKTK that was required for STAT3 inhibition (IC_{50} = 182 μM). PY*L mimic, ISS 610, better disrupted STAT3 DNA-binding activity (IC_{50} = 42 μM) [45, 49], and had increased STAT3 selectivity, (STAT1 IC_{50} = 310 μM; STAT5 IC_{50} = 285 μM) but still had weak intracellular inhibitory properties (IC_{50} = 1 mM), due to poor membrane permeability. The abysmal intracellular performance of the peptide forced the group to employ computational modeling to probe the binding of ISS 610 to the STAT3 SH2 domain, which led to generation of the oxazole-based small molecule S3I-M2001 having increased membrane permeability but similar STAT3 DNA binding inhibition (IC_{50} = 79 μM), loss of specificity (STAT1 IC_{50} = 159 μM), but improved intracellular activity [50]. S3I-M2001 reduced pY-STAT3 levels, DNA-binding, nuclear translocation, and transcriptional activity in NIH3T3/v-Src fibroblasts and human breast carcinoma cells at 50–100 μM. Cell growth inhibition ability was still weak (IC_{50} = 100 μM), including inhibition of cell growth, survival, and metastasis of NIH3T3/v-Src fibroblasts and human breast and pancreatic carcinoma cells with increased pY-STAT3. But importantly, it showed a significant regression of MDA-MB-231 xenografts at 5–20 mg/kg [50].

Another peptide-based approach used pY-peptides derived from STAT3 SH2 domain interacting growth factor or cytokine receptors, e.g. EGFR and gp130, to block SH2-pY-peptide ligand interaction. Shao et al. showed that a phosphododecapeptide (PDP) based on the sequence surrounding Y1068 within the EGFR could directly bind non-phosphorylated STAT3 and inhibit pY-STAT3 DNA binding,

ligand-stimulated STAT3 activation, and TGFα/EGFR-mediated autocrine growth in cancer cells [25]. Examining the structural basis for the specificity of STAT3-SH2 for pYXXQ peptides revealed that only pY-peptides containing +3 Q (not L, M. E or R) bound to wild-type STAT3-SH2 which required its K591 or R609 residues, whose side-chains interact with the peptide pY, and E638, whose amide hydrogen bonds with oxygen within the +3 Q side-chain when the peptide ligand assumes a β turn [25, 51].

Another approach found gp130-derived STAT3-inhibitory pY-peptide Y*LPQTV and several modified versions, including hydrocinnamoyl-Tyr (PO3 H2)-Leu-*cis*-3,4-methanoPro-Gln-NHBn [44, 52], that showed potent inhibition of STAT3 DNA-binding activity (IC_{50} = 0.15–0.29 μM). The peptidomimetic CJ-1383 developed from these, inhibited constitutive pY-STAT3 and inhibited growth of breast cancer cell lines (IC_{50} = 3.6–11.2 μM). PM-73G, another peptidomimetic developed from Y*LPQTV, also showed a low micromolar IC_{50} of pY-STAT3 reduction in cancer cells, inhibited their growth, and blocked xenografts formation [53, 54].

The peptide aptamer APT_{STAT3}-9R, which has a tryptophan zipper scaffold attached to a STAT3-binding peptide and a cell-penetrating motif, was screened from a randomized peptide library [55]; it specifically interacted in SPR assays with the STAT3 dimerization domain (K_d = 231 nM), reduced levels of pY-STAT3, DNA binding, and transcriptional activity [55] and blocked the growth of A549 cells *in vitro* (IC_{50} = 10–20 μM) and *in vivo*. Another aptamer, the recombinant STAT3 inhibitory peptide aptamer (rS3-PA) also decreased pY-STAT3 levels, inhibited growth of cancer cells *in vitro*, and reduced Tu9648 xenograft growth [56–59]. Although partly a peptide, these aptamers differ in their mode of action from peptide inhibitors [47].

A phosphate binder, e.g. Lewis acidic metal–picolylamine complex, was shown to act as a SH2-proteomimetic and disrupt pY-peptide–STAT3 complexes and also was potent in its anti-STAT3 activity (IC_{50} = 15–128 μM) as well it ability to inhibit growth of various cancer cells (IC_{50} = 11–100 μM) [60].

5.3.4.2 Small-Molecules

Despite having potent STAT3-inhibitory activity, peptides and peptidomimetics continue to suffer the limitations of *in vivo* instability and poor membrane permeability. Most of the peptides have not been tested in xenograft models and those that were tried, with the exception of rS3-PA, had to be administered intratumorally (IT), limiting their effective use *in vivo* [47]. Nevertheless, these studies provided the proof of concept that the STAT3-SH2/pY-peptide interaction was amenable to targeting and provided the impetus for many programs engaged in designing small molecules for this purpose.

SH2 inhibitors resulting from rational design or high-throughput screens. A structure-based virtual screening of ~425,000 compounds from four different chemical libraries followed by examination of 100 of the first 200 compounds in an *in vitro* STAT3-luciferase assay identified STA-21, a deoxytetrangomycin, with potent cell growth inhibitory activities (IC_{50} = 12.2/18.7 μM in DU145/PC3, respectively).

Modeling studies suggested that STA-21 binds to the SH2 domain of STAT3 and forms a number of hydrogen bonds with residues that form the pocket that binds the pY residue, including Arg-595, Arg-609, and Ile-634, and thus inhibits STAT3 dimerization, nuclear translocation, DNA-binding, gene transcription, and inhibits growth of breast and soft tissue sarcoma cell lines [61–63] with constitutively activated STAT3. Unexpectedly, STA-21 only minimally reduces levels of constitutively phosphorylated STAT3. The group also identified Compound1, a derivative of STA-21 [61], with similar STAT3 and cell growth inhibitory properties. Another slightly more potent structural analogue LLL-3 had better cellular permeability than STA-21 and inhibited growth of glioblastoma (IC_{50} = 10–20 μM), prostate cancer (IC_{50} = 11.3 μM), and CML cells (IC50 = 6.3 μM). Intratumoral injection of LLL-3 also inhibited intracranial glioblastoma xenografts in nude mice and increased their survival [64]. The acetyl group of LLL-3 was then replaced with sulfonamide to develop another STAT3 inhibitor, LLL-12 [65–73]. LLL-12 reduces pY-STAT3 levels (IC_{50} = 0.16–3.09 μM) and the growth of various cancer cell lines *in vitro* including osteosarcoma cell lines U2Os, SAOS2, and SJSA (IC_{50} = 0.3–0.8 μM,), breast cancer cell lines MDA-MB-231 and SKBR3 (IC_{50} = 0.97–3.1 μM,), pancreatic cancer cell lines HPAC and Panc-1 (IC_{50} = 0.16–0.29 μM), glioblastoma cell lines U87MG and U373MG (IC_{50} = 0.21–0.86 μM) and myeloma cell lines U266 and ARH-77 (IC_{50} = 0.49–1.9 μM), as well as their xenografts [66, 69, 70, 72].

Stattic (*Stat t*hree *i*nhibitory *c*ompound) was another early small molecule STAT3 inhibitor discovered by high-throughput screening of chemical libraries [74]. Stattic selectively inhibited STAT3 binding to pY-peptide (GY*LPQTV; IC_{50} = 5.1 μM) and blocked IL-6-induced STAT3 activation, nuclear accumulation, and DNA-binding activity (IC50 = 20 μM). It efficiently blocked the growth [74–76] of several cancer cell lines with increased levels of pY-STAT3 (IC_{50} = 0.43–5.6 μM), as well as UM-SCC-17B orthotopic xenografts [76]. Stattic was used as an adjuvant to sensitize radioresistant esophageal squamous cell carcinoma (ESCC) cells and xenografts to radiation [77], and to sensitize ovarian cancer cells to cisplatin [78]. A structure-activity relationship (SAR) analysis revealed that saturation of the vinyl sulfone leads to loss in activity. In addition, the presence of 2 mM dithiothreitol (DTT), a nucleophile donor, abrogated STAT3 inhibitory activity of Stattic, suggesting the nucleophilic attack of the sulphonic double bond by a cysteine in the STAT3 SH2 domain [74]. Recently, MS-based studies using high quantities of Stattic (800 μM; 10 μM of pY-STAT3) suggested that eight molecules of Stattic bind to one pY-STAT3 scaffold and identified Cys468 as one possible alkylation site [79]. However, a more recent paper [75] reported covalent binding of nine Stattic molecules to one unphosphorylated core STAT3 protein molecule at a lower concentration (50 μM/10 μM STAT3). Four or five of the nine covalently-modified residues are cysteines, but Cys468 and Cys542 were not among these [75]. A recent report by Sanseverino et al. indicated that Stattic targets other STAT proteins, including STAT1 and STAT5 [80].

Another STAT3 inhibitor resulting from structure-based high-throughput virtual screening of the National Cancer Institute (NCI) chemical libraries was S3I-201/NSC74859. In modeling studies, S3I-201 docked to the pTyr binding site of STAT3-SH2 domain through its salicylic acid moiety, inhibited STAT3

DNA-binding (IC_{50} = 86 μM), and inhibited proliferation of several cancer cell lines, including hepatocellular carcinoma, breast cancer, and prostate cancer albeit with high IC_{50}s (100–300 μM) [81, 82]. However, it successfully inhibited growth of MDA-MB-231 xenografts at a dose of 5 mg/kg [82]. Genetic Optimization for Ligand Docking (GOLD) studies suggested suboptimal interaction between S31-201 and STAT3. To improve this interaction, several molecules were subsequently developed [83, 84], many of which showed higher potency in STAT3 DNA binding inhibition assays (IC_{50} = 18.7–51.9 μM) and disruption of STAT3–pY-peptide interactions (Ki = 15.5–41 μM). S3I-201.1066 (or SF-1066) was the most potent in this series; it was demonstrated to directly bind STAT3 (K_d = 2.7 μM) and to inhibit growth of multiple cancer cell lines with greater potency than S3I-201 (IC_{50} = 35–48 μM) [85, 86]. Sixteen novel sulfonamide analogues of SF-1066 were subsequently characterized; of these, BP-1-102 [87, 88] effectively inhibited STAT3 DNA binding (IC_{50} = 6.8 μM), which was a 5-fold improvement over SF-1066 [83, 84], resulting in better cell growth inhibition (IC_{50} = 10.9–22.7 μM). BP-1-102 was orally bioavailable and effectively limited growth of STAT3-dependent tumor xenografts [88]. Known STAT3 dimerization-disrupting small-molecules, including S3I-201, were then subjected to GOLD analysis and a 3D quantitative structure-activity relationship (QSAR) pharmacophore model adopted to predict optimized STAT3 inhibitors. This analysis identified 2,6,9-trisubstituted purine scaffolds [89] as a promising choice of structural scaffold for projecting functionality into the three corners of the most important SH2-domain subpocket A, which contains the key pTyr705-binding residues and is composed of the polar residues Lys591, Ser611, Ser613 and Arg609 [90]. Select purine scaffolds, e.g. S3I-V3-31, S3I-V3-32, S3I-V3-33, S3I-V3-34, and S3I-V4-01, showed good affinities (K_D, 0.8 – 12 μM) for purified, non-phosphorylated STAT3, inhibited STAT3 DNA-binding (IC_{50} = 27 – 84 μM) and intracellular phosphorylation (IC_{50} = 20 – 60 μM) and suppressed growth of transformed cells (IC_{50} = 41 – 80 μM) with increased constitutive STAT3 activity [89]. Recently, another S3I-201 analog, S3I-1757, was described that was capable of inhibiting STAT3-pYpeptide binding (IC_{50} = 13 μM); however, it had only modest potency for decreasing levels of nuclear pY-STAT3 and STAT3-DNA binding ($IC_{50} \geq 50$ μM) [86].

A library of BP-1-102 analogues containing prodrugs, potential bioisosterses, and salicylic acid mimics was screened for anti-STAT3 and blood-brain barrier permeability properties, which identified 4 inhibitors—SH4-54, SH5-07, SH5-19, and SH5-23. Each had nanomolar IC_{50}s for inhibiting STAT3 binding to pY-peptide [91]. Of these, SH4-54, in which the hydroxyl substituent of the salicyclic acid moiety of BP-1-102 was removed and replaced with hydrogen [91], bound most strongly to STAT3 (K_D = 300 nM). SH4-54 also reduced levels of pY-STAT3 and its downstream transcriptional targets at low nM concentrations and potently targeted glioblastoma brain cancer stem cells (IC_{50} = 0.07–0.2 μM). SH-4-54 crossed the blood–brain barrier, reduced pY-STAT3 levels, and controlled glioma tumor growth *in vivo*. In a more recent study, SH4-54 and SH5-07 were tested in gliomas and breast cancer cells [92]. They were found to have increased ability to inhibit STAT3

DNA binding activity compared to BP-1-102 (IC_{50} = 3.9 and 4.7 μM, respectively) and inhibited DNA-binding in cells at 1–3 μM; however, their ability to reduce levels of pY-STAT3 in cells was much less pronounced (significant reduction not observed below 10 μM) and did not correlate with the ability to block DNA-binding and/or STAT3-regulated gene expression. This lack of correlation within the context of constitutively-active STAT3 was explained by suggesting that disruption of pre-existing STAT3:STAT3 dimers, which directly leads to lower DNA-binding activity, has a non-linear relationship with the turnover of disrupted pY-STAT3 molecules and by suggesting that SH4-54 and SH5-07 could act by binding directly to the STAT3 DBD [92]. In fact NMR data showed that these compounds bind to both SH2 domain and DBD of STAT3, in the later case, most probably to a hydrophobic pocket formed by residues Leu411, Ile386, and Ile439 [92].

Using computer-based ligand screening, our group docked 920,000 compounds from 8 chemical libraries into the p-Y-peptide pocket within the STAT3 SH2 domain and identified three hits, C3, C30, and C188 [93]. C188 demonstrated the greatest activity of the three [93–95] and inhibited STAT3-pY-peptide binding in an SPR-based assay (IC_{50} = 7.5–20 μM; calculated K_i = 37.3 nM), inhibited G-CSF-stimulated increased pY-STAT3 levels in Kasumi-1 cells (IC_{50} = 16.2 μM) and induced apoptosis in pY-STAT3-high breast cancer cells (ED_{50} = 0.7–3.9 μM) [93, 94, 96]. Hit-to-lead strategies focused on C188 [93–96] led to C188-9, which demonstrated improved potency and was non-toxic and orally bioavailable [95–98]. C188-9 binds to STAT3 with high affinity (K_D = 4.7 ± 0.4 nM) in microscale thermophoresis assays and potently inhibited STAT3 binding to its pY-peptide ligand (IC_{50} = 2.5 μM, SPR; K_i = 12.4 nM), inhibited G-CSF-stimulated increased pY-STAT3 levels (IC_{50} = 3.7 μM), and reduced constitutive pY-STAT3 levels (IC_{50} ~ 4 nM) in A549 cells [99].

Shin et al. searched a library of natural compounds using a STAT3-luciferase assay and identified Cryptotanshinone as a STAT3 inhibitor. Cryptotanshinone is derived from the roots of *Salvia miltiorrhiza*, known as Bunge or Danshen. Cryptotanshinone reduced levels of pY-STAT3 in HCT 116 colon cancer cells (IC_{50} = 4.6 μM) and in breast, prostate, and cervical cancer cell lines [100]. Cryptotanshinone inhibited growth of multiple cancer cell lines, including myeloma, glioma, NSCLC, colorectal, and pancreas (IC_{50} = 5.8–15.1 μM) and induced cancer cell apoptosis [100–105]. It was also found to synergize with various drugs, including imatinib and cisplatin in several cancers [103, 106–109]. Binding studies suggested that cryptotanshinone directly interacted with the STAT3 SH2 domain of STAT3 to inhibit STAT3 phosphotyrosylation and prevent STAT3 dimerization and nuclear translocation [100].

Matsuno et al. [110] identified a *N*-[2-(1,3,4-oxadiazolyl)]-4 quinolinecarboxamide derivative, STX-0119, as a novel STAT3 dimerization inhibitor by virtual screening using a customized version of the DOCK4 program and the STAT3 crystal structure. The top 136 hits identified were examined in a STAT3-dependent luciferase reporter gene assay and a fluorescence resonance energy transfer-based STAT3 dimerization assay. STX-0119 inhibited STAT3-reporter activity (IC_{50} = 74 μM), downregulated STAT3-regulated genes, and inhibited growth of multiple hematological cancers (IC_{50} = 1.4–18.3 μM), as well as glioblastoma cell

lines (IC_{50} = 6.6–44.5 μM) but did not affect STAT3 phosphorylation [110–113]. A docking model of STX-0119 [110] bound to the STAT3-SH2 domain revealed that the 2-Ph ring of STX-0119 inserted into a hydrophobic cleft in proximity to the pY-peptide binding pocket. Oral administration of STX-0119 effectively abrogated the growth of human lymphoma and glioblastoma xenografts [112, 113].

In another program, 437 of 7000 compounds that docked to a region of a STAT3 distinct from STAT1 in a previous molecular dynamics simulation [114] were further screened on the basis of favorable binding parameters involving ligand buried surface area (>75 %), and van der Waals and hydrogen bond energies. This resulted in identification of 52 compounds that were tested for the ability to block STAT3 DNA binding by EMSA [79]. Of these 52 compounds, C36 was identified as the most potent hit (IC_{50} = 30–50 μM). Subsequent library-screening using C36 as a template yielded another 48 structurally similar compounds. After further screening for STAT3 DNA binding inhibition and elimination of some leads because of low solubility, C48 emerged as the lead (IC_{50} = 10–50 μM); it reduced constitutive pY-STAT3 levels, DNA binding, and transcription of STAT3 gene targets in breast cancer cells leading to growth inhibition and apoptosis of C3L5 murine breast cancer tumors in a syngeneic mouse model [79]. Site-directed mutagenesis and multiple biochemical experiments showed that C48 is a covalent modifier of STAT3 and alkylates Cys468, a residue at the DNA-binding interface.

Our group used a high-throughput fluorescence microscopy search to identify compounds in a drug-repositioning library (Prestwick library) that block ligand-induced nuclear translocation of STAT3 and identified piperlongumine (PL), a natural product isolated from the fruit of the pepper *Piper longum* [115]. PL inhibited STAT3 nuclear translocation (IC_{50} = 0.9–1.7 μM), inhibited ligand-induced (IC_{50} = 0.9–2.7 μM) and constitutive (IC_{50} = 0.4–2.8 μM) STAT3 phosphotyrosylation, and modulated STAT3-regulated genes. SPR revealed that PL directly inhibited binding of STAT3 to its pY-peptide ligand (Ki 68nM). PL inhibited anchorage-independent growth of multiple breast cancer cell lines with increased levels of pY-STAT3 or total STAT3 (IC_{50} = 0.9–1.7 μM), and induced apoptosis. PL also inhibited mammosphere formation by cancer cells in patient-derived xenografts (PDX) and its anti-cancer activity was linked to its STAT3-inhibiting activity. PL was non-toxic in mice up to a dose of 30 mg/kg/day for 14 days and blocked growth of breast cancer cell line xenografts in nude mice.

SH2 inhibitors identified using fragment-based drug design (FBDD). Most of the above molecules resulted from high-throughput screens (HTS) based on rational design followed by lead optimization. Using biophysical methods like NMR and X-ray crystallography, fragment-based drug design (FBDD) has recently emerged as a successful alternative to HTS-based drug discovery [116–118]. Several groups have combined structural motifs of reported STAT3 inhibitors as part of a fragment-based drug design (FBDD) program to develop more potent STAT3 inhibitors. These and other FBDD STAT3 inhibitor programs are described below.

The intention of one such program was to design peptidomimetics that would bind to the pTyr705-binding site and a side pocket within the STAT3 SH2 domain. A urea linker was used to form H-bonds with residues between the two sites, which are rich in

H-bond acceptors and donors. Ten compounds were designed and XZH-5 emerged as the most promising. The features of XZH-5 were: (i) a carboxylate group that mimics the pTyr705 phosphate group; (ii) a fluorobenzene group able to form hydrophobic interactions with the side pocket; and (iii) a combination of urea and peptidyl linkers that spanned the right distance and were capable of forming H-bonds. XZH-5 was shown in a docking model to bind to the SH2 domain of STAT3 and prevent STAT3 phosphorylation at Tyr705, leading to inhibition of downstream STAT3 activities and apoptosis in multiple cancer cell lines including breast, pancreatic, hepatocellular and rhabdomyosarcoma ($IC_{50} \approx 15–50$ μM) [119–121].

Li et al. used a novel approach combining Multiple Ligand Simultaneous Docking (MLSD), drug scaffolds, and drug repositioning to find potent STAT3 inhibitors. Briefly, their approach consisted of: (i) building a small library of drug scaffolds for the binding hot spots within the STAT3 SH2 domain; (ii) MLSD screening of privileged drug scaffolds to identify optimal fragment combinations; (iii) linking of the fragment hits to generate possible hit compounds as templates; and (iv) similarity searches of template compounds in drug databases [122] to identify existing drugs as possible inhibitors of STAT3. The above process successfully identified two synthetic compounds T2 and T3 and the repositioning search yielded celecoxib. Each reduced the growth of HCT-116 ($IC_{50} = 9.0$, 10.1 or 43.3 μM, respectively). Further lead optimization produced 5 analogues [123] that were more potent in inhibiting cancer cell line growth ($IC_{50} = 6.5$ μM for a breast cancer cell line; 7.6 μM for pancreatic cancer cell lines).

Niclosamide, an FDA-approved anticestodal drug with a very low bioavailability in humans, was identified to inhibit STAT3 activation, nuclear translocation and transactivation [124]. FBDD based on the structure of niclosamide and other STAT3 inhibitors yielded a series of orally bioavailable STAT3 inhibitors including HJC0152 and HJC0123 [125, 126]. HJC0123 inhibited STAT3 activation and promoter activity, growth of breast and pancreatic cancer cell lines *in vitro* ($IC_{50} = 0.1–1.2$ μM) and MDA-MB-231 xenografts [125] and also potentiated doxorubicin- and gemcitabine-mediated killing [119].

More recently Yu et al. developed another STAT3 dimerization inhibitor by utilizing FBDD. They linked the naphthalene-5,8-dione-1-sulphoneamide fragment of LLL-12 (thought to bind to the pTyr705-binding pocket within the STAT3 SH2 domain) to a dimethyl amine that contained various R groups and generated 5 different compounds. LY5, the most potent compound, inhibited growth of U2OS and RD2 cancer cells ($IC_{50} = 0.5–1.39$ μM) better than parent compound LLL-12; it also was easy to synthesize and possessed more drug-like properties than LLL-12 [127].

5.3.5 Inhibitors Targeting the STAT3 DNA-Binding Domain (DBD)

Recognition of specific DNA elements is one of the cardinal features of transcription factors (TFs). The DBD of STAT3 is known to bind two types of DNA elements within promoter sites to mediate its transcriptional activities—serum-inducible

elements (SIE) and gamma-activated sequences (GAS) [22, 128]. Concerted efforts at blocking this interaction have been underway for some time. The following sections describe these efforts (Table 5.3).

5.3.5.1 Decoy Oligonucleotides

Decoy oligonucleotides are double-stranded or duplex DNAs that mimic TF promoter elements. Their use was first described by Bielinska et al. in 1990 as a way of modulating gene transcriptional activity in the cell [129]. Duplex ODNs act by competitively inhibiting TF binding to their endogenous promoter elements. This strategy has been used to target aberrant TF signaling in various diseases and currently represents an active area of research [130, 131]. Following successful demonstration of STAT6 inhibition using this method [132], Leong et al. reported the use of a 15-mer duplex ODN modeled on the c-fos promoter sequence (SIE) to target STAT3 [133]. They demonstrated reduction in STAT3 mediated gene expression that led to growth inhibition of head and neck cancer cells. Other researchers also have shown similar results with other STAT3-associated cancers including, ovarian cancer, glioma, prostate cancer and hepatocellular carcinoma. [134–138]. Although duplex ODNs appeared to have minimal toxicity in primate models [139], instability in plasma was a limitation to their *in vivo* efficacy. To overcome these limitations, the Grandis lab developed a cyclic STAT3 decoy ODN linked to hexa–ethylene glycol. This ODN showed improved stability and retained antitumor efficacy with minimal toxicity when administered intravenously in a preclinical head and neck cancer models [140]. Creating a peptide nucleic acid (PNA) by adding a novel cell-penetrating peptide (CPP) consisting of a glutamate peptide linked to the N-terminus of the nuclear localization signal (NLS) from Oct6 transcription factor, to the minimal 15-mer linear ODN 13410A (Glu-Oct6-13410A) required for inducing cell apoptosis [137, 141] showed better cell-uptake and better apoptosis inducing capacity [141].

5.3.5.2 G-Quartet Oligonucleotides

G-quartet oligonucleotides (GQ-ODN) constitute another approach that is mechanistically analogous to ODNs in inhibiting the transcriptional activity of STAT3. G-quartets oligonucleotides are random coils outside the cell that complex with K^+ ions within the cell form stable box-like structures composed of stacks of 4 G-bases that are hydrogen bonded via hoogensteen pairings [142]. These structures are normally found in telomeres and promoter regions of many genes. G-Quartets are known to associate with DNA binding proteins [143], thus, making them ideal candidates to be used for targeting DNA binding activity of TFs. In 2003, Jing et al. developed a GQ-ODN, that inhibited IL-6 induced DNA binding activity of STAT3 and suppressed expression of STAT3 mediated genes [144]. Subsequent work showed that GQ-ODNs inhibited proliferation in a wide variety of tumor cell lines, including prostrate, breast, head and neck, non-small cell lung cancer, and T-cell leukemia with IC_{50}s ranging

from 5 to 7 μM [145, 146]. Although initial studies predicted that GQ-ODN destabilized dimer formation, the mechanism by which GQ-ODN disrupt and abrogate STAT3 activity remains unclear since subsequent work appeared to show that the GQ-ODN inhibited STAT3 transcriptional activity by preferentially binding to its DNA binding domain rather than the SH2 domain [147]. Nevertheless, it is clear that they show promise as targeted anti-cancer agents. GQ-ODN have not garnered as much interest as small molecules, perhaps due not having properties suitable for systemic delivery. However, this may change as novel nucleic acid delivery systems currently being developed based upon siRNA therapeutics are employed [148].

5.3.5.3 Platinum-Based Inhibitors

The antitumor effects of most platinum compounds are thought to result from their ability to combine with DNA and form complexes that are toxic to cells. In contrast, platinum IV compounds—CPA-1, CPA-7, and platinum (IV) tetra-chloride, were shown to inhibit STAT3 DNA binding activity in an EMSA assay [149]. Importantly, IS3 295, a member of the same group identified from a screen of the NCI 2000 diversity set of compounds, was reported to bind STAT3 and prevent its interaction with specific DNA response elements in a dose dependent manner with an IC_{50} of 1.4 μM [150]. All platinum IV compounds mentioned here preferentially inhibit STAT3 and to some extend STAT1 DNA binding, but showed no activity against STAT5 DNA binding, reducing the possibility that this is a nonspecific DNA targeting effect. The compound suppressed STAT3 dependent gene activation and showed antiproliferative effects against v-Src transformed fibroblast and a variety of breast cancer cells. Of note, CPA-7 also was recently shown to be effective against both gliomas and melanomas in mouse tumor models [151]. Biochemical data also suggests that inhibition of DNA binding by IS3 295 is irreversible, which is not surprising because platinum compounds are known to react with thiol groups [152]. The fact that IS3 295 is selective for STAT3 over STAT5 suggests that covalent modification involves a unique site within STAT3 to which the compounds first binds non-covalently prior to crosslinking. It is important to note that this kind of selectivity implies a "hotspot" within the DNA binding domain [153]. It would therefore be interesting to pinpoint the reactive thiol groups at the DNA interface. This could yield important information that would help drive the development of other compounds directed at STAT3 DNA binding. It remains to be seen what proteins other than STAT3 this class of compounds also targets in order to better assess the possibility of unacceptable levels of off-target effects.

5.3.5.4 Small Molecule Targeting

In contrast to the SH2 binding domain, which presents a well-defined pY binding site that is amenable to targeted small-molecule inhibition, the DNA binding domain has historically been considered challenging, partly due to the belief that

disrupting DNA binding would not achieve the desired level of selectivity necessary to discriminate among TFs. In addition, protein DNA interactions of TFs were conventionally deemed undruggable due to the lack of obvious targetable pockets within their binding interfaces. Using high quality structural data of the DBD of STAT3 [43], Huang et al. applied an improved virtual ligand screen to identify a small molecule called InS3-54 (4-[(3E)-3-[(4-nitrophenyl)-methylidene]-2-oxo-5-phenylpyrrol-1-yl] benzoic acid) that non-covalently binds to the DBD of STAT3, thereby competitively inhibiting its DNA-binding activity [154]. To ensure selectivity towards STAT3, top scoring molecules from the initial screen were docked on to the DBD of STAT1. InS3-54 was selected as the most selective compounds that had the ability to inhibit STAT3 dependent gene expression in a luciferase reporter assay. In addition, InS3-54 was demonstrated to inhibit DNA binding of pY-STAT3 dimer ($IC_{50} = 20$ μM) by non-covalently binding to the DBD of STAT3. Although efficacious in inhibiting proliferation of various cancer cell lines, the IC_{50} (<6 μM) was markedly lower than that for its inhibition of DNA binding, which suggested the possibility of off-target effects. To address this issue, Zhan's group made further activity guided hit-to-lead optimizations that resulted in InS3-54A18, a compound that showed improved IC_{50} for growth inhibition, better specificity, and more favorable pharmacological properties [155]. When orally administered, inS3-54A18 effectively inhibited STAT3 activity in mice leading to a reduction in lung xenograft tumor growth.

Another example of a small molecule presumed to work by the directly targeting the STAT3 DBD is a synthetic analog of curcumin, HO-3867, that has been shown to inhibit DNA binding activity in an ELISA assay [156]. HO-3867 inhibited STAT3 transcriptional activity, was preferentially active in a dose dependent manner in inhibiting growth of cancer vs. normal cell lines, and inhibited xenograft tumor growth. However, this compound appears to have minimal selectivity and was shown to inhibit upstream kinases [157, 158]. To advance further, the specificity of HO-3867 likely will need to be improved.

Galiellalactone, a fungal metabolite from the ascomycete, *Galiella rufa*, inhibited the IL-6/STAT3 signaling pathway [159, 160]. Galiellalactone inhibited STAT3-mediated luciferase induction ($IC_{50} \sim 5$ μM), reduced STAT3-regulated gene induction, and blocked the growth of various cancer cell lines e.g. DU145, *in vitro* ($IC_{50} = 3.4$ μM) and *in vivo* [160–162]. Galiellalactone did not prevent dimerization of the STAT3 monomers and showed no significant inhibition of phosphorylation; it appears to mediate its STAT3 inhibitory effect by covalently modifying residues Cys-367, Cys-468, and Cys-542 in the DBD and directly blocking the binding of STAT3 to DNA [162].

5.3.5.5 Peptides and Aptamers

Like STAT3 SH2-directed aptamers, DBD-directed peptide aptamer DBD-1 and its protein transduction domain (PTD)-fused analog, DBD-1-9R could also target STAT3 and reduce growth of STAT3-dependent cells [163].

5.3.6 Inhibitors Targeting the STAT3 N-Terminal Domain

Although tyrosine phosphorylation precedes STAT3 activation, it has been shown that even nonphosphorylated STAT3 contributes to carcinogenesis through regulation of gene expression [164–166]. In addition, protein–protein interactions between STAT3 and other transcription factors also can affect the repertoire of transcribed genes and contribute to tumorigenesis [167]. The N-terminal domain mediates protein–protein interactions during binding of STAT3 dimers to DNA and in the assembly of the transcriptional machinery, including the interactions between two STAT3 dimers to form a tetramer, as well as with other transcriptional factors and regulators [43, 168, 169]. The N-terminal domain interaction with other transcription factors/cofactors leads to formation of enchanceosomes [170] and its interaction with histone-modifier proteins induces changes in chromatin structure [171]. These complex interactions together maximize STAT3-dependent transcriptional control in normal and cancer cells [167]. Moreover, the NTD also has been implicated in the interaction of STAT3 with peptide hormone receptors and the nuclear translocation of STAT3 [172–174]. Short peptides (Table 5.3) derived from helices within the N-terminal domain, especially helix-2 (ST3-H2A2), recognized and bound to STAT3, but not to other STAT members, and inhibited STAT3 transcriptional activity without affecting levels of pY-STAT3 [169, 175, 176]. The cell-permeable form of this peptide (Hel2K-Pen), generated by its fusion with Penetratin (a protein transduction motif with sequence RQIKIWFPNRR-Nle-KWKK-NH2), selectively induced cell growth inhibition and apoptosis of human MDA-MB-231, MDA-MB-435, and MCF-7 breast cancer cells (IC_{50}~10 μM) through robust induction of pro-apoptotic genes, as a result of altered STAT3 chromatin binding [175–177]. Issues of peptide stability and bioavailability still remain major challenges to be overcome for this unique approach to STAT3 inhibition to advance.

5.3.7 Inhibitors that Target Endogenous STAT3 Negative Regulators

In normal cells, the level and duration of STAT3 activation is controlled by a variety of mechanisms including dephosphorylation of receptor complexes and nuclear STAT3 dimers by protein phosphatases (PTPases), interaction of activated STAT3 with members of the protein inhibitors of activated STAT (PIAS) family, and the actions of suppressor of cytokine signaling (SOCS) protein members that inhibit and/or degrade JAKs [178, 179]. Many different STAT3 inhibitors seem to work through modulating the activity of these endogenous regulators (Table 5.4).

Several protein tyrosine phosphatases, including members of the Src homology 2 (SH2)-domain containing tyrosine phosphatase family (SHP-1 and SHP-2) and protein tyrosine phosphatase 1B (PTP-1B) [180–182] can deactivate STAT3 signaling through direct dephosphorylation of pY-STAT3, thus, are useful targets [183]. In many cancer cells, loss of regulation by these, lead to constitutive STAT3 activation,

Table 5.4 Other STAT3 inhibitors with varying modes of action

Inhibitors	Type	Description	Blocks	IC50 STAT3 inhibition (assay)	IC50 cell growth inhibition, cell	Pre-clinical animal models	Ref
Endogenous STAT3 inhibitor modulators							
AdCN305-cppSOCS3	RV	Recombinant adenovirus encoding SOCS3	pY, DM, DB, GT	NA	NA	5×10^8 PFU/dose, alternate day×4, SW620, BEL7404 xenografts	[206, 207]
Calyculin A	SM	PP2A inhibitor, increases pS-STAT3, decreases pY-STAT3	pY, GT	80 nM[E], constitutive pSTAT3, T lymphoma	NA	NA	[208–210]
SC-1/SC-43/SC-49	SM	Increases SHP1 activity to reduce pSTAT3	pY, DM, DB, GT	1–5[E] μM, constitutive pSTAT3, HCC and breast cancer cells	2–5[E] μM, HCC and breast cancer cells	10 mg/kg, 28d, PO, MDA-MB-468 xenografts	[186, 188, 189]
TPA	SM	Phorbol ester, activates PKC-regulated phosphatase and inhibits pSTAT3	pY, DM, DB, GT	NA	NA	NA	[211]
PF4 (Platelet Factor 4)	CK	Angiostatic cytokine upregulates SOCS3 and inhibits pSTAT3	pY, DB, GT	NA	NA	200 ng, 3/wk, 6 wks, IV, OPM2 xenografts in nude mice	[449, 450]
Nucleotide based inhibitors							
Anti-Sense (AZD9150) (70, NCT01839604)	ASO	16 oligonucleotide antisense molecule (ASO) targeting the 3′ untranslated part of STAT3	pY, DM, DB, GT	0.011 μM, STAT3 mRNA A431	0.05 μM, A431	25 mg/kg, A431 xenografts	[293]
CTLA4[apt]-STAT3 siRNA	AP-ASO	CTLA4[apt] fused to a STAT3-targeting siRNA, internalized into tumor-associated $CD8^+$ T cells and silencing of STAT3	NA	0.5 nM, STAT3 mRNA in CD8 T cells	NA	782.5 pmol/dose/mouse, IV, melanoma, RCC, lymphoma colon carcinoma xenografts	[225]

(continued)

Table 5.4 (continued)

Inhibitors	Type	Description	Blocks	IC50 STAT3 inhibition (assay)	IC50 cell growth inhibition, cell	Pre-clinical animal models	Ref
Other inhibitors with novel mechanism							
Capsaicin, *N*-vanillyl-8-methyl-1--nonenamide)	SM	Hot-pepper ingredient blcoks IL6-stimulated pSTAT3 by translational inhibition of gp130	pY, DM, DB, GT	–5–7^{E} μM, const pSTAT3, U266	0.05 μM, A431	1 mg/kg, 3/wk, IP, U266 xenografts	[232, 235, 451]
PF4 (Platelet Factor 4)	CK	Angiostatic cytokine upregulates SOCS3 and inhibits pSTAT3	pY, DB, GT	~4^{E} μM, const pSTAT3, OPM2, U266	2–4 μM, OPM2, NCI-H929 and U266, growth, MTT	200 ng, 3/wk, 6 wks, IV, OPM2 xenografts in nude mice	[449, 450]
ML116	SM	Novel inhibitor (PubChem CID-2100018) belongs to the thienopyrimidine scaffold	DM, DB, GT	4.2 μM, IL6-stimulated STAT3 luciferase assay	0.8–33.1 μM, glioma cells	15 mg/kg, IP, intracranial GL26 xenografts	[452]

Note: Information for Pre-clinical animal models consist of Dose, route of administration, duration (if available) and animal model used. Information for Pre-clinical animal models consist of Dose, route of administration, duration (if available) and animal model used

Abbreviations: *E* estimated from descriptive data on inhibition, from corresponding reference, *NA* not available, *Ab* antibody, *SM* small molecule, *pY* STAT3 phosphorylation at Tyr-705, *DM* dimerization, *NT* nuclear translocation, *DB* DNA-binding, *GT* gene transcription, *IG* intra-gastrical, *TK* tyrosine kinase, *IC* intra-cranial, *PO* per Os (by mouth), *CK* cytokine, *IP* intra-peritoneal, *AP* aptamer, *ASO* anti-sense oligonucleotide, *RV* recombinant virus

e.g. loss of SHP-1 enhances JAK3/STAT3 signaling in ALK-positive anaplastic large-cell lymphoma and in cutaneous T cell lymphoma [184, 185]. Many chemical agents also appear to up regulate SHP-1 activity/expression. As shown in Table 5.4, sorafenib derivatives lacking Raf-1 kinase activity, e.g. SC-1, SC-43, and SC-49 [186–189], appear to reduce levels of constitutive pY-STAT3 (IC_{50} = 1–5 μM) by upregulation of SHP1 leading to inhibition of cancer cell growth *in vitro* (IC_{50} = 2–5 μM) and inhibition of xenografts growth in mice. Many other known JAK/STAT3 inhibitors e.g. betulinic acid [190], guggulsterone [191], 5-azacytidine [192], SC-2001 [193], sorafenib [194], beta-caryophyllene [195], boswellic acid [196], capillarisin [197]. Honokiol [198], dovitinib [199], 1′-acetoxychavicol [200], gambogic acid [201], dihydroxypentamethoxyflavone [202], butein [203], icariside II (a flavonoid icariin derivative) [204] and 5-hydroxy-2-methyl-1,4-naphthoquinone (a vitamin K3 analogue) [205] can enhance the SHP-1 pathway (either by induction of SHP-1 expression or by increase of SHP-1 activity) and show anti-cancer potential.

Adenovirus mediated transduction of the SOCS3 gene also can reduce levels of pY-STAT3 and thereby reduce SW620 and BEL704 xenograft growth [206, 207]. Other known negative STAT3-regulators also could be modulated in a similar way to reduce STAT3 activity.

Woetmann et al. [208] showed that calyculin A, an inhibitor of serine phosphatases and the protein phosphatases (PPs) PP1yPP2A, induces (i) phosphorylation of STAT3 on serine and threonine residues, (ii) inhibition of STAT3 tyrosine phosphorylation and DNA binding activity, and (iii) relocation of STAT3 from the nucleus to the cytoplasm. Similar results were obtained with other PP2A inhibitors (okadaic acid and endothall thioanhydride) but not with inhibitors of PP1 (tautomycin) or PP2B (cyclosporine). There are other reports of a similar inhibition of STAT3 activity by calyculin A [209, 210] but observations with some of the other PP2A inhibitors [209] could not be repeated.

STAT3 activity is, in part, positively regulated by c-Src and negatively regulated by a PKC-activated PTPase(s) in melanoma cells. The tumor-promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) was shown to inhibit melanoma cell growth by suppression of STAT3 activity through upregulation of PTPase(s) and upregulation of PKC [211], which led to a decrease in STAT3 DNA-binding, STAT3 target gene transcription, and inhibition of growth of melanoma cells [211].

5.3.8 Inhibitors with Other Mechanisms of Action

There are numerous examples of agents (Table 5.4) that inhibit STAT3 activity/oncogenic function, that do not necessarily belong to any of the above groups of indirect or direct STAT3-interacting compounds. These will be discussed in this section.

5.3.8.1 siRNA-Based Inhibitors

Apart from the ODNs, which block the ability of STAT3 DBD to bind the STAT3-responsive sequence containing DNA, there also have been concerted efforts at targeting STAT3 mRNA using siRNA and shRNA based methods as outlined below.

Anti-sense therapy. Many antisense oligonucleotide (ASO)-based drugs, which bind to messenger RNA (mRNAs) and inhibit the production of disease-causing proteins, are at various phases of clinical trials. An ASO complementary to apolipoprotein B-100 mRNA, mipomersen sodium (Kynamro), received FDA approval in January 2013 as an adjunct to statin-based lipid lowering therapy [212, 213]. AZD9150 (ISIS-STAT3Rx or ISIS 481464) is a synthetic ASO against STAT3. Information about its pre-clinical development is scant but its testing in clinical trials is summarized below. RNA interference (RNAi) is a natural post-transcriptional gene-silencing (PTGS) mechanism for silencing unwanted genes. The process is initiated by the presence of double-stranded RNA, not a constituent of the normal cell cytoplasm. The dsRNAs are cleaved by dicer, an endonuclease, into 20–25 nucleotide dsRNAs, referred to as short or small interfering RNAs (siRNAs). The RNA-induced silencing complex (RISC) separates the two strands, and one of these strands then serves as a guide for sequence-specific degradation of complementary mRNA. The utility of this approach is limited due to the short half-life of transfected RNAs. This problem can be circumvented using a DNA-directed RNA interference technique in which a short hairpin RNA (shRNA, a double stranded RNA) is expressed in the cell after insertion of a DNA construct into the nucleus. These shRNAs then enter the RNAi pathway and gene silencing can last for as long as the cell continues to produce the shRNA [214, 215]. This strategy is under evaluation in several clinical trials for the treatment of several diseases including cancers (#NCT01591356, #NCT00363714, #NCT00689065, #NCT00938574). However, data regarding siRNA targeted silencing of STAT genes for cancer therapy are limited to *in vitro* studies and *in vivo* studies of animal models only [216–224].

Intracellular therapeutic targets that define tumor immunosuppression in both tumor cells and T cells remain intractable [225]. Administration of a covalently linked siRNA to an aptamer (apt) that selectively binds cytotoxic T lymphocyte-associated antigen 4 [CTLA4(apt)] allowed gene silencing in exhausted $CD8^+$ T cells and Tregs in tumors as well as CTLA4-expressing malignant T cells [225]. CTLA4(apt) fused to a STAT3-targeting siRNA [CTLA4(apt)-STAT3 siRNA] resulted in internalization into tumor-associated $CD8^+$ T cells overexpressing CTLA-4 [226] and silencing of STAT3, which activated tumor antigen-specific T cells in murine models [225]. Both local and systemic administration of CTLA4(apt)-STAT3 siRNA dramatically reduced tumor-associated Tregs and potently inhibited tumor growth and metastasis in various mouse tumor models [225].

5.3.8.2 Inhibitors Targeting Nuclear Translocation

The role of activated STAT3 as a DNA-binding transcription factor relies on the ability of homodimers to traffic from the cytoplasm to the nucleus [178, 227–231]. Preventing this shuttle of STAT3 dimers could be a way to block STAT3 activity [229]. Importins α3, α5, α7, and β, are involved in passage of STAT3 through the nuclear pore [26, 229]. Once within the nucleus, TC45 dephosphorylates pY-STAT3, which then becomes a substrate for exportin-1–mediated export [229]. Inhibition of exportin 1 by leptomycin B or ratjadone A, has been shown to interfere with nuclear export of STAT3; it reduces pY-STAT3 and STAT3-mediated transcription and causes cells to undergo apoptosis [229]. Although interesting, any small-molecule that inhibits general trafficking across the nuclear membrane is likely to be toxic [229]. Whether an inhibitor of nuclear pore transit can be developed with sufficient STAT3 selectivity remains to be determined.

5.3.8.3 Inhibitor with Novel Modes of Action

There are a few inhibitors, which have very novel mechanisms of action, mostly by way of modulating proteins or pathways indirectly regulating the STAT3 signaling pathway (Table 5.4). E.g. capsaicin has been shown to have anti-carcinogenic effects on various tumor cells through multiple mechanisms including STAT3 inhibition [232–234]. Lee et al. showed that capsaicin treatment of glial tumors induced downregulation of the IL-6 receptor gp130 by translation inhibition, and was associated with activation of endoplasmic reticulum (ER) stress [235]. The depletion of the intracellular pool of gp130 by capsaicin combined with the ER stress inducer led to an immediate loss of the IL-6 response due to short half-life of membrane-localized gp130 [235].

Platelet factor 4 (PF4) is an angiostatic chemokine that suppresses tumor growth and metastasis and is frequently lost in multiple myeloma. Exogenous PF4 treatment not only suppressed myeloma-associated angiogenesis, but also inhibited growth and induced apoptosis in myeloma cells. It has been shown that PF4 negatively regulated STAT3 by inhibiting its phosphorylation and transcriptional activity. Overexpression of constitutively activated STAT3 could rescue PF4-induced apoptotic effects. Furthermore, PF4 induced the expression of SOCS3, an endogenous STAT3 inhibitor, and gene silencing of SOCS3 abolished its ability to inhibit STAT3 activation, suggesting a critical role of SOCS3 in PF4-induced STAT3 inhibition.

5.3.8.4 Other Inhibitors that May Act by Targeting STAT3

There are numerous reports of various compounds, most naturally occurring, that are known to exert powerful anti-tumor effects, through their action on STAT3. However, the mechanistic basis for their anti-STAT3 action is unknown. Some examples are protoepigenone/RY10-4 [236], shikonin [237], paclitaxel [238–240],

vinrelbin [238–240], nifuroxazide [241], icaritin [242–245], and epigallocatechin-3 [246]. These are potent inhibitors that can reduce STAT3 activation and induce growth inhibition and/or apoptosis and in many cases have been proven, in preclinical animal models to reduce tumor growth. Further studies are necessary to elucidate their exact mechanism of action.

In considering this group of compounds, as well as others listed above, it is important to recall that proteases play an important role in STAT3 biochemistry, including its posttranslational modulation [247, 248] and degradation. STAT3 proteases include caspases, calpain, and the proteasome complex. Many compounds induce cell cycle arrest and apoptosis accompanied by reduced pY-STAT3 levels. It is frequently concluded that these compounds target STAT3 but the precise mechanism of STAT3 targeting is not determined. A number of compounds proposed as STAT3 inhibitors exert their antitumor effects by promoting STAT3 protein degradation in cancer cells [249–251]. In addition, pY-STAT3 has been shown to undergo caspase-dependent proteolytic cleavage [252]. Because cysteine proteases, such as caspases and calpain, are well known intracellular effectors of apoptosis, the ability of some purported STAT3 inhibitors to reduce pY-STAT may not be due to direct targeting of STAT3, but rather a reflection of compound-induced apoptosis in which pY-STAT3 levels are reduced by effector proteases within the apoptosis pathway.

5.3.9 Allosteric Effects of STAT3 Inhibitors

Namanja et al. [253] found that pY-peptide interactions with the SH2 domain of STAT3 cause structural and dynamics changes in its LD and DBD. This interdomain allosteric effect likely is mediated by the flexibility within the hydrophobic core of STAT3. In addition, a mutation (I568F) in the LD, identified in a patient with autosomal-dominant hyper IgE syndrome (AD-HIES) induced NMR chemical shift perturbations in the SH2 domain, the DBD and the CCD domain of STAT3, suggesting conformational changes in these domains mediated by a point mutation in a separate domain. Furthermore, they showed that the conformational changes in the SH2 domain seen in the mSTAT3 I568F mutant was accompanied by the reduced affinity of this mSTAT3 for pY-peptide. This effect may help explain the ability of some compounds that bind domains other than the SH2 domain to affect STAT3-pY-peptide binding. The recent paper by Mathew et al. [254] using a rhodium-(II)-catalyzed, proximity-driven modification approach identified the STAT3 coiled-coil domain (CCD) as a novel binding site for a newly described naphthalene sulfonamide inhibitor, MM-206. Despite binding to the CCD, this compound reduces STAT3 binding to pY-peptide and has structural features of C188, previously shown to reduce STAT3 binding to pY-peptide [93, 94, 96], and BP-1-102, thought to bind to the STAT3 SH2 domain. Findings with MM-206 [254] and STAT3 proteins containing substitutions within the CCD, such as Asp170 [174], suggest that the CCD, like the LD, also may engage in interdomain allosteric effects. Based on these findings, one might need to reconsider notions about how STAT3 inhibitors

demonstrated to bind to STAT3 and to reduce STAT3 activity actually mediate their effects and may change our approach to designing drugs to target this oncogene. The fact that selectivity and mechanisms of action of established STAT3 inhibitors continue to be revisited and clarified [255, 256] reinforces this concept.

5.4 Entry of STAT3 Inhibitors into the Clinic

Attempts to develop peptide inhibitors [25, 44, 45, 51, 257] that target the pY-peptide binding pocket within the STAT SH2 domain [45] quickly followed the elucidation of the crystal structure of STAT3β homodimer [43] and confirmation that STAT3 was an oncoprotein [8]. However, due to their lack of membrane permeability and stability, non-peptidic small molecule inhibitors of STAT3 moved to the forefront of this drug discovery area [61]. Although showing promising pre-clinical activity *in vivo*, many compounds in this category show activity in the medium-to-high micromolar range, indicating the need for additional optimization before transitioning to clinical trials involving systemic administration. STA-21 has completed phase I/II trials in patients with psoriasis [258] with effective concentrations being achieved at affected skin sites through topical application. Several agents that systemically target the IL-6R/JAK/STAT3 signaling pathway are at various stages of clinical trials (Table 5.5) for a cancer indication. STAT3 upstream antagonists include the IL-6-neutralizing MAb siltuximab [259], the IL6R-anatgonist MAb tocilizumab [260, 261], the JAK inhibitor ruxolitinib [262–268], AZD1480 [41, 269–272], OPB-31121 [273–278], fedratinib/SAR302503 [279–282], BSE-SFN [283], pacritinib/SB1518 [284, 285], and the dual JAK2/gp130 inhibitors WP1066 [286–290] and OPB-51602 [291]. Direct STAT3 inhibitors include the STAT3-decoys [292] and the STAT3-antisense oligonucleotide based inhibitor ISIS-STAT3Rx (AZD9150) [293]. The third group of compounds includes two re-purposed drugs that also inhibit STAT3—the antiparasitic drug pyrimethamine [283] and the HMG-CoA inhibitor Simvastatin [294–296].

The importance of the IL-6/JAK/STAT signaling pathway in many human malignancies has, in part, spurred development of several IL-6 and IL-6 receptor inhibitors for cancer treatment [297–299]. Siltuximab (CNTO 328), the chimeric anti-IL-6 MAb has been approved by the FDA in 2014 for the treatment of patients with HIV-negative and HHV-8-negative multicentric Castleman's disease (MCD), a lymphoproliferative disorder with germinal center hyperplasia and high morbidity, at a dose of 11 mg/kg every 3 weeks [259, 300]. In a Phase I study, 18 of 23 patients (78%) had complete response, and 12 patients (52%) demonstrated objective tumor response [301]. In a Phase II study, with HIV-negative and HHV-8-seronegative patients with symptomatic MCD (n = 140), durable tumor and symptomatic responses occurred in 18 of 53 patients (34%) in the siltuximab group and none of 26 in the placebo group [302]. A Japanese Phase 1 trial [303] in multiple myeloma patients showed some responses, but in other studies the 11 mg/kg dose did not improve progression-free survival or achieve other measures of response [259]. Out of the 16

Table 5.5 STAT3 inhibitors at various stages of clinical trials

Inhibitor	Target	Indications	Phase	Goals/results	Ref
Siltuximab (CNTO-328)	IL6	Ovarian, pancreatic, colorectal, head and neck, and lung cancer, Castleman's disease, MM[C]	Phase I, Phase II	FDA-approved for Multicentric Castleman's disease (MCD), a lymphoproliferative disorder with germinal center hyperplasia	[259]
Tocilizumab	IL6R	KSHV-associated multi-centric Castleman's disease, MM (combined with allo-SCT), recurrent ovarian cancer (with chemo)	Phase 0, Phase I, Phase II	As both an anti-myeloma therapy and as a method to reduce GvHD, as chemo-sensitizer in recurrent ovarian cancer	[260, 261]
Ruxolitinib (INCB018424)	JAK1/2, STAT3	Chronic myeloproliferative disorders, leukemia, myelodysplastic syndrome, myeloproliferative neoplasms, unspecified childhood solid tumor, metastatic HER2+ BC, TNIBC (with pre-op chemo), HER2- BC (+ capecitabine)	Phase I, Phase II, Phase III	Encouraging results in myelofbrosis, decreasing not only disease symptoms but also JAK2 c.1849G>T (p.V617F) mutation burden. Toxicity remains an issue	[305]
AZD1480	JAK, STAT3	Metastatic cancer, pancreatic cancer, myeloproliferative diseases	Phase I	Pharmacodynamic analysis of circulating granulocytes demonstrated maximum phosphorylated STAT3 (pSTAT3) inhibition. Trial had to be eliminated because of toxicity	[41]
OPB-31121	JAK, STAT3	Advanced solid tumor, Hodgkin's lymphoma, non-Hodgkin's lymphoma, HCC	Phase I	Insufficient antitumor activity for HCC	[273]
Fedratinib (SAR302503)	JAK, STAT3	Advanced cancer, myelofibrosis	Phase I	Fedratinib treatment led to reduced STAT3 phosphorylation but no meaningful change in JAK2V617F allele burden in MF	[280, 282]
BSE-SFN	JAK2, STAT3	Atypical nevi	Phase 0	Evaluation of sulforaphane from Broccoli Sprout Extract (BSE-SFN) as a candidate natural chemopreventive agent able to modulate key steps in melanoma progression and STAT3 mediated gene transcription	[307, 308]
Pacritinib (SB1518)	JAK2, FLT3, STAT3/5	Myelofibrosis, AML (combined with decitabine/cytarabine)	Phase II	Active drug in myelofibrosis. Going in the AML patients for safety and efficacy as a STAT3 inhibitor in combination with decitabine/cytarabine	[284, 285]

WP1066	JAK2, gp130, STAT3	Advanced solid tumor, melanoma and recurrent glioblastoma	Phase I	Find the highest tolerable dose of WP1066 that can be given to patients with recurrent cancerous brain tumors or melanoma that has spread to the brain	[453, 454]
OPB-51602	JAK2, gp130, STAT3	Advanced solid tumor, glioblastoma multiforme, melanoma, relapsed/refractory hematological malignancies	Phase I	Recommended dose 4 mg, rapidly absorbed, accumulated with 4 weeks of treatments. No clear therapeutic response was observed in patients with relapsed/refractory hematological malignancies. Those with relapsed/refractory solid tumors, showed low pSTAT3 in PBMC	[291, 455]
STAT3 decoy	STAT3 DBD	HNSCC	Phase 0	Expression levels of STAT3 target genes were decreased in head and neck cancer patients following intra-tumoral injection	[292]
ISIS-STAT3Rx (AZD9150)	STAT3	Advanced metastatic HCC, Advanced cancer, malignant lymphoma, people with malignant ascites, adult subjects with diffuse large B-cell lymphoma, relapsed metastatic HNSCC (with MEDI4736)	Phase I/ Phase Ib	AZD9150 (ISIS-STAT3Rx) showed single-agent antitumor activity in patients with highly treatment-refractory lymphoma and NSCLC	[293]
Pyrimethamine	STAT3	Relapsed chronic lymphocytic leukemia, small lymphocytic lymphoma	Phase I, Phase II	Phase I: to determine the maximum tolerated dose and recommended Phase II dose of pyrimethamine in relapsed CLL/SLL	[283]
Simvastatin	HMG-CoA, JAK2, STAT3, AKT, ERK	Refractory and/or relapsed solid or CNS tumors of childhood	Phase I	Define toxicity and evaluate cholesterol levels and IL-6/STAT3 pathway changes as biomarkers of patient response	[294–296]

E estimated from descriptive data on inhibition from corresponding reference, *NA* not available, *Ab* antibody, *SM* small molecule, *pY* STAT3 phosphorylation at Tyr-705, *DM* dimerization, *NT* nuclear translocation, *DB* DNA-binding, *GT* gene transcription
C indicates completed, *MM* Multiple Myeloma, *HNSCC* head and neck cell squamous cell carcinoma, *BC* breast cancer, *GvHD* Graft vs Host disease, *BSE-SFN* Brocholi Sprout Extract-sulforaphane

studies undertaken in various cancers with this agent, six have been completed, five are still ongoing, and five have been either terminated or withdrawn because of lack of efficacy. IL-6 signaling inhibition using the IL-6R monoclonal antibody, tocilizumab, has shown promising results in rheumatoid arthritis and related diseases in approximately 230 trials [304] and is being evaluated in patients with cancers, including multiple myeloma, both as an anti-myeloma therapy and as a method to reduce GvHD after allogeneic stem cell transplant (SCT), as well as in recurrent ovarian cancer as adjuvant with carboplatin/doxorubicin [260, 261]. Preliminary analysis of the ongoing trial shows that immune reconstitution was preserved in recipients of tocilizumab and there was a reduced incidence of grade 2–4 acute GvHD [261]. A completed phase I trial combining carboplatin/doxorubicin with tocilizumab and IFNα2b in patients with recurrent epithelial ovarian cancer (EOC) revealed that functional IL-6R blockade is feasible and safe in EOC patients treated with carboplatin/doxorubicin, using 8 mg/kg tocilizumab [260], and the combination was recommended for phase II evaluation based on immune parameters.

Approximately 50 trials with the JAK inhibitor, ruxolitinib, in many different cancer indications are underway and a few completed ones show some encouraging results in myelofibrosis [305], but toxicity remains an issue. In phase III clinical studies, ruxolitinib provided rapid and durable improvement of myelofibrosis-related splenomegaly and symptoms irrespective of mutation status, and was associated with a survival advantage compared with placebo or best available therapy. But because of dose-dependent cytopenias, blood count monitoring and dose titrations were recommended [266]. The JAK2 mutation (c.1849G>T; p.V617F) causes constitutive activation of Janus kinase (JAK)2 and dysregulated JAK signaling in myelofibrosis (MF), polycythemia vera (PV), and essential thrombocythemia (ET). Interestingly, in the phase III Controlled Myelofibrosis Study, patients with MF not only achieved significant reductions in splenomegaly and improvements in symptoms with ruxolitinib vs. placebo but 26/236 patients carrying the allele, also had their mutation burden lowered [306]; 20 achieved partial and 6 achieved complete molecular responses, with median times to response of 22.2 and 27.5 months [306]. The phase I study [41] with AZD1480, a JAK inhibitor, in 38 patients with advanced solid tumors, revealed rapid absorption and elimination with minimal accumulation after repeated daily or twice daily dosing. Pharmacodynamic analysis of circulating granulocytes demonstrated maximal reduction of pY-STAT3 within 1–2 h after dose, coincident with C_{max}, and greater reduction at higher doses. The average reduction in pY-STAT3 levels in granulocytes at the highest dose tested (70 mg daily), was 56 % at steady-state drug levels. Dose-limiting toxicities (DLTs) included pleiotropic neurologic adverse events (AEs), like dizziness, anxiety, ataxia, memory loss, hallucinations, and behavior changes. The trial had to be stopped because of toxicity.

Another JAK inhibitor that showed the best potency in pre-clinical studies, OPB-31121 [274–276], demonstrated insufficient antitumor activity in patients with hepatocellular carcinoma (HCC) in a clinical trial [273]. In an open-label, dose-escalation, and pharmacokinetic study of OPB-31121 in subjects with advanced solid tumor observed that twice-daily administration of OPB-31121 was feasible up to doses of 300 mg. The pharmacokinetic profile, however, was unfavor-

able and no objective responses were observed [273]. A similar study in advanced HCC also came up with the same result [273]. Furthermore, peripheral nervous system-related toxicities were experienced, which may limit long-term administration of OPB-31121 [273].

A very recent interventional study will evaluate the effect of sulforaphane from broccoli sprout extract (BSE-SFN) as a candidate natural chemopreventive agent which is known to modulate key steps in melanoma progression and STAT3 mediated gene transcription [307, 308] in melanocytic and stromal elements of 18 melanoma patients with at least two atypical nevi of ≥4 mm diameter and those who have not received any form of systemic antineoplastic treatment for melanoma within the last year before recruitment, The primary outcomes that will be measured are (i) adverse events associated with oral sulforaphane, (ii) visual changes of atypical nevi size, border and color and (iii) the cellular changes.

Another new trial examines the safety and efficacy of the JAK2 inhibitor, pacritinib, for patients with AML in combination with either decitabine or cytarabine. Pacritinib has been shown to work through inhibition of STAT3 and STAT5 [284]. Pacritinib is an active agent in patients with myelofibrosis (MF), offering a potential treatment option for patients with preexisting anemia and thrombocytopenia. It demonstrated a favorable safety profile with promising efficacy in phase I studies in patients with primary and secondary MF. A subsequent multicenter phase II study demonstrated efficacy [285]. Out of 26 evaluable patients who either had clinical splenomegaly poorly controlled with standard therapies or were newly diagnosed with intermediate- or high-risk Lille score, 8 patients (31 %) achieved a ≥35 % decrease in spleen volume (MRI) and 42 % on the whole attained a ≥50 % reduction in spleen size by physical examination. Grade 1 or 2 diarrhea (69 %) and nausea (49 %) were the most common treatment-emergent adverse events. The study drug was discontinued in 9 patients (26 %) due to adverse events (4 severe).

STAT3-decoy oligonucleotides (ODN) targeting the STAT3 DBD [292] and STAT3 siRNA based formulations [293] are the only direct STAT3 inhibitors that are in clinical trial for a cancer indication. Expression levels of STAT3 target genes were decreased in head and neck cancer patients following intratumoral injection with the STAT3 decoy compared with tumors receiving saline control in a phase 0 trial [292]. While intratumoral administration clearly shows target inhibition, it should be noted that there is no clear evidence that the same level of efficacy would be attained if the ODN were systemically administered. Therefore, it would be interesting to assess the effectiveness of this and the subsequent cyclic ODNs, on tumor STAT3 activity when delivered systemically in patients. Considering that effective and safe systemic intracellular delivery remains a challenge in this field it appears that there still remain some obstacles that have to be overcome before ODNs realize their full clinical potential as STAT3-targeting therapeutic agents.

STAT3 antisense based AZD9150 (ISIS-STAT3Rx) showed single-agent antitumor activity in patients with highly treatment-refractory lymphoma and NSCLC in a phase 1 dose escalation study. Of the 25 patients enrolled (12 advanced lymphoma; 7 with DLBCL, 2 Hodgkin's lymphoma, 2 follicular non-Hodgkin's lymphoma, 1 mantle cell lymphoma), 44 % (11/25) achieved stable disease (SD) or a

partial response (PR); three of six patients (50 %) with treatment-refractory DLBCL had evidence of tumor shrinkage and two patients (33 %) achieved a confirmed durable PR [293]. The only NSCLC patient evaluated showed evidence of near-complete resolution of highly treatment refractory NSCLC liver metastasis upon first restaging, with additional stabilization of mediastinal lymph nodes in response to AZD9150 treatment (3 mg/kg) [293]. The maximum tolerated dose (MTD) of AZD9150 was determined to be 3 mg/kg. A rapidly evolving thrombocytopenia (in the first month of dosing) was observed in two of nine patients at 4 mg/kg and was considered the dose-limiting toxicity (DLT). A more chronic slowly progressing thrombocytopenia also occurred after 4–6 months of dosing at 2 and 3 mg/kg (and for most patients at 4 mg/kg) and was effectively managed with pauses and dose frequency adjustments. The slowly progressing thrombocytopenia seen in patients at or below the MTD is consistent with the reported role of STAT3 in megakaryopoiesis [309, 310], whereas the rapidly progressing thrombocytopenia seen above the MTD was of uncertain etiology. Other drug-related adverse events included aspartate aminotransferase (AST) elevation (44 %), alanine aminotransferase (ALT) elevation (44 %). Responses have also been seen in the DLBCL study. Dose escalation continues in the HCC study and knockdown of STAT3 in peripheral blood mononuclear cells (PBMCs) has been shown. IONIS-STAT3Rx, a variant of AZD9150 is also being examined for safety in patients with advanced cancers.

Tumor-induced STAT3 generates an immunosuppressive microenvironment and, therefore, has become a promising target for cancer therapy. Based on this premise, an ongoing clinical trial is investigating the effects of the antiparasitic drug, pyrimethamine, an inhibitor of STAT3 [283], in chronic lymphocytic leukemia (CLL) patients. Interestingly, pyrimethamine does not affect STAT3 phosphorylation [283] but does affect transcription of STAT3 gene targets.

Another re-purposed STAT3-inhibitor, simvastatin, an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) [294–296] is being tested in a phase I trial in combination with topotecan and cyclophosphamide for refractory and/or relapsed solid or CNS tumors of childhood. HMG-CoA reductase inhibitors, or "statins", lower LDL (low density lipoprotein) cholesterol by inhibiting cholesterol biosynthesis. Statins also have been found to decrease the incidence of cancer [311, 312]. Statins have been shown to inhibit IL-6 mediated STAT3 activation and prevent recruitment of pro-inflammatory cells to injured heart tissue [313].

In conclusion, most of the inhibitors in trial, which target STAT3 in various cancer indications, belong to the upstream and repurposed inhibitors groups. None of the direct small-molecule STAT3 inhibitors under development has entered clinical trials. Since the pharmacokinetic properties of many of these are not well elaborated, it is difficult to comment on their preparedness to go to the clinics. The most promising in this regard is C188-9. Pharmacokinetic (PK) and toxicity studies in mice, rats, and dogs demonstrated that C188-9 provides excellent plasma exposures following oral administration and revealed no toxicity detectable by gross, microscopic or clinical laboratory evaluations when administered up to a dose of 100 mg/kg/day for 28 days in dogs, and up to a dose of 200 mg/kg/day for 28 days in rats [96]. Tumor PK studies of C188-9 in mice at 10 mg/kg demonstrated tumor

levels twice those of plasma levels and nearly 3 times the IC_{50} for pSTAT3 inhibition [96]. C188-9 inhibits growth and survival of many types of cancer cells *in vitro*, including AML [95, 97], NSCLC [99], breast cancer (Dobrolecki et al. 2016, manuscript in preparation), and HNSCC [96] and inhibits the growth of NSCLC and HNSCC xenografts *in vivo* [96, 99].

5.5 Conclusion

Due to the essential contributions of STAT3 to virtually all the hallmarks of cancer, numerous approaches have been applied to identify molecules that effectively block STAT3 signaling to treat and/or prevent cancer, including peptidomimicry, *de novo* rational design, screening chemical libraries *in silico* and *in vitro*, and FBDD. Despite these efforts, few specific and selective STAT3 inhibitors with optimal anti-STAT3 activity have garnered the requisite pharmacokinetic and pharmacodynamic credentials to proceed to clinical trials. Some authors have stated that, unlike small enzymatic clefts, the STAT3:STAT3 dimer represents a protein-protein interaction that involves too large a surface area [86] to be effectively targeted by small, drug-like molecules [314]. These interaction surfaces and others involved in STAT3 protein-protein and protein-DNA interaction also are shallow and relatively featureless, as opposed to the well-defined binding pockets seen in enzyme active sites, thereby making the designing difficult [315]. In addition, the binding regions of STAT3 protein–protein or DNA–protein interactions are often non-contiguous, making mimicry of these domains difficult to accomplish for simple peptides or peptidomimetics [314]. Yet, several small-molecule STAT3 inhibitors are under development, which have good binding affinity for STAT3, potent STAT3 inhibitory activities, and a good safety profile. If these compounds fail to progress into drugs, efforts need to continue in this area of drug development as the impact of having an effective STAT3 inhibitor available in the clinic to treat and/or prevent many cancers will be substantial. Future strategies directed toward the identification of new small-molecule STAT3 probes should combine conventional screening-based strategies with FBDD and structural analytical tools, such as NMR analysis.

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