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## Small Molecule Inhibitors of Signal Transducer and Activator of Transcription 3 (Stat3) Protein

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### 1. INTRODUCTION

Signal transducers and activators of transcription (STATs) are 79–113 kDa proteins playing dual roles as signal transducers and transcription factors. At least seven members have been identified in this family, including Stat1, Stat2, Stat3, Stat4, Stat5A, Stat5B, and Stat6. Although the Stat proteins are structurally related, they participate in different cellular processes.<sup>1,2</sup> Among the members in the STAT family, Stat3 has received particular attention and is the most studied member primarily because of its role in cancer progression, inflammation, cardiomyogenesis, ischemia/reperfusion injury, and stem cell self-renewal.

Stat3 was independently discovered and studied by two research groups and described in 1994. Akira et al. purified and cloned Stat3 from mouse liver nuclear extracts, named it as acute-phase response factor (APRF), and also identified Stat3 as a DNA-binding factor that selectively binds to the IL-6-responsive element within the acute-phase gene promoter.<sup>3</sup> Zhong et al. discovered Stat3 as a DNA-binding protein in response to epidermal growth factor.<sup>4</sup> Since then, multiple Stat3 isoforms have been identified, including the long form Stat3 $\alpha$ , the truncated forms Stat3 $\beta$  and Stat3 $\gamma$ , and a putative novel form Stat3 $\delta$ ,<sup>5</sup> all derived from a single gene located within chromosome 17q21 via alternative splicing of the transcript's 3' end.<sup>5</sup> Stat3 $\alpha$  (p92), a 770 amino acid protein, is the predominantly expressed form of Stat3 in most cell types.<sup>6</sup> Stat3 $\beta$  (p83) is an alternatively spliced RNA form of Stat3 $\alpha$ , in which the 55 C-terminal amino acids of the transactivation domain are replaced by seven distinct amino acids. Stat3 $\beta$  was generally regarded as a dominant negative Stat3 isoform<sup>7</sup> until recent *in vivo* experimental evidence showed that Stat3 $\beta$  rescued the embryonic lethality of a Stat3-null mutation and was capable by itself of activating the expression of Stat3 target genes.<sup>8</sup> Compared with Stat3 $\alpha$  and Stat3 $\beta$ , the physiologic roles of Stat3 $\gamma$  and Stat3 $\delta$  are less clear. Stat3 $\gamma$  (p72) is another C-terminal truncated form of Stat3 $\alpha$  derived post-translationally through limited proteolysis. Stat3 $\gamma$  is primarily activated in terminally differentiated neutrophils.<sup>9</sup> Stat3 $\delta$  exists at low levels and decreases with cell differentiation.<sup>5</sup>

In this review, we summarize the signaling pathways of Stat3, its role in different diseases as well as in stem cell maintenance, and the progress in the design, discovery, and development of Stat3 inhibitors since 2006.

### 2. STAT3 PROTEIN STRUCTURE

The Stat3 $\beta$  structure consists of a coiled coil, a DNA binding, a linker, as well as an SH2 domain and lacks the N-terminal cooperative and C-terminal transactivation domains (Figure 1a). Currently, two crystal structures of mouse Stat3 $\beta$  (1BG1

and 3CWG) are available in the Protein Data Bank.<sup>10,11</sup> 1BG1 consists of a Stat3 $\beta$  homodimer bound to its DNA target sequence with a 2.25 Å resolution (Figure 1b).<sup>10</sup> The phosphorylated Tyr705 along with neighboring residues (702–709) in each monomer (amino acid residues 136–716) is bound to the Src homology 2 (SH2) domain in the other monomer. The SH2 domain comprises three subpockets that can be targeted by small-molecule inhibitors. Residues Lys591, Arg609, Ser611, and Ser613 from the SH2 domain are involved in polar interactions with phospho-Tyr705 (Figure 1c). Leu706 of the phosphopeptide is bound to a hydrophobic pocket of the SH2 domain. Four loops, three from the DNA binding domain and one from the linker domain, form interactions with both DNA strands.<sup>10</sup> Figure 1d shows that the unphosphorylated Stat3 core fragment comprising amino acid residues 136–688 is loosely bound through the SH2 domain (PDB code 3CWG).<sup>11</sup> In the 3CWG crystal structure, Stat3 does not bind DNA because the DNA binding domains are away from each other and are in reverse orientation compared to the 1BG1 structure. The root-mean-square deviation (rmsd) value for C $\alpha$  atoms of these two crystal structures (core fragment of monomers) is only 0.9 Å, suggesting minor conformational changes between the phosphorylated and unphosphorylated forms.<sup>11</sup>

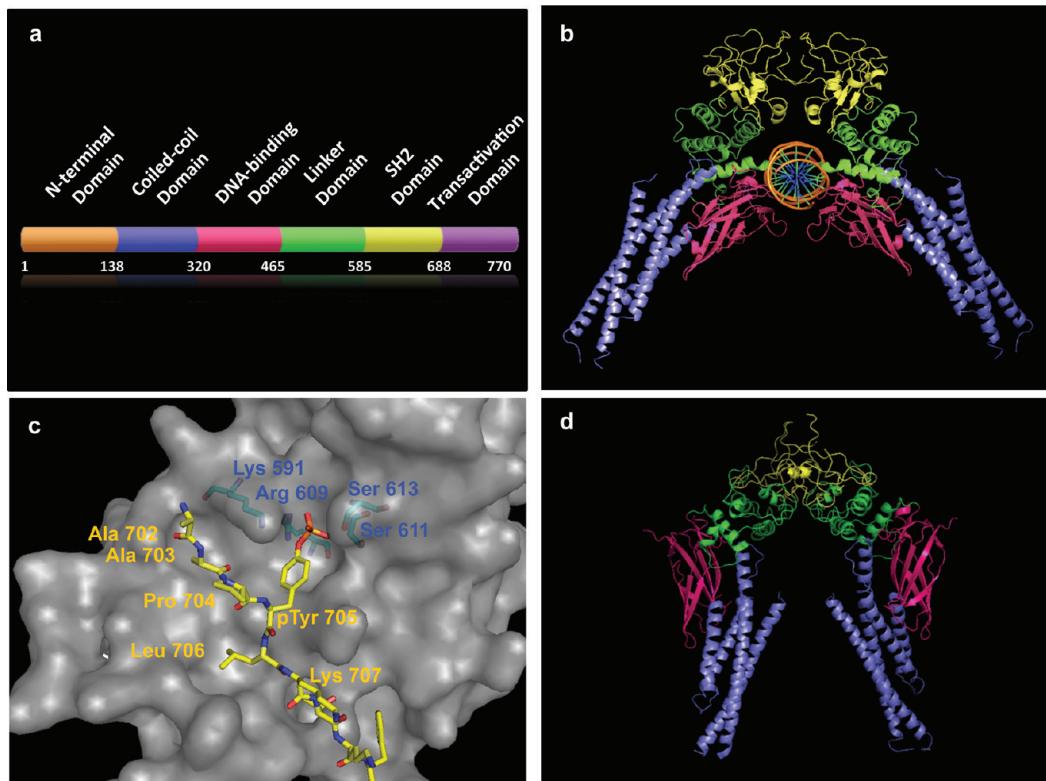
### 3. STAT3 SIGNALING PATHWAY

As part of the Janus kinase (JAK) Stat pathway, Stat3 signaling can be activated by both receptor and nonreceptor tyrosine kinases via the tyrosine phosphorylation cascade (Figure 2). The growth factor receptors that are known to cause the activation of Stat3 include epidermal growth factor receptors (EGFRs), human epidermal growth factor receptor (HER2, also known as NEU), fibroblast growth factor receptors (FGFRs), insulin-like growth factor receptors (IGFRs), hepatocyte growth factor receptors (HGFRs, also known as MET), platelet-derived growth factor receptors (PDGFRs), and vascular endothelial growth factor receptors (VEGFRs). After autocrine or paracrine cytokines/growth factors binding to their respective receptors, they undergo homo- or heterodimerization leading to the subsequent activation of intrinsic receptor tyrosine kinases that culminate in the phosphorylation of Stat3 at Tyr705. For receptors lacking intrinsic tyrosine-kinase activity, ligand engagement induces recruitment and activation of receptor-associated tyrosine kinases, such as JAK and SRC. Subsequently, JAK and SRC proteins phosphorylate certain tyrosine residues in the intracellular domain of the receptor, creating docking sites for cytosolic Stat3 via its SH2 domain.

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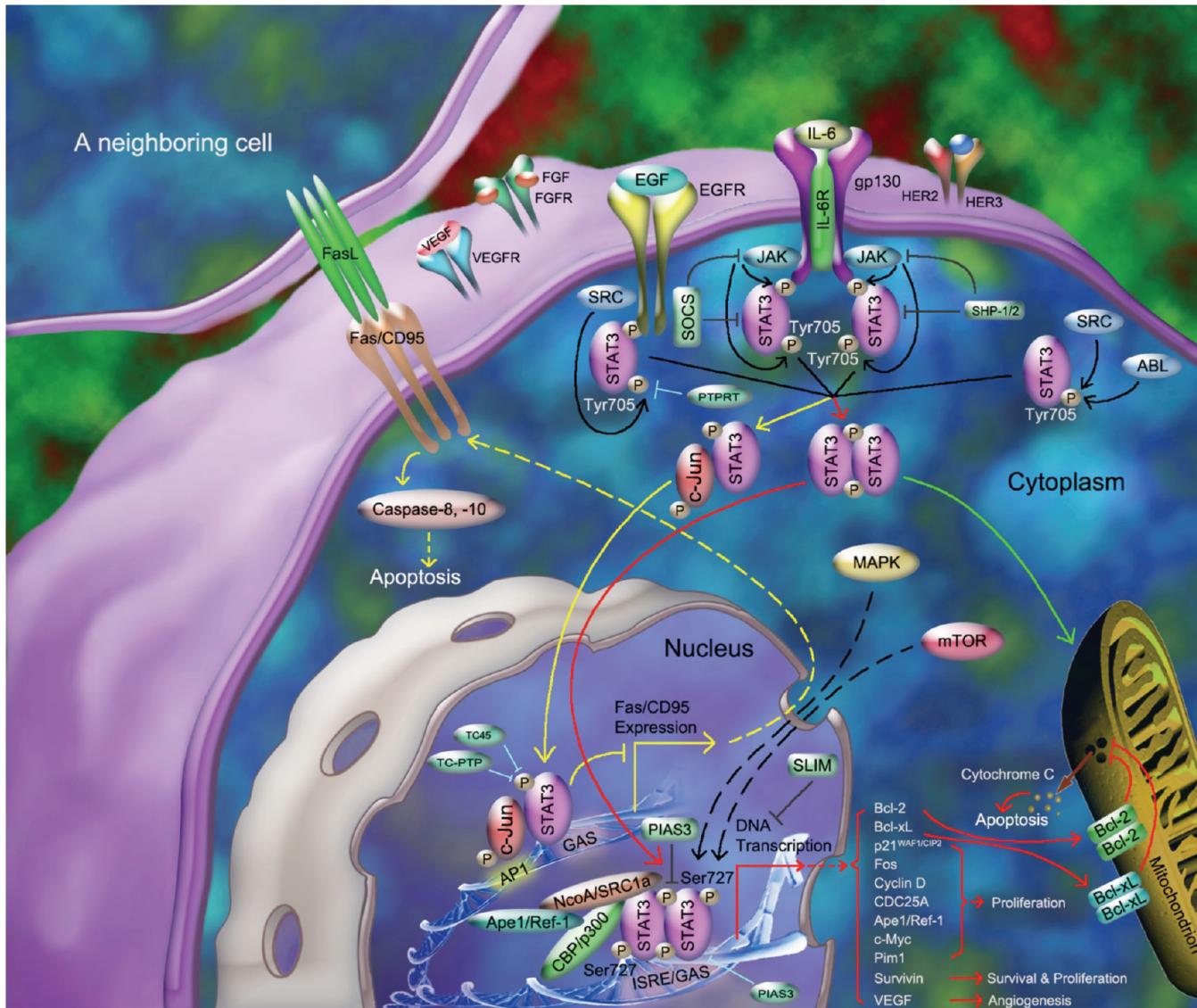
**Figure 1.** Structural organization of Stat3: (a) structural domains of Stat3; (b) structure of Stat3- $\beta$  homodimer bound to its DNA recognition site (PDB code 1BG1); (c) Stat3 phosphopeptide (yellow) bound in the Stat3 SH2; (d) structure of Stat3 core fragment homodimer (PDB code 3CWG).

Once recruited, Stat3 becomes phosphorylated at Tyr705 by JAK or SRC. In addition to JAK and SRC proteins, other classes of nonreceptor protein tyrosine kinases have also been reported to stimulate Stat3 activation. One example is Fes that activates Stat3 in human cells and Sf-9 insect cells, suggesting Stat3 may be a direct substrate for Fes.<sup>12</sup> In transformed cells, Stat3 can also be directly activated by constitutively active nonreceptor tyrosine kinases, such as SRC and ABL.<sup>13</sup>

Tyr705 phosphorylation converts Stat3 from an inactive form to an active form. Following phosphorylation and activation, Stat3 proteins undergo dimerization via the interaction between the phospho-Tyr705 within one monomer and the SH2 domain within the other. Stat3 dimers then disassociate from the receptor and translocate into the nucleus, where they bind to specific DNA sequences such as the cis element IFN-stimulated response element (ISRE) and regulate the transcription of target genes. In addition, Ser727 located in the transactivation domain of Stat3 is further activated by the MAPK or mTOR pathways regulating Stat3 transcriptional activity<sup>14</sup> and is suggested to be necessary for its full activation.<sup>15</sup> Besides phosphorylation at Tyr705 and Ser727, Stat3 activity is also regulated by acetylation at Lys685 that stabilizes Stat3 dimers.<sup>16</sup> Stat3 transcriptional activity requires the association with coactivators such as CREB-binding protein (CBP)/p300, APE1/Ref-1, and NCOA/SRC1a.<sup>17,18</sup> The interaction of Stat3 with c-Jun has also been reported to yield maximal enhancer function for a number of genes, including the gene encoding the interleukin-6 (IL-6) induced acute-phase response protein  $\alpha$ 2-macroglobulin.<sup>19</sup> Additionally, Stat3 activates the transcription of several genes involved in cell cycle progression such as Fos, p21<sup>WAF1/CIP2</sup>, cyclins D1, D2, D3, CDC25A, APE1/Ref-1, c-Myc, and Pim1, genes involved in

angiogenesis such as VEGF, and antiapoptotic genes such as survivin, Bcl-2, and Bcl-xL. Indeed, most Stat3 target genes are key components in the regulation of cell growth, transformation, cell cycle progression, survival, metastasis, and invasion. Apart from gene transcriptional promotion, Stat3 also plays a role in transcriptional suppression. Through its cooperation with c-Jun, active Stat3 down-regulates Fas/CD95 transcription as well as Fas surface expression, which may be mediated by PI3K-AKT signaling.<sup>20</sup> Recently, in vitro and in vivo data further showed that knockdown of Stat3 using Stat3-siRNA in breast cancer resulted in up-regulation of Fas and FADD expression.<sup>21</sup> These findings suggest that Stat3 activities can also be mediated by suppressing the expression of some proapoptotic proteins. It is important to mention that Stat3 not only regulates gene transcription but also participates in some transcription-independent cellular processes.<sup>22</sup> In addition to the well-known localization of Stat3 in the cytosol, associated with receptors at the plasma membrane and in the nucleus, Stat3 was also discovered to be present in isolated mitochondria, suggesting that Stat3 exerts a direct impact on mitochondrial function.<sup>23</sup>

In normal cells, and under physiological conditions, Stat3 activation is a tightly controlled transient process, lasting from 30 min to several hours. Upon inactivation via tyrosine dephosphorylation by nuclear protein-tyrosine phosphatases such as TC-PTP and TC45,<sup>23,24</sup> nuclear Stat3 shuttles back to the cytoplasm through nuclear pores. Apart from the nuclear protein-tyrosine phosphatases, several other negative regulatory mechanisms normally serve to suppress Stat3 activation at different levels of the signal transduction pathway. Upon binding of the correspondent ligands, cytokine or growth factor receptors are rapidly internalized and subsequently recycled or

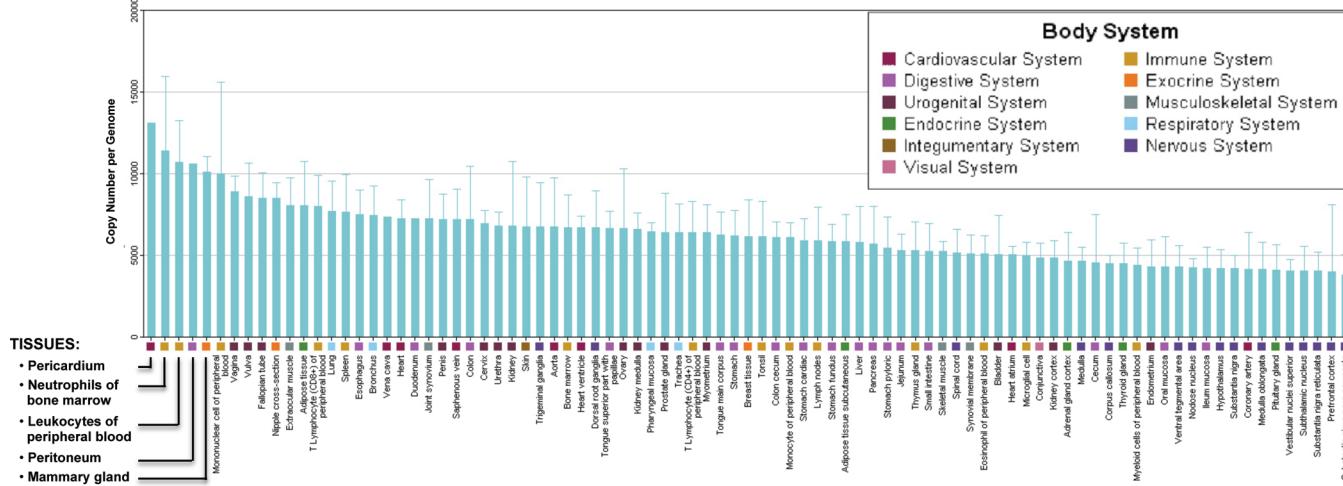
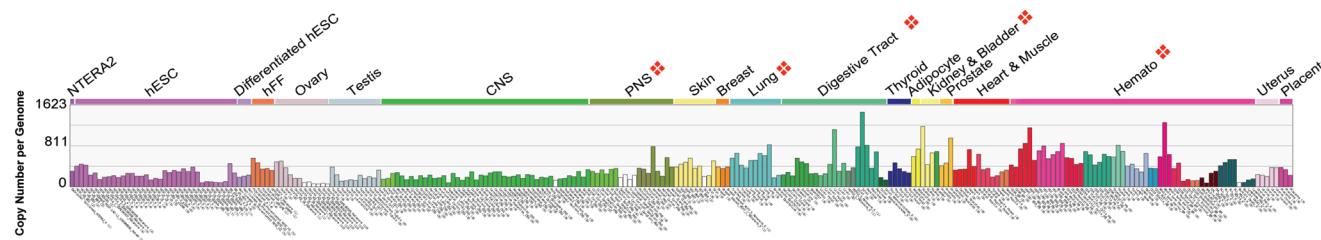
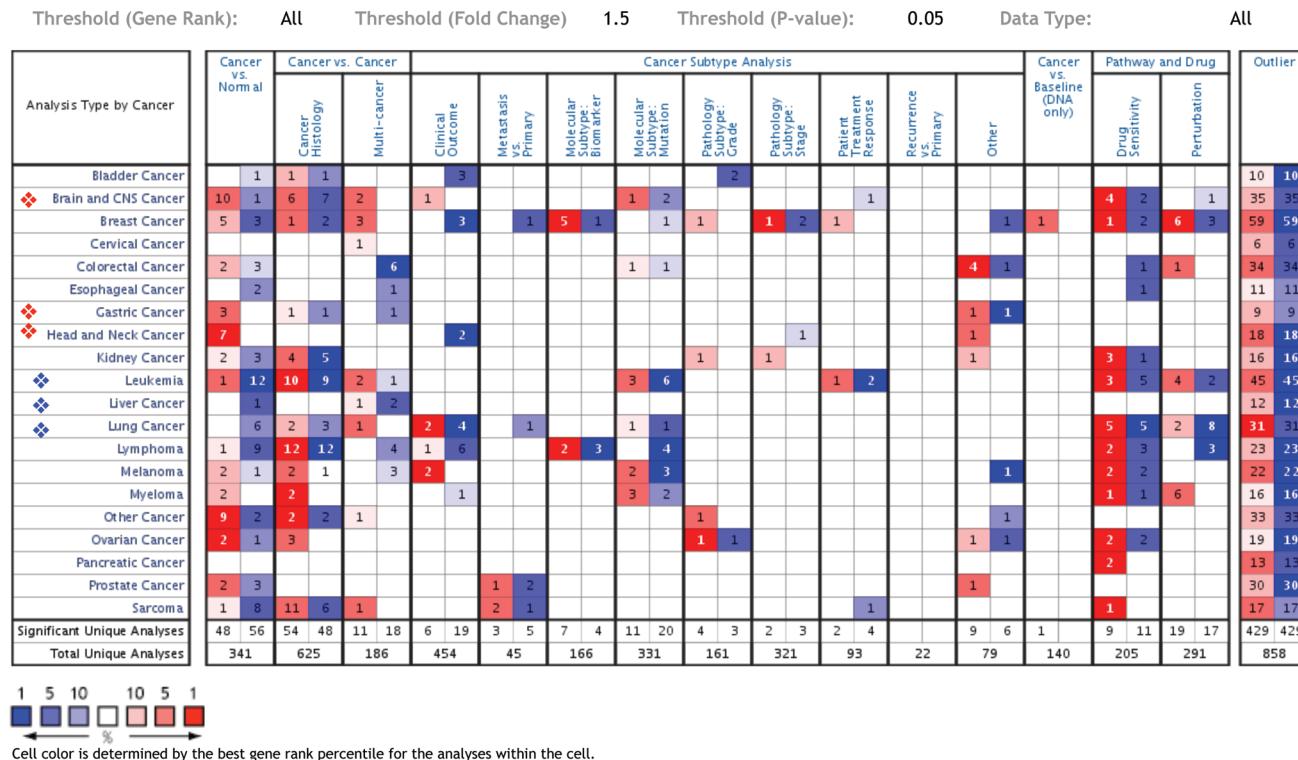


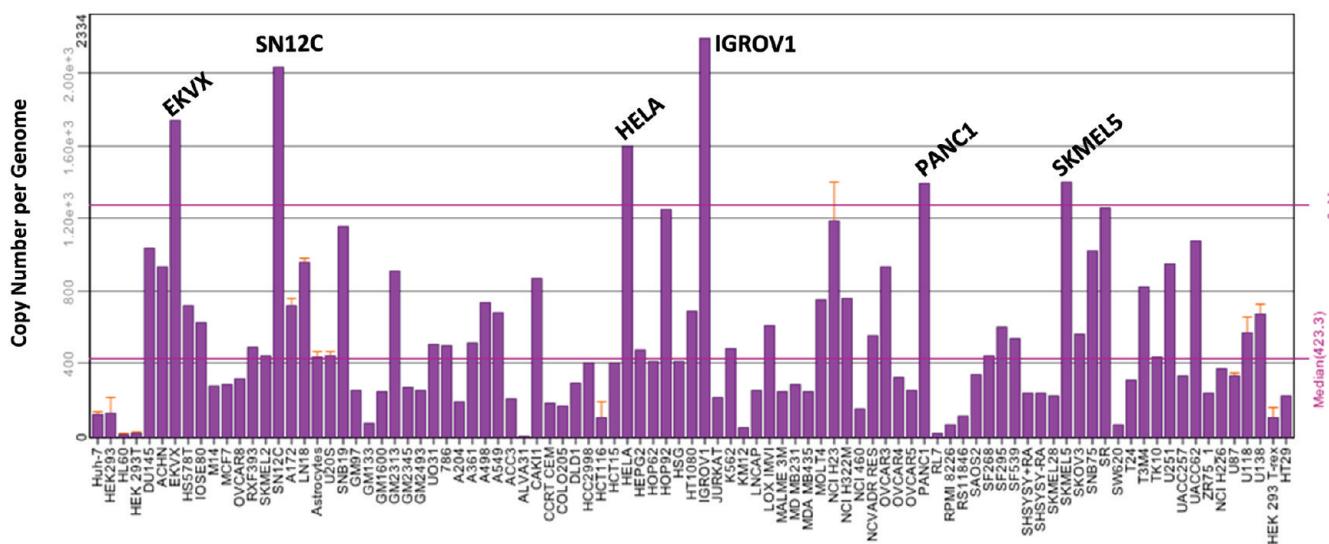
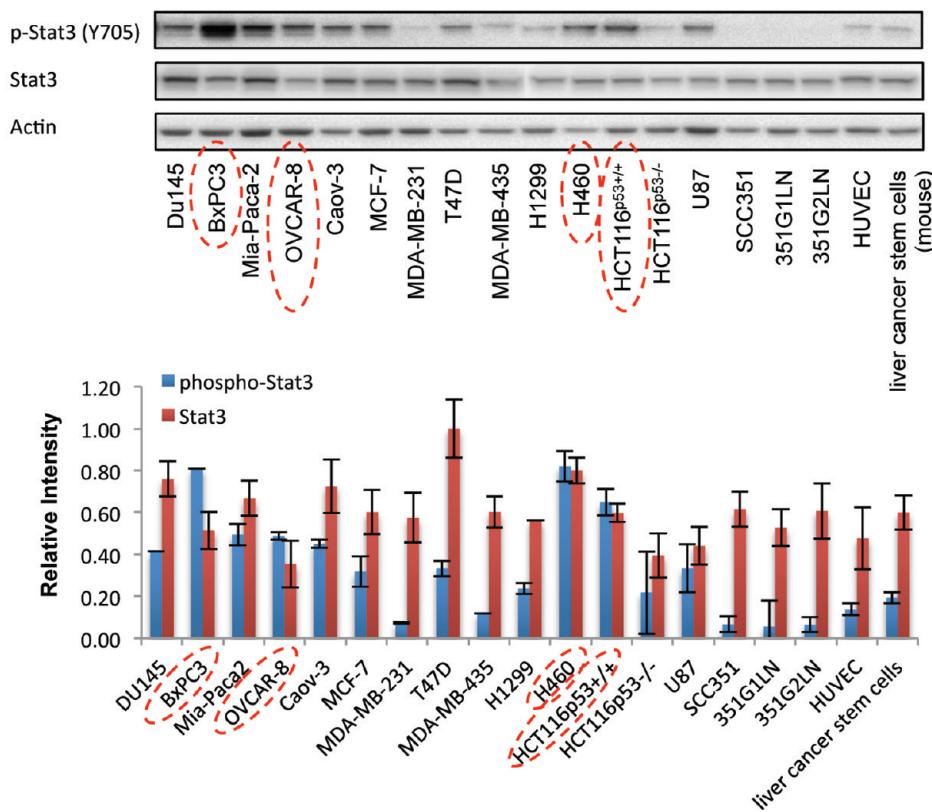
**Figure 2.** Stat3 signaling pathway. Upon cytokines binding to and activating respective receptors, Stat3 is recruited to the cytoplasmic domain of the receptors, where it is phosphorylated and activated. Phosphorylated Stat3 detaches from the cell surface and forms homo- or heterodimers, which translocate to the nucleus and regulate gene expression. Stat3 activation is negatively regulated by SOCS, PIAS, and SHP-1/2.

degraded by the ubiquitin proteasome pathway.<sup>25</sup> The Src homology domain-containing tyrosine phosphatases 1/2 (SHP-1/2) interact with the intracellular domain of cytokine receptors and dephosphorylate and inactivate JAK, Stat3, and the receptor.<sup>26,27</sup> In addition, active Stat3 binds to the promoter regions of suppressor of cytokine signaling (SOCS) genes.<sup>28</sup> Similar to SHP-1/2, SOCS-1 and SOCS-3 interact with the kinase domain of various JAK proteins or the phosphotyrosine residues in the intracellular portion of the respective receptor, leading to the reduction of Stat3 activation. This is another negative feedback mechanism of the Stat3 signaling pathway and its upstream kinases. However, SOCS-3 does not affect SRC-induced Stat3 activation.<sup>29</sup> Besides SHPs and SOCSs, protein inhibitor of activated Stat3 (PIAS3) specifically inhibits the binding of dimerized phosphorylated Stat3 to DNA, thus abolishing the transcription of Stat3 target genes.<sup>30</sup> Recently, the protein tyrosine phosphatase receptor T (PTPRT) has been shown to specifically dephosphorylate Stat3 at Tyr705 and thereby regulates its cellular localization as well as Stat3 target gene expression.<sup>31</sup>

#### 4. STAT3 AND CANCER

Accumulating evidence in the past few years strongly implicates the critical role of aberrant Stat3 activation in malignant transformation and tumorigenesis. In contrast to the transient nature of Stat3 activation in normal cells, overexpression and/or persistent activation of Stat3 has been reported in most human solid and hematological tumors,<sup>32</sup> including ovarian, endometrial, cervical, breast, colon, pancreatic, lung, brain, renal, and prostate cancers, head and neck squamous cell carcinoma (HNSCC), glioma, melanoma, lymphomas, and leukemias. The expression and activation levels of Stat3 in select cancer and normal tissues as well as cell lines are shown in Figure 3. Stat3 is significantly expressed in some normal tissues including pericardium, neutrophils of bone marrow, leukocytes of peripheral blood, peritoneum, and mammary gland (Figure 3a) and in systems including digestive tract, kidney, bladder, hematopoietic, lung, and peripheral nervous system (PNS) (Figure 3b), indicating that Stat3 plays an important role in the physiological processes in these tissues

**a****b****c**

**d****e**

**Figure 3.** Stat3 expression and activation in human normal and cancer tissues and cell lines. Stat3 levels in various body tissues are displayed (a) from highest to lowest (image generated using NEXTBIO, <http://www.nextbio.com><sup>170</sup>) and (b) in groups of tissues (image generated using Amazonia!, <http://amazonia.transcriptome.eu><sup>171</sup>). (c) Comparison of the expression levels of Stat3 in selected cancers and respective normal tissues. Image is generated using Oncomine (Compendia Bioscience, Ann Arbor, MI) analysis and visualization. (d) Comparison of the expression levels of Stat3 in NCI-60 human cancer cell lines. Image is generated using BioGPS, <http://biogps.org/#goto=welcome>.<sup>172</sup> (e) Stat3 expression and phosphorylation at Tyr705 in indicated cell lines analyzed by immunoblotting. Bar indicates SEM.

and organs. Compared with normal tissues, Stat3 is overexpressed in brain and central nervous systems (CNS), head and neck, and gastric cancers but down-regulated in leukemia, liver and lung cancers, lymphomas, and sarcomas (Figure 3c). Among the NCI-60 cell lines, IGROV1, SN12C, EKVX, HELA, PANC1, and SKMEL5 display the highest mRNA levels of Stat3 (Figure 3d). Compared with total Stat3 expression,

phospho-Stat3 (Y705) levels are more functionally related to tumorigenesis. Unlike the transient activation of Stat3 in normal cells, constitutively active Stat3 is present in most human cancer cell lines, especially H460, OVCAR-8, BxPC3, and HCT116<sup>p53+/+</sup> (Figure 3e). Studies have demonstrated that expression of mutant Stat3 is able to induce oncogenesis in cultured cells and nude mice.<sup>33</sup> While the mechanisms of

constitutively active Stat3 in tumor formation continue to be elucidated, studies on different cancers implicate dysregulation of Stat3 as a key participant in cancer cell growth, proliferation, survival, angiogenesis, metastasis, and invasion and are often correlated with a more malignant tumor phenotype. Additionally, the maintenance of multipotency in glioblastoma stem cells requires receptor signaling through Stat3, the inhibition of which results in suppression of cell growth and induction of differentiation as well as apoptosis,<sup>34</sup> suggesting that Stat3 is also essential for cancer stem cell (CSC) survival. Moreover, active Stat3 plays a role in suppressing the host's immune surveillance of cancer, which also contributes to tumor progression.<sup>35,36</sup> On the basis of this evidence, Stat3 has emerged as a promising drug target for cancer treatment.

## 5. STAT3 AND OTHER DISEASES

While Stat3's role in cancer remains a major topic in biomedical research, compelling evidence has shown that activation or suppression of Stat3 signaling has a significant contribution in the progression or remission of a series of other human diseases, including fibrosis in kidney, lung, and liver, cardiovascular diseases, rheumatoid arthritis (RA), Alzheimer's disease (AD), psoriasis, and inflammatory bowel disease (IBD) (Table 1).

**Table 1. Levels of Stat3 in Pathological Phenotypes**

disease condition	stat3 level	ref
cardiomyopathy	↓ pStat3	38
myocardial ischemia/reperfusion injury	↑ pStat3	2
rheumatoid arthritis	↑ pStat3	39
cancer	↑ pStat3	32, 35, 36, 139, 147–165
Alzheimer's disease	??	42
renal fibrosis	↑ pStat3	45
liver fibrosis	↓ pStat3	46, 47
inflammatory lung diseases/pulmonary fibrosis	↑ pStat3	48, 49
psoriasis	↑ pStat3	52, 166, 167
inflammatory bowel disease	↑ pStat3	56
obesity and diabetes type 2	↓ pStat3	57, 58
atherosclerosis	↑ pStat3	60

**5.1. Stat3 and Cardiovascular Diseases.** Stat3 plays an important role in cardioprotection. Increased Stat3 expression was observed in rat myocardium subjected to ischemia compared with sham operated rats. Cardiospecific overexpression of constitutively active Stat3 in transgenic mice provides protection from myocardial ischemia/reperfusion injury,<sup>2</sup> and cardiospecific activation of Stat3 by IL-11 attenuated cardiac fibrosis after myocardial infarction.<sup>37</sup> In contrast, cardiomyocyte-specific deletion of *Stat3* gene causes postpartum cardiomyopathy (PPCM) in female mice, and decreased levels of Stat3 in myocardium are associated with PPCM patients.<sup>38</sup>

**5.2. Stat3 and Rheumatoid Arthritis.** Phosphospecific activation of Stat3 is significantly increased in human peripheral blood fibrocytes derived from RA patients. Survival and abnormal growth of RA synoviocytes are associated with Stat3 activity. Stat3-YF (a dominant negative mutant of Stat3) transduced RA synoviocytes failed to grow and survive with markedly diminished [<sup>3</sup>H]thymidine incorporation.<sup>39</sup> Hyperactivation of Stat3 was observed in RA patients and an RA

mouse model.<sup>40</sup> Mice bearing a single amino acid substitution in gp130 (Y759F) have been reported to exhibit enhanced Stat3 activation that promotes RA development associated with IL-6 stimulation and loss of SOCS3-mediated negative feedback.<sup>41</sup>

**5.3. Stat3 and Alzheimer's Disease.** Tyk2/Stat3 signaling is involved in  $\beta$ -amyloid-induced neuronal cell death in AD. However, whether Stat3 level and activity is up- or down-regulated in AD is still controversial. Elevated levels of phospho-Stat3 (Tyr705) were observed in the cortex and hippocampus of APP/PS1 transgenic mice and in post-mortem brains of AD patients.<sup>42</sup> Overexpression and activation of Stat3 induce  $A\beta$ -mediated neuronal apoptosis and thus cause neuronal cell death.<sup>42</sup> On the other hand, polypeptide humanin and colivelin attenuate AD-related neuronal death by activating Stat3 in vitro. Colivelin ameliorates AD-related memory impairment in vivo by activating Stat3 in the septohippocampal region.<sup>43</sup>

**5.4. Stat3 and Renal Fibrosis.** Activated Stat3 is preferentially overexpressed in rat tubular epithelial cells and myofibroblasts after unilateral ureteral obstruction. Immunohistochemical staining in rat kidney tissues on day 7 showed a 6-fold elevation of Stat3 phosphorylation in tubular epithelial cells and a 2500-fold increase in interstitial cells.<sup>44</sup> Inhibition of Stat3 signaling by a Stat3 inhibitor (blocks Stat3 phosphorylation and dimerization) induced apoptosis in renal fibroblasts of obstructed kidney.<sup>45</sup>

**5.5. Stat3 and Liver Fibrosis.** Stat3 plays a protective role in liver injury and fibrosis in sclerosing cholangitis. Stat3 phosphorylation is significantly down-regulated in cholestatic cirrhosis compared with noncholestatic cirrhosis in adults.<sup>46</sup> Conditional inactivation of Stat3 in hepatic cells was reported to strongly aggravate bile acid induced hepatic injury and fibrosis in mice lacking the multidrug resistance gene 2 (mdr2<sup>-/-</sup>), whereas Stat3-deficient hepatocytes exhibited up-regulation of bile acid biosynthesis and down-regulation of hepatoprotective growth factors.<sup>47</sup>

**5.6. Stat3 and Inflammatory Lung Diseases/Pulmonary Fibrosis.** Several studies have investigated Stat3 in inflammatory lung diseases/pulmonary fibrosis.<sup>48,49</sup> Stat3 was overexpressed in different airway tissues of silica-exposed mice<sup>48</sup> and in lung tissues of chronic obstructive pulmonary disease (COPD) patients.<sup>50</sup>

**5.7. Stat3 and Psoriasis.** Stat3 is involved in epidermal regenerative or stress responses like wound healing and psoriasis.<sup>51,52</sup> During wound healing Stat3 becomes activated, whereas disrupted Stat3 signaling delays healing in mice.<sup>51</sup> On the other hand, Stat3 becomes phosphorylated in lesional keratinocytes in psoriatic patients. Transgenic mice with constitutively active Stat3 in keratinocytes spontaneously developed psoriatic-like skin lesions accompanied by T-cell activation,<sup>52</sup> whereas disruption of Stat3 activity reversed established psoriasis in these transgenic mice.<sup>52</sup> In addition, inhibition of Stat3 by the JAK2 inhibitor WP1066 suppressed psoriasis development in PPAR $\beta/\delta$  transgenic mice.<sup>53</sup>

**5.8. Stat3 and Inflammatory Bowel Disease.** Stat3 regulates intestinal homeostasis. Abnormal Stat3 expression and/or phosphorylation is present in IBD. Stat3 activation is elevated in intestinal epithelial cells from patients suffering from IBD, whereas inhibition of Stat3 delays and suppresses wound healing of intestinal mucosa.<sup>54</sup> There was an over 2-fold increase in the number of phospho-Stat3 positive peripheral blood granulocytes from patients with clinically active IBD

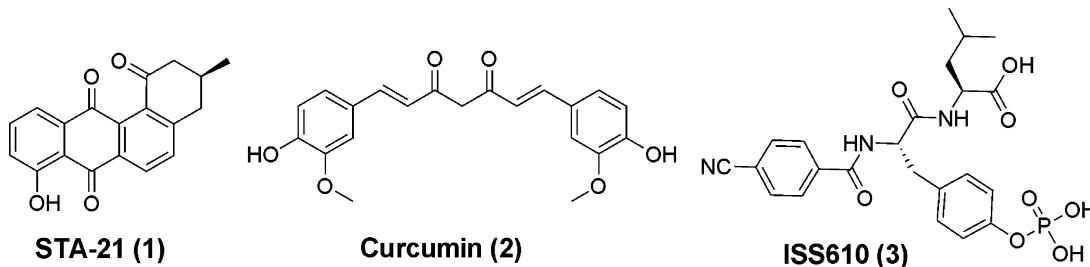


Figure 4. Stat3 inhibitors prior to the year 2006.

compared to those in remission.<sup>55</sup> Elevated expression as well as nuclear translocation of Stat3 is found in Crohn's disease, a type of IBD. Mice treated with Stat3 antisense oligonucleotide showed less colonic tissue damage with reduced proinflammatory cytokines such as TNF- $\alpha$  and INF- $\gamma$  in mucosa in TNBS-induced colitis model.<sup>56</sup>

**5.9. Stat3 and Obesity/Diabetes.** The JAK2/Stat3 pathway in the brain is required for energy homeostasis actions of leptin. Leptin stimulates phosphorylation of Stat3 in hypothalamus. In response to leptin, Stat3 binds the leptin receptor LEPRb and thus is activated to regulate energy homeostasis.<sup>57</sup> Disruption of the Stat3 binding to LEPRb, or deletion of neuronal Stat3, resulted in severe hyperphagia and morbid obesity, as well as hyperglycemia and hyperinsulinemia.<sup>58</sup> Leptin down-regulated fat-mass- and obesity-associated gene in *in vitro* arcuate nucleus of hypothalamus cultures and in mice through activation of the Stat3 signaling pathway.<sup>59</sup>

**5.10. Stat3 and Atherosclerosis.** Elevated phospho-Stat3 level in blood vessels is associated with atherosclerotic lesions. Significantly higher levels of Stat3 activation were observed in endothelium and monocytes in human atherosclerotic lesions.<sup>60</sup> The endothelium-specific Stat3 knockout mice with atherogenic diets exhibited reduced fatty streak formation compared to wild type.<sup>60</sup> Delivery of a human Stat3 gene inhibited aortic structural changes associated with atherosclerosis and down-regulated inflammation/monocyte/macrophage (Mo/MΦ) burden during atherosclerosis, resulting in a significant reduction in atherosclerotic lesions.<sup>61</sup> In addition, there was a significant correlation between pravastatin (a HMG-CoA inhibitor) caused atherosclerotic lesion reduction and decreased Stat3 phosphorylation.<sup>62</sup>

## 6. STAT3 AND STEM CELLS

Stat3 mediates cytokine leukemia inhibitory factor (LIF) induced self-renewal of mouse embryonic stem cells (ESC) in the presence of serum. In the absence of LIF, artificial activation of Stat3 is sufficient to maintain mouse ESC self-renewal.<sup>63</sup> Binding of LIF to its receptor LIFR causes heterodimerization of LIFR and glycoprotein-130 (gp130), resulting in JAK/TK-mediated Stat3 activation and subsequently the expression of Stat3 target genes essential for maintaining the undifferentiated state of mouse ESCs.<sup>64</sup> The interactions between Stat3 and other proteins, such as the transcription factor Nanog<sup>65</sup> and the molecular chaperone heat shock protein 90 (Hsp90),<sup>66</sup> suggest that Stat3 functions in protein complexes to maintain ESC pluripotency. Bone morphogenetic protein (BMP) induction of Id proteins was also documented to collaborate with Stat3 in sustaining ESC self-renewal.<sup>67</sup> Loss of Stat3 function results in ESC differentiation *in vitro* and mouse embryonic lethality (E6.5-7.5) *in vivo*.<sup>68</sup> Recently, Stat3 activation has been demonstrated to be limiting for reprogramming to ground

state pluripotency in both EpiSCs (heterogeneous cell lines established by culture of postimplantation epiblast) and somatic cells.<sup>69</sup> In contrast to the essential role of LIF/Stat3 signaling in mouse ESCs, this pathway fails to maintain self-renewal of human ESC (hESCs) in spite of the presence of LIF and LIFR.<sup>70,71</sup> Besides ESCs, Stat3 has also been documented to regulate hematopoietic stem cell (HSC) regeneration in the early phase,<sup>72</sup> granulocytic differentiation,<sup>5</sup> dendritic cell (DC) development and function,<sup>73</sup> B-cell development,<sup>74</sup> mouse spermatogonial stem cell (SSC) differentiation,<sup>75</sup> and muscle stem cell differentiation.<sup>76</sup>

## 7. STAT3 INHIBITION

As Stat3 plays important pathophysiological roles in various diseases, Stat3 inhibitors are currently under intense development preclinically and clinically in many therapeutic areas. There are two strategies to inhibit the Stat3 signaling pathway: indirectly block the upstream molecules of Stat3 signaling pathway and directly target Stat3 protein. Direct inhibition of Stat3 can be achieved via targeting one of three structural domains of Stat3, (a) SH2 domain, (b) DNA binding domain, and (c) N-terminal domain, which therefore suppresses processes related to Stat3 signaling, such as Stat3 phosphorylation, Stat3 dimerization, nuclear translocation, Stat3-DNA binding, and expression of Stat3 target genes. Ideally, direct Stat3 inhibitors should target Stat3 protein without blocking upstream molecules and other Stat members. Stat3 inhibitors can be broadly classified in two categories: (i) peptide and peptidomimetics; (ii) small molecular nonpeptidic inhibitors.

In our previous review we reported inhibitors of the Stat3 signaling pathway up to the year 2006.<sup>77</sup> In this article we present Stat3 inhibitors not covered in our previous article<sup>77</sup> and new inhibitors reported since 2006. As in our previous review, we have attempted to be inclusive and cite all active inhibitors. Where possible we included pertinent analogues to shed light on potential trends and structure-activity relationships. However, it is important to note that most of the inhibitors need further characterization to be considered true Stat3 inhibitors. An unsettling point is the fact that many of the alleged inhibitors are nonspecific, contain reactive groups, or are not potent in cell-based assays. Future studies will winnow out uninteresting compounds, and a few bona fide inhibitors could be further optimized for clinical use.

**7.1. Small-Molecule Inhibitors.** Small molecules constitute the largest class of Stat3 inhibitors for cancer prevention and treatment.<sup>78-81</sup> To identify novel small molecule inhibitors of Stat3 multiple approaches, such as high-throughput screening of large chemical libraries,<sup>82</sup> virtual screening,<sup>83,84</sup> rational design based on peptides and peptidomimetic inhibitors,<sup>85,86</sup> and fragment-based drug design and drug repositioning using multiple ligand simultaneous docking (MLSD),<sup>87</sup> were used to

disrupt Stat3 phosphorylation, dimerization, nuclear translocation, and/or DNA binding. Several classes of such small molecule inhibitors are discussed below. Unfortunately, there is no uniformity among different publications reporting Stat3 inhibitors in terms of assay systems used to evaluate potency, making comparisons across different classes of inhibitors difficult.

Our previous review covered inhibitors of Stat3 signaling pathway reported prior to 2006.<sup>77</sup> Most of those inhibitors were identified by screening natural product databases, organometallics, peptides, and peptidomimetics. Many of those compounds are not specific inhibitors of Stat3.<sup>77</sup> Figure 4 lists a few selected compounds such as STA-21 (1), curcumin (2), and ISS610 (3)<sup>77</sup> that were further characterized in subsequent years for their Stat3 inhibitory effects or used for lead optimization.

**7.1.1. Compound 1 and Its Analogue.** The first non-peptide small molecule Stat3 inhibitor, compound 1, was discovered through virtual screening.<sup>83</sup> It is a natural product and deoxy analogue of tetragomycin (4) (Figure 5) that was

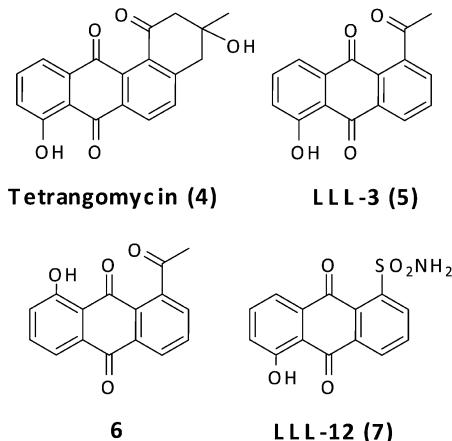


Figure 5. Analogues of compound 1.

originally discovered as an antibiotic isolated from cultures of a variant strain of *Streptomyces rimosus*.<sup>88</sup> Compound 1 inhibited Stat3-dependent luciferase activity in breast and ovarian cancer cell lines. In the MDA-MB-435 cells stably transfected with a Stat3-dependent luciferase reporter pLucTKS3, compound 1 at 20  $\mu$ M inhibited luciferase activity by 5-fold compared to untreated control.<sup>83</sup> It also inhibited Stat3 dimerization, nuclear translocation, Stat3-DNA binding, as well as the expression of Stat3-regulated genes in cancer cells with constitutively active Stat3.<sup>83</sup> Compound 1 at 30  $\mu$ M inhibited Stat3-DNA binding activity in MDA-MB-435s cells.<sup>83</sup> Several analogues (5–7) of compound 1 were also reported to target Stat3 SH2 domain (Figure 5).<sup>89–91</sup> Among these, LLL-3 (5) and 6 along with compound 1 showed antiproliferative activity in cancer cells with persistently active Stat3 (Table 2). The cytotoxic activities of these compounds were directly proportional to Stat3 expression levels from highest in DU145 to lowest or no expression in MCF-7.<sup>89</sup> Acetyl and hydroxyl groups in trans positions favored cytotoxicity. Compound 5 also induced apoptosis in glioblastoma cells by inhibiting Stat3 activities and suppressed tumor growth and metastasis in a glioblastoma xenograft mouse model, resulting in prolonged survival.<sup>92</sup> In addition, compound 5 inhibited cell growth, activated caspases-3/7, and induced apoptosis in K562 cells. It also enhanced the

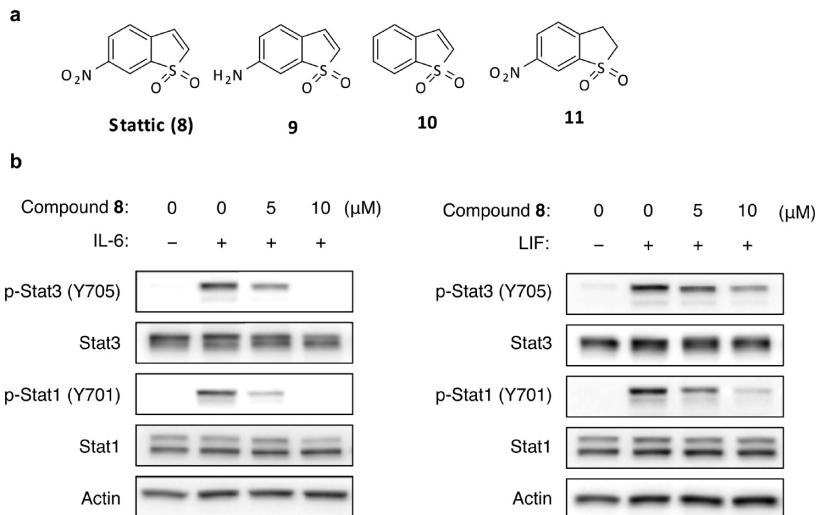
**Table 2. Cytotoxic Activities of Compound 1 and Its Analogs in a Panel of Cancer Cell Lines**

compd	IC <sub>50</sub> ( $\mu$ M)				ref
	DU145	PC3	LNCaP	MCF-7	
1	12.2	18.7	not tested	>100	89
5	16.2	13.4	34.1	88.5	89
6	31.5	32.4	31.5	not tested	89

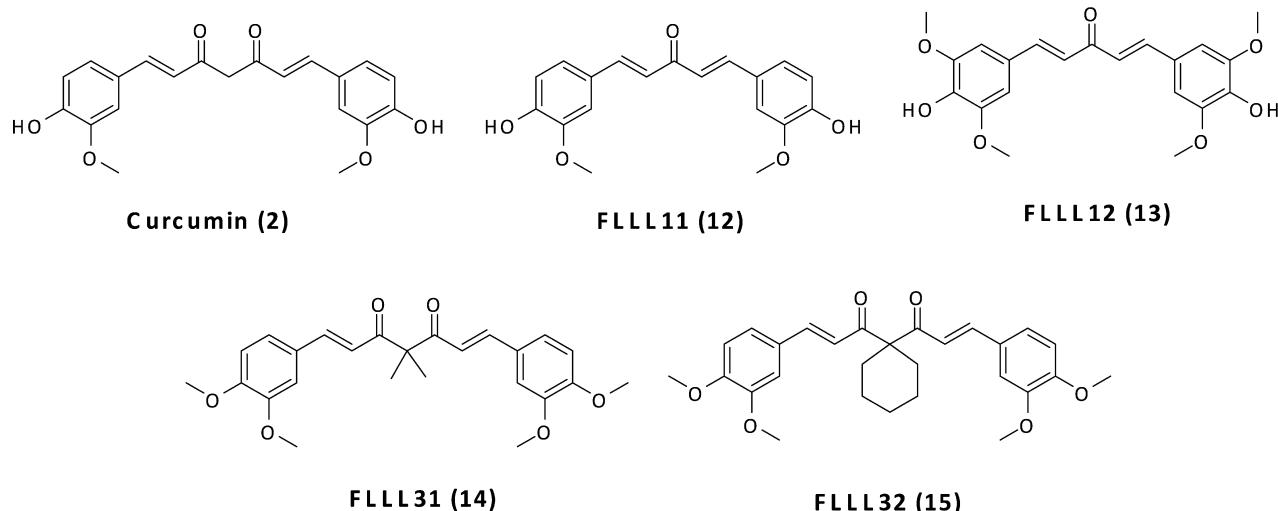
effect of imatinib on BCR-ABL positive cells.<sup>93</sup> Another analogue, LLL-12 (7), selectively inhibited Stat3 phosphorylation in glioblastoma, breast, and pancreatic cancer cells expressing elevated levels of Stat3, without significant effect on kinases such as ERK1/2, mTOR, and Src.<sup>90</sup> Compound 7 also selectively inhibited Stat3-DNA binding and Stat3-dependent transcriptional activities, reduced the secretion of IL-6 and LIF in medulloblastoma and glioblastoma cell lines,<sup>94</sup> and suppressed tumor growth in mouse xenograft models of breast cancer and glioblastoma.<sup>90</sup> In a panel of cancer cell lines, compound 7 at 10  $\mu$ M significantly inhibited Stat3-DNA binding.<sup>90</sup> Compound 7 is more potent than the original lead, compound 1, and they represent a class of selective small-molecule Stat3 inhibitors with druglike properties.

**7.1.2. Stattic (8) and Its Analogue.** Another small molecule, compound 8 (Figure 6a), was discovered as a Stat3-SH2 domain inhibitor via high-throughput screening of a diverse chemical library.<sup>95</sup> Compound 8 exhibited an IC<sub>50</sub> of 5.1  $\mu$ M in a fluorescence polarization (FP) assay and showed selectivity over other proteins containing SH2 domain, including Lck kinase, Stat1, and Stat5. It also inhibited prephosphorylated Stat3 homodimers from binding DNA in electrophoretic mobility shift assays (EMSA) at 10  $\mu$ M. However, no inhibitory effects were observed on prephosphorylated Stat1 homodimers even at higher concentrations. Compound 8 selectively down-regulated phospho-Stat3 in liver carcinoma HepG2 cells and breast cancer MDA-MB-231 and MDAMB-435S cells.<sup>95</sup> By inhibiting Stat3, compound 8 impaired proliferation and neurosphere initiation in tumor-initiating stem cells (TISC) from glioblastoma and sensitized TISCs to temozolomide.<sup>96</sup> However, compound 8 may lack target selectivity because of its simple chemical structure. For example, we demonstrated that compound 8 equally inhibited the phosphorylation of Stat3 and Stat1 induced by IL-6 or LIF in human ovarian cancer OVCAR-8 cells (Figure 6b). Several close analogues of compound 8 were reported in an attempt to optimize the lead (compounds 9–11, Figure 6a). Structure–activity relationship (SAR) analysis of compound 8 and its analogues suggests that replacement of the NO<sub>2</sub> group by NH<sub>2</sub> or H results in loss of Stat3 inhibitory activity in both FP and EMSA assays. Furthermore, saturation of the five-membered ring led to complete loss of activity.<sup>95</sup> The SAR results suggest that the NO<sub>2</sub> group and an unsaturated five-membered ring are important for the Stat3 inhibitory activity of this class of compounds.

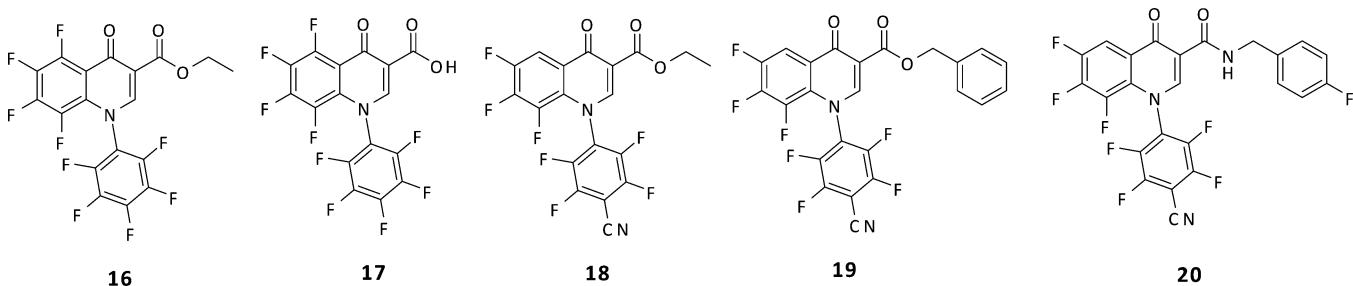
**7.1.3. Curcumin and Its Analogue.** Curcumin, the principal curcuminoid of the Indian spice turmeric, inhibits IL-6-induced Stat3 phosphorylation and nuclear translocation. Several compounds were designed as Stat3 inhibitors based on curcumin (Figure 7).<sup>97–99</sup> FLLL11 (12) and FLLL12 (13) inhibited Stat3 phosphorylation and induced apoptosis in pancreatic cancer cell lines in a dose-dependent manner.<sup>97</sup> Although both compounds inhibited Stat3 phosphorylation, Stat3-DNA binding, and transcriptional activity at 10  $\mu$ M in



**Figure 6.** (a) Compound **8** and its analogues. (b) Compound **8** inhibits Stat3 and Stat1 phosphorylation. Human ovarian cancer OVCAR-8 cells were serum-starved overnight. Cells were then treated with compound **8** at indicated concentrations for 4 h before IL-6 (50 ng/mL) or LIF (50 ng/mL) stimulation for 20 min. Whole cell lysates were prepared for Western blotting analysis using specific antibodies (Cell Signaling Inc.) for indicated proteins.



**Figure 7.** Analogues of curcumin.



**Figure 8.** Analogues of quinolinone.

breast and prostate cancer cells,<sup>98</sup> **13** was more potent than **12** and curcumin.<sup>98</sup> FLLL31 (**14**) and FLLL32 (**15**) were also cytotoxic in several cancer cell lines.<sup>100</sup> Both compounds at 10 μM down-regulated Stat3 phosphorylation, DNA binding, and the expression of Stat3 target genes in MDA-MB-231 and PANC1 cells. Compound **15** showed little inhibitory effect on tyrosine kinases and other non-tyrosine kinases, such as AKT2, CDK-2, EGFR, HER-2, and Met. Both **14** and **15**

demonstrated JAK2 kinase inhibitory activity at 5 μM (approximately 60% and 75% reduction, respectively).<sup>99</sup> Compound **15** specifically inhibited Stat3 phosphorylation at Tyr705 and induced caspase-dependent apoptosis in phospho-Stat3-positive human melanoma cell lines in the low micromolar range.<sup>101</sup>

**7.1.4. Quinolinone Analogs.** Several quinolinones were reported as JAK-Stat3 pathway inhibitors.<sup>102</sup> These compounds

(Figure 8) inhibited Stat3 phosphorylation at Tyr705 (Table 3). SAR analysis revealed that the ester group is important for

**Table 3. Inhibition of Stat3 Phosphorylation by Quinolinone Analogues**

compd	inhibition of Stat3 phosphorylation, EC <sub>50</sub> ( $\mu$ M)	ref
16	4.6	102
17	46	102
18	0.17	102
19	0.13	102
20	0.20	102

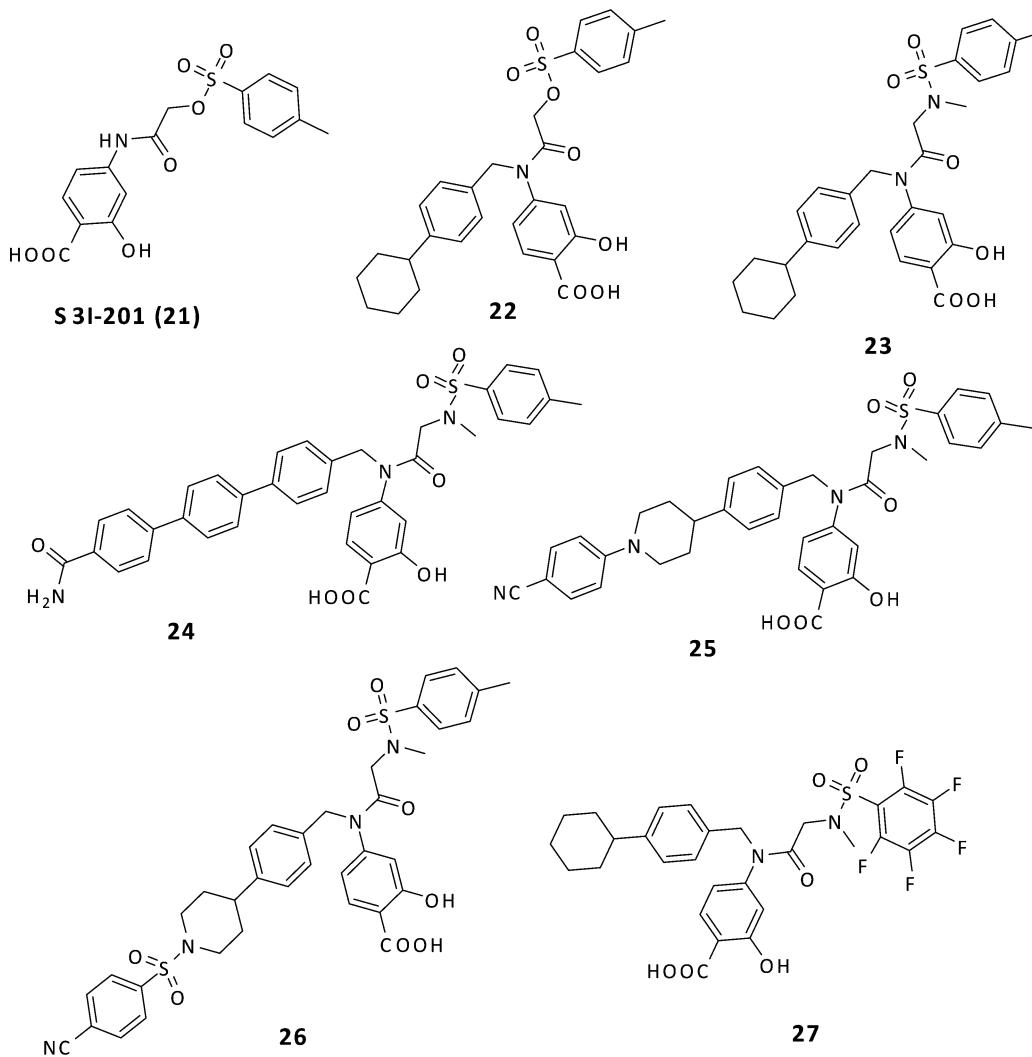
inhibitory activity, as hydrolysis of the ester bond leads to a 10-fold loss of activity (16 vs 17). Introduction of a cyano group (compound 18) leads to a 27-fold increase in potency. Replacement of ethyl ester with a benzyl ester (19) or benzylamino (20) group did not affect activity. Compound 18 inhibited activation of JAKs but did not directly affect the enzymatic activity of JAK1, JAK2, and JAK3.<sup>102</sup> Further investigation is underway to elucidate the actual molecular targets of this class of compounds.

**7.1.5. S3I-201 (21)<sup>84</sup> and Its Analogues.** Structure-based virtual screening of chemical libraries from the National Cancer

Institute led to the identification of a small molecule 21 (Figure 9) as a Stat3 inhibitor.<sup>84</sup> Compound 21 preferentially inhibits the DNA binding ability of Stat3/Stat3 dimer ( $IC_{50} = 86 \mu\text{M}$ ), compared to Stat1/Stat3 dimers ( $IC_{50} = 160 \mu\text{M}$ ) and Stat1/Stat1 dimers ( $IC_{50} > 300 \mu\text{M}$ ).<sup>84</sup>

Compound 21 inhibited proliferation of hepatocellular carcinoma cells with decreased phospho-Stat3(S727) and aberrant TGF- $\beta$  pathway. At 250  $\mu\text{M}$ , it caused 80% inhibition of cell growth in cells with low phospho-Stat3 (S727) and disrupted TGF- $\beta$  signaling, while only 20% inhibition was observed in cells with an intact TGF- $\beta$  pathway. It suppressed tumor growth in mouse xenograft models of hepatocellular carcinoma and breast cancer.<sup>84,103</sup>

In an attempt to optimize compound 21, structure-based modeling resulted in compounds 22–27 (Figure 9) with improved potency.<sup>104,105</sup> Compounds 22–27 inhibited Stat3-DNA binding in a dose dependent manner with  $IC_{50}$  values in the range 19.7–50  $\mu\text{M}$  in EMSA and disrupted Stat3–phosphopeptide binding in FP assays (Table 4).<sup>104–106</sup> Incorporation of hydrophobic substitutions at the amide nitrogen improved Stat3-DNA binding inhibition by 2- to 3-fold over compound 21. Compound 23 at 50  $\mu\text{M}$  inhibited constitutive Stat3 activation in NIH3T3/v-Src, Panc-1, and MDA-MB-231 cells. Compound 23 bound to the SH2 domain



**Figure 9.** Analogue of compound 21.

**Table 4.** Inhibition of Stat3-DNA Binding in EMSA Assay and Stat3–Phosphopeptide Binding in FP Assay by Compound 21 and Its Analogues

compd	EMSA, IC <sub>50</sub> (μM)	FP, K <sub>i</sub> (μM)	ref
21	86	80	104, 168
22	43	50	104
23	35	20, 15	104, 168, 105, 85
24	43	16.7	105
25	45	18.7	105
26	50	21.5	105
27	19.7	12.8	106

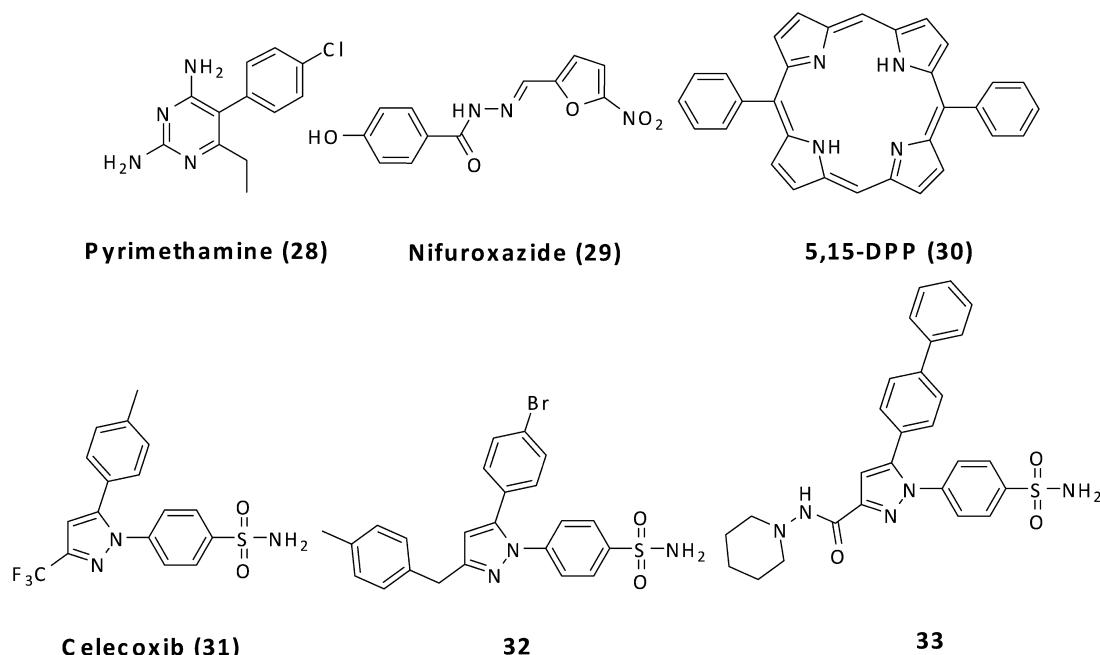
with a  $K_d$  of 2.74 μM and inhibited Stat3-DNA binding with an IC<sub>50</sub> of 35 μM.<sup>86</sup> The SH2 domain comprises three subpockets. Compound 21 only interacts with two subpockets. Additional N-substitution in compound 23 allowed hydrophobic interactions with the third pocket.<sup>86,167</sup> Compounds 22 and 23, in a concentration-dependent manner, disrupted Stat3 interaction with phosphopeptide in a FP assay with IC<sub>50</sub> of 50 and 20 μM, respectively.<sup>104</sup> Compound 23 also significantly inhibited proliferation of malignant cells with aberrant Stat3, such as NIH3T3/v-Src, Panc-1, and MDA-MB-231 cells, compared to cells that do not harbor aberrant Stat3, such as NIH3T3, normal human pancreatic duct epithelial cells (HPDEC), and mouse thymus epithelial stromal cells (TE-71). The IC<sub>50</sub> values in NIH3T3/v-Src, Panc-1, and MDA-MB-231 cells were 35, 48, and 37 μM, respectively, while the IC<sub>50</sub> values were over 200 μM in NIH3T3, HPDEC, TE-71 cells. It also repressed the expression of Stat3 target genes and the growth of human breast tumors in xenograft models.<sup>86</sup> Compound 23 exhibited selectivity toward Stat3 over Stat1 in a FP assay ( $K_i(\text{Stat3}) = 15 \mu\text{M}$  vs  $K_i(\text{Stat1}) \geq 50 \mu\text{M}$ ) and inhibited growth of a panel of leukemia, breast, and prostate cancer cell lines.<sup>105</sup> Compound 27 is a more potent analogue of 23, inhibits Stat3-DNA binding with an IC<sub>50</sub> of 19.7 μM, and binds the Stat3-SH2 domain with a  $K_i$  of 12.8 μM in a FP assay.<sup>106</sup>

**7.1.6. Small-Molecule Stat3 Inhibitors Identified from MLSD, High-Throughput Screening, and Virtual Screening.** Several drugs and bioactive compounds were identified as Stat3 inhibitors using high-throughput screenings (Figure 10). The antimalarial drug pyrimethamine (28),<sup>107</sup> the antidiarrheal agent nifuroxazide (29),<sup>108</sup> and 5,15-diphenylporphyrin (5,15-DPP, 30)<sup>82</sup> showed inhibitory activity against Stat3. Nifuroxazide inhibited Stat3 in a Stat3-dependent luciferase assay using U3A cells ( $\text{EC}_{50} = 3 \mu\text{M}$ ) and down-regulated Stat3 phosphorylation in a dose dependent manner.<sup>108</sup> Porphyrin analogue 30, identified as Stat3-SH2 domain antagonist in a high-throughput α screening assay (inhibition of Stat3–phosphopeptide interaction), selectively inhibited Stat3 (IC<sub>50</sub> = 0.28 μM) over Stat1 (IC<sub>50</sub> = 10 μM).<sup>82</sup>

The COX2 inhibitor celecoxib (31) and other two compounds (32 and 33) were identified as Stat3 inhibitors through fragment-based drug design followed by MLSD and drug repositioning.<sup>87</sup> They inhibited Stat3 phosphorylation in both dose- and time-dependent manners. Phosphorylation of Stat3 was inhibited by compounds 32 and 33 at 10–30 μM and by celecoxib at 25–50 μM.<sup>87</sup>

Various Stat3 inhibitors (34–39, Table 5) targeting the SH2 domain were identified from virtual screening of one million druglike compounds and subsequent in vitro testing.<sup>109</sup> Interestingly, all of these compounds have either free carboxylic acid or carboxylic acid ester. Further optimization through scaffold hopping led to the identification of a small molecule (C188-9, structure not disclosed) with potent activity against Stat3 in acute myeloid leukemia cells.<sup>110</sup>

All these compounds identified from virtual or high-throughput screening need to be further characterized to elucidate their actual molecular targets. A troubling trend is the fact that many of these compounds have  $\alpha,\beta$  unsaturated ketone (Michael acceptor) that could be responsible for their nonspecific interaction with Stat3 as well as with a myriad of other cellular targets. Therefore, at this stage it is too early to classify such compounds as bona fide Stat3 inhibitors. It is not



**Figure 10.** Stat3 inhibitors identified from high-throughput and virtual screening.

Table 5. Selected Examples of Stat3 Inhibitors Discovered by Virtual Screening

Compound	Structure	Stat3/pY-peptide binding assay ( $IC_{50}$ , $\mu M$ )	IL-6-mediated phosphorylation of Stat3 (Immunoblot assay) ( $IC_{50}$ , $\mu M$ )	References
34		447	91	109
35		256	144	109
36		137	63	109
37		30	18	109
38		114	60	109
39		20	73	109

our intention to critically appraise the merit of each compound as a selective Stat3 inhibitor but rather to make the reader aware of the fact that most of these so-called Stat3 inhibitors will eventually fail upon further scrutiny.

Recently, a quinoline analogue STX-0119 (40) was identified as a Stat3 dimerization inhibitor through virtual screening.<sup>111</sup> Table 6 shows several analogues (41–46) of compound 40, highlighting the structural features required for Stat3 inhibition. Compound 40 inhibited Stat3 dimerization as well as transcription but failed to hinder Stat3 phosphorylation. These observations suggest that it inhibits Stat3 through direct binding instead of targeting upstream JAK kinases controlling phosphorylation of Stat3.<sup>111</sup>

**7.1.7. Other Small-Molecule Inhibitors.** Additional compounds have been identified as Stat3 inhibitors that directly or indirectly target Stat3. Figure 11 lists Stat3 inhibitors from different sources. A natural product, cryptotanshinone (47), from *Salvia miltiorrhiza* Bunge (Danshen root) was identified as a Stat3 inhibitor.<sup>112</sup> It inhibits Stat3-dependent luciferase activity in colon cancer cells in a dose-dependent manner with an  $IC_{50}$  of 4.6  $\mu M$  and also selectively blocked Y705 phosphorylation of Stat3 in DU145 cells at 7  $\mu M$ . Cryptotanshinone does not show equal inhibitory activity against upstream tyrosine kinases, such as JAKs, c-Src, and EGFR. However, it inhibits JAK2 kinase at 5  $\mu M$  after 4 h of treatment, while the inhibition of Stat3 phosphorylation occurs within 30 min. Moreover, compound 47 inhibited Stat3

Table 6. Compound 40 and Its Analogues with Inhibitory Activities on Stat3 Transcription and Dimerization

Compound	R <sub>1</sub>	R <sub>2</sub>	Stat3 transcription IC <sub>50</sub> (μM)	Stat3 dimerization		References
				50 μM (%)	10 μM (%)	
40	*—C <sub>6</sub> H <sub>3</sub> O	*—C <sub>6</sub> H <sub>5</sub>	74	62	9	111
41	*—C <sub>6</sub> H <sub>3</sub> O	*—C <sub>10</sub> H <sub>7</sub>	55	70	14	111
42	*—C <sub>6</sub> H <sub>3</sub> O	*—C <sub>4</sub> H <sub>2</sub> S	63	56	18	111
43	*—C <sub>6</sub> H <sub>5</sub>	*—C <sub>6</sub> H <sub>5</sub>	66	60	43	111
44	*—C <sub>6</sub> H <sub>4</sub> C <sub>6</sub> H <sub>5</sub>	*—C <sub>6</sub> H <sub>5</sub>	75	61	25	111
45	*—C <sub>6</sub> H <sub>3</sub> O	*—C <sub>6</sub> H <sub>5</sub>	72	55	8	111
46	*—C <sub>6</sub> H <sub>2</sub> S	*—C <sub>6</sub> H <sub>5</sub>	75	56	36	111

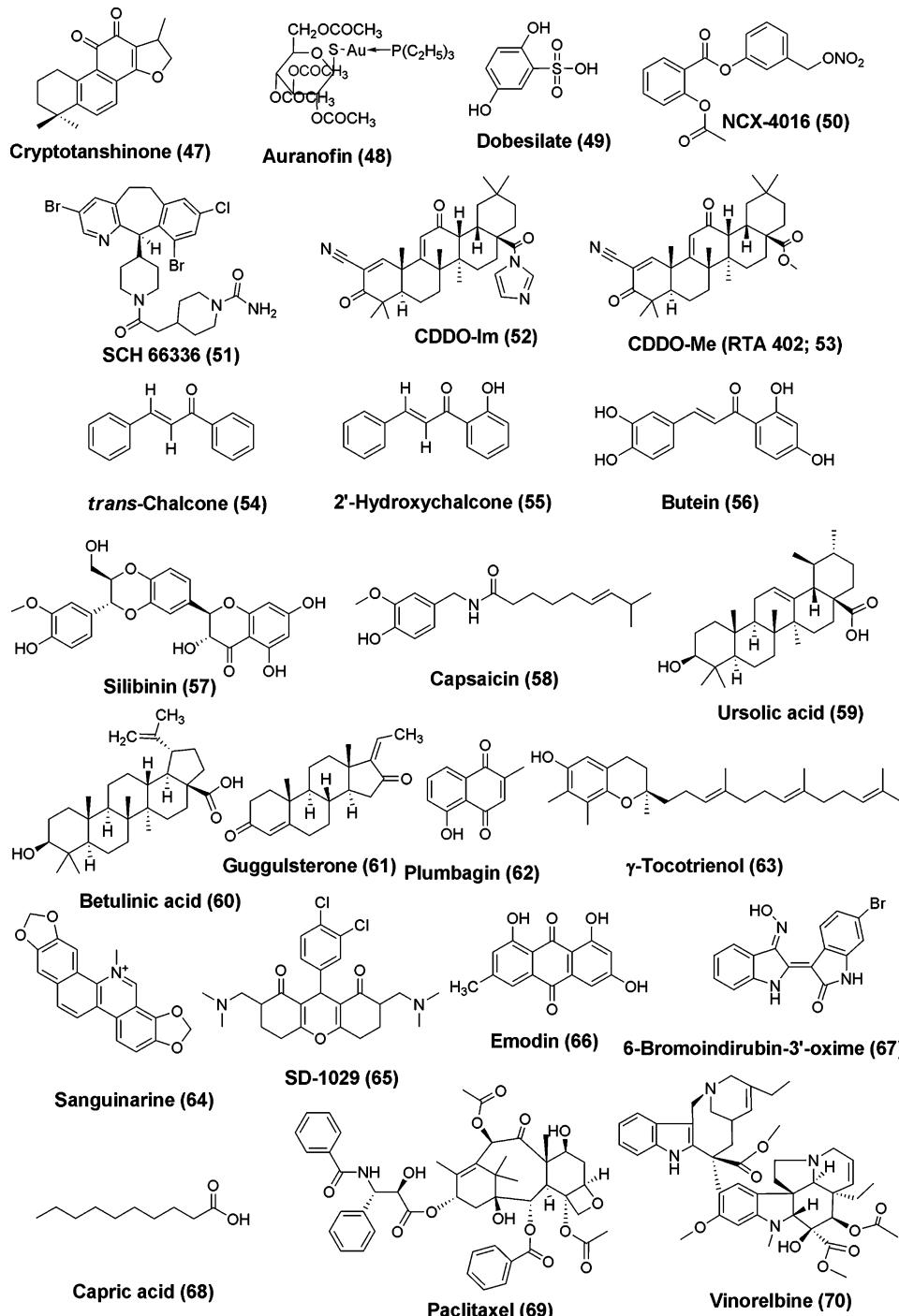
dimerization in EMSA assays by binding to the SH2 domain of the Stat3 monomer.<sup>112</sup>

An antirheumatic sulfur-containing organogold compound, auranofin (48), was reported to inhibit IL6-induced Stat3 phosphorylation in HepG2 cells, fibroblast-like synoviocytes from rheumatoid arthritis patients, human umbilical vein endothelial cells (HUVECs), and rat astrocytes, but the exact molecular mechanism of this compound is unclear.<sup>113</sup> Dobesilate (49), a drug commonly used in the treatment of diabetic retinopathy and chronic venous insufficiency, inhibited Stat3 activation and downstream gene expression in glioma cells.<sup>114</sup> Nitric oxide-releasing aspirin derivative NCX-4016 (50) down-regulated Stat3 protein levels and inhibited phosphorylation of Stat3 at Tyr705 and Ser727 in ovarian cancer cells.<sup>115</sup> An orally bioavailable farnesyl protein transferase inhibitor, SCH 66336 (51), inhibited Stat3 phosphorylation at 4 μM in non-small-cell lung carcinoma (NSCLC) cell lines.<sup>116</sup> A synthetic triterpenoid, 2-cyano-3,12-dioxoolen-1,9-dien-28-oic acid imidazolidine (CDDO-Im, 52) inhibited both constitutive and inducible Stat3 phosphorylation in human cancer cells in a dose- and time-dependent manner and down-regulated Stat3 target genes. It also inhibited Stat5 phosphorylation.<sup>117</sup> Methyl ester of 2-cyano-3,12-dioxoolen-1,9-dien-28-oic acid (CDDO-Me, RTA 402, 53) inhibited Stat3 activity at 500 nM in metastatic murine breast cancer cells. It also inactivated Akt and Src kinases and down-regulated c-Myc by 4-fold.<sup>118</sup> In an *in vivo* murine breast tumor model, compound 53 inhibited tumor cell growth but did not induce apoptosis.<sup>118</sup> Actual mechanisms behind Stat3 inhibition of compound 53 are unclear.

Chalcone (54) and a chalcone derivative (2'-hydroxychalcone, 55) were reported to inhibit IL-6-induced Stat3 phosphorylation in endothelial cells in a dose-dependent

manner. The α,β-unsaturated carbonyl moiety was important for the inhibition of Stat3 phosphorylation, as other flavonoids without this moiety failed to show any inhibitory activity.<sup>119</sup> Another chalcone derivative, butein (3,4,2',4'-tetrahydroxychalcone, 56), inhibited tumor cell proliferation through Stat3 suppression. Compound 56 inhibited both constitutive and IL-6-induced Stat3 activation in multiple myeloma cells through the induction of SHP-1 and also suppressed constitutive activation of JAK1 and c-Src.<sup>120</sup>

Silibinin (57), a flavanone from *Silybum marianum*, inhibited constitutively active Stat3 phosphorylation, activated caspases, and induced apoptosis in DU145 prostate cancer cells in a dose dependent manner.<sup>121</sup> It also suppressed the expression of Stat3 target genes.<sup>121</sup> Capsaicin (58), a vanilloid constituent of green and red peppers, preferentially inhibited constitutive Stat3 phosphorylation in multiple myeloma cells in a dose- and time-dependent manner through the inhibition of JAK1 and c-Src. It down-regulated the expression of Stat3 target genes and inhibited growth of human multiple myeloma cells *in vitro* and *in vivo*.<sup>122</sup> Ursolic acid (59), a natural triterpenoid isolated from different fruits and vegetables, inhibited both constitutive and IL-6-induced Stat3 activation in multiple myeloma cells through the blockade of JAK1, JAK2, and c-Src.<sup>123</sup> It inhibited Stat3 phosphorylation, DNA binding, and the expression of Stat3 target genes. It also induced apoptosis in multiple myeloma cells in a time-dependent manner.<sup>123</sup> Betulinic acid (60), a pentacyclic triterpenoid from the stem bark of the plant *Zizyphus mauritiana*, blocked Stat3 activation in human multiple myeloma cells through the induction of SHP-1.<sup>124</sup> It down-regulated the expression of Bcl-2, Bcl-xL, cyclin D1, and survivin and enhanced apoptosis induced by thalidomide and bortezomib.<sup>124</sup> Farnesoid X receptor antagonist guggulsterone (61) inhibited constitutive as well as IL-6-induced Stat3



**Figure 11.** Other small molecule Stat3 inhibitors.

phosphorylation.<sup>125</sup> Additionally, it suppressed the phosphorylation of JAK2 and c-Src kinases.<sup>125</sup> Inhibition of Stat3 phosphorylation by compound 61 was due to the induction of SHP-1.<sup>125</sup> Moreover, compound 61 down-regulated the expression of Stat3 target genes and inhibited cell proliferation, arrested cells in the sub-G1 phase of cell cycle, and induced apoptosis in multiple myeloma cells.<sup>125</sup>

Plumbagin (62), a vitamin K3 analogue, inhibited Stat3 activation also through the induction of SHP-1. It inhibited Stat3 phosphorylation, nuclear translocation, DNA binding, and the expression of Stat3 target genes in human multiple

myeloma cells. Plumbagin also inhibited the activation of JAK1 and JAK2 and induced apoptosis in tumor cells.<sup>126</sup>

$\gamma$ -Tocotrienol (63), a vitamin E isolated from palm and rice bran oil, showed cytotoxicity through induction of SHP-1, causing inhibition of the Stat3 signaling pathway.<sup>127</sup> It also hindered Stat3-DNA binding as well as the activation of JAK1, JAK2, and c-Src kinases and sensitized tumor cells to anticancer agents.<sup>127</sup>

Sanguinarine (64), a benzylisoquinoline alkaloid extracted primarily from the bloodroot plant, inhibited both constitutive and IL-6 induced Stat3 phosphorylation in prostate cancer cells.<sup>128</sup> It also down-regulated the expression of Stat3 target

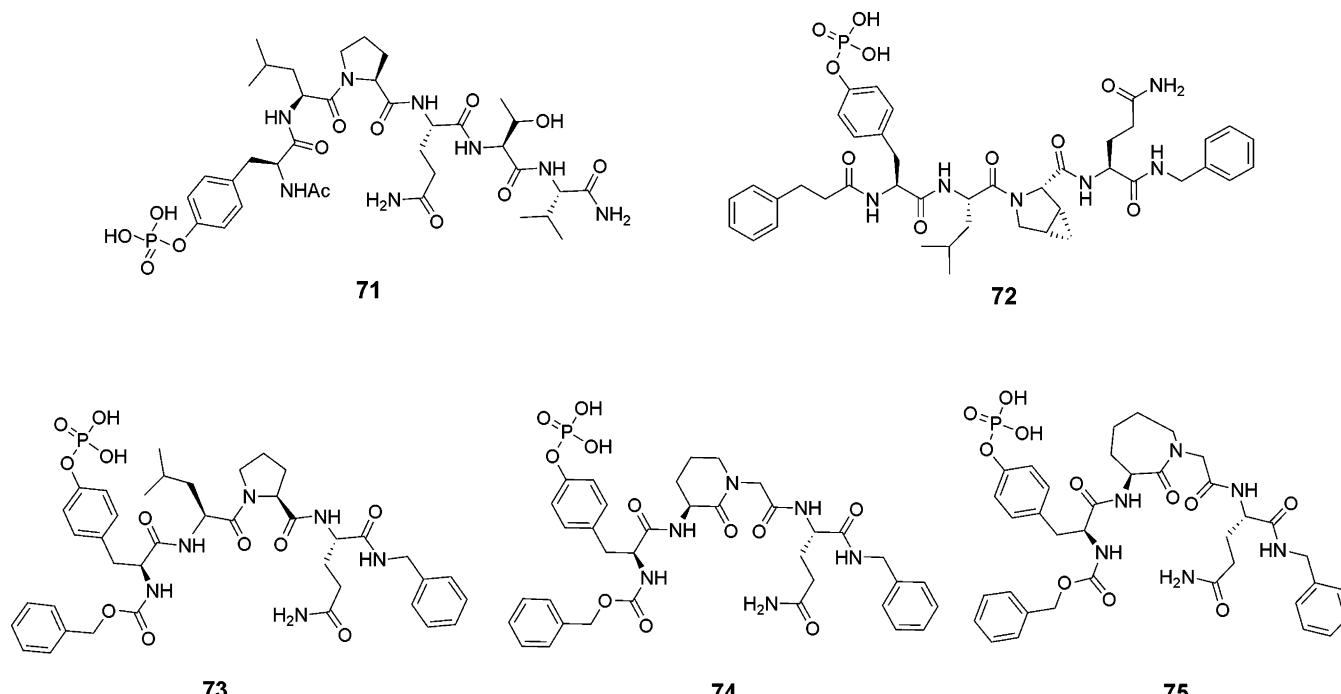


Figure 12. Peptide and peptidomimetic inhibitors.

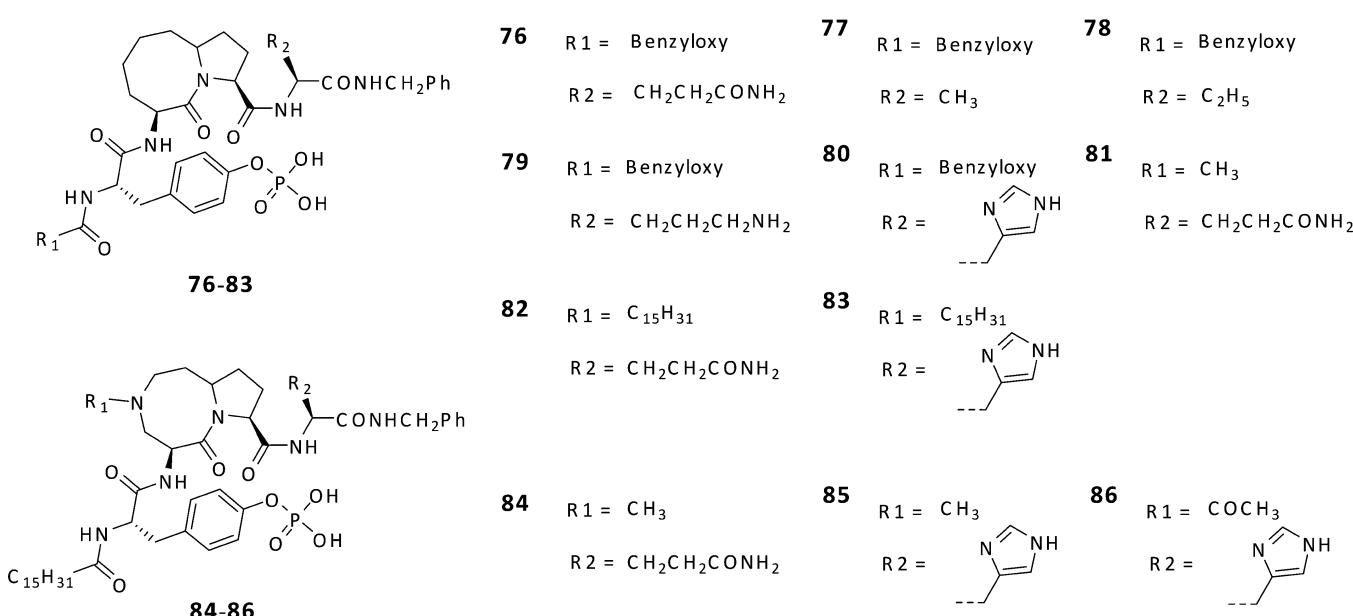


Figure 13. Peptidomimetic Stat3 inhibitors with bicyclic lactam.

genes, such as c-myc and survivin.<sup>128</sup> The NCI bioactive compound SD-1029 (65) was identified as an inhibitor of IL-6 or oncostatin-induced Stat3 nuclear translocation in the micromolar range.<sup>129</sup> Compound 65 inhibited phosphorylation of JAK2, down-regulated Stat3 signaling pathway downstream genes, and induced apoptosis in a panel of breast and ovarian cancer cells.<sup>129</sup>

Emodin (66), a purgative resin isolated from the root and rhizomes of *Rheum palmatum* L., inhibited Stat3 phosphorylation by targeting JAK2.<sup>130</sup> It inhibited cell growth and induced apoptosis in multiple myeloma cells.<sup>130</sup> Another small molecule, 6-bromoindirubin-3'-oxime (67), inhibited Stat3 phosphorylation by blocking JAK/Stat3 signaling and induced

apoptosis in human melanoma cells in a dose- and time-dependent manner.<sup>131</sup> Recently, it was reported that capric acid (68), a saturated fatty acid from coconut oil, palm kernel oil, and milk of various mammals, inhibited nitric oxide production during LPS induced osteoclastogenesis via Stat3 phosphorylation at Ser727 but had no effects on NF-κB, JNK, ERK1/2, and Stat1 pathways.<sup>132</sup>

Microtubule-targeted drugs such as paclitaxel (69) and vinorelbine (70) inhibited Stat3 phosphorylation in cancer cells with constitutively active Stat3 and down-regulated the expression of Stat3 dependent genes.<sup>133</sup> Cytotoxic activity of paclitaxel in breast cancer cells correlates with its Stat3 inhibition.<sup>133</sup>

Purported mechanisms behind these compounds need to be further validated in order to conclude whether these are direct inhibitors of Stat3 or whether they affect pathways leading to Stat3 phosphorylation.

**7.2. Peptide and Peptidomimetic Inhibitors.** Several peptides and peptidomimetics have been reported to inhibit Stat3 (Figure 12). On the basis of truncated gp130 sequence, Ren et al. discovered the phosphopeptide Ac-pTyr-Leu-Pro-Gln-Thr-Val-NH<sub>2</sub> (71) as a Stat3 inhibitor. This peptide inhibited Stat3-DNA binding with an IC<sub>50</sub> of 150 nM in EMSA.<sup>134</sup> Another peptidic inhibitor, hydrocinnamoyl-Tyr-(PO3H2)-Leu-*cis*-3,4-methanoPro-Gln-NHBn (72), binds to the Stat3-SH2 domain and is over 2-fold more potent than peptide 71.<sup>135</sup> In a FP assay, the IC<sub>50</sub> of peptide 72 was 125 nM while that of peptide 71 was 290 nM.<sup>135</sup> Although peptides face several challenges in terms of membrane permeability and stability, they are valuable starting leads for the rational design of peptidomimetic and small-molecule Stat3 inhibitors.

On the basis of the peptide 71, Gomez et al. designed peptidomimetics incorporating Freidinger lactams as Stat3 inhibitors (Figure 12).<sup>136</sup> A shortened version of the gp130 peptide, compound 73, bound to Stat3 with a K<sub>i</sub> of 350 nM in a FP assay. Compounds 74 and 75 were designed based on compound 73's chemical structure by incorporating six- and seven-membered Freidinger lactams, respectively.<sup>136</sup> The six-membered lactam (74, K<sub>i</sub> = 3.13 μM) lost 9-fold potency, while the seven-membered lactam (75, K<sub>i</sub> = 190 nM) gained 2-fold potency over compound 73.<sup>136</sup>

Cyclization of isobutyl group of leucine with a five-membered ring of proline led to bicyclic lactam derivatives (76–86) of compound 73 (Figure 13).<sup>137</sup> Table 7 lists binding affinities and cytotoxic activities of these compounds. A conformationally constrained derivative, compound 76, was 20 times more potent than compound 73. Various structural changes in the glutamine residue resulted in derivatives (77–80) less potent than compound 76. This suggests that the

glutamine residue is structurally important for the binding affinity. Replacement of carbobenzoyloxy (Cbz) with an acetyl group resulted in compound 81 without any loss of activity. Compounds 76–81 showed moderate to high activity in FP-based binding assays but failed to show cellular activity up to 100 μM probably because of their poor cellular uptake. Substituting the acetyl group of compound 81 with a lipid chain led to a cell-permeable derivative, compound 82, which exhibited a K<sub>i</sub> of 10 nM in a binding assay and inhibited the growth of breast cancer cells in a dose-dependent manner. In an effort to improve cell permeability, the glutamine was replaced with a histidine (compound 83), resulting in a loss of binding affinity but an increase in cellular activity. Introduction of a positively charged nitrogen atom in the seven-membered ring to neutralize the negatively charged phosphotyrosine group resulted in compounds 84 and 85 retaining binding activity. Compound 85 showed high potency in cell growth inhibition with IC<sub>50</sub> of 11.2 and 3.6 μM in MDA-MB-231 and MDA-MD-468 cells, respectively.<sup>137</sup>

Peptidomimetics with glutamine mimics incorporated into the phosphopeptide were reported as Stat3 inhibitors targeting the SH2 domain (87–89) with nanomolar potency in FP assays (Figure 14).<sup>138</sup> A series of small-molecule analogues (compounds 90–94, Figure 15) were designed based on the peptidomimetic Stat3 inhibitor 3,<sup>85</sup> which were capable of inhibiting Stat3-DNA binding with IC<sub>50</sub> < 100 μM (Table 8). SAR analysis of these analogues revealed that a thiazole moiety is more preferable than an oxazole moiety for the inhibition of Stat3-DNA binding, as thiazole analogues (92 and 94) were at least 2- to 3-fold more potent than their oxazole analogues (91 and S3I-M2001 (93)). Compound 93 preferentially inhibited Stat3-DNA binding over Stat1 in EMSA assays with nuclear extracts or lysates from Sf-9 cells and also inhibited Stat3 phosphorylation in MDA-MB-231 and MDA-MB-435 cells.<sup>139,140</sup> Moreover, 93 inhibited the migration of malignant tumor cells and repressed the growth of human breast cancers in xenograft models.<sup>139,140</sup>

A novel class of hybrid peptidomimetics was designed by conjugating compound 3 with high-affinity peptides from gp130 (Figure 16). Compound 95 strongly interacted with Stat3 (K<sub>d</sub> = 205 nM) and disrupted Stat3 interaction with phosphopeptide (K<sub>i</sub> = 9 μM) in a FP assay. It also exhibited time-dependent inhibition of constitutively active Stat3 in NIH3T3/v-Src cells. A nonphosphorylated analogue (compound 96) binds (K<sub>d</sub> = 17 μM) Stat3 but fails to disrupt Stat3–phosphopeptide interaction.<sup>141</sup> This observation suggests that these compounds act through different mechanisms: compound 95 binds to the SH2 domain, while compound 96 binds a different site.

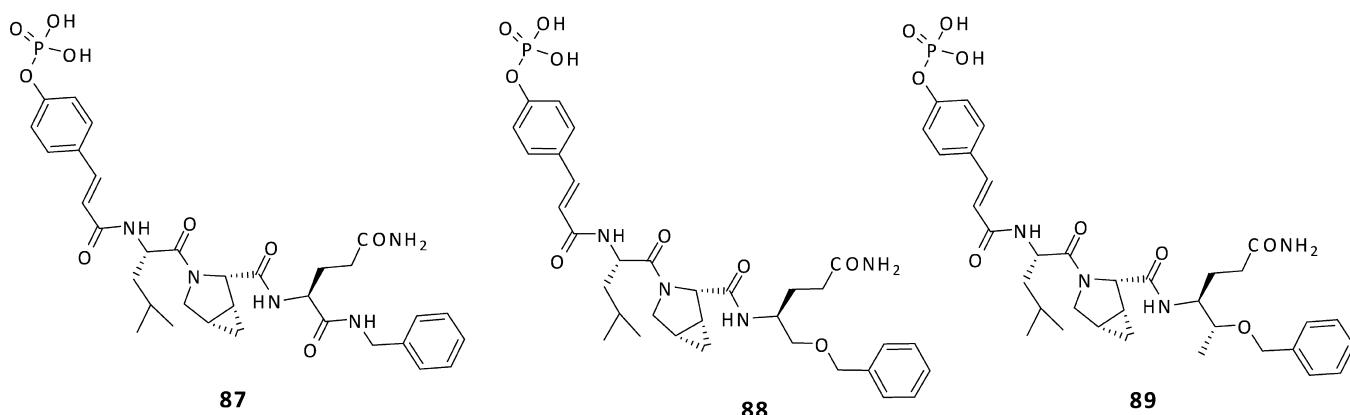
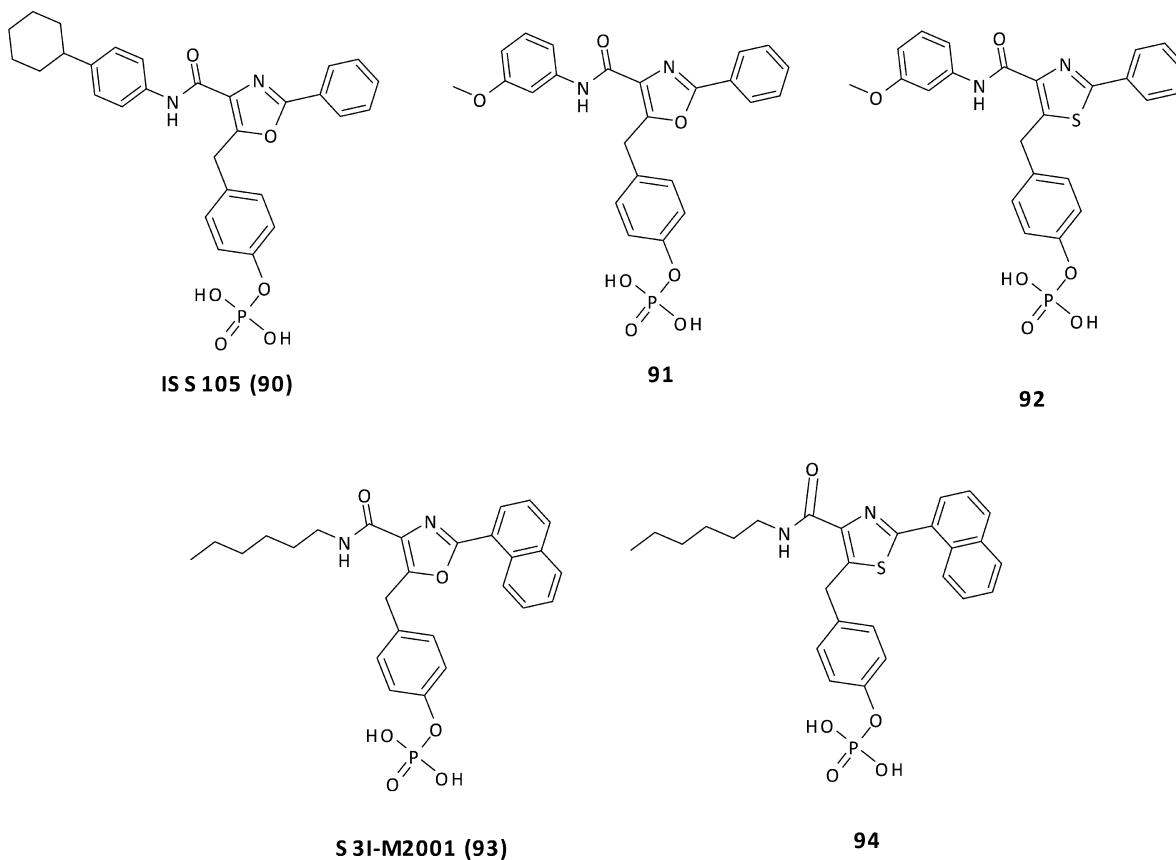
## 8. STAT3 INHIBITORS IN CLINICAL TRIALS

Several Stat3 inhibitors are in early phase clinical trials (Table 9). Compound 1 has completed phase I/II clinical trials for the treatment of psoriasis.<sup>142</sup> However, no results are publicly available at this time. Pyrimethamine is in phase I/II trials for the treatment of chronic lymphocytic leukemia and small lymphocytic leukemia.<sup>143</sup> Another Stat3 inhibitor, OPB-31121 (structure not disclosed), is currently in phase I in patients with advanced solid tumors.<sup>144</sup> NF-κB/Stat3 inhibitor, compound 53, is currently undergoing phase I/II clinical testing for pancreatic cancer.<sup>145</sup> A phase I trial with compound 53 has been completed in patients with solid tumors and lymphoid malignancies. It was well tolerated with a maximum tolerated

**Table 7. Stat3 Binding Affinity and Cytotoxic Activities of Select Peptides and Peptidomimetics in Breast Cancer Cells**

compd	K <sub>i</sub> <sup>a</sup> (μM)	IC <sub>50</sub> <sup>b</sup> (μM)		ref
		MDA-MB-231	MDA-MB-468	
71	0.29	NA	NA	135
72	0.125	NA	NA	135
73	0.35	>100	>100	136, 137
74	0.19	NA	NA	136
75	3.13	NA	NA	136
76	0.017	>100	>100	137
77	3.23	36	52.5	137
78	8.57	>100	>100	137
79	0.21	>100	>100	137
80	0.44	>100	>100	137
81	0.015	>100	>100	137
82	0.010	25.6	35	137
83	0.46	50.4	70	137
84	0.030	42.6	20.6	137
85	0.95	11.2	3.6	137
86	0.76	41.5	36.1	137
87	0.068	NA	NA	138
88	0.094	NA	NA	138
89	0.069	NA	NA	138

<sup>a</sup>FP-based binding assay. <sup>b</sup>NA: not available.

**Figure 14.** Peptidomimetic Stat3 inhibitors.**Figure 15.** Analogues of compound 3.**Table 8. Inhibition of Stat3-DNA Binding in EMSA Assay by Compound 3 and Its Analogues**

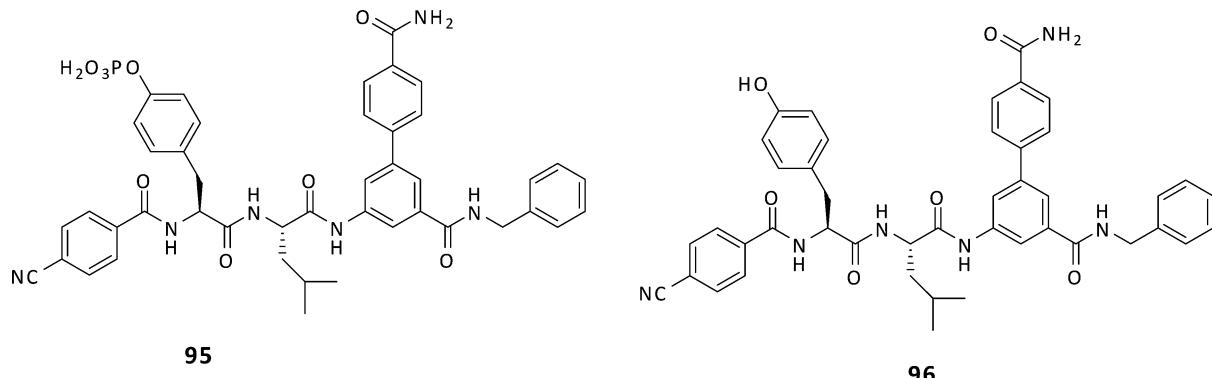
compd	EMSA, IC <sub>50</sub> ( $\mu$ M)	ref
3	42	139, 140
90	33	85, 169
91	90	85, 169
92	28	85, 169
93	58, 79	85, 139, 140, 169
94	25	85, 169
95	5	141

dose of 900 mg/day with prolonged exposure up to 12 months. Partial clinical efficacy was observed in a patient with thyroid cancer. Furthermore, a phase II trial with compound 53 is

recruiting patients with solid tumors and lymphoid malignancies.<sup>146</sup>

## 9. CONCLUSION

Our understanding of the involvement of Stat3 in numerous signaling pathways has grown exponentially in the past decade, reinforcing Stat3 as an important and a valid therapeutic target for many diseases. There is a great potential for the development of Stat3 inhibitors as therapy for cancer and other diseases, such as cardiovascular, rheumatoid arthritis, psoriasis, renal and pulmonary fibrosis, and Alzheimer's disease. After a decade of experience with preclinical evaluations of Stat3 inhibitors, limited translational studies are currently in progress.

**Figure 16.** Hybrid analogues of compound 3.**Table 9. Stat3 Inhibitors in Clinical Trials**

Agent	Structure	Trial phase	Indication	References
<b>1</b>		Phase I/II	Psoriasis	<sup>142</sup>
Pyrimethamine		Phase I/II	Chronic lymphocytic leukemia / Small lymphocytic lymphoma	<sup>143</sup>
OPB-31121	Structure not disclosed	Phase I	Advanced solid tumor	<sup>144</sup>
<b>53</b>		Phase I/II	Pancreatic cancer	<sup>145</sup>
		Phase II	Solid tumors and lymphoid malignancies	<sup>146</sup>

Virtual and high-throughput screenings as well as structure- and ligand-based molecular modeling yielded little success in identifying highly selective Stat3 inhibitors compared to other upstream or downstream targets in the Stat3-signaling pathway. The cellular targets of the putative Stat3 inhibitors identified from different virtual and high-throughput screening are unclear and require further validation and triaging. Moreover, many purported inhibitors possess biologically labile groups that may react with multiple targets, emphasizing the need to develop more selective inhibitors. Nevertheless, the diverse set of compounds reported in this article can be used for the optimization of leads via ligand-based and structure-based modeling and scaffold hopping approaches. For example, peptides and peptidomimetics should be further explored to develop physiologically stable small molecule Stat3 inhibitors. Several interesting inhibitors, such as compounds 7, 8, and 27, could be utilized for lead optimization and subsequent clinical development. Although there has been some progress in the development of Stat3 SH2 domain binding inhibitors, almost no progress has been made in the development of selective DNA-binding and N-terminal domain inhibitors. Medicinal chemistry efforts are warranted to develop inhibitors for these two domains.

The design and discovery of truly specific and potent Stat3 inhibitors have been very challenging. Therefore, there is a need to reassess the ongoing strategies to develop clinically useful drugs. Robust high-throughput screens using reliable assays and rational computational approaches are being considered to improve upon existing inhibitors. In the near future it will become clear that transcription factors like Stat3 are indeed druggable, opening a whole new field for medicinal chemists to affect patient care.

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## ■ ABBREVIATIONS USED

APRF, acute-phase response factor; CBP, CREB-binding protein; CDDO-Im, 2-cyano-3,12-dioxolen-1,9-dien-28-oic acid imidazolide; CDDO-Me, methyl ester of 2-cyano-3,12-dioxolen-1,9-dien-28-oic acid; EGFR, epidermal growth factor receptor; EMSA, electrophoretic mobility shift assay; EpiSC, epiblast stem cell; FGFR, fibroblast growth factor receptor; FP, fluorescence polarization; HER2, human epidermal growth factor receptor 2; HGFR, hepatocyte growth factor receptor; Hsp90, heat shock protein 90; HUVEC, human umbilical vein endothelial cell; IGFR, insulin-like growth factor receptor; ISRE, IFN-stimulated response element; LIF, leukemia inhibitory factor; MLSD, multiple ligand simultaneous docking; NSCLC, non-small-cell lung carcinoma; PDGFR, platelet-derived growth factor receptor; SAR, structure–activity relationship; SH2, Src homology 2; SHP-1/2, Src homology domain-containing tyrosine phosphatases 1/2; SOCS, suppressor of cytokine signaling; Stat3, signal transducer and activator of transcription 3; VEGFR, vascular endothelial growth factor receptor

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