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TARGETING STAT3 IN CANCER INHIBITION

Palma, G. [1,6]; D'Aiuto, M.[4]; Petrillo, A.[9]; Dallemagne, P.[7]; Sinicropi, M.S.[2]; Rodriquez, M.[5]; Longo, P.[3]; Mariconda, A.L.[8]; Arra, C.[1]; De Martino, F.[3]; Capasso, A.[5]; Saturnino, C.[5*]

[1]Istituto dei Tumori di Napoli Fondazione "G. Pascale" - IRCCS- , 80131, Naples, Italy
[2]Dipartimento di Farmacia e Scienze della Nutrizione, Università della Calabria, Arcavacata di Rende, 87036 (CS), Italy.
[3]Dipartimento di Chimica, Università di Salerno, Via Giovanni Paolo II n°132, 84084 Fisciano (SA), Italy
[4]Istituto dei Tumori di Napoli Fondazione "G.Pascale" - IRCCS-, 80131, Naples, Italy
[5]Dipartimento di Farmacia, Università degli Studi di Salerno, via Ponte don Melillo, 84084, Fisciano (SA), Italy
[6]Istituto Endocrinologia e Oncologia Sperimentale – Consiglio Nazionale delle Ricerche, Via Pansini, 80131, Naples, Italy
[7]CERMN, UFR des Sciences Pharmaceutiques, Université de Caen Basse-Normandie, Bd Becquerel, 14032 Caen, France
[8]Dipartimento di Ingegneria Industriale, Università di Salerno, 84084 Fisciano (SA), Italy
[9]Struttura Complessa di Radiodiagnostica 1, Istituto dei Tumori di Napoli Fondazione "G.Pascale" - IRCCS-, 80131, Naples, Italy

*saturnino@unisa.it

Abstract

Signal transducer and activator of transcription 3 (STAT3) regulates many critical functions in human normal and malignant tissues, such as differentiation, proliferation, survival, angiogenesis and immune function. Constitutive activation of STAT3 is implicated in a wide range of human cancers. As such, STAT3 has been studied as a tumor therapeutic target. The last approach using small molecule STAT3 inhibitors has been the most examined so Heterocyles and Carbazole. Heterocyles are an essential class of molecules, assuming a role in many aspect of our life. Indeed heterocyclic nucleus is a common feature of several biomolecule and bioactive compounds including agrochemical products and drugs. In this review we focused on two important class of heterocyclic compounds: carbazoles and NHCs (N-heterocyclic carbenes) and there implications in cancer inhibitions. Carbazoles, prevalent as structural motifs in various synthetic materials and naturally occurring alkaloids, as is known, have many applications especially as promising bioactive compounds due to their biological properties, known since 1965.
Furthermore NHCs are a class of stable carbenes that over the last few years have entered the field as "new" ligands for bioactive coordination compounds. It has been demonstrated that metal NHC complexes can be used to develop highly efficient metal based drugs with possible applications in the treatment of cancer or infectious diseases.

Keyword: STAT3, targeting, cancer

Introduction

Heterocycles are a class of compounds, making up more than half of all known organic compounds. Heterocycles are prevalent in a broad variety of drugs, vitamins, natural products, biomolecules, and biologically active compounds, including anticancer, antibiotic, anti-inflammatory, antimalarial, anti-HIV, antimicrobial, antibacterial, antifungal, antiviral, anti-diabetic, herbicidal, fungicidal, and insecticidal agents. Heterocycles are also of considerable interest because of their synthetic utility as synthetic intermediates, protecting groups, chiral auxiliaries, organ catalysts, and metal ligands in asymmetric catalysts inorganic synthesis. Therefore, substantial attention has been paid to develop efficient new methods to synthesize heterocycles. They are common structural units in marketed drugs and in medicinal chemistry in the drug discovering process. The majority of pharmaceuticals and biologically active agrochemicals are heterocyclic while countless additives and modifiers used in industrial applications from cosmetics, reprography, information storage and plastics are heterocyclic in nature. Many natural drugs [1,2,3,4] such as papaverine, theobromine, quinine, emetine, theophylline, atropine, procaine, codeine, reserpine and morphine are heterocycles. Almost all the compounds known as synthetic drugs such as diazepam, chlorpromazine, isoniazid, metronidazole, azidothymidine, barbiturates are also heterocycles. Synthetic heterocycles have therapeutic applications such as antibacterial, antifungal, anti-mycobacterial, trypanocidal, anti-HIV activity, anti-tubercular, antimalarial, analgesic, anti-inflammatory, muscle relaxants, anticonvulsant, anticancer and lipid peroxidation inhibitor, hypnotics, antidepressant, anti-tumoral, anthelmintic and insecticidal agents [5,6,7,8,9]. In fact, heterocyclic motif is present in the structures of all top 10 brand name small molecule drugs (Figure 1). Moreover heterocycles are used as bioisosteres for a variety of functional groups in drug candidates. The pharmacological benefits of employing heterocycles for better potency and specificity can in many cases be explained by their ability to participate in hydrogen bonding with the target protein, where the heterocycle can play the role of either H-acceptor as in heteroaromatic compounds or H-donor as in saturated N-heterocycles. Hydrogen bonding is relevant not only for pharmacological properties, but also for physicochemical and transport properties of drug molecules. Carbazoles are a class of aromatic heretocyclic nuclei (Figure 2), isolated first from coal tar in 1872 by Graebe and Glazer. In 1965, Chakraborty et al. described the isolation and biological properties of murrayanine from Murraya koenigii Spreng. The isolation of murrayanine was the first report of a naturally occurring carbazole alkaloid. Since then there has been a strong interest in this compounds due to the alluring structural features and promising biological activities exhibited by many carbazole alkaloids. They are prevalent as structural motifs in various synthetic materials and naturally occurring alkaloids. Most carbazole alkaloids have been isolated from the taxonomically related higher plants of the genus Murraya, Glycosmis, and Clausena from the family Rutaceae. The genus Murraya represents the richest source of carbazole alkaloids from terrestrial plants. The lower plants from which carbazole alkaloids have been isolated include several different Streptomyces species. Further natural sources for carbazole alkaloids are, for example, the blue-green algae Hyella caespitosa, Aspergillus species, Actinomadura species, and the ascidian Didemnum granulatum [10]. A large number of carbazoles derivate from plants are endowed with profound biological activities, which include antitumor, psychotropic, anti-inflammatory, antihistaminic, antibiotic, and anti-oxidative activities. The structural attributes of such carbazole-based natural products are multifarious. One of their most important features is the presence of nuclear hydroxyl groups, as well as the quinone functionality. In addition, prenyl groups are often found in some natural carbazoles. A large group of bioactive carbazole natural products and synthetic derivatives are found to contain annulated rings such as those in ellipticine derivatives (1), staurosporine (2),carbazomycin B (3), carbazomadurin A (4), clausenamine A (5), etc. Moreover, the carbazole moiety is considered as one of the pharmacophores in the cardiovascular pharmaceuticals carvedilol (6), and carazolol (7), which are used in the treatment of hypertension, ischemic heart disease, and congestive heart failure [19] (Figure 3). Considering the evidences of carbazoles bioactivities, several synthetic strategies has been reported in the literature.

In recent years, the synthesis of these carbazole alkaloids has been extensively reviewed by Knolker et al. [12,13]. Traditionally, the synthesis of carbazoles could be carry out upon nitrene insertion, Fischer indolization, Pummerer cyclization, Diels-Alder reaction, dehydrogenative cyclization of diarylamines, etc. In more recent years, transition metal-mediated C-C and C-N bond formation,

cyclotrimerization, benzannulation, Suzuki-Miyaura coupling, ring-closing metathe- sis, etc. have been investigated. These reactions follow two modes: (i) the formation of an A or C ring from substituted indole derivatives and (ii) the formation of a B ring from benzene derivatives. Many of these strategies are continuously innovated to address issues related to region-chemical selectivity and efficiency [11]. Several working hypotheses have been proposed to account for the biogenesis of carbazole alkaloids, but no evidences have been observed. A comparison of the structural features of the carbazole alkaloids isolated from higher plants suggests that 3-methylcarbazole may represent the key intermediate in their biosynthesis. On the other hand, 2-methylcarbazole appears to be the common biogenetic precursor of the conventional tricyclic carbazoles isolated from lower plants. However, the biogenetic precursor of the carbazole nucleus in nature is not clear. The isolation of several 3-methylcarbazole derivatives from higher plants [14] and of carbazole from Glycosmis pentaphylla [15] shows that the aromatic methyl group can be eliminated oxidatively from the key intermediate 3-methylcarbazole via -CH2OH, -CHO, and -COOH functionalities [16]. The isolation of 3-methylcarbazole from the genus Clausena [17] the co-occurrence of murrayafoline A (2), koenoline (3), murrayanine (4), and mukoeic acid (5) in M. koenigii, as well as the subsequent isolation of mukonine (6) and mukonidine (9) and the discovery of 2-hydroxy-3-methylcarbazole (7) and mukonal (8) in M. koenigii support the hypothesis of biomimetic hydroxylation of 3-methylcarbazole. Congeners that differ in the oxidation state of the C-3 methyl group, i.e. -CH2OH, -CHO, -COOH, and -COOMe, were found for various alkaloids, a fact which indicates an *in vivo* oxidation of carbazole alkaloids (Figure 4). The occurrence of heptaphylline (10) [18] and murrayacine (11) [19] (Fgiure 5) in Clausena heptaphylla is circumstantial evidence for the origin of the pyran ring from the prenylated congener. This explains the formation of pyranocarbazoles from 2-hydroxy-3-methylcarbazole as shown by Popli and Kapil [20] The co-occurrence of 2-hydroxy-3-methylcarbazole (7) [21] mukonal (8), [22] and mukonidine (9) [23] provides clear evidence for the *in vivo* oxidation of the methyl group in 2-hydroxy-3-methylcarbazole. All these findings strongly suggest 3-methylcarbazole as the key precursor for the carbazoles isolated from higher plants.

A classical method that has been often utilized For the synthesis of aromatic carbazoles is the dehydrogenation of 1,2,3,4-tetrahydrocarbazoles prepared by Fischer-Borsche synthesis. Many carbazole alkaloids were synthesized from a variety of indole precursors or by oxidative cyclization of diarylamines. Carbazole, the active compound of coal tar and its N-alkyl derivatives, decreased IL-6-stimulated STAT3 activation and DNA-binding activity in embryonic kidney or human monocytic leukaemia cells through undefined mechanisms, without affecting phosphorylation of STAT3 [24, 25]. Data published by Saturnino et al, can reinforce the idea of a role of carbazoles as inhibitors activity of STAT3 in cancer [133 - 137].

Material and Methods

STAT proteins: novel molecular targets for cancer drug discovery

Signal Transducers and Activators of Transcription (STATs) are a family of cytoplasmic proteins with roles as signal messengers and transcription factors that participate in normal cellular responses to cytokines and growth factors. Frequently, however, abnormal activity of certain STAT family members, particularly Stat3 and Stat5, is associated with a wide variety of human malignancies, including hematologic, breast, head and neck, and prostate cancers (Table 1). STATs were originally discovered as latent cytoplasmic transcription factors that mediate cellular responses to diverse cytokines and growth factors [26, 27, 28]. STATs are activated by tyrosine phosphorylation following the binding of cytokines or growth factors to cognate receptors on the cell surface. Tyrosine kinases that mediate STAT activation include growth factor receptors and cytoplasmic tyrosine kinases, particularly Janus kinase (JAK) and Src kinase families. Once tyrosine phosphorylated, two STAT monomers form dimers through reciprocal phosphotyrosine-SH2 interactions, translocate to the nucleus, and bind to STAT-specific DNA-response elements of target genes to induce gene transcription. There are seven STAT family members identified in mammals, designated Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b and Stat6 . STATs have diverse normal biological functions, which include roles in cell differentiation, proliferation, development, apoptosis, and inflammation [29, 30]. STAT proteins are involved in some fundamental cellular processes, including cell growth and differentiation, development, apoptosis, immune responses and inflammation [31]. STAT1 signaling is activated in response to interferon (IFN) stimulation and supports immune function by controlling the growth and apoptosis of immune cells [32]. STAT1 signaling regulates T helper type 1 (TH1)

cell-specific cytokine production that alters both immune function and inflammatory responses by shifting the balance between TH1 and TH2 cells [33]. Indeed, STAT1 deficiency abrogates IFN responsiveness, leading mice to succumb to bacterial and viral infections [34]. Furthermore, the loss of responsiveness to IFNγ due to STAT1 deficiency provides malignant cells with a growth advantage and leads to increased tumor formation [32]. This outcome suggests that STAT1 has a tumor-suppressive function; although recent data indicate that the protein has a more complex role in carcinogenesis [32]. STAT2 signaling is important for the induction of antiviral effects. STAT2-null mice and STAT2-null cell lines have defective antiviral responses to IFNα and IFNβ, as well as blunted apoptotic effects to IFNα and IFNβ [35]. Evidence further suggests that altered STAT2 signaling may partly contribute to carcinogenesis through the up regulation of interleukin-6 (IL-6) production, which promotes STAT3 activation [35]. Notably, STAT2-deficient mice showed markedly lower levels of IL-6, which was associated with diminished tumor progression [35]. By contrast, the reconstitution of STAT2 in the null background up regulated IL-6 production and increased the levels of pSTAT3.

STAT3 function is essential for early embryonic development, which is clearly demonstrated by the death on day 8.5 of STAT3-deficient mouse embryos [36]. The biological importance of STAT3 was further studied using tissue-specific STAT3-deficient mice [37]. In vitro cultured STAT3-deficient T cells did not respond to IL-6 stimulation and could not be rescued by IL-6 from apoptotic cell death, indicating that STAT3 functions are essential for IL-6-mediated anti-apoptotic responses [38]. Furthermore, STAT3-deficient keratinocytes showed a poor wound- healing response in vitro and in vivo, which was due to the limited migration of the cells [39]. STAT4 is a crucial mediator of IL-12 function that regulates the differentiation of TH1 cells and their inflammatory responses [33]. Accordingly, STAT4 signaling is associated with autoimmune diseases, such as experimental autoimmune encephalomyelitis (EAE) induction, an animal model of multiple sclerosis [52]. Additionally, STAT4-null lymphocytes showed decreased proliferation and had an impaired response to IL-12 [53]. Treatment with a STAT4-specific antisense oligonucleotide (ASO) led to an improvement in the systemic lupus erythematosus (SLE) phenotype, severe lupus nephritis in mouse models, which suggests that STAT4 also has an important role in SLE, even though STAT4 knockout had no effect on the clinical presentation of the disease in mouse models [53].

STAT5 has two isoforms, STAT5A and STAT5B, which are encoded by distinct genes and share 96% sequence homology, with notable differences occurring in the transactivation domain [54]. Normal STAT5 signaling is important in mammary gland development and milk production, and in hematopoiesis [55]. Mice deficient in both STAT5A and STAT5B show defects in IL-2 receptor-α expression in T lymphocytes [37]. By contrast, mice that are deficient in STAT5B only showed loss of sexual dimorphism of body growth rate, which was accompanied by a decreased expression in male-specific liver genes [56] and the attenuation of the cytolytic activity of natural killer cells [57]. Constitutive STAT5 activation is also implicated in the pathogenesis of HNSCC, chronic myelogenous leukaemia (CML) [58], breast [59], prostate and uterine cancers [44]. The aberrant STAT5 activation by BCR–ABL in CML43 is particularly noteworthy. It is widely recognized that STAT5 and STAT3 share similar functions in promoting cancer, including the induction of pro-proliferative and anti-apoptotic genes [60,61].

STAT6 signaling is induced by IL-4 and IL-13 and supports immune function, notably regulating the balance between inflammatory and allergic immune responses[62,63,64,65]. Beyond the immune system, STAT6 signaling promotes luminal mammary epithelium development and is implicated in the pathology of lung and airway disease, including the promotion of airway hyperactivity and mucus production in the lung epithelium, and the regulation of allergic skin inflammation [62, 66, 67, 68].

Role of STATs in oncogenesis

Studies of the molecular basis of oncogenesis by oncoproteins like v-Src have provided insights into changes in intracellular signaling proteins that participate in malignant transformation. The initial finding that Stat3 is constitutively activated in v-Src transformation [69, 70] suggested that aberrant STATs may have key roles in oncogenesis [71, 72]. Moreover, a constitutively activated Stat3 mutant alone is sufficient to induce transformation, and cells transformed this way can form tumors in nude mice [73], providing genetic evidence that Stat3 has oncogenic potential. Certainly, these evidences confirm that the abnormal Stat3 activation can induce permanent changes in gene expression programs that ultimately lead to the malignant phenotype. Hence, constitutive Stat3 signaling contributes to transformation by oncogenic tyrosine kinases. Accumulating evidence strongly implicates

the critical role of aberrant STAT3 activation in malignant transformation and tumorigenesis. Among the earliest clues that STATs contribute to oncogenesis were the findings that STAT3 is constitutively activated in Src-transformed cell lines and that interrupting STAT3 signaling blocks the transformation of mouse fibroblasts by the Src oncoprotein [123]. The first direct links between STATs and human cancer came from the findings that constitutive STAT3 activity is required for the growth of head and neck cancer [131] and of acute myelocytic leukemia and chronic myelocytic leukemia cells [132]. In addition to v-Src, other transforming tyrosine kinases, such as v-Eyk [74], v-Ros [75], v-Fps [76], Etk/ BMX [77], and Lck [78], all activate Stat3 in the context of oncogenesis.

Furthermore, constitutive Stat3 activation is also associated with transformation induced by tumor viruses, including HTLV-1 [79], polyomavirus middle T antigen [76], EBV [80], and herpesvirus saimiri [78], that directly or indirectly activate JAKs or Src family tyrosine kinases. Due of its central position in the signaling pathways from protein tyrosine kinases, aberrant Stat3 activity is a key mediator in the transforming process induced by oncogenic tyrosine kinases. In contrast, Stat3-independent mechanisms mediate transformation by oncoproteins that are not tyrosine kinases themselves or do not activate tyrosine kinase signaling pathways, including v-Ras and v-Raf [76].

With regard to other STATs, constitutive activation of both Stat1 and Stat5 accompanies transformation of pre-B lymphocytes by the v-Abl tyrosine kinase [81]. The transforming BCR-Abl fusion protein also activates Stat1 and/or Stat5 [82] and constitutive Stat5 activity is essential for BCR-Abl-induced transformation [83, 84]. Consistent with these findings, mutational activated forms of Stat5 are sufficient to induce certain properties of transformed cells [85]. That being so, it can be resumed that Stat3 and Stat5 are the STAT family members with intrinsic oncogenic potential and most strongly associated with human cancer.

Mounting evidence gives credence to Stat3 as a bona fide mediator of oncogenesis that participates in human malignancies. The dysregulation of the role of STAT3 in keratinocyte physiology is thought to contribute to the induction of skin carcinogenesis [40]. STAT3 function is often aberrant in the context of cancer, and this abnormality represents an underlying mechanism of STAT3 for promoting malignant transformation and progression.

Of clinical significance, constitutively active STAT3 Is detected in numerous malignancies, including breast, melanoma, prostate, head and neck squamous cell carcinoma (HNSCC), multiple myeloma, pancreatic, ovarian, and brain tumor [42, 43, 44, 45]. Aberrant STAT3 signaling promotes tumorigenesis and tumor progression partly through dysregulating the expression of critical genes that control cell growth and survival, angiogenesis, migration, invasion or metastasis [41, 42, 43, 44]. These genes include those that encode p21WAF1/CIP2, cyclin D1, MYC, BCL-XL, BCL-2, vascular endothelial growth factor (VEGF), matrix metalloproteinase 1 (MMP1), MMP7 and MMP9, and surviving [41, 42, 43, 44]. Evidence also supports a role of STAT3 in the suppression of tumor immune surveillance [46, 47]. Consequently, the genetic and pharmacological modulation of persistently active STAT3 was shown to control the tumor phenotype and to lead to tumor regression in vivo [43, 44, 45, 48, 49]. Of further clinical significance, mutations within the SH2 domain-coding sequence in STAT3 occur in patients with a rare primary immunodeficiency, hyperimmunoglobulin E syndrome, and in large granular lymphocytic leukaemia [50]. These mutations reportedly increase the stability of the functional STAT3–STAT3 dimers and are associated with the disease pathogenesis [60, 61].

STAT proteins have been shown to play important roles in cell proliferation induced by cytokines. The first evidence towards the role of STAT3 in survival was that STAT3 activation is essential for gp130-induced proliferation of the IL-3-dependent pro-B hematopoietic cell line, BAF/B03 cells [125]. In breast carcinoma cells, autocrine mediated STAT3 activation was found to correlate with cell proliferation [126]. Activated STAT3 promotes proliferation primarily by stimulating transcription of key cancer genes linked with proliferation of tumor cells, such as cyclin D1, cyclin B and cdc2,which are involved in the regulation of cell cycle [124]. The active form of STAT3 was found to promote the G1/S phase transition of the cell cycle through the expression of cyclin D1, which can associate with cdk4 or cdk6 and control progression from G1 to S phase in gastric [127] and colorectal [128] cancer cells.

One of the first indications that STAT3 signaling contributes to malignancy by preventing apoptosis pathway came from studies in multiple myeloma cells showing that increased expression of Bcl-xL, a member of the anti-apoptotic Bcl-2-family, is dependent on constitutively activated STAT3 [123]. Several anti-apoptotic proteins, such as surviving and members of the Bcl family (Bcl-xL, Bcl-2 and Mcl-1), which are known to be crucial for tumor cell survival,

are direct target genes of STAT3 and are down-regulated as a consequence of STAT3 inhibition [129]. STAT3 has also been found to be involved in colorectal cancer cell growth, survival, invasion, and migration through regulation of gene expression, such as Bcl-2, p21waf1/cip1, p27kip1, E-cadherin, VEGF, and MMPs [130]. In the context of human cancer, there is a high frequency of activation of Stat1, Stat3 and Stat5 (Table 2), with higher incidence of abnormal Stat3 activation in almost all the tumors studied [87].

Approaches to inhibit STAT proteins

Since the discovery of the first peptide inhibitor of a STAT protein [61], the achievement to target STAT signaling for therapeutic purposes continue.

Several inhibitory strategies against STAT signaling and function are being pursued. A validated model for the inhibition of STATs pathways is represented by small-molecule dimerization disruptors (SMDDs) or phospho-peptidomimetic inhibitors (PPMIs) that target the phospho-Tyr-SH2 domain interaction at the interface of dimers of signal transducer and activator of transcription (STAT) proteins. This leads to the disruption of STAT–STAT dimers and the formation of STAT–SMDD or STAT–PPMI heterocomplexes, leading to a suppression of STAT signaling and function. Instead the binding of ligands, such as growth factors and cytokines, to their cognate receptors on the cell surface induces STAT tyrosine phosphorylation and activation. Tyrosine kinases that mediate STAT phosphorylation, leading to STAT–STAT dimer formation, are the targets of small-molecule tyrosine kinase inhibitors. The overall effect of these modulators is to block the induction of STAT phosphorylation and signaling [69]. Most of these focus on inhibiting STAT dimerization using peptides or peptidomimetics generated through structure-based design, small molecules identified by molecular modelling, virtual or library screening, or natural products. STAT dimerization and signaling can also be blocked by inhibiting tyrosine kinases that phosphorylate STAT proteins, or by inducing phosphatases that dephosphorylate STAT proteins.

Other approaches, include the use of oligodeoxynucleotide (ODN) decoys as specific STAT DNA-binding domain inhibitors and ASOs that interfere with STAT mRNA [69]. The knockdown of the STAT3 protein by antisense RNA or small interfering RNA approaches has been demonstrated in studies, which showed the induction of tumor cell apoptosis and tumor regression [138-140].

Targeting STAT3

Several human cancers could benefit from therapeutics that target aberrantly active STAT3. Owing to its therapeutic significance, STAT3 is the target in many drug discovery research efforts.

An important factor determining STAT3 functional heterogeneity is probably the existence of two alternatively spliced isoforms: the full-length STAT3α and the truncated STAT3β, which lacks the C-terminal activation domain and is generally considered a dominant negative form [86,87]. Mice specifically lacking STAT3β are hypersensitive to endotoxin-induced inflammation and undergo both up- and down regulation of gene subsets, indicating specific functions for the STAT3β isoform [88]. To assess the specific functions of STAT3α and STAT3β, V. Poli and co. [99] generated mice that can produce only one isoform or the other. They confirmed that STAT3β is not required for viability but is involved in inflammation. In contrast, STAT3α-deficient mice died within 24 h of birth, showing that this isoform is required for postnatal functions but that STAT3β can rescue the embryonic lethality of a complete STAT3 deletion. Indeed, their data indicate that STAT3β is not a dominant negative factor, as it can activate specific STAT3 target genes. However, STAT3α has non-redundant roles in modulating IL-6 signaling and mediating IL-10 functions.

Several studies highlighted the oncogenic importance of Stat3 and established a direct link to tumor progression. Aberrant Stat3 signaling is obligatory for growth and survival of various human tumor cells, including multiple myelomas (MM), breast carcinomas, head and neck squamous cell carcinomas (HNSCC), the T cell lymphoma mycosis fungoides, and large granular lymphocyte (LGL) leukemia [90, 91]. STAT3 drug discovery research has mostly focused on targeting the pTyr-SH2 domain interaction [92, 93] given its importance in promoting STAT3 dimerization and function, and these efforts have generated various inhibitory agents. However, there are many reported STAT3 inhibitors that may induce their effects through multiple mechanisms.

Peptides and peptidomimetics

A semi-rational, structure-based design approach identified the first SH2 domain-binding peptides and peptidomimetics that disrupt the STAT3 pTyr-SH2 domain interactions and STAT3–STAT3 dimerization [92, 93, 94]. The native, parent pTyr peptide, PY*LKTK (where Y* stands for pTyr) and its modified forms blocked the DNA-binding and transcriptional activities of STAT3 at high micromolar concentrations

[94]. Peptidomimetic and non-peptide analogues, including ISS-610 and S3I-M2001, exhibited improved potency against STAT3 activity in vitro and against diverse malignant cells harbouring aberrantly active STAT3 [48, 93, 94]. S31-M2001 inhibited the growth of human breast tumor xenografts, while ISS-610 inhibited cell growth and induced apoptosis in vitro [48]. Furthermore, phosphopeptide binding sequences with the primary structure pTyr-Xxx-Xxx-Gln (where Xxx represents any amino acid) derived from leukaemia inhibitory factor (LIF), IL-10 receptor, epidermal growth factor receptor (EGFR), granulocyte colony-stimulating factor (GCSF) receptor or glycoprotein 130 (gp130), similarly inhibited STAT3 activation. In these studies, the peptidomimetic Ac-pTyr-Leu-Pro-Gln-Thr-Val-NH2 was derived, which inhibited STAT3 activity (IC50 values of 150 nM) [95, 96]. Moreover, a 28-mer native peptide identified as SPI, derived from the STAT3 SH2 domain, inhibited the STAT3 pTyr-SH2 domain interaction and signaling. The activity of SPI was moderate, but it did suppress cell viability and induced apoptosis of human breast, pancreatic, prostate and non-small cell lung cancer (NSCLC) cells in vitro [98]. Peptidomimetics have better pharmacokinetic properties than peptides. As a result investigators employed the afore-mentioned peptide, XpYL as the basic structural scaffold to develop their peptidomimetic compounds. Out of these, ISS610 [114] and S31-M2001 had superior pharmacokinetic profiles [115]. Similarly several other peptidomimetic molecules have been developed from the basic scaffold of compound [115-1120]. Among these CJ-1383 showed promising results with IC50= 3–11 μM in two breast cancer cell lines containing high levels of phosphorylated STAT3 [116]. Despite hard work of several investigators, these agents need substantial improvement with regards to their in vivo metabolic susceptibility and cellular permeability prior to clinical testing. For the same reason no promising STAT5 dimerization inhibitor could be developed from this class.

Small molecules

Many small-molecule inhibitors of STAT3 activity have been identified through computational modelling, docking studies and virtual screening of chemical libraries. In most cases, these agents function as disruptors of STAT3–STAT3 dimerization. STA-21 (also known as NSC628869) was identified from the screening of the National Cancer Institute (NCI) chemical library as an inhibitor of STAT3 dimerization, DNA-binding activity and transcriptional function in breast cancer cells, with a potency of 20 μM [99]. Its structural analogue, LLL-3 (which showed improved membrane permeability), decreased cell viability in vitro and intracranial tumour size *in vivo* in glioblastoma animal models [100]. A catechol (1,2-dihydroxybenzene) compound was identified from Wyeth's proprietary small-molecule collection as a STAT3 SH2 domain inhibitor, and this agent was active at 106 μM against a multiple myeloma cell line [101]. Furthermore, a structure-based, virtual screen of the NCI chemical library targeting the STAT3 SH2 domain discovered S3I-201 (also known as NSC74859) as a STAT3–STAT3 dimerization disruptor, with a potency of 60–110 μM [48]. S3I-201 inhibited STAT3 DNA-binding and transcriptional activities, induced growth inhibition and apoptosis of tumor cells harboring constitutively active STAT3, and suppressed the growth of human breast cancer xenografts [48]. Several of its derivatives, including S3I-201.1066, BP-1-102 and S3I-1757, showed improved potencies, with IC50 values of 35 μM (S3I-201.1066), 6.8 μM (BP-1-102) and 13.5 μM (S3I-1757), and inhibited cell growth, malignant transformation, survival, migration and invasiveness in vitro of malignant cells harboring aberrantly active STAT3.

Virtual ligand screening identified Cpd30 (4-(5-((3-ethyl-4-oxo-2-thioxo-1,3-thiazolidin-5-ylidene)methyl)-2-furyl)benzoic acid), which selectively inhibited STAT3 with moderate potency, blocked STAT3 nuclear translocation upon IL-6 stimulation, and induced apoptosis in breast cancer cells harbouring constitutively active STAT3 [60]. The related agent, Cpd188 (4-((3-((carboxymethyl)thio)-4-hydroxy-1-naphthyl)amino)sulphonyl)benzoic acid), in combination with docetaxel, decreased tumor growth in chemotherapy-resistant human breast cancer xenograft models [61].

Stattic, a non-peptide small molecule discovered through virtual screening of chemical libraries, targets the STAT3 SH2 domain and inhibits STAT3 signaling at 10 μM [102]. It induced apoptosis of STAT3-dependent breast cancer and HNSCC cells and inhibited growth of orthotopic HNSCC tumour xenografts [102]. STX-0119, another STAT3 SH2 domain antagonist, induced antitumor cell effects in vitro and antitumor effects in vivo in a human lymphoma model, possibly by disrupting STAT3–STAT3 dimerization, with little effect on STAT3 phosphorylation [103]. Fragments of STX-0119 and static were chemically fused to generate HJC0123 [104], which suppressed STAT3 phosphorylation and transcriptional activity in breast cancer cells and

induced antitumor cell effects against breast and pancreatic cancer cells in vitro at IC50 values of 0.1–1.25 μM. The oral administration of HJC0123 led to inhibition of growth of human breast cancer xenografts [104].

Furthermore, an agent identified as OBP-31121 was reported to inhibit STAT1, STAT3 and STAT5 phosphorylation. OBP-31121 induced loss of viability and apoptosis in vitro and inhibited tumor growth in vivo in gastric cancer models, and it further sensitized gastric cancer cells to cisplatin and 5-fluorouracil [105]. OBP-31121 has progressed to a Phase I clinical trial to determine the maximum tolerated dose. Matsuno et al. recognized STX-0119 (IC50 = 74 μM), a derivative of N-[2-(1, 3, 4-oxadiazolyl)]-4- quinolinecarboxamide by virtual screen using a customized version of DOCK4 program with the crystal structure of STAT3. Oral administrations of STX-0119 arrested the growth of human lymphoma cells in a SCC-3 subcutaneous xenograft model through inhibition of STAT3 activity [121].

Unfortunately, these molecules are still not potent enough or drugable to be examined in clinical studies.

Natural product inhibitors

Natural products have been an important resource in STAT3 inhibitor discovery and these efforts have yielded several lead candidates. In many of these cases, however, the mechanism of action of these candidates with regard to STAT3 activity are unclear. It is possible that they inhibit STAT3 indirectly and are likely to block several targets.

Curcumin, a phenolic compound derived from the perennial herb Curcuma longa was shown to suppress JAK–STAT signaling at 15μM, induce cell cycle arrest and inhibit cell invasion in vitro in a small cell lung cancer model [106]. In mice bearing gastric cancer xenografts, treatment with curcumin inhibited IL-6 production by IL-1β-stimulated myeloid-derived suppressor cells, which was associated with decreased activation of STAT3 and nuclear factor-κB (NF-κB).

Curcumin analogues with improved bioavailability and stability, such as FLLL32, had enhanced potency (IC50 values of 0.75–1.45μM) in suppressing both pSTAT3 and total STAT3, and they also induced STAT3 ubiquitylation and possible proteasomal degradation in canine and human osteosarcoma cells in vitro[107]. Another curcumin analogue, HO-3867, similarly down regulated STAT3 signaling in cisplatin-resistant human ovarian cancer cells, thereby promoting enhanced sensitivity to cisplatin [108]. HO-3867 also induced apoptosis in BRCA1-mutated human ovarian cancer cells that harbour aberrantly active STAT3 [109].

Studies suggest that LLL12, another small-molecule inhibitor of STAT3 signaling that is based on curcumin [110], might suppress STAT3 activation by blocking its recruitment to the receptor and thereby preventing phosphorylation by tyrosine kinases, and by interfering with dimerization [111]. LLL12 suppressed cell viability, induced apoptosis, and repressed colony formation and migration *in vitro* in studies of glioblastoma, osteosarcoma and breast cancer cells84,86. It also inhibited angiogenesis, tumour vasculature development, and tumor growth *in vivo* in osteosarcoma xenograft models[111, 112]. Except for the finding that the analogues FLLL32 and FLLL62 may interact with the STAT3 SH2 domain, questions remain regarding how curcumin or its analogues inhibit STAT3 signaling. Resveratrol (3,5,4′-trihydroxystilbene), a polyphenolic compound found in red grapes and several other plants, was reported to inhibit STAT3 signalling at high micromolar concentrations, and it is likely that this effect contributes to the antitumor cell responses to this agent.

Constitutive and IL-6-induced STAT3 activation in multiple myeloma, leukaemia and other tumor cell types were inhibited on resveratrol treatment, leading to a decreased expression of BCL-2 and other anti-apoptotic proteins, and apoptosis induction in vitro [112]. The resveratrol derivative LYR71 suppressed breast cancer cell viability (IC50 value of 20μM), and inhibited STAT3-mediated MMP9 expression [113]. Reports also showed that the inhibition of the JAK–STAT3 pathway by resveratrol or its analogue, piceatannol (3,3′,4,4′-transtrihydroxystilbene), decreased BCL-XL and BCL-2 expression and sensitized lung carcinoma, multiple myeloma, prostate and pancreatic cancer and the glioblastoma multiforme patient-derived CD133-positive cells to radiation or chemotherapy in vitro. Despite these observations, the mechanisms for the inhibition of STAT3 signaling by resveratrol or its analogues remain poorly understood.

Shin et al. searched within the natural compounds using a dual-luciferase assay to describe novel and specific inhibitor of STAT3. Cryptotanshinone, derived from roots of Salvia miltiorrhiza Bunge (Danshen, a Chinese herb), was identified as a potent STAT3 inhibitor. Cryptotanshinone inhibited STAT3 activity in a dose dependent manner in HCT116 colon cancer cells with an IC50 value of 4.6μM. Activity of STAT3 was also inhibited in breast, prostate and cervical cancer cell lines. Study of binding mechanism

revealed that cryptotanshinone directly interact with SH2 domain of STAT3 to inhibit Tyr705 phosphorylation and prevents STAT3 dimerization and nuclear translocation [122].
Carbazole, the active compound of coal tar and its N-alkyl derivatives, decreased IL-6-stimulated STAT3 activation and DNA-binding activity in embryonic kidney or human monocytic leukaemia cells through undefined mechanisms, without affecting phosphorylation of STAT3 [24, 25].

Conclusion

STAT proteins were originally discovered in the context of normal cytokine signaling and were not considered as potential targets for cancer therapy at that time. Recent studies provide evidence for persistent STAT activation at high frequency in clinical tumor samples, so that STAT3 now emerges as an attractive target for tumor treatment. Persistent STAT3 activation in tumor cells seems to disrupt the normal physiological roles in controlling cell growth, survival, angiogenesis and immune function while conversely, blockade of STAT3 in cultured tumor cells was found to induce apoptosis, inhibit cell proliferation, suppress angiogenesis and stimulate immune responses. Inhibiting STAT3 at the source of activating signaling pathways would be challenging because healthy cells would also be affected, unless there is away to accurately target only tumor cells. As STAT3 is activated by dimerization and then binds DNA to perform its functions, specific inhibitors targeting the disruption of their protein–protein interactions or DNA binding activity, are more promising agents. Inhibitors may target upstream kinases, such as JAK2 or Src, or act as inducers of Src homology 2 phosphatases. After a decade of preclinical evaluation of STAT3 inhibitors, limited translational studies are currently in progress. The design and discovery of truly specific and potent STAT3 inhibitors is a challenging process. There is an abundance of natural compounds with inhibitory properties against this transcription factor that could act as lead compounds for the synthesis of more efficient molecules. The most important hurdle for the development of a STAT3 inhibitor as potential therapeutics will be the demonstration of their anti-tumor efficacy in in vivo systems and lack of toxicity in relevant animal models of human cancer.

Competing interest

The authors have no relevant competing interests.

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Table 1. STAT activation in normal functions and diseased.

STAT	CELULAR FUNCTIONS	MAJOR DISEASED
1	Cell growth and apoptosis Th1 cell-specific cytokine production Antimicrobial defence	Atherosclerosis Infection Immune disorder
2	Mediation of IFN a/b	Cancer Infection Immune disorder
3	Cell proliferation and survival Inflamation Immune response Embryonic development Cell motility	Cancer
4	Th1 cell differentiation Inflammatory reponses Cell proliferation	Systemic lupus erythematosus
5 A	Cell proliferation and survival IL2R exepression Mammary gland development	Cancer Chronic myelogenous leukemia
5B	Cell proliferation and survival NK cell cytolytic activity	Cancer Chronic myelogenous leukemia
6	Inflammatory and allergic immune response B cell and T cell proliferation Th2 cell differentiation	Asthma Allergy

Table 2. Activation of STAT proteins in tumors

Tumor Type	**Actived STATs**
Breast	STAT1 and STAT3
Head and Neack	STAT3
Brain Tumors	STAT1, 3, 5
Multiple Myeloma	STAT1, 3
Lymphomas	STAT3, 5
Leukemia	STAT1, 3, 5
Other Cancer	
Prostate	STAT3
Pancreatic	STAT3
Adenocarcinoma	STAT3
Ovarian Carcinoma	STAT3
Melanoma	STAT3

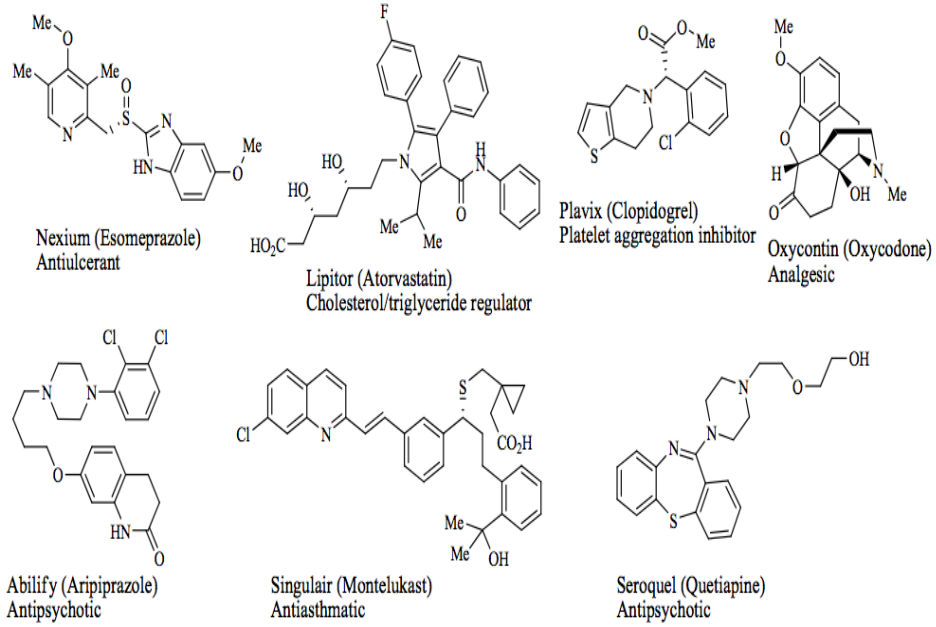


Figure 1. All top 10 brand name small molecule drugs

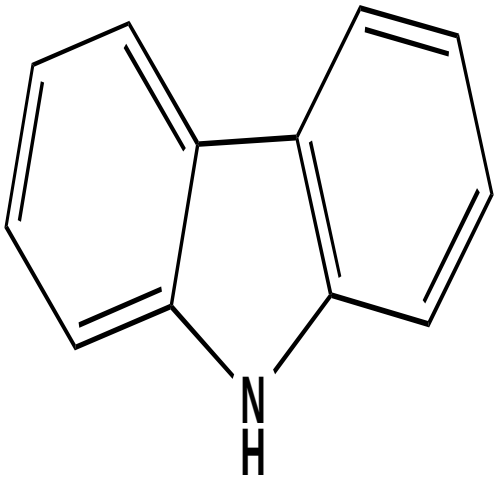


Figure 2. Structure of carbazole

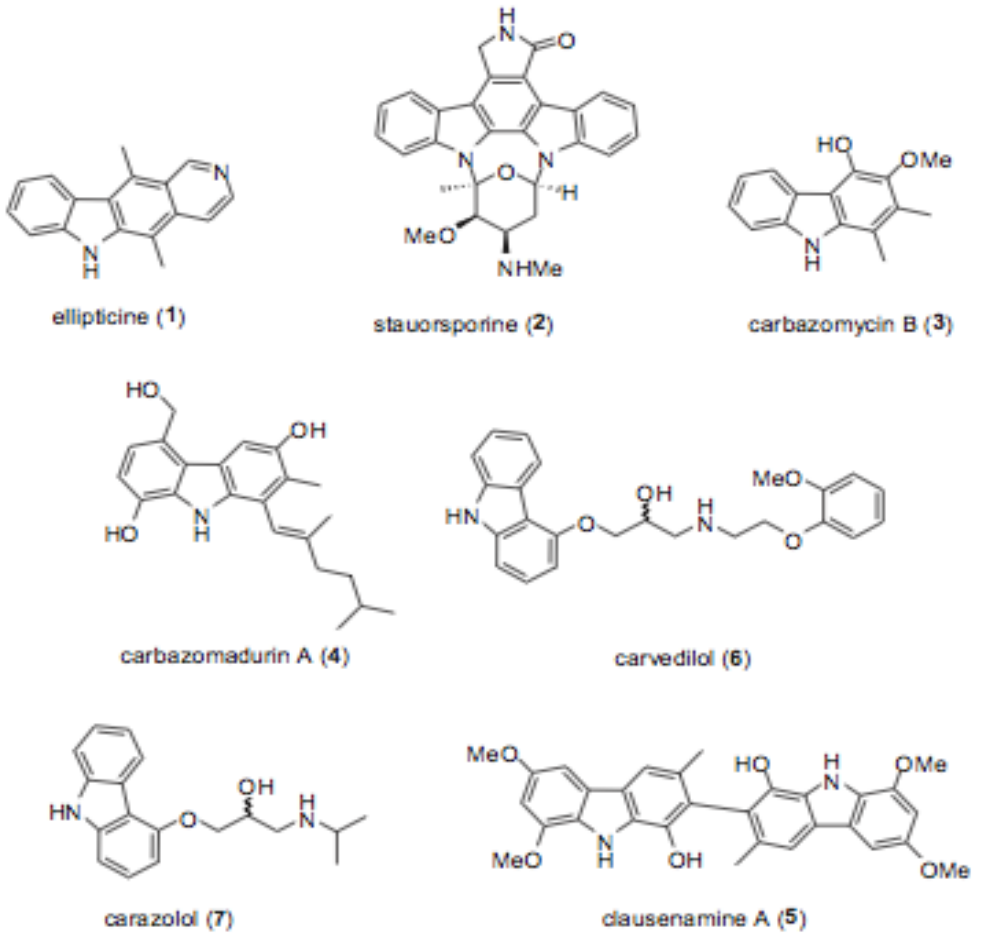
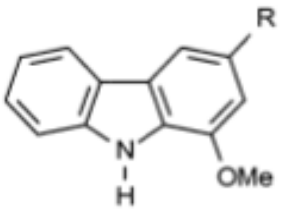
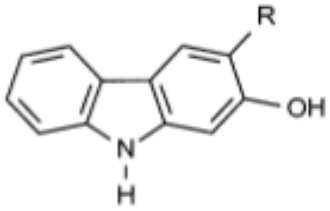


Figure 3. Bioactive carbazole natural products and synthetic derivatives

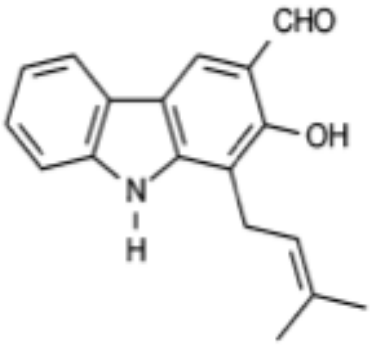




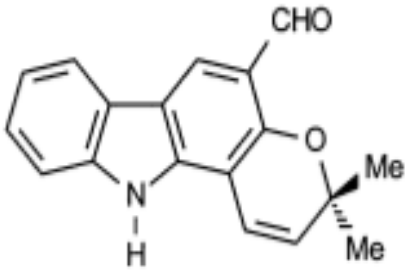
2 Murrayafoline A
R = Me
3 Koenoline
R = CH_2OH
4 Murrayanine
R = CHO
5 Mukoeic acid
R = COOH
6 Mukonine
R = COOMe

7 2-Hydroxy-3-methylcarbazole
R = Me
8 Mukonal
R = CHO
9 Mukonidine
R = COOMe

Figure 4. Carbazole alkaloids (1)



10 Heptaphylline



11 Murrayacine

Figure 5. Carbazole alkaloids