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Targeting STATs in neuroinflammation: the Road Less Traveled!

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Short title: STAT inhibitors in neuroinflammation

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Graphical abstract
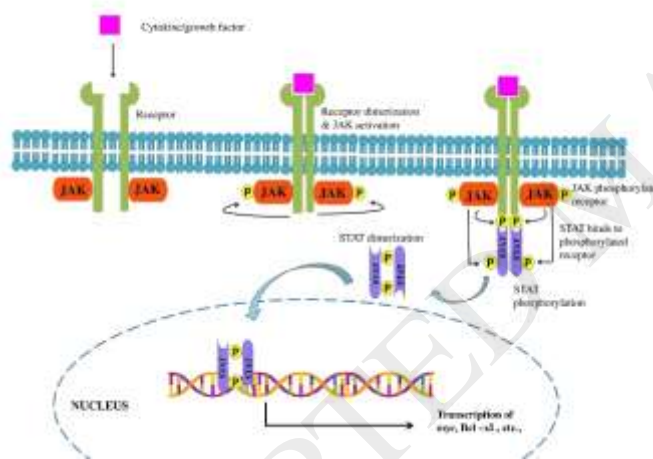
Abstract

JAK/STAT transduction pathway is a highly conserved pathway implicated in regulating cellular proliferation, differentiation, survival and apoptosis. Dysregulation of this pathway is involved in the onset of autoimmune, haematological, oncological, metabolic and neurological diseases. Over the last few years, the research of anti-neuroinflammatory agents has gained considerable

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attention. The ability to diminish the STAT-induced transcription of inflammatory genes is documented for both natural compounds (such as polyphenols) and chemical drugs. Among polyphenols, quercetin and curcumin directly inhibit STAT, while *Berberis vulgaris* L. and *Sophora alopecuroides* L extracts act indirectly. Also, the Food and Drug Administration has approved several JAK/STAT inhibitors (direct or indirect) for treating inflammatory diseases, indicating STAT can be considered as a therapeutic target for neuroinflammatory pathologies through direct and indirect interaction. Considering the encouraging data obtained so far, clinical trials are warranted to demonstrate the effectiveness and potential use in the clinical practice of STAT inhibitors to treat inflammation-associated neurodegenerative pathologies.

1. Introduction

Neuroinflammation is a very complex process comprising all the inflammatory processes that occur within the central nervous system (CNS) and involving multiple cell types and mediators depending on the cause of origin and evolution. The inflammatory response is under tight control by both pro- and anti-inflammatory signals and mediators. In general, transient and acute inflammatory response is beneficial, inducing an adaptive response that helps the host to be defended against pathogens. On the contrary, chronic neuroinflammation is deleterious leading to neuronal dysfunction and tissue damage. Thus, inflammation of the CNS is an essential

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process in protecting the brain against insults, but it can also contribute to worse many brain and neurological diseases, including brain injuries, cancer, epilepsies and neurodegenerative diseases mainly Parkinson's or Alzheimer's diseases [1,2]. The inflammatory process in the CNS is mainly mediated by the activation of the microglia, resident macrophages and astrocytes and the release of many mediators including cytokines, chemokines or growth factors [3].

STATs (Signal transducers and activators of transcription) are transcription factors found within the cytoplasm in an inactive or latent state. STAT and Janus kinase (JAK) constitute the ubiquitous and highly conserved JAK/STAT pathway, which is involved in transducing signals from membrane-localized receptors to cell nucleus. When cytokines, hormones and growth factors bind to their own receptor, conformational changes are induced leading to activation of JAKs. Activated JAKs phosphorylate a tyrosine residue in the cytokine receptor, providing a docking site for STATs. Then, STATs bind to the cytokine receptor through SH2 (Src 2 homology) domains, and are rapidly activated upon autophosphorylation on a tyrosine residue existing in the C-terminal domain (CTD). Phosphorylated STATs are released from the receptor, undergo homo-dimerization (and in some cases hetero-dimerization) and translocate into the cell nucleus where they could bind to target genes promoters, thereby engaging diverse elements of the transcriptional apparatus and stimulating gene expression [4-7]. STATs are DNA-binding proteins downstream of the engagement of JAK and is responsible for regulating diverse cellular processes related to cell metabolism, inflammation, apoptosis and immune response [6,8-10]. Moreover, an elevated gene expression of STAT pathway, derived from hyperactivation of the JAK/STAT pathway, is present in many neurological disorders contributing to disease pathogenesis [11]. Therefore, a dysregulation of this pathway is implicated in various diseases, including autoimmune, haematological, oncological, metabolic and neurological diseases [12-15].

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Conversely, genetic dysfunctions/mutations of JAK/STAT pathway are implicated in inflammatory diseases, erythrocytosis, leukemias, as well as in the predisposition to obesity or type 2 diabetes mellitus (T2DM) [16]. This scenario highlights the deep implication of JAK/STAT in the cell homeostasis.

Many natural and synthetic substances have the capability to interfere with JAK/STAT pathways. Thus, they could represent a therapeutic option among other treatments available for neuroinflammatory diseases. These include natural compounds chiefly, polyphenols, extracted from plants with potential to develop as nutraceuticals, synthetic drugs and metallic compounds. This review aims to report data from literature regarding the role of STATs in neuroinflammation and the interaction of certain molecules and plant extracts with JAK/STAT pathways being able to become therapeutic option for neurodegenerative pathologies.

2. The STAT family: origin, structure and function

In mammals, the JAK family consists of 4 members: JAK1, JAK2, JAK3, and Tyk2 whereas the STAT family is comprised by seven members to date: STAT1-4, STAT5A-B and STAT6, all of which retain a tyrosine residue near C-terminal target of phosphorylation by JAKs [17,18]. JAK/STAT pathway is schematically represented in figure 1. Depending on the cytokine or growth factor signals, diverse combinations between JAKs and STATs can be produced with an elevated grade of specificity [13]. The seven STAT proteins derive from gene duplication and random genetics changes. In this sense, Souza-Neto et al. using comparative genomics confirmed the conservation of JAK-STAT pathway between insects and mammals [19]. The STAT molecule STAT92E from *Drosophila melanogaster* is homologue to human STAT5 with an identity of 37% [20]. Through evolution the ancestral STAT genes duplicated into A- and B-

type STATs [21]. The D-STAT, Ag-STAT, STATs 5A, 5B and 6 comes under A-type STATs. Among these D-STAT and Ag-STAT are insect STATs found in *D. melanogaster* and *Anopheles gambiae*. The STATs 5A, 5B and 6 are vertebrate STATs. B-type STATs, which include STATs 1, 2, 3 and 4, belong to vertebrate. Since no B-type STAT is observed in insect genomes, it has been predicted that the duplication of STAT genes happened previous to the evolutionary separation between vertebrates and insects [22]. The sequencing of STAT homologues in vertebrate showed the presence of conserved motif of C-terminal serine phosphorylation in STAT1, STAT3 and STAT4, whereas the STAT5s contains a PSP (Pro-Ser-Pro) motif. Except STAT2, all the B-type STATs contain PMSP (Pro-Met-Ser-Pro) motif. STAT2 is found in species like zebrafish or *Xenopus laevis*, which are distantly related to humans. The sequence analysis suggests that after the duplication of STAT gene and appearance of B-type STATs, the PMSP motif was acquired [23]. The available information suggests the STAT gene duplication and its functional diversification occurred to their evolutionary ancestors. This diversification process causes various functional role played by STAT in cell biology.

The STATs genes in human are located on chromosome 2 (STAT 1 and STAT 4), chromosome 12 (STAT 2 and STAT 6), and chromosome 17 (STAT 3, STAT 5a, and STAT 5b) with an average length of 750-850 amino acids and a size range from 90–115 kDa [24]. Although from a functional point of view the STATs proteins show different specific function, they share a common structure with remarkable homologies [25-27]. Six different domains are highly conserved within STATs, including an N-terminal domain (ND), coiled-coil domain (CCD), and the DNA-binding domain (DBD) located in the midst of the protein, the linker domain (LD), the SH2 domain, and, finally, a transcriptional activation domain (TAD) in the CTD. A schematic illustration of STATs structure is depicted in Fig. 2.

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The ND is a highly-conserved area containing the amino acidic sequences responsible for protein-protein interactions which mediate oligomerization of STAT [28]. STAT dimers interaction is cooperative, and cease following of the ND deletion [29]. The CCD, with an amino acidic region comprised between 130 and 315, bridges the ND with the DBD. This domain, possessing alfa-helical structures, is generally responsible for interactions with regulatory proteins [30-32]. The DBD determines the recognition of specific DNA sequence elements for each STAT and is generally located in the region comprised between 320–475 amino acids [32,33]. From a structural perspective, it shows similarity to an immunoglobin-like fold with β-sheets very close to the observed in NF-kB or p53 [32]. The LD, possessing mostly an alfa-helical structure sits between the DNA binding domain and the SH2 domain and it is also implicated in the transcription by interaction with the transcriptional machinery of the cell. The SH2 domain is located in the amino acid region between 600 and 700 [34]. It plays a key role in recognizing phosphorylated tyrosine residues and, therefore, mediates the interplay between STATs and other phosphorylated proteins, such as growth factors, JAKs, and other members of the STATs family to form dimer [35,36]. [37,38]. Differences within the SH2 domains are responsible for selectivity towards different specific receptors. Nearby the SH2 domain is located the key tyrosine residue needed for STAT activation. In addition to Tyr phosphorylation necessary for dimerization and migration into the nucleus, phosphorylation a Ser residue, with the exception of STAT2 and 6, is also required to start transcriptional activity [39,40]. This conserved Ser residue, is located into the TAD in the STATs C-terminal region (amino acidic residues 661–851) [40,41].

STATs are very versatile transcription factors that modulate a number of activities of cellular growth, survival and differentiation [42,43]. STAT members also govern four main effector

responses, in particular STAT1, STAT2 and STAT4 are responsible for antiviral type1 response, STAT3 is responsible for antibacterial and antifungal type 3 response, STAT6 is responsible for anti-helminth type2 response, and STAT5 is important in the initiating inflammation processes [16]. Overall, STATs are crucial for optimal immune expression, otherwise could lead to immunodeficiency or autoimmune disorders [44,45]. Moreover, deregulation of STATs has also been reported to trigger angiogenesis and in turn promote tumors survival [46-50].

STAT1 and STAT2 have shown a crucial role in the host defense in case of hepatitis C virus (HCV) infection and interferon (IFN)-α treatment-induced HCV clearance [51,52]. The HCV-infected hepatocytes lead to the generation of IFN-β, as a result, STAT1 and STAT2 activation occurred through modulation of IFN-α/β receptor and thus cause the upstream regulation of several antiviral proteins in the surrounding uninfected environment that stop further proliferation of infection [53-57]. STAT1 mutations have been associated with a progressive multifocal leukoencephalopathy (PML) produced by the polyomavirus JC virus, which typically occurs only in immune-compromised patients [35]. In addition, the activity and expression of STAT1 is deregulated in both cancer and insulin resistance disorders [36]. STAT3 is considered a proto-oncogene constitutively activated in several human cancers and plays a crucial role in survival of tumor cells, cell proliferation, migration, metastasis and angiogenesis [58]. STAT3 has reported to be required for optimal function of the electron transport chain in a transcriptional-independent manner [59-62]. In STAT3−/− cells it has been evidenced defects in mitochondrial complexes I and II [60]. However, the mechanism by which STAT3 modulates complexes I and II remains to be elucidated. Moreover, STAT3 was reported to support oncogenic transformation that maintains mitochondrial respiratory chain activity and blocks the transition pore of mitochondrial permeability [63]. STAT3 over-expression also showed a direct

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association with the development of arthritis [64,65]. STAT4 is distinct from other STAT member family since its expression is limited to T lymphocytes, myeloid cells and spermatozoa [66,67]. STAT4 was first observed to be activated in response to treatment with interleukin-12 (IL-12) of T cells [68] and studied for its role as central protective mediator during immune responses and immune-mediated diseases [69]. STAT5A and 5B share almost 95% of sequence identity and the functions of these proteins appear to be dependent on hormone and tissue in a context-dependent manner [70]. Recently, the participation of STAT5 signaling pathway in modulating maternal and feeding behaviors has been suggested [71]. STAT6 has been reported to be activated by IL-4 and IL-13 and modulates the expression of genes that regulate allergic inflammatory responses mediating the pathogenesis of allergic disorders such as asthma, atopic dermatitis and food allergy [72,73]. Additionally, a chimeric decoy oligodeoxynucleotides significantly down regulated lung inflammation through STAT6 modulation [74].

Finally, an association between diverse polymorphisms in STATs and the development of some inflammatory diseases has been reported. In this sense, a strong gene-gene interaction of STAT-4 T90089C, STAT-6 G2964A, and IFN-γ T874A has been found to cause an up-regulation of asthma in a Chinese population [75]. STATs gene polymorphisms STAT3 C4796793G and STAT5b C6503691T favor the appearance of rheumatic heart diseases and could be an effective biomarker for the prognosis of individuals with high risk of rheumatic heart diseases [76].

Cytokines, growth factors and hormones are the main activators of STATs family. STAT1 has been reported to be activated by IFN-α, IFN-β or IFN-γ and several interleukins (IL-6, IL-7, IL-10 and IL-27) in immune cells [77-82], and this STAT1 activation was reduced by Zn^{2+} chelation [77,78]. Recently, estrogen treatment has been also shown to up-regulate STAT1 mRNA expression in THP-1 and K562 cells [83]. In an animal model of injury-induced inflammation,

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STAT2 mRNA levels were up-regulated in astrocytes in a process mediated by NF-κB signaling [84]. In human artery endothelial cells, oxidized low density lipoprotein (oxLDL) treatment was able to increase the STAT1/2 mRNA levels [85]. Cytokines such as IL-6, IL-1β, and TNFα increased STAT3 mRNA levels in pancreatic [86] and lung cells [87,88]. β-catenin/T-cell factor (TCF) pathway has evidenced to increase mRNA STAT3 expression human esophageal squamous carcinoma cells [89]. STAT3 was also increased in ob/ob livers after long-term 17β-estradiol treatment [90]. STAT4 activation in mediated by IFN-α/β and by diverse interleukins (IL-2, IL-12, IL-13, and IL-17) [91-93]. STAT4 can also undergo epigenetic regulation by DNA hypermethylation, since higher levels of STAT4 mRNA were found after treatment with demethylating drugs in human primary T cells [94]. STAT5 is activated by prolactin, erythropoietin and growth factors [95]. Cytokines such as IL-2, IL-3, IL-4, IL-5, IL-6 and IL-7 could induce STAT5 A/B expression in different immune cell types [96-101]. STAT6 activation is mediated by IL-4 and IL-13 [102]. In fact, IL-4 facilitates the direct gene-specific demethylation in the differentiation of innate immune cells through a mechanisms that involve activation of STAT6 [103] and IL-13 acts as a central effector cytokine in ulcerative colitis by activating the STAT6 [104].

Recent studies have identified three molecules that negatively regulate JAK/STAT signaling including protein inhibitors of activated stats (PIAS), suppressors of cytokine signaling (SOCS), and protein tyrosine phosphatases (PTPs). PIAS, characterized by a zinc-binding ring-finger domain in the central portion, bind the activated STAT dimers preventing them from binding DNA, or promoting SUMOylation which can modulate STATs phosphorylation and dimerization [105,106]. SOCS proteins contain a central SH2 domain which directly binds to phosphorylated tyrosines of activated JAKs, causing the JAK kinase activity to be blocked,

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preventing the recruitment of the STATs [105,107]. PTPs are a group of distinct proteins that dephosphorylate JAKs at the level of tyrosine residues reversing the JAK-STAT activity [108]. miRNA are important gene expression modulators, and several miRNA are found to control the expression of STAT family of transcription factors. In this context, Kohanbash and Okada summarized the reciprocal regulations between STATs and miRNA in the context of diseases [109].

STATs can also interact with other transcription factors (STAT co-activators) modulating gene transcription in an indirect way. Some transcription factor such as orphan nuclear hormone receptor, USF1 (upstream stimulatory factor-1), ZXDC (zinc finger X-linked duplicated family member), ETS (erythroblast transformation-specific), p300/cAMP-responsive element-binding protein (CBP), AP-1 (activator protein-1), and Sp1 (specificity protein 1) potently drive target gene expression of STAT1 [110,111]. The transcriptional activity of STAT3 can be enhanced by its association with HNF-3 (hepatocyte nuclear factor 3), NcoA/SRC1a (steroid receptor coactivator 1), GR (glucocorticoid receptor), Crif1 (CR6-interacting factor 1), Sp1, p300/CBP and c-jun [112,113]. The interaction of STAT5 with p300/CBP, high-mobility group N2 protein (HMGN2), CPAP (centrosomal P4.1-associated protein), Nmi (N-Myc interactor), Sp1, YY1 (Ying Yang-1), and C/EBPβ increase STAT5 target gene expression [114-116]. The transcriptional activation of STAT6 requires the interaction with p160/SRC (NCoA) coactivator family, C/EBP and p100 [117-119].

3. Role of STATs signaling in neuroinflammation

Microglia and astrocytes are the main CNS-resident cells responsible for the immune and inflammatory responses. Activation of these cells induces inflammation and the release of

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multiple inflammatory mediators including cytokines and growth factors. In this scenario, the JAK-STAT pathways are implicated in the regulation of the inflammatory response since they are activated by some of these cytokines/growth factors but also by other inflammatory signals such as LPS, gangliosides and thrombin [120,121]. Upon exposure to pro-inflammatory signals (cytokines, cellular or bacterial debris), resident macrophages and microglia become polarized towards a pro-inflammatory M1 phenotype and release inflammatory mediators and chemokines, in a process mediated by STATs [122,123]. Under physiological conditions, when the CNS is damaged, the JAK-STAT pathway plays an important role in the neuronal regeneration process as well as in the formation of glia scars around the injured area. In accordance, it has been observed that after nerve injury, STAT3 expression is induced and activated by phosphorylation in regenerating neurons promoting the regrowth of damaged axons [124]. Moreover, the deletion of SOCS3 in diverse mutant mouse models of optical nerve injury induces the regeneration of injured axons [125]. Diverse cytokines and oxidative stress have been reported to activate mainly the STAT1 and STAT3 isoforms leading to the induction of inflammation-associated and survival-associated gene expression [126,127]. Although CNS inflammation is an important mechanism to protect the brain against injuries, when it occurs excessively or prolonged, inflammation can contribute to the appearance or worsen various diseases that affect the brain. Since the JAK-STATs mediate the inflammatory responses, a dysregulation of these pathways is related to neuroinflammation and neuroglial survival. In addition, depending of the STAT activated and the site of activation the effects on inflammation and cell survival could be not identical.

Although evidences report the participation of STAT3 in brain inflammation, the exact role is not clear and still controversial. The activation of this pathway results in the expression of genes

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that sequentially repress the transcription of pro-inflammatory genes contributing to the resolution of the inflammatory process [128]. However, it has been evidenced that STAT3 is capable to promote cell apoptosis and brain damage or favor cell survival. Specifically, diverse studies have evidenced that STAT3 excessive activation contributes to neuronal damage, whereas in other studies the deleterious effects are associated with the disruption of STAT3 signaling [129-131]. On the contrary, the role of STAT1 is clearer as it induces cell death [132]. STAT3 activation also induces the expression of STAT3 itself, thus promoting a positive feedback loop of STAT3 that if not properly regulated can lead to neuronal damage [133]. It was also evidenced that STAT3 is necessary for the expression of miR-155 in cultured Th17 cells, indicating a function in the etiology of inflammatory disorders inducing the expansion of Th17 cells [134]. Mutations in STAT proteins that lead to excessive activation of the pathways are not frequent and the excessive activity is generally triggered by the overproduction of cytokines, and/or deregulation of endogenous negative modulators of the JAK/STAT [10,11]. The negative feedback molecules, especially SOCS1 and 3 that are expressed in immune cells and cells of the CNS, are an important point of interest since they act as suppressors of cytokine signaling, limiting the cellular cytokine response [135]. In accordance, many anti-inflammatory drugs, such as aspirin, exert some of their effects via SOCS proteins [136]. It has been reported that SOCS1 limits chemokine-induced migration of immune cells within the brain and ameliorates the release of chemokines and pro-inflammatory mediators by inhibiting the STAT and the NF-kB activation [137-139]. Similarly, SOCS3 inhibits the signalling of a wide range of immune molecules such as IL-6, IL-10, IFNs, LPS, among others [137,140,141].

It is interesting to mention that de-regulated JAK/STAT pathways are found in neurodegenerative disorders. For example, in a model of Parkinson's disease developed in rats

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(by viral mediated overexpression of α-synuclein), the pharmacological inhibition of the JAK/STAT pathway by the JAK inhibitor AZD1480 , significantly prevented neuroinflammation and neurodegeneration [142]. The activation of the JAK/STAT pathway in Parkinson's disease is related to the elevated levels of IFN-γ and IL-6 [142]. Multiple sclerosis is a devastating demyelinating disease that curses with chronic inflammation and neuronal degeneration. In patients with this pathology, it has been evidenced that immune cells from subjects suffering from relapsing-remitting multiple sclerosis express [141]more phosphorylated STAT3 during relapse than in remission [143]. Moreover, diverse studies concentrate on the induction of SOCS3, which leads to the inhibition of STAT1 and STAT3 in humans and in experimental autoimmune encephalomyelitis animal model [144,145]. On the contrary, in Alzheimer's disease the levels of phosphorylated STAT3 in hippocampal neurons were reduced in a mice model of Alzheimer's disease (Tg2576) and in Alzheimer's disease patients [146]. In this study, pharmacological inhibition of the JAK2/STAT3 pathway caused memory impairment in Tg2576 mice, whereas colivelin, a derivate of the neuroprotective peptide humanin, totally recovered cognitive function in an Alzheimer's disease model (Tg2576) via activation the JAK2/STAT3 pathway [146]. Also, nicotinic acetylcholine receptors have been reported to reduce Aβ neurotoxicity by activating the JAK2/STAT3 pathway [147].

In addition, neural damage resulting from infections, trauma and deposition of abnormal proteins like β-amyloid and transactive response (TAR) DNA binding protein, can induce secondary response such as neuroinflammation aggravating the neuronal impairment far more than the primary injury [148].

4. POLYPHENOLS

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4.1 Direct inhibitors of STAT

Although diverse polyphenols have been evidenced diminish the ability of STAT to signal transcription of inflammatory genes, the most studied are quercetin, curcumin, resveratrol and luteolin (figure 3). Quercetin directly inhibits the phosphorylation of STAT and has been found effective in preventing apoptosis in dopaminergic cells through down regulation of pro-apoptotic genes like Bax and up-regulation of anti-apoptotic gene Bcl-2 [149]. Kumar and colleagues showed that quercetin possesses neuroprotective effects in diabetes induced neuro-degeneration and inflammation, partly due to its STAT inhibition [150]. Coupling quercetin with nanoparticles like β-cyclodextrin-dodecylcarbonate increases its permeability through blood-brain barrier making it a suitable drug for STAT intervention and prevention of neuroinflammation [151].

Curcumin also presents its anti-inflammatory properties partly by suppressing JAK/STAT pathway [152]. It has been shown to decrease activation of microglial cells by preventing STAT 1 and 3 phosphorylation indirectly through activation of SHP-2, a tyrosine phosphatase which negatively regulates JAK, thereby weakening the inflammatory action of microglia. [153]. Curcumin also suppresses Oncostatin M (a cytokine from interleukin 6 family) induced p-STAT1 [154]. Curcumin and 1,25 dihydroxyvitamin-D3 at dosages of 20 μg/ml and 250 nM respectively, independently prevent the development of autoimmune encephalomyelitis, leading to multiple sclerosis (MS-EAE) by inhibiting the differentiation of neural antigen specific Th1 cells, through blockage of IL-12 mediated STAT3/4 activation [155,156].

Resveratrol is a wine-derived polyphenol that suppresses LPS (lipopolysaccharide) induced inflammation in part by inhibiting phosphorylation of STAT1/3 which mediates the release of pro-inflammatory cytokines [157]. It significantly inhibits the activation of STAT1 in a lower dose than 5-aminosalicylic acid (25μM for resveratrol vs 500 μM for 5-aminosalicylic acid) in

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HT-29 cells [158] However, the co-treatment with the two inhibitors did not exert synergistic effect against the expression of pro-inflammatory mediators in cytokine-stimulated cells mediated by the JAK-STAT pathway [158]. However, resveratrol does not interfere with the MAPK mediated negative feedback mechanism of STAT showing its STAT1 specificity [158].

Luteolin supplementation leads to decreased IL-6, a microglial cell activator, indirectly suppressing STAT activation in children with autism spectrum disorders (ASD) which resulted in improvement in their communication and living skills [159], thus preventing microglial activation associated with an anti-inflammatory phenotype at dose of luteolin of 100 U/ml [160,161].

In addition to these four polyphenols, genistein, kaempferol and daidzein have been reported to inhibit the activation of STAT-1 and NF-κB pathways [162]. Hämäläinen et al. [162], analyzed the effects of 36 naturally occurring flavonoids as anti-inflammatory agents. Within these compounds, the above-mentioned polyphenols were capable to inhibit the activation of STAT-1 in macrophages stimulated with LPS. A recent study also reported that kaempferol 7-*O*-β-D-glucoside, isolated from *Cudrania tricuspidata*, inhibits the phosphorylation of STAT-1 in LPS-stimulated macrophages [163].

Extracts from a number of herbal plants have been found to target JAK/STAT pathway, and could present as a therapeutic regime for neuroinflammation. *Agrimonia pilosa* has long been used in traditional Chinese medicine for its anti-inflammatory properties as it is rich in flavonoids like Agrimonolide, which at a dose of 80 µM has been found to inhibit LPS-induced JAK/STAT pathway directly by preventing phosphorylation of JAK1 and consequently STAT1 and 3, while indirectly by down regulating IL-6 expression [164]. Plumbagin, a compound extracted from *Plumbago zeylanica,* directly blocks the phosphorylation of STAT1/4 and STAT3

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to downregulate differentiation of Th1 and Th17 cells respectively (at 2 mg/kg in experimental autoimmune encephalomyelitis-EAE), to control encephalitis [165]. Progression of disease is substantially controlled when plumbagin is administered pre-immunization in the model [165]. Similarly, tripchlorolide (T4) a constituent of *Tripterygium wilfordii Hook F*. extract reduces the disease severity of EAE in the same manner by blocking the JAK/STAT along with ERK1/2-NF-kB involved in Th1 and 17 proliferation at a mere dose of 40 µg/kg [166]. A compound extracted from *Cornus officinalis,* cornel iridoid glycoside (CIG) halts multiple sclerosis progression by suppressing the JAK/STAT1 and JAK/STAT3 and subsequently IL-6 in a dose dependent manner, also returning the microglial population to normal at 120 mg/kg in EAE animal model [167].

4.2. Indirect inhibitors of STAT

Berberis vulgaris L derived Berbamine extract indirectly stimulates STAT4 degradation through STAT-interacting LIM protein (SLIM) without affecting its phosphorylation, leading to decreased inflammatory cytokine assembly in EAE [168]. Berberine at the dose of 50 mg/kg targets JAK1/2 and STAT1/3/4 activation among many others to halt the differentiation of T helper cells Th1 and 17 in the EAE [169]. A novel caffeic acid derivative [(*E*)-2-cyano-*N*-[(*S*)-1-phenylethyl]-3-(pyridin-2-yl)acrylamide], which resembles in structure like AG490, a developed JAK inhibitor (also called Jakinibs), has been found to significantly down regulate levels of pSTAT3 and its dependent genes in glioma cells in a long term manner by acting on JAK1/2 activators, rendering low phosphorylation levels and inducing apoptosis at half the dose of AG490 (i.e., 25 µM). AG490 at 50 µM only slightly lowers p-JAK levels [170].

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Sophora flavonone G extracted from *Sophora alopecuroides* partly exhibits its anti-neuroinflammatory properties by suppression of JAK1/2 and STAT1/3/5, their phosphorylation was significantly reduced in LPS induced inflamed microglial cells treated with the compound [171]. In the same manner, *Serenoa repens* is another phyto-therapeutic compound which along with inhibiting neuroinflammation by blocking the inflammation regulator (STAT3), also decreases growth of glioma cells; this effect of *Serenoa repens* is synergized by the JAK inhibitor AG490, where the co-treatment of the two compounds at 1 μl/ml and 20 nM respectively, markedly increased apoptotic processes in glioma cells [172]. Moreover, it down regulated both basal p-STAT and IL-6 induced p-STAT, facilitating the chemotherapeutic activity of docetaxel in treatment of myelomas by increasing apoptosis, however, lower cancer cell viability is also evidenced after co-treatment with docetaxel and AG490 [173].

5. DRUGS

Certain JAK/STAT inhibitory drugs have been approved by FDA for use in treatment of neuro-inflammatory diseases while other potential suppressors are vigorously under investigation and could be developed as standard intervention strategy for inflammatory and autoimmune disorders of nervous system.

5.1. Direct STAT inhibiting drugs

Approved STAT inhibitors include laquinimod, glatiramer acetate, and fumarates. These halt multiple sclerosis progression by blocking STAT1/3 activation leading to decreased cytokine production and increased population of macrophages and type II dendritic cells; where they

additionally block the differentiation of Th1 and Th17 cells [174]. Laquinimod and fumarates have the potential to reverse EAE at the doses of 25 mg/kg and 70 μM respectively [175,176].

AG490 is an established JAK inhibitor and is used for blocking JAK/STAT pathway in many inflammatory diseases including multiple sclerosis and cancers like the glioblastoma, where it down-regulates transcription of a number of inflammatory cytokines like IL-2, 6 and 12. In the LPS induced microglial apoptosis, AG490 (10 μM) prevents cellular death and inflammation by preventing JAK2/STAT induced NO release and protects endothelial cell mediated blood brain barrier destruction [177]. It is also used as a standard when there is a need for comparing the JAK inhibitory potential of novel synthetic drugs and natural compounds under study.

Among others, Cucurbitacin-I (JSI-124) inhibits both JAK2/STAT3 directly by blocking STAT's phosphorylation via JAK and also preventing STAT dimerization [178]. Sorafenib and WP1066, both are known STAT inhibitors in CNS. Sorafenib at 10 μM blocks the phosphorylation of STAT3 in glioma cells inducing apoptosis, the upstream JAK1 and 2 inhibition by Sorafenib is reversible by mutant STAT3, and its phosphatase activity is revoked by tyrosine phosphatase inhibitor [179]. A novel molecule structurally similar to AG490, WP1066, which at the concentration of 10 μM significantly inhibits STAT3 and exhibits anti-cancer apoptotic properties in glioma cells and in vivo conditions, and is found to be almost 17 times more cytotoxic to U87-malignant glioma cell line than AG490 [180]. It also blocks JAK2/STAT3 to abolish growth of multiple brain tumor sphere cultures (BTSC) and their xenografts irrespective of the mutations they carry [181].

AZD1480 suppresses STAT1/4 in Th1 cells and STAT3 in Th17 cells of EAE models at 25 mg/kg to inhibit their differentiation and release of other pro-inflammatory cytokines thereby preventing neuronal inflammation in multiple sclerosis [182], it also target JAK/STAT pathway

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to inhibit dopaminergic degeneration in PD cell line. Acute administration of 10 μM AZD1480 exhibits anti-inflammatory properties by suppressing α-synuclein induced immune system activation [183]. AZD1480 supplied to 50 mg/kg induce apoptosis and reduce proliferation in human and murine glioblastoma tumor cells by suppressing basal and induced JAK1/2 and STAT3 [184].

A JAK3/STAT5 specific inhibitor ZM39923 (ZM) completely abolishes the activation of this pathway at 30 μM [185] and also inhibits the growth of brain tumor initiating cells (BTIC) at a dose of merely 1 μM by blocking JAK3/STAT3 [186]. The drug Pacritinib (also called as SB1518) is a JAK2/STAT3 inhibitor which significantly decreases cell viability and neurosphere formation in heterogeneous BITC populations at 5 μM concentration and increases the sensitivity of BITC cultures without affecting normal astrocytes in human [187]. A synthetic molecule ORL-NIH001 is a STAT3 suppresser which prevents the progression of inflammation in experimental uveitis by halting STAT mediated IL-6 expression which suppresses upstream differentiation of $CD4^+T$ cells into Th17 cells, also blocking Th17 proliferation, entry into neuro-retina and subsequent release of inflammatory cytokines; it can also be used in the management of multiple sclerosis due to its Th17 inhibitory activity [188]. Similarly, another synthetic molecule BRD0476 has a novel mechanism of action against JAK/STAT pathway where it decrease the levels of IFN-γ- induced p-STAT1 without affecting the kinase activity of JAK2 by inducing a competition between its phosphorylation and ubiquitination, where ubiquitination is performed by inducing deubiquitinase ubiquitin-specific peptidase 9X (USP9X) [189]. In methamphetamine induced neuro-inflammation models, Asiatic acid has the capability to return the methamphetamine induced abnormal phosphorylation of STAT3 by JAK2 to standard levels at 20 μM, blocking the expression and release of inflammatory IL-6 as the DNA binding activity

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of STAT is also attenuated by Asiatic acid [190]. Pyridone 6 (P6) also inhibits the phosphorylation of JAK2/STAT3 and blocking the release of its mediated pro-inflammatory cytokines, specifically TNFα and the iNOS (inducible nitric oxide synthase) to attenuate the destructive function of activated microglial cells, which arise in neuroinflammation and various neuropathological conditions like Parkinson's disease and amyotrophic lateral sclerosis [191].

5.2. Indirect STAT inhibiting drugs

Tofacitinib, ruxolitinib and baricitinib are three developed JAK1/JAK2 inhibitors FDA approved for use in autoimmune and anti-inflammatory diseases like Rheumatoid arthritis, psoriasis and lupus; and could present as an effective therapeutic regime for encephalitis and other autoimmune inflammatory disorders of nervous system. Tofacitinib is a JAK1/3 inhibitor where ruxolitinib and baricitinib suppress the activity of JAK1/2 and their downstream STATs in the signaling cascade [192]. Ruxolitinib inhibits JAK/STAT to indirectly prevent oxidative stress-induced neuroinflammation which is potentiated by IL-13, and subsequent death of dopaminergic neurons in cultured cells and animal models of Parkinson's disease [193].

Ionomycin and PMA (phorbol 12-myristate 13-acetate) inhibits IL-6 induced STAT3 promptly by activating MAPK, ERK (extracellular signal regulated kinase) family of MPAK is antagonistic to STAT in certain conditions, which is reversible on the suppression of the latter by ERK specific kinase inhibitors. However, ionomycin and PMA have no effect on IFN-α stimulated STAT3 activation, showing their specificity and potential for use in IL-6 mediated inflammatory pathways like those involved in EAE, ASD, uveitis, etc. [194]. Similarly, phenylephrine at 100 μM can also reversibly inhibit STAT3 activation through MAPK dependent pathway [195].

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Among other Jakinibs, tyrphostin B42 is a JAK2 specific inhibitor reducing MS severity by diminishing IL-12 induced JAK phosphorylation and thereby, reducing Th1 proliferation [196]. A new JAK3 specific inhibitor CP-690,550, has negative potency for JAK 1 and 2; and could be used therapeutically in multiple sclerosis for its ability to target IL-2 and 12 stimulated T cell populations [197]. JAK3 blockage sufficiently prevents against autoimmune and alloimmune actions [198]. Dimethoxy-quinazolines (WHI-P154 and WHI-P131), two novel quinazoline derivatives are JAK3 inhibitors, where the latter also blocks OSM induced STAT1 phosphorylation. In a familial ALS mouse model with Cu, Zn-superoxide dismutase (SOD1) mutation, which leads to ROS (reactive oxygen species) accumulation, causing STAT mediated release of pro-inflammatory cytokines, WHI-P131 treatment inhibits STAT and increases neuronal survival in transgenic mice and improves pathological condition [154,198].

Various other JAK inhibitors possessing affinity for specific JAK target molecule are being successfully tested in clinical trials (primary and advanced stages) of numerous cancers including those of nervous system where they prevent inflammation and increases apoptosis of malignant cells; these Jakinibs include but are not limited to TG101348 specific for JAK2, NS-018 (JAK2), XL019 (JAK2), CEP-701 (JAK2), CYT-387 (JAK1/2), LY2784544 (JAK2/STAT5), BMS-911543 (JAK2/STAT1), lestaurtinib (JAK2/3) [199-206]. Lestaurtinib is a blocker of STAT5 phosphorylation and its activity in CNS has led to its use in treatment of neuroblastoma [207].

6. Conclusions and future prospects

In conclusion, the role of STAT in brain inflammation is still controversial. In fact, on the one hand, many investigations showed that STAT3 activation contributes to neuronal damage, and, on the other hand, the disruption of STAT3 signaling resulted be linked to deleterious effects

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[129-131]. In particular, deregulation of the JAK/STAT pathway found in neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, and multiple sclerosis. Therefore, over the last few years, modulation of STATs caused by many natural and synthetic substances has become a hot topic in the treatment of human diseases including those linked to neuroinflammation. The mechanisms at the basis of the modulation of STATs are different and very complex being both direct and indirect interactions depending on the type of substance. In addition, growing evidence suggests the importance of JAK inhibitors possessing affinity for specific JAK target molecule. Some JAK inhibitors resulted to be effective in treating cancers inCNS where they decrease inflammation and increase apoptosis of malignant cells.

The natural substances, characterized by low adverse and secondary effects, and with the capability to exert specific effects on JAK/STAT should be evaluated and developed for the treatment neuroinflammatory disorders opening a new horizon of hope for patients. The intervention of JAK/STAT pathway by small molecules could be a feasible therapeutic choice against many neuroinflammatory diseases due to their low cytotoxicity, usually high target specificity, low IC_{50}, and sufficient potential of crossing the blood brain barrier.

Considering the encouraging data obtained so far, clinical trials are requested to show the effectiveness and use in the clinical practice of JAK/STAT pathway inhibitors for the treatment of inflammation-based neurodegenerative pathologies. In addition, considering that the mechanisms of action of these inhibitors still remain to be fully elucidated, further studies are strongly requested to understand these aspects.

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Figures and figure legends

Figure 1.   Schematic representation of JAK/STAT signaling pathway JAK, Janus kinase; STAT, signal transducer and activator of transcription; P, phosphorylated molecule.
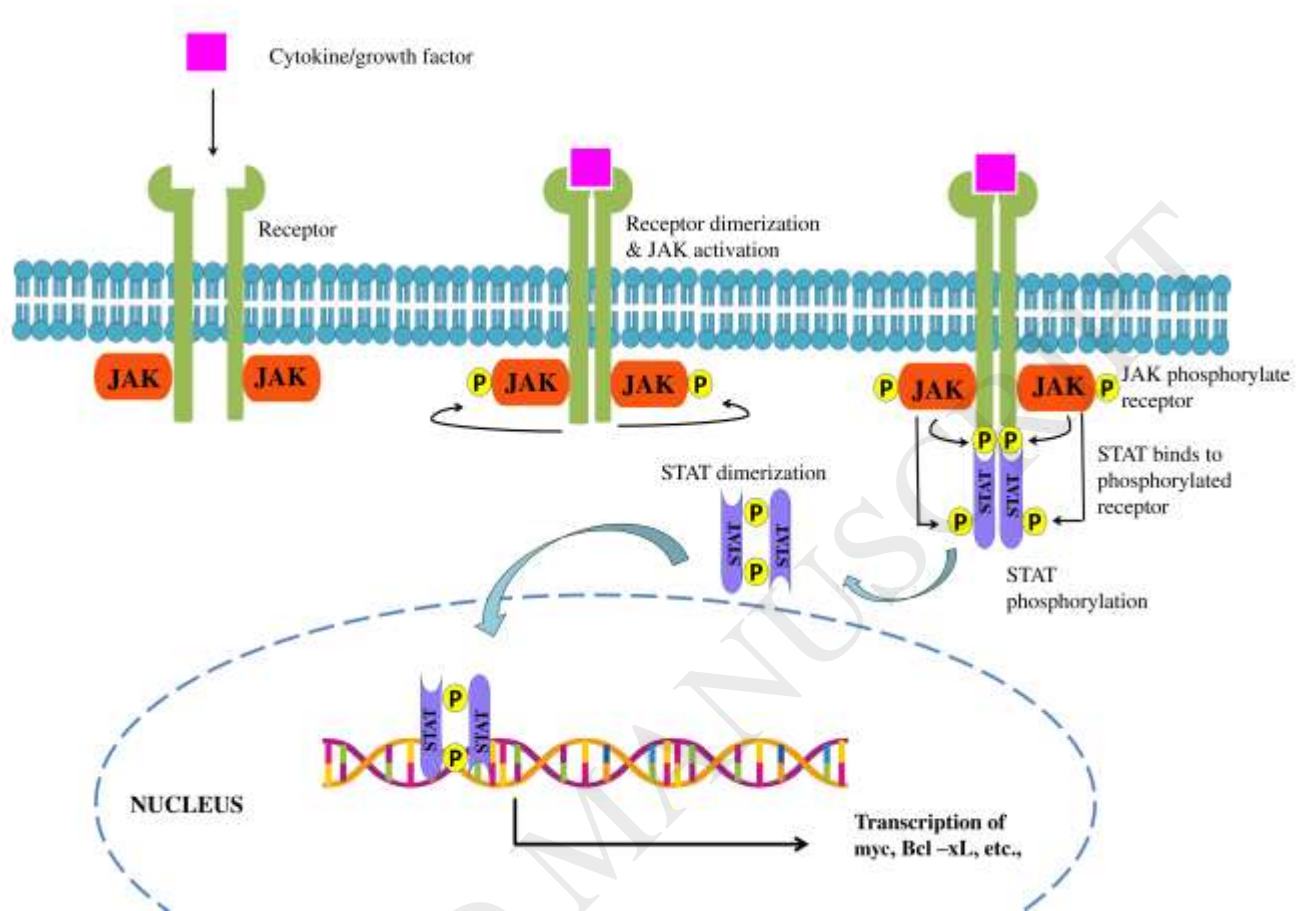
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Figure 2. Commonly conserved structure domains of the STATs family protein: N-terminal domain (ND); coiled-coil domain (CCD); DNA-binding domain (DBD); linker domain (LD); Src homology 2 domain (SH2); Tyrosine residue (Tyr) crucial for phosphorilation (P); transcriptional activation domain (TAD).
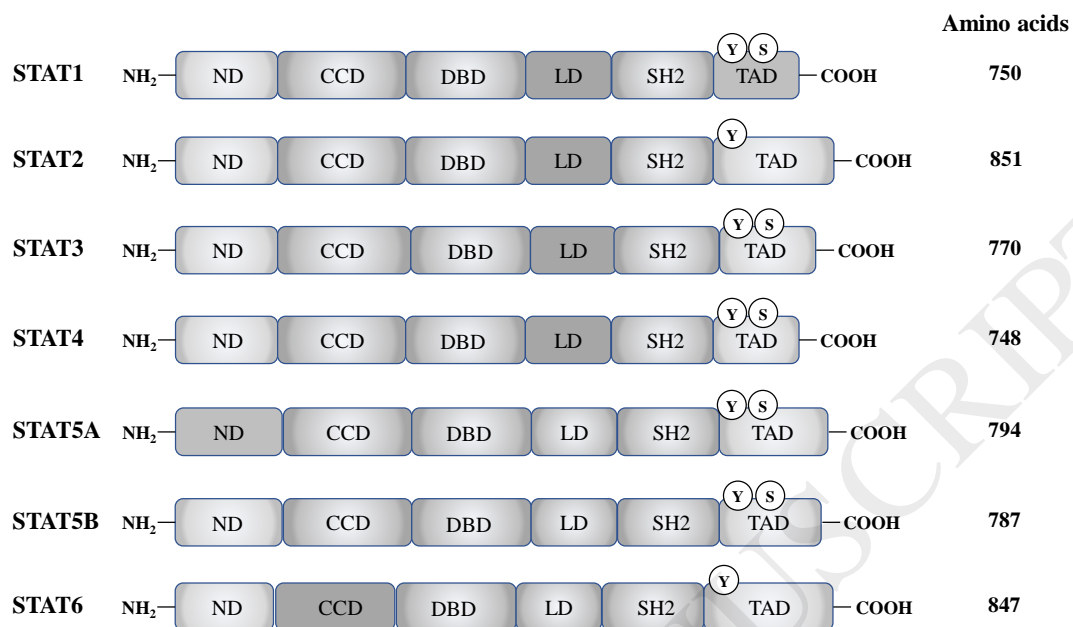
Figure 3. Chemical structure of curcumin (a), quercetin (b), plumbagin (c), luteolin (d) and resveratrol (e)

a) Curcumin

b) Quercetin

c) Plumbagin

d) Luteolin

e) Resveratrol