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JAK inhibitors in immune-mediated rheumatic diseases: From a molecular perspective to clinical studies

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ABSTRACT

The Janus Kinase signalling pathway is implicated in the pathogenesis of immune-related diseases. The potency of small-molecule Janus Kinase inhibitors in the treatment of inflammatory diseases demonstrates that this pathway can be successfully targeted for therapeutic purposes. The outstanding relevant questions concerning drugs' efficacy and toxicity challenge the research to enhance the selectivity of these drugs. The promising results of computational techniques, such as Molecular Dynamics and Molecular Docking, coupled with experimental studies, can improve the understanding of the molecular mechanism of Janus Kinase pathway and thus enable the rational design of new more selective inhibitor molecules.

1. Introduction

The Janus Kinase (JAK) Signal Transducers and Activators of Transcription (STAT) pathway mediate signals of more than fifty cytokines, growth factors and hormones [1]. Available genomic tools have provided a large amount of information on disease-related mutations in the JAK-STAT pathway and novel insights into its regulatory mechanism.

The analysis of the JAK FERM and SH2 sequence domains revealed a high degree of sequence conservation among the JAK family members, suggesting that all JAKs interact with their receptors by same main domains. However, due to the poor expression and solubility of JAKs [2], it has not been possible yet to crystallographically characterize the entire protein at an atomic-level resolution. The lack of complete structural models of JAK has limited the present understanding of its activation mechanisms. Nevertheless, fragments of JAK proteins have been satisfactorily resolved. Structural models of the those fragments have indicated that JAK enzyme is a multi-domain protein composed of seven domains called Janus Homology domains (JH1-7), characterized by a similar N-terminal region (including JH7 and the first 19 amino acids of JH6) and a C-terminal region (JH3-JH6), which differs between receptors in the same JAK family. Structural biology studies have also provided important information on JAK FERM domains (JH4 to JH7), packaged in a canonical trilobed FERM architecture, and the SH2 domain (JH3-JH4) identified as the receptor binding site. As far as the regulation and functionality of the protein are concerned, JH1 and JH2 have been identified as the JAK tyrosine kinase and tyrosine kinase-like domains, respectively. It is noteworthy that the latter has been shown to be a mutational hotspot

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for clinical JAK mutations causing immunologic and neoplastic diseases [3]. In this context, it is worth mentioning that the understanding of pathogenic molecular mechanisms leading to immune-mediated rheumatic diseases is still limited because of our incomplete knowledge of JAK-STAT regulatory mechanisms. A deeper understanding of the above-mentioned regulatory behaviour represents a key challenge in the development of next-generation therapies targeting the JAK-STAT pathway.

Progress in the field of rational drug discovery has been accelerated by molecular modelling approaches such as *Molecular Mechanics* (MM), *Molecular Dynamics* (MD), *Molecular Docking* and *Virtual database Screening* (VS) [4], which have been generally recognized as powerful tools that offer support for wet-lab experiments. The field of JAK family inhibitor [5] discovery/design is no exception in this regard.

Substantial progress has been made in the area of pathogenesis of inflammatory rheumatic diseases, thanks to the innovations both in basic and translational research [6]. Biomolecular modelling has been successfully employed to unravel subcellular pathways and molecular targets implicated in the regulation of the immune system physiology [7–9]. The JAK-STAT pathway [10] is recognized as playing a pivotal role in the pathogenesis sub-serving various immune-mediated diseases. Currently, clinical JAK-STAT inhibition is mainly focused on the treatment of *Myeloproliferative Neoplasms* (MPNs) [11] and *Rheumatoid Arthritis* (RA) [12], while clinical trials involving JAK inhibitors for other immune-mediated rheumatic diseases are ongoing [13]. The major challenge in the development of these novel drugs is that their efficacy in the treatment of inflammatory arthritis and other immune-mediated diseases remains to be fully optimized [10]. Furthermore, it is worth stressing that the insufficient selectivity of this novel group of drugs complicates their clinical use and many pharmaceutical strategies are employed in order to address this aspect [14]. Moreover, the existing limitations on the selectivity of the improved JAK drugs have been recognized and dosing regimens remain an open issue.

This review intends to summarize the main far-reaching contributions of computational techniques, such as Molecular Dynamics and Molecular Docking, in the discovery and development of JAK inhibitors in the context of immune-mediated diseases. In this scenario, MM and MD can provide very useful insights into the molecular mechanism of action in terms of interactions taking place in the formation of the JAK-ligand complex. Additionally, Molecular Docking can be used to complement experimental approaches with the objective of designing and optimizing novel JAK inhibitors. Studies considered in the present review paper represent an attractive benchmark for a better understanding of the biological functions and mechanisms involved in the JAK-STAT pathway and also for investigations related to rational design of selective and specific inhibitors targeting JAK family members.

2. JAK-STAT signal transduction mechanism

JAK proteins are key upstream factors of the Janus kinase, signal transducer of the JAK-STAT pathway [15]. This pathway provides a straightforward communication from transmembrane receptors to the cell nucleus through an architecturally simple and remarkably efficient mechanism [16]. Fig. 1 shows the events' concatenation, which leads to the signal transduction cascade. JAKs, through their N-terminal domain, are associated with a proline-rich membrane-proximal domain of the cytokine receptors.

Upon ligand binding, cytokine receptors dimerize, inducing the mutual approach of the two associated JAKs, which can cross-phosphorylate and become activated [17]. Activated JAKs, in turn, phosphorylate the tyrosine residues within the intracellular domain of the receptor chains, creating the docking sites to accept

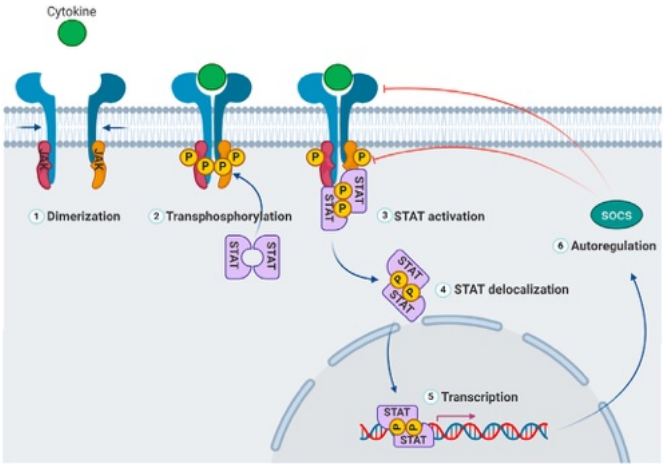


Fig. 1. Upon cytokine binding (green), the cytokine receptor (blue) undergoes a conformational switch and dimerizes (1). The dimerization induces receptor's constitutively associated JAKs (orange and red) kinase activity and transphosphorylation, which also phosphorylates the cytoplasmic domain of the receptor on tyrosine amino acids (2). The signal proceeds due to recruitment of inactive dimers of STAT (violet) at the tyrosine phosphate sites on the receptor; at this stage STATs get activated by JAKs through further phosphorylation (3). The activated STAT dimer delocalizes to the nucleus (4), where it binds the DNA and promotes transcription of specific target genes (5). Most often, one of the expressed target genes codes for SOCS (turquoise) (6), an inhibitor of the JAK-STAT pathway, which promotes cytokine receptor and JAK degradation, thereby enabling a negative feedback cycle, regulating signal transduction. In the illustration, phosphorylation sites on tyrosine residues appear as yellow-circled capital Ps. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

STAT proteins. At that point, JAKs mediate the phosphorylation of the recruited STATs [13]. In their activated form, STATs dissociate from the receptor and form a dimer. The resulting bi-phosphorylated STAT dimer transfers to the nucleus where it combines to particular DNA sequences, promoting transcription of genes. Some of these target genes code for a suppressor called Suppressor of Cytokine Signalling (SOCS), which helps in the regulation of the molecular signal transduction, through a negative feedback mechanism.

This demonstrates that the JAK-STAT pathway has an extremely finely regulated mechanism in which extracellular factors affect gene expression and thus can modulate cell functions [18].

JAK-STAT pathway is implicated in a wide spectrum of physiological processes. Pathogenic mechanisms of this pathway are linked to the development of MPNs, such as *Primary Myelofibrosis* (PMF), *Essential Thrombocythemia* (ET), and *Polycythemia Vera* (PV). Moreover, JAK-STAT malfunctions can also compromise immune modulation and cause inflammation, which can contribute to the onset of inflammatory diseases, such as RA, *Psoriatic Arthritis* (PsA), *Systemic Lupus Erythematosus* (SLE) and *Inflammatory Bowel Disease* (IBD).

The JAKs isoforms expressed in the mammalian members can be divided into four groups, namely JAK1, JAK2, JAK3 and Tyrosine Kinase 2 (TYK2) [19–21]. JAKs are kinase proteins associated to the type I and type II cytokine receptors. This cytokine receptor family binds many cytokines, growth factors and hormones. Type I cytokine receptors are elements of a family that shares distinct structural domains, such as the IL-6 gp130 family, the IL-2 g chain, the IL-23 p40 subunit and the IL-12 and some haemopoietic cytokines b chain cytokine receptors. Type II cytokine receptors are elements of the IFN and IL-10. Both types of receptors (I and II), transduce signals intracellularly via transphosphorylation of the JAK protein family proteins, which can be present in both homodimers and heterodimers (Fig. 2). At any rate, each JAK selectively binds to

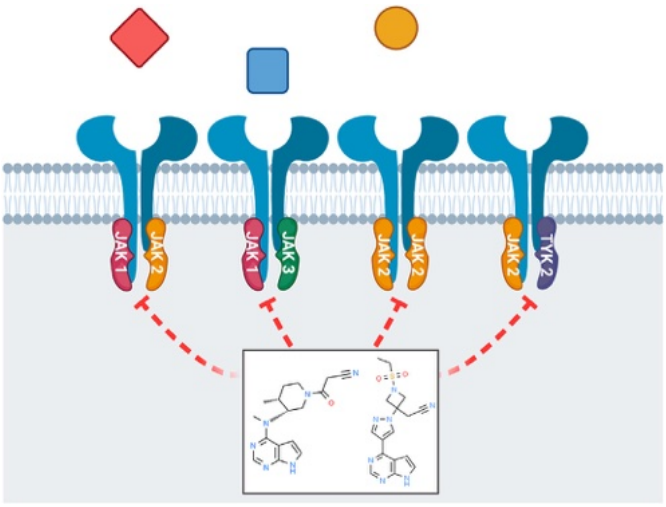


Fig. 2. JAK isoforms JAK1, JAK2, JAK3 and TYK2 are kinases coupled to the receptors of cytokines type I and II, shown in blue. These receptors have many endogen substrates such as interleukins, colony-stimulating factors, hormones and neuropeptides, all shown as shapes on the top. JAKs are inhibited by a diverse set of drugs; in the figure, two of them are shown: Tofacitinib and Baricitinib. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

different cytokine receptor chains, and the different pairing of JAKs is significant in regard to the signalling conducted.

The importance of this protein is evident also when observing various JAK knockout phenotypes, for example JAK1 Knockout (KO) mice, which exhibit perinatal mortality [22] while the defective erythropoiesis of JAK2 knockout animals is lethal to the embryotic stage [23]. JAK3 KO animals display severe immunodeficiency and impaired survival [24], a similar condition is caused by JAK3 mutations in humans, too [25,26]. The reduced IFN response in vital TYK2 KO mice, make them susceptible to viral infection [27]. At this point, it is evident how a complete inhibition of any of the JAK isoforms is not viable, due to its potentially negative effects on immune response and homeostasis. The preferential strategy of inhibition needs to allow a fine modulation of the activity of one or more isoforms of JAK. Adopting this modulatory approach, it would be possible to precisely and in a timely manner inhibit JAKs, when needed, using an instantaneous activation/inhibition mechanism.

3. JAK inhibitors in rheumatology

3.1. The immunological and clinical perspective

In recent years, considerable progress has been made in the knowledge regarding pathogenesis of *inflammatory rheumatic diseases*, such as RA, PsA and SLE [28,29]. Numerous new pharmacological entities have been tested and introduced into clinical practice. New drugs have not only allowed to expand the possibilities of therapeutic choice but have played a crucial role in clarifying uncovered pathogenic pathways [30]. With the introduction of new drugs, awareness of the importance of the management strategy of rheumatic diseases has emerged. Starting from the experience gained in diseases such as hypertension or diabetes mellitus, the *Treat To Target (T2T) strategy* was also proposed for RA [31]. According to this approach, the fundamental principle of patient management must be the achievement of remission or Low Disease Activity (LDA) along with the prevention of disability caused by the disease and the improvement of health-related Quality of Life (QoL). From this point of view, remission or LDA are associated with optimal control of disease activity, with a reduction of the risk of undesirable side effects of therapies and with a minimization of the risk of progression of osteo-structural damage. Once this objective has been established, patient management, follow-up strategy and pharmacological choices are reduced based on the achievement of the objective [32]. Growing evidence exists, including randomized clinical trials, which have already demonstrated the superiority in terms of outcomes of the T2T strategy compared to the traditional management of RA [32]. Based on the experience gained in RA, recommendations have been developed to manage other systemic inflammatory rheumatic diseases such as PsA, *Axial Spondylarthritis* (axSpA), SLE [33,34]. To make possible a T2T approach in these pathologies, a great effort is being made to define and monitor the optimal target, which is different for each pathology [35,36]. However, despite the progress made in recent years, up to now optimal disease control has been achieved only for a few patients [37–39].

3.2. The pharmacological and therapeutic perspective

The development of the therapeutic scenario over the last 20 years has been dominated by *monoclonal antibodies* or *fusion proteins* directed against single cytokines or cell surface elements. Recently, the possibility of modulating the inflammatory response in a new way, acting on the intracellular signalling, has come to the attention of the scientific community and clinicians. Within this perspective, the JAK-STAT pathway is implicated in a large number of autoimmune diseases [10] and inflammatory conditions, such as rheumatic diseases. With regards to their pivotal role, JAKs have emerged as the major therapeutic target for these cytokine-related disorders. The strategy adopted consists in the inhibition of JAKs' kinase domain to interrupt the cascade of the JAK-STAT signalling process described above.

As previously introduced, JAKs subtypes form different heterodimers, so the single kinase cannot be unequivocally associated with a distinct kinase receptor and hence cannot be referred to as a single pathway. Since JAKs are shared among different cytokines, JAK inhibitors spread their inhibitory capacity to a wide spectrum of immune active molecules. This aspect makes JAK inhibitors unique in the cytokine-targeting therapy scenario. Today, JAK inhibitors can be subdivided into two different categories: *Pan-JAK inhibitors* and *Selective inhibitors*. As suggested by the name, the first group includes those ligands which are competitive with the ATP-cleft of different JAK family members, whereas selective ligands distinguish the kinase activity domain among JAK family members. In order to highlight the clinical course of this kind of drugs, Pan-JAK and selective inhibitors can be labelled as *First Generation* or *Second Generation*, respectively.

3.2.1. First generation JAK inhibitors drugs

First Generation Inhibitors exploit the high homology degree present in the ATP-binding site to target different JAKs. To date, the Pan-JAK inhibitors either approved or under evaluation in systemic rheumatic diseases are *Tofacitinib* [40] and *Baricitinib* [41]. Tofacitinib (or CP-690550) is a non-selective JAK inhibitor, which inhibits mainly JAK1 and JAK3, and to a lesser extent JAK2, with a minimal activity on TYK242. Tofacitinib was approved by the Food and Drug Administration (FDA) and, only recently, by the European Medicines Agency (EMEA), in combination with methotrexate (MTX) or monotherapy, for the treatment of patients with moderate to severe RA [43] and PsA [44] who have responded inadequately to, or who are intolerant to one or more Disease-Modifying Antirheumatic Drugs (DMARDs) [45,46]. Baricitinib is a JAK1 and JAK2 inhibitor, approved by FDA and EMEA only for RA [47]. Phase III clinical trials demonstrated the efficacy of Tofacitinib in Ulcerative

Colitis. Clinical trials on Tofacitinib are in Phase III for Juvenile RA and Plaque Psoriasis, in Phase II for Atopic Dermatitis, in Phase I/II for Diffuse Scleroderma and in Phase I for Dermatomyositis [48]. Baricitinib is in phase III for Atopic Dermatitis and in Phase II for Giant Cells Arteritis and SLE. The other pan-JAK inhibitor *Peficininb* is in phase III for RA. Lastly, another inhibitor for JAK1 and JAK2, called *Ruxolitinib*, was designed, but its development was discontinued [48].

Finally, Tofacitinib has demonstrated that the idea of referring to JAK inhibitors as a new *treatment paradigm* for rheumatic diseases is not only consistent but also clinically effective. Fig. 3 shows the Tofacitinib binding site inside the JAK1 ATP cleft.

3.2.2. Second generation JAK inhibitors drugs

Given the success of Pan-JAK compounds in the treatment of rheumatic diseases and their lack in specificity, the development of a second generation of pharmacological inhibitors has become particularly important, leading to the emergence of a new class of therapeutics targeting JAKs. First-generation drugs inhibit multiple cytokines, leading to relatively low efficacy and adverse side effect profiles of these drugs, both linked to their mechanism of action. Thus, there is a strong interest in the design of more selective drugs, especially in the field of long-term use for immune-related diseases.

Tofacitinib has shown to be more selective for JAK1/JAK2/JAK3 than TIK2, suggesting that it is possible to achieve a selective JAK inhibition [49], although the differences in the JAK's ATP cleft are subtle. A structure-based drug design aimed at targeting specific amino acid interactions could achieve higher levels of selectivity.

In the past several years, a second generation of selective JAK1 or JAK3 or JAK3/Syk inhibitors has been developed. The major challenge of this second generation of JAK inhibitors is their *selectivity* [3,14]. This issue opens relevant questions concerning efficacy and toxicity [50]. In fact, it is possible to speculate that the selectivity towards a single type of JAK and the consequent reduction of the number of inhibited cytokines may be associated with a reduction of the undesirable side effects. Nevertheless, it remains to be verified that the greater selectivity is also not associated with a reduction in efficacy, due to a reduction in the number of antagonized pathways. From an immunologic point of view, it may be important that the selectivity for JAK1 or JAK3 excludes the inhibition of the Interleukin 12 (IL12)/IL23 axis [51], which relies on JAK2/TYK2 heterodimers.

Filgotinib, a selective JAK1 inhibitor, is in Phase III clinical trials for RA, Crohn's disease and Ulcerative colitis [52]. The drug development is in Phase II for SpA, PsA, Lupus nephritis, Cutaneous lupus erythematosus, Uveitis and Sjogren's syndrome. *Upadacitinib*, another selective JAK1 inhibitor has been approved for RA and is in Phase III trials for PsA, Ulcerative Colitis and Crohn's disease and in Phase II for SpA and Atopic Dermatitis [53]. The drug development for rheumatology of the other selective JAK1 inhibitors *Solcitinib*, *Itacitinib* and of the JAK3 inhibitors *Decernotinib* and *R333* was discontinued [48]. Fig. 4 and Table 1 summarize the main JAK inhibitor drugs used for immune-mediated disease treatment and reports their 2D structure and their JAK specificity.

4. Molecular modelling aimed to JAKs functional characterization and drug discovery

4.1. Molecular structural models of JAKs

JAKs are 120–130 kDa multidomain enzymes, belonging to the tyrosine kinase family. These unique proteins have a multidomain nature consisting of seven well-defined Janus Homology domains (JH) numbered from the C-terminus to the N-terminus as shown in Fig. 5.

JH1 and JH2 are not only the most structurally relevant regions, but they are also responsible for JAK physiological functions. On the one hand, JH1 (also called the *kinase domain*) presents the ATP-binding cavity where the proper kinase catalytic activity occurs. There, tyrosine residues become phosphorylated triggering the cascade of events in the JAK-STAT signalling process. On the other hand, JH2 is called *pseudokinase domain* due to the high similarity with JH1 with a simultaneous lack of enzymatic activity. Adjacent to JH2, the Src Homology 2 (SH-2) domain is located and it includes JH3, JH4, which do not represent a conventional phosphotyrosine-binding domain. Instead, they are believed to stabilize the large structure of the protein.

Lastly, the N-terminal region or FERM domain, includes JH5 to JH7 and contributes to the association of JAKs with cytokine receptors or with the JH1 domains of other kinases. The term FERM stands for Frequency band 4.1, Ezrin, Radixin and Moesin [54].

The ATP binding site in the JH1 domain comprises 4 different

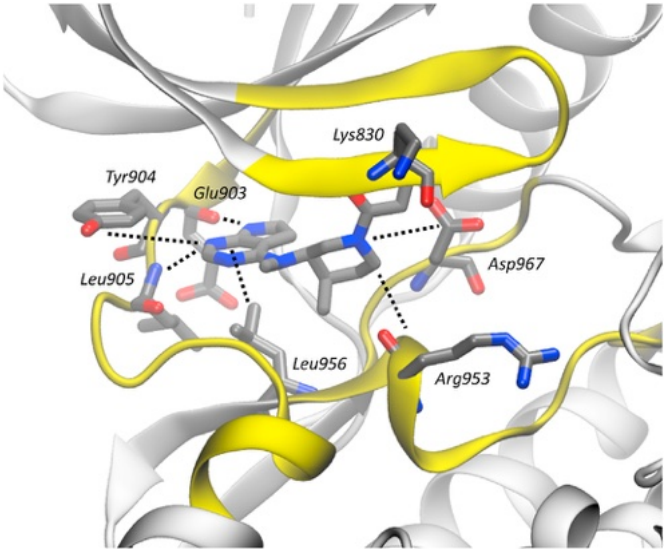
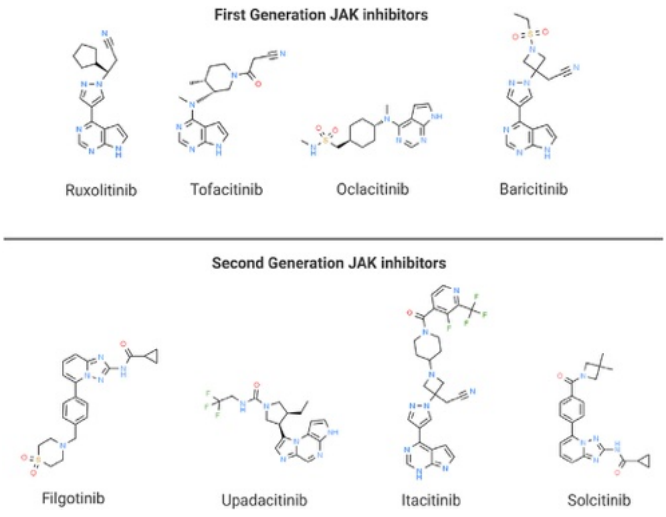


Fig. 3. Tofacitinib in the ATP-binding site of JAK1 (PDB: 3EYG). In the figure, the main interactions of the molecule with the hinge and the glycine-rich loop are highlighted.



Figs. 4. 2D depiction of known JAK inhibitors for immune-mediated diseases treatment.

Table 1
First and second-generation JAK inhibitors assessed in clinical trials for rheumatic diseases treatment.

	Name	JAK Specificity
1st **Generation Drugs**	*Tofanicib*	JAK3/JAK1 > JAK2, TYK2
	Bariticinib (INCB28050)	JAK1/JAK2
	Peficininb	Pan-JAK (some selectivity for JAK3)
	Ruxolitinib (INCB18424)	JAK1/JAK2 > TYK2
2nd **Generation Drugs**	*Filgotinib*	JAK1
	Upadacitinib	JAK1
	Solcitinib	JAK1
	Itacitinib	JAK1
	Decernotinib	JAK3

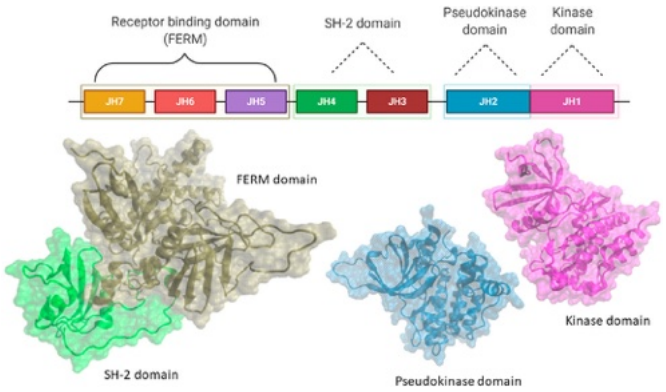


Fig. 5. The seven domains of JAK proteins: the Kinase domain (JH1), the Pseudokinase domain (JH2), the SH-2 domain (JH3, JH4) and the FERM domain (JH5, JH6, JH7). JH stands for JAK Homology domain.

regions as shown in Fig. 6: the glycine-rich loop, the hinge region, the catalytic and activation loops. It is noteworthy that JAKs' ATP

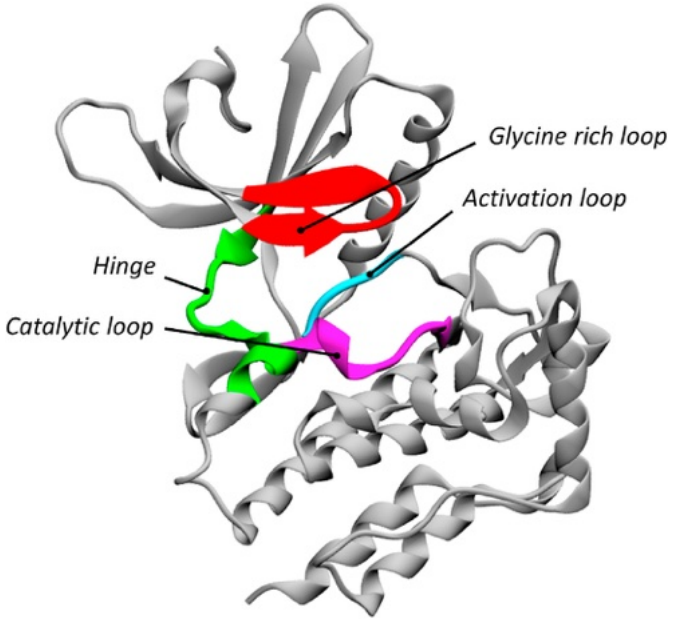


Fig. 6. A close-up view of the JH1 domain showing the ATP cleft position, made of 4 regions: the glycine-rich loop (highlighted in red), the hinge region (highlighted in green), the catalytic loop (highlighted in purple), and the activation loop (highlighted in light blue). 3EYG entry (Protein Data Bank) was used to obtain this figure and missing domains were reconstructed by homology modelling. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

binding sites not only conserve tyrosine residues (essential for the phosphorylation function) but are characterized by a large sequence identity. Table 2 identifies for each region stated above, which residues are in common and which are not, concluding that 60% of them are present indistinctly from the JAK isoform type [49]. At present, several experimentally-determined kinase structures are available for download in the Protein Data Bank (PDB). Protein structural models of the full-length JAK protein are not yet available [55]. However, single domains (the kinase domain, the pseudokinase domain and the FERM-SH2 domain) have been separately resolved for both JAK1[56–58] and JAK2[56,59–64]. The kinase and pseudo-kinase complex (JH1-JH2) has been solved for TYK2 protein [65].

4.1.1. JAK1

JAK1 is the target for several cytokines that bind to the common receptor subunit γ chain (γc), gp130, granulocyte colony-stimulating factor (G-CSF) and IFNs [15]. It is involved also in IL-2R and IL-4R families as well as class II receptor family. The absence of JAK1 leads to the lack of T and B cell production and profound defects in response to IFNs [66]. For this reason, it is essential to have a structural model of the JAK1 kinase domain to fully understand the kinase-receptor interaction mechanism. In the past, when an X-ray model was not available, alternative solutions were tried. Starting from the PDB models of the JAK2 and JAK3, a comparative model of the JAK1 was constructed using the Insight II software package [67]. Proteins with sequence and folding patterns comparable to the JAK1 were used to construct a homology model in a similar way, and the quaternary structure was derived by superimposing the model on the JAK2 framework [68]. In 2009, two high-resolution crystal structures of the JAK1 kinase domain in the active form were obtained [56]. These structures represent the domain in the presence of one inhibitor, namely CP-690,550 (PDB: 3EYG - resolution: 1.9 Å) and CMP6 (PDB: 3EYH - resolution: 2 Å) [56]. These crystallographic representations represent the starting point for the Molecular Docking drug design attempted in recent years. Currently, JAK1 kinase domain has been largely present in PDB as a complex with various inhibitors (as explained in Section 4.1.3). Finally, JAK1 pseudokinase domain (PDB: 4L00 - resolution 1.8 Å) [57] and FERM-SH2 domain (PDB: 5L04 - resolution 2.1 Å) [58] have been solved and deposited in the PDB.

4.1.2. JAK2

JAK2 is the most carefully investigated system among the JAK family and it is involved in the signal transduction cascade mediated by a variety of cytokines such as Prolactin (PRL), Growth Hormone (GH), Thrombopoietin (TPO), Erythropoietin (EPO) and other cytokines that use the receptors IL-3 and gp130[15]. JAK2, in mice, exhibited also embryonic lethality due to failure of erythropoiesis, and is involved in IFN-c and GM-CSF signalling [69]. Initially, a computational structure model of JAK2 was assembled

Table 2
Amino acid alignment of the ATP-binding site for the 4 JAK isoforms. Not conserved residues are highlighted in bold (red for the glycine-rich loop, green for the hinge one, purple for the catalytic one and light blue for the activation one).

	Glycine Rich Loop	Hinge Loop	Catalytic Loop	Activation loop
JAK1 (3EYG.pdb)	LG**E**G**H**FG**K**V**E** [881–890]	ME**FL**P**S**G**S**L**KEY**L [956–968]	HR**D**LA**A**RN**V**L [1001–1010]	**G**DFG [1020–1023]
JAK2 (4D0X.pdb)	LG**K**GNFG**S**V**E** [855–864]	ME**YL**P**Y**G**S**L**RDY**L [929–941]	HR**D**LA**T**RNI**L** [974–983]	**G**DFG [993–996]
JAK3 (3LXK.pdb)	LG**K**GNFG**S**V**E** [828–837]	ME**YL**P**S**G**C**L**RDF**L [829–837]	HR**D**LA**A**RNI**L** [947–956]	**A**DFG [966–969]
TYK (4OLI.pdb)	LG**E**G**H**FG**K**V**S** [903–912]	ME**YV**P**L**G**S**L**RDY**L [978–990]	HR**N**LA**A**RN**V**L [1021–1030]	**G**DFG [1040–1043]

by means of homology modelling methods and coevolution data [70,71] in order to augment the unique X-ray JH1 crystal structure available. However, thanks to experimental works performed in the last few decades, JAK2 kinase domain currently presents different X-ray structures such as PDB: 2B7A (resolution of 2 Å) [59], PDB: 3E62 (resolution of 1.9 Å), PDB: 3FUP (resolution of 1.8 Å) [60], PDB: 4AQC (resolution 1.9 Å) [61] and PDB: 4D0X (resolution 1.8 Å) [62]. Moreover, JAK2 pseudokinase domain (PDB: 5UT3 - resolution 1.5 Å) [63] and FERM-SH2 domain (PDB: 6E2Q - resolution 2.6 Å) [64] have been solved and deposited in the RCSB PDB.

4.1.3. Homology models of JH1 and JH2 domains

Homology modelling is a computational technique used to determine 3D structures of proteins for which an experimental crystal structure is not available, provided the primary sequence exists of a similar protein for which the crystal structure is already present [72]. Homology techniques are considered the most accurate computational tools for predicting structures, solving small gaps of crystallographic models and generally enhancing the quality of a model. Lastly, homology modelling in drug discovery has a large number of benefits such as a large scope of applications, rapid model generation, ease of use and low cost [73,74].

Although modern experimental techniques are well developed, the pseudokinase-kinase domains in complex (JH1-JH2) in the JAK family has not yet been fully solved (except for a TYK2 family member [65]). Likewise, the full-length crystal structure of any JAK family member has not yet become available. Despite the fact that JH2 is not directly involved in the catalytic activity, it has been predicted to play a critical role in concert with JH1 with respect to JAK functions. Numerous experiments have proven that the presence of JH2 is essential for preserving a low baseline level of JH1 activity [75]. Indeed, it has been recently hypothesized that JH2 regulates JH1's kinase activity through other phosphorylation processes [60,61]. As evidence of this assumption, it has been proved that mutations within JH2 can influence the overall JAK kinase activity. Nevertheless, this molecular mechanism is far from being fully elucidated. In this framework, the influence of JH2 on JH1 is impossible to derive studying the domain alone. Therefore, homology modelling offers a valid alternative to overcome these limitations. In 2001 Lindauer and co-workers proposed a first homology-based model for the JH1-JH2 complex of JAK2[71]. Using this model, they showed that two main interactions occur between JH1 and JH2: the first between two α-helices (one in JH1 and the other in JH2) and the second between two β-strands of JH2 and the activation loop of JH1. In line with experimental data, they predicted that these sites have an inhibitory effect on the kinase capacity of the protein. Furthermore, they also corroborated the results obtained by a model developed using mutual information-based evaluation of MD simulations (MutInf) [76]. In that model JH2, present in an active conformation, stabilizes JH1, which appears in an inactive conformation through hydrophobic contacts and electrostatic interactions [77,78].

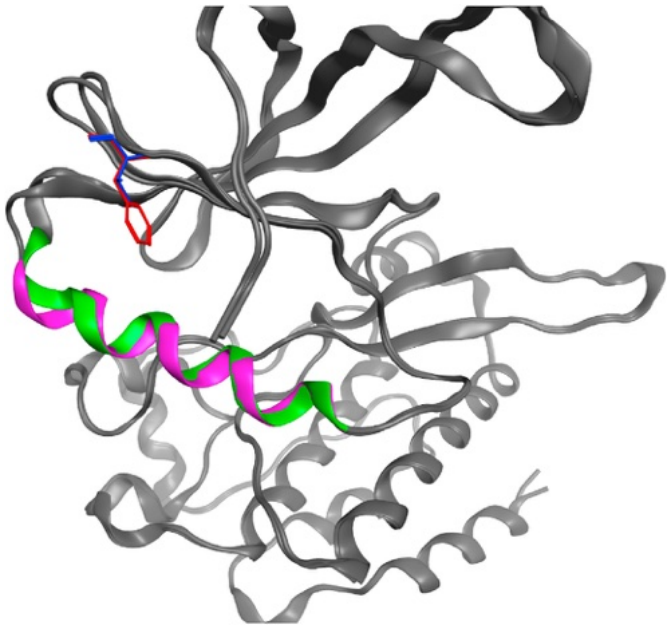
Another JH1-JH2 model for JAK2 was proposed in 2012 by Wan and Coveney [79]. It consists of an antisymmetric dimer (called Kromer's model) in which the JH1 N and C lobes interact with the JH2 C and N lobes, respectively. MD simulations showed that the two dissociated domains are necessary for JAK2 activation and with JH1 in active conformation.

Silvennoinen and co-authors, in 2013, performed biochemical and functional analysis on JAK2 pseudokinase domain, revealing that it behaves as an active protein kinase and phosphorylates Ser523 and Tyr570 (negative regulatory sites, thus facilitating JH1-JH2 inhibitory mechanism) [80]. This behaviour is unique to JAK2. It has also been shown that, to preserve basal autoinhibition, JH2 displays low catalytic activity and autophosphorylates two negative regulatory sites in the SH2-JH2 domain linker and in JH2[66]. Finally, in 2015 Min et al. investigated the JH2 pseudokinase domain of TYK2, revealing that JH2 actually binds ATP, but the catalytic activity is off [81]. ATP seems to take on the role of stabilizing JH2 and indirectly modulating the TYK2 activity. Since TYK2 pseudokinase domains have similar structure with respect to JAK1 and JAK2, this domain offers interesting possibilities to become a novel pharmacological target for drug development of rheumatoid therapeutics.

4.2. The role of mutations

Disease-related mutations in JAK2 have been attracting increasing interest of the research community. Three different structures, with the PDB: 4FVP (structure of JH2-Wild Type, without nucleotide, resolution of 2 Å), PDB: 4FVQ (JH2-Wild Type, with Mg-ATP, resolution of 1.75 Å) and PDB: 4FVR (JH2-V617F mutation, with Mg-ATP, resolution of 2 Å) have been deposited in the Protein Data Bank [78] (Fig. 7). The three cited structures are those of JAK2 JH2 domain, with and without the pathogenic mutant V617F, respectively, which is involved in myeloproliferative neoplasms. Most of the disease-related JAK mutations are found in the pseudo-kinase domain and underline the importance of elucidating the molecular mechanism of natural and pathogenic JAK signalling. This understanding is important in order to provide new and improved therapies, and for being able to treat each type of mutation-linked disease efficiently.

In general, JAKs play key roles in development and growth, but are especially important in the regulation of haematopoiesis and the immune system. Furthermore, JAKs are key players in many diseases due to their specific mutations. Understanding the consequences of the mutation mechanism is a complex problem and molecular dynamics (MD) simulations are a powerful tool able to clarify at a molecular level the interactions governing the above-mentioned phenomena. MD is an *in-silico* methodology employed to investigate biological systems at the nanoscale adopting the principles and methods of classical and statistical mechanics [82,83]. MD employs the analysis of a system at an atomistic level in terms of positions, velocities and forces. The evolution of individual atomic trajectories provides deep insights into the properties of the system in terms of structure and interactions with the surrounding environment (water or different solvent molecules). Over the past decades, MD has been successfully applied to improve our understanding of complex phenomena at atomistic scales [84–91] such as molecular recognition [92–98], protein folding [99–102], as well as transport of ions, peptides and nanoparticles across membranes



Figs. 7. 3D visualization of the PDB structures 4FVR and 4FVQ superimposed on each other. It has been demonstrated that the V617F mutation in 4FVR enhances the stiffness of the α helix C, facilitating transphosphorylation of the partner JAK. The mutated phenylalanine of 4FVR is coloured in red, the WT valine of 4FVQ is coloured in blue. In this picture, the two α helixes are depicted in purple for 4FVR and in green for 4FVQ. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

[99,103–106]. Finally, this computational approach provided by MD is widely used for multiscale analysis thanks to its capacity to bridge information at the nanoscale level to related properties and behaviour at larger scales (e.g. effects of point mutations in a protein and protein structure-function relationships [107,108]).

Focusing on JAK regulatory mechanisms related to the pseudokinase domain of JAK2, it is possible that many of them are situated in the proximity of the interface between JH1 and JH2. Mutations occurring there can either reinforce or reduce the interactions between JH1 and JH2 domains and therefore control the activation of JAK2 kinase domain. The catalytic activities and effects induced by the presence of mutations have been interpreted in view of the interactions of JH2 with JH1, which influence the configuration of the A-loop and αC-helix placed in the JH1 domain [71,109–111]. Additional research has been conducted on kinases, and in particular on JAK2, in order to gain further insight into their roles and regulation mechanisms.

Due to its relatively high frequency of occurrence, JAK2 V617F is by far the most studied of these mutations. Lee and co-workers, through 60 ns of unbiased MD simulation, investigated the autoinhibition mechanism of JAK2, strongly influenced by the V617F mutation [110]. In fact, JH1 domain, in its wild type form, is in contact with JH2 domain, which stabilizes the inactivated form of JH1 thanks to several JH2 residues, including S591, F595 and V617. In contrast, in the presence of a mutation, JH1 is always in the active form (Fig. 7) [110]. Subsequently, mutations K539L in exon 12, R564L, L579F, H587 N, S591L in exon 13, deletion of exon 14, H606Q, V617I, V617F, C618R in exon 14 and L624P in exon 15 have showed similar results. In particular, the wild type model shows strong van der Waals contact interactions leading to the absence of space for ATP ligands in the active site of JH1 domain, that remains inactive [111]. Simulations on long time scales (20 μs) of the JH2-JAK2 domain have been used to support experimental data. Specifically, in the wild-type it has been observed that the Phe617 residue interacts with the residues Phe595 and Phe594 stabilizing the alpha-helix, assuming however an indirect mechanism, because the interactions are transitory in time [78].

The observation that the V617F mutation in JAK2 causes a continuous activation of the JAKs pathway, it may be useful as a model to study inhibition of JAKs pathological constitutively active signalling. However, understanding the exact molecular mechanism of JAK2 autoinhibition is not trivial and requires a complete, full-length model of the protein. Therefore, much effort has been invested in obtaining a reliable structure. In Kromer's model, as explained in Subsection 4.1.3, the JH1 and JH2 domains produce an antisymmetric dimer. Hereafter, the term "dimer" is used when considering the union of the two kinase (-like) domains, independently of which JAK2 molecule they come from. Conversely, the JAK2 dimer described in the literature [112], usually stands for a complex made of two full-length JAK2 proteins. The structure of the complex constituted by the union of JH1-JH2 was first modelled using a proposed dimeric form of Fibroblast Growth Factor Receptor (FGFR) as a template [113]. Recently, an asymmetric model, characterized by an activator domain and a receiver domain has also been proposed [114]. Both the regulation and activation of JAK2 depend on the extent to which the JH1 domain occupies the receiver position. Under physiological conditions, the JH2 domain assumes a receiver position and keeps JAK2 catalytically inactive [79]. JH1 tends to assume a receiver position more easily if mutations occur in the JH2 domain causing a weakening of the interactions formed between the two domains of interest [79].

Wan and coworkers developed and subsequently tested experimentally a molecular model for the self-inhibiting interaction between the tyrosine kinase domain (JH1) of JAK2 and the pseudokinase domain (JH2) through extended time MD simulations. Despite employing an MD approach, molecular modelling required decisions to be made based on biochemical and structural information on JAK2. This then appeared to be not consistent as a "turnkey" method to be used for *ab initio* modelling of protein-protein interactions. However, it also highlighted the possibilities of MD simulations as an effective tool for the structural investigation of such interactions [79]. The autoinhibitory mechanism mediated by JH2 described above would reduce transphosphorylation of JAK molecules linked to either heterodimeric receptors put together through ligand binding, or with preformed homodimeric receptors (e.g., the Epo receptor) redistributed by ligand binding. It is worth noting that JAK2 is the only JAK molecule associated with preformed homodimer receptors, phosphorylation of Ser523[56,61,78] and Tyr570[56,59,77]. All these aspects seem to provide additional mechanisms in JH2-JH1 stabilization. In recent years, there has been enormous interest seen in the design of the V617F-specific inhibitors of the JAK2 used in the treatment of MPNs. The knowledge of how JH2 and JH1 in their ground state behave together could lead to screening and design of small molecules, which could be detected as new therapeutic inhibitors of V617F or other JAK2 pathogenic mutants and, at the same time, they could strengthen the interaction between the two parts [115]. Shan and co-authors, through an MD unbiased simulation on protein-protein docking, created a structural model for the self-inhibiting interaction between JAK2 JH1 and JH2, which showed that JH1 is stabilized in an inactive state by JH2 and exhibits a molecular mechanism that may explain the hyperactivity of V617F, the main JAK2 MPN mutation [116].

4.3. Molecular Docking and Virtual Screening

Nowadays, computer-based methods strongly contribute to enhancing and accelerating the drug design and discovery processes. These supporting tools constitute the Computer-Aided Drug

Design (CADD) technology. Among them, Molecular Docking plays a key role. This method assesses the optimal conformation and orientation (pose) of a ligand relative to a targeted receptor. From the resulting poses, it is possible to establish the strength of interaction as well as to locate the atoms (or residues) involved in the binding mechanism. Virtual Screening (VS) is another technique widely applied in drug discovery. Once a receptor site is provided, this methodology screens large *in silico* compound databases in order to identify potential hits that are predicted to inhibit the target receptor [116–118]. Quantitative Structure-Activity Relationship (QSAR) is an ensemble of mathematical models, which quantitatively describe drugs' biological activity as a function of physicochemical and structural features of the compounds investigated. The principle behind the formalism is that changes in structural properties cause corresponding variations in biological activities of the molecules. Modern approaches introduce the possibility to build 3D models of a molecule (3D-QSAR) [119]. 3D-QSAR is a valuable predictive tool, employed in the design of pharmaceuticals. It considerably decreases the number of compounds to be synthesized by simplifying the choice of the most promising candidates [120,121].

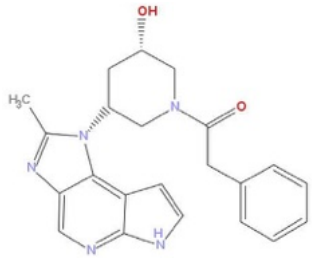
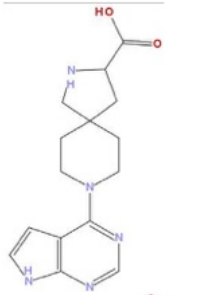
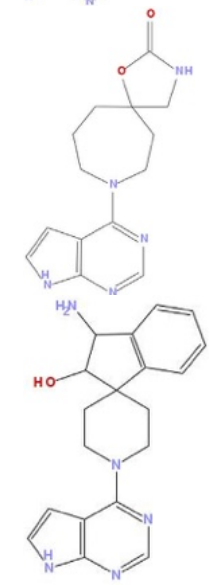
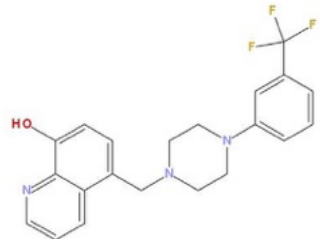
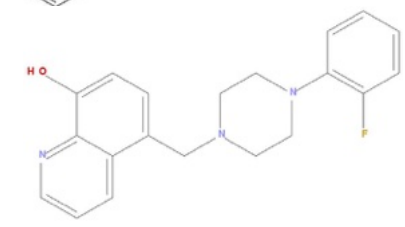
Molecular docking and VS studies have been used to augment experimental approaches and to develop and optimize novel JAK inhibitors in an effective and economic way. In this context, these tools have demonstrated that they can significantly contribute to the research effort and lead to the improvement of selective second-generation hits. On the one hand they can maximize their efficacy, on the other hand they can minimize undesired off-target effects. Indeed, structure-guided and residues-target strategies have demonstrated to possibly overcome the high homology and similarity of ATP binding sites [122]. A detailed study of molecular interactions of imidazo-pyrrolopyridine derivative with JAK1 was performed involving 3D-QSAR, MD simulations and Molecular Docking in order to design novel molecules with enhanced anticancer activity [121]. The designed compounds showed better-predicted activity than reference ones, which indicates QSAR models generated have a good predictive ability to design potent inhibitors [121,123].

Through a docking-based virtual screening and a subsequent experimental testing, spirocyclic (4-position) pyrrolopyrimidines [124] were identified as a promising starting point for the development of novel JAK1-specific inhibitor class since they demonstrated a slight preference for JAK1 over JAK2. The same template was used to design specific inhibitors for JAK3, exploiting a covalent bond with the unique Cys909 residue. The results were crystallographically resolved and are available in PDB: 5 TT, PDB: 5TTV and PDB: 5TTU [125]. Similarly, Duan et al. identified a promising lead through the rational design of the pyrrolo [1,2-b] pyridazine-3-carboxamide template. The resulting compound emerged as a potent inhibitor of JAK3, and to a lesser degree TYK2, relative to JAK2 (PDB: 4RIO) [126]. Recently, a pyrrolopyridazine diol emerged as another inhibitor of JAK1 and JAK3 and a phosphate prodrug was added in order to enhance its PK properties. The pyrrolopyridazine diol can be analysed using the PDB: 6NY4. Additionally, the same pyridine was considered as an initial template to obtain a molecule with a TYK2-selective profile, which is 7-fold more potent than JAK1 and 6-fold more potent than JAK2 (PDB: 4GIH and PDB: 4GMY) [127]. In the same way, 8-hydroxyquinoline [128] (with phenyl-piperazine/benzyl-piperidine moieties in the 5 position) was found as another starting point to optimize a hinge-binding molecule with a view to achieve selective inhibition of JAK1. Another issue emerging in the search of second-generation molecules is the generation of an inhibitor able to distinguish between JAK2 and JAK1. This fact is due to the high level of conservation of amino acids within the ATP binding cavity and among the different residues, only Gln853-Arg879 and Asp939-Glu966 (JAK2-JAK1) have sidechains orientated towards the binding site (thus exploitable by potential inhibitors) [129]. The drug design and discovery is carried out through virtual screening customized protocols and ligand-based pharmacophore methods (usually starting from Pan-JAK inhibitors). A noteworthy work from Simov et al. [130] followed this type of approach, targeting Arg879 and Glu966 and optimizing a benz(imidazole) scaffold. During the research, the PDB: 5HX8 was utilized.

Another remarkable example is a recent work in which by structurally modifying Tofacitinib molecule, JAK1 inhibitor was pursued (PDB: 6BBU). This molecule, PF-04965842, has already progressed into a phase 1 clinical study [131]. Kim et al. [132] optimized a flavonoid structure primarily mimicking Tofacitinib and secondly by adjusting some functionalities made it selective for JAK1. Their promising result demonstrated a remarkable JAK1 selectivity: more than 12 times over JAK2 and more than 10 times over JAK3. A flavonoid derivative was previously used to obtain also a JAK3 inhibitor. Another positive result was achieved by the means of an *in-silico* protocol based on Molecular Docking, MD and free energy evaluations: a thienopyridine derivative turned out to be more selective towards JAK2 versus other JAK family members [133]. Even indazole-based compounds [129] and other hits [134,135] demonstrated wide margins in the design of molecules with a high degree of JAK2 potency. Furthermore, the dehydrocrenatidine compound, extracted from ZINC database using Virtual Screening, showed a significant inhibitory behaviour with regards to JAK2136. Finally, a candidate drug (AZD4205) has been recently reported after a structure-based design, resulting in a strong affinity for JAK1 over JAK2 (>214 folds) and a favourable DMPK. During the discovery process, which led to this result, PDB: 6S58 was released. It is noteworthy that the selectivity among those results are impossible to compare since the experimental protocols used were different between the research groups. Nevertheless, even if the previously mentioned works brought new contributions to second-generation JAK inhibitors, a high degree of selectivity towards a single JAK has not yet been reached. Interestingly, most of the molecules approved for treatment can target different kinases at the same time. However, this characteristic opens new therapeutic opportunities since recent developments in CADD have also revealed that the single-target drugs may not always induce the desired effect across the entire biological system, even if they successfully inhibit or activate a specific target [137]. Finally, a new approach merging the first and second generation of inhibitors has been attempted by some researchers. This approach aims to discover dual inhibitors whose effect can be simultaneously reflected both in JAK1 and JAK2137,138. For instance, through an approach of pharmacophore-based screening and docking simulations, 15 dual JAK1/2 inhibitors were discovered for the inhibition of both enzymes. The hydrogen bonding interactions between inhibitors and Glu957, Leu959 and Glu930, Leu932 amino acid residues were found crucial for JAK1 and JAK2 enzyme inhibition, respectively [137]. Finally, Field et al. found another interesting approach in the JAK inhibitor discovery scenario [139]. They found that PF-956980, in addition to being a pan-JAK inhibitor, also downregulates the production of JAK2 and JAK3, keeping unaffected JAK1 and TYK2 proteins. The possibility of designing a new therapeutic strategy consisting in modulating the expression of receptors together with inhibiting them is worthy of further analysis. Table 3 summarizes the main molecules identified through molecular docking studies, showing their 2D structure, their JAK specificity and the corresponding study in which they were discovered.

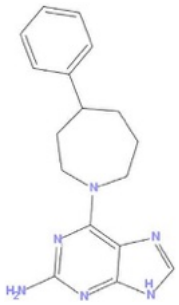
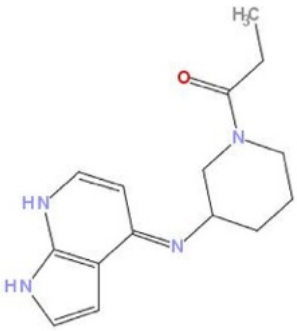
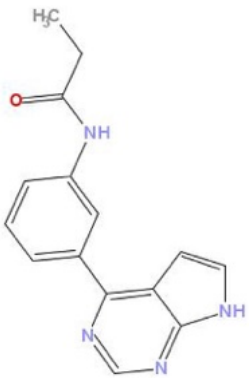
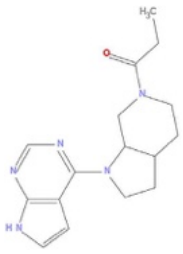
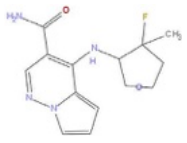
To conclude, *in-silico* studies can pave the way for the rational design of specific and selective potent inhibitors of JAK family

Tables 3
2D depiction of molecules identified through molecular docking and virtual screening studies.

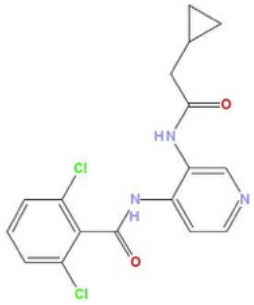
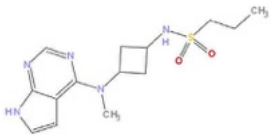
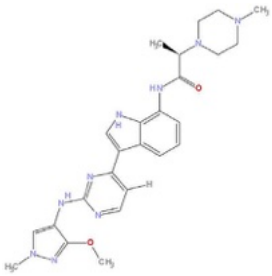
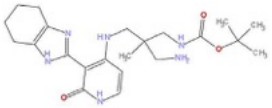
2D structure	JAK Specificity	Reference
	JAK1	The represented compound is R1[121].
	JAK1/JAK2	The represented compounds are B59, B61 and B62[124].
		
	JAK1	The represented compounds are R81 and R85[128].
		

(*continued on next page*)

Tables 3 (*continued*)

2D structure	JAK Specificity	Reference
	JAK1	The represented compound is one example of the 6 compounds listed in the work by Bajusz et al. [129]
	JAK3	The represented compound is cmp4 (PDB: 5TTS, resolution: 2.34 Å).
	JAK3	The represented compound is cmp6 (PDB: 5TTV, resolution: 1.93 Å).
	JAK3	The represented compound is cmp7 (PDB: 5TTU, resolution: 1.72 Å).
	JAK3/TYK2	The represented compound is 5g (PDB: 4RIO, resolution: 2.69 Å).

Tables 3 (*continued*)

2D structure	JAK Specificity	Reference
	TYK2/JAK2	The represented compound is cmp46 (PDB: 4GIH, resolution: 2 Å).
	JAK1	The represented compound is cmp25 (PDB: 6BBU, resolution: 2.08 Å).
	JAK1	The represented compound is cmp211[40].
	JAK1	The represented compound is cmp23[130].

members as well as a consistent and effective platform for the investigation of the role in disease development individual JAK isoforms may play [5].

4.4. Signalling pathway modelling

Several modelling approaches have been used in recent years to analyse complex biological systems such as signalling pathways. These methods range from traditional mathematical methods based on differential equations [141] to probabilistic and graph theory models [142,143]. Examples of integrative models in which Machine Learning techniques have been included exist, too [144]. Computational pathway models are methodologies useful in the study of detailed signalling processes. They are used together with experimental studies, helping to elucidate subcellular dynamics and to formulate new hypotheses, most often about the mechanism of interactions [145]. JAK-STAT pathway shows substantial behavioural complexity. A traditional mathematical model of this pathway, based on differential equations, can be found in the study by Gambin and co-authors [145]. A kinetic model of the JAK-STAT pathway was developed and validated by Yamada and coworkers [146].Guerriero and co-workers created a computational model of the gp130/JAK/STAT signalling pathway, which is consistent with biological observations on the system dynamics, by successfully developing a descriptive, high-level language enabling readily executable codes for the characterization of the involved biological pathways [147,148]. Simulations were carried out to validate the model and finally sensitivity analyses were carried out to identify significant parameters for the dynamic behaviour of the system, i.e. nuclear compartmentalisation and the phosphorylation state of STAT. In addition, the model shows that alternative signal attenuation mechanisms affect the system at different times. The above-mentioned narrative model of the gp130/JAK/STAT pathway represents an interesting practical example of the usefulness of high-level computational approaches, through which researchers can model biological systems without explicitly considering mathematical theories and formal biological notations (as typically used to be the case in biochemical modelling), being nevertheless able to obtain sensible simulation and analysis results. From this model and from the results of the sensitivity analysis it has been possible to characterize the parameters most affected by perturbations. Moreover, the results, which turned out to agree with the existing theoretical models of the gp130/JAK/STAT path, are a possible validation of the model and the approach itself.

5. Future perspectives and conclusions

Over the last decade, the JAK-STAT pathway has increased its role as a therapeutic focus for a large variety of immune-mediated diseases. Although elucidation of JAK-STAT regulatory mechanisms has grown steadily, molecular understanding of JAK activation and inhibition is still incomplete and JAK inhibitor drugs have not been demonstrated always effective or without side-effects. As a matter of fact, one of the major challenges in designing these drugs is making them highly selective, guaranteeing a desired reduction of side-effects. Moreover, another aspect to be improved is the

evaluation and characterization of dosing and scheduling strategies.

A detailed understanding of the mechanisms of JAK-STAT pathway activation and inhibition will be of fundamental importance for the development of novel modulators of JAK activity (with different modes of action with respect to already existing drugs). All current JAK inhibitors target the catalytic ATP-binding site of JAK kinase domain (JH1), which presents a similar structure between all JAKs, posing a challenge in regard to the specificity of these inhibitors. Furthermore, current inhibitors cannot differentiate between mutated JAKs and wild-type ones causing potential side effects.

In silico methodologies have already demonstrated being an effective supportive tool to identify features for highly selective compounds for specific JAK isoforms. In addition, molecular modelling could be exploited to indicate novel inhibitory strategies which do not directly address the homologous ATP binding site (e.g. the influence of JH2 upon JH1). Current efforts are aimed at identifying highly selective compounds for each JAK isoform and even for pathological JAK mutations. Designing improved inhibitors that possess greater selectivity and specificity properties, will allow to lower the therapeutic dose hence limiting side effects and toxicity and enhancing efficacy in clinical applications.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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