If you cannot win them, join them: understanding new ways to target STAT3 by small molecules

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ABSTRACT: Signal transducer activator of transcription 3 (STAT3) is among the most investigated oncogenic transcription factors, as it is highly associated with cancer initiation, progression, metastasis, chemoresistance, and immune evasion. The evidence from both preclinical and clinical studies have demonstrated that STAT3 plays a critical role in several malignancies associated with poor prognosis such as glioblastoma and triple-negative breast cancer (TNBC), and STAT3 inhibitors have shown efficacy in inhibiting cancer growth and metastasis. Constitutive activation of STAT3 by mutations occurs frequently in tumour cells, and directly contributes to many malignant phenotypes. Unfortunately, detailed structural biology studies on STAT3 as well as target-based drug discovery efforts have been hampered by difficulties in the expression and purification of the full length STAT3 and a lack of ligand-bound crystal structures. Considering these, molecular modelling and simulations offer an attractive strategy for the assessment of “druggability” of STAT3 dimers and allow investigations of reported activating and inhibiting STAT3 mutants at the atomistic level of detail. In the present study, we focused on the effects exerted by reported STAT3 mutations on the protein structure, dynamics, DNA binding and dimerisation, thus linking structure, dynamics, energetics, and the biological function. By employing atomistic molecular dynamics (MD) and umbrella sampling (US) simulations to a series of human STAT3 dimers, which comprised wild-type protein and four mutations; we explained the modulation of STAT3 activity by these mutations. Counter-intuitively, our results show that D570K inhibitory mutation exerts its effect by enhancing rather than weakening STAT3-DNA interactions, which interferes with the DNA release by the protein dimer and thus inhibits STAT3 function as a transcription factor. We mapped the binding site and characterised the binding mode of a clinical candidate napabucasin/BBI-608 at STAT3, which resembles the effect of D570K mutation. Our results contribute to understanding the activation/inhibition mechanism of STAT3, to explain the molecular mechanism of STAT3 inhibition by BBI-608. Alongside the characterisation of the BBI-608 binding mode, we also discovered a novel binding sites amenable to bind small molecule ligands, which may pave the way to design novel STAT3 inhibitors and to suggest new strategies for pharmacological intervention to combat cancers associated with poor prognosis.

INTRODUCTION

Signal transducer activator of transcription 3 (STAT3) protein has emerged as a prominent target in tumour progression due to its pivotal role in cell signalling. The activation of STAT3 protein has been also related with drug resistance, with the expression of other anti-apoptotic proteins or with the inflammatory processes in tumour development, among others. Although its importance in cancer progression the pharmacological drugging of STAT3 is still a challenge that demands clarification. Although many strategies have been described in literature to inhibit STAT3, just few inhibitors are still going through clinical trials (e.g. TTI-101 [ClinicalTrials.gov Identifier: NCT03195699] or napabucasin [BBI-608]† and [ClinicalTrials.gov Identifier: NCT03647839]) and STAT3 has become one of the most challenging cancer-related protein to target by small molecule.

Gaining insights into the atomistic level nature of STAT3 would permit the identification of novel strategies to interact with this protein by small molecules and targeting oncogenic pathways indirectly. Human STAT3 monomer is composed by six highly specialised domains (i.e. N-terminal coiled-coil domain, DNA binding domain, linker-domain, SH2 domain, and C-terminal transactivation domain). Particularly, the DNA-binding domain (residues: 320-494) is responsible for the DNA binding when STAT3 is in the dimeric form. An alpha-helix linker domain joins this latter with the SH2-domain, which is essential for the binding of STAT3 to phosphorylated receptors and for its dimerisation (residues: 493-583). The SH2 domain has traditionally been the main target for drug design, mostly accompanied by computational studies relying on molecular docking calculations, despite no crystallographic data available up to date to support them.
The conservation of the three-dimensional structure of the whole protein is crucial for the activity of STAT3. Experimental data have demonstrated that point mutations in the linker domain suggest contacts with both the DNA binding and SH2 domains that could cause structural changes that severely affect STAT3 activity \(^ {12}\). Alanine scanning demonstrated that the modification of inter-domain hydrogen bonds can produce a significant decrease (i.e. K551A, W546A) or increase (D570K) in STAT3-DNA binding compared to the wild type protein. Understanding the effect of point mutations on STAT3 activity at the atomistic level could provide significant information about novel binding sites, unveiling new ways to target STAT3 by small molecule ligands.

The lack of a ligand-bound crystallised structure hinders the path for effective structure-based ligand design for both computational and medicinal chemists. Only a few moderate-resolution crystal structures of mouse STAT3 (PDB id: 1BG1, 3CWG and 4E68) are available, and none of them presents a bound ligand. A small number of STAT3 inhibitors are in clinical trials, but their binding sites at STAT3 and hence their binding modes remain unknown. Among them, napabucasin (BBI-608) is a first-in-class cancer stemness inhibitor that targets STAT3\(^5\), which is being tested (Phase 3) as a treatment in advanced colorectal cancer\(^6\). Ji and coworkers reported that napabucasin binds in a pocket between the linker and DNA binding domain in a STAT3 crystal structure\(^6\), but the crystal structure has not been disclosed.

In this study, we addressed “druggability” of STAT3 dimer at the atomistic level of detail by performing equilibrium all-atom molecular dynamics (MD) and umbrella sampling (US) simulations to determine the behaviour of the wild-type and mutated STAT3. Monitoring the time evolution of the different domains at hundreds of nanoseconds time scale revealed their differential behaviour in terms of molecular flexibility and their ability to bind DNA when point mutations were introduced. The examination of mutations-induced structural changes directed us to new sites amenable to inhibition by small molecule ligands. Furthermore, construction of a homology model of ligand-bound STAT3 along with MD and US simulations helped us understand the behaviour of a ligand that interacts with a newly identified “druggable” region of STAT3.

**METHODS**

**Molecular modelling of human STAT3 dimer.** The initial models of dimeric human STAT3 (wild type and mutants) in complex with DNA were created using crystal structure of unphosphorylated mouse STAT3B (PDB code: 4E68), which spans residues 136-716. Loops spanning the residues 184-194 and 688-702 were modelled using MODELLER interface\(^7,8\) in UCSF Chimera\(^9\). The N-terminal domain (residues: 1-135) was modelled using human STAT1 as the template (PDB code: 1YVL\(^10\); sequence identity: 55%). The model has been built using SWISS-MODEL, a fully automated protein structure homology-modelling server, accessible via the ExPASy webserver\(^11,12\). Both models were then joined in UCSF Chimera and these were quality checked – the conformations of interfacial side chains were assessed for steric clashes. The final model was subjected to 20,000 cycles of steepest descent energy minimisation.

The STAT3 mutations (Figure 1), which were selected following the study by Mertens and coworkers\(^12\), were introduced in UCSF Chimera by swapping side chains to the target residues and adjusting new conformations using Dunbrack rotamer library integrated within UCSF Chimera.

**Modelling of ligand-bound STAT3.** The crystal structure of ligand-bound STAT3 is yet not available, therefore we built the most similar model possible with the accessible data. Starting from the dimer model of wild-type STAT3 described in the previous section, molecular docking calculations were performed with MOE\(^13\), using napabucasin (BBI-608) as the ligand. To ensure the scoring function accuracy with the target and the best possible fit both blind (full dimer) and targeted docking (pocket described) were performed. 200 different conformations of the ligand were scored per each run using Triangle Matcher, and London ∆G for the first scoring function. Thereafter, the top 100 conformations were rescored using Induced Fit and GBVI/WSA (Generalized-Born volume integral/weighted surface area) score \(^1\). From the final poses obtained, one for each monomer was selected based on score,
interactions and consistency with the experimental data available.

Figure 2 Description of the STAT3 umbrella sampling (US) simulations performed in this study. After a 10ns of equilibrium MD production run, DNA is pulled out of the dimer and a series of windows are selected that will be simulated for 5ns to compute potential of mean force (PMF) of DNA.

Molecular dynamics and umbrella sampling simulations. In molecular dynamics studies on the effects of mutations in the STAT3 dimer, N-terminal domains (residues 1-136) were omitted. Structural hydrogens were added and the following protein parametrisation was performed using the Gromacs 2016.03 suite with AMBERFF99SB-ILDN force field. Before pulling the DNA from the complex, the systems were relaxed with a short equilibrium MD production run. Hence, a 1 nm cubic box was centred on the structure and the system is solvated with TIP3P waters. Sodium and chloride ions were added to a concentration of 0.1 M. Bonds were constrained using the LINCS algorithm. The electrostatic interactions were calculated using particle-mesh Ewald method, with a non-bonded cut-off set at 0.1 nm. All structures were minimised via the steepest descent algorithm for 20,000 steps of 0.02 nm, and minimisations were stopped when the maximum force fell below 1000kJ/mol/nm using the Verlet cut-off scheme. After minimisation, NVT equilibration was performed for 100ps with a time step of 2fs with position restraints applied to the backbone. The temperature coupling was set between the protein and the non-protein entities by using a Berendsen thermostat, with a time constant of 0.1 ps, and the temperature set to reach 300K with the pressure coupling off. Sequentially, a pressure NPT equilibration was performed followed by 100ps of an NVT equilibration, the following 100ps of NPT equilibration, and a production run of 10 ns. Temperature was set constant at 300K by using a modified Berendsen thermostat (τ = 0.1 ps)\(^2\). Pressure was kept constant at 1 bar by Parinello-Rahman isotropic coupling (τ = 2.0 ps) to a pressure bath\(^3\).

For the umbrella sampling simulation, the complex had its principal rotation axis aligned to the z-axis of the simulation box. A pull sampling was used using a constant force approach (k = 1000 kJ/mol/nm, with a rate of 0.01 nm) between the centres of masses of SH2 domain and the DNA double helix, along the described path shown in Figure 2. From each corresponding pull simulation, a series of conformations have been selected in order to sample the process of entering-exiting the DNA-binding site. Each selected conformation has been through a 1ns NPT equilibration run, followed by a 5ns NPT distance restrained production run, using the previously described protocol and parameters. Afterwards, the potential of mean force (PMF) curve of the studied scenario has been calculated with the Weighted Histogram Analysis Method (WHAM) tool available in Gromacs\(^3\).

RESULTS AND DISCUSSION

The mutations directly affect the sampled configurations of STAT3 dimer. In the study by Mertens and coworkers, several mutations were indicated as crucial to control DNA retention time within its respective binding cleft at STAT3\(^1\). These mutations occurred either in DNA-binding domain, or in the inter-domain region.

Figure 3 MD simulations show conformational changes between WT (blue) and D570K (red) STAT3 dimers (A). In the D570K mutant, one of the monomers is shifted (B), changing the conformational landscape of the dimer. Panel (C) shows how the position of the DNA duplex is shifted downwards in D570K mutant compared to WT.

One of the most significant configurational changes occurred within D570K mutant, as the DNA double helix shifted downwards (Figure 3). This was most likely caused by the electrostatic effects at the residue located in the interface between linker and DNA-binding domain. The modification of the side chain charge from negative (D) to positive (K) increased favourable protein interaction with the negatively charged nucleic backbone. This tightened the DNA binding, resulting in a higher average DNA RMSD when compared to the crystal structure. It strongly indicates that the end-point
configurations of the protein-DNA complexes play a significant role in their binding free energy, since the protein-nucleotide interactions change significantly between different mutants.

We assessed whether the conformational changes induced by D570K mutation were observed in other mutations. Figure 4 shows root-mean-square deviation (RMSD) plots of all STAT3 considered in this study as well as WT protein. Except D570K, there were no large differences in protein RMSD between the mutated STAT3 dimers and WT. This gap between D570K and other mutants is likely to arise from the combination of electrostatic and steric effects (all other mutations replaced large and polar residue with smaller and apolar alanine), which affects intrinsic dynamics of the Coiled-Coiled (CC) domain. Hence, the dynamics of the CC domain might “tune” DNA-STAT3 interactions by allowing adjacent SH2 and DBD domains to improve their structural “fit” to the DNA.

To follow up on the effects of the mutations which control DNA retention at the STAT3 on the structure, dynamics, and energetics of STAT3-DNA complexes, we carried out a set of umbrella sampling (US) simulations, where DNA has been pulled from STAT3 dimer. This process was studied using 20 US windows and simulated for 5ns each.

The potential of mean force (PMF) calculated via weighted histogram analysis method (WHAM) were consistent with the results reported by Mertens and coworkers14 in most cases. Experimental results showed a drop in DNA-binding for the tested inter-domain mutations (EE434/435AA, W546A and K551A) through time and an extraordinary high retention time for D570K, with a 100% DNA-binding even after two hours.12 The higher the binding free energy difference, the better is the affinity of DNA with the dimer. The WT STAT3 had a higher energy barrier to reach its unbound state in comparison to both W546A and K551A mutants (Table 1). Both mutants showed a lower retention time, which indicated that inter-domain interactions between mutated residues and E434 are crucial for STAT3-DNA binding. Therefore, disrupting these interactions could represent an attractive strategy to target STAT3 by small molecules.

The equilibrium MD simulations as well as PMF showed that D570K binding affinity to DNA is more favourable than of any other mutant, and more than twice of WT STAT3 (Table 1). This indicated that this mutation promotes a very tight binding between STAT3 and DNA, with much higher energy gap for DNA release upon pulling. The PMF curve showed that DNA pulling from D570K required a higher energy gap to release DNA from the STAT3 dimer. Experimental retention time correlated with the simulations when compared to WT STAT3. The data showed that DNA binding to the D570K was persistent through time, it did not drive transcription and resisted dephosphorylation, thus prevented STAT3 to exert its function12.

Collectively, these results strongly indicate that D570K promotes a very tight DNA binding, so much that the bound duplex stays “locked” between the dimers, which effectively inhibits STAT3 by preventing it from releasing DNA and exerting its function as a transcription factor.

The only discrepancy between our simulations and experimental data was observed for the EE434/435AA double mutant. In the simulations, the mutant showed a higher PMF value than WT, which indicated that its DNA binding affinity should be higher, while experimental data showed that its behaviour resembled that of K551A and W546A mutants, which have shown a considerable drop of DNA-binding through time. Analysis of the final US windows indicated that the middle of the DNA duplex interacted favourably with the DNA-binding domain of one STAT3 monomer, but not another. Therefore, the US curve of EE434/435AA mutant displayed higher values arising from these interactions (DNA-STAT3 monomer) rather than from favourable interactions DNA-STAT3 dimer, as it was for D570K mutant.
Arginine R414 acts as “gatekeeper” for DNA binding. All simulations indicated significant conformational changes of the arginine R414, which were required to release DNA (or to allow the DNA binding to the STAT3 dimer). R414 is at close distance from DNA and its initial position did not allow the DNA exit from the dimer. By acting as a “gatekeeper” of DNA binding, R414 exerted a key role in controlling opening and closing of STAT3 dimer, as well tuned the dynamics of STAT3 monomers by modulating intra-domain DBD-SH2, CC-SH2, DBD-LD, and CC-LD interactions.

Inhibition of STAT3 by napabucasin (BBI-608). The results of simulations of apoSTAT3 (WT and mutants) highlighted a set of inter-domain residues, explaining their effect on STAT3 behaviour and function at the atomistic level of detail. These observations may pave the way to novel strategies for STAT3 inhibition using small molecule ligands. Traditionally, structure-guided ligand design for STAT3 inhibition was based on targeting the SH2 domain, which intended to prevent STAT3 dimerisation. Although several inhibitors have been described to bind to SH2 domain, there is no crystal structure available to confirm it, and all studies focusing on SH2 domain binders relied solely on semi-rigid molecular docking calculations, which may raise the question whether SH2 domain is the binding site.

Recently, Ji and coworkers reported that napabucasin (BBI-608), which is one of the few STAT3 inhibitors in advanced clinical trials (Phase 3), binds to the small pocket between the linker and DNA binding domain in a STAT3 crystal structure. Since the crystal structure has not been released to the public domain, we have assessed the druggability of this segment of STAT3, identified the putative pocket, and subsequently built the model of BBI-608 bound to STAT3 using molecular docking approach, and subsequently validated the obtained binding mode by the atomistic MD and US simulations.

Molecular docking was performed by MOE for each STAT3 monomer separately, trying to generate the most plausible conformation relying the limited data available. Both blind (whole monomer) and targeted (residues of the identified pocket) docking calculations resulted in a set of conformations with favourable energy scores and highly-populated cluster located within the DBD site pocket, in close contact with residues H332, P333, R335, K573 and D570 (Figure 7). Two conformations, matching the published data, were found: both were assessed and validated.

To validate the binding mode of BBI-608, MD simulations of WT STAT3-DNA-BBI-608 complex were performed for 100 ns. Subsequently, the ligand affinity has been calculated. The ligand docked either of SAT3 monomers remained bound through the whole simulation. Interaction energies, calculated by MMPBSA analysis (g_mmpbsa module) resulted in -18.1 ±2.6 kcal/mol, showing a favourable binding.

US simulations, performed using the same protocol as for STAT3-DNA complexes, started the pull from the most populated cluster. The calculated binding affinity has been overestimated (-160 kcal/mol), nevertheless it showed very tight binding. Compared with the results obtained for protein-DNA complexes described in the previous section, it implies that the presence of BBI-608 enhances DNA binding with a similar effect to D570K mutation. As such, BBI-608 inhibits the function of STAT3 in a similar manner to D570K mutation, which does not drive transcription and resists phosphorylation. Since D570 has been annotated by Ji et al as the BBI-608 binding site residue, we concluded that BBI-608 binding to WT STAT3 generated a similar DNA-protein interaction pattern and retention time than D570K mutation.
**BBI-608 binding promotes STAT3 dimerisation.** We performed MD simulations of BBI-608-bound WT STAT3 without DNA, to study the influence of the ligand on the protein behaviour in the absence of DNA. BBI-608 was bound to each STAT3 monomers and three 50 ns replicas showed a variation of results. In two out of the three replicas, the BBI-608 molecules remained bound in their pockets through the whole trajectory, while DBD domains of both STAT3 monomers moved closer to each other, reaching the point of forming inter-domain hydrogen bonds. In one simulation both ligand molecules dissociated from the pockets, which caused an opening of the STAT3 dimer.

To assess ligand-induced conformational changes within the DNA entry through both DBD domains, distances between some of the residues involved in H-bonding (e.g. Q344-G342 and T412-Q344) were measured and analyzed in all three replicas and compared to the simulation of the WT STAT3 dimer without DNA and/or BBI-608 bound (Figure 8).

STAT3 dimer closed further down in the presence of BBI-608. This was particularly pronounced in one of the replicas, in which the distance between monomers reduced to ~5 Å. These results indicate that BBI-608 binding to apoSTAT3 is likely to trigger conformation changes that would prevent DNA from binding. In the replica simulation, where both ligands dissociated from STAT3, the distance between monomers increased upon ligand dissociation, as both ligands exit via the gap formed between DBD domains of STAT3 monomers.

Protein-ligand interaction energy was calculated and a correlation between ligand dissociation, dimer separation and poor ligand affinity could be observed.

The simulations also indicate that ligand binding to one of the STAT3 monomers is more favorable than binding to another one. While interaction energies are favourable for both monomers, the more favorable shows the interaction energy values twice as favourable as for another monomer. Although the allosteric effects within STAT3 were beyond the scope of this study, these results strongly suggest that such effects may occur in STAT3 dimers and contribute to the modulation of STAT3 by inhibitors.

**Identification of a novel “druggable” binding site.** Experimental results combined by the simulations strongly indicated that targeting the interface between STAT3 monomers may trigger similar response to the inhibition by ligands binding to the DBD domain, and therefore be explored in structure-based ligand design efforts. With most STAT3 ligands being designed for the SH2 domain and just a few for the DBD domain, the identification of new “druggable” pockets for STAT3 inhibition is of a great interest.
Yet very challenging drug target.

As such, we scanned STAT3 for the presence of potential binding sites with two pocket detection tools: fpocket and MOE’s Site Finder. Upon selection of the dimer model and different clusters from its MD simulation trajectories we confirmed the binding site identified for BBI-608, which has been identified by both tools as their top-ranked site. Interestingly, that site was detected by both fpocket and SiteFinder for all analysed structures (Figure 9). In addition, a new pocket within DNA binding domain (close contact with E434 and E435) was identified. The main difference between results obtained by both tools is that fpocket was more prone to detect SH2 domain sites as pockets (Figure 9, B2-3) while SiteFinder identified a novel “druggable” pocket close to R414 (Figure 9, A1). Ligand binding to the R414 pocket could result in a possible DNA release/binding impediment.

CONCLUSION

Using computational approaches based on atomistic molecular dynamics simulations, enabled us to understand the effects of specific STAT3 mutations, which were described in the literature, and to explain their modulation of the STAT3 activity. Consistently with Mertens and coworkers; D570K mutation exerted its effect by enhancing interactions between STAT3 and DNA, which interfered with the DNA release by the STAT3 dimer and thus inhibited the protein’s function by not driving transcription and resisting dephosphorylation. Subsequently, recent identification of a plausible binding site for small molecule STAT3 inhibitor nababucasin (BBI-608) helped us to deconvolute its inhibition mechanism, with resembled the effect exerted by D570K mutation. The identification of the putative binding site for BBI-608 around the DNA binding domain may contribute to novel potent and selective STAT3 inhibitors. Mutated inter-domain residues E435, W546 and K551 unveil a poor binding to DNA, leading to another way of targeting STAT3 and pointing towards novel allosteric binding sites. Structure-based ligand design targeting these novel pockets, coupled with novel methodologies, such as employing recently the developed FragLite; is likely to expand a set of chemotypes active towards STAT3 and contribute to the development of novel inhibitors of this important yet very challenging drug target.

Figure 9 “Druggability” of STAT3 dimer. Sitefinder A) and fpocket B) were used to identify new potential pockets for structure-based drug design. In both cases the BBI-608 DBD site was identified along with novel DBD pockets.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding Sources

This work was funded by School of Natural and Environmental Sciences, Newcastle University (scholarships to F.S.Z. and J.V.S.).

Notes

The authors declare no competing interests.

ACKNOWLEDGMENT

We are grateful to Dr. M. Garner for technical assistance, and to Dr. V. I. Korolchuk for critical review of the manuscript and constructive feedback.

ABBREVIATIONS

STAT3, Signal transducer activator of transcription 3; MD, molecular dynamics simulation; US, umbrella sampling simulation; DBD, DNA-binding domain; CC, coiled-coil domain; LD, linker domain; MM-PBSA, Molecular Mechanics Poisson-Boltzmann Surface Area; WHAM, weighted histogram analysis method.

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