Targeting STAT3 with proteolysis targeting chimeras (PROTACs) and next generation antisense oligonucleotides

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Abstract

Signal transducer and activator of transcription 3 (STAT3) has been recognized for its key role in the progression of cancer, where it is frequently upregulated or constitutively hyperactivated, contributing to tumor cell proliferation, survival, and migration, as well as angiogenesis and suppression of anti-tumor immunity. Given the ubiquity of dysregulated STAT3 activity in cancer, it has long been considered a highly attractive target for the development of anti-cancer therapies. Efforts to target STAT3, however, have proven to be especially challenging, perhaps owing to the fact that transcription factors lack targetable enzymatic activity and have historically been considered "undruggable". Small molecule inhibitors targeting STAT3 have been limited by insufficient selectivity and potency. More recently, therapeutic approaches that selectively target STAT3 protein for degradation have been developed, offering novel strategies that do not rely on inhibition of upstream pathways or direct competitive inhibition of the STAT3 protein. Here, we review these emerging approaches, including the development of STAT3 proteolysis targeting chimera (PROTAC) agents, as well as preclinical and clinical studies of chemically stabilized antisense molecules, such as the clinical agent AZD9150. These therapeutic strategies may robustly reduce the cellular activity of oncogenic STAT3 and overcome the historical limitations of less selective small molecules.

Keywords

STAT3; PROTACs; ASOs; cancer; therapeutics

Introduction

Signal transducer and activator of transcription (STAT) proteins comprise a family of transcription factors involved in cell growth, survival, and differentiation.[1] Highly conserved in structure, the seven members of the STAT family (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, STAT6) are critical mediators of intracellular signaling, relaying and integrating extracellular stimuli from cytokines and growth factors into responses that alter levels of gene transcription in accordance with their respective functions.[2] Perhaps the most

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well-studied STAT, STAT3 is frequently dysregulated and implicated in cancer development and progression. Numerous studies have provided evidence linking overexpression and/or hyperactivation of STAT3 to tumor growth, invasion, and angiogenesis via transcription of target genes encoding positive regulators of the cell cycle and negative regulators of apoptosis signaling.[3–7] Additionally, STAT3 promotes tumor cell production of immunosuppressive cytokines, and STAT3 activation in infiltrating immune cells acts, in general, to suppress anti-tumor immunity.[8] Analyses of clinical specimens from many solid tumor cancers have demonstrated an association between aberrant STAT3 activation in the tumor microenvironment and poor patient prognosis.[9] Collectively, these factors identify STAT3 as a promising therapeutic target in cancer.

Canonical activation of STAT3 involves phosphorylation of a specific carboxyl-terminal tyrosine, Y^{705}, by upstream kinases, including Janus kinases (JAKs), epidermal growth factor receptor (EGFR), c-Src, and BCR/ABL. In the well-characterized IL-6/JAK/STAT3 signaling pathway, IL-6 binds to its cognate cell surface receptor, forming a heterohexameric IL-6/IL-6R/gp130 complex. Formation of this complex leads to activation of associated members of the JAK family of tyrosine kinases, which directly phosphorylate STAT3.[8] Receptor tyrosine kinases such as EGFR undergo autophosphorylation following ligand binding, and these phosphorylation sites then serve as docking sites for the Src-homology 2 (SH2) domain of STAT3, facilitating EGFR-mediated phosphorylation of STAT3.[10,11] EGFR, and other receptor tyrosine kinases, also induce activation of c-Src, leading to phosphorylation of STAT3 by this non-receptor tyrosine kinase.[12] Phosphorylation of STAT3 Y^{705} leads to homodimerization that is facilitated by the SH2 domain of each monomer interacting with the phosphorylated Y^{705} residue on the other partner. Once dimerized, STAT3 translocates into the nucleus and binds to a response element in the promoter regions of target genes, inducing the transcription of these genes. Serine phosphorylation of STAT3, most frequently on S^{727}, also modulates STAT3 transcriptional activity.[13]

More recently, studies of non-canonical pathways of STAT3 activation and signaling have identified the potential of these alternate mechanisms to contribute to cancer development.[14] Regulation of mitochondrial function is one area in which STAT3 has been discovered to play a role. Translocation of STAT3 to the mitochondria is dependent on S^{727} phosphorylation, and leads to interaction with the electron transport chain to regulate cellular respiration. Mitochondrial STAT3 is observed in non-transformed cells, but dysfunction of its normal role in the mitochondria, resulting in increased respiration, has been shown to contribute to RAS-mediated cellular transformation.[14,15]

Additional studies of non-canonical STAT3 signaling have indicated that dimerization can occur in the absence of Y^{705} phosphorylation, and that unphosphorylated STAT3 (uSTAT3) dimers and monomers can interact with interferon gamma-activated sequence (GAS) DNA-binding sites.[16–18] Interestingly, uSTAT3 has been shown to regulate the transcription of a distinct set of genes, including cyclin B1, E2F1, c-MET and M-RAS, and also interacts with unphosphorylated NF-κB to activate a set of NF-κB-dependent genes responsive to the uSTAT3/NF-κB complex.[14,16,17,19,20] Due to the oncogenic nature of the uSTAT3 regulated proteins c-MET and M-RAS, and the aberrant co-expression of both uSTAT3 and phosphorylated STAT3 in cancer cells, emerging evidence indicates that unphosphorylated

and phosphorylated STAT3 work in concert through canonical and non-canonical pathways to promote cancer development.[14] Hence, therapeutic approaches that target both the phosphorylated and unphosphorylated forms of STAT3 may be necessary to efficiently reduce STAT3-mediated oncogenic signaling in cancer cells. In this regard, antisense and PROTAC approaches, which target the expression of STAT3, may be particularly advantageous compared with small molecule competitive inhibitors.

Proteolysis targeting chimeras (PROTACs)

Proteolysis targeting chimera (PROTAC) technology has emerged as a novel approach that enables modulation of protein expression levels via targeted degradation.[21–23] PROTAC drugs show particular promise in that their mode of action is rapid and catalytic, and they degrade rather than inhibit the targeted protein of interest (POI) to effectively and holistically reduce the activity of the protein in the cell.[21–23] Structurally, PROTAC drugs are heterobifunctional molecules comprised of a "warhead" ligand that recruits the POI, connected by a linker to a ligand that recruits an E3 ubiquitin ligase (Figure 1A). The technology exploits the eukaryotic ubiquitin-proteasome system, such that the PROTAC molecule brings the POI and the E3 ubiquitin ligase moiety into close proximity, whereupon the E3 ligase recruits an E2 ubiquitin-conjugating enzyme that subsequently transfers ubiquitin to the target POI. The ubiquitinated POI is then targeted for degradation by the 26S proteasome (Figure 1B).

The concept of PROTACs was introduced in 2001 by Deshaies, Crews, Sakamoto and colleagues who created the first PROTAC molecule, consisting of an angiogenesis inhibitor, ovalicin, targeting the enzyme methionine aminopeptidase-2 (MetAP-2) linked to a phosphopeptide that recruits the SCF$^{\beta\text{-TRCP}}$ E3 ubiquitin ligase.[22,24] While studies with this first-generation PROTAC faced limitations in cell permeability, they provided proof-of-concept evidence that spurred the development of PROTACs incorporating non-peptidic ligands for E3 ligases. The use of small molecules (as opposed to peptides) as warheads or E3 ligase ligands for PROTAC drugs is advantageous due to their low molecular weight, lack of labile peptide bonds, and potential for greater cell permeability and potency.[23] A key advancement occurred when Bradner and colleagues successfully employed the small molecule phthalimide as a ligand recruiter for cereblon E3 ubiquitin ligase.[25] PROTAC drugs have now been designed to recruit an expanding repertoire of E3 ligases, including the E3 ligases MDM2, CRBN, SCF$^{\beta\text{-TRCP}}$, VHL, and IAPs, representing only a fraction of the vast family of E3 ubiquitin ligases found in the human genome.[21,22]

A key strength of PROTAC approaches lies in the rapid and catalytic nature of the process. The binding of a PROTAC drug to a target POI is a transient interaction, and once a successful ubiquitin transfer has been catalyzed, leading to destruction of the POI, the PROTAC molecule can dissociate to participate in another reaction (Figure 1B).[22] The catalytic nature of PROTAC drugs means, in principle, that efficient potency can be achieved using lower drug concentrations and shorter exposure times than would be needed for a typical competitive inhibitor. The ability to utilize low doses of PROTAC drugs may limit nonspecific adverse toxicities. Studies have shown that PROTACs can achieve near complete depletion of the target protein in as little as several hours after treatment. PROTACs targeting

the estrogen receptor alpha (ERα) demonstrated efficient depletion of the protein one hour after treatment of cells in culture at nanomolar concentrations.[26–28] Remarkably, not only is depletion of target proteins generally sustained after only a single treatment, but downstream signaling is also reduced. Depletion of ERα was sustained for up to 48 hours after treatment and downstream estrogen signaling was inhibited, while PROTAC degraders of anaplastic lymphoma kinase (ALK) showed persistent inhibition of ALK downstream signaling for at least 24 hours following a 2-hour treatment with 30 nM of drug.[26,27,29]

Currently, a number of PROTAC-based cancer therapeutics have been developed by groups in both academia and industry. These efforts include, but are not limited to, PROTACs targeting ALK (implicated in lymphomas), ERα (implicated in estrogen receptor-positive breast cancer), androgen receptor (AR; implicated in prostate cancer), eukaryotic translation initiation factor 4E (eIF4E; a critical regulator of cell proliferation), and BCR-ABL (a prominent driver of chronic myelogenous lymphoma).[21,27,29,30] Efforts by the pharmaceutical company Arvinas, Inc. have propelled two PROTACs into Phase I clinical studies, ARV-110 and ARV-471, which act to target AR and ER, respectively (NCT03888612; NCT04072952).[31,32] Preliminary results indicate a generally positive safety profile and tolerability. Although challenges remain in the development of PROTAC drugs (see below), they are emerging as promising therapeutic agents for targets that have proven difficult to treat by conventional methods.

PROTACs targeting STAT3

Recently, Bai *et al.* reported the development of a small-molecule PROTAC targeting STAT3.[28] Known as SD-36, this PROTAC potently and selectively degraded STAT3 protein in acute myeloid leukemia (AML) and anaplastic large-cell lymphoma (ALCL) cells, while also mediating complete tumor regression in AML and ALCL mouse xenograft models.

The warhead for SD-36 was the small molecule SI-109 (Figure 2A), a STAT3 SH2 domain inhibitor reported to bind to STAT3 with high affinity ($K_i = 9$ nM).[28] To generate the SD-36 PROTAC, SI-109 was attached via a six carbon linker to a ligand analog of lenalidomide, which acts to recruit the CRBN E3 ligase (Figure 2B). A modified version, SD-36Me, containing a methyl group on the lenalidomide analog moiety, was also generated; SD-36Me lacked the ability to recruit CRBN and was used as a negative control (Figure 2C).

SD-36 demonstrated efficient reduction of STAT3 levels in human hematopoietic cell lines, promoting 90% degradation in the AML cell line MOLM-16 after 4-hour treatment at 250 nM and 50% in the ALCL cell lines SU-DHL, DEL, and KI-JK after 7-hour treatment at the same concentration. The small molecule inhibitors SI-109 and SD-36Me, on the other hand, failed to promote STAT3 degradation.

Evaluation of the SD-36 selectivity revealed that treatment of MOLM-16 cells for 4 hours, or SU-DHL cells for 18 hours, with 0.3 μM SD-36 resulted in nearly complete degradation of STAT3.[33] By contrast, treatment with concentrations as high as 10 μM had little impact on other expression levels of other STAT family members, including STAT1, STAT2, STAT4, STAT5A/B, and STAT6. Interestingly, binding analyses determined that SD-36

exhibits a Kd of 44 μM for STAT3, and also binds with moderately high affinities to STAT1 and STAT4 (Kds ~ 1 μM).[33] Hence, while SD-36 binds to all three of these STAT proteins, it is exquisitely selective for degradation of STAT3. This unique selectivity underscores a promising, yet complex, aspect to the development of selective PROTAC degraders. Specifically, the degradation selectivity of a PROTAC will be determined by the precise physical structure of the ternary complex. The promise lies in the fact that highly selective degraders can be generated, even when limited by a warhead ligand that is not entirely specific for the POI, as in the case of SD-36. The complexity, or difficulty, lies in how to design the PROTAC molecule to generate a ternary complex with selective degradation capability. Currently, a great deal of effort is being expended to empirically optimize linker length and composition, as well as the ligands for E3 ligases, although this can be a daunting task in the process of developing PROTACs selective for different POIs.

Additional studies determined that SD-36 efficiently degraded mutated STAT3 proteins containing the D661Y and K658R mutations that are seen in hematologic cancers such as T-cell large granular lymphocytic leukemia as well as an engineered Y705F mutant.[34,35] The ability of SD-36 to stimulate degradation of the Y705F mutant indicates the capacity of this PROTAC to degrade STAT3 that may be involved in non-canonical signaling pathways. Indeed, SD-36 induced reduction of STAT3 levels in the mitochondria. Inhibition of leukemia and lymphoma cell growth by SD-36 was associated with G_1 arrest and downregulation of c-Myc.

Treatment of mice harboring MOLM-16 or SU-DHL-1 tumors with a single intravenous dose of SD-36 (25 mg/kg) resulted in nearly complete depletion of tumoral STAT3 after 6 hours, and this maximal reduction was sustained for up to 24 hours. Treatment with a maximum dose of 100 mg/kg up to three times per week for up to 4 weeks led to complete and long-term tumor regression in MOLM-16, SU-DHL-1, and SUP-M2 xenograft models. Tumor regression after the last dosing persisted for at least 2 weeks for MOLM-16 and >100 days for SU-DHL-1 and SUP-M2 xenograft models. Evaluation of spleen, liver, heart, and kidney in immunocompetent mice showed no substantial toxicity from treatment with 100 mg/kg SD-36, and red and white blood cell counts remained relatively constant. Thus, SD-36 was well tolerated in immunocompetent mice.

Collectively, these results have identified PROTAC approaches, including treatment with SD-36, as advantageous and promising therapeutic strategies for eliminating the cellular activity of STAT3, a transcription factor that has historically evaded efficacious molecular targeting. Importantly, SD-36 appears to be capable of degrading STAT3 that is activated via non-canonical pathways, where phosphorylation of Y^{705} is not required. As emerging evidence indicates that these alternate, non-canonical pathways contribute alongside canonical STAT3 signaling to promote tumor progression, PROTACs offer a unique advantage by robustly inhibiting the total pool of cellular STAT3 protein. In view of the important role that STAT3 plays in the development of both solid and hematologic malignancies it is likely that considerable effort will be invested in generating and optimizing new STAT3-targeting PROTAC molecules in the near future. It is also worth considering, however, given the complex position STAT3 holds within the immune system, if deleterious effects may arise from complete elimination of STAT3. An immunodeficiency

disorder leading to increased plasma IgE levels and recurring staphylococcal abscesses and pneumonia, hyperimmunoglobulin E syndrome (HIES), results from expression of dominant-negative mutations in STAT3, emphasizing the need for meticulous evaluations of toxicity.[36,37]

Current Challenges to Development of PROTAC Drugs

Current challenges in the development of PROTAC drugs include minimization of nonspecific binding and off-target effects, optimization of warhead-linker-E3 ligand combinations, and efficient delivery to tumor cells. The elaborate process of employing the cellular ubiquitin-proteasome system for selective degradation necessitates the need for precise and specific drug design conducive to the formation of a productive ternary complex; structural design of PROTAC molecules will thus require intensive studies to optimize drug function and pharmacological properties for their specific target proteins.[38]

The linker is crucial to the biological function of the PROTAC. In addition to properly situating the POI and E3 ubiquitin ligase for the formation of a ternary complex, it also affects physicochemical properties including propensity for cellular uptake, stability, and aqueous solubility.[38] However, optimization of the linker and linkage site is often a difficult and demanding process.[39] Ongoing efforts to identify small-molecule ligands for E3 ligases that will enable recruitment of the more than 600 E3 ligases present in the human genome represents another area that requires extensive development and optimization. The differential expression of E3 ubiquitin ligases in tissues and variation in subcellular localization and regulation may present the potential for tissue-specific as well as spatially and temporally confined protein degradation.[40,41] Moreover, the use of different E3 ligases will modify the range of proteins that can be targeted for degradation.[42] These considerations highlight the complex nature of PROTAC design, which due to its modularity, presents both challenges and potential opportunities for the development of efficacious and highly specific PROTACs.

An inherent consequence of the design of current PROTAC molecules is their relatively large molecular size, which adversely impacts a number of parameters, including metabolism, stability, and uptake.[38] Optimization of linker design and length, as well as optimization of other components contributing to the large size, should help to improve the physicochemical properties of PROTACs and efforts to optimize pharmacokinetic parameters are intensively ongoing.[40] Interestingly, due to their catalytic mode of action and ability to initiate several cycles of degradation, PROTACs show sub-stoichiometric activity.[41] For example, *in vivo* studies of PROTACs targeting RIPK2 demonstrated lasting pharmacodynamic efficacy at low doses even after levels of the PROTAC were no longer measurable.[43] Evidence of the lower and infrequent dosages required by PROTACs may prove to be advantageous despite somewhat unfavorable physicochemical properties, although the therapeutic benefit of PROTACs needs to be determined through comprehensive clinical investigations.

Antisense Oligonucleotides

Antisense oligonucleotides (ASOs) are synthetic oligonucleotides that selectively target the expression of mRNAs. ASOs typically consist of short, single-stranded DNA or RNA sequences approximately 20 base pairs in length that bind to single-stranded RNA sequences in a complementary fashion.[44] Contingent on their specific design, ASOs are capable of altering protein expression by reducing mRNA levels or by modulating alternative splicing patterns.[45] ASOs can operate through several mechanisms, including targeting mRNA for degradation by the cellular endonuclease ribonuclease H (RNase H) or by binding and blocking pre-mRNA splice sites to alter splicing and generation of the final mRNA product. Hybridization of ASOs to AUG translation start sites can also prevent the recruitment of ribosome translational machinery and, hence, subsequent protein expression.[44,45]

A multitude of chemical modifications aimed at enhancing intracellular delivery and stability have advanced the development of ASOs as therapeutic agents. Prominent limitations to ASOs include rapid degradation by cellular nucleases and potential toxicities due to their immunostimulatory properties. The poor ability of negatively charged oligonucleotides to cross cell membranes also poses a problem to clinical application. One modification that increases the resistance of ASOs to degradation is the replacement of a non-bridging oxygen atom of the phosphodiester linkage by a sulfur atom to produce a phosphorothioate backbone (Figure 3). This modification reduces the sensitivity of ASOs to nucleolytic degradation, stabilizes the longevity of ASOs in tissues, and improves binding to plasma proteins.[45,46] Non-specific binding to proteins can, however, induce toxicity, while immune stimulation, likely due to CpG motifs in phosphorothioate oligonucleotides, has also been reported.[47,48] ASOs containing modifications to the phosphate backbone that are aimed at increasing resistance to nucleases are generally referred to as first-generation ASO therapeutics. The toxicity of first-generation ASOs containing phosphorothioate backbones led to the development of second-generation ASOs containing modifications to the 2' position of the ribose sugar. Common additions produce 2'-O-methyl (2'-OME) and 2'-O-methoxyethel (2'-O-MOE) nucleotides (Figure 3) that have been shown to increase nuclease resistance and binding affinity to target RNAs while lowering toxicity by reducing non-specific binding.[46,49] As modifications to the 2' position of the ribose sugar reduce the activity of RNase H, these modifications may be particularly valuable in instances where altering of mRNA splicing, as opposed to degradation of the target mRNA, is the goal. In cases where degradation is the primary goal, the strategic use of a gapmer design comprising a stretch of 2' modified oligoribonucleotides flanking either side of an internal DNA segment has been shown to restore RNase H-mediated degradation.[49] It is critical that the internal DNA segment, modified or not, is capable of being recognized by RNase H when hybridized.

Finally, various other modifications have been implemented to further enhance the stability and binding affinity of first- and second-generation ASOs. These include the substitution of a morpholine ring in place of the deoxyribose in the sugar-phosphate backbone to form neutrally charged phosphorodiamidate morpholino oligonucleotides (PMO) composed of phosphorodiamidate linkages (Figure 3).[50] ASOs containing this modification do not facilitate RNase H recruitment and are thereby primarily used to inhibit translation of the

target mRNA or alter splicing events through steric blocking at a splice junction sequence.[46] A conformational constraint introduced by bridging the 2' oxygen and 4' carbon of the ribose moiety in RNA, referred to as locked nucleic acid (LNA), considerably increases binding affinity while also augmenting nuclease resistance (Figure 3).[51] A constrained ethyl (cEt) nucleotide formed from the addition of a methyl group to LNA residues has also demonstrated improved binding affinity and resistance to nucleases (Figure 3). Despite auspicious improvement in hybridization, LNA- and cEt-based ASOs have been observed to induce liver toxicity due to partial complementary binding to off-target transcripts, emphasizing the importance of ASO selection and design.[50,52,53] In many cases, chimeric approaches utilizing a combination of modified residues may be employed to refine the activity of ASOs for their intended use, as well as help to compensate for detrimental effects caused by the use of a particular modification.

ASOs target mRNA for degradation through binding to target RNA and forming a RNA-DNA duplex. These hybrid duplexes are recognized by RNase H, which subsequently cleaves and degrades the RNA strand.[50] Around 6–7 nucleotides of an RNA-DNA complex are generally required to hybridize for recognition by RNase H, and successful targeting by the ASO causes downregulation of the target gene protein product.[44] This approach has been employed in diseases associated with hyperactivated or mutated proteins. One such example is Inotersen, a second-generation RNase H-recruiting ASO that decreases the production of transthyretin, a protein that can accumulate in deposits in peripheral nerves, kidney, and gastrointestinal tract, causing transthyretin amyloidosis. Inotersen has advanced to Phase III clinical trials, where it has been shown to reduce TTR levels and improve quality of life.[54]

ASOs that modulate splicing to alter the RNA product, and subsequently the protein product, have been applied to diseases that manifest from aberrant splicing events. Strategic design of ASOs targeting splice sites can produce modifications of mRNA that include exon skipping or regulation of the population of mRNA variants. Eteplirsen, developed as a therapy for Duchenne muscular dystrophy, is a PMO-modified ASO that facilitates skipping of exon 51 in dystrophin pre-mRNA to form a splice variant that encodes a protein which retains partial activity. Following a Phase II clinical trial and follow-up study, Eteplirsen was provisionally approved by the Food and Drug Administration (FDA). Another FDA-approved ASO that alters splicing is Nusinersen, developed as a therapeutic for spinal muscular atrophy. Nusinersen induces inclusion of exon 7 during splicing of the survival motor neuron 2 pre-mRNA to restore a functional protein.[55]

ASOs targeting STAT3

Early antisense approaches against STAT3 utilized unmodified oligonucleotide sequences designed and selected for low overall free energy between the ASO and STAT3 mRNA.[56] The more effective of these unmodified STAT3 ASOs, AS10, when transfected into A549 non-small cell lung cancer (NSCLC) cells downregulated expression of total STAT3, phospho(Y^{705})-STAT3 (p-STAT3), and the STAT3 target gene encoding BCL-XL. Furthermore, cell proliferation was inhibited in a dose-dependent manner and induction of apoptosis was observed at a rate significantly higher than the control group.[56] STAT3 AS-ON, another early STAT3 ASO, was shown to inhibit expression of STAT3 protein and cell

proliferation in HEp-2 human laryngeal carcinoma cells.[57] In an *in vivo* study evaluating the role of STAT3 in trinitrobenzene sulfonic acid-induced colitis, intracolonically administered STAT3 ASO lowered the levels of inflammatory cytokines produced and increased apoptosis of lamina propria mononuclear cells.[58] Limitations of these unmodified STAT3 ASOs, however, included modest potencies and nonspecific toxicities.[56]

Extensive studies on modifications to ASOs resulting in improved potency led to the development of AZD9150, a chemically modified ASO designed to target STAT3 mRNA.[59] Based on second-generation 2'-O-methoxyethel (2'-O-MOE)-based ASOs, AZD9150 is a generation 2.5 (Gen 2.5) ASO containing 2' and 4' constrained ethyl (cEt) residues. AZD9150 employs a gapmer approach where a central segment of 8–10 deoxynucleotides containing phosphorothioate backbones is flanked by a few cEt residues on either side. The mechanism of action involves recognition by RNase H and cleavage of the target RNA within the resulting RNA-DNA hybrid, effecting a downregulation of STAT3 expression.[59]

AZD9150 demonstrated 10 to 20 times greater potency than its second generation ASO counterpart in human epidermoid cancer A431 cells in the absence of transfection agent, achieving IC_{50} values for knockdown of STAT3 mRNA in the nanomolar range and selective and near-complete reduction of STAT3 protein over STAT1 and STAT5.[59] In human ALCL and AML cell lines exhibiting hyperactivation of STAT3, AZD9150 selectively depleted STAT3, inhibited proliferation, and induced apoptosis. Notably, downstream STAT3 target genes such as MCL-1, c-MYC, BCL-6, and cyclin D1 were downregulated in lymphoma cells while MCL-1 and other oncogenes were downregulated in leukemia cells.[59,60] Leukemia and myelodysplastic stem cells treated with AZD9150 also demonstrated uptake of the ASO and increased erythoid and myleloid differentiation.[60] Inhibition of STAT3 expression in neuroblastoma cells impaired growth in colony formation assays and decreased cell migration.[61]

In *in vivo* mouse studies, AZD9150 was successful at reducing STAT3 expression and inhibiting the growth of lung and lymphoma xenograft tumors, although no change was observed in established neuroblastoma xenograft tumors.[59,61] However, treatment with AZD9150 did decrease STAT3 expression in mice harboring neuroblastoma xenograft tumors and strongly inhibited the formation of secondary tumors.[61] Furthermore, while AZD9150 alone did not impair growth of established neuroblastoma tumors, the addition of AZD9150 to cisplatin treatment significantly sensitized the tumors to cisplatin and significantly lengthened survival time in comparison to mice treated with either agent alone. This suggests a potential clinical benefit of AZD9150 in patients who have developed cisplatin resistance, as commonly occurs in lung, ovarian, colorectal, and head and neck cancers.[61,62] Similarly, in studies investigating radioresistance, a considerable clinical problem, mice bearing HNSCC tumors showed increased survival and impaired tumor growth in response to the combination of radiation and AZD9150.[63]

Preclinical studies assessing the safety profiling of AZD9150 in mice and cynomolgus monkeys showed toxicity levels comparable to previous second-generation ASOs, likely due to the increased protein binding generated by phosphorothioate-modified residues.[64] However, no new harmful effects were observed, and AZD9150 entered Phase Ib clinical

testing in patients with relapsed or treatment refractory lymphoma (NCT01563302).[64,65] Of the 30 patients enrolled with non-Hodgkin's lymphoma, all had undergone previous systemic treatments and the majority had diffuse large B-cell lymphoma (DLBCL), for which activated STAT3 expression has been observed to accompany poor overall survival.[66] The initial cycle began with a loading dose of thrice weekly treatments of 2 mg/kg administered via intravenous infusion, with subsequent weekly doses escalating to 3 mg/kg if disease progression occurred. Two complete responses, two partial responses, and one stable disease response were observed among the patients with DLBCL, amounting to a 13% clinical benefit. AZD9150 was found to be well-tolerated with no adverse events sufficiently serious to be cause for premature discontinuation. Common grade 1 and 2 adverse events consisted of transaminitis, fatigue, nausea, anemia, and thrombocytopenia, while some grade 3 and 4 events involving thrombocytopenia also occurred.[65] It is probable that the reduction of platelets causing thrombocytopenia is an on-target effect resulting from inhibition of STAT3, requiring the recommended Phase II dose to remain at 3 mg/kg.[59,65]

Studies evaluating pharmacokinetic properties showed AZD9150 to be comparable to previous second-generation ASOs.[59] In contrast to dosing based on ideal body weight, more recently, population pharmacokinetic analysis of AZD9150 proposed pharmacokinetic exposure to be minimally affected by a change to flat dosing at 200 mg weekly.[67] This analysis was congruent with preliminary results from an ongoing Phase Ib/II clinical trial assessing AZD9150 in patients with advanced solid tumors receiving a flat dose of 200 mg (D5660C00016, Study 16).[67]

Overall, in the clinic, AZD9150 appears to be well-tolerated and provides a therapeutic benefit for a subset of patients with treatment refractory DLBCL. Further investigations to understand the basis for the differential responses among patients is merited. Moreover, concerns remain over dose-limiting toxicities of AZD9150, such as the thrombocytopenia observed in treated subjects.[65] Compelling evidence for AZD9150 as an advantageous therapy lies in its superior potency over previous ASO iterations in both preclinical and clinical settings and demonstrable efficacy in inhibiting STAT3 in multiple preclinical models. *In vivo* studies involving combination therapies of AZD9150 with cisplatin and radiation have also proven promising. Furthermore, AZD9150 targets STAT3 directly at the level of expression, theoretically extending its effects to non-canonical STAT3 signaling, including oncogenic signaling via uSTAT3.

Small molecule competitive inhibitors of STAT3

Efforts are also ongoing to develop competitive inhibitors of STAT3, a few of which have advanced to the stage of clinical testing, although limited efficacy remains a significant obstacle. The small molecule inhibitors OPB-31121 and OPB-111077, developed by Otsuka Pharmaceutical Company, bind with high affinity to the STAT3 SH2 domain to directly inhibit STAT3 activity and have been tested in Phase I trials in patients with solid tumors. [68,69] OPB-31121 was found to have dose-limiting toxicities of vomiting and diarrhea in addition to observation of disease progression (NCT00955812).[70] OPB-111077 was found to be better tolerated; however, only modest anti-tumor activity was observed (NCT01711034).[68] C188–9, another small molecule inhibitor, binds to the STAT3 SH2

domain with a Kd of ~5 nM and is undergoing Phase I testing in patients with advanced cancers (NCT03195699).[71,72]

Napabucasin (BBI608) is a small molecule inhibitor of STAT3 that was demonstrated to be well-tolerated in a Phase I trial in Japanese patients with advanced gastric cancer when administered in combination with paclitaxel.[73] A Phase III trial evaluating the efficacy of napabucasin plus best supportive care versus placebo plus best supportive care in advanced colorectal cancer revealed no difference in overall survival between treatment groups (NCT01830621).[74] A Phase III study of napabucasin in addition to nab-paclitaxel with gemcitabine is currently ongoing in patients with pancreatic adenocarcinoma (NCT02993731).[75]

A STAT3 decoy oligonucleotide consisting of a double-stranded 15-base pair sequence corresponding to the STAT3 genomic response element, competitively inhibits binding of activated STAT3 to target gene promoters, abrogating expression of these genes, and demonstrated pharmacodynamic activity following intratumoral delivery in a Phase 0 trial. [76,77] A chemically stabilized version of the decoy (cyclic STAT3 decoy) exhibited anti-tumor effects when delivered systemically to tumor-bearing mice and was well tolerated. [77,78] Future studies with the cyclic STAT3 decoy will address pharmacology and toxicology, prior to evaluation in human clinical trials.

Indirect targeting of STAT3

The challenge of directly targeting STAT3 has resulted in substantial attention being paid to indirect targeting of STAT3 via inhibition of the upstream activators of STATs, Janus kinases (JAKs). Several JAK inhibitors have been developed and tested in clinical trials in patients with solid tumors, with two inhibitors currently approved by the FDA for other indications. [79,80] Ruxolitinib, a JAK1/2-selective inhibitor, is one such FDA approved agent, and clinical studies of ruxolitinib in solid tumors have been reported.[80] Although a Phase II study of ruxolitinib in combination with capecitabine in patients with metastatic pancreatic cancer showed greater overall survival in a subset of patients with elevated C-reactive protein (CRP) levels (NCT01423604), the overwhelming majority of clinical trials with ruxolitinib have been terminated due to lack of efficacy.[81] Phase III trials studying ruxolitinib in patients with pancreatic cancer and in patients with elevated CRP levels were terminated due to a lack of difference observed in overall or progression-free survival between treatment groups (NCT02117479) (NCT02119663).[82] In a Phase II study of ruxolitinib in triple-negative breast cancer, no objective responses despite demonstration of inhibitory activity against STAT3 led to termination of the trial (NCT01562873). A Phase I/II trial of ruxolitinib and the EGFR inhibitor erlotinib demonstrated that this combination was well-tolerated but lacked clinical activity in patients with lung adenocarcinoma (NCT02155465). [83,84]

Other JAK inhibitors have also suffered from inadequate anti-tumor activity when advanced to clinical trials in solid tumors. INCB052793, which selectively inhibits JAK1, entered a Phase I/II trial in patients with advanced malignancies that was prematurely terminated as the drug was shown to be ineffective (NCT02265510).[85] Similarly, despite promising

preclinical results, a Phase I clinical trial of AZD1480, an ATP-competitive inhibitor selective for JAK1/2, was halted due to lack of efficacy in addition to dose-limiting neurotoxicities.[86] This dose-limiting toxicity exemplifies another limitation exhibited by JAK inhibitors; a Phase Ib study of the JAK1/2 inhibitor, momelotinib, when administered in combination with erlotinib in patients with EGFR-mutated metastatic NSCLC, was terminated due to dose-limiting toxicities of diarrhea and neutropenia (NCT02206763)[87].

Thus, despite extensive efforts, efficacious indirect targeting of STAT3 in clinical studies using JAK inhibitors has proven challenging, with disappointing results stemming from lack of clinical activity and observation of adverse toxicities. Hence, direct targeting with agents such as SD-36 and AZD9150, or small molecule competitive inhibitors, may be advantageous in their capacity to selectively and more completely eliminate expression or function of STAT3 protein, increasing the specificity of effects and decreasing toxicity due to off-target activity. The PROTAC approach of SD-36, in particular, may be favorable due to its catalytic mode of action that may, in theory, lower the amount of drug required and potentially reduce risk of toxicities.

Targeting STAT3 in the tumor microenvironment

Although tightly regulated in normal cells, STAT3 signaling in cancer cells is often hyperactivated due to aberrantly active receptor tyrosine kinases (eg. EGFR, c-MET) or non-receptor tyrosine kinases (c-Src and BCR-ABL), or inactivation or downregulation of critical negative regulators such as protein inhibitor of activated STAT (PIAS), suppressor of cytokine signaling (SOCs) proteins, protein tyrosine phosphatases (PTPases), and members of the protein tyrosine phosphatase receptor (PTPR) family.[8,88] Hyperactivation of STAT3 in tumor cells leads to production and secretion of immunosuppressive cytokines and growth factors, including VEGF, IL-10, and IL-6.[8,88] These factors can act in a paracrine fashion to further propagate STAT3 activation in the tumor microenvironment. Activation of STAT3 in tumor infiltrating immune cells acts to suppress anti-tumor immunity by inhibiting the differentiation of dendritic cells (DCs), downregulating T helper (Th1 and Th2) cell activity, and contributing to the polarization of macrophages to an M2 immunosuppressive phenotype.[89,90] Furthermore, T regulatory cells (Tregs) are positively modulated by STAT3 and have been observed to accumulate in tumors. The levels of myeloid-derived suppressor cells (MDSCs), associated with a number of immunosuppressive properties including impairment of $CD4^+$ and $CD8^+$ T cell activation, are also enhanced by STAT3. Accordingly, STAT3 mediates a number of immune evading and suppressing events that contribute to cancer progression.[89–91]

It is thus important to bear in mind the immunosuppressive role of STAT3 activation within the tumor microenvironment in order to better understand the anti-cancer activity of STAT3 inhibitors. The reduction of immunosuppressive Tregs levels following treatment with STAT3 inhibitor has been shown to augment the efficacy of radiation therapy in head and neck cancer.[63] Given the role of STAT3 in the tumor microenvironment, it is possible that downregulation of STAT3-induced pro-inflammatory cytokines such as IL-6 and immunosuppressive MDSCs may generate immunostimulatory responses that enhance clinical benefit. Thus, identifying relationships between STAT3 inhibition and responses

within the tumor microenvironment may prove valuable to designing targeted therapeutic strategies with optimal efficacy.[92] A Phase Ib/II clinical trial evaluating the efficacy of AZD9150 in combination with durvalumab, an FDA-approved antibody that inhibits binding of PD-L1 to PD-1, and AZD9150 in combination with durvalumab plus chemotherapy is currently ongoing (NCT03421353).[93]

Conclusions

The development of PROTAC drugs and ASOs targeting STAT3 presents promising therapeutic strategies for inhibiting STAT3 expression, a transcription factor that has long been a challenging and highly pursued target. Notably, the mechanisms of action of these two therapies result in elimination of STAT3 at the level of expression, offering a more holistic approach to targeting STAT3, particularly in light of emerging evidence implicating non-canonical STAT3 signaling in the progression of cancer. PROTAC and ASO therapeutics may thus be advantageous over small molecule inhibitors operating through inhibition of upstream pathways or competitive inhibition of STAT3 protein, which are often limited by poor potency and the development of resistance.

SD-36, a novel PROTAC targeting STAT3 protein, has demonstrated robust and selective depletion of STAT3 protein in leukemia and lymphoma cell lines as well as complete and persisting tumor regression in mouse xenograft models. Encouragingly, SD-36 was well-tolerated in immunocompetent mice, although rigorous studies to further assess its safety profile will be important before clinical application is possible. Furthermore, although these initial studies show promising evidence of tumor regression in hematopoietic malignancies, it will be imperative to examine the activity of SD-36 in solid tumors and also investigate the potential deleterious effects that may arise from potently eliminating STAT3 expression in normal tissues. The ASO AZD9150 is generally well-tolerated and has shown potency at inhibiting STAT3 expression in a number of preclinical models as well as clinical benefit in a subset of patients with treatment refractory lymphoma. Phase II/III trials further evaluating the efficacy of AZD9150 in a range of tumor types are ongoing or pending. The potential for combining novel PROTAC and ASO agents targeting STAT3 with conventional chemotherapy and radiation represents a promising avenue for tumor reduction or eradication in the clinic.

Acknowledgements

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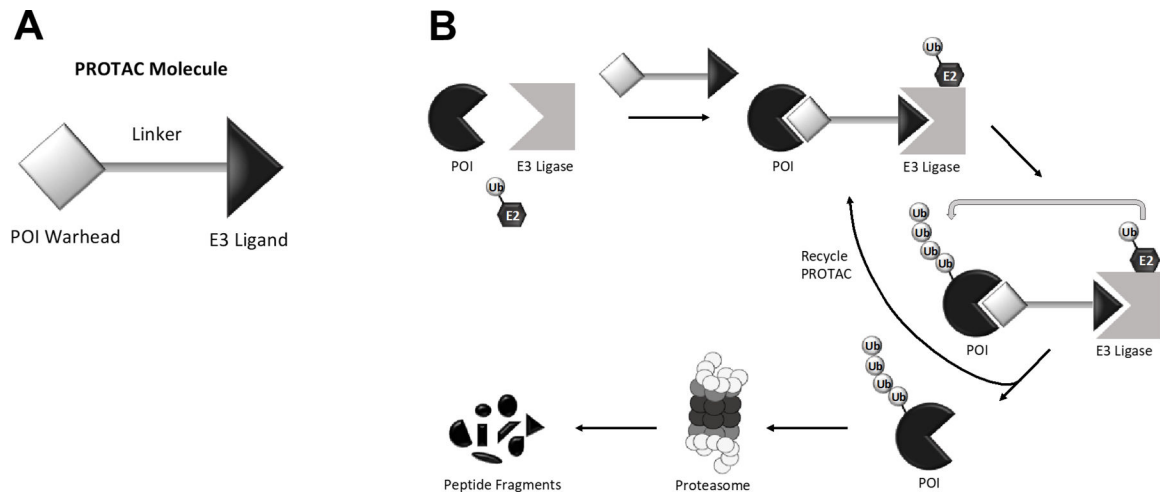
Figure 1. The structure and mechanism of PROTACs.
A) The general structure of a PROTAC molecule consists of a "warhead" ligand that recruits the protein of interest (POI), a linker, and an E3 ligand that recruits an E3 ubiquitin ligase. **B**) The "warhead" ligand and the E3 ligand bring into close proximity the protein of interest (POI) and an E3 ubiquitin ligase, respectively. The E3 ubiquitin ligase, in concert with an E2 ubiquitin-conjugating enzyme, promotes polyubiquitination of the POI. Polyubiquitination of the POI results in degradation by the 26S proteasome. This process is catalytic, meaning that the freed PROTAC molecule can participate in another reaction. Figure adapted from Pei *et al.* and Sun *et al.*[21,22]
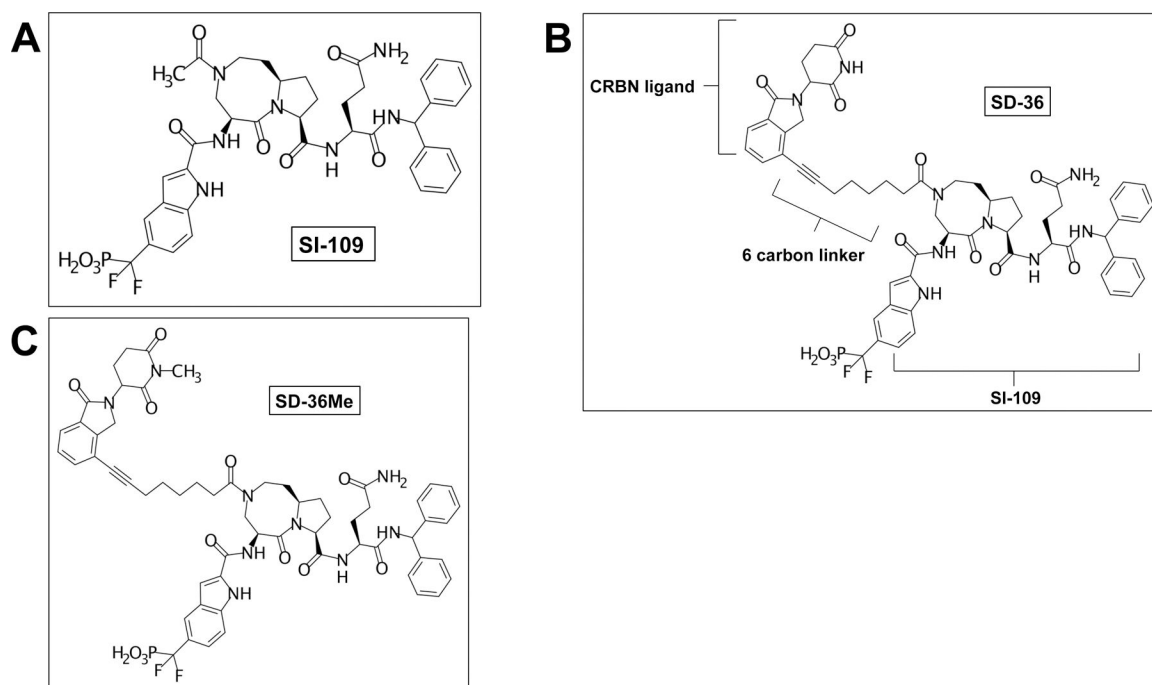
Figure 2. Chemical structures of STAT3 inhibitor, PROTAC, and PROTAC control.
A) Chemical structure of the STAT3 SH2 domain inhibitor SI-109. **B)** Chemical structure of SD-36. **C)** Chemical structure of SD-36Me, which contains an additional methyl group on the lenalidomide analog moiety. SD-36Me is deficient in recruiting the E3 ligase and acts as a negative control.
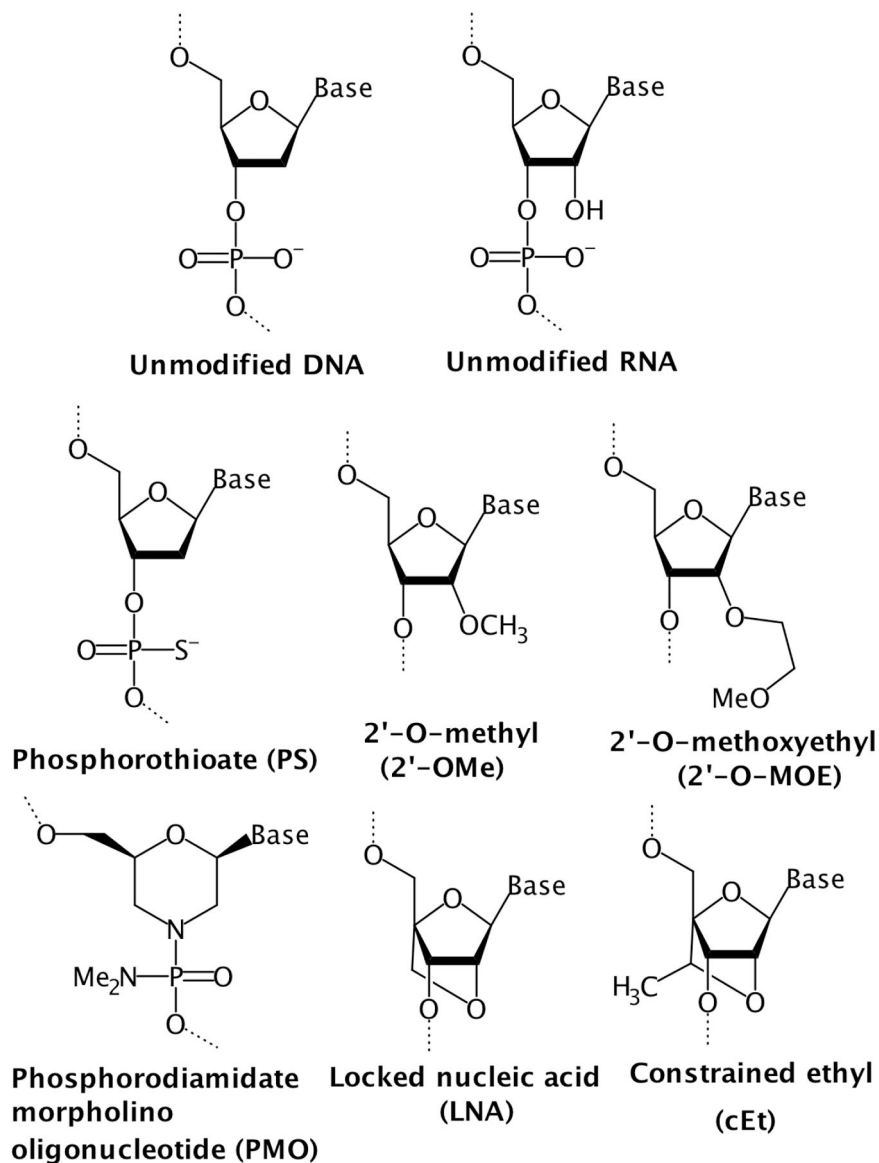
Figure 3. Common structural modifications of ASOs.
First-generation ASOs incorporating modifications of the phosphate backbone include phosphorothioate (PS) oligonucleotides, while second-generation ASOs harboring 2' ribose modifications include 2'-O-methyl (2'-OMe) and 2'-O-methoxyethyl (2'-O-MOE) residues. Additional modifications incorporated in constrained ASOs include locked nucleic acid (LNA) and constrained ethyl (cEt) modifications.