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Small-molecule modulators of tumor necrosis factor signaling

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Tumor necrosis factor (TNF) is a pleiotropic cytokine with a major role in immune system homeostasis and is involved in many inflammatory and autoimmune diseases, such as rheumatoid arthritis (RA), psoriasis, Alzheimer's disease (AD), and multiple sclerosis (MS). Thus, TNF and its receptors, TNFR1 and TNFR2, are relevant pharmacological targets. Biologics have been developed to block TNF-dependent signaling cascades, but they display serious side effects, and their pharmacological effectiveness decreases over time because of their immunogenicity. In this review, we present recent discoveries in small molecules targeting TNF and its receptors and discuss alternative strategies for modulating TNF signaling.

Keywords: tumor necrosis factor; tumor necrosis factor receptors; inflammatory diseases; small molecules

Introduction

TNF and TNFR activation

TNF is an essential cytokine in the regulation of immune function, inflammation, response to infections, synaptic transmission and plasticity, and numerous other processes.[1] It is generated as a transmembrane protein (mTNF) that can be cleaved by a disintegrin and metalloprotease domain 17 (ADAM17), also known as TNF alpha-converting enzyme (TACE), to shed the extracellular soluble portion of TNF (sTNF).[2] Both forms of TNF have biological activity and signal as homotrimers via the cognate receptors TNFR1 (also named TNFRSF1A, CD120a, or p55) and TNFR2 (also named TNFRSF1B, CD120b, or p75).[3,4] TNF trimers engage receptor monomers to induce the formation of receptor homotrimers, where each receptor monomer binds at the interface between the three TNF protomers. TNFR1 is widely expressed and is activated by both sTNF and mTNF.[5] By contrast, TNFR2 is mainly restricted to immune cells, endothelial cells, and glia and, although it can bind to both sTNF and mTNF, it is only activated by mTNF.[6,7]

TNFR1 signaling

Activation of TNFR1 can initiate signal transduction via two major intracellular complexes (Figure 1). Complex I is responsible for activation of the proliferative and cytokine-producing canonical nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways and complex II is responsible for programmed cell death.[8] TNF binding initiates a conformational change in an intracellular death domain (DD) of TNFR1, which leads to the recruitment of a TNFR1-associated death domain (TRADD), TNFR-associated factor 2 or

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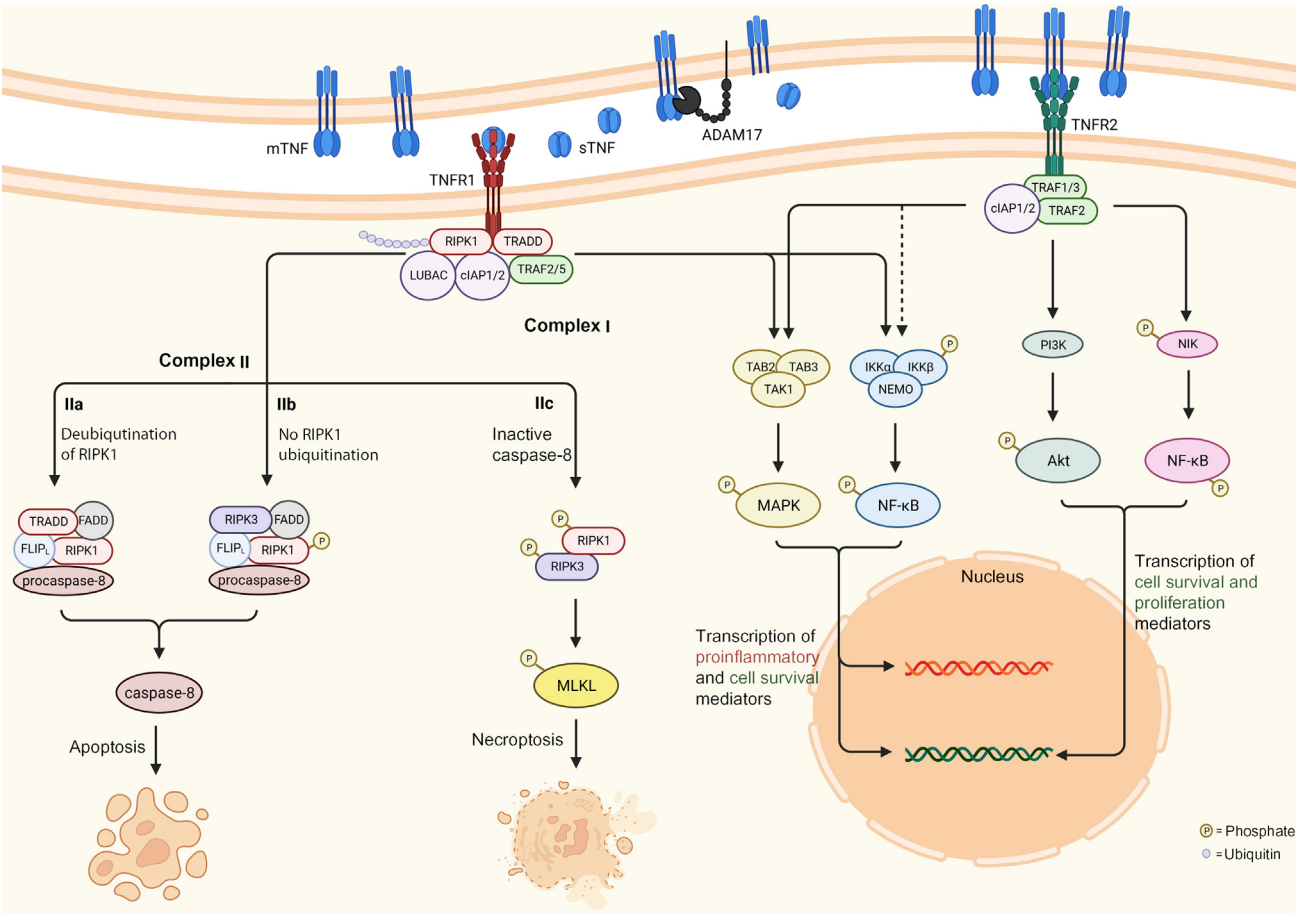


FIGURE 1

Tumor necrosis factor receptor (TNFR) activation and signaling. Activation of TNFR1 leads to the formation of complex I, which is responsible for activating the nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways, resulting in the transcription of proinflammatory and cell survival mediators. Complex IIa and IIb formation depends on the ubiquitination state of receptor-interacting serine/threonine-protein kinase 1 (RIPK1). These two complexes activate caspase 8, resulting in apoptosis. If caspase 8 is inactive, complex IIc can be formed, activating mixed-lineage kinase domain-like (MLKL) protein, resulting in necroptosis. Activation of TNFR2 leads to the binding of TNFR-associated factors (TRAFs), which can activate an NF-κB pathway different from that activated by TNFR1, resulting in transcription of cell survival and proliferation mediation. Activation of TNFR2 has also been associated with downstream signaling, similar to complex I of TNFR1. Abbreviations: ADAM17, a disintegrin and metalloprotease 17; Akt, protein kinase B; cIAP1/2, cellular inhibitor of apoptosis protein 1 and 2; FADD, FAS-associated death domain protein; $FLIP_L$, FLICE-like inhibitory protein; IKKα/β, IκB kinase α and β; LUBAC, linear ubiquitin assembly complex; mTNF, membrane-bound tumor necrosis factor; NEMO, NF-kappa-B essential modulator; NF-κB essential modulator; NIK, NF-κB-inducing kinase; PI3K, phosphoinositide 3-kinase; sTNF, soluble tumor necrosis factor; TAB2/3, MAP3K7-binding protein 2 and 3; TAK1, TGFβ-activated kinase 1; TRADD, TNFR1-associated death domain. Created with BioRender (BioRender.com).

5 (TRAF 2/5), and receptor-interacting serine/threonine-protein kinase 1 (RIPK1), which form complex I (Figure 1).[1] The complex is characterized by ubiquitination of RIPK1 by cellular inhibitor of apoptosis protein 1 and 2 (cIAP1/2) and linear ubiquitin assembly complex (LUBAC).[9,10] Activation of complex I recruits the IκB kinase (IKK) complex and the TGFβ-activated kinase 1 (TAK1) complex, leading to activation of the NF-κB and MAPK pathways, respectively, both initiating transcription of proinflammatory and cell survival-encoding proteins.[11,12] The actual mechanism behind the complex I activation is still unclear because ubiquitination of RIPK1 has been suggested to not be essential.[13]

Complex II is formed in the cytosol when RIPK1 ubiquitination is absent or removed. Three different subtypes of complex II have been suggested; in both complex IIa and IIb, RIPK1 dissociates from the membrane with other scaffold proteins and activates caspase 8, resulting in caspase cascade activation and apoptosis.[14] Complex IIc is formed when caspase 8 is nonaccessible or insufficiently activated. Then, phosphorylated RIPK1 forms a necrosome with RIPK3, which further activates mixed-lineage kinase domain-like (MLKL) protein, leading to necroptosis (Figure 1).[15] *In vivo* studies suggest that complex I and complex IIc are responsible for chronic inflammation conditions in autoimmune diseases and that caspase 8 activity can dampen the inflammatory response.[16,17]

TNFR2 signaling

The outcome of TNFR2 activation is primarily cell proliferation and cell survival (Figure 1).[1] TNFR2 lacks a DD and thus does not interact with TRADD. Instead, TRAF proteins form a complex

with the intracellular part of TNFR2 and recruit cIAP1/2.[18] Multiple signaling pathways are engaged downstream of TNFR2 activation, including the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway,[19,20] the p38 MAPK pathway,[21] and the non-canonical NF-κB pathway via activation of NF-κB-inducing kinase (NIK), which initiates transcription of genes that differ from those transcribed by TNFR1-dependent NF-κB activation.[22,23] In addition, ubiquitination of the TNFR2 complex has been associated with canonical NF-κB activation, suggesting a proinflammatory response similar to the output of complex I formation from TNFR1 activation (Figure 1).[24]

TNF and TNFR imbalance in disease pathogenesis

TNF is excessively produced in autoimmune diseases, such as RA, psoriasis, and Crohn's disease (CD) resulting in joint erosion, fibrosis, and structure formation.[25] Neuroinflammatory diseases, such as MS and Alzheimer's disease (AD), are also characterized by elevated concentrations of TNF, among other proinflammatory cytokines.[26,27] Imbalance in receptor expression can also contribute to disease pathogenesis. TNFR2 can be considered an oncogene and is found in immunosuppressive cells and specific tumor cells.[28] It appears that the receptor has a vital role in the tumor microenvironment.[29] By contrast, there is evidence that TNFR2 has a neuroprotective role in neurological diseases, such as MS and AD.[30] Moreover, its stimulation with selective TNFR2-activating biologics, for example NewSTAR2 or EHD2-scTNFR2, promotes central nervous system (CNS) repair.[31,32].

Anti-TNF biologics as current treatment

Several biologics targeting TNF and its receptors have been generated, especially soluble receptor proteins and monoclonal antibodies (mAbs). In 1998, the US Food and Drug Administration (FDA) approved the first two anti-TNF biologics, infliximab (Remicade®) and etanercept (Enbrel®) for CD and RA treatment, respectively. Infliximab is a recombinant chimeric mAb and Etanercept is a fusion protein comprising the TNFR2 extracellular domain and the human IgG1 Fc domain.[25] These biologics bind to TNF and thereby block the activation of both TNFR1 and TNFR2. Other biologics have been marketed and there are currently five anti-TNF biologics available.[25] One of these anti-TNF biologics is the recombinant human mAb, adalimumab (Humira®), which has been one of the top-selling drugs for the past decade.[33] However, given that the core patents for the anti-TNF biologics are expiring, the number of approved biosimilars is increasing.[34]

The need for better treatment options

Anti-TNF biologics are the only marketed drugs for targeting TNF signaling, but they are not ideal drugs because they require complex manufacturing procedures and temperature-controlled supply chains, resulting in expensive production costs. One major problem, especially with mAbs, is the inconsistent response. Lack of initial response is observed for 10–30% of rheumatology patients, which has been suggested to be due to mutations in apoptosis-related proteins, such as caspase 9, or the nonselective inhibition of both TNFR1 and TNFR2 signaling.[35,36] In addition, time-dependent loss of response (LOR) is observed in many patients. A LOR of 13.1% was observed for Infliximab after 54 weeks in patients with CD.[36] Generally, LOR is due to immunogenicity, including anti-drug antibodies. Hypersensitivity reactions are also common using mAbs, spanning from local skin irritations at the injection site to potentially fatal anaphylaxis.[37] Furthermore, rare but severe thrombocytopenia has been reported as a consequence of treatment with anti-TNF biologics.[38] The pharmacokinetic properties of biologics are also problematic because the molecular size limits oral absorption, whereby the drugs must be administered by invasive routes. This requires assistance from healthcare professionals and is a problem when used to treat people with needle phobia and in pediatrics. Additionally, these drugs are not distributed to the CNS; thus, they do not provide a solution for treating neurodegenerative diseases.[39]

Targeting TNF with small molecules

The trimeric structure of TNF is key for receptor activation, because only the trimer can trigger intracellular signaling. Hence, for small molecules targeting TNF, trying to disrupt the TNF trimer has become one of the main strategies to impact TNF inflammatory signaling. The first molecule reported to disturb the structure of the TNF trimer was suramin, in 1992 (Table 1).[40] It was shown in an ELISA that suramin induces deoligomerization of the sTNF trimer into inactive monomeric subunits. The other main breakthrough in this field was the discovery by Sunesis Pharmaceuticals of SPD-304 (Table 1), which leads to disruption of the TNF trimer. They showed by X-ray crystallography that SPD-304 displaces one subunit of the TNF trimer, forming a complex with a TNF dimer [Protein Data Bank (PDB) ID: 2AZ5]. This TNF-SPD-304 complex is then unable to bind TNFR1.[41] However, SPD-304 showed metabolic instability, toxicity, and low solubility, so it was not useful for clinical applications.[42] Recently, researchers from UCB Pharma identified compound UCB-9260 (Table 1) via fragment-based surface plasmon resonance (SPR) screening and optimization guided by X-ray crystallography.[43] UCB-9260 is capable of distorting the TNF trimer in a way that leads it to bind only two TNFR1 subunits instead of three (PDB ID: 6OP0). This prevents propagation of inflammatory signaling through TNFR1 both *in vitro* and *in vivo*.[44] This is the first study to show inhibition of TNF signaling via allosteric regulation by a small molecule that stabilizes an inactive form of the TNF trimer. It led to the development of the orally available small molecule, SAR441566, in collaboration with Sanofi,[45] which has now finished a Phase I clinical trial for treatment of psoriasis (NCT05453942). Scientists from Bristol Myers Squibb aimed to develop a more potent inhibitor of TNF using UCB-9260 as a starting point.[46] They produced compound 42 (Table 1), with low nM IC_{50} in a homogeneous time-resolved fluorescence (HTRF) assay, via scaffold hopping and structure-based drug design (PDB ID: 7JRA). The TNF inhibition mechanism of compound 42 is similar to that of UCB-9260, and the molecule showed biological-like efficacy in a collagen antibody-induced arthritis (CAIA) mouse model. Having already developed the antibody adalimumab, AbbVie scientists also aimed to find a small-molecule alternative to their TNF treatment. They developed an orally efficacious allosteric inhibitor of TNF, compound 12 (Table 1), using fragment-based drug discovery via nuclear magnetic resonance (NMR) and SPR screening followed by opti-

TABLE 1

Activity and binding affinity of small-molecule modulators of TNF signaling.

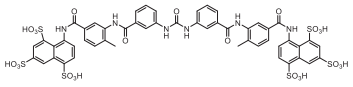
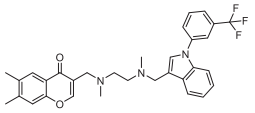
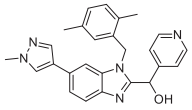
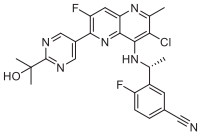
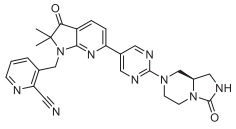
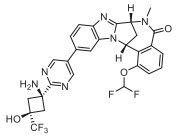
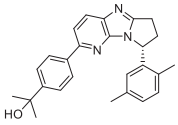
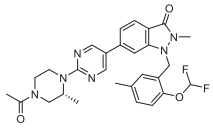
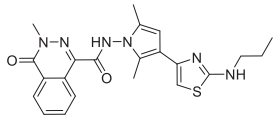
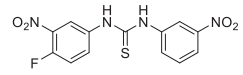
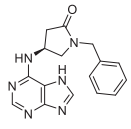
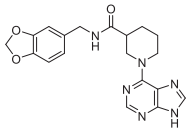
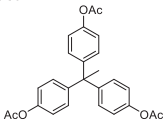
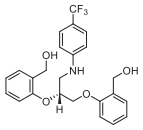
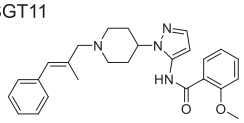
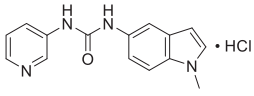
Structure	IC_{50}	Assay	K_D	Assay	Target	Application	Refs
 Suramin	0.65 mM	ELISA	n.d.	n.d.	TNF	Reduction of TNF-induced inflammation in clinic but serious adverse effects	40
 SPD-304	22 μM	ELISA	5.36 μM	FP	TNF	Limited use because of poor solubility and selectivity	41
 UCB-9260	116 nM	L929 TNF cell-based assay	13 nM	SPR	TNF	Efficacy in CAIA mouse model	43
 Compound 42	27 nM	HTRF	n.d.	n.d.	TNF	Efficacy in CAIA mouse model	46
 Compound 12	96 nM	L929 TNF cell-based assay	6.8 nM	SPR	TNF	Efficacy in mouse GPI-induced paw swelling model	47
 Example 12	<10 nM	Reporter gene assay	n.d.	n.d.	TNF	Patented by UCB/Sanofi as modulator of TNF activity	48
 Example 37	<1 μM	Reporter gene assay	n.d	n.d.	TNF	Patented by Bristol Myers Squibb as modulator of TNF activity	49
 Example 20.4	<1 μM	Fluorescence polarization competitive assay	n.d.	n.d.	TNF	Patented by Abbvie as modulator of TNF activity	50
 TIM1c	~25 μM	ELISA	95 μM	SPR	TNF	Suppresses symptoms of CIA in mice	51
 Compound 2	6.5 μM	ELISA	n.d.	n.d.	TNF	Inhibits TNF production from LPS-activated THP-1 cells	52
 Benpyrine	0.109 μM	ELISA	82.1 μM	MST	TNF	Efficacy in LPS-induced endotoxemia in mice	53

TABLE 1 (*CONTINUED*)

Structure	IC_{50}	Assay	K_D	Assay	Target	Application	Refs
 R1	>200 μM	TNF-induced L929 cell death	16 μM	SPR	TNFR1	Inhibits TNF-mediated cytotoxicity on L929 cells	[57]
 F002	27.2 μM	TNF-induced L929 cell death	0.45 μM	ITC	TNFR1	Efficacy on CIA in mice	[58]
 SGT11	5.5 μM	Luciferase reporter assay	n.d	n.d	TNFR1	Reduced neuroinflammation from traumatic brain injury in mice	[59]
 DSA114	25.9 μM	Luciferase reporter assay	45.7 μM	SPR	TNFR1	Inhibition of NF-κB activation in HEK293 cells	[63]
 SB 200646 hydrochloride	6.8 μM (EC_{50})	Luciferase reporter assay	75 μM	SPR	TNFR1	Increased NF-κB activation in HEK293 cells	[64]

mization of the hits guided by X-ray crystallography (PDB ID: 6X86).[47] The molecule leads to the formation of an asymmetric form of the sTNF trimer, preventing signaling through TNFR1. It also showed activity in a glucose-6-phosphate isomerase (GPI)-induced arthritis model in mice. Several patents have been deposited for TNF small-molecule inhibitors by UCB/Sanofi, Bristol Myers Squibb, and Abbvie, with a diverse range of structures (Table 1).[48–50] There have also been several recent studies on the disruption of the TNF trimer by small molecules. For example, compound TIM1c was developed as an inhibitor of TNF by using the X-ray structure of TNF in complex with SPD-304 (PDB ID: 2AZ5) for virtual screening.[51] This compound appears to bind the TNF monomer and displays activity in a murine collagen-induced arthritis (CIA) model. In addition, two other studies used the same X-ray structure (PDB ID: 2AZ5) for virtual screening and discovered compounds that bind to TNF.[52,53] Notably, compound 2 was discovered among 10 000 compounds from natural and synthetic origins (Table 1). Compound 2 has an IC_{50} of 6.5 μM toward TNF in ELISA.[52] Another virtual screening strategy led to the discovery of benpyrine (Table 1), which was characterized by microscale thermophoresis (MST).[53] Benpyrine prevents the formation of the TNF trimer similarly to SPD-304, and by ELISA it was shown that this prevents TNFR1 from binding to TNF. Other compounds showing activity in cellular assays against TNF have also been reported, but many lack characterization of their binding affinity to TNF.[54–56].

Targeting TNFRs with small molecules

Virtual screening has become an important tool for finding starting points for identifying novel small-molecule inhibitors for specific receptor targets, including the TNFRs. Recently, Chen *et al.* used an *in silico* (molecular docking and pharmacophore)-based screening strategy followed by biophysical (SPR) and cell-based cytotoxicity assays to identify novel antagonists that bind to TNFR1.[57] Even though the final compound R1 was not binding with high affinity (Table 1), their virtual screening method could encourage future researchers to search for potent TNFR1 antagonists this way. In another study, Murali *et al.* virtually screened a library of commercially available compounds to identify ligands that induce a conformational change of tryptophan (W107) in the WP9 loop.[58] Approximately 20 hits based on DOCK score and visual examination were subject to molecular dynamics simulation. Among these, the binding of compound F002 (Table 1) to TNFR1 was biophysically evaluated using isothermal titration calorimetry (ITC), suggesting an entropically driven hydrophobic interaction between F002 and TNFR1. An intrinsic tryptophan fluorescence assay and mutational studies supported F002 as an allosteric inhibitor. Furthermore, F002 was shown to inhibit TNF-mediated signaling *in vitro* and prevent cartilage destruction dose dependently *in vivo*.[58] Later, structure–activity relationship (SAR) studies of F002 led to the discovery of a novel TNFR1 inhibitor, SGT11 (Table 1). These allosteric modulators block the TNFR1 pathway leading to NF-κB inhibition, modulate post-traumatic sleep, and reduce traumatic brain injury-induced neuroinflammation.[59] Selective small-molecule TNFR2 agonists or antagonists are of particular interest because of their potential for targeting autoimmune diseases and restoring the T cell balance in cancer.[60] Shaikh *et al.* performed a virtual screening of 400 000 natural products by Glide docking. The top scoring ligands that bind at the extracel-

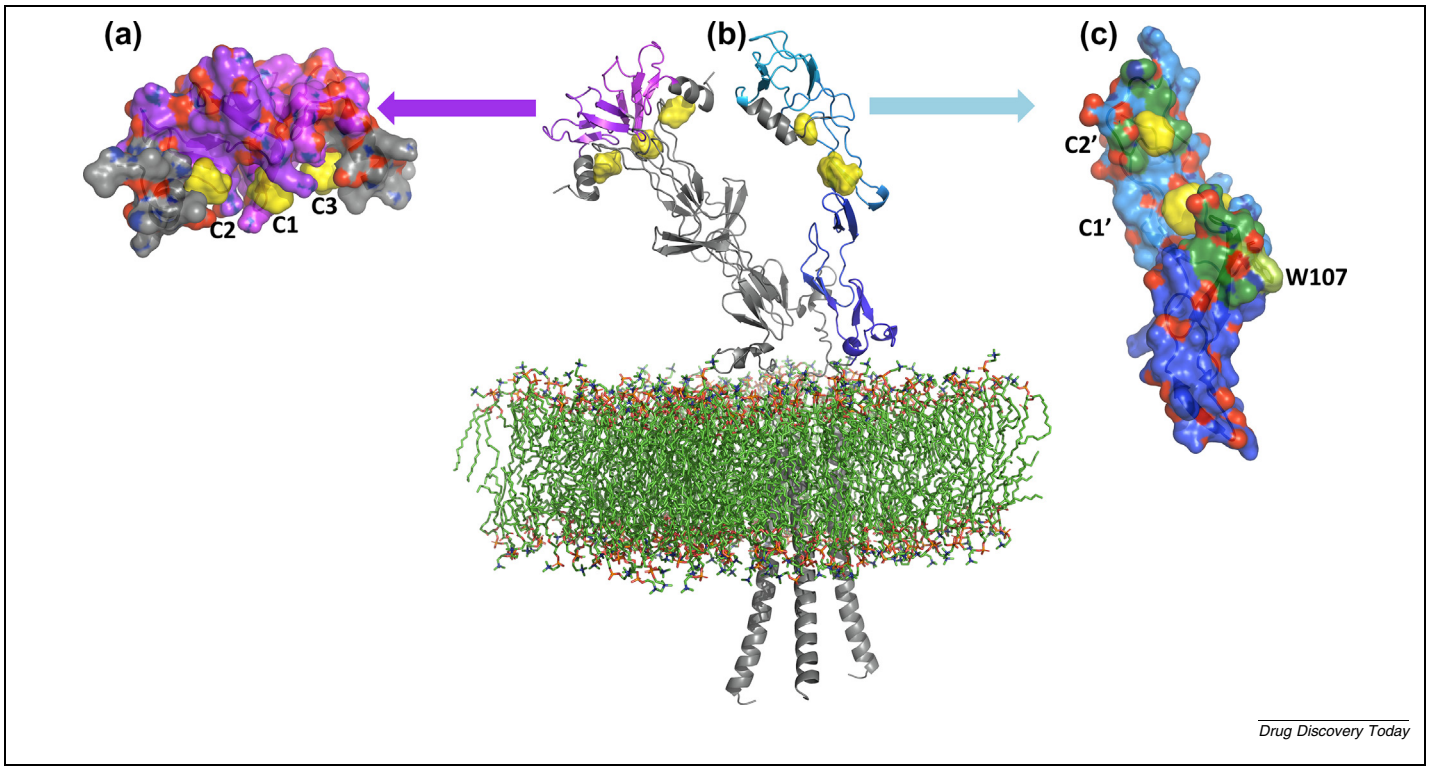


FIGURE 2

Druggability hot spot analysis mapped to the AlphaFold tumor necrosis factor receptor 1 (TNFR1) structure. **(a)** FTMap hot spots C1–3 in yellow are assigned to the TNFR1 AlphaFold dimer. The surface of the cysteine-rich domain 1 (CRD1) (magenta) is shown in a cartoon and surface representation. **(b)** The dimer is shown in gray except for CRD1, which is in magenta, the nondimerized subunit is in blue for CRD1–4 and in cartoon, membranes are green and orange sticks, and FTMap pockets are in yellow. FTMap hot spots C1′–C2′ found in TNFR1 (PDB ID: 1TNR; TNF-β is removed during FTMap analysis) are assigned to the nondimerized subunit of the AlphaFold TNFR1 structure. **(c)** The surface of the extracellular CRD1–3 (blue) is highlighted in a cartoon and surface representation, with Chen[57] (close to C2′) and Murali[58] (close to C1′) pockets in green and the tryptophan residue (W107) at the Murali pocket is shown as light-green sticks.

lular domains of TNFR2 were visually inspected and subjected to molecular dynamics simulation assessing the stability of the virtual hits in the TNFR2 cavity.[61] However, no experimental assay validation has yet been performed on the top hits.

Experimental high-throughput screening has also been applied for finding small-molecule TNFR1 ligands. Lo *et al*. used a time-resolved fluorescence energy transfer (TR-FRET)-based assay to screen three different libraries of a total of 51 726 compounds against TNFR1.[62–64] The initial hits were assessed by FRET assay and further validated by SPR and cell-based assays measuring IκBα and NF-κB activity.[63,64] An exploratory SAR analysis resulted in noncompetitive TNFR1 inhibitors with DSA114 as the lead compound able to inhibit NF-κB activation in cells (Table 1).[63] In addition, a small-molecule TNFR1 activator, SB 200646 hydrochloride (SBH), was found to bind TNFR1 and increase NF-κB activation in cells by stabilizing an active conformation of the receptor complex (Table 1).[64]

Pocket analysis of TNFR1

An important early step in drug discovery is to evaluate whether the intended drug target is druggable (i.e., does it have pockets that can accommodate small-molecule drug-like structures). For TNFRs, an additional intriguing key question is whether such binding will inhibit the interaction with TNF or affect receptor activation via other mechanisms, for example by preventing receptor assembly. Alternatively, small-molecule agonists may bind and activate the receptors. To shed light on this question, a 3D full-length structure of TNFR1 was generated using AlphaFold[65] (Figure 2). This structure represents the dynamic feature of TNFRs, where a preligand assembly domain (PLAD)–PLAD interaction initiates the unliganded TNFR1 dimerization (Figure 2b) and, thus, facilitates TNF-induced TNFR1 activation through the formation of trimer–trimer or higher-order oligomers.[63,66] Thus, in this model, the three receptor subunits exist as a monomer/dimer equilibrium (Figure 2b), and, upon TNF binding, the active trimer–trimer complex is formed. The pockets in the AlphaFold TNFR1 structure can be visualized by FTMap (Figure 2a), an open-source program that identifies and ranks hot spots in proteins and thereby suggests the most likely ligand-binding pockets.[67] The three top hot spots (C1–3) are located in the PLAD region of the TNFR1 dimer (Figure 2a), while FTMap does not reveal any hot spots in the nondimerized subunit of the AlphaFold TNFR1 structure. However, FTMap identifies two hot spots (C1′ and C2′) in TNFR1 from the TNFR1–TNF-β X-ray crystal structure (PDB ID: 1TNR[68]) that align closely with the pockets suggested by Chen[57] and Murali[58] (Figure 2c). Overall, there are several potential pockets for small-molecule binding to TNFR1, and different ways ligands can be envisioned to

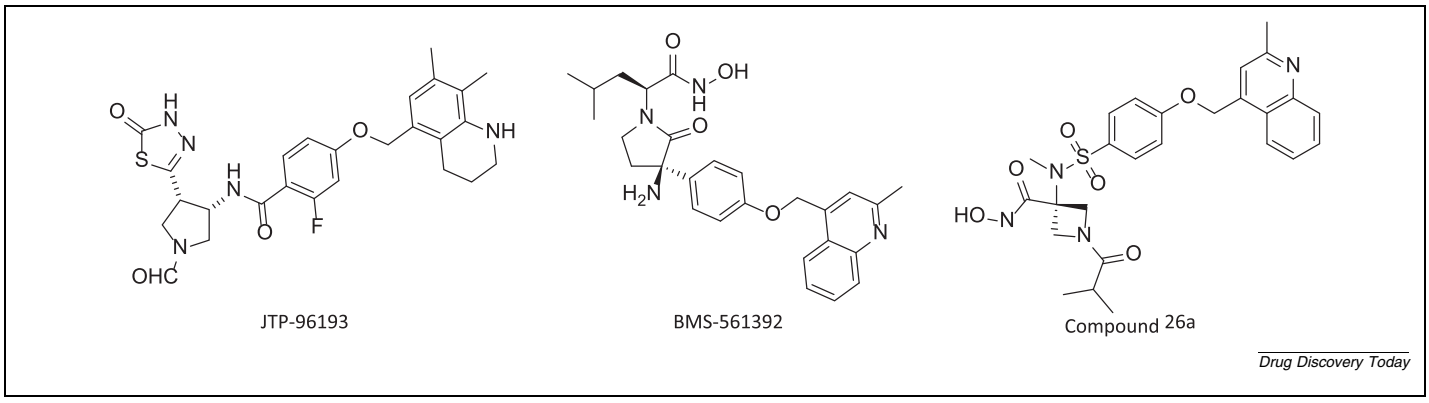


FIGURE 3
Structures of recently disclosed a disintegrin and metalloprotease domain 17 (ADAM17) inhibitors.

impact the TNFRs. For example, TNFR1 ligands bound to C1′ or C2′ (Figure 2c) might affect TNF binding directly or allosterically modulate (e.g., via W107[58]) receptor complex assembling or downstream TNFR1 signaling events. Likewise, small molecules binding the C1–3 pockets (Figure 2a) could induce inhibition by affecting receptor complex assembling or TNF binding.

Other strategies to disrupt TNF signaling

Small-molecule inhibitors of ADAM17

Even though small molecules directly targeting the TNF trimer or the TNFRs are emerging as alternatives to biologics, other ways of altering the TNF inflammatory signaling have been explored. Notably, the inhibition of ADAM17 has been studied for years.[69] Given that ADAM17 is responsible for the release of sTNF, the enzyme is a relevant target for the treatment of inflammation. Inhibition of ADAM17 would reduce TNFR1 activation without affecting TNFR2 activation, thus resulting in a more target-specific reduction in the inflammatory state. However, there are many challenges to overcome for ADAM17 to become a therapeutic target for inflammation. First, ~30 ADAMs are identified in mammals and some of them share identical binding motifs with ADAM17, hence finding selective inhibitors is a challenge. In addition, ADAM17 cleaves ~80 different substrates; thus, inhibition will interfere with several pathways.[70] Nonetheless, there have been recent discoveries of ADAM17 inhibitors for various clinical applications (Figure 3). A group from Japan Tobacco Inc. discovered JTP-96193, a selective ADAM17 inhibitor with potential use in type 2 diabetes mellitus and diabetic peripheral neuropathy.[71] JTP-96193 was identified using an enzymatic assay and is an analog of BMS-561392 (also named DPC-333), another ADAM17 inhibitor (Figure 3).[72] JTP-96193 inhibits ADAM17 with an IC_{50} of 5.4 nM and ~1800-fold selectivity over ADAM10, ADAMTS13, and MMP-14. Importantly, it was shown in a rat LPS-induced TNF production assay that the compound is efficient in reducing the production of TNF in a dose-dependent manner. Recently, the discovery of the clinical candidate 26a as an inhibitor of ADAM17 for the topical treatment of psoriasis was reported.[73] Again, they used BMS-561392 as a starting point and tested the potency of the analogs through inhibition of TNF in human peripheral blood mononuclear cells (hPBMCs). After multiple optimizations and SAR studies, they got to 26a with an IC_{50} of 9 nM toward TNF in hPBMCs and an IC_{50} of 4 nM in an ADAM17 inhibition assay. The compound showed good selectivity for ADAM17 over other ADAMs and alleviates oxazolone-induced chronic skin inflammation in mice.

Biopharmaceuticals

The study of TNF receptor-mediated protein therapeutics has led to the discovery of several proteins, including the sTNF blocker XPro1595, TNFR1 antagonists (ATROSAB and TROS) and TNFR2 agonists (TNC-scTNFR2, EHD2-scTNFR2, and NewSTAR2), used as exploratory drugs to treat neuroinflammatory diseases.[74] The development of peptide inhibitors of TNF signaling has also been studied intensively. A group from Jinan University mimicked TNF-binding residues to create a peptide inhibitor that would block TNFR1.[75] This led to the discovery of RMP16, a 31-mer peptide comprising 20 amino acids from TNF (residue 75–94), linked to a 7-mer peptide to improve human serum albumin (HSA) binding. The linker is a tetrapeptide sensitive to proteolysis by the plasma factor Xa, and allows the slow release of the peptide after injection. RMP16 is selective, with a K_D of 21.3 nM against TNFR1 compared with a K_D of 8.64 μM against TNFR2 in SPR experiments. The peptide showed efficacy in inhibiting tumor growth and angiogenesis, with better properties in mice compared with natural TNF. A 29-mer peptide has also been reported to disrupt the TNF trimer into monomeric TNF.[76,77] The peptide was based on a 58-mer three-helix Z domain mini-protein scaffold that binds to TNF. It was improved by reducing the number of amino acids, incorporating a disulfide bridge and six unnatural amino acids. The peptide showed an IC_{50} of 9.6 nM in a TNF–TNFR1 competitive ELISA and was shown to bind to a TNF monomer when co-crystallized with TNF (PDB ID: 7TA6). Recently, the 8-mer peptide 17.1A capable of binding to TNFR1 and altering the TNF binding of the receptor was reported.[78] It was designed to mimic the binding residues of PGLYRP1 (also known as Tag7), a protein that can compete with TNF for TNFR1 binding. The peptide 17.1A showed an IC_{50} of 0.3 nM in a TNF cytotoxicity assay and was able to reduce inflammation symp-

toms in the complete Freund's adjuvant (CFA)-induced arthritis mouse model.

Concluding remarks

TNF is one of the most studied targets for a large variety of clinical applications, ranging from inflammatory to neurodegenerative diseases. TNF antibody therapies are still the main option for clinical applications even though they give rise to multiple side effects. An alternative strategy for targeting TNF signaling is the use of small molecules that disrupt the trimeric assembly of the cytokine after it has been cleaved by ADAM17. Recently, several such small molecules capable of modulating TNF signaling have been reported, and some of them might be useful starting points for the development of future drug candidates. The fact that compound SAR441566 has finished Phase I clinical trials is an important step forward, and shows that making oral small molecules to prevent protein–protein interactions, even though challenging, is possible. Other strategies have also been tested, for example, the targeting of TNF receptors or ADAM17. Selectively activating or inhibiting TNF receptors would be a particularly useful way of modulating TNF signaling and preventing inflammatory response; hence, small-molecule binders of TNFR1 and TNFR2 could have multiple potential therapeutic applications.

Declaration of interests

The authors declare no conflicts of interest.

Data availability

Data will be made available on request.

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