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TNF Superfamily Protein–Protein Interactions: Feasibility of Small-Molecule Modulation

Yun Song¹ and Peter Buchwald^{1,2,*}

¹Department of Molecular and Cellular Pharmacology, Miller School of Medicine, University of Miami, Miami, Florida, USA

²Diabetes Research Institute, Miller School of Medicine, University of Miami, Miami, Florida, USA

Abstract

The tumor necrosis factor (TNF) superfamily (TNFSF) contains about thirty structurally related receptors (TNFSFRs) and about twenty protein ligands that bind to one or more of these receptors. Almost all of these cell surface protein-protein interactions (PPIs) represent high-value therapeutic targets for inflammatory or immune modulation in autoimmune diseases, transplant recipients, or cancers, and there are several biologics including antibodies and fusion proteins targeting them that are in various phases of clinical development. Small-molecule inhibitors or activators could represent possible alternatives if the difficulties related to the targeting of protein-protein interactions by small molecules can be addressed. Compounds proving the feasibility of such approaches have been identified through different drug discovery approaches for a number of these TNFSFR-TNFSF type PPIs including CD40-CD40L, BAFFR-BAFF, TRAIL-DR5, and OX40-OX40L. Corresponding structural, signaling, and medicinal chemistry aspects are briefly reviewed here. While none of these small-molecule modulators identified so far seems promising enough to be pursued for clinical development, they provide proof-of-principle evidence that these interactions are susceptible to small-molecule modulation and can serve as starting points toward the identification of more potent and selective candidates.

Keywords

CD40; costimulation; druggability; OX40; tumor necrosis factor

1 INTRODUCTION

As drug research and discovery are facing increasing challenges and difficulties in identifying innovative new therapeutics [1, 2], small-molecule modulation of protein-protein interactions (PPIs) has emerged as a possible alternative approach that might be challenging, but could offer particularly valuable solutions. Since most biological processes are not performed by stand-alone single proteins, but by complexes consisting of different interacting proteins, the modulation of these PPIs could lead to novel and more function-specific therapeutic applications. This potential is well illustrated by the current interest in

*Correspondence to: Peter Buchwald, Diabetes Research Institute, Miller School of Medicine, University of Miami, 1450 NW 10 Ave (R-134), Miami, FL 33136, USA, Tel.: 305 243-9657, pbuchwald@med.miami.edu.

biotechnology products such as antibodies and fusion proteins, which can be particularly potent and specific PPI modulators.

Whereas antibodies (immunoglobulins) have the advantage of being highly specific for their targets and quite stable in human serum, they usually cannot reach intracellular targets [3] and, as all other protein therapies, are often hindered by solubility, route of administration, distribution, and stability problems as well as by the possibility of a strong immune response mounted against them [4]. For immunomodulatory biologics, a high incidence of additional unwanted adverse reactions (including serious infections, malignancy, cytokine release syndrome, anaphylaxis, hypersensitivity as well as immunogenicity) always looms as a hindrance in their development [5]. While some of these are due to on-target interactions (i.e., exaggerated pharmacology), some (e.g., generation of neutralizing anti-drug antibodies and hypersensitivity reactions) are due to the inherent immunogenicity of biologics [5]. For example, out of the 40 licensed immunomodulatory biologics, 18 have been associated with serious infections, including reactivation of bacterial, viral, fungal, and opportunistic infections. As we discussed before [6], “traditional small-molecule drugs could provide a convenient alternative; however, small molecules were considered unlikely to be effective PPI inhibitors because these interactions usually involve relatively large protein surfaces (1,500–3,000 Å²) that also lack the well-defined binding pockets present on traditional targets of most existing drugs (G-protein coupled receptors – GPCRs, ion channels, enzymes). Hence, for a long time, small molecules were not pursued at all as possible PPI inhibitors.”

During the last two decades, it has become increasingly clear that, at least in certain cases, small molecules can act as effective PPI modulators. Sufficiently effective small-molecule inhibitors have been identified for a few important PPIs and several candidates are in advanced clinical development [6–13]. Binding pockets on protein–protein interfaces suitable for binding small molecules have been shown to be considerably smaller than the binding pockets of traditional protein–ligand interactions [14]. According to one quantitative estimate, whereas, marketed drugs typically target a single binding pocket with an average occupied volume of ~270 Å³, existing PPI inhibitors typically target three to five much smaller pockets (~100 Å³) [14]. As pointed out [6], since “pocket size limits the achievable binding energy [15], in most cases, PPIs can achieve adequate affinity only by being large enough to simultaneously reach a sufficient number of smaller pockets.” Therapeutic applicability usually requires sufficient activity – i.e., nanomolar potency [16, 17]; however, for many PPIs, modulation with such nanomolar potency is likely achievable only by compounds that have larger molecular sizes, typically larger than desired for drug-like structures and especially for good absorption properties (e.g., ‘rule-of-five’ [18]). A study comparing the structures of identified PPI inhibitors to those of FDA approved drugs found that PPI inhibitors tend to be larger than enzyme inhibitors, ion channel modulators, or receptor ligands [19]. Accordingly, as we noted [6], they also “tend to have a larger molecular size, a more hydrophobic nature, and structures with more rigid, aromatic scaffolds in combination with charged or polar functionalities. A number of recent studies confirmed a poor correspondence between the chemical spaces of existing screening libraries and known PPI inhibitors [19–21].”

Nevertheless, certain structures clearly show good protein-binding ability, and a chemical space with privileged structures for protein binding might very well exist [22]. A number of studies suggested various scaffolds, many of them based on flat, aromatic structures, for designing effective small molecule PPI inhibitory structures [23–25]. Planar or partially planar aromatic and hydrophobic residues are indeed over-represented at PPI interfaces [26, 27]. Recently, even promiscuous small-molecule PPI inhibitors have been identified [28, 29]. The ability to target PPIs could considerably enlarge the number of feasible therapeutic targets. As discussed before [17], “currently existing small-molecule drugs target only about 1% of the human proteome,” which contains only about 20,500 unique proteins [30, 31], a number that has also been confirmed by recent draft maps of the human proteome [32, 33]. It is estimated that only about 10% of these proteins (i.e., approximately 3,000) are druggable (i.e., contain protein folds that can interact with drug-like compounds at possible ligand binding sites) [3, 34, 35]. Furthermore [17], “only about 10% of all genes seem to be disease modifying genes. Hence, only the intersection of these two subsets represents true potential drug targets with only an estimated 500–1,500 members [34, 35].” According to the latest update of the Therapeutic Target Database (2012), the number of current successful drug targets is 364 with an additional 286 clinical trial drug targets and 1331 research targets [36]. PPIs, if druggable, could represent a large number of alternative targets. The number of such interactions has been estimated to be in the range of 300,000 [37] to 650,000 [38] for humans (human interactome). There is already documented experimental evidence for about 80,000 PPIs in the human protein interactome with a distribution well characterized by a power law, i.e., a few proteins that have very high number of interactions and many that have only a few [39]. For the druggability aspects it is encouraging that a computational analysis aiming to extract so-called small-molecule inhibitor starting points (SMISPs) from protein-ligand and protein-protein complexes in the Protein Data Bank (PDB) suggested that nearly half of all PPIs may be susceptible to small-molecule inhibition [40]. Hence, despite all inherent difficulties, a fair number of targets involved in PPIs could still be suitable for small-molecule modulation [41].

As we highlighted it before, PPI modulators, as any therapeutic agent in general, “can be either *orthosteric* – directly interfering with critical hot spots on the interface and competing with the original protein ligand; or *allosteric* – binding at some different site, but causing conformational changes that are sufficient to interfere with the binding of the protein ligand” [6]. Most PPI modulators are PPI inhibitors (antagonists) and not agonists that enhance binding or stimulate activity; however, a few examples of agonists do exist. As mentioned [42], “by all accounts, identification of small-molecule PPI stimulators is even more challenging than that of PPI inhibitors since they, in addition to binding, also need to trigger the downstream activation cascade [43]. Only a very limited number of small-molecule PPI ‘agonists’ (i.e., enhancers or stabilizers) have been identified.” Direct evidence of PPI stabilization is demonstrated by tacrolimus (FK506) and sirolimus (rapamycin) [44, 45]. In their absence, the immunophilin protein FKBP12 is unable to bind calcineurin and mTOR. However, these compounds can bind FKBP12 and then form a complex with calcineurin and mTOR, respectively [46–48]. Another example of PPI agonist is represented by the adenylyl cyclase (AC) binding forskolin [43]. Some other examples of stabilizers have been found for

PPIs in which protein 14-3-3 is involved [49–51] and a possible small-molecule activator of TRAIL receptor DR5 that will be discussed later [52].

Here, we will review small-molecule modulators targeting PPIs within the TNF superfamily, which contains a large number of cell surface protein receptor-ligand interactions that represent highly valuable therapeutic targets. For TNFSF PPIs where small-molecule modulators have been published, a brief review of relevant structural and signaling aspects will be included with the description of the modulators.

2 TNF SUPERFAMILY

The tumor necrosis factor (TNF) superfamily (TNFSF) contains about thirty structurally related receptors (TNFSF-R) and about twenty protein ligands that bind to one or more of these receptors [53–58]. TNFSF ligands are soluble or membrane-anchored trimers that cluster their cell surface receptors to initiate signal transduction; a set of representative ligand-receptor interacting trimer structures obtained from corresponding crystal structures are shown for illustration in Figure 1. These interactions are integral to communication and signaling systems involved in numerous physiological functions essential to inflammatory signaling, to the functioning of the immune and nervous system, to bone development, and others. The development of protein-based biologics inhibiting the binding of TNF to its receptors, which have been shown to be effective in reducing the inflammation associated with several autoimmune diseases and have become some of the best selling drugs, is one of the few recent immunopharmacology success stories [59]. Following this success, considerable attention has been focused on the therapeutic potential of modulating other TNFSF interactions, and there are biologics in clinical development for almost all of these interaction pairs [57, 58]. Currently, there are five biologics blocking TNF (TNFSF2) or LT α (TNFSF1) that are approved for treating various autoimmune and inflammatory disorders including rheumatoid arthritis (RA), psoriatic arthritis, juvenile idiopathic arthritis, psoriasis, ankylosing spondylitis, Crohn's disease and ulcerative colitis: etanercept (LT α , TNFSF1 and TNF, TNFSF2), infliximab, adalimumab, certolizumab pegol, and golimumab (TNF, TNFSF2). There are also biologics targeting other TNFSF members approved for clinical use: brentuximab vedotin (CD30L, TNFSF8) for Hodgkin's lymphoma and systemic anaplastic large cell lymphoma (sALCL); denosumab (RANKL, TNFSF11) for osteoporosis, and belimumab (BAFF, TNFSF13B) for systemic lupus erythematosus (SLE) and RA [58].

Costimulatory and coinhibitory signaling play crucial roles in T cell biology as they determine the functional outcome of the T cell receptor (TCR) signaling [60]. Several TNFSF ligand-receptor pairs provide important co-signaling (costimulatory as well as coinhibitory signaling) interactions that regulate T cell activation and, therefore, are promising therapeutic targets in autoimmune diseases, in transplant recipients as well as in cancers [53, 55, 60, 61]. Costimulatory blockade has emerged as a particularly valuable target for immune modulation both in transplant recipients and in autoimmune diseases since it might avoid the broad suppression of immunity caused by all existing immunosuppressive agents [62–67]. Cell surface costimulatory receptor-ligand interactions belong to two main families: the immunoglobulin superfamily (CD28–CD80/86 and ICOS–

ICOS-L) and the TNF–TNFR superfamily. Costimulation of T-cell activation has been reported for several members of the TNFR–TNF superfamily [53, 66, 68–70], and all its members play important roles in various aspects of the immune response [53, 55, 61]. Because members of this family are expressed only upon T-cell activation (with the exception of CD27), they are generally considered to play a particularly important role in the effector and memory phases of the immune response [66]. Interaction of TNF family ligands with their corresponding receptors leads to recruitment of TNFR-associated factors (TRAF), which initiate signaling cascades resulting in T-cell activation [69]. While many different receptor-ligand pairs are now recognized to contribute to the activation of T cells, their interplay is still incompletely understood. In the beginning, the main therapeutic focus has been on the CD28–CD80/CD86 and the CD40–CD154 pathways [62–65], but there is an increasing recognition of the role played by other members as well [66].

3 TNF–TNF-R

The name of this entire superfamily is derived from TNF, the premier member of this superfamily and an important cytokine involved in systemic inflammation and immune regulation. TNF signaling is an obvious therapeutic target for many diseases, and the development of biologics inhibiting the binding of TNF to its receptors is one of the few recent drug discovery/immunopharmacology success stories [59]. Accordingly, there is considerable interest in corresponding small-molecule inhibitors, but only limited success has been achieved so far. In fact, none of the small-molecule TNF inhibitors currently identified have made it into animal testing most likely due to their low potencies. These approaches have been reviewed in detail recently [71]; therefore, we will include here only a very brief overview of the main structures and their mechanism of action as early, proof-of-principle evidence for the feasibility of the approach and as context for the discussion of the small-molecule modulators of other TNFSF PPIs that will be presented in more detail.

3.1 Small-molecule modulators

3.1.1 Suramin and its analogs—Suramin (**1**, Figure 2), a symmetric polysulfonated naphthylamine-benzamide urea derivative, was the first small molecule found to inhibit TNF action and do so by inducing deoligomerization of the TNF trimer [72, 73]. Suramin is approved for the prophylactic treatment of African sleeping sickness (trypanosomiasis) and river blindness (onchocerciasis) caused by parasitic infections. It shows strong polypharmacology at mid-micromolar concentrations; for example, it is a known P2 (ATP/UTP purine receptor) antagonist ($IC_{50} \approx 5\text{--}10\text{ }\mu\text{M}$) [74], a known inhibitor of the binding of a range of tumor growth factors, and it has various other biological activities as well [75, 76]. Based on this work, a number of organic dyes such as Trypan blue and Evans blue (**2**), which are structural analogs of **1** (Figure 2), have also been found to inhibit the TNF–TNFR1 interaction using a solid-phase TNFR1 ELISA assay [77]. None of these were very potent however: the two best compounds, **1** and **2**, having IC_{50} s of only 650 and 750 μM , respectively [77].

3.1.2 SPD304—The first promising small-molecule inhibitor of the TNF- α –TNFR1 interaction with known mechanism of inhibition was discovered by using a fragment

screening approach [78]. SPD304 (**3**; PubChem CID 16079006) is one of the best inhibitors identified until now, but it still has a relatively low affinity ($K_d \approx 15 \mu\text{M}$). Mechanistic studies revealed that **3** binds in the core of the TNFR1 trimer interface and is able to disrupt the structure to the extent that one of the monomers is fully ejected leading to a complex in which the compound binds to the exposed core of a dimeric form of TNFR1. TNFSF ligands have relatively low sequence similarities (10%–30%); however, these are mostly confined to internal aromatic residues responsible for trimer assembly [53]. Therefore, achieving good specificity within TNFSF with small molecule modulators could be challenging. Indeed, **3** has been shown to also inhibit RANKL mediated osteoclastogenesis *in vitro*, possibly due to its interference with the hydrophobic interfaces of RANKL monomers [79].

3.1.3 Other compounds—Several other compounds have been identified that showed some inhibitory activity, but none yet of sufficient promise to undergo further development. All of the most recent advances have been through disruption of the TNF trimer, and, as mentioned, a detailed recent review is available [71]. For example, two natural products with either pyrazole-linked quinuclidine (**4**, Figure 2) or indolo-[2,3-*a*]quinolizidine structure inhibited the TNF- α –TNFR1 interaction [80]. These two compounds were identified via a high-throughput, ligand-docking-based virtual screening using the structure of the TNF- α dimer with **3** (PDB: 2AZ5) from a library containing 20,000 natural products or natural-product like structures. Functional studies suggested that both inhibited the binding of TNF- α to TNFR1 with an IC_{50} of 50 μM in a preliminary ELISA and $\sim 5 \mu\text{M}$ in NF- κB luciferase induction assay in HepG2 cells for **4** (Figure 2), the most promising compound in cell assays [80]. A somewhat similar approach of scoring function-based screening of a filtered 240,000 compound library and testing of the top-scoring commercially available structures identified compounds containing pyrimidine-2,4,6-trione moieties such as **5** that showed inhibition against lipopolysaccharide-activated Raw264.7 macrophage cells [81]. It should also be mentioned that the promiscuous PPI inhibitors we have identified in our organic dye-based library, such as erythrosine B (**6**, Figure 2), an FDA approved food colorant, also inhibited the TNF–TNFR1 interaction in an ELISA set-up quite potently (IC_{50} of 5 μM) as well as the TNF α –induced JNK (c-Jun N-terminal kinase) phosphorylation in THP-1 cells (at 50 μM) [28]. This, however, is of no particular value as **6** promiscuously inhibits PPIs within TNFSF as well as outside of it with a remarkably consistent IC_{50} in the 2–20 μM (approximately 2–20 mg/L) range.

4 CD40–CD40L

This PPI is an important receptor-ligand interaction and one of the most extensively studied TNFSF member. CD40 (TNFRSF5) is a ~ 48 kDa type I transmembrane glycoprotein constitutively expressed on antigen presenting cells (APCs) including B-cells, monocytes, macrophages, dendritic cells and non-immune (thymic epithelia, endothelial) cell types [82]. CD40L (CD154, TNFSF5) exists both as a ~ 30 kDa type II transmembrane protein as well as soluble forms with molecular weight of 14, 18, and 29 kDa in the plasma [83, 84]. Membrane bound CD40L can be found on T cells, B cells, mast cells, natural killer cells, macrophages, endothelial cells, vascular smooth muscle cells, and activated platelets;

whereas, soluble CD40L is predominantly platelet-derived and biologically active [85]. The broad expression profile of this PPI pair is an indication of its versatile physiological role and its unequivocal association with immune and non-immune pathologies such as type 1 diabetes (T1D), autoimmune thyroiditis, inflammatory bowel disease (IBD), psoriasis, multiple sclerosis (MS), RA, SLE [67] and cardiovascular disease [86]. CD40–CD40L is a therapeutic target in these diseases due to its involvement in driving inflammatory events and autoimmunity, and the therapeutic effects of its inhibition are mainly due to the suppression of T and B cell mediated immune responses.

4.1 Structure

Like most ligands within the TNFSF, CD40L shares little sequence similarity with other members (20–30%) [54], but its sequence is more conserved across species (e.g., 78% AA similarity between human and mouse). One CD40L monomer folds like a sandwich of two anti-parallel β -sheets with a Greek key topology. Crystallography studies indicate that the biologically active CD40L is a truncated pyramid-like trimer [87]. The extensive interface between the trimer subunits is formed mainly by aromatic and hydrophobic residues. The extracellular region of CD40 consists of three cysteine-rich domains (CRD), each having two to three disulfide bridges running parallel and conferring stability to its elongated ladder-like structure. All three CRD domains are directly involved in CD40L binding (Figure 1) [88].

The CD40–CD40L binding interface consists of a mixture of both hydrophilic and hydrophobic residues. Upon binding, two CD40L subunits interact with the extracellular domain of one CD40 subunit most of the binding energy coming from the hydrophilic and charge interactions [89]. Mutations in CD40L that prevent its binding to CD40 resulting in the so-called hyper IgM syndrome (HIGM) are mapped on the protein core, trimerization interface, and area of direct CD40 contact [88, 90]. Interestingly, point mutation of Ser132 on CD40L does not abrogate its binding ability to CD40, but is able to selectively silence p38- and ERK-dependent signaling initiated by CD40 binding, whereas JNK-dependent signaling is not affected [88]. Further investigations suggested a spatial conflict in the Ser132 loop as a Ser132 mutation can inhibit the complete activation of CD40 even with intact binding of CD40L.

4.2 Signaling

CD40 signaling culminates in a cell-dependent pattern of gene expression of cytokines, chemokines, and cell adhesion molecules among other mediators of its various cell-type specific functions. The main signal transduction pathways involved are NF- κ B (nuclear factor- κ B), stress activated protein kinase (SAPK), MAPK (mitogen-activated protein kinase), ERK (extracellular signal-regulated kinase), and STAT3 (signal transducers and activators of transcription-3) [55, 91, 92]. Association of CD40L with p53 induces translocation of ASM (acid sphingomyelinase) to the cell membrane causing its activation and the resultant formation of a ceramide-enriched platform for the clustering of CD40L. The formed CD40L trimers associate with CD40 to induce its trimeric clustering [93]. This facilitates the binding of the receptor's intracellular domain to adaptor proteins called TRAFs (TNF receptor associated factors). In addition to the TRAFs, JAK3 can also directly

associate with CD40 to activate STAT3 and PI3K pathways that are relevant in delivering anti-apoptotic signals in B-cells [94–96]. Notably, some small-molecule inhibitors of the CD40–TRAF6 interaction that can improve obesity-associated insulin resistance have been identified recently [97] as well as some small-molecule inhibitors of the CD40-mediated NF- κ B signaling pathway (through HTS using NF- κ B luciferase reporter assay in BL2 cells activated with trimerized CD40 ligand) [98]; the latter, however, seem more likely to be possible downstream regulators than direct CD40–CD40L inhibitors. Detailed information on these is beyond the scope of the present review.

4.3 Biologics

CD40 and CD40L were the first TNFSF costimulatory molecules to be identified. Blocking of this PPI is a proven highly effective immunomodulatory therapy [92, 99–101]. Hence, there is considerable ongoing interest in targeting CD40L and/or CD40, and multiple antibodies have been developed and reached different phases of preclinical or clinical testing. Corresponding biologics in clinical development include PG102, ASKP1240/4D11, lucatumumab (HCD122), dacetuzumab (SGN 40), Chi Lob 7/4, and CP 870893 [58]. For example, there are ongoing clinical studies with the antagonistic anti-CD40 antibody ASKP1240 in kidney transplant recipients (NCT01780844) and in patients with moderate to severe plaque psoriasis (NCT01585233) as well as with the agonistic antibody Chi Lob 7/4 to treat advanced malignancies refractory to conventional anti-cancer treatment (NCT01561911). As we discussed [102], “clinical trials of an anti-CD40L humanized antibody (ruplizumab, hu5c8)” for SLE, MS, and kidney transplant looked promising; however, they “have been halted because of thrombotic side effects [103–105], and development is no longer supported [106]. Activated platelets express CD154; however in platelet-rich plasma, the 5c8 antibody by itself did not induce platelet aggregation *per se* and did not significantly affect maximal aggregation. It has been suggested that CD154 expression produced by physiological or pathophysiological platelet activation can sustain a pro-aggregatory effect of the antibody by a mechanism involving the mAb Fc domain [107].” Along these lines [102], a “cyclic heptapeptide (CLPTRHMAC) capable of blocking the CD40–CD154 interaction did not prime human platelet activation and aggregation in *in vitro* platelet activation studies contrary to the anti-CD154 mAb tested [108].” Accordingly, one might be able to avoid this restrictive side effect by using small-molecule inhibitors. More recently, another CD40-targeting peptide (VLQWAKKGYYTMKSN), which was designed from the CD40L domain critical for interaction with CD40, has been shown to be effective in preventing T1D in NOD mice, a well-known animal model of this disease [109].

4.4 Small-molecule modulators

4.4.1 Naphthalenesulphonic acid derivatives—The first small-molecule capable of inhibiting the CD40–CD154 interaction published in the literature was suramin (**1**, Figure 2), which we have identified after testing it on the basis of its activity in the TNF system [110]. In a very encouraging manner, **1** was found to inhibit the binding of human CD40–CD40L with an IC₅₀ of 15 μ M in a cell-free ELISA-type assay. It showed about similar inhibitory efficacy in the murine CD40–CD40L system (with an IC₅₀ of about 3 μ M), but more importantly, **1** was considerably (about 30-fold) more active in inhibiting the CD40–CD40L interaction than the human TNFR1–TNF- α (IC₅₀ of 500 μ M in a similar

experimental setting). Further functional studies have shown **1** to concentration-dependently inhibit CD40L-induced human B cell proliferation and activation as assessed by BrdU assays and quantification of the expression levels of CD86, CD80, CD40, and MHC-II [110]. Suramin (at 100 μ M) was also shown to block CD40L-induced proinflammatory cytokine (IL-6, IL-8, and IFN- γ) release by human pancreatic islet cells. Hence, even if **1** is well-known to show considerable polypharmacology at these concentrations, its interference with the positive costimulatory interaction might provide a possible mechanism for its ability to suppress T cell activity and induce immunosuppression.

After probing the chemical space of structural analogs of **1**, we have identified a series of organic dye compounds that were able to disrupt the CD40–CD154 interaction with low micromolar potency [102, 111]. Both inhibitory activity and specificity over several other ligand receptor pairs within the TNFSF of these compounds were confirmed in cell-free assays. Several of the compounds including direct red 13, direct red 80 (**7**), and direct fast red B (**8**) (Figure 3) have been shown to concentration dependently inhibit CD40L-induced CD86 and CD54 expression in human B cells. In addition, CD40L-mediated expression of CD54, CD40, and MHC-II was inhibited in human myeloid THP-1 cells by these compounds in a concentration-dependent and specific manner, as neither PMA- nor SAC-induced marker expressions were affected. Although there is no information about potential binding sites of these compounds, binding-partner assays indicated CD40L and not CD40 as the likely binding partner of these compounds [102, 111]. Computational simulations using molecular docking provided some further support as they also indicated an allosteric site on the CD154 trimer as the likely binding site [112].

Further screenings within the same chemical space of organic dyes, led to the identification of mordant brown 1 (**9**, Figure 3), another naphthalenesulphonic acid derivative that is an even more potent and specific CD40-CD40L inhibitor [113]. Compound **9** inhibited CD40–CD154 binding with an IC_{50} of 0.13 μ M, more than an order of magnitude more potent than the previous set of compounds, and it concentration-dependently blocked CD40L-induced CD40 and MHC-II expression in THP-1 cells. It also showed improved selectivity toward CD40-CD40L compared to other TNFSF ligand-receptor pairs with >100-fold difference compared to all other TNFSF interactions tested (except RANK–RANKL with 70-fold selectivity) [113]. Even if such compounds are unlikely candidates to become drugs, the chemical space of organic dyes seems to provide a feasible starting point that should not be overlooked in the search for possible small molecule PPI modulators. As we discussed [111], “once an active structural scaffold could be identified, the color-related problems might be avoidable” by standard medicinal chemistry approaches – “the same way as suramin is, in fact, a ‘colorless dye’ since it is structurally related to polysulfonated azo dyes such as trypan blue or Evans blue, but it does not contain the aryl azo moiety causing their vivid color” (*cf.* **1** and **2** in Figure 2).

4.4.2 BIO8898—BIO8898 was discovered as a CD40-CD40L inhibitor by mass spectrometry after rapid separation from unbound library members by gel filtration [27]. It has a relatively large structure with a 4, 4'-bipyridine core and four arms: two 2-(N-pyrrolidinomethyl) pyrrolidine, one 2-cyclohexyl-2-aminoacetic acid, and one biphenyl arm

(**10**, Figure 3). Compound **10** was shown to inhibit CD40-CD40L binding in dissociation-enhanced lanthanide fluorescence immunoassay (DELFI) with an IC_{50} of 25 μ M. It inhibited CD40L-induced cell death in baby hamster kidney (BHK) cell lines with a somewhat lower potency that could not be fully quantified due to solubility limitations, but seemed to be within three-fold of the 25 μ M measured in the binding assay. The crystal structure of the bound complex was determined, and, surprisingly, it indicated that **10** binds not on the surface of the protein, but in a deep pocket between two subunits of CD40L disrupting the native three-fold symmetry of the trimer (Figure 4) [27]. Binding occurs with a 1:1 molar ratio to the CD40L trimer. The contact area between **10** and the protein is mostly hydrophobic (~80%). Unlike the interaction between **3** and TNF- α , which was completely by hydrophobic interactions [78], **10** also makes several hydrogen bonds with subunit C of CD40L. These H-bonds are thought to direct the orientation of the molecule, which is lacking in the TNF- α binding of **3**. To characterize the mechanism of inhibition, the ability of **10** to displace the subunits of the CD40L trimer was tested in a setting similar to that done for **3**. Failure to inhibit CD40 binding to singly biotinylated CD40L when a washing step is introduced after compound incubation revealed that binding of **10** is reversible and does not cause ejection of CD40L subunits. Experiments also indicated that high affinity binding between CD40 and CD40L requires cooperative binding of at least two CD40 monomers to two adjacent binding sites on the CD40L trimer. Since only one binding site is extensively disrupted in the complex of CD40L with **10**, these results suggest that inhibition of CD40-CD40L binding by **10** occurs not by direct (orthosteric) interference at the CD40-CD40L binding site, but due to allosteric disruption of the overall CD40 binding sites adjacent to the area where the compound binds (Figure 4). Hence, allosteric mechanisms might provide promising alternative routes to PPI inhibition, which is of particular interest for TNFSF, where the ligand and the receptors are trimers susceptible to disruption or distortion.

5 BAFFR-BAFF

The BAFF signaling is a complicated network consisting of two ligands and three receptors. The ligands BAFF (BLyS, CD257, TNFSF13B) and APRIL (CD256, TNFSF13; a proliferation inducing ligand) are expressed on surfaces of dendritic cells (DCs), monocytes, neutrophils, activated T cells, malignant B cells, and epithelial cells [114–118]. While BAFF mostly exists as a soluble homotrimer in plasma that binds to BAFFR (CD268, TNFRSF13C) and BCMA (B cell maturation antigen; CD269, TNFRSF17), the multimerized form of BAFF is necessary for TACI (transmembrane activator and calcium modulator ligand interactor; CD267, TNFRSF13B) binding. APRIL binds to BCMA with a higher affinity than to BAFF in the human system, and it binds to TACI as a multimerized form as well [119–121]. Both BAFFR and BCMA deliver positive signals to B cells, whereas TACI acts as an inhibitor receptor for the BAFF signaling [122]. The BAFF-BAFFR axis is an important therapeutic target primarily due its regulatory role in B cell mediated autoimmune diseases and cancer [123–125], and several biologics targeting BAFF are in clinical development.

5.1 Structure

BAFF is a Type II membrane-bound protein that can be released as a soluble trimeric form upon proteolytic cleavage. Like other ligands within the TNFSF, the BAFF monomer consists of two antiparallel β -sheets with Greek-key topology as confirmed by crystal structures of both unbound and BAFFR-bound ligand [126–128]. BAFF has a low level of sequence homology with other TNFSF members: 21.5%, 17.4%, and 20.1% with TNF- α , CD40L, and TRAIL, respectively [54]. Biologically active BAFF is a trimer. The core of the protein is mostly hydrophobic with residues such as Trp168 and Phe279 well conserved in the TNFSF. A few buried polar residues also exist in the core contributing to polar interactions such as the hydrogen bond between His210 and Tyr201. The monomer–monomer interface is primarily composed of hydrophobic residues characterized by Tyr192, Phe194, Tyr196, Tyr246, and Phe278. In addition, three Gln234 residues form a polar cluster on the three-fold axis near the top of the trimer. Interestingly, a virus-like assembly made of 60 soluble BAFF monomers has been solved and found to be biologically active [127, 129].

BAFFR is a Type III membrane protein due to its lack of a signal peptide. Unlike other receptors within the TNFSF, BAFFR contains a single CRD, which interacts extensively with one monomer of BAFF. Two key residues of the DxL motif in CRD (Asp26 and Leu28) are involved in BAFF binding. Furthermore, ionic interactions also contribute to BAFFR–BAFF binding. This is characterized by a large number of negatively charged residues on the binding surface of BAFFR that are thought to complement positively charged residues on BAFF. A notable difference of BAFFR–BAFF binding interface is that the buried surface is significantly smaller than those of other TNFSF members (approximately half of that in TNFR1–TNF- α and a third of that in DR5–TRAIL) [128]. This might confer an edge for the design of small-molecule modulators of the BAFFR–BAFF interaction.

5.2 Signaling

The BAFF–BAFFR signaling is delivered primarily through the alternative NF- κ B2 pathway [130]. Upon BAFF ligation, TRAF3 is recruited to the cytoplasmic domain of BAFFR via a PVPAT binding site [131] and degraded. This event rescues the proteasomal degradation of NF- κ B inducing kinase (NIK) [132], which is mediated by a functional complex consisting of TRAF2, TRAF3, and the cellular inducer of apoptosis proteins 1 or 2 (cIAP1/2). Subsequent activation of NIK and I κ B kinase 1 (IKK1) leads to the proteolytic processing of p100 into p52, which translocates into the nucleus and regulates gene expression such as anti-apoptotic protein Bcl-2 [133, 134]. In addition, recent evidence has shown the possibility of the interaction between BAFFR and TRAF1, which might contribute to the degradation of TRAF3 and NF- κ B2 activation [135, 136]. Besides TRAF mediated NF- κ B activation, BAFFR has been shown to activate PI3K [137, 138], which can lead to the downstream activation of AKT, Btk and PKC β [139, 140]. In addition, the synergy between BCR and BAFFR signaling is strengthened by a recent finding that in conjunction with BCR signal, BAFF stimulation is able to phosphorylate Syk and thus deliver BAFFR mediated survival signals through the ERK and PI3K pathways [141].

5.3 Biologics

Several biologics targeting BAFF (e.g., belimumab, tabalumab, atacicept, briobacept, and blisibimod) as well as APRIL (atacicept) are in clinical development mostly for indications including SLE, RA, and MS [58]. Development of most of these BAFF antagonistic biologics was relatively slow mainly due to the low response rate of patients. Even in the case of belimumab, which was approved in 2011 in the US, Europe, and Canada for the treatment of SLE, only about half of the patients categorized as displaying B cell dysfunction in the form of circulating antinuclear antibodies were responsive to the drug in phase III clinical trials [142]. Data also suggested that targeting BAFF and APRIL has potential as cancer therapy due to their role as B cell growth factors that may directly contribute to B cell tumor growth, and trials are ongoing with tabalumab in multiple myeloma.

5.4 Small-molecule modulators

5.4.1 Guanidine derivatives (KR33426)—Following the synthesis and testing of a series of carbonylguanidine derivative compounds that showed inhibitory effect against the sodium/hydrogen exchanger (NHE) [143], they were also screened against BAFF on the basis of their potential to interfere with the binding of biotinylated human BAFF-murine CD8 (BAFF-muCD8) to BAFFR on the WIL2-NS human B lymphoblast cell surface [144]. Five compounds showed inhibitory activity toward this interaction as characterized by a decreased level of BAFF-muCD8 signal in flow cytometry. The putative target of these compounds does not seem to be BAFF. Pre-incubation of these compounds with BAFF-muCD8 barely reduced fluorescence intensity, whereas pre-incubation with BAFFR expressing WIL2-NS cells showed considerable inhibitory effect as indicated by the decreased fluorescence signal from the bound BAFF-muCD8. One exception was KR33426 (**11**, Figure 5), which inhibited BAFF-muCD8 binding in both experimental settings. The inhibitory effect is achieved at low micromolar range – a concentration level that showed no apparent cytotoxicity as assessed by MTT assay in the same cell line. Further functional studies suggested that these compounds are able to inhibit BAFF mediated cell survival in both WIL2-NS cells and splenocytes with **11** being the most potent inhibitor. In addition, failure of these guanidine compounds to inhibit TNF- α or FasL-mediated cell death and CD40L-mediated cell proliferation suggested target specificity toward BAFFR–BAFF.

The most promising compound, KR33426 (**11**), was further characterized regarding its BAFFR–BAFF binding inhibitory potential [145]. It has been shown to inhibit the BAFF induced increase of WIL2-NS cell density. To address whether **11** can interfere with BAFF mediated B cell survival, it has been shown that it can attenuate BAFF-induced anti-apoptotic activity on splenocytes. In addition, anti-IgM antibody- and BAFF-stimulated WIL2-NS cell proliferation level has been reduced by **11** with reduced BAFFR level on WIL2-NS cells as well. A SLE mouse model (MRL^{lpr/lpr} mice) was also explored to test the potential effect of **11** mediated inhibition of BAFFR–BAFF *in vivo*. Renal swelling in glomerulus and lymphocyte proliferation in splenic white pulp can be recognized in 13 week old MRL^{lpr/lpr} mice, and these phenotypes were ameliorated in mice injected intraperitoneally with **11** for 4 weeks. The biological targets of these effects seem to be a variety of B cell populations. Together, these results support the ability of compound **11** to

target certain B cell populations in an SLE mouse model through interference with the BAFFR–BAFF interaction.

6 TRAIL–DR5

TRAIL (tumor necrosis factor related apoptosis-inducing ligand; CD253, TNFSF10), is a 32.5kDa type II transmembrane protein expressed on activated T cells, natural killer (NK) cells, monocytes, and dendritic cells [146, 147]. TRAIL participates in modulating various immune functions [148]. Its ability to selectively induce apoptosis through the activation of death receptors in virus infected or tumor cells while having little effect on normal cells [149–151] makes it a promising therapeutic target in cancer treatment. TRAIL has five known receptors. In TRAIL signaling, the binding of TRAIL with two death receptors, DR4 (TRAILR1, CD261, TNFRSF10A) and DR5 (TRAILR2, CD262, TNFRSF10B), can induce cell apoptosis [152–154]. Another class of TRAIL receptor includes two ‘decoy’ receptors DcR1 and DcR2 that lack death domains [155]. DcR1 is only expressed on normal cells, but not cancer tissues, and DcR2 is found on fetal liver and adult testis tissue. Both DcR1 and DcR2 can competitively bind to TRAIL and prevent TRAIL-induced cell death in normal cells [149]. The third class is a soluble protein osteoprotegerin (OPG). Although OPG has a weaker affinity to TRAIL, the binding of TRAIL and OPG can also inhibit TRAIL induced cell apoptosis [149, 156–158].

6.1 Structure

TRAIL is a type II transmembrane protein, which can be cleaved to a soluble TRAIL species through the action of metalloproteases. Both soluble and membrane bound TRAIL are biologically active [159]. Like most members within the TNFSF, TRAIL forms a trimeric structure with three monomers containing two antiparallel β sheets (Figure 1). TRAIL exhibits a high content of aromatic residues providing a hydrophobic platform for extensive edge-to-face interactions between adjacent monomers [160]. A distinctive feature of TRAIL compared to other TNFSF ligands is an insertion of 12–16 amino acids in the AA' loop near the N terminus [160]. This elongated loop forms an extensive contact with DR5 and is required for the TRAIL–DR5 binding, which suggests a possible strategy to confer specificity for molecular recognition [161]. Another feature of TRAIL is the presence of a solvent inaccessible zinc-binding site located near the tip of the trimerization interface [161, 162]. Zinc was found to coordinate to three Cys230 residues from each subunit and bind to the TRAIL trimer in a 1:1 stoichiometry.

The structure of DR5 has been well studied. The crystal structure of the TRAIL–DR5 complex (Figure 1) showed that DR5 contains two organized CRD regions that contribute equally to the binding of TRAIL [163]. Alanine-scanning mutagenesis studies have identified five key residues on TRAIL that are important for receptor binding and biological activity of DR5 [164]: Gln205, Val207, Tyr216, Glu236, and Tyr237 with Gln205 and Tyr216 being the most critical residues. Interestingly, alanine substitutions of Asp218 or 269 resulted in 3–5-fold increases in apoptotic activity, although neither mutation significantly affected receptor binding. In addition, residues 131–135 of the AA' loop that penetrate into the central binding interface of the TRAIL–DR5 complex have been shown to contribute to several specific polar interactions that are important for DR5 binding [161].

6.2 Signaling

The apoptosis signal transduction pathway can be triggered by the specific binding of TRAIL to the extracellular domain (ECD) of death receptors DR4/DR5 on the target cell surface. The binding of TRAIL promotes the clustering of these receptors. Then, the cytoplasmic death domains of DR4/DR5 bind to the death domain of the adaptor protein Fas-associated protein with death domain (FADD). FADD in turn recruits procaspase-8 through its death effector domain (DED) in the N terminal. The formed DR4/DR5/FADD/procaspase-8 death-inducing signaling complex (DISC) then leads to the cleavage of procaspase-8 and the formation of active caspase-8 [165–168]. Two different pathways to transmit apoptosis signals are activated by caspase-8: one extrinsic and one intrinsic (mitochondria-dependent) pathway. In the extrinsic pathway, caspase-8 directly activates downstream effector caspase-3, caspase-6, and caspase-7 inducing apoptosis. In the intrinsic pathway, caspase-8 promotes the cleavage of Bid and then activates truncated Bid (tBid) ultimately leading to mitochondrial mediated apoptosis [169].

6.3 Biologics

Because ligation of death domain containing TNFRSF members (such as TNF-R, DR4, and DR5) can induce apoptosis in a p53-independent manner, targeting of these interactions has been considered as an alternative to conventional chemotherapy especially since p53 mutations are frequently found in various tumor types (ranging from 10% to 80%). The TRAIL-DR4/DR5 apoptosis pathway has been actively explored for anti-tumor purposes; a recent detailed review is available [170]. Several biologics including both recombinant TRAIL (e.g., dulanermin, AMG 951) and agonistic antibodies targeting DR4 or DR5, such as mapatumumab (HGS-ETR1), conatumumab (AMG 655), drozitumab (apomab, PRO95780), lexatumumab (HGS-ETR2), tigatuzumab (CS-1008, TRA-8), and others, are in various stages of clinical trials [58, 170].

6.4 Small molecules

6.4.1 Bioymifi—To date, one small-molecule TRAIL-mimetic that targets DR5 has been identified – it was designated as bioymifi (**12**, PubChem CID 70678419; Figure 5) [52]. A library of about 200,000 drug-like compounds was screened in T98G human glioblastoma cells to search for leads capable of promoting cell death in combination with Smac mimetics, which have been shown to increase the cell-killing efficiency of TRAIL [171–173]. One compound, A2C2, was found to show the most robust synergy with the Smac mimetic in inducing cell death with relatively low cytotoxicity. A2C2 turned out to be a mixture of compound A2 and C2 in a molar ratio of 1:9, and the cell death inducing effect of A2C2 seemed to be a result of the combined activity of A2 and C2. Using a structure-activity relationship approach, an A2C2 related compound with enhanced cell-killing activity toward T98G cells was identified and named bioymifi (**12**, Figure 5). Contrary to A2C2, **12** was found to promote cell death without the need for the Smac mimetic in T98G cells and other human cancer cell lines tested. Cell death induced by **12** could be blocked by the pan-caspase inhibitor Z-VAD, suggesting that **12** induces apoptosis. In addition, **12** activated both caspase-3 and caspase-8 while knockdown of caspase-8, but not caspase-9 rescued cells from **12**-induced apoptosis suggesting a critical role of the extrinsic apoptotic

pathway in bioymifi-induced cell death. Subsequent knockdown experiments of different death receptors in T98G cells indicated DR5 as the direct target of **12**. These results suggest that **12** is able to activate DR5 in a manner similar to the TRAIL induced apoptotic pathway. To test if TRAIL is involved in **12**-induced cell death, a TRAIL neutralizing antibody was applied before **12** or soluble recombinant TRAIL. It was found to block TRAIL-, but not **12**-induced cell death. Knockdown of TRAIL by siRNA also could not block the activity of **12** suggesting that **12**-induced apoptosis is independent of TRAIL. Isothermal titration calorimetry experiments demonstrated that **12** binds directly to the ECD of DR5 with a K_d of 1.2 μM , whereas it has little affinity toward the ECD of DR4. Formation of a larger polymer of DR5 upon treatment with **12** was observed in both SDS-PAGE and size-exclusion chromatography, suggesting that oligomerization of DR5 was induced by this compound. Hence, **12** serves as a potential lead for the development of small-molecule TRAIL mimics targeting DR5 for cancer therapy.

7 OX40–OX40L

The OX40-OX40L costimulatory interaction is the latest TNFSF PPI with an identified small molecule modulator. OX40 (CD134, TNFRSF4) was discovered as a cell surface antigen found on activated rat T cells [174]. It was found to be primarily expressed on activated T cells, including different effector CD4^+ T cell populations (Th1, Th2, and Th17) and CD8^+ T cells. In addition, mouse natural $\text{CD4}^+\text{Foxp3}^+$ regulatory T cells constitutively express OX40, whereas its expression can only be induced on human Foxp3^+ regulatory T cells. OX40L (CD252, TNFSF4) was identified on human T leukemia virus type 1 transformed cells [175, 176] and was later found to be expressed on APCs, endothelium, and activated CD4^+ T cells. The main function of the OX40-OX40L ligation initiated signal has been shown to be the promotion of the expansion and survival of conventional T cells. A detailed review of the critical role of OX40 in immunity has been published recently [177]. Due to the importance of OX40-mediated T cell response, blockade of the OX40-OX40L interaction has been shown to control the development of inflammation in several mouse models including asthma, colitis, graft-versus-host disease (GVHD), T1D, MS, RA, transplantation, and leishmaniasis [178–183].

7.1 Structure

The OX40L monomer is a brick shaped jelly-roll β -sandwich that packs together to form flowerlike trimers. It has the lowest level of sequence homology with other TNFSF members (~10%–15%) and is also very compact with only about 132 residues in the entire extracellular region of human OX40L versus about 195 residues for the other TNFSF members [54]. Because no proteolytic site can be found in the linker between the ECD and the transmembrane helix, OX40L is expected to exist only in a membrane bound state [184, 185]. There are two striking structural differences between OX40L and other TNFSF members [186]. First, the OX40L trimer interface is more ‘open’ due to the splayed out monomer arrangement. An approximately 45° angle with respect to the trimer axis, which differs from other TNF ligands by $\sim 15^\circ$ rotation of the monomer, leads to much smaller trimer interfaces ($\sim 2,600 \text{ \AA}^2$) than in other structurally characterized TNF ligands (e.g., $\sim 12,000 \text{ \AA}^2$ in the BAFF trimer). Second [186], “the characteristic ‘tiles’ of alternating

aromatic or hydrophobic residues along the trimer axis first seen in the structure of TNF [187, 188]” are lacking, and “the trimer interface of murine and human OX40L is formed by a very short layer of generally hydrophobic residues from the C strand (Leu102), F strand (Leu138), and the C-terminal tail (Gln175).”

The extracellular domain of OX40 is composed of three full CRD regions and a partial, fourth C-terminal CRD. Three copies of OX40 bind to the trimeric ligand to form the OX40–OX40L complex (Figure 1). While both CRD2 and CRD3 form extensive contacts with OX40L upon binding, additional contacts are made between CRD1 and OX40L. The ligand portion of this interface has been found to be even more discontinuous with 31 human OX40L residues from 11 different secondary structure elements including the unusual C-terminal tail. Such extensive contacts including novel interactions mediated by CRD1 of OX40 and the OX40L C-terminal tail have not been observed in other TNFRSF members. Mutagenesis studies have shown that Phe180 and Asn166 (conserved between human and murine OX40L) are the most critical residues that contribute to receptor binding [186]. Phe180 and Asn166 are located at diagonally opposite ends of the monomer-monomer interface of the OX40L trimer. While Phe180 interacts with a hydrophobic region on hOX40 CRD1, Asn166 forms hydrogen bonds to the backbone of hOX40 residues Trp86 and Cys87 at the juncture of CRD2 and CRD3. In addition, mutational analysis has also shown that the binding energy in the hOX40–hOX40L interface is not concentrated in one location, but is spread out to at least two areas, similar to what was seen for the TRAIL–DR5 interaction [161–163].

7.2 Signaling

Binding of OX40L to OX40 leads to trimerization of OX40 and recruitment of TRAF2, 3, and 5 at its cytoplasmic tail [189, 190] through a QEE motif that is present in other family members [191]. From here, OX40 can act either as an independent receptor or as a classic costimulatory molecule dependent on the presence or absence of the TCR signal. As an independent receptor, the complex of the OX40 and TRAF proteins is able to activate the NF- κ B1 pathway, which leads to the expression of several antiapoptotic Bcl-2 family members including Bcl-2, Bcl-xL, and Bfl-1 [189, 190, 192, 193]. When the TCR signal is present, OX40 is able to synergize with TCR to promote Akt activation as well as NFAT accumulation [194].

7.3 Biologics

Oxelumab, a fully humanized anti-human-OX40L neutralizing antibody (huMAb) has completed efficacy and safety testings for the treatment of allergen-induced mild asthma in a Phase II clinical trial. On the other hand, OX40 stimulation has been explored as a possible cancer treatment due to its stimulatory activity on T and NK cells [70]. Antitumor responses have been observed in both OX40 monotherapy [195–197] and in combination approaches [198–201]. Particularly, OX40 stimulation combined with cyclophosphamide has been shown to induce tumor regression even in the poorly immunogenic B16 murine melanoma [202]. Currently, two Phase II clinical trials are in progress to evaluate OX40 agonists as both a monotherapy for breast cancer in patients with metastatic lesions (NCT01862900)

and combination therapy with stereotactic radiation and/or cyclophosphamide in patients with progressive metastatic prostate cancer (NCT01303705).

7.4 Small-molecule modulators

7.4.1 CVN and related compounds—During the exploration of possible small-molecule inhibitors of OX40-OX40L interaction, we have recently identified four compounds, including **13** and **14** (Figure 5) capable of blocking the OX40–OX40L binding in a modified ELISA assay [42]. These compounds not only successfully inhibited the binding of recombinant hOX40L protein to the plate bound hOX40 with IC₅₀s in the low micromolar range (~2 μM), but also showed acceptable target specificities when compared to several other ligand–receptor pairs within the TNFSF (RANK-RANKL, 4-1BB-4-1BBL, CD40-CD40L, and TNFR1-TNF-α) in similar experimental settings as the OX40-OX40L binding assay. Subsequent studies in an OX40 expressing NF-κB reporter HEK cell line, similar to those used previously [29, 203], indicated that **13** acts as a partial agonist [204, 205]. It produces only a lower maximum activation than the natural ligand OX40L, and in the presence of sufficiently high OX40L, it actually slightly decreases activation. Data from this experiment could be nicely fitted with a generalized minimal ‘two-state theory’ (del Castillo–Katz) model for receptor activation [42]. Together, these results suggest that **13** binds to OX40 to interfere with its binding to OX40L, but it also stimulates OX40 to initiate downstream signaling by itself; i.e., it acts as a partial agonist. It was also shown [42] that “under polarizing conditions based on TGF-β *in vitro*”, 50 μM of **13** (a concentration that is close to the concentration able to achieve maximum level of NF-κB activation by itself) “successfully mimicked the effect of an agonistic anti-OX40 antibody in suppressing regulatory T-cell generation and diverting CD4⁺CD62L⁺Foxp3[−] cells to TH9 phenotype,” but to a lesser extent.

8 CONCLUSIONS

There are many cell surface PPIs within the TNF superfamily that represent high-value therapeutic targets with several biologics in various phases of clinical development. Small-molecule inhibitors or activators could represent possible alternatives, and compounds proving the feasibility of such approaches have been identified through different drug discovery approaches for a number of these PPIs including CD40-CD40L, BAFFR-BAFF, TRAIL-DR5, and OX40-OX40L. While none of these small-molecule modulators identified so far seems promising enough to be pursued for clinical development, they provide proof-of-principle evidence that these PPIs are susceptible to small-molecule modulation and can serve as starting points toward the identification of more potent and selective candidates.

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Abbreviations

CRD cysteine-rich domains

ECD	extracellular domain
GPCR	G-protein coupled receptors
HTS	high-throughput screening
IBD	inflammatory bowel disease
JNK	c-Jun N-terminal kinase
MS	multiple sclerosis
PPI	protein-protein interaction
RA	rheumatoid arthritis
SLE	systemic lupus erythematosus
T1D	type 1 diabetes
TCR	T cell receptor
TNF	tumor necrosis factor
TNFSF	TNF superfamily
TRAF	TNFR-associated factor

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Figure 1.

Three-dimensional structures showing the interacting trimeric structures for human CD40–CD40L, TRAIL–DR5, and OX40–OX40L from two different perspectives – a side view (top row) and a 90°-rotated top view (bottom row). Ribbon rendering of crystal structures are shown for PDB IDs 3QD6, 1D4V, and 2HEV, respectively with the ligands shown in reddish and the receptors shown in blueish colors. The crystal structure of CD40–CD40L is lacking one of the CD40 monomers.

Figure 2.
Some of the small-molecules shown to inhibit the TNF-TNFR PPI.

Figure 3.
Small-molecules shown to inhibit the CD40-CD40L costimulatory PPI.

Figure 4.

Structure of the CD40L (CD154) trimer as distorted by binding of **10**, an intercalating small-molecule disruptor (PDB structure 3KLJ [27]). Such intercalation between two surfaces might allow much better interaction along the entire surface of the molecule than possible at the relatively small pockets on the transient PPI surfaces; hence, it could make possible much more adequate and specific binding. If sufficient conformational change is caused to hinder binding of the protein partner (here, CD40), effective PPI inhibition is achieved via an allosteric mechanism.

BAFFR-BAFF

11

KR33426

TRAIL-DR4/5

12

bioymifi

OX40-OX40L

13

chlorazol violet N

14

direct black 36

Figure 5.
Selected small-molecules modulators of the BAFFR-BAFF, TRAIL-DR5, and OX40-OX40L PPIs.