Small-Molecule Probes for the Modulation of Ligand-Receptor Interactions within the TNF Superfamily

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SMALL-MOLECULE PROBES FOR THE MODULATION OF LIGAND-RECEPTOR INTERACTIONS WITHIN THE TNF SUPERFAMILY

By

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A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Coral Gables, Florida

May 2015
A dissertation submitted in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

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The activation and clonal expansion of T cells requires not just engagement of the T cell receptor (TCR), but also a functionally defined second co-stimulatory signal. Interactions between several co-stimulatory molecules within the tumor necrosis factor (TNF) superfamily (TNFSF) expressed by T cells and various other immune and non-immune cell types are central to T-cell function. Modulation of these interactions holds remarkable therapeutic promise as confirmed by various animal models of inflammatory and autoimmune diseases, transplant rejections, and cancer. Following the recent identification of the first small-molecule inhibitors of the CD40–CD40L co-stimulatory protein-protein interaction (PPI) in our group, the present work focuses on the identification of compounds with modulatory activity towards several other important ligand–receptor interactions within the TNF superfamily and, in particular, the OX40–OX40L interaction.

Using a series of ELISA-type screening assays with recombinant human TNFSF proteins of interest, compounds capable of inhibiting the OX40–OX40L, RANK–RANKL, 4-1BB–4-1BBL, TNFα–TNF-R1, GITR–GITRL, and TRAIL-R1–TRAIL interactions have been identified. To follow up on the promising activity obtained in the OX40–OX40L assay, structure–activity relationships have been
investigated in more detail, including computational screening experiments using available three-dimensional OX40 structures, and promising lead compounds have been identified.

To elucidate the mechanism of the observed OX40–OX40L inhibition in this cell-free assay, the nature and mechanism of the binding were investigated, and these indicated a competitive and reversible binding with OX40 and not OX40L as the binding partner.

To confirm and characterize activity and specificity, OX40-transfected sensor cells with NF-κB reporters were constructed and used. Interestingly, several compounds that inhibited OX40-OX40L binding in the cell-free assay, showed a concentration-dependent enhancement of the OX40L-induced NF-κB activation in these sensor cells, but not in the other TNFSF ligand sensors tested (i.e., TNFα and CD40L). Intriguingly enough, these compounds were able to cause NF-κB activation in concentration-dependent manner even without the presence of OX40L, but not to the same maximum activation level as can be achieved by OX40L. Hence, these cell assays indicated these compounds are partial agonists with low micromolar potency and adequate selectivity. Furthermore, the activity of our most promising compound (chlorazol violet N, CVN) has been confirmed in ex vivo T cell polarization assays, where it successfully mimicked the effects of an agonistic anti-OX40 antibody in suppressing regulatory T-cell generation and in diverting CD4+CD62L+Foxp3− cells to TH9 phenotype in vitro. CVN has also shown some activity in preventing hyperglycemia in an exploratory NOD mouse study, a widely used animal model
for type 1 diabetes (T1D). Taken together, these results have validated our organic dye–based small molecule library as a reasonable starting point to identify small-molecule modulators for immunologically relevant protein-protein interactions within the TNFSF. Furthermore, the present discovery of the first small-molecule partial agonists for OX40 provides proof-of-principle evidence for the feasibility of small-molecule modulation of the OX40-OX40L co-stimulatory interaction, and should lead to new pharmacological tools to study OX40 mediated immune responses.
ACKNOWLEDGEMENTS

I thank my father He Song, a physics teacher, for inspiring me with the curiosity of science since my childhood. I thank my mother Shuqiong Dai and my father for being the pillar of strength. I dedicate this work to Dr. Peter Buchwald. I thank him for his mentorship, which not only shaped my rigorous attitude towards conducting scientific research, but also helped the improvement of my personality that will definitely benefit my future career. I thank all the family members in Buchwald lab for their support to my scientific research, especially Dr. Emilio Margolles-Clark, Dr. Lakshmi Ganesan, and Dr. Sirlene Cechin for their counseling and good moment we have shared. I am grateful to Dr. Allison Bayer for her coaching and generous support to the mouse regulatory T cell experiment. I thank all the members in Slepak lab for their support to my protein purification project, especially Dr. Konstantin Levay for sharing his invaluable experience. I thank Dr. Bonnie Blomberg and Dr. Ana Marie Landin for giving me the opportunity to learn and conduct human B cell experiment. I thank my committee members, Dr. Charles Luetje, Dr. Grace Zhai, and Dr. Bonnie Blomberg for their guidance to my research project. Many thanks to Dr. Charles Luetje for his detailed revision of my Ph.D. thesis. I am grateful to Dr. Michelle Arkin for being my external committee member and her invaluable suggestions to my scientific reasoning. I also thank my girlfriend Dr. Xiaoyu Wang for all the things we shared that has transformed into strength indispensable to my Ph.D. study. Finally, I feel fortunate to have everyone who has helped me on my journey to pursue my doctoral degree.
# TABLE OF CONTENTS

LIST OF FIGURES...........................................................................................................................................vi

CHAPTER 1  BACKGROUND AND SIGNIFICANCE.......................................................................................... 1
  1.1 SIGNIFICANCE ................................................................................................................................. 1
  1.2 PROTEIN–PROTEIN INTERACTIONS: THE PROMISE OF ALTERNATIVE DRUG TARGETS ..................... 2
    1.2.i Challenges in small-molecule modulation of PPIs ........................................................................ 2
    1.2.ii Targeting protein–protein interactions using small molecules............................................. 4
  1.3 TNF SUPERFAMILY AND COSTIMULATORY SIGNALING................................................................. 9
    1.3.i TNF superfamily – a valuable therapeutic target ................................................................. 9
    1.3.ii Costimulatory signaling ........................................................................................................ 11
    1.3.iii CD40–CD40L ....................................................................................................................... 13
    1.3.iv BAFFR–BAFF ....................................................................................................................... 17
    1.3.v TRAIL–DR5 ........................................................................................................................... 21
    1.3.vi OX40–OX40L ....................................................................................................................... 25
  1.4 SMALL MOLECULE MODULATION OF TNFSF .............................................................................. 31
    1.4.i TNF-α–TNFR1 inhibitors ......................................................................................................... 31
    1.4.ii CD40–CD40L inhibitors ......................................................................................................... 35
    1.4.iii BAFF–BAFFR inhibitors ....................................................................................................... 39
    1.4.iv TRAIL–DR5 (DR5 activators) .............................................................................................. 42
  1.5 EXPLORATION OF THE CHEMICAL SPACE OF ORGANIC DYES ................................................. 43

CHAPTER 2 MATERIALS AND METHODS................................................................................................. 49
  2.1 SMALL-MOLECULE SCREENING ASSAY ....................................................................................... 49
  2.2 COMPUTATIONAL METHODS ....................................................................................................... 50
    2.2.i Virtual screening of small molecule libraries toward OX40 .................................................... 50
  2.3 BIOCHEMICAL ASSAYS ............................................................................................................... 51
    2.3.i Identification of the binding partner ....................................................................................... 51
    2.3.ii Gaddum-Schild EC$_{50}$ shift ................................................................................................ 51
    2.3.iii Binding Kinetics ................................................................................................................. 52
  2.4 CELL-BASED ACTIVITY ASSAYS ................................................................................................. 53
    2.4.i Toxicity assays ....................................................................................................................... 53
    2.4.ii Cell transfection ................................................................................................................... 54
    2.4.iii NF-κB reporter assay .......................................................................................................... 55
    2.4.iv THP-1 cell activation assay ................................................................................................ 56
2.4.v TNF-α mediated JNK phosphorylation ................................................................. 57
2.4.vi Human T cell assay .......................................................................................... 58
2.4.vii Mouse T cell assay .......................................................................................... 59
2.5 NOD MOUSE TREATMENT ........................................................................... 61
2.6 DATA FITTING AND STATISTICS....................................................................... 62

CHAPTER 3 SEARCH FOR SMALL MOLECULE MODULATORS WITHIN TNFSF ........................................................................................................................................... 64
3.1 SCREENING FOR MODULATORS OF LIGAND-RECEPTOR PAIRS WITHIN THE TNFSF .................................................................................................................................................. 64
3.1.i Inhibitory activity of selected ligand-receptor pairs within the TNFSF ......... 64
3.2 INHIBITION OF THE OX40–OX40L BINDING ..................................................... 68
3.2.i Inhibitory activity towards OX40–OX40L interaction .................................... 68
3.2.iii Virtual screening of drug-like small molecule libraries toward OX40–OX40L interaction ...................................................................................................................................................... 74

CHAPTER 4 DEVELOPMENT OF FUNCTIONAL ASSAYS FOR SMALL MOLECULE MODULATOR ACTIVITY VERIFICATION ........................................................................... 78
4.1 CYTOTOXICITY ASSESSMENT ........................................................................ 78
4.1.i BrdU incorporation assay .................................................................................. 78
4.1.ii DAPI exclusion assay ....................................................................................... 79
4.2 CONSTRUCTION OF TNFSF-ACTIVATED NF-ĸB CELLS .............................. 80
4.3. OX40 SIGNALING ENHANCES MOUSE NATURE REGULATORY T CELL PROLIFERATION IN VITRO ................................................................................................................................. 81

CHAPTER 5 IDENTIFICATION OF THE FIRST SMALL MOLECULE OX40 PARTIAL AGONISTS ......................................................................................................................... 85
5.1 BINDING PARTNER IDENTIFICATION ................................................................... 85
5.2 MECHANISM OF OX40–OX40L BINDING INHIBITION ................................ 85
5.3 EFFECTS IN SENSOR CELLS WITH NF-ĸB REPORTER ................................. 88
5.3.i Identification of OX40L mediated NF-ĸB activation enhancement by small molecules in OX40 sensor cell system ......................................................................................................................... 87
5.3.ii Identification of OX40-specific small molecule agonists ............................ 88
5.4 NAIVE CD4+ T CELL POLARIZATION IN VITRO .......................................... 90
5.4.i CVN mimics the effect of OX86 to block iTReg induction ......................... 90
5.4.ii CVN mimics the effect of OX86 to enhance Th9 cell generation ............... 91
5.5 EXPLORING THE AGONISTIC NATURE OF OX40 SMALL MOLECULE AGONIST ........................................................................................................... 93
5.6 PREVENTION OF T1D WITH AGONISTIC OX40 SIGNALING: EXPLORATORY NOD STUDY ........................................................................... 97

CHAPTER 6  DISCUSSION ............................................................................... 99
6.1 FEASIBILITY OF SMALL MOLECULE MODULATION OF PPI ............... 99
6.2 IDENTIFICATION OF THE FIRST SMALL MOLECULE OX40 AGONISTS 105
6.3 SUMMARY ................................................................................................. 113
REFERENCES ................................................................................................. 116
LIST OF FIGURES

CHAPTER 1
Figure 1-1: Receptor–ligand interactions vs. protein–protein interactions .......... 4
Figure 1-2: Representative ligand-receptor interactions within the TNF superfamily ........................................................................................................................................ 11
Figure 1-3: Two-step OX40L costimulation model ............................................. 26
Figure 1-4: Chemical structures of some of the small-molecules shown to inhibit the TNF-TNFR PPI ........................................................................................................................................ 34
Figure 1-5: Small-molecules shown to inhibit the CD40-CD40L costimulatory PPI ................................................................. 37
Figure 1-6: Structure of the CD40L (CD154) trimer as distorted by binding of 1-10, an intercalating small-molecule disruptor (PDB structure 3KLJ (Silvian, Friedman et al. 2011)) ........................................................................................................... 40
Figure 1-7: Small-molecules modulators of the BAFFR-BAFF and TRAIL-DR5 PPIs ........................................................................................................................................ 43
Figure 1-8: Representative chemical structures for active compounds identified so far in our organic dye-based chemical library showing good PPI inhibitory activity ........................................................................................................................................ 45

CHAPTER 2
Figure 2-1: ELISA based screening assay .......................................................... 49

CHAPTER 3
Figure 3-1: Concentration-dependent inhibition of the BAFF–BAFF-R PPI ......... 65
Figure 3-2: Concentration-dependent inhibition of the TNFα–TNFR1 PPI .......... 65
Figure 3-3: Concentration-dependent inhibition of the 4-1BBL–4-1BB PPI .......... 66
Figure 3-4: Concentration-dependent inhibition of the RANKL–RANK PPI ....... 66
Figure 3-5: Concentration-dependent inhibition of the GITRL–GITR PPI ........... 67
Figure 3-6: Concentration-dependent inhibition of the TRAIL–TRAILR PPI ....... 67
Figure 3-7: Concentration-dependent inhibition of the OX40L–OX40 PPI ........... 68
Figure 3-8: Consensus motifs of active compounds for the inhibition of OX40–OX40L binding ........................................................................................................................................ 69
Figure 3-9: Inhibition of selected human TNFSF PPIs by active compounds with consensus motif A ........................................................................................................................................ 70
Figure 3-10 Inhibition of mouse OX40–OX40L PPI by active compounds with consensus motif A ........................................................................................................................................ 71
Figure 3-11 Inhibition of selected human TNFSF PPIs by active compounds with consensus motif B ........................................................................................................................................ 72
Figure 3-12: Predicted druggable sites for OX40 and the OX40–OX40L complex ........................................................................................................................................ 75
Figure 3-13: Glide-XP docking and scoring of pancratistatin at the binding site of OX40–OX40L complex

CHAPTER 4
Figure 4-1: Cell toxicities of the present compounds assessed by a standard proliferation assay (BrdU)
Figure 4-2: Cell toxicities of the present compounds assessed by a standard apoptosis assay (DAPI exclusion)
Figure 4-3: Chimerical constructs of OX40 HEK-Blue sensor cells
Figure 4-4: Specificity of NF-κB activation in OX40 sensor cell line
Figure 4-5: OX40 signaling enhances the survivability and proliferation of natural Treg (nTreg) in vitro

CHAPTER 5
Figure 5-1: Compounds 3-1–3-4 bind to OX40, not OX40L
Figure 5-2: Schild analysis of the interference with the OX40–OX40L binding for compound 3-4 (CVN)
Figure 5-3: Confirmation of activity for the present compounds of interest in OX40-expressing NF-κB reporter sensor cells
Figure 5-4: Selective stimulatory activity of 3-1 (DB36) and 3-4 (CVN) in OX40-transfected NF-κB sensor cells (a) as compared to total lack of such effect in similar CD40 (b) and TNF-R1 (c) sensor cells
Figure 5-5: CVN inhibits iTreg generation
Figure 5-6: CVN enhances the induction of TH9 cells
Figure 5-7: Activation of OX40 NF-κB reporter cells by OX40L and 3-4 combined at various concentrations
Figure 5-8: Quantification of receptor activation for competitive partial agonists in the presence of a full agonist using the minimal two-state receptor model
Figure 5-9: Results of the pilot NOD diabetes prevention study with OX40 agonists as adjuvants

CHAPTER 6
Figure 6-1 Illustration of the effects produced by combinations of a full and a partial agonist at different concentrations (C1 and C2, respectively)
CHAPTER 1 BACKGROUND AND SIGNIFICANCE

1.1 SIGNIFICANCE

Type 1 diabetes mellitus (T1DM) is a chronic autoimmune disease during which the insulin-secreting pancreatic β-cells are selectively destroyed. Insulin administration is required to maintain life. So far, all clinical trials investigating the possibility of stopping or reverting the decline of the residual β-cell mass through induction of immunologic tolerance have failed (Skyler and Ricordi 2011) and a cure is difficult because of the significant loss of β-cells that already occurred by the time of clinical manifestation. In certain patients, islet transplantation from healthy donors could serve as an alternative therapy to control disease progression through the normalization of metabolic control (Ricordi and Strom 2004). However, it requires life-long immunosuppression, and the deleterious side effects of existing therapies, including an increased risk of opportunistic infection, malignancy, and metabolic disorders have limited such procedures to the most severe forms of diabetes. In addition, although the short-term allograft survival rates have been significantly improved, chronic allograft loss is still unavoidable. T-cell mediated alloimmune response is a key factor that drives pathological development of chronic rejection in islet transplantation and co-stimulatory molecules of the tumor necrosis factor (TNF) superfamily (TNFSF) have been shown to play key roles in the T cell response following alloantigen presentation (Adams, Larsen et al. 2002, Li, Rothstein et al. 2009). Thus, co-stimulatory modulation within the TNFSF seems to hold promise for the treatment of chronic rejection primarily through the prevention of T cell activation. Indeed,
currently numerous active clinical trials are ongoing in various therapeutic fields, including transplant rejection, inflammation, autoimmunity, oncology, and bone diseases to assess the therapeutic value of modulating almost every TNFSF receptor–ligand pair (Tansey and Szymkowski 2009). A pivotal advantage of TNFSF modulation is related to the inducible expression nature of several molecules including OX40 and 4-1BB during the active phase of diseases, as this should allow more specific targeting avoiding the side effects associated with most existing therapies. Essentially all current trials are using biologics (i.e., monoclonal antibodies – mAbs), which bear unavoidable risks such as those due to risk of acute anaphylaxis and cytokine storm. Therefore, I am conducting research to identify possible small-molecule modulators of immunologically relevant protein-protein interactions (PPIs) within the TNFSF to achieve improved immunosuppression in islet transplantation as well as other possible therapeutic applications.

1.2 PROTEIN–PROTEIN INTERACTIONS: THE PROMISE OF ALTERNATIVE DRUG TARGETS

1.2.i Challenges in small-molecule modulation of PPIs

Protein–protein interactions (PPIs) are central to most biological processes. In a given organism, the size of the PPI interactome is thought to be the best indicator of biological complexity, and the human interactome is estimated to include as many as ~650,000 distinct pair-wise interactions (Stumpf, Thorne et al. 2008). PPIs have started to be recognized as therapeutic targets of greater chemical and structural diversity than kinases and proteases that
represent most classical drug targets in the biological system. Despite the obvious therapeutic importance of targeting PPIs, this task represents a major challenge due to the composition and topography of PPI interfaces that made PPIs to be traditionally classified as “undruggable”. A protein target is usually defined as “druggable” by its ability to bind a drug-like small molecule. This is characterized by the structural and chemical features of the target binding site, and is often known as “ligandability” (Edfeldt, Folmer et al. 2011). Protein-protein interfaces in this respect are traditionally considered “unligandable” because of the following aspects:

1) PPIs involve large (~1600 Å²) buried interfaces (Jones and Thornton 1996, Lo Conte, Chothia et al. 1999) compared with those that are involved in protein–small molecule interactions (~300–1000 Å²) (Smith, Hu et al. 2006, Cheng, Coleman et al. 2007);

2) on the contrary to enzyme-substrate interaction scenario, natural PPI binding partners do not provide direct clues to the design of small molecules, and the key interacting residues are often ambiguous;

3) the binding regions of PPI interfaces are often discontinuous, flat, and relatively featureless making it much harder to simultaneously form a large number of individual inter-atomic interactions required to achieve high affinity (Whitty and Kumaravel 2006).
Traditional drug targets have well-defined binding pockets of sufficient concavity to concentrate a large number of interactions over a small surface area. PPIs have flat and featureless topologies exhibiting great conformational dynamics and surface adaptivity to accommodate one or more protein partners, whose interactions distribute over large surface areas. (Whitty and Kumaravel 2006).

1.2.ii Targeting protein–protein interactions using small molecules

1.2.ii.a Small molecule inhibitors

Identification of small-molecule inhibitors of PPIs using conventional drug-discovery processes has proved to be challenging mainly due to the different ‘chemical space’ of traditional drug molecules. However, several examples of potent inhibitors of PPI have been reported recently. MDM2 (murine double minute 2) is an ubiquitin E3 ligase responsible for the degradation of p53. Targeting PPI of the MDM2/p53 complex should lead to increased p53 level and activity. A family of potent and selective small-molecule antagonists of MDM2
was identified from a high-throughput screen with the most potent derivative Nutlin-3 showing an IC_{50} (half maximal inhibitory concentration) value of 90 nM. Crystal structures of complexes showed that these compounds bind MDM2 in the p53-binding pocket, and subsequent assays have suggested the ability of these inhibitors to activate the p53 pathway in cancer cells, leading to cell cycle arrest, apoptosis, and growth inhibition of human tumor xenografts in nude mice model (Garcia-Echeverria, Chene et al. 2000, Vassilev, Vu et al. 2004). In a similar approach, researchers at Johnson & Johnson Pharmaceuticals have identified a benzodiazepinedione family able to disrupt the p53/MDM2 PPI and decrease the proliferation of tumor cells with high affinity for MDM2 at an IC_{50} value of 420 nM (Parks, Lafrance et al. 2005, Raboisson, Marugan et al. 2005). In all cases, these ligands were able to reproduce the spatial projection and functionality of the anchoring indole ring of the p53 peptide and at least one other recognition side chain. The MDM2/MDMx (murine double minute x) heterodimer has also been shown to be involved in the degradation of p53. Results indicated that selectivity of binders toward MDM2 over MDMx could contribute to improved efficacy and overcoming resistance in certain circumstances (Khoo et al, 2014). Existing examples of MDM2 binders also included a new family of spirooxindoles identified by Wang and co-workers using virtual screening with MI-219 as the most potent compound of the series with a K_i (inhibition constant) value of 5 nM and a 10,000-fold selectivity in targeting the MDM2 protein over MDMX (Ding, Lu et al. 2005, Yu, Qin et al. 2009) and a potent and selective piperidinone MDM2 inhibitor (IC_{50} ≈ 1 nM) with good oral bioavailability identified using structure-
based rational design by Sun and coworkers (Rew, Sun et al. 2012). More recently, after performing binding pocket prediction on the VEGFR-1 D2 domain, Gautier and coworkers have identified potent VEGFR-1–VEGF interaction inhibitors using virtual screening. These small compounds contain (3-carboxy-2-ureido) thiophen units and have IC$_{50}$ values in the low micromolar range, with the most potent compound able to inhibit the VEGF-induced VEGFR-1 transduction pathways (Gautier, Miteva et al. 2011).

Fragment-based screening represents a modern drug discovery tool, and it is perceived as particularly useful for the identification of PPI modulators (Hajduk and Greer 2007). Compound Ro26-4550 was initially designed as a peptidomimetic of interleukin 2 (IL-2); later, it was found to bind to IL-2 and block the binding of IL-2 receptor with an IC$_{50}$ of 3-6 μM (Tilley, Chen et al. 1997, Emerson, Palermo et al. 2003). Structure and thermodynamic studies have shown that the structure of the binding site of Ro26-4550 to IL-2 is inherently flexible, and a binding groove is captured upon accommodating Ro26-4550 (Mott, Baines et al. 1995, Arkin, Randal et al. 2003, Hyde, Braisted et al. 2003, Thanos, Randal et al. 2003). Thus, unlike structure-based design, fragment-based screening methods have advantages in targeting protein surfaces with adaptable regions. Later, by employing a novel “tether” technique, James Wells’ group has further developed a series of strong IL-2 binders with SP-4206 having the best affinity with an IC$_{50}$ value of 60 nM (Erlanson, Braisted et al. 2000, Braisted, Oslob et al. 2003). Researchers at Abbott Laboratories used a fragment-based SAR (structure-activity relationship) coupled with an NMR spectroscopic method
to generate a library of inhibitors for the Bcl-xL/Bak PPI. Among the generated compounds, ABT-737 was found to bind in the Bak binding cleft with a high affinity for Bcl-xL ($K_i$ value of 0.6 nM) (Bruncko, Oost et al. 2007). Improvements to the oral efficacy of ABT-737 resulted in the identification of ABT-263 as a potent orally bioavailable analog with affinity for Bcl-2 family proteins ($K_i < 1$ nM) (Tse, Shoemaker et al. 2008), and recently reported optimized derivatives of this inhibitor have shown to inhibit cell growth in cancer lines with IC$_{50}$ values of 60–90 nM (Zhou, Aguilar et al. 2012, Zhou, Chen et al. 2012). A newly published report by Stephen Fesik’s group showed another example of using fragment-based method for the design of replication protein A (RPA) N-terminal domain PPI inhibitors. Fragment hits that bind to two adjacent sites in the basic cleft of RPA70N have been identified by NMR spectroscopy. Subsequent high-resolution X-ray crystal structures of RPA70N-ligand complexes revealed how these fragments bind to RPA and guided the design of linked compounds that simultaneously occupy both sites with sub-micromolar affinity and minimal disruption of the interaction of RPA with ssDNA (Frank, Feldkamp et al. 2013).

1.2.ii.b Small molecule agonists

The 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. Until now, only a very limited number of small-molecule PPI ‘agonists’ (i.e., enhancers or stabilizers) has been identified. Two different modes of action have been recognized so far for direct PPI stabilizers (Thiel, Kaiser et al. 2012).
1) The stabilizing molecule first binds to one of the proteins thereby creating or modifying the interaction surface for the second protein. This stabilizing effect can be so strong that two proteins that do not bind to each other in the absence of these molecules can be induced to dimerize. This extreme case is observed for the FK506 binding protein (FKBP) binding natural compounds FK506 (Harding, Galat et al. 1989, Siekierka, Hung et al. 1989, Griffith, Kim et al. 1995, Kissinger, Parge et al. 1995) and rapamycin (Choi, Chen et al. 1996, Banaszynski, Liu et al. 2005).

2) The stabilizing molecule directly binds to the edge of an already established protein–protein interface and increases the binding affinity of the corresponding complex. Such a binding mode represents the molecular basis of action of small molecules such as forskolin (Sunahara, Dessauer et al. 1997, Tesmer, Sunahara et al. 1997, Zhang, Liu et al. 1997), fusicoccin A, epibestatin, and pyrrolidone (Wurtele, Jelich-Ottmann et al. 2003, Ottmann, Marco et al. 2007, Rose, Erdmann et al. 2010).

In addition, other PPI agonists with uncharacterized molecular mechanisms have also been documented. One example is the small-molecule nonpeptidyl-mimic of granulocyte-colony-stimulating factor (GCSF) identified by a high-throughput cell-based screen. This compound (SB247464) is an agonist of the GCSF protein with 30% efficacy in a luciferase assay. Like G-CSF, SB247464 caused tyrosine phosphorylation of both JAK1 and JAK2 as well as of G-CSFR, but not of the interleukin-3 (IL-3) receptor, indicating specific recognition of the target (Tian, Lamb et al. 1998). Further studies showed that SB247464
stimulates primary murine bone marrow cells to form granulocytic colonies and elevates peripheral blood neutrophil counts in mice. Collectively, these data demonstrated that SB247464 acts as a mimetic of G-CSF to activate G-CSF receptors by targeting a different domain of G-CSFr. Another compound, L-783281 – a natural product from a fungal extract, was identified as an agonist of the human insulin receptor tyrosine kinase (IRTK). It was selected from a 50,000-compound library including synthetic compounds and natural products and was able to induce 50% of the maximal effect of insulin on IRTK activity at low concentration (3-6 μM). The in vivo efficacy of L-783281 was tested in mice, and it showed a significant decrease in blood glucose levels (Hubbard 1997).

1.3 TNF SUPERFAMILY AND COSTIMULATORY SIGNALING

1.3.i TNF superfamily – a valuable therapeutic target

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 (Locksley, Killeen et al. 2001, Bodmer, Schneider et al. 2002, Aggarwal 2003, Bossen, Ingold et al. 2006, Tansey and Szymkowski 2009, Croft, Benedict et al. 2013). 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 1-2. 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. Biologics including a wide range of products such as vaccines and recombinant therapeutic proteins are used to treat a variety of medical conditions. 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 immune-pharmacology success stories (Palladino, Bahjat et al. 2003). 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 (Palladino, Bahjat et al. 2003, Tansey and Szymkowski 2009, Croft, Benedict et al. 2013). Currently, there are six biologics blocking TNF (TNFSF2) or LTα (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 (LTα, TNFSF1), infliximab, etanercept, 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 (Croft, Benedict et al. 2013).
Co-stimulatory and co-inhibitory signaling play crucial roles in T cell biology as they determine the functional outcome of the T cell receptor (TCR) signaling (Chen and Flies 2013). Several TNFSF ligand–receptor pairs provide important co-signaling (co-stimulatory as well as co-inhibitory signaling) interactions that regulate T cell activation and, hence, are promising therapeutic targets in autoimmune diseases, in transplant recipients as well as in cancers (Locksley, Killeen et al. 2001, Aggarwal 2003, Chen and Flies 2013, Yao, Zhu et al. 2013). Co-stimulatory 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 (Gao, Demirci et al. 2003, Vincenti and Luggen 2007, Weaver, Charafeddine et al. 2008, Li, Rothstein et al. 2009, Peters, Stunz et al. 2009). Cell surface co-stimulatory receptor-ligand interactions belong to two main families: the immunoglobulin superfamily (CD28–CD80/86 and ICOS–ICOS-L) and the TNF–TNFR superfamily. Co-stimulation of T-cell activation has been reported for several members of the TNFR–TNF super-family (Locksley, Killeen et al. 2001, Croft 2003, Watts 2005, Croft 2009, Li, Rothstein et al. 2009), and all members play important roles in various aspects of the immune response (Locksley, Killeen et al. 2001, Aggarwal 2003, Yao, Zhu et al. 2013). Because most 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 (Li, Rothstein et al. 2009). Interaction of the TNF family ligands with their corresponding receptors leads to recruitment of TNFR-associated factors (TRAF), which initiate signaling cascades resulting in T-cell activation (Watts 2005). 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–CD40L (CD154) pathways (Gao, Demirci et al. 2003, Vincenti and Luggen 2007), but there is an increasing recognition of the role played by other members as well (Li, Rothstein et al. 2009).
1.3.iii 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 (Foy, Aruffo et al. 1996). 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 (Graf, Muller et al. 1995, Mazzei, Edgerton et al. 1995). 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 (Henn, Slupsky et al. 1998). 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 (Peters, Stunz et al. 2009) and cardiovascular disease (Pamukcu, Lip et al. 2011). 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.
1.3.iii.a Structure

Like most ligands within the TNFSF, CD40L shares little sequence similarity with other members (20–30%) (Bodmer, Schneider et al. 2002), 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 (van Kooten and Banchereau 2000). 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 1-2) (An, Kim et al. 2011).

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 (Singh, Garber et al. 1998). Mutations in CD40L that prevent its binding to CD40 and result in so-called hyper IgM syndrome (HIGM) are mapped on the protein core, trimerization interface, and area of direct CD40 contact (Karpusas, Hsu et al. 1995, An, Kim et al. 2011). 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 (An, Kim et al. 2011). Further investigations suggested a spatial conflict in the Ser132 loop as Ser132 mutation can inhibit complete activation of CD40 even with intact binding of CD40L.

1.3.iii.b 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-κB (nuclear factor-κB), stress activated protein kinase (SAPK), MAPK (mitogen-activated protein kinase), ERK (extracellular signal-regulated kinase), and STAT3 (signal transducers and activators of transcription-3) (Bishop and Hostager 2001, Aggarwal 2003, Elgueta, Benson et al. 2009). 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 (Grassmé, Bock et al. 2002). 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 B-cells in delivering anti-apoptotic signals (Bishop and Hostager 2003, Dempsey, Doyle et al. 2003, Chatzigeorgiou, Lyberi et al. 2009). Notably, some small-molecule inhibitors of the CD40–TRAF6 interaction that can improve obesity-associated insulin resistance have been identified recently (Chatzigeorgiou, Seijkens et al. 2014) as well as some small-molecule
inhibitors of the CD40-mediated NF-κB signaling pathway (through HTS using NF-κB luciferase reporter assay in BL2 cells activated with trimerized CD40 ligand) (Li, Diogo et al. 2013); the latter, however, seem more likely to be possible downstream regulators than direct CD40-CD40L inhibitors.

1.3.iii.c Biologics targeting CD40–CD40L

CD40 and CD40L were the first TNFSF co-stimulatory molecules to be identified. Blocking of this PPI is a proven highly effective immunomodulatory therapy (Burkly 2001, Daoussis, Andonopoulos et al. 2004, Quezada, Jarvinen et al. 2004, Elgueta, Benson et al. 2009). 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, CP 870893 (Croft, Benedict et al. 2013). 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). 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 thrombolic side effects (Kawai, Andrews et al. 2000, Koyama, Kawai et al. 2004, Roth, Zuckermann et al. 2004), and development is no longer supported (Couzin 2005). Activated platelets express CD40L, but in
platelet-rich plasma, this antibody by itself did not induce platelet aggregation per se and did not significantly affect maximal aggregation. It has been suggested that CD40L 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 (Mirabet, Barrabes et al. 2008). Along these lines, a cyclic heptapeptide (CLPTRHMAC) capable of blocking the CD40–CD40L PPI did not prime human platelet activation and aggregation in in vitro platelet activation studies contrary to the anti-CD154 mAb tested (Deambrosis, Lamorte et al. 2009). Hence, this restrictive side effect might be avoidable 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 (Vaitaitis, Olmstead et al. 2014).

1.3.iv 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 (Moore, Belvedere et al. 1999, Schneider, MacKay et al. 1999, Yu, Boone et al. 2000, Mackay, Silveira et al. 2007, Mackay and Schneider 2009). 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 (Medema, Planelles-Carazo et al. 2003, Dillon, Gross et al. 2006, Bossen, Cachero et al. 2008). Both BAFFR and BCMA deliver positive signals to B cells, whereas TACI acts as an inhibitor receptor for the BAFF signaling (Mackay and Schneider 2008). The BAFF–BAFFR axis is an important therapeutic target primarily due its regulatory role in B cell mediated autoimmune diseases and cancer (Ryan and Grewal 2009, Liu and Davidson 2011, Vincent, Saulep-Easton et al. 2013), and several biologics targeting BAFF are in clinical development.

1.3.iv.a 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 (Karpusas, Cachero et al. 2002, Liu, Xu et al. 2002, Kim, Yu et al. 2003). 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 (Bodmer, Schneider et al. 2002). 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 (Liu, Xu et al. 2002, Cachero, Schwartz et al. 2006).

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) (Kim, Yu et al. 2003). This might confer an edge for the design of small-molecule modulators of the BAFFR–BAFF interaction.

1.3.iv.b Signaling

The BAFF–BAFFR signaling is delivered primarily though the alternative NF-κB2 pathway (Gardam and Brink 2014). Upon BAFF ligation, TRAF3 is recruited to the cytoplasmic domain of BAFFR via a PVPAT binding site (Morrison, Reiley et al. 2005) and degraded. This event rescues the proteasomal
degradation of NF-κB inducing kinase (NIK) (Liao, Zhang et al. 2004), 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 IκB 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 (Batten, Groom et al. 2000, Do, Hatada et al. 2000). 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-κB2 activation (Lavorgna, De Filippi et al. 2009, Zheng, Kabaleeswaran et al. 2010). Besides TRAF mediated NF-κB activation, BAFFR has been shown to activate PI3K (Patke, Mecklenbrauker et al. 2006, Otipoby, Sasaki et al. 2008), which can lead to the downstream activation of AKT, BtK and PKCβ (Shinners, Carlesso et al. 2007, Khan 2009). 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 (Schweighoffer, Vanes et al. 2013).

1.3.iv.c Biologics targeting BAFFR–BAFF

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 (Croft, Benedict et al. 2013). 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 (Stohl and Hilbert 2012). 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.

1.3.v 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 (Wiley, Schooley et al. 1995, Pitti, Marsters et al. 1996). TRAIL participates in modulating various immune functions (Finnberg, Klein-Szanto et al. 2008). 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 (Huang and Sheikh 2007, Fox, Humphreys et al. 2010, Gerspach, Pfenizmaier et al. 2011) 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, CD26, TNFRSF10A) and DR5 (TRAILR2, CD262, TNFRSF10B), can induce cell apoptosis (Nagata 1997, Ashkenazi and Dixit 1998, Wilson, Dixit et al. 2009). Another class of TRAIL receptor includes two ‘decoy’ receptors DcR1 and DcR2 that lack death domains (Degli-Esposti, Dougall et al. 1997). 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 (Huang and Sheikh 2007). The third class is 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 (Emery, McDonnell et al. 1998, Sandra, Hendarmin et al. 2006, Huang and Sheikh 2007, Peng, Liu et al. 2011).

1.3.v.a 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 (Testa 2010). Like most members within the TNFSF, TRAIL forms a trimeric structure with three monomers containing two antiparallel β sheets (Figure 1-2). TRAIL exhibits a high content of aromatic residues providing a hydrophobic platform for the extensive edge-to-face interactions between adjacent monomers (Cha, Kim et al. 1999). 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 (Cha, Kim et al. 1999). This elongated loop forms extensive contact with DR5 and is required for the TRAIL–DR5 binding, which suggests a possible strategy to confer specificity for molecular recognition (Cha, Sung et al. 2000). Another feature of TRAIL is the presence of a solvent inaccessible zinc-binding site located near the tip of the trimerization interface (Hymowitz, Christinger et al. 1999, Cha, Sung et al. 2000). 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 1-2) showed that DR5 contains two organized CRD regions that contribute equally to the binding of TRAIL (Mongkolsapaya, Grimes et al. 1999). Alanine-scanning mutagenesis studies have identified five key residues on TRAIL that are important for receptor binding and biological activity of DR5 (Hymowitz, O'Connell et al. 2000): 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 (Cha, Sung et al. 2000).

1.3.v.b Signaling

The apoptosis signal transduction pathway can be triggered by the specific binding of TRAIL to the extracellular domain (ECD) of death receptor 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
(Kischkel, Lawrence et al. 2000, Sprick, Weigand et al. 2000, Peter and Krammer 2003, Varfolomeev, Maecker et al. 2005). 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 (Green 2005).

1.3.v.c 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 (Micheau, Shirley et al. 2013). 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 (Croft, Benedict et al. 2013, Micheau, Shirley et al. 2013).
1.3.vi OX40–OX40L

1.3.vi.a The OX40–OX40L interaction in effector T cell function

OX40 (CD134) is one of the most important TNF superfamily members. It was discovered as a cell surface antigen found on activated rat T cells (Paterson, Jefferies et al. 1987). The ligand for OX40 (gp34, OX40L, CD252, TNFSF4) was identified on human T leukemia virus type 1 transformed cells (Tanaka, Inoi et al. 1985, Miura, Ohtani et al. 1991, Baum, Gayle et al. 1994) and was later found to be expressed on APCs, endothelium, and activated CD4+ T cells. OX40 is not expressed by resting T cells, including naive and most memory T cells (Croft 2010). The expression of OX40 on CD4 and CD8 T cells is induced by TCR signaling, and peaks starting from 48 hours after *in vitro* and *in vivo* antigen stimulation, respectively (Calderhead, Buhlmann et al. 1993, Gramaglia, Weinberg et al. 1998, Mousavi, Soroosh et al. 2008, Salek-Ardakani, Moutaftsi et al. 2008). Unlike other co-stimulatory receptors and their ligands, such as CD28, which is constitutively expressed by resting T cells, different expression kinetics for CD28 and OX40 suggest that the main role of OX40 is to promote the expansion, proliferation, and survival of effector T cells – whereas CD28 signals are essential for the activation of naive T cells and the generation of effector T cells (Figure 1-3) (Croft 2010).
The interaction between OX40 and OX40L occurs during the T-cell–DC interaction, one to three days after Ag recognition. After leaving DCs, the OX40-expressing T cell may interact with an OX40L-expressing cell other than DC, and receive an OX40 signal from the cell, which may provide essential signals for the generation of memory T cells, the enhancement of Th2 response, and the prolongation of inflammatory responses. Thus, the optimal interaction between OX40 and OX40L might be formed in two steps. After (Ishii, Takahashi et al. 2010).

While initial IL-2 production from naive CD4 T cells is largely controlled by CD28, the signal generated upon OX40-OX40L binding can strongly promote IL-2 production from effector T cells (Rogers, Song et al. 2001). In addition, OX40-OX40L interactions are able to influence the cytokine environment and thus direct the differentiation of naive T cells into different effector populations. Under neutral conditions, OX40 stimulation can direct naive CD4 T cells to Th2 cell
phenotype as driven by autocrine IL-4 and calcium/NFATc signaling pathway (Flynn, Toellner et al. 1998, Ohshima, Yang et al. 1998, Ito, Wang et al. 2005, So, Song et al. 2006). However, the secretion of IFN-α or IL-12 can overcome this TH2 driven force and result in TH1 cell generation as regulated by OX40 in the same time window (De Smedt, Smith et al. 2002, Ito, Amakawa et al. 2004, Ito, Wang et al. 2005). In vitro, engagement of OX40 under TH9 cell polarization environment has been shown to enhance the production of TH9 cells, and stimulation of OX40 in vivo has resulted in IL-9-dependent allergic lung inflammation (Xiao, Balasubramanian et al. 2012). In another in vitro system, OX40 antagonized TH17 cell production (Li, Li et al. 2008); however, this action does not correlate with in vivo data showing that OX40 is necessary for TH17 cell mediated diseases such as experimental autoimmune encephalomyelitis (EAE) (Weinberg, Wegmann et al. 1999, Ndhlovu, Ishii et al. 2001) or in models of rheumatoid arthritis (RA) (Yoshioka, Nakajima et al. 2000, Horai, Nakajima et al. 2004). Again, these data taken together emphasize the ability of the signal delivered through the OX40-OX40L interaction to influence effector cell differentiation.

1.3.vi.b The OX40–OX40L interaction in the function and development of regulatory T cells

The OX40–OX40L interaction has a diverse effect on the induction, proliferation, and suppressing function of Foxp3+ regulatory T cells (Tregs), possibly due to the different differentiation state of these cell populations. Antigen-responding naive CD4 T cells will acquire Foxp3 expression in a TGF-β
dependent manner. Compelling evidence suggests that the OX40 signal is able to inhibit Treg generation from CD25−Foxp3− naive CD4+ T cells (So and Croft 2007, Vu, Xiao et al. 2007, Piconese, Valzasina et al. 2008). In the presence of OX40 signal delivered through either an anti-OX40 agonistic antibody or OX40L, induction of Foxp3 in naive CD4+ T cells is strongly suppressed (Xiao, Balasubramanian et al. 2012). In the natural Treg (nTreg) scenario, evidence has shown that, in the presence of high amount of IL-2, OX40 signals can promote survival and proliferation of CD4+Foxp3+ nTreg both in vivo and in vitro (Elpek, Yolcu et al. 2007, Hippen, Harker-Murray et al. 2008). In addition, the effect of OX40 signal on the suppressing activity of Foxp3+ Treg has been addressed by several studies. Meanwhile, both in vitro and in vivo data have shown that the ligation of OX40 can reduce suppressor activity, and direct engagement of receptors on the Treg has been proposed to be the mechanism of the inhibition of suppressive function (Takeda, Ine et al. 2004, Valzasina, Guiducci et al. 2005, Kroemer, Xiao et al. 2007, Vu, Xiao et al. 2007, Piconese, Valzasina et al. 2008).

1.3.vi.c Structure

The OX40L monomer is a brick shaped jelly-roll β-sandwich that packs together to form flower-like 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 (Bodmer, Schneider et al. 2002). Because no proteolytic site can be found in the linker between the extracellular domain (ECD) and the transmembrane helix, OX40L is expected to
exist only in a membrane bound state (Baum, Gayle et al. 1994, Godfrey, Fagnoni et al. 1994). There are two striking structural differences between OX40L and other TNFSF members (Compaan and Hymowitz 2006). 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 ~15° rotation of the monomer, leads to much smaller trimer interfaces (~2,600 Å²) than in other structurally characterized TNF ligands (e.g., ~12,000 Å² in the BAFF trimer). Second, the characteristic ‘tiles’ of alternating aromatic or hydrophobic residues along the trimer axis first seen in the structure of TNF (Eck and Sprang 1989, Jones, Stuart et al. 1992) are lacking. Instead, the trimer interface of 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 1-2). 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 seen in most of 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 (Compaan and Hymowitz 2006). 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 (Hymowitz, Christinger et al. 1999, Mongkolsapaya, Grimes et al. 1999, Cha, Sung et al. 2000).

1.3.vi.d Signaling

Binding of OX40L to OX40 leads to trimerization of OX40 and recruitment of TRAF2, 3, and 5 at its cytoplasmic tail (Arch and Thompson 1998, Kawamata, Hori et al. 1998) through a QEE motif that is present in other family members (Ye, Park et al. 1999). From here, OX40 can act either as an independent receptor or as a classic co-stimulatory 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-κB1 pathway, which leads to the expression of several antiapoptotic Bcl-2 family members including Bcl-2, Bcl-xL, and Bfl-1 (Arch and Thompson 1998, Kawamata, Hori et al. 1998, Rogers, Song et al. 2001, Song, So et al. 2008). When the TCR signal is present, OX40 is able to synergize with TCR to promote Akt activation as well as NFAT accumulation (So, Song et al. 2006).
1.3. vi.e 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 the possible cancer treatment due to its stimulatory activity on T and NK cells (Croft 2009). Antitumor responses have been observed in both OX40 monotherapy (Weinberg, Rivera et al. 2000, Jensen, Maston et al. 2010, Weinberg, Morris et al. 2011) and in combination approaches (Gough, Crittenden et al. 2010, Watanabe, Hara et al. 2010, Garrison, Hahn et al. 2012, Redmond, Triplett et al. 2012). Particularly, OX40 stimulation combined with cyclophosphamide has been shown to induce tumor regression even in the poorly immunogenic B16 murine melanoma (Hirschhorn-Cymerman, Rizzuto et al. 2009). 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).

1.4 SMALL MOLECULE MODULATION OF TNFSF

1.4.i TNF-α–TNFR1 inhibitors

1.4.i.a Suramin and its analogs

Suramin (1-1, Figure 1-4), 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 (Alzani, Corti
et al. 1993, Alzani, Cozzi et al. 1995). Suramin is approved for the prophylactic treatment of African sleeping sickness (trypanosomiasis) and river blindness (onchocerciasis) caused by parasitic infections. Suramin shows strong polypharmacology at mid-micromolar concentrations; for example, it is a known P2 (ATP/UTP purine receptor) antagonist (IC$_{50}$ = 5–10 μM) (Ralevic and Burnstock 1998), a known inhibitor of the binding of a range of tumor growth factors, and it has various other biological activities as well (Stein 1993, Voogd, Vansterkenburg et al. 1993). Based on this work, a number of organic dyes such as Trypan blue and Evans blue (1-2), which are structural analogs of suramin (Figure 1-4), have also been found to inhibit the TNF-TNFR1 interaction using a solid-phase TNFR1 ELISA assay (Mancini, Toro et al. 1999). None of these were very potent however: the two best compounds, 1-1 and 1-2, having IC$_{50}$s of only 650 and 750 μM, respectively (Mancini, Toro et al. 1999).

1.4.i.b 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 (He, Smith et al. 2005). SPD304 (1-3; PubChem CID 16079006) is one of the best inhibitors identified until now, but it still has a relatively low affinity ($K_d$ ≈ 15 μM). Mechanistic studies revealed that 1-3 binds in the core of the TNFR1 trimer interface and is able to disrupt the structure to the extent that one monomer is fully ejected leading to a complex in which the compound binds to the exposed core of a dimeric form of TNFR1.
1.4.i.c 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 (Davis and Colangelo 2013). For example, two natural products with either pyrazole-linked quinuclidine (1-4, Figure 1-4) or indolo-[2,3-a]quinolizidine structure inhibited the TNF-α-TNFR1 interaction (Chan, Lee et al. 2010). These two compounds were identified via a high-throughput, ligand-docking-based virtual screening using the structure of the TNF-α dimer with SPD304 (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\textsubscript{50} of 50 µM in a preliminary ELISA and ~5 µM in NF-κB luciferase induction assay in HepG2 cells for 1-4 (Figure 1-4), the most promising compound in cell assays (Chan, Lee et al. 2010). 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 1-5 that showed inhibition against lipopolysaccharide-activated Raw264.7 macrophage cells (Choi, Lee et al. 2010). It should also be mentioned that the promiscuous PPI inhibitors we have identified in our organic dye-based library, such as erythrosine B (1-6, Figure 1-4), an FDA approved food colorant, also inhibited the TNF-TNFR1 interaction in an ELISA quite potently (IC\textsubscript{50} of 5 µM) as well as the TNFα–
induced JNK (c-Jun N-terminal kinase) phosphorylation in THP-1 cells (at 50 μM) (Ganesan, Margolles-Clark et al. 2011). This, however, is of no particular value as 1-6 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.

![Chemical structures of some of the small-molecules shown to inhibit the TNF-TNFR PPI](image)

**Figure 1-4:** Chemical structures of some of the small-molecules shown to inhibit the TNF-TNFR PPI
1.4.ii CD40–CD40L inhibitors

1.4.ii.a Naphthalenesulphonic acid derivatives

The first small-molecule capable of inhibiting the CD40–CD154 interaction published in the literature was again suramin (1-1, Figure 1-4), which we have identified after testing it on the basis of its activity in the TNF system (Margolles-Clark, Jacques-Silva et al. 2009). In a very encouraging manner, 1-1 was found to inhibit the binding of human CD40- CD40L with an IC$_{50}$ of 15 μM in a cell-free ELISA-type assay. Suramin showed about similar inhibitory efficacy in the murine CD40–CD40L system (with an IC$_{50}$ of about 3 μM), but, more importantly, it was considerably (about 30-fold) more active in inhibiting the CD40-CD40L interaction than the human TNFR1–TNF-α (IC$_{50}$ of 500 μM in a similar experimental setting). Further functional studies have shown 1-1 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 (Margolles-Clark, Jacques-Silva et al. 2009). Suramin (at 100 μM) 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 suramin is well-known to show considerable polypharmacology at these concentrations, its interference with the positive co-stimulatory interaction might provide a possible mechanism for its ability to suppress T cell activity and induce immunosuppression.

After probing the chemical space of suramin-like compounds, we have identified a series of organic dye compounds that were able to disrupt the CD40–
CD154 interaction with low micromolar potency (Margolles-Clark, Umland et al. 2009, Buchwald, Margolles-Clark et al. 2010). 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 (1-7), and direct fast red B (1-8) (Figure 1-5) 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 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 (Margolles-Clark, Umland et al. 2009, Buchwald, Margolles-Clark et al. 2010). Computational simulations using molecular docking also provided some further support as they also indicated an allosteric site on the CD154 trimer as the likely binding site (Ganesan, Vidović et al. 2012).

Further screenings within the same chemical space of organic dyes, led to the identification of mordant brown 1 (1-9, Figure 1-5), another naphthalenesulfonic acid derivative that is an even more potent and specific CD40-CD40L inhibitor (Margolles-Clark, Kenyon et al. 2010). Compound 1-9 inhibited CD40–CD154 binding with an IC$_{50}$ of 0.13 μM, more than an order of magnitude more potent than the previous set of compounds. It was also more selective toward CD40-CD40L compared to other TNFSF ligand-receptor pairs.
Compound 1-9 has also been shown to concentration-dependently block CD40L-induced CD40 and MHC-II expression in THP-1 cells with a less than 10 μM IC₅₀. 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. Once an active structural scaffold is identified, the color-related problems might be avoidable by standard medicinal chemistry approaches – the same way as suramin is, in fact, a ‘colorless dye’ that 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. 1-1 and 1-2 in Figure 1-5).

![Chemical structures of compounds](image)

**Figure 1-5:** Small-molecules shown to inhibit the CD40-CD40L costimulatory PPI
1.4.ii.b BIO8898

BIO8898 was discovered as a CD40-CD40L inhibitor by mass spectrometry after rapid separation from unbound library members by gel filtration (Silvian, Friedman et al. 2011). 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 (1-10, Figure 1-5). BIO8898 was shown to inhibit CD40-CD40L binding in dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA) with an IC$_{50}$ of 25 µM. 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 µM measured in the binding assay. The crystal structure of the bound complex was determined, and, surprisingly, it indicated that 1-10 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 1-6) (Silvian, Friedman et al. 2011). Binding occurs with a 1:1 molar ratio to the CD40L trimer. The contact area between 1-10 and the protein is mostly hydrophobic (~80%). Unlike the interaction between 1-3 and TNF-α, which was completely by hydrophobic interactions (He, Smith et al. 2005), 1-10 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 1-3. To characterize the mechanism of inhibition, the ability of 1-10 to displace the subunits of the CD40L trimer was tested in a setting similar to that done for 1-3. Failure to inhibit CD40 binding to singly biotinylated
CD40L when a washing step is introduced after compound incubation revealed that binding of 1-10 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 BIO8898-CD40L complex, these results suggest that inhibition of CD40–CD40L binding by 1-10 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 1-6). 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.

1.4.iii BAFF–BAFFR inhibitors

1.4.iii.a 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) (Lee, Yi et al. 2009), 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 (Moon, Yi et al. 2011). 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 seems not to be BAFF. Pre-incubation of these compounds with BAFF-muCD8 barely reduced fluorescent
Figure 1-6: Structure of the CD40L (CD154) trimer as distorted by binding of 1-10, an intercalating small-molecule disruptor (PDB structure 3KLJ (Silvian, Friedman et al. 2011)).

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.

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 (1-11, Figure 1-7), 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 1-11 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 (1-11), was further characterized regarding its BAFFR–BAFF binding inhibitory potential (Lee, Oh et al. 2011). It has been shown to inhibit the BAFF induced increase of WIL2-NS cell density. To address whether 1-11 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 1-11 with reduced BAFFR level on WIL2-NS cells as well. A SLE mouse model (MRL<sup>lpr/lpr</sup> mice) was also explored to test the potential effect of 1-11 mediated inhibition of BAFFR–BAFF <i>in vivo</i>. Renal swelling in glomerulus and lymphocyte proliferation in splenic white pulp can be recognized in 13 week old MRL<sup>lpr/lpr</sup> mice, and these phenotypes were ameliorated in mice intraperitoneally injected with 1-11 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 1-11 to target certain B cell populations in an SLE mouse model through interference with the BAFFR–BAFF interaction.
1.4.iv TRAIL–DR5 (DR5 activators)

1.4.iv.a Bioymifi, the first small-molecule agonist identified within the TNFSF

To date, one small-molecule TRAIL-mimetic that targets DR5 has been identified – it was designated as bioymifi (1-12, PubChem CID 70678439; Figure 5) (Wang, Wang et al. 2013). 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 (Li, Thomas et al. 2004, Petersen, Wang et al. 2007, Wang, Du et al. 2008). 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 (1-12, Figure 1-7). Contrary to A2C2, 1-12 was found to promote cell death without the need for the Smac mimetic in T98G cells and other human cancer cell lines tested. Bioymifi–induced cell death could be blocked by the pan-caspase inhibitor Z-VAD, suggesting that 1-12 induce apoptosis. In addition, 1-12 activated both caspase-3 and caspase-8 while knockdown of caspase-8, but not caspase-9 rescued cells from 1-12-induced apoptosis suggesting the 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 1-12. These results suggest that 1-12 is able to activate DR5 in a manner similar to the TRAIL induced apoptotic pathway. To test if TRAIL is involved in 1-12-induced cell death, a TRAIL neutralizing antibody was applied before 1-12 or soluble recombinant TRAIL. It was found to block TRAIL-, but not 1-12-induced cell death. Knockdown of TRAIL by siRNA also could not block the activity of 1-12 suggesting that 1-12-induced apoptosis is independent of TRAIL. Isothermal titration calorimetry experiments demonstrated that 1-12 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 1-12 was observed in both SDS-PAGE and size-exclusion chromatography suggesting that oligomerization of DR5 was induced by this compound. Hence, 1-12 serves as a potential lead for the development of small-molecule TRAIL mimics targeting DR5 for cancer therapy.

![Figure 1-7: Small-molecules modulators of the BAFFR-BAFF and TRAIL-DR5 PPIs](image)

1.5 EXPLORATION OF THE CHEMICAL SPACE OF ORGANIC DYES

Although several cases of PPI small-molecule modulators within the TNFSF have been reported, the progress of this field is still relatively slow,
possibly due to the fact that a different small-molecule chemical space is required to achieve effective PPI modulation. For example, the physico-chemical properties of existing PPI modulators normally violate Lipinski’s ‘rule of five’ due to their increased structural rigidity and larger molecular weight as compared to small-molecule modulators of traditional drug targets (Wells and McClendon 2007). Thus, to tackle with interfaces of PPIs, an expanded chemical space in addition to that of traditional drug targets is necessary. Our group has been exploring the chemical space of organic dyes as part of our search for PPI inhibitors due to their good protein binding ability (Figure 1-8), and several scaffolds have been identified that exhibit promising activity and specificity for their targets. Some of these compounds are well-known polyionic sulfo-dyes that are available in a large structural variety and are relatively nontoxic. For example, 1-7, crocein scarlet 7B (1-13, CS7B), and mordant brown 1 (1-9) were shown to be quite specific for CD40–CD154 (Margolles-Clark, Jacques-Silva et al. 2009, Margolles-Clark, Umland et al. 2009, Buchwald, Margolles-Clark et al. 2010, Margolles-Clark, Kenyon et al. 2010). Other compounds were found to be non-specific, i.e., promiscuous PPI inhibitors (e.g., erythrosine B 1-6) (Ganesan, Margolles-Clark et al. 2011), whereas others such as tartrazine (1-14) were inactive in all assays.

Organic dyes are small molecules consisting of a core chromophore (such as azo dyes, anthraquinone, or phathalocyanin) structure that can contain carboxyl, amino, chloride, or sulfonic groups and can also be coupled with a
triazine ring (reactive dyes). Most dyes also contain nitrogen either inside or outside their aromatic ring systems.

Figure 1-8: Representative chemical structures for active compounds identified so far in our organic dye-based chemical library showing good PPI inhibitory activity

Organic dyes are well known for their ability to bind various proteins. The interaction between the dye and proteins can be mediated through a combination of forces including electrostatic, hydrophobic, hydrogen bonding, electron-transfer, and π-π stacking. Acid dyes with sulfonic acid groups, such as most of the structures considered here, are negatively charged at all pH values and they can form ionic interactions with the positively charged amino acids on the protein surface with binding being further enhanced by polar, van der Waals, and
hydrogen bond interactions (Buchwald 2010). Indeed, selective and reversible binding ability of dyes to most of the proteins has been widely applied to protein purification purposes (Denizli and Piskin 2001). In addition, to improve the binding specificity of organic dyes to certain target proteins, biomimetic dyes have been designed through computational approaches, which has led to significant improvement of the purification outcome of corresponding target proteins (Clonis, Labrou et al. 2000).

Organic dyes are less appealing as possible therapeutic agents (drugs). Dye compounds are not a likely choice to advance in clinical trials because of possible coloration effects. Exceptions exist, as, for example, FP-21399, a bis(disulphonaphthalene)-azo compound was selected from a screening program of Fuji compounds originally developed for photography for its potential in the treatment of HIV infections (as a possible inhibitor of the gp120-mediated fusion), and it was even advanced into clinical trials (Ono, Wada et al. 1997). Furthermore, once an active structural scaffold for PPI modulation is identified, color-related problems can be avoided through medicinal chemistry approaches as the case seen with suramin (1-1).

Suramin is, in fact, a “colorless dye”, since it is structurally related to certain polysulfonated azo dyes such as trypan blue or Evans blue (1-2), but it does not contain the chromogenic aryl azo moiety. Nevertheless, it has been shown to retain protein binding activity. Suramin, like its structural predecessors, has found use in treating trypanosomiasis, onchocerciasis, and also to inhibit P2 (ATP/UTP purine receptor) and recently by our group to inhibit the CD40–CD154
co-stimulatory interaction (Margolles-Clark, Jacques-Silva et al. 2009). Inspired by the previous discoveries in our lab and encouraged by the promising clinical outcomes through modulating PPIs within the TNFSF, the present work aimed at assessing potential modulatory activity of our established chemical library towards several important ligand-receptor interactions within TNFSF. The present study also aimed at validating the activity and clarifying mechanisms of action of several OX40–OX40L small-molecule modulators identified during our preliminary study.
CHAPTER 2 MATERIALS AND METHODS

2.1 SMALL-MOLECULE SCREENING ASSAY

Microtiter plates (Nunc F Maxisorp; 96-well) were coated overnight at 4°C with 100 µL/well of Fc-conjugated receptors diluted in PBS 7.2. This was followed by blocking with 200 µL/well of blocking solution (PBS 7.2, 0.05% Tween-20, 1% BSA) for 1 hour at RT. Then plates were washed twice using washing solution (PBS 7.4, 0.05% Tween-20) and tapped dry before the addition of the appropriate FLAG tagged / biotinylated ligands along with different concentrations of tested dyes diluted in binding buffer (100 mM HEPES, 0.005% BSA pH 7.2) to give a total volume of 100 µL/well. After 1 h incubation, anti-FLAG HRP conjugate was used to detect the bound FLAG-tagged ligand. Plates were washed three times before the addition of 120 µL/well of HRP substrate TMB (3,3’,5,5’-tetramethylbenzidine) and kept in the dark for 15–30 min. The reaction was stopped using 30 µL of 1M H₂SO₄, and the absorbance value was read at 450 nm. The concentrations of receptors used were 0.3 µg/mL for CD40, TNF-R1, TRAIL-R, BAFF-R and RANK;

Figure 2-1: ELISA based screening assay
0.6 μg/mL for OX40, GITR and 4-1BB (all from Enzo Life Sciences, Plymouth Mtng, PA). The concentrations of the ligands were fixed at 0.02 μg/mL for CD40L, TNF-α, TRAIL and RANKL; 0.06 μg/mL for OX40L, BAFF and GITRL, 0.1 μg/mL for 4-1BBL. All binding analyses were done in duplicate or triplicate per plate and repeated at least three times; the averaged data was normalized and used for data fitting and analysis. Binding data were fitted using the standard log inhibitor versus response model (- Eqn 1) using GraphPad Prism 5.04 (La Jolla, CA) (Margolles-Clark, Umland et al. 2009).

2.2 COMPUTATIONAL METHODS

2.2.i Virtual screening of small molecule libraries toward OX40

Small molecule libraries from National Cancer Institute (NCI-Divset and NCI-Natprod), ChemNavigator, and ChemDiv were used. They were prepared for docking using LigPrep version 2.5 (Schrödinger, LLC, New York, NY), which has built-in functionalities for generating ionization states at target pH 7.0 ± 2.0 and enumerating tautomers using Epik version 2.2 (Schrödinger, LLC), generating 3D structures, enumerating stereo centers (as needed), and generating conformers employing OPLS 2005 force field (MacKerell, Feig et al. 2004). The OX40 monomer structure obtained from 2HEV was processed using Protein Preparation Wizard (Schrödinger Suite 2011; Epik version 2.2, Impact version 5.7, Prime version 2.3; Schrödinger, LLC). Grid-boxes for OX40 monomer were constructed using the receptor grid generation tool, Glide version 5.7 (Schrödinger, LLC). They were centered on druggable sites as predicted by FTMAP and QsiteFinder. Finally, virtual screening workflow was performed using
standard precision (SP) scoring as the first step. Compounds with top 10 SP scores were chosen and subsequent virtual screening workflow with extraprecision (XP) scoring mode was performed using Glide version 5.7 (Schrödinger, LLC).

2.3 BIOCHEMICAL ASSAYS

2.3.1 Identification of the binding partner

To determine whether OX40 inhibitors bind to the receptor OX40 or OX40L, 96-well plates coated with OX40:Fc (0.6 μg/mL) or OX40L:Fc (0.06 μg/mL) were first incubated with different concentrations of compounds for 1 hour at RT. Then, after three washes with washing solution, the plates were incubated for 1 h with the corresponding tagged ligands (OX40L or OX40, respectively). Plates were then washed three times with washing solution and the amount of bound partner was detected with secondary antibody anti-tag–peroxidase HRP conjugate followed by reaction with HR-Peroxidase substrate TMB in the dark for 15–30 min. The reaction was stopped using 30 μL of 1M H₂SO₄, and the absorbance value was read at 450 nm.

2.3.2 Gaddum-Schild EC₅₀ shift

Schild analysis was performed using the same ELISA setup described above for the PPI inhibition assay. The plot was constructed by fixing the concentration of OX40 at 0.6 μg/mL. The concentration of OX40L was varied from 0.001 to 10 μg/mL by serial dilution to obtain concentration–response curves in the absence or the presence of increasing concentrations (0.04 to 5.0
µM) of test compound (CVN and CBR). Binding data were fitted with a unified model as described below in the data fitting subsection. Binding data at five different inhibitor concentrations were fitted using the Gaddum / Schild EC_{50}-shift model (Eqn 2). The model was used with unified $K_d$ and $K_i$ values (equilibrium dissociation constants characterizing the CD40–CD154 and the inhibitor bindings, respectively) and Hill ($n_H$) and Schild slopes ($n_S$) were set to unity. All fittings were done with GraphPad Prism 5.04 (La Jolla, CA) (Tallarida 2007).

2.3.iii Binding Kinetics

To determine the on/off rate of OX40 binders (CVN and DB36), 96-well plates coated with OX40:Fc (0.6 µg/mL) were first incubated with compounds at concentrations serial diluted from 100 µM to 0.01 µM for 1 h at RT. Then, after three washes with washing solution, the plates were incubated with 300 µL of binding buffer at different time points for 1, 2, 4, 8, and 24 hours with the corresponding tagged ligands (OX40L or OX40, respectively). Then plates were washed three times with washing solution and 0.06 µg/mL of OX40L was added for 1 h incubation. The amount of bound OX40L was detected with secondary antibody anti-tag–peroxidase HRP conjugate followed by reaction with HR-Peroxidase substrate TMB in the dark for 15–30 min. The reaction was stopped using 30 µL of 1M H$_2$SO$_4$, and the absorbance value was read at 450 nm.
## 2.4 CELL-BASED ACTIVITY ASSAYS

### 2.4.i Toxicity assays

#### 2.4.i.a BrdU incorporation assay

THP-1 human myeloid cells obtained from American Type Culture Collection (ATCC; Manasses, VA) were cultured in RPMI-1640 medium (Invitrogen, CA) with 10% FBS (v/v; Invitrogen) and 1% penicillin-streptomycin (v/v; Invitrogen). THP-1 human myeloid cells were centrifuged and re-suspended in the same medium without FBS and added to a 96-well microtiter plate at a density of 50,000 cells/well in the absence or presence of various concentrations of compounds diluted in the same media. The plate was incubated at 37°C for 24 h. BrdU incorporation level was determined using the BrdU cell proliferation kit from Roche (Mannheim, Germany) according to the manufacturer’s protocol. Briefly, BrdU was added to the culture after treatments, and cells were incubated at 37°C for another 6 h. Then, fixing/denaturing buffer was added followed by the addition of HPR-conjugated detection anti-BrdU antibody. BrdU levels were measured using a plate reader at 450 nm after color development with TMB solution and addition of a stopping solution of H₂SO₄.

#### 2.4.i.b MTT assay

THP-1 human myeloid cells obtained from American Type Culture Collection (ATCC; Manasses, VA) were cultured in RPMI-1640 medium (Invitrogen, CA) with 10% FBS (v/v; Invitrogen) and 1% penicillin-streptomycin (v/v; Invitrogen). THP-1 human myeloid cells were centrifuged and re-suspended in the same medium without FBS and added to a 96-well microtiter plate at a
density of 50,000 cells/well in the absence or presence of various concentrations of compounds diluted in the same media. The plate was incubated at 37°C for 24 h and cells were re-suspended in 200 μL of growth media per well with 10 μL of MTT added. After 4 h incubation, the supernatant was carefully removed and the violet crystal formed was mixed with 150 μL of detergent solution. After 2 h incubation with the detergent solution, the absorbance value was measured using a plate reader at 570 nm.

2.4.i.c DAPI exclusion assay

HEK293 cells were seeded at a density of 1×10^6 cells/mL in the absence or presence of test compounds for 18 h. Viability upon treatment was determined using a BD LSR II Flow Cytometer (BD Biosciences, San Jose, CA) and the software FlowJo version 7.2.2 (Ashland, OR). The number of live cells was quantified after gating out 4’, 6-diamidino-2-phenylindole (DAPI), a nuclear stain and cell debris assessed on the basis of forward and side scatter properties of the untreated samples as reference.

2.4.ii Cell transfection

HEK293 cells containing a reporter gene under the control of a minimal promoter fused to NF-κB binding sites (HEK-Blue TNFα/IL-1b cells) were acquired from Invivogen (San Diego, CA) and further modified to express native or hybrid TNF super family receptors (TNFRSF) of interest such as CD40, OX40, or 4-1BB. The coding regions of the CD40, OX40, and 4-1BB genes were amplified by PCR using as template cDNA of activated CD19^+ B cells, activated CD8^+ T-cells, and activated CD4^+ T-cells, respectively. Primers were designed
from published nucleic acid sequences of CD40 (BC012419), OX40 (BC105072), and 4-1BB (BC006196). Hybrids of receptors OX40, 4-1BB, and CD40 were generated via overlapping PCR by fusing the corresponding extracellular regions including signal sequences of OX40 (amino acids 1–214) or 4-1BB (amino acids 1–186) to a region of the CD40 containing the trans-membrane and intracellular domains (amino acids 193–277). After cloning the amplified sequences into the vector pcDNA 3.3–TOPO TA (Invitrogen, San Diego, CA), the resulting plasmids were transfected into HEK-Blue TNFα/IL-1β cells. Stable lines resistant to 0.6 mg/mL of Geneticin (G418; Invitrogen, San Diego, CA) were analyzed by flow cytometry for expression of TNF receptors.

2.4.iii NF-κB reporter assay

To evaluate nuclear factor kappa B (NF-κB) transactivation via the specific activation of the OX40 pathway, we used our previously engineered OX40 sensor cells. An un-transfected line expressing TNF-R, and two transfected lines with either CD40 or 4-1BB expression was used as controls. The HEK-Blue reporter cells were cultivated in Dulbecco’s Modified Eagle (DMEM) media supplemented with 4.5 g/L glucose, 10% v/v FBS, 50 U/mL penicillin, 50 μg/mL streptomycin, 100 μg/mL Normocin, and 2 mM L-glutamine. The cells were centrifuged and re-suspended in the same medium with 1% FBS, added to a 96-well microtiter plate at a density around $1 \times 10^5$ cells/well in the absence and presence of various concentrations of compounds diluted in the same media. For ligand mediated stimulation, final concentrations of recombinant human TNFα (20 ng/mL), CD40L (20 ng/mL), 4-1BBL (400 ng/mL), or OX40L (40 ng/mL) were
maintained in the wells for this purpose. After 18 h incubation at 37°C, 20 μL supernatant of each well were taken and added to another 96-well microtiter plate containing 180 μL/well of QUANTI-Blue (InvivoGen, CA). The level of SEAP was determined after 30-minute incubation at 37°C by reading at 625 nm using a spectrophotometer.

2.4.iv THP-1 cell activation assay
THP-1 cells were cultivated as before (section 2.4.i.a). Serum-starved THP-1 human monocytic leukemia cells were plated in medium without FBS having a density around 1×10^6 cells/mL and were stimulated with 0.5 μg/mL soluble CD154 in the absence and presence of various concentrations of test compounds diluted in the same medium. After 48 h of stimulation at 37°C and 5% CO₂, the cells were washed with FBS stain buffer (BD Biosciences, CA) and stained with the antibodies for any of the following cell surface markers CD40, HLA–DR, and CD54, tagged with fluorochromes R-phycoerythrin (PE; Ex/Em = 496 nm / 578 nm), fluorescein isothiocyanate (FITC; Ex/Em = 494 nm / 520 nm), and allophycocyanin (APC; Ex/Em = 650 nm / 660 nm) respectively (BD Biosciences, CA) as described previously (Margolles-Clark, Umland et al. 2009). The fluorochromes were pre-determined not to interfere with either the organic dyes tested or with each other. Cell surface marker expression was analyzed using a BD LSR II flow cytometer (BD Biosciences, CA). Staining using DAPI (Ex/Em = 350 nm / 470 nm) was used to assess cell viability before quantifying surface marker expression.
2.4. v TNF-α mediated JNK phosphorylation

THP-1 cells were starved overnight in 25 cm² tissue culture flasks at a density of 8×10⁵ cells/mL in serum free RPMI supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin. They were stimulated with 20 ng/mL recombinant human TNFα (Enzo Life Sciences, PA). Following an initial time-course investigation of the phosphorylation pattern of JNK in THP-1 cells with stimulation times of 5 min, 10 min, 30 min, 1 h, 2 h, 4 h, and 8 h, the 10 min time-point was selected for the inhibition experiments. In these, TNFα was added immediately after the addition of 50 mM test compounds or 2 µg/mL / 13 nM anti-human TNFα monoclonal antibody (R&D Systems, MN). The JNK (c-Jun N-terminal kinase) inhibitor anthrax- (1, 9-cd) pyrazol-6 (2H)-one (SP600125, Sigma–Aldrich) was also used as a positive control (50 mM, 30 min before TNFα ligand induction). At the indicated time points, cells were rapidly washed with ice-cold PBS and lysed with a chilled lysis buffer (10 mM Trizma base, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM sodium orthovanadate, 10 mg/mL leupeptin, 25 mg/mL aprotinin, 1.25 mM NaF, 1 mM sodium pyrophosphate and 10 mM n-octyl-β-D-glucopyranoside) for 15 min on ice. Cell debris was removed by centrifugation at 14,000 rpm for 15 min at 4 - 8°C, and the supernatant immediately transferred to a fresh tube. Solubilized proteins were separated using SDS–PAGE and transferred to a nitrocellulose membrane. p-JNK and JNK (loading control) were visualized with monoclonal antibodies #9251 (Cell Signaling Technology, MA) and AF1387 (R&D Systems
MN), respectively. Protein quantification was performed with NIH ImageJ software (http://rsb.info.nih.gov/ij/)

2.4.vi Human T cell assay

2.4.vi.a Human Naive CD4+ T cell isolation

Whole blood from healthy donor was diluted 4× with sterile PBS7.4 and 35 mL of diluted blood was carefully layered over 15 mL of Ficoll-Paque (GE Healthcare, Little Chalfont, UK) in a 50 mL conical tube. Mononuclear cells were obtained by subsequent density gradient centrifugation. After separation with Ficoll-paque, the cell suspensions were pooled incubated with 10 μL of Human Naive CD4+ T cell Biotin-Antibody Cocktail II (Miltenyi Biotec, Bergisch Gladbach, Germany) per 10^7 total cells for 5 minutes at 4 °C. Then, 20 μL of Naive CD4+ T Cell MicroBead Cocktail II per 10^7 total cells were added and incubated for an additional 10 minutes in the refrigerator (4°C). The cell mixture was passed through a column for magnetic separation and flow-through containing unlabeled cells, representing the enriched naive CD4+ T cell was collected according to the manufacturer’s protocol. Cells are fluorescently stained with CD45RA-FITC and CD4-APC, naive CD4+ t cells represented by the CD4+CD45RA+ double positive population was typically above 95% pure.

2.4.vi.b Human CD4+ T cell IL-4 secretion assay

96-well plates were coated overnight with anti-CD3 antibody (1 μg/mL; PharMingen, San Diego, CA) or PBS 7.4 at 4°C overnight. 1×10^6 cells/well of isolated naive CD4+ T cells were cultured either alone or with the addition of 0.5
µg/mL or 5 µg/mL of OX40L per well. The cultures were incubated with 2 µL of anti-CD3 anti-CD28 magnetic beads (Life Technologies, Carlsbad, CA) in RPMI 1640 medium containing L-glutamine supplemented with 10 U/mL penicillin/100 mg/mL streptomycin and 5% FBS at 37°C with 5% CO₂ for three days. At the required time points, the cultures were harvested and washed thoroughly to remove antibodies. The cultures were restimulated with anti-CD3 mAb for 4 h at 37°C as described above in the presence of GolgiStop according to the manufacturer’s protocol (Biolegend, San Diego, CA) for 4 h. The level of IL-4 was measured by intracellular IL-4 staining using BD LSR II Flow Cytometer (BD Biosciences, San Jose, CA) and data were analyzed with FACSDiva software. IL-4 ELISA experiments were also performed in parallel according to the manufacturer’s protocol (Biolegend) to determine the level of secreted IL-4.

2.4.vii Mouse T cell assay

2.4.vii.a Mouse CD4⁺ T cell IL-17 secretion assay

Spleen cell suspensions were passed through a nylon wool column. Then, the flow-through fraction was incubated with mouse CD4⁺ T cell magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and passed through a magnetic cell sorting column (Miltenyi Biotec), and the negative fraction was collected (CD4⁺ T cells, >95%). CD4⁺ T cells (2×10⁵ cells per well) were cultured in a plate coated with an anti-CD3 mAb (1 µg/mL) alone, a mixture of anti-CD3 mAb (1 µg/mL) plus anti-OX40 mAb (OX86; BD Pharmingen), or a mixture of anti-CD3 mAb (1 µg/mL) plus different concentrations of test compounds CVN and TZ
(negative control). The culture was incubated for 96 h, and IL-17 levels in culture supernatants were determined by ELISA (Biolegend).

2.4.vii.b Natural regulatory T cell (Treg) expansion assay

Natural Tregs (CD4+GFP+ (Foxp3+)) obtained from GFP-Foxp3 reporter mice were sorted by flow cytometry, then 1×10^5 cells per well were stimulated with anti-CD3 and anti-CD28 conjugated Dynabeads (6 μL/well; Invitrogen) supplemented with 2,000 IU/ml rhIL-2 (Peprotech) in complete medium, which consisted of 10% FBS, nonessential amino acids, 0.5 mM sodium pyruvate, 5 mM Hepes, 1 mM glutamax in DMEM base. At certain conditions, 1 μg/mL of an agonistic anti-OX40 mAb OX86 and different concentrations of test compounds (CVN and TZ) were added into the culture. The whole culture was monitored daily and maintained at 0.7–1×10^6/mL by diluting with IL-2-supplemented complete medium for 8–12 days. At the end of the culture, intracellular staining for Foxp3 and Ki67 was performed and the expression of Foxp3 and Ki67 was assessed by flow cytometry experiments.

2.4.vii.c Polarization of naive CD4+ T Cells in vitro

Naive CD4+ T cells (CD4+CD62L+Foxp3−) obtained from GFP-Foxp3 reporter mice were sorted by flow cytometry, then 1×10^5 cells per well were activated with anti-CD3 and anti-CD28 conjugated Dynabeads (3 μL/well; Invitrogen). The induction of iTreg cells and T H9 cells was done in the presence of 3 ng/mL human TGF-β1 (R&D Systems) and 10 ng/mL IL-2 (PeproTech) for induction of iTreg cells, and for T H9 polarization, 3 ng/mL human TGF-β1 and 10
ng/mL IL-4 (PeproTech) were used. CD4+ T cells cultured for 3 days under those polarizing conditions were collected and cell polarization was assessed by intracellular staining and flow cytometry.

For intracellular cytokine staining, CD4+ T cells activated under Th9 polarizing condition were restimulated for 4 h with phorbol 12-myristate 13-acetate (50 ng/mL) and ionomycin (500 ng/mL; Sigma-Aldrich) in the presence of GolgiStop (Biolegend). After staining with Live/Dead yellow (Sigma Aldrich), cells were fixed and made permeable with Cytofix/Cytoperm solution (BD Bioscience; San Jose, CA) and were stained with fluorochrome-conjugated anti-IL-9 (RM9A4; BioLegend). In addition, for iTreg cell conversion experiment, DAPI staining (Sigma Aldrich) was used to distinguish live/dead cells and iTreg cells were identified by the expression of GFP. All samples were acquired with BD LSR II Flow Cytometer (BD Biosciences, San Jose, CA) and data were analyzed with FACSDiva software (BD Biosciences).

2.5 NOD MOUSE TREATMENT

Six-week old female NOD mice (Taconic, Hudson, NY) were divided into six groups with five mice per group. Mice in group two, five, and six were immunized intranasally with insulin B:9-23 peptide (Biorbyt, San Francisco, CA) at 40 mg/immunization (three times a week for the first two weeks; once a week for five more weeks and once every two weeks until the mice reached 25 weeks of age). Mice in group one were treated with PBS 7.4 both intranasally and subcutaneously. Mice in group four and six received three subcutaneous injections (200 mg/injection) of anti-OX40 agonistic antibody (clone OX86;
BioXcell, West Lebanon, NH) on days 0, 2, and 4. Mice in group three and five were treated with 1 mg/injection of CVN subcutaneously for 21 consecutive days. Blood glucose level was monitored once per week starting at week twelve and twice per week starting at week fifteen. Mouse were considered hyperglycemic following two consecutive readings of >250 mg/dL.

2.6 DATA FITTING AND STATISTICS

All binding analyses were done in duplicate per plate and repeated at least three times; the averaged data was normalized and used for data fitting and analysis. Binding data were fitted using the standard log inhibitor versus response model in GraphPad Prism (GraphPad, La Jolla, CA):

$$B = 100 \frac{[C]}{[C] + IC_{50}} = 100 \frac{1}{1 + 10^{\log IC_{50} - \log[C]}}$$  \hspace{1cm} (Eqn. 1)

For the Schild analysis, a common Gaddum/Schild EC$_{50}$-shift model was used to fit all binding data obtained at five different inhibitor concentrations:

$$B = B_{bottom} + \left(B_{top} - B_{bottom}\right) \frac{[L]^{n_H}}{[L]^{n_H} + \left\{K_i \left[1 + \left(\frac{[L]}{K_i}\right)^{n_S}\right]\right\}^{n_H}}$$  \hspace{1cm} (Eqn. 2)

The model was used with Hill and Schild slopes constrained to unity ($n_H = 1$, $n_S = 1$). All fittings were done with GraphPad Prism. Cytotoxicity and reporter assay data were analyzed by one-way repeated-measures analysis of variance (ANOVA) followed by Dunnett’s multiple comparison post hoc test for individual
differences using GraphPad Prism and a significance level of $p < 0.05$ for all comparisons.

The NF-κB activation data obtained in the sensor cell assays was fitted with a general quantitative modeling of activation for competitive partial agonists obtained using the minimal ‘two-state theory’ (del Castillo–Katz) model for receptor activation (Del Castillo and Katz 1957, Jenkinson 2003, Bodor and Buchwald 2012) (mathematically equivalent with the Black and Leff operational model (Black and Leff 1983, Kenakin 2006)) when two ligands ($L_1$, $L_2$) of different affinities ($K_{d1}$, $K_{d2}$) and efficacies ($\varepsilon_1$, $\varepsilon_2$) are present simultaneously. The final equation used for fitting was:

$$E = E_{\text{max}} \frac{\varepsilon_1 \frac{[L_1]}{K_{d1}} + \varepsilon_2 \frac{[L_2]}{K_{d2}}}{1 + (1 + \varepsilon_1) \frac{[L_1]}{K_{d1}} + (1 + \varepsilon_2) \frac{[L_2]}{K_{d2}}}$$

(Eqn. 3)
CHAPTER 3 SEARCH FOR SMALL MOLECULE MODULATORS WITHIN TNFSF

3.1 SCREENING FOR MODULATORS OF LIGAND-RECEPTOR PAIRS WITHIN THE TNFSF

3.1.i. Inhibitory activity of selected ligand-receptor pairs within the TNFSF

Following the recent success of the Buchwald lab in the discovery of the first small molecule inhibitors of the CD40–CD154 interaction (Margolles-Clark, Jacques-Silva et al. 2009), I have performed screening assays using our dye-based library for a number of important receptor-ligand interactions, and I considered compounds with IC\textsubscript{50}s below 100 \( \mu \)M toward a particular ligand-receptor interaction as possible hits. Following a pre-screening at two different concentrations (5 and 50 \( \mu \)M), detailed concentration-responses were assessed for the promising hits for each receptor-ligand pair separately. Within my expectation, I observed relatively high rates of hit identification, about 10 hits per ligand-receptor pair within our ~250 dye based small molecule library toward seven ligand-receptor interactions screened that included BAFF–BAFF-R, TNF\( \alpha \)–TNFR1, 4-1BBL–4-1BB, RANKL–RANK, GITRL–GITR, TRAIL–TRAILR, and OX40L–OX40 (Figure 3-1 – Figure 3-7).
Figure 3-1: Concentration-dependent inhibition of the BAFF–BAFF-R PPI
Dose-response curves of top ten small-molecule compounds ranked by their IC$_{50}$ values.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC$_{50}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calamine Orange 2RS</td>
<td>4.3</td>
</tr>
<tr>
<td>Supranol Orange RA</td>
<td>6.3</td>
</tr>
<tr>
<td>Remazol Brilliant Blue R</td>
<td>7.6</td>
</tr>
<tr>
<td>Direct Yellow 27</td>
<td>9.7</td>
</tr>
<tr>
<td>Acid Blue 129</td>
<td>10.5</td>
</tr>
<tr>
<td>Acid Green 23</td>
<td>18.3</td>
</tr>
<tr>
<td>Chlorazol Violet N</td>
<td>19.3</td>
</tr>
<tr>
<td>Direct Blue 71</td>
<td>20.2</td>
</tr>
<tr>
<td>Direct Black 36</td>
<td>23.4</td>
</tr>
<tr>
<td>Mordant Black 25</td>
<td>37.2</td>
</tr>
</tbody>
</table>

Figure 3-2: Concentration-dependent inhibition of the TNFα–TNFR1 PPI
Dose-response curves of top ten small-molecule compounds ranked by their IC$_{50}$ values.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC$_{50}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reactive Blue 4</td>
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</tr>
<tr>
<td>Chlorazol Black</td>
<td>18.0</td>
</tr>
<tr>
<td>Acid Orange 63</td>
<td>37.1</td>
</tr>
<tr>
<td>Acid Blue 113</td>
<td>37.2</td>
</tr>
<tr>
<td>Direct Blue 15</td>
<td>38.4</td>
</tr>
<tr>
<td>Direct Yellow 27</td>
<td>59.8</td>
</tr>
<tr>
<td>Calamine Dark Green BG</td>
<td>45.2</td>
</tr>
<tr>
<td>Brilliant Blue R</td>
<td>45.6</td>
</tr>
<tr>
<td>Chlorazol Violet N</td>
<td>48.4</td>
</tr>
<tr>
<td>Chlorazol Black 8H</td>
<td>62.2</td>
</tr>
</tbody>
</table>
Figure 3-3: Concentration-dependent inhibition of the 4-1BBL–4-1BB PPI

Dose-response curves of top nine small-molecule compounds ranked by their IC$_{50}$ values.

Figure 3-4: Concentration-dependent inhibition of the RANKL–RANK PPI

Dose-response curves of top ten small-molecule compounds ranked by their IC$_{50}$ values.
Figure 3-5: Concentration-dependent inhibition of the GITRL–GITR PPI

Dose-response curves of top seven small-molecule compounds ranked by their IC₅₀ values.

Figure 3-6: Concentration-dependent inhibition of the TRAIL–TRAILR PPI

Dose-response curves of top six small-molecule compounds ranked by their IC₅₀ values.
3.2 INHIBITION OF THE OX40–OX40L BINDING

3.2.i Inhibitory activity towards OX40–OX40L interaction

With my focus on the OX40–OX40L interaction, I have analyzed the structure–activity relationship (SAR) information from the binding experiments and found two consensus motifs for the active compounds toward OX40–OX40L interaction. The first motif (motif A) consists of a central aromatic system that is phenyl, biphenyl, or naphthyl, flanked by an azo linker. The two arms also contain naphthalene or other bulky aromatic groups with polar substitutions (Figure 3-8a). The second motif (motif B) has a central alizarin core and one extended arm with or without the triazine group (Figure 3-8b).
3.2.i.a Inhibitory activity towards OX40–OX40L interaction

Several compounds sharing consensus motif A have shown particularly good activity and selectivity in our ELISA based cell-free screening assays (Figure 3-9). Compounds 3-1–3-4 concentration dependently inhibited the OX40–OX40L co-stimulatory interaction with low micromolar potency. As before, tartrazine (1-14) was included as a negative control in all assays since it is a polysulfonated dye with a chemical structure somewhat related to those of 3-1–3-4 that, however, consistently showed no activity in any of these assays. Median inhibitory concentrations (IC$_{50}$s) obtained by fitting the data with a standard binding model were 1.8, 3.8, 1.4, and 3.2 µM for 3-1, 3-2, 3-3, and 3-4, respectively and are summarized in Table 3-1. As before for the CD40–CD40L system (Margolles-Clark, Umland et al. 2009), in addition to the human proteins, the inhibitory assay was also performed with mouse OX40 and OX40L. Whereas the human antibody inhibited only the human system, the small molecules showed similar inhibitory activity in both systems (Figure 3-10).
Figure 3-9: Inhibition of selected human TNFSF PPIs by active compounds with consensus motif A

Concentration-dependent inhibition of the OX40–OX40L and other TNFSF ligand–receptor bindings by the small-molecule compounds (3-1–3-4) of the present study with structures as shown (see Table 3-1 for the corresponding IC50 values). Data are average ± SD (normalized to percent binding) for n = 3 independent experiments with duplicates for each condition.
Concentration-dependent inhibition of the mouse OX40–OX40L PPI by the small-molecule compounds (3-1–3-4). Both tartrazin (TZ) and anti-human OX40L antibody served as negative controls here did not block mouse OX40–OX40L interaction.

I also selected the most promising compounds sharing consensus motif B from results of two-concentration screening and assessed their inhibitory activity and selectivity toward OX40–OX40L in a wider dose range. Compounds 3-5–3-7 concentration dependently inhibited the OX40–OX40L interaction with low micromolar potency. Median inhibitory concentrations (IC₅₀s) obtained by fitting the data with a standard binding model were 0.4, 0.07, and 3.4 μM for 3-5, 3-6, and 3-7, respectively and are summarized in Table 3-1. Interestingly, while 3-6 has the best inhibitory activity towards the binding of OX40–OX40, 3-7 shows the best selectivity simply due to the difference of three methyl groups on the phenyl ring.
Figure 3-11: Inhibition of selected human TNFSF PPIs by active compounds with consensus motif B

Concentration-dependent inhibition of the OX40–OX40L and other TNFSF ligand–receptor bindings by the small-molecule compounds (3-5–3-7) of the present study with structures as shown (see Table 3-2 for the corresponding IC₅₀s).

3.2.ii.b Specificity (within TNFSF)

Besides activity, selectivity toward the desired target also has to be defined. Accordingly, for compounds of interest, in addition to OX40–OX40L, I have quantified inhibitory activities toward several other ligand–receptor pairs within the TNF superfamily, such as TNF-R1–TNFα, CD40–CD40L, RANK–RANKL, and 4-1BB–4-1BBL. While 3-1, 3-4, and 3-7 showed good (>10–30-fold) selectivity toward OX40–OX40L interaction when compared to all other TNFSF interactions tested, 3-2, 3-3, and 3-5 showed similar activity toward CD40–CD40L and RANK–RANKL, respectively as they inhibited these interactions with IC₅₀s comparable to that for OX40–OX40L (Figure 3-9, 3-11, Table 3-1).
Table 3-1: Median inhibitory concentrations (IC\textsubscript{50}) and their 95% confidence intervals (CI, shown in smaller font and in square brackets) for compounds 3-1–3-7 for different TNFSF interactions as indicated

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC\textsubscript{50} ((\mu M))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OX40–OX40L</td>
</tr>
<tr>
<td></td>
<td>human</td>
</tr>
<tr>
<td>3-1 (DB36)</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>[1.0–2.6]</td>
</tr>
<tr>
<td>3-2 (DB71)</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>[2.2–3.2]</td>
</tr>
<tr>
<td>3-3 (DB15)</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>[1.2–2.0]</td>
</tr>
<tr>
<td>3-4 (CVN)</td>
<td>1.9</td>
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<tr>
<td></td>
<td>[1.5–2.6]</td>
</tr>
<tr>
<td>3-5 (PB)</td>
<td>0.4</td>
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<tr>
<td></td>
<td>[0.1–1.0]</td>
</tr>
<tr>
<td>3-6 (AB129)</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>[0.05–0.09]</td>
</tr>
<tr>
<td>3-7 (AB25)</td>
<td>3.4</td>
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<td></td>
<td>[1.7–6.8]</td>
</tr>
<tr>
<td>1-14 (TZ)</td>
<td>&gt;2,000</td>
</tr>
<tr>
<td>Negative Control</td>
<td></td>
</tr>
<tr>
<td>1-6 (ErB)†</td>
<td>1.7</td>
</tr>
<tr>
<td>Positive Control</td>
<td></td>
</tr>
<tr>
<td></td>
<td>[1.2–2.6]</td>
</tr>
</tbody>
</table>

Notation for small-molecule compounds – 3-1: DB36 (direct black 36); 3-2: DB71 (direct blue 71); 3-3: DB15 (direct blue 15); 3-4: CVN (chlorazol violet N, direct violet 1); 3-5: PB (procion blue mx-r, reactive blue 4); 3-6: AB129 (acid blue 129); 3-7: AB25 (acid blue 25); 1-14: TZ: tartrazine; 1-6: ErB: erythrosine B (†values obtained in (Ganesan, Margolles-Clark et al. 2011) except for the last column, which was measured here). *IC\textsubscript{50} values obtained in the binding partner assay. N/A, not available.
3.2.iii Virtual screening of drug-like small molecule libraries toward OX40–OX40L interaction

Although the sequence identity of receptor members within the TNF receptor superfamily is around 30%, their quaternary structure is well conserved and quite similar (Bodmer, Schneider et al. 2002). Particularly, they are characterized by the presence of four cysteine-rich domains (CRDs). While for a given receptor, only CRD2 and CRD3 are involved in ligand binding, interestingly, CRD1 in OX40 offers extensive contacts in the ligand–receptor interaction, and as such has not been seen in most of the other TNFRSF members (Compaan and Hymowitz 2006). By taking advantage of this structural uniqueness, we attempted to identify small molecules that could specifically target the CRD1 region of OX40 and achieve activity and selectivity toward OX40–OX40L interaction within the TNFSF. As part of a computational screening strategy in collaboration with the Center for Computational Studies at the University of Miami, PDB files of both the OX40 monomer and OX40–OX40L monomer-monomer complex were submitted to both FTMap server (http://ftmap.bu.edu) and QsiteFinder (http://www.modelling.leeds.ac.uk/qsitefinder) to predict potential druggable sites in both OX40 and OX40–OX40L by means of solvent clustering. Multiple druggable sites were predicted in both structures (Figure 3-10). Of particular interest are sites located in the CRD1 region of OX40 alone and CRD1 region included in the binding interface of OX40-OX40L.
Figure 3-12: Predicted druggable sites for OX40 and the OX40–OX40L complex

Crystal structures of OX40 monomer and OX40L–OX40 monomer complex (PDB ID 2HEV) were used for FTMAP study. A number of low energy conformations of small organic molecule probes were clustered (shown in orange) and ranked on the basis of the average binding energy, which represented potential druggable sites. Red circles highlight clusters located in CRD1 in both structures.

To target potential druggable sites in CRD1 regions involved in OX40–OX40L binding, 1990 compounds from the ‘diverse’ set library and more than 200 compounds from a natural products library provided by the National Cancer Institute were used for virtual screening experiments. All compounds and proteins were prepared using Ligprep and Protein Preparation Wizard in the Schrodinger Suite Software before screening. More than 20 compounds with the best docking scores were selected by using the virtual screening workflow in the
Schrodinger suite with pancratistatin as one of the best compounds having the lowest Glide extra precise (XP) Score of -8.9 docked at the interface of OX40–OX40L binding (Figure 3-11). Comparing Glide XP scores of these hits generated from virtual screening to that of a strong CD40L binder BIO8898 (a Glide XP score of -23 (Ganesan, Vidović et al. 2012)) suggested these hits are not strong binders. The lack of good hits from the virtual screening may be due to the fact that these compound libraries have been skewed towards smaller molecules for traditional drug targets, which may not be ideal to target PPI interfaces due their unique characteristics as discussed before. Indeed, most of these hits showed no inhibitory activity towards OX40–OX40L interaction as accessed in our ELISA based binding experiment.
Figure 3-13: Glide-XP docking and scoring of pancretatin at the binding site of OX40–OX40L complex

Glide-XP docked pose of pancretatin on the OX40–OX40L monomer complex, with dark purple highlighting interacting surface of OX40–OX40L. Corresponding ligand interaction 2D diagrams of pancretatin with neighboring residues involved in hydrogen bond and hydrophobic interactions are also shown on the right.
4.1 CYTOTOXICITY ASSESSMENT

4.1.i BrdU incorporation assay

For the promising compounds identified in the cell-free ELISA assays, cytotoxicity evaluations were performed in THP-1 (human monocytic leukemia) cells. As shown in one of the representative cell proliferation assays, none of the compounds showed cytotoxicity with IC$_{50}$s less than 100 $\mu$M (well above the range of OX40–OX40L inhibitory activities); however, DB71 (3-2) inhibited cell proliferation at the highest concentration of 500 $\mu$M (Figure 4-1). Interestingly, some compounds such as DB36 and DB15 increased BrdU incorporation indicating a possibly increased proliferation rate.

![Figure 4-1: Cell toxicities of the present compounds assessed by a standard proliferation assay (BrdU)](image)

Compounds 3-1–3-4 plus tartrazine (1-14, TZ) as assessed by a standard proliferation assay (BrdU) in THP-1 cells. Data are average ± SD for $n = 3$ independent experiments with triplicates for each condition.
4.1.ii. DAPI exclusion assay

DAPI exclusion experiment was performed in THP-1 cells to further evaluate potential cytotoxicity of our promising compounds. Consistent with the results in the previous BrdU incorporation assay, while ErB, a positive control which showed significant cytotoxicity at both 500 μM and 200 μM, none of the promising compounds showed cytotoxicity with IC_{50}s less than 100 μM (Figure 4-2). Again, DB71 (3-2) slightly decreased the viability of THP-1 cells at both 500 μM and 200 μM.

Figure 4-2: Cell toxicities of the present compounds assessed by a standard apoptosis assay (DAPI exclusion)

Compounds 3-1–3-4 plus tartrazine (1-14, TZ), as a negative control and erythrosine B, ErB, as a positive control as assessed by standard apoptosis assay (DAPI exclusion analyzed by flow cytometry) in THP-1 cells.
4.2 CONSTRUCTION OF TNFSF-ACTIVATED NF-κB CELLS

To develop a more biologically relevant assay to assess the specificity and activity of my most promising molecular probes, I created transgenic cell lines with inducible (NF-κB) reporter genes (Figure 4-3). These constructs were based upon the characteristics that a large number of the TNF receptors is capable of integrating downstream signal transduction upon ligand binding through different TRAFs (TNFR-associated factors) eventually leading to the activation of NF-κB. We have overexpressed OX40 either with the wild-type receptor or with their ectodomains fused to the intracellular part of CD40 receptor in HEK-Blue sensor cells from InvivoGen (San Diego, CA). Similarly, I have overexpressed the corresponding ligands OX40L in Jurkat cells (human T cell lymphoblast-like cell line) (ATCC, Manassas, VA). In preliminary experiments, co-culturing of stimulator and responder cells (Figure 4-4a) or stimulation of responder cells with their corresponding ligand (Figure 4-4b) has successfully led to NF-κB activation as indicated by secretion of SEAP. In addition, secretion of SEAP was specifically induced in the presence of the corresponding ligand-receptor pair (OX40–OX40). Therefore, the inhibitory activity of our hits identified though binding assays can be confirmed by testing their ability to inhibit SEAP secretion when a proper stimuli is present (either soluble OX40L or OX40L Jurkat cells).
NF-κB sensor cells, which can respond to TNF-α and IL-1β stimulation were transfected with a chimerical receptor bearing extracellular domain of OX40 combined with intracellular domain of CD40. The engineered OX40 sensor cell line was able to respond to stimulation by either human recombinant OX40L or OX40L expressing Jurkat cells leading to NF-κB activation.

4.3. OX40 SIGNALING ENHANCES MOUSE NATURE REGULATORY T CELL PROLIFERATION IN VITRO

Several lines of evidence have shown that in the presence of OX40 signal, enhanced expansion of nTregs has been observed both in vivo and in vitro (Elpek, Yolcu et al. 2007, Hippen, Harker-Murray et al. 2008). To develop and optimize a more physiological relevant assay able to validate the modulatory activity of the OX40–OX40L interaction by my most promising compounds, in
collaboration with Dr. Allison Bayer, an assay using Foxp3-RFP mice as the source of nTregs was set up and used to evaluate the potential effect of OX40 delivered signal to enhance nTreg proliferation in vitro. In the presence of anti-OX40 agonistic antibody (OX86) which can stimulate OX40 receptor, an enhancement of both survivability and proliferation of expanded nTregs in vitro was observed (Figure 4-6). This is represented by the lower percentage of DAPI negative cell population (Figure 4-6a, d) and higher Ki67 positive cell population (Figure 4-6c, f). In addition, more viable nTregs maintained their phenotype (17.4% v.s. 9.2%) (Figure 4-6b, e) in culture when OX86 was present as compared to the control condition. Thus, this assay can be used to characterize the modulatory activity of OX40–OX40L interaction by selected compounds of interest (either attenuate nTreg proliferation by inhibitors or enhance proliferation by activators).
Figure 4-4: Specificity of NF-κB activation in OX40 sensor cell line

OX40 sensor cells were stimulated with (a) different TNFSF ligand expressing Jurkat cells and, (b) different recombinant TNFSF ligands. In both scenarios, OX40 sensor cells only responded to OX40L delivered signal.
CD4⁺CD25⁺Foxp3-GFP⁺ nTregs were sorted from Foxp3-RFP⁺ mice and cultured in the presence of anti-CD3, anti-CD28, and high level of IL-2 in vitro for nine days. The percentage of viable cells, Tregs, and proliferating Tregs were determined by assessing DAPI negative (a, d), CD25⁺RFP⁺ double positive (b, e), and Ki67 positive (c, f) cell populations, respectively. Percent values of (b) and (e) shown are relative to viable CD4⁺ cells, while percent values of (c) and (f) are relative to viable CD25⁺Foxp3⁺ Tregs. In the presence of OX86, an OX40 agonistic antibody (d, e, f), both cell survivability and proliferation were enhanced as compared to anti-CD3, anti-CD28, and high level of IL-2 culture conditions (a, b, c).
CHAPTER 5 IDENTIFICATION OF THE FIRST SMALL MOLECULE OX40 PARTIAL AGONISTS

5.1 BINDING PARTNER IDENTIFICATION

To elucidate the mechanism of the observed PPI inhibition in ELISA, experiments were conducted to assess whether these compounds bind to the receptor (OX40) or its ligand partner (OX40L). Similar experiments with the previously identified CD40–CD40L PPIIs showed that the most active inhibitors bind to the surface of CD154, but not CD40 (Buchwald, Margolles-Clark et al. 2010). Plate-coated proteins (OX40:Fc or OX40L:Fc, respectively) were incubated for 1 h with increasing concentrations of the test compounds, and after three washes to eliminate the unbound small molecules, the abilities to still bind the corresponding protein partner (OX40L or OX40, respectively were assessed. Results (Figure 5-1) indicate OX40 as the binding site for all of these compounds as they showed inhibition with IC\textsubscript{50}s in general agreement with those obtained for their previous interaction inhibitory activity (Figure 3-9, Table 1). None of the compounds tested bound OX40L, except for the promiscuous PPI inhibitor erythrosine B (Ganesan, Margolles-Clark et al. 2011).

5.2 MECHANISM OF OX40–OX40L BINDING INHIBITION

As a further mechanistic evaluation, I performed a Schild analysis with CVN (3-4) as a representative OX40–OX40L inhibitor to assess the competitive/reversible nature of this binding. Concentration-dependency curves were generated by using a fixed concentration of OX40 and varying concentrations of OX40L in the presence of increasing concentrations of 3-4.
Data from this complex binding assay (Figure 5-2) could be fitted well with the unified Gaddum–Schild model (Eqn. 2) indicating a competitive/reversible nature for the inhibition of the OX40–OX40L binding by 3-4. The fit indicated a $K_d$ of approximately 2 nM for the binding of the protein receptor-ligand pair (OX40–OX40L) and a $pA_2$ value of 6.3 (corresponding to $K_i$ of 0.5 $\mu$M) for the inhibitory activity of 3-4, in acceptable agreement with data obtained in the previous binding experiment (Table 3-1).

![Figure 5-1: Compounds 3-1–3-4 bind to OX40, not OX40L](image)

Identification of the binding partner (OX40 or OX40L) by assessing the amount of OX40L (top) or OX40 (bottom) bound after incubations of the candidate compounds with OX40 or OX40L, respectively and addition of the protein binding partner (OX40L or, OX40) only after a wash-out. All tested compounds bind to OX40 while only the promiscuous PPI inhibitor eythrosine B (ErB, 1-6) binds to OX40L. Data are average ± SD (normalized to percent binding) for $n = 3$ independent experiments with triplicates for each condition.
Figure 5-2: Schild analysis of the interference with the OX40–OX40L binding for compound 3-4 (CVN).

Data show the amount of OX40L bound in the presence of increasing concentrations of the small-molecule compound 3-4 as indicated in the legend. Symbols indicate experimental data and lines the fit obtained with the unified Gaddum/Schild EC<sub>50</sub>-shift model (Eqn. 2).

5.3.i Identification of OX40L mediated NF-κB activation enhancement by small molecules in OX40 sensor cell system

To confirm the activity of the identified inhibitors in a cell-based model, compounds identified through ELISA based screening were tested in the OX40 sensor cell system discussed in the previous chapter. Intriguingly, for compounds 3-1–3-4, which all inhibited OX40-OX40L binding in the cell-free assay, I could not see inhibition. Rather, they all showed a concentration-dependent enhancement of the OX40-linked activation of NF-κB in these sensor cells in the presence of a fixed concentration (100 ng/mL ≈ 3.3 nM) of recombinant OX40L with 3-1 and 3-4 showing the most potency (Figure 5-3). This, in combination
with the previous results, suggested the possibility that these tested compounds can not only compete for binding to OX40 with its innate OX40 ligand, but might also have the potential to activate the OX40 signaling downstream.

5.3 EFFECTS IN SENSOR CELLS WITH NF-κB REPORTER

Figure 5-3: Confirmation of activity for the present compounds of interest in OX40-expressing NF-κB reporter sensor cells.

Compounds 3-1–3-4 all enhanced the OX40L mediated stimulation of NF-κB. Anti-OX40L blocking mAb and tartrazine (TZ) were used as positive and negative controls, respectively. Data (average ± SD for n = 3 experiments with triplicates for each condition) were analyzed by ANOVA with Dunnett's post hoc test and asterisks indicate \( p < 0.05 \) (*), \( p < 0.01 \) (**), and \( p < 0.001 \) (***) as compared with cells activated with OX40 ligand.

5.3.ii Identification of OX40-specific small molecule agonists

To test whether the presence of OX40 ligand is required for the stimulation of OX40 signaling by the small-molecule compounds, OX40 sensor cells were incubated with increasing concentrations of 3-1 and 3-4 and quantified NF-κB activation via SEAP. Interestingly, both compounds clearly increased SEAP secretion in concentration-dependent manner even without the presence
of recombinant OX40L; however, they could not produce the same maximum activation. Furthermore, the activation by 3-1 and 3-4 was target specific as they did not activate the downstream NF-κB pathway when incubated with either TNF-R1 or CD40 expressing sensor cells (Figure 5-4).

Figure 5-4: Selective stimulatory activity of 3-1 (DB36) and 3-4 (CVN) in OX40-transfected NF-κB sensor cells (a) as compared to total lack of such effect in similar CD40 (b) and TNF-R1 (c) sensor cells.
Data (average ± SD for \( n = 3 \) independent experiments with triplicates for each condition) were analyzed by ANOVA with Dunnett’s multiple comparison post hoc test. Asterisks denote \( p < 0.01 \) (**\) and \( p < 0.001 \) (***) as compared to control (cells incubated without any stimulation).

5.4 NAIVE CD4+ T CELL POLARIZATION IN VITRO

5.4.i CVN mimics the effect of OX86 to block iTreg induction

Regulatory T cells (T\(_{\text{reg}}\)) are known to play important immune-modulatory roles, and OX40 agonist signaling has been shown to suppress the generation of induced regulatory T cells (iT\(_{\text{reg}}\)) (So and Croft 2007, Xiao, Balasubramanian et al. 2012). To validate the stimulatory activity on OX40 signaling of our small-molecule agonists, iT\(_{\text{reg}}\) conversion experiments were conducted using flow cytometry sorted naïve CD4+ T\(_{\text{conv}}\) cells (CD62L\(^+\) Foxp3\(^-\)) from Foxp3-GFP reporter mice. They were activated in vitro under iT\(_{\text{reg}}\) polarizing conditions (TGF-\(\beta\) plus IL-2) and then assessed for Foxp3 induction. After three-day incubation, approximately 30% of CD4\(^+\) T\(_{\text{conv}}\) cells were converted into Foxp3\(^+\) iT\(_{\text{reg}}\) cells in the presence of anti-CD3 and anti-CD28 magnetic beads (Dynabeads) plus TGF-\(\beta\) and IL-2. The agonistic anti-OX40 monoclonal antibody (mAb) (OX86) used as a positive control indeed inhibited iT\(_{\text{reg}}\) conversion, and small-molecule 3-4 also showed a less potent, but concentration-dependent inhibition with significant inhibition at 50 \( \mu \text{M} \) (Figure 5-5).
Figure 5-5: CVN inhibits iTreg generation

(a) Naïve wild-type CD4⁺ T cells were activated for three days (Dynabeads plus iTreg polarizing cytokines, IL-2 + TGF-β) by increasing concentrations of CVN. Agonist OX40 antibody (OX86) and tartrazine (TZ) were added as positive and negative controls, respectively. Inhibitory effect on the induction of iTreg cells were accessed by gating on the live CD4⁺ T cell population. (b) Summary of all experiments showing average ± SD of three independent replicates. Data were analyzed by ANOVA with Dunnett’s post hoc test with asterisks denoting $p < 0.05$ (*) and $p < 0.01$ (**) as compared to Treg polarizing conditions (IL-2 + TGF-β).

5.4.ii CVN mimics the effect of OX86 to enhance TH9 cell generation

TH9 cell is a recently defined T helper cell subset (Dardalhon, Awasthi et al. 2008, Veldhoen, Uyttenhove et al. 2008) that preferentially produces interleukin 9 (IL-9). TH9 cells function as a double edged sword. Results have indicated their protective role in a N. brasiliensis infection model (Licona-Limon,
Henao-Mejia et al. 2013) and in a melanoma tumor model (Lu, Hong et al. 2012, Purwar, Schlapbach et al. 2012) as well as their pathogenic role in allergy, colitis, and EAE in vivo (Schmitt, Klein et al. 2014). T\_h9 cell can be differentiated in vitro via TCR stimulation in the presence of both TGF-\(\beta\) and IL-4 (Chang, Sehra et al. 2010, Staudt, Bothur et al. 2010). In addition, other stimuli have been shown to contribute to T\_h9 cell development, such as co-stimulatory/co-inhibitory signals delivered through OX40 (Xiao, Balasubramanian et al. 2012) and programmed cell death ligand (PD-L2) (Kerzerho, Maazi et al. 2013). Importantly, enhanced IL9 gene expression via OX40 co-stimulation is mediated by the non-canonical NF-\(\kappa\)B pathway, which is contrary to TCR induced IL9 gene expression through the canonical NF-\(\kappa\)B pathway in cooperation with NFAT\_c2 (Jash, Sahoo et al. 2012).

As a test of the potential effect of my OX40 agonist to modulate T\_h9 cell production, 3-4 was incubated with naive CD4\(^+\) T\_conv cells in the presence of anti-CD3 and anti-CD28 conjugated beads plus T\_h9 cell polarization conditions (TGF-\(\beta\) plus IL-4) for three days, restimulated with PMA/ionomycin for 4 h in the presence of Golgi stop, and assessed IL-9 levels by intracellular staining. The OX40 agonistic antibody (OX86) significantly enhanced T\_h9 cell percentage (by approx. 35%; Figure 5-6). Compound 3-4 also caused less prominent, but statistically significant enhancement of the T\_h9 cell population: approx. 15% at 50 \(\mu\)M vs. approx. 5% of control (TGF-\(\beta\) plus IL-4 only) (Figure 5-6).
93

Figure 5-6: CVN enhances the induction of TH9 cells

(a) Naïve wild-type CD4+ T cells were activated for three days (Dynabeads plus iTreg polarizing cytokines, IL-4 + TGF-β) by increasing concentrations of 3-4 (CVN). Agonist OX40 antibody (OX86) and tartrazine (TZ) were added as positive and negative controls, respectively. Stimulatory effect on the induction of TH9 cells were accessed by gating on the live CD4+ T cell population. (b) Summary of all experiments showing average ± SD. Data were analyzed by ANOVA with Dunnett’s post hoc test with asterisks denoting \( p < 0.05 (*) \) and \( p < 0.001 (****) \) as compared to TH9 polarizing conditions (IL-4 + TGF-β).

5.5 EXPLORING THE AGONISTIC NATURE OF OX40 SMALL MOLECULE AGONIST

As a further step, to clarify the agonistic nature of these compounds as observed in our sensor cell system, the concentration-dependence of the NF-κB activation caused in these sensor cells was quantified in detail by combinations of CVN 3-4 and the natural ligand OX40L at various concentrations. In the
absence of OX40L (or in the presence of low concentrations of OX40L), 3-4 was able to produce concentration-dependent increase in NF-κB activation, but with a maximum that even at saturation was only a fraction of that produced by the natural ligand OX40L (Figure 5-7b,c). In the presence of sufficiently high OX40L (1000 ng/mL ≈ 33 nM), 3-4 actually produced a slight decrease of activation in a concentration-dependent manner (Figure 5-7a), a behavior typical for partial agonists (Jenkinson 2003, Kenakin 2006).

To verify this, a quantitative modeling of the activation was performed using a generalization of the minimal ‘two-state theory’ (del Castillo–Katz) model for receptor activation (Del Castillo and Katz 1957, Jenkinson 2003, Bodor and Buchwald 2012) (mathematically equivalent with the Black and Leff operational model (Black and Leff 1983, Kenakin 2006)) for the case of two ligands (L1, L2) of different affinities (Kd1, Kd2) and efficacies (e1, e2) simultaneously present. Fitting of the model to the data (Figure 5-8) shows that assumptions of a partial agonist with an efficacy of about 70% of that of OX40L can indeed account well for the behavior seen, including the concentration-dependent decrease of activation produced in the presence of the highest concentration of the natural ligand OX40L. The affinity (Kd) values derived from this cell-based assay as fitted with a unified model are somewhat lower but still in acceptable agreement with those obtained in previous cell-free assays (approx. 11 nM for OX40L and 17 μM for 3-4 obtained by fitting an equation equivalent to eq. 3, but with efficacies incorporated into different Emaxs).
Figure 5-7: Activation of OX40 NF-κB reporter cells by OX40L and 3-4 combined at various concentrations.

(a) OX40 sensor cells with NF-κB-induced SEAP were incubated overnight with a combination of both serial diluted OX40L and 3-4. Results are represented as the absorbance value of collected cell supernatant at the wavelength of 650 nm. Asterisks denote statistically significant differences compared to CVN 0 within each OX40L group (*p < 0.05, ***p < 0.001); double daggers denote ‡‡‡p < 0.001 between the maxima of OX40L alone (OX40L 33.3 nM, CVN 0) vs. CVN alone (OX40L 0, CVN 100 μM) (ANOVA with Dunnett’s multiple comparison post hoc test). As compared to OX40L (b), 3-4 (CVN) alone (c) can only achieve a maximum activation of the OX40-triggered response that is about 70% indicating a partial agonist type response in this system.
Figure 5-8: Quantification of receptor activation for competitive partial agonists in the presence of a full agonist using the minimal two-state receptor model

Three-dimensional surface representation of the activation effect on OX40 by a combination of full agonist OX40L and partial agonist 3-4 (CVN) in sensor cells with an NF-κB reporter (a). Purple dots represent experimental data and the surface the calculated values by the corresponding fitted model. Concentrations are on the horizontal axes and are on log scales. The bottom figure (b) shows a corresponding two-dimensional representation (essentially equivalent with a cross-sectional view of the top figure from left, front) with a linear-scaled concentration scale.
5.6 PREVENTION OF T1D WITH AGONISTIC OX40 SIGNALING: EXPLORATORY NOD STUDY

Antigen-specific therapies are possibly one of the safest approaches to prevent type 1 diabetes (T1D). Recently, it has been shown that treatment of non-obese diabetic (NOD) mice with an OX40 agonistic antibody (OX86) reduced T1D incidence primarily though boosting the immune suppressing function of a subpopulation of regulatory T cells (Bresson, Fousteri et al. 2011). To evaluate the ability of my OX40 small-molecule partial agonist to mimic the activity of the non-depleting anti-OX40 agonist antibody (OX86) in preventing T1D progression, I treated NOD mice early (six-week old) with a combination of InsB9:23 peptide, OX86, and CVN and followed disease development in a classic NOD diabetes prevention design (Chaparro and Dilorenzo 2010).

T1D was significantly delayed and the incidence was reduced after both anti-OX40 treatment and anti-OX40 treatment in conjunction with InsB9:23 peptide therapy (100% protection in both treatments versus 20% for both the control group and peptide treatment) at 32 weeks post-treatment (Figure 5-9). More importantly, treatment with our OX40 partial agonist CVN alone or in conjunction with InsB9:23 peptide also led to 80% and 60% protection, respectively. Although CVN mediated protection was not significant in this pilot study in which only five mice were used in each treatment group, the result showed a clear trend indicating that the small-molecule CVN mediated OX40 partial agonism can indeed lead to the prevention of T1D disease progression in
NOD. One possible mechanism of CVN mediated protection might be attributed to boosting regulatory T cell function, which might worth further study.

**Figure 5-9: Results of the pilot NOD diabetes prevention study with OX40 agonists as adjuvants**

Six-week old female NOD mice were immunized intranasally with the insulin B-chain 9-23 (B:9-23) (40 µg) peptide alone or in conjunction with subcutaneously injection of either the anti-OX40 agonistic mAb OX86 (200 µg; day 1, 3, and 5) or CVN (1 mg, one injection/day for 21 days). Diabetes was defined as two consecutive blood glucose values >250 mg/dL.
CHAPTER 6 DISCUSSION

6.1 FEASIBILITY OF SMALL MOLECULE MODULATION OF PPI

During the last two decades, it has become increasingly clear that, small molecules can act as effective PPI modulators. Sufficiently effective small-molecule inhibitors have been identified for a few important PPIs and more encouragingly, several candidates are in advanced clinical development (Berg 2003, Arkin and Wells 2004, Fry 2006, Whitty and Kumaravel 2006, Wells and McClendon 2007, Buchwald 2010, Mullard 2012, Arkin, Tang et al. 2014). Binding pockets on protein–protein interfaces that are suitable to bind small molecules have been shown to be considerably smaller than the binding pockets of traditional protein–ligand interactions (Fuller, Burgoyne et al. 2009). According to one quantitative estimate, whereas, marketed drugs typically target a single binding pocket with an average occupied volume of \( \sim 270 \, \text{Å}^3 \), existing PPI inhibitors typically target three to five much smaller pockets (\( \sim 100 \, \text{Å}^3 \)) (Fuller, Burgoyne et al. 2009). As pocket size limits the achievable binding energy (Buchwald 2008), 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 (Bodor and Buchwald 2001, Overington, Al-Lazikani et al. 2006); 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’ (Lipinski, Lombardo et al. 1997)). A study comparing the
structures of identified PPI inhibitors to those of FDA approved drugs found that PPI inhibitors indeed tend to be larger than enzyme inhibitors, ion channel modulators, or receptor ligands (Neugebauer, Hartmann et al. 2007). They also tend to be more hydrophobic and tend to contain more rigid, aromatic scaffolds in combination with charged or polar functionalities. Along these lines, a number of recent studies confirmed a poor correspondence between the chemical spaces of existing screening libraries and known PPI inhibitors (Neugebauer, Hartmann et al. 2007, Reynes, Host et al. 2010, Sperandio, Reynes et al. 2010).

Nevertheless, certain structures clearly show good protein-binding ability, and a chemical space with privileged structures for protein binding might very well exist (Hajduk, Bures et al. 2000). A number of studies suggested various scaffolds, many of them based on flat, aromatic structures, for designing effective small molecule PPI inhibitory structures (Che, Brooks et al. 2006, Fletcher and Hamilton 2006, Hershberger, Lee et al. 2007). Planar or partially planar aromatic and hydrophobic residues are indeed over-represented at PPI interfaces (Glaser, Steinberg et al. 2001, Silvian, Friedman et al. 2011). Recently, even promiscuous small-molecule PPI inhibitors have been identified (Ganesan, Margolles-Clark et al. 2011).

Using an iterative activity screening/structural analog search procedure, our group has identified a series of small molecules with adequate CD40L–CD40 inhibitory activity and selectivity confirmed in cell assays along with evidence of selectivity in binding assays compared to the closely related ligand-receptor pairs within the TNFSF member despite low sequence identities (~10%-30%) that
indicate good structural similarities (Margolles-Clark, Jacques-Silva et al. 2009, Margolles-Clark, Umland et al. 2009, Buchwald, Margolles-Clark et al. 2010, Margolles-Clark, Kenyon et al. 2010). My work focused on identifying potential small-molecule modulators toward several important ligand-receptor interactions within TNFSF with a particular focus on the OX40L–OX40 axis. After a preliminary two-concentration screening of our dye-enriched library towards interactions including BAFF–BAFF-R, TNFα–TNFR1, 4-1BBL–4-1BB, RANKL–RANK, GITRL–GITR, TRAIL–TRAILR, and OX40L–OX40, a series of compounds with promising inhibitory activity towards OX40L–OX40 were identified. These compounds showed both good activity (with IC50s from high nano-molar to low micro-molar range) and specificity (above at least 10 fold selectivity) tested across a wide panel of other ligand-receptor interactions within the TNFSF (Table 3-1). As pointed out earlier, PPIs are distributed over a large surface area, (lower binding energy/unit volume). Therefore, often a sufficiently large molecule is required to simultaneously engage with multiple sub-pockets to achieve desired potency. This is reflected in the average ligand efficiency (LE) of known PPI inhibitors (~0.24 kcal/mol/non-hydrogen atoms [NHA]) as compared to higher average LEs for traditional small molecule compounds such as protease inhibitors (~0.25–0.35 kcal/mol/NHA) and protein-kinase inhibitors (~0.3–0.4 kcal/mol/NHA) (Wells and McClendon 2007). For the active compounds identified here, a pattern of LE can be observed based on different consensus motifs of these molecules. While compounds 3-1–3-4 with consensus motif A (Figure 3-8) showed relative low LE (an average of 0.15 kcal/mol/NHA),
compounds 3-5–3-7 showed high LE (Table 6-1). This is primarily due to the consistently low molecular weight (M.W. of ~400 Da–600 Da) for compounds possessing consensus motif B.

**Table 6-1: Ligand efficiency of active OX40L–OX40 binding inhibitors**

<table>
<thead>
<tr>
<th>Compound</th>
<th>M.W. (Da)</th>
<th>IC$_{50}$ (µM)</th>
<th>LE (kcal/mol/NHA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-1 DB36</td>
<td>721</td>
<td>1.2</td>
<td>0.176</td>
</tr>
<tr>
<td>3-2 DB71</td>
<td>966</td>
<td>2.7</td>
<td>0.122</td>
</tr>
<tr>
<td>3-3 DB15</td>
<td>993</td>
<td>1.5</td>
<td>0.136</td>
</tr>
<tr>
<td>3-4 CVN</td>
<td>729</td>
<td>1.9</td>
<td>0.167</td>
</tr>
<tr>
<td>3-5 PB</td>
<td>637</td>
<td>0.4</td>
<td>0.218</td>
</tr>
<tr>
<td>3-6 AB129</td>
<td>460</td>
<td>0.07</td>
<td>0.323</td>
</tr>
<tr>
<td>3-6 AB25</td>
<td>416</td>
<td>3.4</td>
<td>0.273</td>
</tr>
</tbody>
</table>

Notations: M.W, molecular weight; IC$_{50}$, half maximum inhibitory concentration; NHA, non-hydrogen atom; LE, ligand efficiency, calculated as Δg/number of NHA; notation for compounds, see table 3-1.

One issue that has to be addressed for the identification of small-molecule PPI modulators is the possibility of polymolecular conglomeration (Roterman, No et al. 1993, Stopa, Gorny et al. 1998) or aggregation mediated inhibition (McGovern, Caselli et al. 2002, Shoichet 2006). To differentiate our hits from aggregators, OX40–OX40L binding assays in the presence of a non-ionic detergent (Triton-X 100, 0.05% and 0.5%) as recommended for the detection of promiscuous inhibitors (Feng and Shoichet 2006) were performed to test selected active compounds having consensus motif A. No significant change on
IC50s for any of the tested compounds was observed (data not shown) suggesting that consensus motif A compounds mediated OX40–OX40L binding inhibition was not due to polymolecular conglomeration or aggregation. In other words, they are likely to be true hits. Although similar experiments were not conducted to test active compounds possessing consensus motif B, the inactivity of these compounds in cell-based assays suggests that they are false hits; thus, their inhibitory activity towards OX40–OX40L binding is probably due to aggregation mediated non-specific inhibition.

The ability to target PPIs could considerably enlarge the number of feasible therapeutic targets. Currently existing small-molecule drugs target only about 1% of the human proteome, which contains only about 20,500 unique proteins (Clamp, Fry et al. 2007, Dunham, Kundaje et al. 2012), a number that has also been confirmed by recent draft maps of the human proteome (Kim, Pinto et al. 2014, Wilhelm, Schlegl et al. 2014). 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 as possible ligand binding sites) (Hopkins and Groom 2002, Russ and Lampel 2005, Verdine and Walensky 2007). Furthermore, 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 (Hopkins and Groom 2002, Russ and Lampel 2005). 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 (Zhu, Shi et al. 2012). PPIs, if druggable, could represent a large number of alternative targets. 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 (Klapa, Tsafou et al. 2013). For the druggability aspects it is encouraging that a computational analysis that attempted 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 (Koes and Camacho 2012).

Although PPIs could represent a biological goldmine for drug discovery purposes, existing small-molecule compound libraries are relatively populated with compounds that are collected to target traditional drug pockets, which are skewed towards smaller molecules. This is an obvious disadvantage for PPI oriented lead identification (Buchwald 2010, Mullard 2012). In the present study, a diversity-set (Divset) small-molecule library and a natural product library both from National Cancer Institute with a combined ~2,500 compounds were screened towards OX40–OX40L crystal structure through computational approaches. Although failure to identify any true hits from virtual screening might be ascribed to the limitation of the crystal structure used, Divset compounds selected from 140,000 compounds and ~1,000,000 possible pharmacophores (http://dtp.nci.nih.gov/index.html) filtered to match ‘Lipinski’s rule of 5’ were obviously biased towards targeting traditional drug targets, which could also
account for the extremely low success rate as discussed before. Therefore, an alternative approach would be to use libraries with more complicated molecules and are more tailored to PPI modulation such as those in the TIMBAL database (Higueruelo, Schreyer et al. 2009) and iPPI-DB (Labbe, Laconde et al. 2013). In addition, the discoveries of existing PPI modulators indicated that large variability exists among different PPI interfaces and existing small-molecule inhibitors, so that each discovery project requires a case-by-case approach (Mullard 2012). The fact that a high rate of hit identification towards PPIs within the TNFSF was observed during the screening of our organic-dye based library suggests that the structures of organic dyes are likely to be skewed to target certain PPIs, at least PPIs within the TNFSF. Thus our organic-dye based library is likely to contain a series of “privileged” structures to target PPIs within the TNFSF, and once a promising scaffold is identified, medicinal chemistry approaches can be employed to avoid “dye-related” problems and refine the structure of existing scaffold to systematically derive better compounds with better activity and specificity.

6.2 IDENTIFICATION OF THE FIRST SMALL MOLECULE OX40 AGONISTS

As discussed, the tumor necrosis factor receptor superfamily (TNFRSF) currently consists of about 30 identified transmembrane receptors. Members of TNFRs contain an extracellular domain responsible for ligand binding, and a number of reported structures of ligand-receptor complexes revealed that a 3:3 symmetrical interaction at the extracellular region is the biologically active synapse required for downstream signal activation (Bodmer, Schneider et al.
Upon ligand binding, most TNFRs, such as CD40, OX40, 4-1BB, and TNFR, can activate nuclear factor κB (NF-κB) through recruitment of TRAF proteins to their intracellular domains. By taking advantage of this specific TNFR mediated integration of NF-κB activation, NF-κB sensor cells (including OX40 sensors, CD40 sensors and 4-1BB sensors) were constructed to provide a convenient cell-based validation platform to further assess the functionality of identified modulators in my ELISA-based binding assays. To my surprise, several inhibitors identified in the OX40–OX40L binding assay did not block OX40L mediated NF-κB activation in the established OX40 sensor system. On the contrary, two of the compounds, 3-1 and 3-4, activated NF-κB in OX40 sensor cells with EC₅₀s around 5 μM and 10 μM respectively, and this NF-κB activation was found to be target specific to OX40 as they were unable to activate NF-κB in both TNFR sensor cells and CD40 sensor cells. Thus, the constructed TNFRSF NF-κB sensor system has proved itself as an effective and valuable lead validation platform to verify modulation of selected TNFRs by small-molecule leads generated in ELISA-based screening assay. In addition, by employing this system, the first small-molecule OX40 agonists have been identified. This is remarkable not only because PPIs have been traditionally considered undruggable with small-molecule inhibitors – with some success achieved only within the last decade or so (Arkin and Wells 2004, Whitty and Kumaravel 2006, Wells and McClendon 2007, Buchwald 2010, Arkin, Tang et al. 2014), but also because small-molecule PPI agonists are considered even less likely to exist
since, as mentioned, in addition to binding, they also need to be able to trigger the downstream activation.

Conflicting data regarding the role of OX40 agonists on the persistence and function of Tregs have revealed that OX40 signaling plays a complex role in the physiology of Tregs (Ito, Wang et al. 2006, Kroemer, Xiao et al. 2007, So and Croft 2007, Vu, Xiao et al. 2007, Duan, So et al. 2008, Piconese, Valzasina et al. 2008, Redmond, Ruby et al. 2009, Ruby, Yates et al. 2009, Griseri, Asquith et al. 2010, Kinnear, Wood et al. 2013). Nevertheless, there is compelling evidence supporting a suppressive effect of OX40 agonism on the development of inducible Tregs (iTregs) in vitro (Xiao, Balasubramanian et al. 2012). Consistent with the finding that OX40 stimulation abrogates iTreg generation from naive CD4+ T cells in vitro, CVN (3-4), a representative small-molecule OX40 agonist has been shown here to successfully mimic the OX40 stimulating signal to enhance Treg generation in vitro.

The development of helper T cells that express discrete subsets of cytokines is critical for the development of effective immunity to cope with a variety of foreign bodies. The cytokine micro-environment is a principal trigger that promotes the differentiation of naive CD4+ helper T cells that commit their T helper cell fate and acquire the ability to secrete a proper panel of cytokines. As a pivotal finding, it has been shown that engagement of the receptor OX40 on T cells increases expression of the transcription factor TRAF6, activates the non-canonical transcription factor NF-κB pathway, and induces the production of interleukin 9 (IL-9) of TH9 cells (Xiao, Balasubramanian et al. 2012). Consistent
with the finding that small-molecule OX40 agonist CVN 3-4 is able to activate NF-kB in our established OX40 sensor cells, 3-4 again, successfully increased Th9 cell generation in an *in vitro* cytokine environment polarized to Th9 cell fate. By all accounts, the observed suppression of Treg generation and enhancement of Th9 cell production *in vitro* has further validated our identified small-molecule OX40 agonism as represented by CVN 3-4.

As a further note, although targeting PPIs has gained significant momentum during the last two decades, limited data demonstrating *in vivo* efficacy of existing PPI modulators have been reported (Arkin, Tang et al. 2014). This is also the case among PPI modulators of TNFSF identified so far. In addition, albeit data suggested that OX40 signal is able to regulate Treg responses (Croft 2010, Ishii, Takahashi et al. 2010), and *in vivo* and *ex vivo*-expanded Tregs are currently under clinical evaluation to restore long-term tolerance in type 1 diabetes (T1D) (Masteller, Tang et al. 2006, Luo, Herold et al. 2010, Orban, Farkas et al. 2010), only one report suggested direct evidence that OX40 signaling can mediate T1D disease progression (Bresson, Fousteri et al. 2011). Thus, my preliminary results with CVN 3-4 in an NOD mouse diabetes prevention model show particular promise for our ability to modulate T1D progression *in vivo* by small-molecule OX40 agonism – consistent with the finding that anti-OX40 agonistic antibody is able to ameliorate T1D progression in NOD mouse primarily through the enhancement of the proliferation and function of a subtype of regulatory T cells (Bresson, Fousteri et al. 2011).
To further dissect CVN 3-4 mediated OX40 agonism, the concentration-dependence of the NF-κB activation observed in OX40 sensor cells was assessed in detail by combinations of CVN 3-4 and the natural ligand OX40L at various concentrations. To quantify activation, the assumptions of the minimal ‘two-state theory’ (Del Castillo-Katz) model (Del Castillo and Katz 1957, Jenkinson 2003) as described before (Bodor and Buchwald 2012) have been used, but all results are mathematically equivalent with the operational (Black and Leff) model (Black and Leff 1983, Kenakin 2006). To account for partial agonism, these models incorporate both affinity (to describe receptor binding via a $K_d$ dissociation constant) and efficacy ($\varepsilon$; to describe receptor activation) into the model assuming that not all occupied receptors are active:

![Diagram of receptor states: vacant, inactive, active, occupied, LR, LR*](image)

The corresponding constants $K_d$ and $\varepsilon$ are defined in terms of concentrations (denoted with square brackets) as:

$$K_d = \frac{[LR]}{[LR]}$$

(Eqn4)

$$\varepsilon = \frac{[LR^*]}{[LR]}$$

(Eqn5)

The fractional response (effect) is obtained via the expression of the total receptors present with the corresponding concentration, $[R_{tot}]$, being the sum of that of free and occupied receptors:
So that the fractional response produced can be expressed as

\[
f_{\text{active}} = \frac{E}{E_{\text{max}}} = \frac{[LR^*]}{[R_{\text{tot}}]} = \frac{\varepsilon \left[ L \right]}{1 + \left(1 + \varepsilon \right) \left[ L \right] / K_d}
\]  
(Eqn 7)

This is equivalent with the more frequently used form either in the minimal two-state theory or the operational model to describe the effect \( E \) as a function of ligand concentration ([L]):

\[
E = E_{\text{max}} \frac{\varepsilon [L]}{1 + \varepsilon [L] / K_d}
\]  
(Eqn 8)

If two ligands (L\(_1\), L\(_2\)) of different affinities (\( K_{d1} \), \( K_{d2} \)) and efficacies (\( \varepsilon_1 \), \( \varepsilon_2 \)) are present simultaneously competing for binding simultaneously at the same receptor, the situation can be summarized below with total effect observed being a sum of those produced by the two ligands:

In this case, the total number (concentration) of receptors [\( R_{\text{tot}} \)] can be written as:
\[
[R_{tot}] = [R] + [L_1R] + [L_1R^*] + [L_2R] + [L_2R^*]
= [R] + \frac{[L_1R]}{K_{d1}} + \varepsilon_1 \frac{[L_2R]}{K_{d2}} + \varepsilon_2 \frac{[L_2R]}{K_{d2}}
\]

(Eqn9)

With this, the total effect produced can be expressed as a function of the \([L_1]\) and \([L_2]\) concentrations present as (see also eq. 6.79 in reference (Kenakin 2006) for the same result obtained in the formalism of the operational model):

\[
f_{active} = \frac{E}{E_{max}} = \frac{[L_1R^*] + [L_2R^*]}{[R_{tot}]} = \frac{\varepsilon_1 \frac{[L_1]}{K_{d1}} + \varepsilon_2 \frac{[L_2]}{K_{d2}}}{1 + (1 + \varepsilon_1) \frac{[L_1]}{K_{d1}} + (1 + \varepsilon_2) \frac{[L_2]}{K_{d2}}}
\]

(Eqn10)

Assuming that the efficacies are quite different (\(\varepsilon_1 < \varepsilon_2\)), the partial agonist \(L_1\) can produce either concentration-dependent activation (when \(L_2\) concentrations are low compared to \(K_{d2}\)) or inactivation (when \(L_2\) concentrations are high compared to \(K_{d2}\)) as illustrated by the figure below.

For a competitive agonist \(L_2\) (I) that produces no effect upon binding, \(\varepsilon_2 = 0\), the above equation can reproduce the well-known Gaddum equation for competitive agonists (assuming a highly efficacious ligand \(L\) (\(L_1\)) so that \([LR^*] = [LR]\) and \(\varepsilon_1\) can be eliminated from the model):

\[
E = E_{max} \frac{[L]}{[L] + K_d \left(1 + \frac{[L]}{K_d}\right)}
\]

(Eqn11)
As a final note, the fully general version of the equation for competing partial agonists can also incorporate a Hill slope, \( n_H \) (see reference (Kenakin 2006)), so that:

\[
\frac{f_{active}}{E_{max}} = \frac{E}{\frac{E_{max}}{1 + \left( \frac{L_1}{K_{d1}} \right)^{n_H} + \left( \frac{L_2}{K_{d2}} \right)^{n_H}}} = \frac{E}{\frac{E_{max}}{1 + \left( \frac{L_1}{K_{d1}} \right)^{n_H} + \left( \frac{L_2}{K_{d2}} \right)^{n_H}}}
\]

(Eqn12)

Figure 6-1 Illustration of the effects produced by combinations of a full and a partial agonist at different concentrations (\( C_1 \) and \( C_2 \), respectively)

Whereas the full agonist alone can produce 100% activation (back, left corner), the partial agonist can produce only a fraction of this even at saturation (here, set to 40%; front, right corner). Under certain conditions (\( C_2/K_{d2} > C_1/K_{d1} \)), it can displace the full agonist and actually reduce the response (back, right corner). The 2D graphs in the bottom show serial cross sections corresponding to different \( C_1 \) or \( C_2 \) concentrations, respectively.
6.3 SUMMARY

As part of the ongoing search for potentially therapeutically useful small-molecule modulators of TNFSF co-stimulatory signaling, I have now identified a first set of compounds capable of interfering with the OX40–OX40L PPI that also seem to act as partial agonists at the OX40 receptor (Song, Margolles-Clark et al. 2014). TNF superfamily has many high-value therapeutic targets, and biologics targeting almost all of the more than twenty known ligand–receptor pairs in this superfamily are now in clinical development for autoimmune diseases and cancer, respectively (Tansey and Szymkowski 2009, Yao, Zhu et al. 2013). In the meantime, only very few small-molecule PPI inhibitors have been identified for TNFSF; they include one for TNF-R–TNFα (SP307) (He, Smith et al. 2005) as well as some polyaromatic inhibitors for the CD40–CD40L costimulatory interaction including those by our group (Margolles-Clark, Umland et al. 2009, Margolles-Clark, Kenyon et al. 2010) and another, quite different scaffold (BIO8898) from Biogen (Silvian, Friedman et al. 2011). For the scenario of PPI activators, even fewer cases of small-molecule PPI ‘agonists’ were reported.

By starting from the chemical space of organic dyes that provided our first CD40–CD40L PPIs, I have now identified what seem to be the first small-molecule disruptors of the OX40–OX40L PPI that also act as partial agonists of the OX40 receptor with low micro-molar potency. The activity of 3-4 (CVN, Figure 3-10) has been confirmed in sensor cell assays (Figure 5-4) as well as in T cells, including regulatory T cells (Tregs) and TH9 cells (Figure 5-6, Figure 5-6). OX40 (CD134), a member of the TNF receptor superfamily, is mainly expressed on
activated T cells. Under proper cytokine microenvironment, OX40 stimulation has been demonstrated to suppress regulatory T cell (Treg) production while inducing TH9 cell generation (Xiao, Balasubramanian et al. 2012). Consistent with these findings, my small-molecule OX40 partial agonist 3-4 showed less prominent, but similar activity to that of the OX40 agonistic mAb (OX86) when tested in these models relying on the induction of Treg and TH9 cells from naive CD4+ T cells.

Preclinical data from tumor models and clinical data from breast cancer patients have suggested the beneficial effect of OX40 agonists (either OX40L:Ig fusion protein or OX40 agonist antibody) on tumor progression (Weinberg, Morris et al. 2011). Both CD4+ and CD8+ T cells seem to be involved in the therapeutic efficacy of OX40 agonists. Due to the inducible and time-limited expression of OX40 on these cells, therapies targeting OX40 might produce less toxicity and reduced systemic side effects than existing immunomodulatory therapies in clinical use. Consequently, several biological candidates targeting this pathway are in various stages of clinical development (Tansey and Szymkowski 2009, Sheridan 2013, Yao, Zhu et al. 2013). Furthermore, and of particular interest for our autoimmune disease-focused work, recently it has been shown that OX40 stimulation can promote Treg expansion in vivo in both mouse models of diabetes and colitis, which in turn prevented diabetes onset and suppressed colitis (Griseri, Asquith et al. 2010, Bresson, Fousteri et al. 2011). The dual function of OX40 agonists to increase T-cell effector function as well as regulate Treg function selectively in different pathological conditions renders OX40 agonist a promising therapeutic possibility for the prevention of these diseases.
My pilot animal study of OX40 small-molecule partial agonist CVN on T1D progression also showed OX40 signal could lead to disease protection, and this is possibly due to enhanced Treg function. These proof-of-principle results identifying the first small-molecules that can act as disruptors of the OX40–OX40L PPI as well as (partial) OX40 agonists represent a first step toward identifying new pharmacological tools to investigate this interaction that could also lead to possible therapeutic alternatives complementing the current biologics under development.
REFERENCES


