Small Molecule Inhibition of CD40–CD154 Co-stimulatory Interaction

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SMALL MOLECULE INHIBITION OF THE CD40–CD154 CO-STIMULATORY INTERACTION

By

Lakshmi Ganesan

A DISSERTATION

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

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SMALL MOLECULE INHIBITION OF THE CD40–CD154 CO-STIMULATORY INTERACTION

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The CD40-CD154 protein-protein interaction (PPI) is an important tumor necrosis factor superfamily (TNFSF) interaction that mainly provides co-stimulatory signals to T-cells consequent to antigen presentation in adaptive immunity. Blockade of this PPI can cause immune unresponsiveness (anergy) and is, therefore, an important therapeutic target for immunosuppression. Following our recent discovery of the first small-molecule inhibitors of this PPI, the present work focuses on the identification of compounds with improved ligand efficiency and the confirmation of their activity in cell-based assays. Our best inhibitors identified to date are organic dyes that show low micro-molar activity in inhibiting CD40–CD154 binding and whose activity was also confirmed in several immune cell activation assays, for example, using THP-1 human monocytic leukemia cells or splenocytes from BDC2.5 / NOD mice activated with the corresponding antigen. Based on the analysis of the accumulated activity data, we have established clear structure-activity guidelines needed for CD154 inhibitory activity for this class of compounds.
I have performed a series of computational docking experiments using available three-dimensional CD154 structures. Among our inhibitors investigated, docking scores (*Glide*, Schrödinger LLC) distinguished binders (specific and non-specific) from non-binders most at an allostERIC site at the interior core of the CD154-trimer reiterating the established druggable nature of this site among TNFSF ligands (Ganesan *et al.* 2012).

During this search for specific inhibitors, I have also identified a set of xanthene-based promiscuous inhibitors (erythrosine B, rose Bengal) that are the first promiscuous PPI inhibitors described in the literature. We found erythrosine B (ErB) to be a non-specific promiscuous inhibitor of a number of PPIs within the tumor necrosis factor superfamily (TNF-R–TNFα, CD40–CD154, BAFF-R–BAFF, RANK–RANKL, OX40–OX40L, 4-1BB–4-1BBL) as well as outside of it (EGF-R–EGF) with a remarkably consistent median inhibitory concentration (IC\textsubscript{50}) in the 2–20 μM (approximately 2–20 mg/L) range. This was a novel, somewhat unexpected finding since small-molecule inhibition of PPIs is notoriously difficult due to the relatively large and flat nature of the interacting surfaces that do not have pockets suitable for the efficient binding of drug-like molecules (Ganesan *et al.* 2011).

To identify the mechanisms of inhibition, extracellular CD154 was expressed with a histidine-tag in *E. coli* and purified using nickel affinity and size exclusion chromatography. The *metachromatic shift* exhibited by most of these inhibitors, which are organic dyes, upon protein binding (i.e., the shift in the absorption maxima, \(\lambda_{\text{max}}\)) was exploited to determine the binding partner and
derive binding constants such as affinity ($K_d$) and stoichiometry ($n$). While the spectra of DR80, a specific inhibitor, shifted ($\Delta \lambda_{\text{max}} \sim 10\text{nm}$) only upon incubation with CD154; that of erythrosine B (ErB), a promiscuous non-specific inhibitor, shifted upon incubation with both CD154 and CD40 indicating that it indeed bound both proteins. Stoichiometry analyses (Job’s plot) showed that proteins bind multiple ErB molecules. These results can now provide the basis for a more focused, structure-guided drug discovery effort to identify sufficiently efficient small-molecule CD40–CD154 inhibitors as well as to identify the mechanism of the unexpected promiscuous PPI inhibition we observed for the food colorant ErB.
ACKNOWLEDGEMENTS

I thank my parents Geetha and V.V. Ganesan for being a pillar of strength. I am immensely grateful to my mentor, Dr. Peter Buchwald for all his care in allowing my learning. I admire his infinite patience and perennial smile and whole-heartedly dedicate this work to him. Many thanks to the Buchwald lab for providing me a home: Dr. Emilio Margolles-Clark for all the early lessons at the bench and his counseling and support, and Yun (Eric) Song for all the good times. I am indebted to my committee for their valuable contributions in enriching this project. I am grateful to Dr. Vladlen Slepak for training me in his laboratory in protein purification. I thank the Slepak lab for considering me as one of their own: Dr. Konstantin Levay for making me appreciate the art of bench-work, Darla Karpinsky for being a supportive friend, and Kelly Wang for her warmth. I thank Dr. Stephan Schürer for facilitating my learning of many computational tools and Dr. Dušica Vidović for the various discussions and invaluable help in finishing my second manuscript. I thank Dr. Wasif Khan for all his advice and the Khan lab for their help in conducting animal studies. I thank Dr. Alberto Pugliese for his prompt support and constant interest in my progress. Many thanks to Dr. Arun Malhotra and Brad Schmier for their help with setting up initial crystal screens, and Dr. Zhibin Chen and Jun Suzuki for their help with the BDC2.5 drug screens. I also thank Dr. Georg Petroianu for graciously agreeing to serve as my external examiner. Finally, I feel fortunate to have this thirst for knowledge and thank everyone who has nurtured it by teaching me a thing or two.
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CHAPTER 1 BACKGROUND

1.1 SMALL MOLECULE INHIBITION OF PROTEIN–PROTEIN INTERACTION

Protein-protein interactions (PPI) are ubiquitous elements in the human interactome. Defining their roles both in physiological and pathological states has uncovered useful therapeutic targets to alter the course of many important diseases. However, they are less susceptible to modulation by small molecules than traditional drug targets (e.g., G-protein coupled receptors, ion channels and enzymes) that have well defined pockets for binding small molecule therapeutics. This is because PPIs are typically characterized by flat and featureless topologies, large surface area of interaction (1500 - 3000 Å) exhibiting surface complementarity and high degree of structural adaptability (Figure 1-1) (Arkin et al. 2004; Buchwald 2010) all of which makes difficult the design of inhibitors using just the apo-structure as a starting point (Eyrisch et al. 2007; Wells et al. 2007).

For the above reasons, the original inhibitors of PPIs were antibodies, including monoclonal antibodies that had the advantage of high target specificity characterized by their nanomolar potencies, but suffered due to size related adverse effects typical of biopharmaceuticals including poor solubility, poor adsorption and bioavailability, metabolism and the possibility of precipitating an immune response, being foreign / immunogenic proteins. Evidence for this is the withdrawal from the clinical trials of Antova / ruplizumab (anti-CD154 monoclonal antibody) and TGN1412 (anti-CD28 superagonist monoclonal antibody)
(Suntharalingam et al. 2006), which caused thrombolysis and cytokine storms respectively in treated patients (Buchwald 2010). Efforts therefore were refocused on developing strategies to generate small molecules that can provide safer alternative to antibodies and peptide-based therapeutics and as a result, two drugs that can be considered PPI inhibitors (tirofiban, Aggrastat®) and maraviroc, Selzentry®) are already approved for use in the market (Fuller et al. 2009) (Table 1-1).

![Figure 1-1: Receptor–ligand interactions vs. protein–protein interactions](image)

<table>
<thead>
<tr>
<th>[i] Receptor-Ligand Interactions</th>
<th>[ii] Protein-Protein Interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interface Topology:</td>
<td>Concave</td>
</tr>
<tr>
<td>Surface dynamics:</td>
<td>Limited</td>
</tr>
<tr>
<td>Interaction volume:</td>
<td>Small (200-300 Å³)</td>
</tr>
<tr>
<td>Binding energy / unit volume</td>
<td>Large</td>
</tr>
<tr>
<td>Ligand efficiency</td>
<td>~1.5 KJ/NHA</td>
</tr>
</tbody>
</table>

**Figure 1-1: Receptor–ligand interactions vs. protein–protein interactions**

Traditional drug targets having well-defined binding pockets with sufficient concavity to concentrate a large number of interactions over a small surface area [i]. PPIs having 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 [ii] (Whitty et al. 2006).
The existing approaches to small-molecule PPI inhibition can be classified as target (protein)-based or ligand-based. Protein-centric approaches use the knowledge of critical residues on the PPI interface that contribute the most to the binding energy or are indispensable for the PPI. Identification of these so-called ‘hot spots’ is now a necessary first step in small-molecule design for PPI inhibition. Hot spots are by nature highly flexible, adaptable regions on the protein surface that can easily be cherry-picked by superimposing the apo- and small-molecule bound forms of available crystal structures.

Table 1-1: Examples of PPI inhibitors

<table>
<thead>
<tr>
<th>PPI/ Target class</th>
<th>Antibody</th>
<th>Inhibitor</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>αIIβ3 integrin – fibronectin (Anti-platelet)</td>
<td></td>
<td>Tirofiban (IC₅₀=27μM)</td>
<td>Mimics the Arg-Gly-Asp (RGD) motif present in the αIIβ3 integrin’s natural ligands (Fry 2006)</td>
</tr>
<tr>
<td>IL-2–IL-2R (Immuno-suppression)</td>
<td>Basiliximab Daclizumab</td>
<td>SP4206 (Kᵢ=60nM)</td>
<td>Binds IL-2 by mimicking key side chain interactions made by its receptor (IL-2R) and induces conformational change upon binding (Raimundo et al. 2004)</td>
</tr>
<tr>
<td>CCR5–gp120 (HIV entry inhibition)</td>
<td></td>
<td>Maraviroc (IC₅₀=5nM)</td>
<td>Allosteric modulator of chemokine receptor CCR5 that prevents its association with HIV gp120 protein and hence its entry into CD4⁺ cells (Dorr et al. 2005).</td>
</tr>
<tr>
<td>HDM2–p53 (Anti-cancer)</td>
<td></td>
<td>Nutlin-3 (Kᵢ=90nM)</td>
<td>Binds to HDM2 at the sub-pockets normally occupied by the hydrophobic side-chains of p53 (Shen et al. 2011)</td>
</tr>
<tr>
<td>Bcl-XL – Bak (Anti-cancer)</td>
<td></td>
<td>ABT-737 (Kᵢ=1nM)</td>
<td>Competitive inhibition by mimicry of aliphatic residues in the binding pocket (Tse et al. 2008)</td>
</tr>
<tr>
<td>TNFR–TNFα (Anti-inflammatory)</td>
<td>Infliximab Adalimumab</td>
<td>SP304 (Kᵢ=15μM)</td>
<td>Dissociates active TNFα trimer to inactive monomer (He et al. 2005)</td>
</tr>
<tr>
<td>CD40–CD154 (Immuno-suppression)</td>
<td>Ruplizumab</td>
<td>Suramin (IC₅₀=15μM) Direct red 80 (IC₅₀=6μM) Mordant brown M (IC₅₀=0.13 μM) BIO8898 (IC₅₀=25μM)</td>
<td>BIO8898 causes subunit fracture and hence disrupts trimer integrity of CD154 (Margolles-Clark et al. 2009; Margolles-Clark et al. 2009; Margolles-Clark et al. 2010; Silvian et al. 2011)</td>
</tr>
</tbody>
</table>
In the absence of available structures, various computational algorithms have been developed for use in identifying critical consensus motifs that can serve as starting points for a drug discovery initiative. The existing algorithms for druggable pocket detection can be broadly classified as geometry-, evolution-, or energy-based (Perot et al. 2010). Geometry-based methods identify surface cavities and measure complexity or roughness (e.g., LIGSITE, Pocket, Surfnet, PASS, and Q-sitefinder). Evolutionary algorithms map putative druggable sites by comparison with related structures having annotated ligand binding sites (Henrich et al. 2010). Energy-based methods focus on pockets that are hit mainly by chemically diverse probe molecules with relatively high binding affinity (Hajduk et al. 2005).

However, all these methods suffer from the limitation that they use as starting material a crystal structure, which but represents the time-averaged coordinates of one of the many possible protein conformations. An alternative to account for the inherent dynamics of protein conformation is to use a large number of snapshots taken using a computationally intensive molecular dynamics (MD) simulation as starting material for the above pocket-finding algorithms (Eyrisch et al. 2007). These identified pockets can then serve as templates for high-throughput virtual screening (HTVS) via docking and scoring to yield several thousands of likely hits from libraries of several millions of molecules.
Ligand-centric approaches use the “central similarity principle” that states that similar molecules exhibit similar properties, to predict the activity of a test set of compounds based on similarity (or distance) to a set of actives taken as reference. Many algorithms have been developed to compare two- or three-dimensional molecular descriptors using classification schemes such as recursive partitioning, Bayesian statistics, and neural networks among other methods (Meireles et al. 2011). This approach has the advantage of being convenient and requiring little starting information, as a single active compound can be used to screen an entire library for ‘relatedness’. ‘Similarity’ (or) ‘relatedness’ is computed using either two dimensional (topological) descriptors, like atom types, inter-atomic distances, presence of functional groups, number of bonds, and bond length (PubChem Similarity search) (Willett 2006) or using three dimensional information like volume, represented as a continuous function (Gaussian) for comparison of shapes (ROCS, OpenEye Scientific Software, Inc.) (Hawkins et al. 2007), or a combination of both (Vidovic et al. 2011). The above descriptors are pre-computed for a test set of compounds that can be compared by means of Tanimoto distance (see sections 2.2.i.a, 2.2.i.b) to a reference.

Docking algorithms suffer from several limitations. Some of these are inadequate treatment of electrostatics, lack of accounting for entropy changes upon binding, insufficient ligand conformer sampling, inadequate sampling of tautomers and ionization states of proteins and ligands, and, most importantly, the rigid receptor approximation. On the other hand, ligand-based virtual screening (either structure or shape-based) using a reasonable staring point can
be considered to be more straightforward and computationally less intensive. Although, screening using the docking approach does recognize actives and predicts reasonably their binding modes, they work less well in discriminating false-positives. Ligand-based approaches on the other hand suffer from the inverse issue of generating false negatives, as they can easily miss actives that are radically different in size, shape or chemistry. Another point of contrast is that while docking method works best using a co-crystal pose rather than the apo-structure due to the manifold conformational changes that occur upon ligand binding, shape similarity searches have been shown to work reasonably well even in the absence of a bioactive ligand pose, by using the lowest-energy conformations of the ligand as queries (Hawkins et al. 2007). Pharmacophore searches, either based on protein or ligand structures, have proven the most productive in virtual screens yielding hit rates of 1 – 20% for selected datasets and this may further increase when used in a complementary fashion (Meireles et al. 2011).

Screening using virtual libraries of drug-like compounds for PPI inhibition still suffers from the limitation that many repositories are largely populated with the compounds that targeted and therefore customized to traditional drug pockets. While they are skewed towards smaller and flatter molecules, most PPI inhibitors tend to be larger, more rigid, and three-dimensional (Buchwald 2010; Mullard 2012). To cater to the requirements of PPI inhibition, approaches have evolved to use as starting point protein-structure mimetics and chemical spaces that contain compounds that are ‘privileged’ for protein binding (Hajduk et al. 2011).
Two such libraries have been built by FORMA Therapeutics, Inc., one consisting of over 150,000 molecules derived from diversity-oriented synthetic approaches covering novel chemical subspaces and another built using protein-mapping and interfacial analysis. Ensemble Therapeutics has built a library of natural-product like small molecule macrocycles (MW\(^1\) - 500 - 1000 Da) containing over 4 million molecules using a DNA-programmed chemistry approach. This library has yielded hits for a number of PPIs, notable among them being for the interaction between interleukin 17 (IL-17) and its receptor (Mullard 2012).

NMR-based screens have also revealed that biphenyl scaffolds and carboxylic acids (–COOH) are among the structures that frequently figure among protein binders, and that a high hit rate of a target protein by such molecules is a direct indication of its druggability. Therefore, libraries that are enriched with compounds containing such groups will constitute a privileged space for PPI inhibition (Hajduk \textit{et al.} 2000). With accumulating information, databases of small-molecule PPI inhibitors and related protein structures are also being built. A few notable examples include the TIMBAL database (http://www-cryst.bioc.cam.ac.uk/databases/timbal) (Higuерuelo \textit{et al.} 2009), and the 2P2I database (http://2p2idb.cnrs-mrs.fr/) (Bourgeas \textit{et al.} 2010).

\footnote{\textup{MW – Molecular weight}}
The potency or activity of small molecules in a given assay or test is typically expressed as median effective dose (EC\textsubscript{50}) / inhibitory constant (IC\textsubscript{50}) or dissociation constant (K\textsubscript{d}) in a molar scale. For therapeutic applicability, it is desirable for small molecules to have affinity comparable to that of the protein partner, which falls on an average in the sub-micromolar range (~20nM). As PPIs typically involve large surface areas without adequate pockets (lower binding energy per unit volume), a sufficiently large molecule is required to interact simultaneously with multiple pockets to achieve desired potencies. Additionally, only a fraction of the small molecule's surface is engaged at the protein interface and not most of the surface as it happens when inside a well-defined pocket. Thus, PPI inhibitors tend to violate the size constraints of the Lipinski's "rule of five", a well-established guide needed to achieve oral bioavailability (Lipinski \textit{et al}. 2001), more often than traditional drugs.

To limit this tendency, a better metric to potency in the lead optimization process is one that normalizes the activity to molecular size. This index, called ligand efficiency (LE or \(\Delta G\)) is defined as the binding energy (\(\Delta G = -RT\ln K_d\)) per non-hydrogen atom (NHA) (Hopkins \textit{et al}. 2004; Abad-Zapatero \textit{et al}. 2005; Abad-Zapatero \textit{et al}. 2010). Comparing LE can better highlight differences among classes of binding sites and / or that among small molecules. For example, the average LE of PPI inhibitors was found to be 1.0 kJ/atom, which is less than that of inhibitors of traditional ligand-receptor interactions (LE = 1.5 kJ/atom). In other words, a small molecule with a LE of 1.0 kJ/NHA will require >45 heavy atoms to achieve a \(K_d\) of 20 nM.
On the other hand, those with an LE of 1.5 kJ/NHA can achieve the same potency using fewer heavy atoms. This analysis further highlights the need for good protein binders to fulfill the requirements of PPI inhibition within the constraints of “druggability” (Buchwald 2010).

1.2 CD40–CD154 PPI–FUNCTION

The CD40-CD154 PPI is an important co-stimulatory interaction in adaptive immunity and an inflammatory mediator. It is a proven target for immunosuppressant and anti-inflammatory drug development and is of particular interest to our group as its blockade has potential in achieving immune-modulation in islet-cell transplantation (Kenyon et al. 1999; Ricordi et al. 2004).

1.2.i Specific immunity (Co-stimulation)

T-cell activation marks the beginning of the adaptive immune response, and its expansion and differentiation into distinct subsets is required for its sustenance. While antigen presentation by antigen presenting cells (APC) as an MHC-peptide complex (signal 1) initiates T-cell activation, this has to be accompanied by confirmatory signals by a constellation of cell-surface proteins (signal 2) called co-stimulatory molecules (Figure 1-2). These co-stimulatory interactions signal for the clonal expansion, survival and differentiation of the T-cells to yield a productive immune response. The lack of signal 2 leads to T-cell unresponsiveness (anergy) that can lead to antigen-specific tolerance.
Thus co-stimulatory blockade is an effective strategy to achieve immunosuppression. Such immunosuppression is also immune-activation specific, useful both in autoimmune and allograft settings (van Kooten *et al.* 2000; Schonbeck *et al.* 2001; Quezada *et al.* 2004; Li *et al.* 2009).

Co-stimulatory molecules vary both spatially and temporally in their expression patterns. While some are constitutively expressed (CD28–B7-1), many are induced upon activation at different time-points and, hence, are classified as early (CD40–CD154) or late-expressed (e.g., OX40–OX40L, 4-1BB–4-1BBL) co-stimulatory interactions. Moreover, the same co-stimulatory molecules may have different effects on different subsets of T-cells.

Based on their function, co-stimulatory molecules can also be classified as positive co-stimulatory molecules (CD28–B7, CD40–CD154, OX40–OX40L), which promote immune cell activation, and negative co-stimulatory molecules (CTLA4–B7, PD-1–PD-L), which represent a physiological means of dampening the immune response and inducing peripheral tolerance. At any given time, it is the balance of positive and negative co-stimulatory molecules that determines the fate of the adaptive immune response. Also, since co-stimulatory molecules exhibit functional redundancy, one that can express later can compensate the blockade of an early-expressed interaction. Therefore, blocking the right combination of co-stimulatory molecules (polypharmacology) could hold the key to effective therapy.
Figure 1-2: The two signal theory of immune activation
Presentation of antigen peptide as a complex with major histocompatibility complex (MHC) by the antigen presenting cells (APC) and its recognition by the T-cell receptor (TCR) of the naïve T-cell [Signal 1]. Confirmatory signals from the engagement of co-stimulatory molecules (CD40–CD154) [Signal 2].

Co-stimulatory molecules belong to three main families - the immunoglobulin (Ig) superfamily, the tumor necrosis factor superfamily (TNFSF), and the T-cell Ig domain and mucin domain (TIM) family (Li et al. 2009). Our main focus, the CD40–CD154 protein-protein interaction (PPI) is an important receptor–ligand interaction between two members of the TNFSF. TNFSF comprises several ligands and receptors whose interactions are essential for development, homeostasis, and adaptive immunity (Bodmer et al. 2002). A clear evidence for the importance of the CD40–CD154 interaction is provided by the fact that mutations in CD154 that prevent its binding to CD40 result in so-called
hyper IgM syndrome (HIGM), which is characterized by defective immunoglobulin class switching (Schonbeck et al. 2000; Quezada et al. 2004). Blockade of the CD40–CD154 PPI using anti-CD154 antibodies (e.g., 5c8) has been shown to be therapeutically effective in both autoimmune and allograft settings in rodent and non-human primate models (Kenyon et al. 1999; Karpusas et al. 2001). These findings established this PPI as a target for immunosuppressant drug development. Particular relevance has been found in type I diabetes as on the one hand its blockade has proven effective in islet transplantation where it has consistently allowed engraftment and long-term functioning of transplanted islets, leading to antigen-specific immune-modulation (Kenyon et al. 1999; Cardona et al. 2006) and on the other hand it has also been shown to effectively prevent the development of the autoimmune disease itself (Wagner et al. 2002; Bour-Jordan et al. 2004).

1.2.ii Inflammation

Eventually, as the broader expression profiles of both CD40 and CD154 were discovered, their pro-inflammatory roles in non-hematopoietic cells via the release of mediatory cytokines, chemokines, and cell-adhesion molecules have been revealed (Antoniades et al. 2009). CD154, exists both as a 39 kDa type II transmembrane protein (expressed on CD4+T-cells, B-cells, mast cells, basophils, eosinophils, natural killer cells, macrophages, endothelial cells, vascular smooth muscle cells and activated platelets) and as active trimeric (predominantly platelet-derived) soluble form in the plasma (Henn et al. 1998). Its cognate receptor CD40 is a 48 kDa type I transmembrane glycoprotein found on
immune (B-cells, monocytes, macrophages, dendritic cells) and non-immune (thymic epithelia, endothelial) cell types (Lutgens et al. 2007). The broad expression profile of this PPI pair points to its versatile physiological role and its consequent association with immune and extra-immune pathologies. A striking evidence of this is the consistent elevation of CD154 levels in atherosclerotic lesions, in patients with hypercholesterolemia and acute coronary syndromes (Pamukcu et al.; Vishnevetsky et al. 2004). Its elevation reliably indicates increased risk of cardiovascular events, and hence, has acquired a biomarker status in their diagnosis and prognosis (Lievens et al.; Unek et al.; Lutgens et al. 2007). Furthermore, conventional therapeutic agents (antiplatelet drugs, statins, and ACE inhibitors) that decreased the plasma CD154 levels improved cardiovascular disease outcomes better than those that did not affect the same (Vishnevetsky et al. 2004).

The role of this PPI in cardiovascular pathology predominantly stems from its role as an inflammatory mediator. Upon interaction with CD40 on various cell types, CD154 induced pro-inflammatory chemokines (MIP1α, MIP1β, RANTES, SDF1, and MCP), cytokines (IL12, IL15, and IFNγ), cell-adhesion molecules (E/P-selectins, VCAM1, and ICAM-1), and matrix metalloproteinases (Antoniades et al. 2009). Cell-adhesion molecules act as pro-atheromatous factors leading to the association of immuno-competent cells to the endothelium. CD154-mediated angiogenesis (via VEGF and possibly IL-8, IL-5 and Cox2) supports the growth of atheromatous lesions (Andre et al. 2002). The pro-inflammatory chemokines trigger the expression of tissue factor (an initiator of the extrinsic pathway of
coagulation), without affecting that of its inhibitor (TFPI), while decreasing that of thrombomodulin, the anticoagulant receptor for thrombin (Schonbeck et al. 2000). Thus, they shift the balance in favor of coagulation contributing to the thrombogenicity of plaques (Schonbeck et al. 2001). A possible pro-apoptotic role for CD154 ligation in the formation of a necrotic core remains to be elucidated. Additionally, platelets constitutively express CD40 and its ligand, which further increases in acute coronary syndromes. Ligation of CD40 on platelets increases expression of P-selectin (CD62P), α-granule and dense granule release, causing its activation (Lievens et al.; Henn et al. 1998; Andre et al. 2002; Inwald et al. 2003). CD154, independent of CD40, also binds to αIIbβ3 integrin receptors on platelets causing thrombus formation and platelet spreading. Inhibition of this PPI using anti-CD154 monoclonal antibody has not only been useful in elucidating the role of this PPI in cardiovascular pathophysiology, but has also indicated potential therapeutic value. In the setting of experimentally induced hypercholesterolemia in mice, anti-CD154 treatment limited the atherosclerotic lesional size by 59%, its lipid content by 79%, and its titer of immune and inflammatory mediators by 70% (Mach et al. 1998). As a result, established lesions do not progress to form vulnerable plaques. Clinical development of the anti-CD154 antibody clone 5c8 was hampered due to thromboembolic events in clinical trials (Nakamura et al. 2006). However, this is likely due to its large size or a pro-aggregatory effect mediated by its Fc domain (Mirabet et al. 2008).
1.3 **CD40–CD154 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), extracellular signal-regulated kinase (ERK), and STAT3 (Signal Transducers and Activators of Transcription-3) (Bishop *et al.* 2001; Aggarwal 2003; Elgueta *et al.* 2009).

Association of CD154 with p53 causes 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 CD154. The formed CD154 trimers associate with CD40 to induce its trimeric clustering (Grassme *et al.* 2002). This facilitates the binding of the receptor’s intracellular domain to adaptor proteins called TRAFs (TNF Receptor Associated Factors). TRAFs 2, 3, and 6 bind directly and TRAF 1 and 5 indirectly through heterodimerization with TRAF 2 and 3, respectively. The TRAFs consist of a conserved C-terminus that binds to CD40 and a less conserved N-terminus having one (TRAF 1) or more (TRAF 2-6) zinc finger domains that associate with secondary messengers (Elgueta *et al.* 2009). Act1 (NF-κB Activator-1) is an adaptor protein that links the TRAF proteins to TAK1 (Transforming growth factor-Beta Activated Kinase-1) /IκK (I-κB kinase) to activate NF-κB/I-κB (Inhibitor of κ Light Chain Gene Enhancer in B-Cells) and MKK (Mitogen-Activated Protein Kinase Kinase) complex to activate JNK, p38 MAPK, and ERK1/2. Upon phosphorylation of IκB
by IκK, it gets ubiquitinated and degraded by the proteosome, thereby liberating the NF-κB for nuclear translocation and transcriptional activation of pro-survival and pro-inflammatory genes (Figure 1-3). 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 et al. 2003; Dempsey et al. 2003; Chatzigeorgiou et al. 2009).

**Figure 1-3: CD40–CD154 signaling**
The CD154trimer (T-cells) induces trimeric clustering of receptor CD40 (B-cells). Adaptor proteins TRAFs mediate CD40 signaling intra-cellularly. TRAFs activate kinase cascades of the PI3K, AKT, and JNK pathways resulting in the activation of AP1 transcriptional complex, NF-κB pathways. These suppress apoptosis; enhance cell proliferation through growth factors and cytokine secretions.
1.4 STRUCTURE OF CD40 AND CD154

The gene for CD40 is located in the q12-q13.2 region of the human chromosome. Its mRNA is 1.5 kb, encoding a 277 amino-acid-long type I transmembrane glycoprotein that is 48 kDa in size. Its extracellular region 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 (van Kooten et al. 1996; van Kooten et al. 2000) (Figure 1-4.i). The CD154 gene is located on the X-chromosome, region q26.3 - q27.1, spans approximately 13 kb, and encodes a 261 amino acid polypeptide. Alongside the full length 39 kDa type II transmembrane platelet-derived active trimeric soluble forms of CD154 of molecular weights 31, 18, and 14 kDa active trimeric soluble form in the plasma have also been described (van Kooten et al. 1996; Schonbeck et al. 2000; van Kooten et al. 2000; Schonbeck et al. 2001). The crystal structure of the extracellular region corresponding the soluble CD154 (residues 116 – 261; 18 kDa) shows it to form a trimer the shape of a truncated pyramid having a three-fold symmetry axis parallel to beta strands of the monomers. Each monomer is a sandwich of two anti-parallel beta sheets having the topology of a jellyroll or a Greek key. The interface between the subunits comprises Tyr 170, His 224, and Leu 261 from each monomer that cluster at the core of the three-fold symmetry axis. The hydrophobic core of the CD154 trimer is essential for holding it together in its tertiary structure and hence the residues that comprise it are important for the stability of the trimer (Figure 1-4.ii) (Karpusas et al. 1995).
Figure 1-4: Structures of CD40 and CD154

CD40, (type-I transmembrane protein) having three extracellular cysteine rich domains (CRD1, 2, and 3 colored blue, orange and maroon respectively) [i]. CD154, (type-II transmembrane protein) with extracellular TNF homology domain. Subunits (A, B and C are colored gray, red and green respectively) forming trimers the shape of a truncated pyramid. Residues at the interior core of the CD154 trimer (Y172, Y170, H224) and the subunit interface (Y145, Y146) [ii] (An et al. 2011).
1.4.i CD40–CD154 interaction

The binding site of CD40 on CD154 is in the groove at the interface of the monomers. A recent crystal structure showed CD40 to bind only at two of the three potential binding sites present in each of the subunit interfaces of the trimeric CD154 (An et al. 2011). This stoichiometry is unusual among known TNFSF complexes that typically have a stoichiometry of three receptors per ligand trimer.

This distinction arises due to the asymmetric distribution of CD40 on the two CD154 subunits with which it interacts (611 Å² with one monomer CD154 and 378 Å² with neighboring monomer CD154*). This asymmetry is attributed to a disulfide bridge in CRD3 between Cys 111 and Cys 116 that is unique to CD40, which distorts the ladder pushing it away from one (CD154*) and more towards the other interacting CD154 subunit.

All the three CRDs are involved in the interaction with CD154 subunits, while CRD1 and the first half of CRD2 distributes equally between the CD154 subunits, the second half of the CRD2 and CRD3 predominantly interact with CD154. The complex is held together mainly by charge complementarity between negatively charged residues of CD40 (Glu 74, Asp 84, Glu117) and positively charged residues of CD154 (Lys 143, His 249, Arg 203, Arg 207) (Bajorath 1998; Singh et al. 1998) (Figure 1-5).
Figure 1-5: CD40–CD154 interaction

Binding of CD40 at the CD154 subunit interface: Acidic (D84, E64, E66, E117) residues of CD40 interact with the basic residues (K143, R203, R207) of CD154. The CD154 subunits A, B, and C are colored gray, red and green respectively. The CD40 cysteine rich domains (CRD 1, 2, and 3) are colored blue, orange, and maroon respectively (An et al. 2011).

1.4.ii Small molecule inhibition of CD40–CD154 PPI

Early demonstrations of CD40–CD154 blockade were done using the anti-CD154 monoclonal antibody (5c8, ruplizumab), which bound at the interface of the CD154 monomers, thus competitively inhibiting it’s binding with the cognate receptor CD40 (Karpusas et al. 2001). However, despite success in various animal models, the blockade of the CD40–CD154 interaction using the antibody
failed in human clinical trials due to the associated thrombocytopenic complications suspected to stem from size-related attributes typical of biopharmaceuticals (Nakamura et al. 2006). We hypothesized that such problems could be overcome by using small molecule inhibitors. Recently, an intriguing possibility that seems particularly suitable for disrupting PPIs of the TNFSF by small molecules has been suggested: dissociation of the trimeric configuration of the protein ligand (or the receptor) making it unsuitable for adequate binding to its cognate partner (Silvian et al. 2011). For example, SP307, a small molecule TNFR–TNFα inhibitor achieves this by binding to TNFα and causing the ejection of one of the subunits (He et al. 2005). BIO8898, a recently reported inhibitor (IC$_{50}$ = 25 μM) of CD40–CD154 PPI also acts similarly by accessing the interior core of the CD154 trimer by intercalating between two monomeric subunits (A and C) making extensive hydrophobic contacts (Figure 1-6.i).

CD154 modified its structure largely to accommodate BIO8898 as observed when compared to the native structure. This involves intermediate and global changes in the protein structure caused by conformational changes in side and main chain atoms to accommodate specific portions of the inhibitor. Global changes involve the translational split between A and C subunits of CD154. The three tyrosines (Tyr 170) separate axially from the three histidines (His 224) at the central core of the trimer to form a deep lateral cavity deviating from the original closely packed native structure to snugly accept the projecting biphenyl (arm 4) of BIO8898. A split between Leu 259 from subunits A and C
accommodates the pyrrolidonemethyl pyrrolidine (arm 2) group of BIO8898. AA’
loop of subunit C that includes Tyr 145 and Tyr 146 reorganizes to form a flap
folding over the other pyrrolidonemethyl pyrrolidine and cyclohexylaminoacetic
acid (arms 1 and 3 respectively) chains and partially shield them from the solvent
(Figure 1-6.ii). Further, the core amino acid residues that undergo structural
adaptation both in TNFα and CD154 can be thus considered as ‘druggable’ and
are over-represented by aromatic amino acids, especially tyrosines that play a
privileged role in small-molecule binding. Additionally, these residues are all
conserved across the ligands of the TNFSF and thereby may constitute a
possible generic hot spot for small-molecule attack within the family.

Figure 1-6: BIO8898–CD154 interaction
Structure alignment of apo (line representation) and bound (tube representation)
structures of CD154 with BIO8898 showing subunit fracture resulting in a
translational split between subunits A and C when compared to the intact apo form
[i]; Ligand interaction diagram (LID) showing the two-dimensional representation
of the interaction of BIO8998 with residues of CD154 [ii] (Silvian et al. 2011).
1.5 EXPLORATION OF THE CHEMICAL SPACE OF ORGANIC DYES

Our group has explored the chemical space of organic dyes for PPI inhibition due to their good protein binding ability (Figure 1-7) and has found some to exhibit specificity for their targets. For example, direct red 80 (DR80), crocein scarlet 7B (CS7B), and mordant brown 1 (MB1) are quite specific CD40–CD154 inhibitors (Margolles-Clark et al. 2009; Margolles-Clark et al. 2009; Buchwald et al. 2010; Margolles-Clark et al. 2010). Other compounds were found to be non-specific or promiscuous (e.g., erythrosine B, ErB) (Buchwald et al. 2010; Ganesan et al. 2011). Some of these compounds are well-known poly-ionic sulfo-dyes, a large structural variety of which are easily available, and are relatively nontoxic. Acid dyes, such as most of the structures considered here, 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 et al. 2010).

Several evidences point to the protein-binding ability of dyes and their potential for being specific: tissue staining and dye-ligand affinity chromatography largely exploit this (Denizli et al. 2001). Modern day medicinal chemistry dates back to the early days’ dye research of Paul Ehrlich, who is considered the father of chemotherapy. Ehrlich doctoral thesis work lead to the discovery of mast cells using aniline dyes as staining tools (Crivellato et al. 2003). His experiments using dyes also revealed the anti-malarial potential of dye methylene blue and the usefulness of trypan blue in the treatment of trypanosomiasis (Schwartz 2004). The visual appeal of organic dyes lead him to coin the term “magic bullet”, which
he defined as “a compound that can highlight and target disease-causing organisms for destruction”. This term has now come to refer to “drugs” in general (Strebhardt et al. 2008). He also instilled this enthusiasm in his students Pappenheim and Michaelis, of which Leonor Michaelis studied extensively color change in dyes resulting from their interaction with cellular components - a phenomenon called “metachromasy” meaning “altered color” – again a term coined by Ehrlich (Bergeron et al. 1958). Thus, this class of compounds also presents an additional property that can be exploited to study its interactions with proteins (Bergeron et al. 1958; Klunk et al. 1999) and thereby makes an interesting addition to the chemical space of possible small molecule PPI modulators.

Unfortunately, the very property that allows for their investigation is not as appealing for their development as drugs. Dye compounds are not a likely choice to advance in clinical trials because of possible coloration effects. Exceptions exist as FP-21399, a bis(disulphonaphthalene)-azo compound selected from a screening program of Fuji compounds originally developed for photography, found potential in the treatment of HIV infections (as a possible inhibitor of the gp120-mediated fusion). This was advanced into clinical trials relatively recently (Ono et al. 1997). Furthermore, once an active structural scaffold could be identified, color-related problems may be avoided in many cases as seen with suramin.
Suramin is, in fact, a “colorless dye”, since it is structurally related to certain polysulfonatedazo dyes such as trypan blue or Evans blue, but it does not contain the chromogenic aryl azo moiety, however 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 costimulatory interaction (Margolles-Clark et al. 2009).

Figure 1-7: Organic dyes as a chemical space for protein-binding compounds
Privileged substructures that are consistently found among protein binders [inner circle] (Hajduk et al. 2000) are well represented among organic dye scaffolds tested for their protein binding ability and PPI inhibition [outer circle] (Buchwald et al. 2010; Ganesan et al. 2011).
1.5.i Specific inhibitors

During the search for small molecule inhibitors for the CD40–CD154 interaction, we discovered that suramin, a previously known low-potency TNFR–TNFα inhibitor, also inhibits CD40–CD154 and that it does so with an approximately thirty fold higher potency (IC$_{50}$ = 15 μM) compared to TNFR–TNFα (Margolles-Clark et al. 2009). Subsequently, various structural analogs were tested, which identified several low micromolar inhibitors, which were organic dyes such as direct red 80 (DR80; IC$_{50}$ = 3 μM), and crocein scarlet 7B (CS7B; IC$_{50}$ = 6 μM) (Buchwald et al. 2010; Buchwald et al. 2011). In the present study, we have also included two other compounds for comparison purposes: erythrosine B (ErB; IC$_{50}$ = 3 μM), a food colorant we found to be a promiscuous PPI inhibitor (Ganesan et al. 2011), and tartrazine (TZ; IC$_{50}$> 1000 μM), another food colorant that was consistently found to have no PPI inhibitory effect and was used as negative control (Figure 3-1).

1.5.ii Non-specific Inhibitors

![Structure of erythrosine B](image)

**Figure 1-8: Structure of erythrosine B**

Chemical structure of erythrosine B - tetraiodinated xanthene derivative [i]; ball and stick representation of erythrosine B, also showing van der Waals spheres of atoms [ii].
A further proof of the adequacy of the chemical space of organic dyes to provide small molecule PPI inhibitors is the intriguing promiscuous behavior of organic dyes such as erythrosine B. Erythrosine B (ErB; FD & C red no. 3; Figure 1-8) is a poly-iodinated xanthene dye, which was first identified for its potential to inhibit various PPIs by our group. It is used as a coloring agent for food and cosmetics, biological stain and a fluorescent probe for membrane proteins. The accepted daily intake (ADI) for the dye as a food colorant is 0.1 – 0.25 mg/kg body weight. The fraction of dye absorbed by the gastrointestinal tract upon oral administration is negligible (0.013% of the administered dose/liter of serum). The dye showed slight mutagenicity in *E.coli* at 0.05 g/mL, but not in other models (Rogers et al., 1988). It is non-teratogenic and non-tumorigenic to rats, but causes long-term effects of growth retardation only seen at high doses (5% body weight).

ErB, along with other halogenated xanthene analogs are among the promiscuous inhibitors that consistently show up in several drug screens with comparable inhibitory constants (Table 1-2) suggesting a common underlying mechanism (McGovern *et al.* 2002). Early associations were made of ErB with the Feingold hypothesis that food colorants caused hyperkinetic behavior in children due to several of its observed effects on the central nervous system (Lafferman *et al.* 1979; Logan *et al.* 1979; Mailman *et al.* 1980). ErB was found to inhibit dopamine uptake (Lafferman *et al.* 1979; Logan *et al.* 1979). Since this inhibitory effect varied with the concentration of membrane protein used in the preparation, it was considered a non-specific artifact due to the interaction of the
anionic dye with the lipid membrane altering its surface charge (Mailman et al. 1980). Consistent with this, the dye was observed to alter the resting membrane potential by increasing $K^+$ conductance in molluscan neurons, a character that was correlated with the lipophilicity of the dyes within the xanthene family (Levitan 1977; Colombini et al. 1981; Levitan et al. 1984; Gatto et al. 1993). Additionally, ErB also inhibited the cholinesterase and catecholamine mediated hyperpolarization in the amphibian sympathetic ganglia (Smith et al., 1984). However, the above effects required ErB concentrations much higher than would be found upon oral administration at the current ADI levels.

Several effects of ErB on the components of the metabolic machinery have been reported. Xanthenes are known inhibitors of catechol-O-methyl transferase with sub-millimolar IC$_{50}$s. ErB was found to prevent de-iodination of thyroid hormones through the inhibition of the mono-deiodinase enzyme. ErB and halogenated xanthene analogs were suggested to act as pseudo-substrates for this enzyme due to their structural closeness to the substrate thyroxine (Cody, 1985). ErB inhibits aromatase (CYP19), the enzyme responsible for the conversion of testosterone to estradiol (Satoh et al. 2008). Due to its competition with estradiol for estrogen receptor binding and consequent promotion of estrogenic activity even in the absence of the hormone, the dye is regarded as a xenoestrogen (Dees et al. 1997). It also inhibits the sulfotransferase enzyme for ethynyl estradiol and estrone. The above three effects could account for its classification as an endocrine disruptor. The widely expressed cytochrome-P450 isozyme, CYP3A4 is also subject to inhibition by ErB, and so are UGT1A6 and
the drug efflux pump P-glycoproteinas well. It was suggested that light-irradiation of the dye causes the release of superoxide anions (O₂⁻). These can cause photo-damage to the metabolic enzymes as no inhibition was seen in the presence of ¹O₂ quenchers (Kuno et al. 2005). Since the characterization of fluorescein dyes as pseudo ATP analogs, these dyes have been applied to label the nucleotide binding sites of relevant enzymes (Neslund et al. 1984). P-type ATPases are another class of enzymes whose activities are not spared by the ErB (Colombini et al. 1981; Gatto et al. 1993). These generally have two different ATP binding sites per enzyme. A derivative of ErB, EITC (erythrosine isothiocyanate) has the ability to selectively label through non-covalent binding the low affinity site in Na⁺ K⁺ ATPase and the catalytic site of sarcoplasmic reticulum Ca²⁺ ATPase, albeit leaving the secondary structures of the enzymes unaltered (Mignaco et al. 1996; Mignaco et al. 1996). This has made it a useful probe for the identification of the active site residues through the analysis of the dye-bound tryptic digests (Linnertz et al., 1998). The dye was also used to ascertain the function of loops in the mitochondrial ADP/ATP carrier (Majima et al. 1998) Active site-binding was also found to be the mechanism of inhibition by rose Bengal (IC₅₀ ~ 3-30µM) among other halogenated xanthenes of the human protein tyrosine phosphatase isoforms (Xie et al. 2003). Poly-iodinated xanthenes were additionally also found to inhibit insulin binding to its receptor. Such binding was shown to occur at multiple sites where one such may coincide with the site of the PPI thereby causing inhibition (Schlein et al. 2001).
Table 1-2: Published inhibitory activities of erythrosine B

<table>
<thead>
<tr>
<th>Protein / Enzyme</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine release</td>
<td>≈ 10</td>
<td>(Augustine et al. 1980)</td>
</tr>
<tr>
<td>Catecholamine (dopamine) uptake</td>
<td>≈ 50</td>
<td>(Lafferman et al. 1979; Logan et al. 1979; Mailman et al. 1980; Silbergeld et al. 1982)</td>
</tr>
<tr>
<td>ATPases</td>
<td>10–30</td>
<td>(Swann 1982; Neslund et al. 1984)</td>
</tr>
<tr>
<td>Galactokinase</td>
<td>≈ 10</td>
<td>(Wierenga et al. 2008)</td>
</tr>
<tr>
<td>Sulfotransferase</td>
<td>5–10</td>
<td>(Bamforth et al. 1993)</td>
</tr>
<tr>
<td>ADP transport via the ADP/ATP carrier</td>
<td>≈ 1</td>
<td>(Majima et al. 1998)</td>
</tr>
<tr>
<td>Insulin–insulin receptor</td>
<td>11</td>
<td>(Schlein et al. 2001)</td>
</tr>
<tr>
<td>ATP-dependent glutamate uptake</td>
<td>35</td>
<td>(Bole et al. 2005)</td>
</tr>
<tr>
<td>Protein tyrosine phosphatases (PTP1B, TC-PTP, YPTP1)</td>
<td>5–25</td>
<td>(Shrestha et al. 2006)</td>
</tr>
<tr>
<td>DNA – anti-DNA antibody (IgG2b) interaction</td>
<td>≈ 10</td>
<td>(Ben-Chetrit et al. 1988)</td>
</tr>
<tr>
<td>Aromatase (CYP19)</td>
<td>≈ 0.3</td>
<td>(Satoh et al. 2008)</td>
</tr>
<tr>
<td>UDP glucuronosyltransferase (UGT1A6)</td>
<td>50</td>
<td>(Furumiya et al. 2008; Mizutani 2009)</td>
</tr>
<tr>
<td>Cytochrome P450 (CYP3A4)</td>
<td>8</td>
<td>(Furumiya et al. 2008; Mizutani 2009)</td>
</tr>
<tr>
<td>P-glycoprotein (P-gp)</td>
<td>16</td>
<td>(Furumiya et al. 2008; Mizutani 2009)</td>
</tr>
</tbody>
</table>

Consequent to the above discoveries, the present work was thus aimed at improving the specific inhibitors’ activity, specificity and ligand efficiency and clarifying their mechanisms of action. It was also directed at eludication of the mechanism of inhibition by the promiscuous inhibitor ErB, to help better understand the factors that may contribute to its observed non-specificity.
2.1 SMALL-MOLECULE SCREENING ASSAY

Microtiter plates (Nunc F Maxisorp; 96-well) were coated overnight at 4-8°C with 100 μL/well of Fc chimeric 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 h at RT. The plates were then washed twice using the washing solution (PBS 7.4, 0.05% Tween-20) and dried before the addition of the appropriate FLAG tagged/ biotinylated ligands along with different concentrations of test compounds diluted in binding buffer (100mM HEPES, 0.005% BSA pH 7.2) or protein-containing media (IMDM medium supplemented with 5% FBS) to give a total volume of 100 μL/well. Either anti-FLAG-HRP or streptavidin-HRP conjugated antibodies were used to detect the bound FLAG-tagged or biotinylated ligands, respectively. Plates were washed thrice before the addition of 120 μL/well of HRP substrate TMB (3, 3, 5, 5-tetramethylbenzidine) and kept in the dark for 30 min. The reaction was stopped using 30 μL 1M H₂SO₄, and the absorbance was read at 450 nm (Figure 2-1).

Figure 2-1: ELISA screen
Schematic representation of the ELISA screening assay in a 96-well format
All binding analyses were done in duplicate or triplicate per plate and repeated at least twice; 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 2) using GraphPad Prism 5.04 (La Jolla, CA) (Margolles-Clark et al. 2009; Margolles-Clark et al. 2009; Buchwald et al. 2010; Ganesan et al. 2011).

2.1.1 Specificity panel

The specificity panel consisted of PPIs within the TNFSF (TNFR1–TNFα, RANK–RANKL, BAFFR–BAFF, OX40–OX40L, and 4-1BB–4-1BBL) and outside of it (EGF-R–EGF). The concentrations of receptors used were: 0.3 µg/mL (CD40, TNF-R1, and RANK), 0.6 µg/mL (BAFF-R, OX40, and 4-1BB), and 2 µg/mL (EGF-R). The concentrations of the ligands were fixed at 0.02 µg/mL (CD154, TNFα, and RANKL), 0.2 µg/ml (BAFF, OX40L, 4-1BBL), and 0.3 µg/mL (EGF) (Margolles-Clark et al. 2009; Margolles-Clark et al. 2009; Buchwald et al. 2010; Ganesan et al. 2011)

Saturable binding model:

\[
\frac{[LR]}{[LR]_{max}} = \frac{[L]^{n_h}}{[L]^{n_h} + K_d^{n_h}}
\]  - Eqn 1

Log(inhibitor) Vs. response model:

\[
\frac{[LR]}{[LR]_{max}} = 1 - \frac{1}{1 + n_h 10^{log IC_{50} - log[L]}}
\]  - Eqn 2

- \([LR]/[LR]_{max}\) - receptor occupation by ligand as a fraction of maximum
- \([L]\) - Concentration of the unoccupied ligand / protein agonist (M)
- \(K_d\) - Dissociation constant of the ligand – receptor / protein–protein interaction (M)
- \(IC_{50}\) - Concentration of inhibitor causing half maximal response (M)
- \(n_h\) - Hill slope
2.1.ii Calculation of ligand efficiency

A desired attribute in lead compounds is increased potency and specificity that can be achieved by the smallest possible structure. Therefore, ligand efficiency, which normalizes activity to molecular weight, serves as a better comparator than activity alone, which tends to increase with molecular size (section 1.1). The experimental IC$_{50}$s were converted to K$_i$ using the Cheng-Prusoff equation (-Eqn 3). Gibbs free energy ($\Delta G$) was then computed using the obtained K$_i$ -Eqn 4), which was then normalized to the number of heavy (non-hydrogen) atoms in the compound to derive its ligand efficiency (LE (or) $\Delta g$; -Eqn 5)$^{3}$ (Hopkins et al. 2004; Abad-Zapatero et al. 2005; Abad-Zapatero et al. 2010; Tanaka et al. 2010).

\[
K_i = \frac{IC_{50}}{1 + \frac{[L]}{K_d}}
\]  
- Eqn 3

\[
\Delta G = -RT\ln K_i
\]  
- Eqn 4

\[
\Delta g = \frac{\Delta G}{NHA}
\]  
- Eqn 5

$^-3$K$_i$ - Dissociation constant for inhibitor binding to protein (M)
- $\Delta g$ - Ligand efficiency (J/Mol/Atom)
- $\Delta G$ - Gibbs free energy of small molecule binding to protein (J/Mol)
- NHA - Number of heavy (non-hydrogen) atoms
- $R$ - Ideal gas constant ($\approx$8.314 J/Mol K)
- $T$ - Temperature (K)
2.2 COMPUTATIONAL METHODS

2.2.i Pharmacophore search algorithms

2.2.i.a Structure similarity search

Virtual screening of molecular libraries small molecule repository (MLSMR) of ~340,000 compounds (PubChem Compounds Database) by the PubChem similarity search algorithm was done using the 2D structures of the best compounds identified as query. The compounds were scored and ranked using Tanimoto coefficient for structure similarity (S1:-Eqn 6)\(^4\) as a measure of similarity after setting a threshold of above 80% (Willett 2006).

2.2.i.b Shape similarity search

Shape similarity search was performed by virtual screening of molecular libraries small molecule repository (MLSMR) of 31 million compounds (PubChem Compounds Database) by ROCS, version 3.0 (OpenEye Scientific Software, Inc., Santa Fe, NM, 2010) (Hawkins et al. 2007; Tawa et al. 2009) using the 3D structures of BIO8898 and CS7B as query. While the co-crystal pose of BIO8898 was fed as input, input poses of CS7B were optimized for either stability or bioactivity. Starting structures were prepared using QuacPac 1.1.0 (OpenEye Scientific Software, Inc., Santa Fe, NM, USA, 2010), which has modules to generate tautomers, assign pK\(_a\) and partial charges to the input structures. Input

---

\(^4\)A – Number of descriptors in compound A (query)
- B - Number of descriptors in compound B (test)
- AB – Number of descriptors common to A and B
poses of the compounds for shape similarity search were selected on the basis of stability (top 50 lowest energy conformers generated by OMEGA 2.4.3, OpenEye Scientific Software, Inc.) and bioactivity (Top 50 lowest energy docking poses at the BIO8898 binding site in 3LKJ generated by FRED 2.2.5, OpenEye Scientific Software, Inc.). Top 200 hundred compounds were rank ordered based on computed Tanimoto coefficient for shape similarity (S2:-Eqn 7)\(^5\)

\[
S1 \left( \% \right) = 100 \times \frac{AB}{A + B - AB} \quad \text{-Eqn 6}
\]

\[
S2 \left( \% \right) = 100 \times \frac{O_{f,g}}{I_f + I_g - O_{f,g}} \quad \text{-Eqn 7}
\]

2.2.\text{ii} Computational docking experiments

2.2.\text{ii.a Prediction of druggable binding pockets}

The structures of CD40 (chain s) and CD154 (chains a, b, c) alone and that of the CD40–CD154 complex (chains a, b, c, s, r), were prepared from the CD40–CD154 co-crystal structure (PDB ID: 3QD6) obtained from RCSB Protein Data Bank. These were submitted to the FTMAP web server (http://ftmap.bu.edu/) (Brenke et al. 2009) to identify druggable pockets.

\[^{5}f, g – \text{Characteristic functions (Gaussian volumes) of two entities (test and query)}\]
\[^{6}I_f, I_g – \text{Self overlap of volume functions f and g}\]
\[^{7}O_{f,g} – \text{Overlap between f and g}\]
2.2.ii.b Preparation of ligands for docking

Two-dimensional structures of the ligands to be docked were generated using ACD/ChemSketch 12.01 (ACD/Labs, Toronto, ON, Canada). 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 2004).

2.2.ii.c Preparation of proteins for docking

The CD154 structures used were obtained from RCSB Protein Data Bank (PDB ID: 3QD6, 3LkJ), and they were further processed using Protein Preparation Wizard (Schrödinger Suite 2011; Epik version 2.2, Impact version 5.7, Prime version 2.3; Schrödinger, LLC). CD154 prepared from 3QD6 (CD40–CD154 complex) was used for docking at the competitive site, whereas the structure 3LkJ (BIO8898–CD154) was used for docking at the allosteric site. The 3QD6 structure had no water molecules, and all water molecules were removed from the 3LkJ structure to facilitate docking at the allosteric site. Final minimization was performed employing the OPLS 2005 force field (Mackerell 2004).

2.2.ii.d Docking at the competitive site

A grid-box for 3QD6 was constructed using the receptor grid generation tool, Glide version 5.7 (Schrödinger, LLC). It was centered on the residues critical for CD40 binding, which were also identified as druggable by FTMAP [Arg 203,
Ile 204, Leu 205, Arg 207 (Subunit A); Ile 127, Gly 144, Tyr 145, Tyr 251, Gly 252 (Subunit C). Ligand docking was performed for BIO8898, CS7B, MB1, ErB, and TZ without any constraints and scored using standard precision (SP) and extra-precision (XP) modes using Glide version 5.7 (Schrödinger, LLC) (Friesner et al. 2004; Friesner et al. 2006). Induced fit docking was done similarly using the SP and XP modes (Glide version 5.7, Prime version 3.0; Schrödinger, LLC) (Sherman et al. 2006).

2.2.ii.e Docking at the allosteric site

For docking at the allosteric site, a grid-box was constructed for 3LKJ centered on the workspace ligand BIO8898. Ligand docking was performed for BIO8898, CS7B, MB1, ErB, and TZ without any constraints and scored using standard precision (SP) and extra-precision (XP) modes using Glide version 5.7. Induced fit docking was done similarly using the SP and XP modes.

2.3 CELL-BASED ACTIVITY ASSAYS

2.3.i Toxicity assays

2.3.i.a Inhibition of cell-proliferation

For the BrdU assay, THP-1 human myeloid cells from American Type Culture Collection (Manassas, VA, USA) were cultivated in Roswell Park Memorial Institute (RPMI) media 1640 supplemented with 10% v/v fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin, and 50 mM β-mercaptoethanol. The cells were centrifuged and re-suspended in the same medium without FBS and added to a 96-well microtiter plate at a density of
100,000 cells/well in the absence or presence of various concentrations of inhibitors diluted in the same media. The plate was incubated at 37°C, 5% CO₂ for 24 h. BrdU incorporation was determined using the BrdU cell proliferation kit from Roche (Mannheim, Germany) according to the manufacturer’s protocol (Ganesan et al. 2011).

2.3.i.b Inhibition of cell viability

For the DAPI assay, THP-1, Jurkat, and HEK293T cells were seeded at a density of 0.5 x 10⁶ cells/mL in the absence or presence of test compounds for 12 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.

2.3.ii NF-κB reporter assay

To evaluate nuclear factor kappa B (NF-κB) transactivation via the specific activation of the CD40 pathway, we used the HEK-Blue™ reporter cell line (InvivoGen, CA) transfected with CD40. A

Figure 2-2: NF-κB reporter assay
CD154-mediated activation of NF-κB pathway in HEK-Blue™ reporter cells expressing CD40, resulting in the secretion of reported enzyme serum alkaline phosphatase (SEAP)
un-transfected line expressing TNF-R was used as a control. The reporter cell-line was generated by stable transfection of HEK293 cells with the human CD40 gene and an NF-κB-inducible SEAP (serum alkaline phosphatase) construct. The SEAP construct consists of the SEAP reporter gene under the control of the IFN-β minimal promoter fused to five NF-κB binding sites. Secretion of SEAP by HEK-Blue™ cells is specifically induced by CD154 in the cells transfected with CD40 receptor and by TNF-α and interleukin-1β (IL-1β) in both transfected and un-transfected lines. 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 without FBS, added to a 96-well microtiter plate at a density around 1 x 10^6 cells/well and stimulated with 0.1 µg/mL CD154 in the absence or presence of various concentrations of inhibitors diluted in the same media. After 24-h incubation at 37°C and 5% CO₂, SEAP level was determined by adding QUANTI-Blue™ reagent, whose change in color intensity from pink to purple/blue is proportional to the enzyme’s activity. The level of SEAP was determined quantitatively using a spectrophotometer at 620-655 nm (Figure 2-2).

2.3.iii Immune cell activation assay (flow cytometry)

THP-1 cells were cultivated as before (section 2.3.i.a). Serum-starved THP-1 human monocytic leukemia cells were plated in medium without FBS having a density around 1 x 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 = 496nm/578nm), fluorescein isothiocyanate (FITC; Ex/Em = 494nm/520nm), and allophycocyanin (APC; Ex/Em = 650nm/660nm) respectively (BD Biosciences, CA) as described previously (Margolles-Clark 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.

2.3.iv TNF-α mediated JNK phosphorylation

THP-1 cells were starved overnight in 25 cm² tissue culture flasks at a density of 8 x 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 (Axxora, CA). 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 2.0 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 Image J software (http://rsb.info.nih.gov/ij/)

2.3.v Lymphocyte activation assay using BDC 2.5 auto-antigen

Splenocytes (1x10^6) from BDC 2.5/ NOD mice (Chen et al. 2005) were cultured with 2 ng/mL of BDC peptide and compounds of indicated concentrations in 96well plates. After 24 h (37°C, 5% CO2) plates were pulsed with tritiated [3H] thymidine whose incorporation was measured 16 h afterwards. The anti-CD154 monoclonal antibody (clone 5c8; R&D Systems, MN) was used as a positive control. Tartrazine (TZ) was used as a negative control for non-specific effects that may arise from the compound addition.
2.4 EXPRESSION AND PURIFICATION OF RECOMBINANT HUMAN HISTIDINE-TAGGED EXTRACELLULAR CD154

2.4.i Cloning of extracellular CD154 into pET-15b bacterial expression vector

Nucleotides 357-812 from the cDNA of the full-length huCD154 (corresponding to amino acids 108-261 of extracellular domain) were PCR amplified using as forward primer 5’ CTAGCATATGGAAAACAGCTTTGAAATGC 3’ and as reverse primer 5’ CTAGGGATCCTCAGAGTTTGAGTAAGCC 3’. This fragment was cloned into the multiple cloning site (MCS) of pET-15b vector (Novagen, WI) under a T7 promoter and N-terminal sequences for (His)$_6$ – tag and thrombin cleavage. The restriction enzymes used were BamHI and Ndel (Promega, WI) (Figure 2-3).

Figure 2-3: Cloning of extracellular CD154 into the pET15b vector
Vector map of pET–15b showing the sequence of extracellular (EC) CD154 flanked by BamHI and Ndel restriction sites with N-terminal sequences for (His)$_6$ – tag and thrombin cleavage, all under the T7 promoter. Also shown is the coding sequence that confers ampicillin resistance (bla CDS).
2.4.ii Expression of histidine–tagged CD154 in *E. coli*

BL21 (pLys) S competent cells (Promega, WI) were transformed with the cloned plasmid pET-15b described above and plated on Miller’s LB agar (Difco, BD biosciences, NJ). Colonies were selected for ampicillin resistance and introduced into terrific broth media (Affymetrix, CA) for large-scale protein expression. The culture was grown in a bacterial shaker at 37°C and 250 rpm until the cells reached a density (OD$_{600}$) of 0.5 and switched to 30°C and 300 rpm before the addition of 1M isopropyl-β-D-1-thiogalactopyranoside (IPTG, Sigma–Aldrich), a lactose analog used to drive the expression of CD154 from the T7 promoter. After 4-6 h of expression, the bacterial cells were centrifuged, washed and re-suspended in phosphate buffered saline (20 mM phosphate buffer, 500mMNaCl) containing 20 mM imidazole and complete EDTA-free protease inhibitor cocktail (Roche, IN) before their lysis by sonication.

2.4.iii Purification of histidine–tagged CD154 by Ni-NTA chromatography

The bacterial cell lysate was passed slowly through a column packed with nickel nitrilotriacetic acid (Ni-NTA) agarose resin (Qiagen, CA). After collecting the flow-through, the column was washed with phosphate buffered saline (20 mM phosphate buffer, 500 mM NaCl) containing 20 mM imidazole until no protein emerged as observed using a coomassie spot test. The column was progressively eluted with gradients of imidazole to eliminate non-specifically bound bacterial proteins having lower affinity. Pure (His)$_6$ – tagged CD154 emerged upon addition of ~ 250-300 mM imidazole, and the eluate was collected until no further protein emerged even after washing the column with 500 mM
imidazole. The purity and identity was verified by SDS–PAGE (protein degradation products <10% of total) and by western blotting respectively (both using anti-CD154 monoclonal antibody (clone 40804, R&D Systems, MN) and anti-(His)_6-tag antibody (Abcam, MA). The concentration (C) of the pooled purified fractions was ascertained from absorbance (A) measured using NanoDrop 1000 spectrophotometer (Thermo-Fisher Scientific, MA) by setting the percent extinction coefficient (absorbance of 1 % (E_{1%} (w/v)) solution) of the protein at 280 nm be 6.98 (Eqn 8) (Mazzei et al. 1995).

\[
C (mg/ml) = \frac{A}{E_{1\%}} \times 10
\]

-Eqn 8

2.4.iv Purification of histidine – tagged CD154 by size exclusion chromatography

The fractions from nickel chromatography were further purified through a Superdex-200 size exclusion column (GE Healthcare Life Sciences, NJ). This was connected to ÄKTA™ fast protein liquid chromatography (FPLC) system (GE Healthcare Life Sciences, NJ). The protein was exchanged into 20 mM phosphate buffer, 500 mM NaCl and 10% glycerol, used as the mobile phase at a flow rate of 0.5 mL/min to completely remove imidazole. Fractions were detected using an in-line UV detector set at wavelengths of 210, 280, and 260 nm that detect absorbance by peptide bonds, amino acids (Trp, Tyr, Cys), and nucleic acids respectively. The apparent molecular weight of the purified (His)_6–tagged CD154 was ascertained from a calibration curve constructed using gel filtration low molecular weight calibration kit (GE Healthcare Life Sciences, NJ).
2.5 BIOCHEMICAL ASSAYS

2.5.i Gaddum-Schild EC$_{50}$ shift

Schild analysis was done using the same ELISA setup described above. The plot was constructed by fixing the concentration of CD40 at 1 µg/mL. The concentration of CD154 was varied from 0 to 10 µg/mL to obtain dose–response curves in the absence or the presence of increasing concentrations (0.08 to 10 µM) of direct red 80 (DR80), and anti-CD154 antibody (0.13 to 3.25 nM), which was used as a positive control for competitive behavior. Binding data at five different inhibitor concentrations were fitted using the Gaddum / Schild EC$_{50}$-shift model (Eqn 9). 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) (Jenkinson et al. 1995; Cheng 2001; Tallarida 2007).

$$\frac{[LR]}{[LR]_{\text{max}}} = \frac{[L]^{n_h}}{[L]^{n_h} + \left[K_d \left\{ 1 + \left[ \frac{I}{K_i} \right]^{n_s} \right\}\right]^{n_h}}$$

-Eqn 9

2.5.ii Metachromatic shift

An inherent property of certain dye molecules is the shift in their absorption maxima upon binding with protein, called metachromasy. This property was exploited to identify the protein-binding partner, to derive binding constants and stoichiometry of the dye-protein complex (D–P).
The absorption spectra of the various concentrations of dye without and with added protein were measured by spectrophotometry. A difference spectrum was constructed by plotting the differences in optical density (OD) units of the spectral trace of free dye and that obtained with added protein. The difference in OD between the maximum and minimum points on the difference spectra was used as a quantitative measure of dye-protein complex formation for use in constructing binding curve and in analysis of stoichiometry (Bergeron et al. 1958).

2.5.ii.a Binding curves

Dye-protein complex (D–P) was quantified from the difference spectra as described above. Corresponding binding curves for different concentrations of added protein [P] and dye [D] were analyzed with a unified log dose vs. response model (Eqn 10) (GraphPad Prism version 5.04, San Diego, CA) to obtain binding constant ($K_d$) (Klunk et al. 1999).

$$\frac{[D-P]}{[D-P]_{max}} = \frac{1}{1 + 10^{n_h \left(\log K_d - \log[D]\right)}}$$

- Eqn 10

2.5.ii.b Stoichiometry

Dye-protein complex (determined from the difference spectra at complementary molar ratios of dye and protein, keeping the total molar concentration constant) was plotted against dye / protein concentrations to obtain the Jobs plot. Tangents were drawn from either side of the resultant plot to determine the point of maximum complex formation to give the stoichiometry ($n$) (Ingham 1975; Huang 1982; Schlein et al. 2001; Olson et al. 2011).
2.5.iii Crystallization screening

Initial crystal screen trials were set up using Crystal Screen kit™ from Hampton Research (Riverside, CA) using the hanging drop vapor diffusion method with a 1:1 volume ratio of protein (10 mg/ml purified (His)_6 - tagged CD154 in 20 mM phosphate buffer, 500 mM NaCl, 10% Glycerol) and reservoir solution 20-25% polyethylene glycol (PEG) 3350, 50mM HEPES pH 7-8, 0.2M sodium citrate at room temperature according to the published conditions (Karpusas et al. 1995; Karpusas et al. 2001; Silvian et al. 2011).
CHAPTER 3 SEARCH FOR SMALL MOLECULES

3.1 INHIBITORY ACTIVITY AND SELECTIVITY

During the early phases of our search for small molecule inhibitors for the CD40–CD154 interaction using our ELISA-based screening assay (section 2.1), we discovered that suramin, a previously known low-potency TNFR–TNFα inhibitor, also inhibits CD40–CD154 and it does so with an approximately thirty fold higher potency (IC$_{50}$ = 15μM) compared to TNFR–TNFα (Margolles-Clark et al. 2009). Subsequently, we tested various structural analogs for CD40–CD154 inhibition as well as other PPI within the TNFSF (OX40–OX40L, RANK–RANKL, 4-1BB–4-1BBL, BAFFR–BAFF, TNFR1–TNFα) and outside of it (EGF-R–EGF) as described previously (section 2.1.i). We identified the first low micromolar inhibitors, which were organic dyes such as direct red 80 (2, DR80; IC$_{50}$ = 3 μM), crocein scarlet 7B (4, CS7B; IC$_{50}$ = 6 μM), mordant brown 1 (5, MB1; IC$_{50}$ = 0.13 μM) (Margolles-Clark et al. 2009; Buchwald et al. 2010; Margolles-Clark et al. 2010), and pontamine diazo blue (3, PDB; IC$_{50}$ = 0.2 μM). In the present study, we have also included two other compounds for comparison purposes: erythrosine B (6, ErB IC$_{50}$ = 3 μM), a food colorant we found to be a promiscuous PPI inhibitor (Ganesan et al. 2011), and tartrazine (7, TZ; IC$_{50}$> 1000 μM), another food colorant that was consistently found to have no PPI inhibitory effect (Figure 3-1, Table 3-1).
Compounds were selected not just for activity and specificity for CD40-CD154 inhibition, but also for ligand efficiency ($\Delta g$) as described before (section 2.1.ii). Ligand efficiency (LE, $\Delta g$), is obtained by dividing the former with a measure of molecular size, such as molecular weight or the number of non-hydrogen atoms, $NHA$ (Hopkins et al. 2004). Our smaller, more promising compounds show significant improvement in ligand efficiency compared to suramin (Margolles-Clark et al. 2009) or the recently reported BIO8898 (1) (Silvian et al. 2011). The azo-dyes DR80 (2) and PDB (3) showed greater than hundred fold specificity for CD40–CD154 inhibition over that of closely related TNFR1–TNF$\alpha$, but PDB (3, MW 992 Da; LE = 0.69) showed a greater LE than DR80 (2, MW 1372 Da; LE = 0.40) due to its smaller molecular size (Figure 3-2, Table 3-1).

From the active and selective structures obtained from the screens, we were able to derive minimal structural requirements for selective inhibition represented by a consensus motif as depicted (Figure 3-1). All active compounds possessed a central aromatic group that comprised of a phenyl, biphenyl as seen with the initial compounds DR80 (2) or a napthyl group found in the newer and more potent ones PDB (3). These were flanked by azo or the isosteric amide groups. Terminal naphthalene with polar acidic substituents was attached either symmetrically to the azo-groups or on one side, with a bulky aromatic group attached to the other. Polar substitution on the terminal aromatic groups was indispensable for activity (Figure 3-1).
The consensus motif required for CD40-CD154 inhibition as established in our SAR studies [A] is shown together with BIO8898 (1), a known inhibitor of CD40-CD154 PPI and the organic dyes we have identified as showing the most promising CD40-CD154 PPI inhibitory activity (2-5) [B]. Erythrosine B (6), a promiscuous PPI inhibitor [C], and tartrazine (7), a compound with no PPI inhibitory activity and used as negative control is also included [D] (see Figure 3-2 for details).
Figure 3-2: Classification of small molecules

Classification of compounds based on PPI inhibitory activity within the TNFSF (CD40–CD154, OX40–OX40L, 4-1BB–4-1BBL, RANK–RANKL, BAFFR–BAFF, and TNFR–TNFα) and outside of it (EGF–R–EGF): Potent and selective compound (2, DR80) [A], efficient and selective compound (3, PDB) [B], inactive compound (7, TZ) [C]; and a promiscuous PPI inhibitor (6, ErB) [D] (see Table 3-1 for details).
Table 3-1: Activity, specificity and ligand efficiency

<table>
<thead>
<tr>
<th>SN (Ref Figure 3-1)</th>
<th>Compound</th>
<th>MW (kDa)</th>
<th>IC₅₀ (µM)</th>
<th>Fold specificity (TNFR-α)</th>
<th>LE (Δg) (CD40-CD154; kJ/atom)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>BIO8898</td>
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<td></td>
<td>0.44</td>
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<td>6</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>3</td>
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<td>145</td>
<td>&gt;500</td>
</tr>
<tr>
<td>4</td>
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<tr>
<td>7</td>
<td>TZ</td>
<td>468</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

3.2 VIRTUAL SCREENING OF DRUG-LIKE LIBRARIES OF COMPOUNDS

Several of the organic dyes tested showed non-specific binding at concentrations not very far from IC₅₀ possibly due to their predominant hydrophobic character. In the hope of identifying compounds devoid of the structural attributes that might contribute to such non-specificity, while retaining those features necessary for sufficient binding affinity to the target protein, shape similarity searches were performed as described under materials and methods (section 2.2.i.b) using the prepared three dimensional structures of our best compounds CS7B (4) and MB1 (5) as query against the PubChem Compounds repository. Top 200 compounds were selected based on computed Tanimoto coefficients (S2) to obtain for use in our ELISA-based screening assay (Figure 3-3, Figure 3-4, and Figure 3-5).
**Figure 3-3: Structures similar in shape to co-crystal pose of BIO8898**
The top 5 structures similar in shape to BIO8898 (1) (using its pose from the co-crystal structure 3LKJ and shown as a tube representation) obtained by virtual screening of molecular libraries small molecule repository (MLSMR, PubChem Compounds Database) by ROCS, version 3.0. (OpenEye Scientific Software, Inc., Santa Fe, NM, 2010) are shown in wire representations together with their corresponding PubChem Compound IDs and names.

**Figure 3-4: Structures similar in shape to the stability-optimized CS7B pose**
Top 5 structures similar in shape to the stability-optimized pose of CS7B (4) (OMEGA, version 2.4.3, OpenEye Scientific Software, Inc., shown as tube representation) obtained by virtual screening of PubChem Compounds Database by ROCS, version 3.0, OpenEye Scientific Software, Inc., are shown in wire representations together with their corresponding PubChem Compound IDs and names.

**Figure 3-5: Structures similar in shape to the bioactivity-optimized CS7B pose**
Top 5 structures similar in shape to the bioactivity-optimized pose of CS7B (4) (FRED, version 2.2.5, OpenEye Scientific Software, Inc., shown as tube representation) obtained by virtual screening of PubChem Compounds Database by ROCS, version 3.0, OpenEye Scientific Software, Inc., are shown in wire representations together with their corresponding PubChem Compound IDs and names.
3.3 TRANSLATION OF INHIBITION OF BINDING TO FUNCTION

3.3.i Inhibition of immune cell activation

For compounds that showed promising activity in binding assays, the translation of this activity into inhibition of CD40-CD154 function in cell-based models was tested. The concentrations of compounds that were non-toxic to the cell-line being used were selected based on evaluation of cytostatic activity using BrdU incorporation (Figure 3-6). Inhibition constants in the functional assays were correlated with those obtained from the binding assays performed using protein-containing media (media with 10% FBS).

Figure 3-6: Cytostatic activity on human monocytic leukemia (THP1) cells
Cytostatic activity on THP1 cells as measured by standard proliferation assay (BrdU incorporation) in the absence and presence of indicated concentrations of DR80 (2), PDB (3), acid orange 79 (AO79), and acid blue 113 (AB113). Data (average ± SD, quadruplicate for each condition) were analyzed by ANOVA with Tukey’s post hoc test and asterisks (*) indicate statistically significant reduction (p< 0.001) versus no inhibitor treatment (0).
Inhibition of expression cell surface markers CD54 (ICAM), HLA-DR (MHC class-II), and CD40 upon CD154 stimulation on monocytic leukemia cells (THP1), which are stable lines that are naturally express many cell-surface receptors like CD40 (Tsuchiya et al. 1980), was used as a preliminary test to assess functional translation of inhibitory activity. PDB (3) showed dose-dependent reduction of at least two of the three markers tested. The anti-CD154 antibody, used as a positive control inhibited the expression of all the three markers tested (Figure 3-7).

Figure 3-7: Inhibition of cell-surface marker expression.
The expression of MHC-II (HLA-DR) on human monocytic leukemia (THP-1) cells, in the absence and presence of CD40L (CD154; 0.5 μg/mL) [A], and upon additional treatment with indicated concentrations of PDB (3) obtained from flow cytometry analysis as shown in the histogram [B]. The expression of three different cell surface markers (CD40, HLA-DR, and CD54) in the presence of indicated concentrations of anti-CD154 antibody (mAb) [C]; and PDB (3) [D] (NS indicates non-stimulated controls).
3.3.ii Inhibition of BDC2.5 transgenic lymphocyte proliferation

As an initial attempt to assess the relevance of the compounds in autoimmunity, the BDC 2.5 transgenic mouse was used, which is a model for autoimmune (type 1) diabetes. It is a CD4⁺ T-cell restricted strain carrying a transgenic T-cell receptor that specifically recognizes BDC 2.5; a pancreatic auto-antigen (Ji et al. 1999; Chen et al. 2005). This results in the reactivity of a vast majority of the T-cells against the pancreatic islets, causing a sharp and synchronous onset of insulitis and highly regulated islet destruction.

Proliferation of splenic T-lymphocytes was measured upon the BDC2.5 antigen stimulation in the absence and presence of the small molecule compounds as described previously (section 2.3.v). DR80 (2, EC₅₀ = 227 μM) and PDB (3, EC₅₀ = 100 μM) dose-dependently inhibited the proliferation, while TZ (7) that did not show inhibition in the binding assays did not also inhibit the proliferation of T-cells (Figure 3-8). The inhibitory concentrations were well below the toxic levels for these compounds and correlated with the concentration range that produced inhibitory effects in the binding assays performed in the presence of protein-containing media.
Figure 3-8: Inhibition of T-lymphocyte proliferation

Agonist peptide-induced proliferation of transgenic T-lymphocytes in the splenocytes isolated from BDC2.5 mice in the presence of indicated concentrations of DR80 (2), PDB (3), and TZ (7). Data (average ± SD, triplicates for each condition) were analyzed by ANOVA with Tukey’s post hoc test and asterisks (*) indicate statistically significant reduction ($p < 0.001$) versus no inhibitor treatment (0).
CHAPTER 4  SPECIFIC INHIBITION

4.1 IDENTIFICATION OF BINDING PARTNER

The elucidation of inhibitory mechanism through the identification of the binding partner (CD40 or CD154) and subsequently the binding site as well as the nature of the binding interactions was considered an important goal because it should provide important structural information needed for the design of improved small molecule inhibitors. Furthermore, it could identify a druggable pocket on the protein that can be targeted for achieving specificity.

An inherent property of dye molecules is a shift in the absorption maxima upon binding with protein called metachromasy. This property was exploited to establish whether DR80 (2) binds to CD40 or CD154. Upon addition of CD40 to DR80 (2) at a 1:2 ratio, no changes were observed in the absorption spectra of the dye, whereas, upon addition of CD154 at the same ratio, a red shift of the $\lambda_{\text{max}}$ could be observed. This confirms that the dye indeed shows preferential binding to CD154 (Figure 4-1-A). Difference spectra were constructed by plotting the differences in optical density (OD) units of the spectral trace of free dye and that obtained with added protein. A bimodal difference spectra resulting from a red-shifted absorption spectra was seen only upon addition of CD154, and not with CD40 (Figure 4-1 -B).
Figure 4-1: Identification of the protein binding partner
The absorbance spectra of DR80 (2) without and with added proteins (light and dark pink, respectively) (A: i. CD40, ii: CD154) and the corresponding difference spectra (B) showing that spectral shift occurs with added CD154, but not with added CD40.\(^6\)\(^7\).

\(^6\)OD – Optical density
\(^7\)\(\Delta\text{OD} = \text{OD}_{\text{Bound}} - \text{OD}_{\text{Free}}\)
4.2 EXPRESSION AND PURIFICATION OF RECOMBINANT HUMAN HISTIDINE-TAGGED EXTRACELLULAR CD154

To have a sufficient amount of protein to perform mechanistic studies, CD154 was expressed with a hexa-histidine ((His)$_6$) –tag in *Escherichia coli* and purified using nickel-NTA affinity chromatography as described under materials and methods (section 2.4). The purity of the CD154 was verified by SDS-PAGE (protein degradation products <10% of total), and the concentration was determined using nanodrop setting percent extinction coefficient ($\varepsilon_{1%}$) of the protein to be 6.98 (see section 2.4.iii). A concentration of 13.6 mg/ml of over 95% pure CD154 protein was obtained (Figure 4-2).

---

**Figure 4-2: Expression and purification of histidine-tagged CD154**
Expression of histidine - tagged CD154 in E. coli strain BL21 (pLys) S, 2 h post IPTG induction (A) and corresponding western blot using anti-CD154 monoclonal antibody (mAb; 5c8) confirming CD154 expression (B) [i]. Eluted fractions of pure histidine-tagged CD154 after Ni-NTA chromatography [ii].
To ascertain the molecular size and hence the oligomer status of the purified CD154, the pure fractions collected from the nickel column were subjected to size exclusion chromatography, which separates proteins based on their hydrodynamic radius (section 2.4.iv). The apparent molecular weight inferred from the standard curve indicated it to be a trimer (~50KDa) (Figure 4-3). Evidence in the literature consistently shows CD154 to form active stable trimers (Mazzei et al. 1995).

Figure 4-3: Verification of oligomer status of histidine-tagged CD154
Confirmation of the identity (apparent molecular weight) and purity of the hexa histidine-tagged huCD154 purified here by size-exclusion chromatography using a Sephacryl S200 column (GE Healthcare Biosciences, PA) with UV detection and a coomassie stained gel showing corresponding fractions [ii]; (inset showing calibration [i]).
The purified histidine-tagged CD154 bound CD40 in a dose-dependent manner in an ELISA-based binding assay when using an anti-histidine tagged antibody for detection of bound CD154 (Figure 4-4A). The ability of the purified histidine-tagged CD154 to activate CD40-mediated NF-κB transactivation was confirmed using HEK-Blue cell line that was transfected with CD40 as previously described (section 2.3.ii). The absence of activation of a CD40-null line was used as a control to demonstrate the specificity of activation (Figure 4-4B).

![Figure 4-4: Activity of the purified histidine-tagged CD154
Binding of hexa histidine-tagged huCD154 to chimeric CD40: Fc receptor in an ELISA-based assay [A]; NF-κB transactivation in HEK-Blue sensor cells that overexpress CD40 (dark blue) and in the null line (light blue) [B].](image)

4.3 SCHILD PLOT FOR SURMOUNTABLE INHIBITION

In previous studies, our lab has shown that whereas most other organic dye small molecule inhibitors identified seem to bind to the surface of CD154 and not CD40, the non-specific inhibitor, ErB (6) binds with low micromolar affinity to both
(Buchwald et al. 2010). As a preliminary evaluation of the mechanism of inhibition, i.e., as a first test for the competitive / reversible nature of its inhibitory effect, the Schild analysis was performed as described in materials and methods (section 2.5.i). Dose-response curves were generated using a fixed concentration of CD40 and varying concentrations of CD154 in the presence of increasing concentrations of inhibitors.

As a positive control, an anti-CD154 monoclonal antibody (mAb) was used; its binding data (Figure 4-5 A) could be fitted well with the unified Gaddum-Schild model (Eqn 9), and the best fit was obtained with a $K_d$ of 1 nM for the CD40–CD154 binding and a $pA_2$ value of 9.57 (corresponding to $K_i$ of 0.27 nM) for the mAb inhibition – in good agreement without previous results from straightforward inhibition experiments (e.g., IC$_{50}$ of 0.25 nM ~ 0.04 μg/mL (Margolles-Clark et al. 2009; Margolles-Clark et al. 2009; Buchwald et al. 2010; Ganesan et al. 2011).

ErB (6), as well as DR80 (2), a relatively specific inhibitor for CD40–CD154 (Margolles-Clark et al. 2009), showed similar profiles (Figure 4-5B and C, respectively) indicating competitive/reversible binding with $pA_2$ values of 6.0 and 5.7 (corresponding to $K_i$s of 1.0 and 1.8 μM), again in good agreement with our previous results from inhibition experiments (IC$_{50}$s of 2–3 μM for both ErB (6) and DR80 (2), respectively.)
Figure 4-5: Schild analysis for surmountable inhibition
Dose-response curves showing the binding of CD154 to CD40 in the absence and presence of indicated concentrations of anti-CD154 antibody (mAb), used as a positive control [A], as well as DR80 (2) [B] and ErB (6) [C].
4.4 COMPUTATIONAL ASSESSMENT OF THE NATURE OF BINDING

Using the FTMAP server, consensus-binding sites were identified both on CD40 and CD154 as described under methods (section 2.2.ii.a). The largest consensus sites were on CD154, while CD40 had several smaller pockets. The consensus sites on CD154 were at two distinguishable regions, one at the CD40-binding interface was labeled as the competitive (orthosteric) site [CS: Arg 203, Ile 204, Leu 205, Arg207 (Subunit A); Ile 127, Gly 144, Tyr 145, Thr 251, Gly252 (Subunit C)]. Two pockets identified at the interior core of the trimer were labeled as the allosteric sites [AS1:Gly 174, Arg 209, Asn 210, Ser 122, Ile 223, His 224 (Subunits A, B, C) and AS2: Gly 121, Ile 122, Leu 259, Lys 260, Lys 261, Tyr 170 (Subunits A, B, C) (Figure 4-6)

**Figure 4-6: Identification of druggable sites by FFTMap**
A, B, and C (shown in gray, red, and green, respectively) are the three subunits of CD154. CS designates the competitive (orthosteric) site at the CD40-CD154 PPI interface, whereas AS1 and AS2 designate the two allosteric sites at the interior core of the trimer.
4.4.i Docking at the competitive site

Small molecules of interest, including BIO8898 (1), CS7B (4), MB1 (5), ErB (6), and TZ (7), were docked at the CD40 binding site of CD154 (prepared from 3QD6) that was also identified as the competitive site (CS) by FTMAP as described previously (section 2.2.ii.a). For further analysis, the Glide docking scores were used (the lower, the better) rather than ligand efficiencies due to the comparable size of our test compounds. Glide docking scores at competitive site were only modest (Table 4-1), and the scores generated by both standard (SP) and extra precision (XP) mode at the competitive site were very similar for both binders and non-binders and, therefore, not in agreement with the observed experimental specificities. Induced-fit docking was also performed to allow some the protein flexibility; nevertheless, the corresponding docking scores produced no significant differentiation between binders and non-binders (Table 4-1).

4.4.ii Docking at the allosteric site

Docking at the allosteric site was done using the co-crystal structure (3LKJ) of CD154 with BIO8898 (1). Re-docking of the co-crystallized ligand BIO8898 (Silvian et al. 2011) was done to test the performance of Glide at the allosteric site. The generated docking pose was very similar to the co-crystallized pose; in addition, it scored the highest among tested ligands. Glide SP and XP modes were used to dock and score the four tested small molecules as previously described (section 2.2.ii.e). CS7B (4) and MB1 (5) ranked the highest followed closely by the promiscuous inhibitor ErB (6) by both Glide modes. Although Glide in the standard precision (SP) mode predicted only a slightly
lower score for the inactive compound TZ (7) at this site, the extra precision (XP)
mode was able to better discriminate it as a poor binder (Figure 4-7, Table 4-1).
Scores obtained by induced fit in the SP mode showed little difference among the
different compounds, similar to Glide SP. Induced fit in the XP mode showed
better correspondence with experimental activity, albeit not as marked as Glide
XP (Ganesan et al. 2012).

Table 4-1: Glide docking scores at the competitive and allosteric sites

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<th>Allosteric Site (AS)</th>
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<td>Induced fit docking scores</td>
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<tr>
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Figure 4-7: Glide-XP docking and scoring at the allosteric site of CD154
Glide-XP docked pose of 4, CS7B [A.1], and 7, TZ [A.2]. The A, B, and C subunits of CD154 are shown in tube representation in gray, red, and green respectively, hydrophobic residues are colored by Li-Tang-Wingreen scheme. Corresponding ligand interaction diagrams of CS7B and TZ with neighboring residues involved in hydrogen bond and hydrophobic interactions are also shown in B.1 and B.2, respectively (blue – polar residues, green – hydrophobic residues; gray – glycine; pink – negatively charged residues; yellow – solvent exposure; solid pink line – backbone H-bond).
CHAPTER 5 NON-SPECIFIC INHIBITION

5.1 ERYTHROSINE B– A UNIQUE FOOD COLORANT

During our search for specific CD40–CD154 inhibitors, we noticed that erythrosine B (ErB, 6) an FDA approved food colorant inhibited several PPI with very similar activity (i.e., it behaved as a non-specific, promiscuous inhibitor). I followed up with this finding using more detailed investigations. A total of fourteen food colors (Figure 5-1) approved in the USA, Europe, or Japan (Glória 2006) – including all seven FDA-approved food colorants – were tested for their activity to inhibit a number of PPIs mainly within the TNF superfamily (TNFSF), but also outside of it (Table 5-1; Figure 5-2). As before, a blocking antibody was included as a positive control in a number of assays (e.g., TNF-R–TNFα, CD40–CD154, BAFF-R–BAFF, and EGF-R–EGF), and in all cases their inhibitory activities with IC\textsubscript{50}s in the nanomolar (nM) range have been confirmed. Whereas ErB (6) consistently showed inhibition in all these assays with a remarkably similar activity, IC\textsubscript{50}s in the 2-20 μM range (approx. 2-20 mg/L), none of the other food colors 7–19 showed any significant (i.e., IC\textsubscript{50} < 50 μM) inhibitory activity. Interestingly, ErB (6) seems to maintain considerable inhibition even in the presence of protein containing media (Figure 5-3, Figure 5-4), a condition that resulted in about ten-fold loss in activity in these assays for azo-containing organic dyes (Margolles-Clark et al. 2009; Margolles-Clark et al. 2009), and also a complete loss of activity (IC\textsubscript{50} > 500 μM) for the food colorants 7–19 (Table 5-1).
Figure 5-1: Chemical structure of food colorants and xanthenes (See Table 5-1 for details).
Figure 5-2: Inhibition of PPI by FDA-approved food colorants
Concentration-dependent inhibition of human TNFR1–TNFα [A]; CD40–CD154 [B]; and EGF-R–EGF [C] interactions by FDA–approved food colorants (See Table 5-1 for the corresponding IC50s). Insets show inhibition with corresponding antibodies.
Figure 5-3: Inhibition of TNF-R1–TNFα binding by all tested food colors (See Table 5-1 for corresponding IC₅₀s).
Figure 5-4: Inhibition of CD40–CD154 binding by all tested food colors (See Table 5-1 for corresponding IC$_{50}$s).
Table 5-1: Data for food colorants included in the present study

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<th>ADI (mg/kg)</th>
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5.2 CELLULAR EFFECTS OF PROMISCUOUS INHIBITION

5.2.i TNFα-mediated JNK phosphorylation

To test the cellular effects of the toxicity of ErB (6), we also evaluated its effect on TNFα–induced JNK (c-Jun N-terminal kinase) phosphorylation in THP-1 cells by Western blotting as described before (section 2.3.iv). JNK is a member of the mitogen activated protein kinase (MAPK) family, and it is a stress-response kinase activated by pro-inflammatory cytokines and growth factors coupled to membrane receptors or by various stimuli, such as heat shock, UV irradiation, protein synthesis inhibitors, and elevated levels of reactive oxygen intermediates (ROI), through non-receptor pathways. TNFα (20 ng/mL) clearly caused activation of this pathway significantly increasing the phosphorylated fraction of JNK (predominantly that of one isoform (Dreskin et al. 2001; Ferlito et al. 2001)) even after a short incubation (5 min), and this was reversed by the anti-TNFα antibody (2.0 mg/mL) as well as by ErB (6; 50 μM) and the known JNK inhibitor SP600125, but not TZ (7), which was used as a negative control (Figure 5-5). This assay was selected because it can be due to cytotoxicity following longer incubations at these concentrations.

5.2.ii Cytotoxicity in cells of various histological origin

To test whether this promiscuous in vitro protein inhibitory activity translates into toxic effects, the cytotoxic potential of ErB was determined in THP-1 (human monocytic leukemia cells), Jurkat (immortalized T lymphocyte cells), and HEK293T (human embryonic kidney derived cells) cells compared to other control organic dyes. Toxic effects were assessed both by a cell
proliferation assay (BrdU incorporation, section 2.3.i.a) and an apoptosis assay (DAPI staining analyzed by flow cytometry, section 2.3.i.b). Whereas both promiscuous inhibitors ErB (6) and RB (20) clearly showed signs of cytotoxicity with IC₅₀s of less than 100 μM for ErB (6) and even less for RB (20), the corresponding comparators used, including DR80 (2), which has a similar Kᵢ for the CD40–CD154 inhibition (Margolles-Clark et al. 2009), and ALR (9), which is the other FDA-approved red food colorant, showed no toxic effect up to 500 μM concentrations (Figure 5-6) – a clear indication of the possible detrimental effects related to the nonspecific inhibitory activity of ErB (6) and RB (20).

Figure 5-5: Inhibition of TNFα-mediated JNK phosphorylation
Western blot showing JNK phosphorylation in THP-1 cells in the presence of indicated treatments: TNFα (20 ng/ml); mAb – anti-TNFα antibody (~13 nM); SP – SP600125 a competitive inhibitor of JNK 1 and 2 (50 μM); ErB (6; 50 μM); and TZ (7; 50 μM). Data (fraction of JNK phosphorylated, normalized with TNFα treatment as reference; average ± SD for n = 3 independent experiments) were analyzed by ANOVA with Tukey’s post hoc test, and †† and ** indicate statistically significant differences (p< 0.01) versus untreated control and TNFα treatment, respectively.
Figure 5-6: Cytotoxic effects of food colorants

Cell toxicities of ErB (6), ALR (9), TZ (7), RB (20), and DR80 (2) as assessed by a standard proliferation assay (BrdU; A.i) and an apoptosis assay (DAPI exclusion) in THP1 cells [A], Jurkat cells [B] and HEK293T cells [C]. Data (average ± SD for \( n = 3 \) independent experiments with triplicates for each condition) were analyzed by ANOVA with Tukey’s post hoc test and asterisk (*) indicates statistically significant differences (\( p < 0.001 \)) versus untreated controls (0).
5.3 MECHANISMS OF NON-SPECIFIC INHIBITION

5.3.i Photo-enhanced inhibition by erythrosine and rose Bengal

The presence of bromine or iodine atoms in phosphorescent molecules such as ErB (6) and RB (20) enhances the yield of intersystem crossing to the reactive triplet state upon absorption of photons. When energy from the reactive triplet state transfers to molecular oxygen, it results in the formation of singlet oxygen species ($^1\text{O}_2$) (Ben Amor et al. 2000). Singlet oxygen can oxidize accessible amino acids (His, Cys, Phe, Tyr) in proteins thereby causing photo-damage.

To investigate if photo-damage could account for the inhibition by the promiscuous xanthenes, CD40–CD154 inhibition assay with dark and light conditions was performed in parallel and found the activity of ErB (6: IC$_{50}$ (light) = 3 μM; IC$_{50}$ (dark) = 27 μM) and RB (20: IC$_{50}$ (light) = 5 μM; IC$_{50}$ (dark) = 23 μM) to diminish in the dark about 5 to 10-fold, whereas those of DR80 (2: IC$_{50}$ (light) = 6 μM; IC$_{50}$ (dark) = 10 μM) and anti-CD154 antibody (IC$_{50}$ (light) = 0.05nM; IC$_{50}$ (dark) = 0.04nM) to remain virtually unaffected (Figure 5-7). Hence, photosensitization accounts for part of the observed inhibition by these halogenated xanthenes.
Figure 5-7: Photo-enhanced inhibition of PPI by erythrosine B and rose Bengal

CD40–CD154 inhibitory effect of ErB (6) and RB (20) in light [A] and dark [B] conditions (with DR80 (2), and anti-CD154 monoclonal antibody (mAb), as controls). Data (average ± SD for n = 3 independent experiments with triplicates for each condition) Comparisons of pIC50s in light vs. dark using paired t-tests, and asterisk (*) indicates statistically significant differences (light versus dark; p<0.05) [C].
5.3.ii Structure activity relationships (SAR) of substituted xanthenes

Since ErB (6) has a unique chemical structure among FDA-approved food colorants, I also tested several close structural analogs including polyhalogenated xanthene analogs such as RB (20), EO (21), and PHL (22), as well as non-halogenated xanthene analogs such as FL (23) and GLN (24) to assess the structural basis of the promiscuous inhibitory activity across the spectrum of these PPIs (Table 5-2). Whereas ErB (6) and RB (20) showed consistent and promiscuous inhibitory activity, this was considerably diminished in the polybrominated analogs EO (21) and PHL (22) and essentially lacking in the non-halogenated parent xanthene FL (23). The hydroxyl-substituted xanthene GLN (24) showed inhibitory activity only in selected interactions (CD40–CD154, RANK–RANKL, and OX40–OX40L) (Figure 5-8, Table 5-2).

Since the structure of ErB is hydrophobic and rigid with the iodine substituents contributing to its large molecular size, it is likely that binding with many targets occurs due to van der Waals and hydrophobic interactions. When inhibitory activity of substituted xanthenes was plotted against various physicochemical descriptors, greatest correlation was obtained with molecular weight (MW; \( r^2 = 0.85 \)) and octanol-water partition coefficient (log\( P; r^2 = 0.89 \)), a measure of hydrophobicity, in support of this hypothesis (Figure 5-9).
Figure 5-8: Inhibition of PPI by xanthene analogs of erythrosine B
Inhibition of protein-protein interactions in the TNF superfamily (TNFR1–TNFα [A], CD40–CD154 [B], RANK–RANKL [C], OX40–OX40L [D], BAFFR–BAFF [E], 4-1BB–4-1BBL [F]) and outside of it (EGF-R–EGF [G]) by tested xanthene dyes (see Table 5-2 for the corresponding IC50s).
The inhibitory activity of xanthene analogs of ErB (6) (n = 5; see Figure 5-1) for a representative PPI (TNFR1–TNFα) expressed as pIC50 correlates well with molecular weight (MW) [A], and log octanol-water partition coefficient (logP), a commonly used index of hydrophobicity [B].

Table 5-2: Median inhibitory concentrations of xanthene analogs of erythrosine B

<table>
<thead>
<tr>
<th>No. (Refer Figure 5-1)</th>
<th>Legend</th>
<th>CD40–CD154</th>
<th>TNFR1–TNFα</th>
<th>RANK–RANKL</th>
<th>OX40–OX40L</th>
<th>BAFF–BAFF</th>
<th>4-1BB–4-1BBL</th>
<th>EGFR–EGF</th>
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<tr>
<td>1</td>
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<td>5</td>
<td>2</td>
<td>2</td>
<td>12</td>
<td>11</td>
<td>3</td>
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<td>2</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>21</td>
<td>PHL</td>
<td>17</td>
<td>161</td>
<td>8</td>
<td>7</td>
<td>365</td>
<td>20</td>
<td>64</td>
</tr>
<tr>
<td>22</td>
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<td>&gt;579</td>
<td>&gt;47</td>
<td>&gt;45</td>
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<td>17</td>
<td>4</td>
<td>63</td>
<td>57</td>
<td>398</td>
</tr>
</tbody>
</table>
5.3.iii Characterization of ErB binding

Various studies have explained the competitive binding of poly-iodinated xanthenes to P-type ATPases and mono-deiodinase enzymes to arise from their structural relatedness to the enzymes’ substrates adenosine mono/diphosphates and thyroxine respectively. However, although the ball and stick structures (top) seem to allow for some possible overlap, the CPK structures clearly reveal very different molecular sizes mainly due to the presence of iodine substituents (Figure 5-10). The overall flexibility of these molecules is also very different.

Figure 5-10: Comparison of erythrosine B and adenosine monophosphate (AMP)
Comparison of ball and stick [A] and CPK [B] structures of ErB (6) (darker colors) and AMP (lighter colors).

To study the direct interaction of ErB (6) with its many targets, I performed binding experiments exploiting its innate tendency to exhibit a spectral shift upon protein binding (metachromasy) (Prento 2009). Increased concentrations of added protein BSA₈ and CD154 produced a progressive bathochromic⁹ shift of ErB’s spectrum (from 526-527nm with no added protein to 536 nm and 531nm

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⁸ BSA – bovine serum albumin
⁹ Bathochromic shift – shift to a longer wavelength
with 25 µM BSA and CD154 respectively). Addition of BSA caused greater bathochromic shift to the ErB’s spectra compared to that seen with CD154. A hypochromic\(^{10}\) shift was also observed upon addition of BSA, which was more pronounced in the case of CD154 (Figure 5-11, Figure 5-13). Metachromatic shift was used to quantify the complex formation between ErB (6) and the proteins BSA and CD154 as described under materials and methods (section 2.5.ii.a). The binding constant \((K_d)\) for ErB (6) binding to BSA using this method was found to be \(~14\) µM (Figure 5-12), while that to CD154 was found to be \(~20\) µM (Figure 5-14). The binding curves had hill slopes \((n_h)\) of 2.1 and 2.4 for ErB–BSA and ErB–CD154 respectively indicative of cooperativity. To follow-up with the evidence of cooperative binding, I wanted to confirm whether ErB (6) indeed had multiple binding sites using the Job’s analysis (Schlein et al. 2001). This analysis is based on the principle that for mixtures of varying compositions, maximum complex formation occurs at the right stoichiometry. The Job plot for ErB–BSA binding was constructed using complementary molar ratios of dye and protein such that the total molar content remained constant. The ErB–BSA complex at every point was quantified from spectral data as described previously (section 2.5.ii.b). The point of maximum complex formation was derived from the tangents drawn from both ends. The corresponding molar ratio for the ErB–BSA complex was found to be \(~85:15\) (5–6 ErB molecules per BSA molecule; Figure 5-15), while that for ErB–CD154 was found to be \(~90:10\) (8–9 ErB molecules per CD154 monomer; Figure 5-16).

\(^{10}\) Hypochromic shift – shift to a lower intensity
5.3.iii.a Binding constant (ErB–BSA)

Figure 5-11: Metachromatic shift due to erythrosine B–BSA binding
Absorbance spectra of different concentrations of ErB (6) with 0 μM BSA [A]; 5 μM BSA [B]; 7 μM BSA [C]; 11 μM BSA [D]; 16 μM BSA [E]; and 25 μM BSA [F] showing spectral shift from a λ_max of 527 nm obtained without added protein (0 μM BSA)\(^\text{11}\).

\(^{11}\) Normalized OD – OD normalized to OD_{max} obtained with free dye
Figure 5-12: Erythrosine B–BSA binding curve
Difference spectra of ErB (Bound - Free) with 25 μM BSA [A]; 16 μM BSA [B]; 11 μM BSA [C]; 7 μM BSA [D]; and 5 μM BSA [E] and the corresponding binding curve with different concentrations of added BSA together with the binding constants obtained by fitting with a log dose vs. response model (inset showing binding normalized to B max fitted using shared $K_d$ and $n_h$) [F] (GraphPad Prism version 5.04, San Diego, CA).
5.3.iii.b Binding constant (ErB–CD154)

**Figure 5-13: Metachromatic shift due to erythrosine B–CD154 binding**

Absorbance spectra of different concentrations of ErB (6) with 0 μM CD154 [A]; 5μM CD154 [B]; 7 μM CD154 [C]; 11 μM CD154 [D]; 16 μM CD154 [E]; and 25 μM CD154 [F] showing spectral shift from a $\lambda_{\text{max}}$ of 526 nm obtained without added protein (0 μM CD154).
Figure 5-14: Erythrosine B–CD154 binding curve
Difference spectra of Er B (Bound - Free) with 25 μM CD154 [A]; 16 μM CD154 [B]; 11 μM CD154 [C]; 7 μM CD154 [D]; and 5 μM CD154 [E] and the corresponding binding curve with different concentrations of added CD154 together with the binding constants obtained by fitting with a log dose vs. normalized response model (inset showing binding normalized to B max fitted using shared $K_d$ and $n_h$) [F] (GraphPad Prism version 5.04, San Diego, CA).
5.3.iii.c Stoichiometry (ErB–BSA)

Figure 5-15: Jobs plot-derived stoichiometry of erythrosine B–BSA binding
Difference spectra of protein BSA with no added dye ErB (50 μM BSA + 0 μM ErB) [A], protein BSA with added dye ErB (30.5 μM ErB + 19.5 μM BSA) [B], and dye ErB with no added protein BSA (50 μM ErB + 0μ M BSA) [C] with insets showing the corresponding absorbance spectra; Jobs plot of ErB–BSA binding using complementary molar ratios of ErB and BSA, keeping the total molar content constant to obtain stoichiometry (n) [D].
5.3.iii.d Stoichiometry (ErB-CD154)

Figure 5-16: Jobs plot-derived stoichiometry of erythrosine B–CD154 binding
Difference spectra of protein CD154 with no added dye ErB (50 μM CD154 + 0 μM ErB) [A], protein CD154 with added dye ErB (30.5 μM ErB + 19.5 μM CD154) [B], and dye ErB with no added protein CD154 (50 μM ErB + 0 μM CD154) [C]. Insets show the corresponding absorbance spectra; Jobs plot of ErB–CD154 binding using complementary molar ratios of ErB and CD154, keeping the total molar content constant to obtain stoichiometry (n) [D].
CHAPTER 6  DISCUSSION

6.1 SEARCH FOR SMALL-MOLECULE INHIBITORS

Peptides interfering with the CD40–CD154 interaction have been reported recently in the literature with activities ranging from quite high (IC$_{50}$s of 50–100 nM for relatively large cyclic structures, (MW 2500Da), with trimeric symmetry (Fournel et al. 2005) through moderate (IC$_{50}$s of 100 µM for two end-group blocked peptides (Allen et al. 2005) and 50 µM for a cyclic heptapeptide (Deambrosis et al. 2009) to very low (IC$_{50}$ < 1µM for three recombinant phage proteins (Kitagawa et al. 2005). A series of dipyridine derivatives were claimed in a patent (Zheng et al. 2007) and a co-crystal structure with CD154 has been obtained recently using a related compound BIO8898 (Silvian et al. 2011).

Using an iterative activity screening/structural analog search procedure, our group was the first to report a series of active small molecules (some with MW < 600 Da) with adequate CD40–CD154 inhibitory activity confirmed in cell assays along with evidence of selectivity in binding assays compared to the closely related TNFSF member TNF-R1–TNFα which despite a low sequence identity (~17 %) shows a good overall structural similarity (Margolles-Clark et al. 2009; Margolles-Clark et al. 2009; Buchwald et al. 2010). My work focused on identifying compound with greater ligand efficiency and their characterization in *in-vitro* binding and activity assays.
After identification of the most active compounds in each round of in vitro testing, I used a Tanimoto coefficient-based structural similarity search of available chemicals using these structures as lead. I have presented here, a representative sample of the identified structural analogs after several iterative steps focusing on the most active, smaller molecular weight compounds.

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 pockets to achieve desired potencies. Additionally, since only a fraction of the small molecule's surface can interact at the protein interface than when inside a well-defined pocket, PPI inhibitors have greater likelihood of violating the molecular size constraints laid out by the Lipinski’s “rule of five” (an established guide to evaluate oral bioavailability) (Lipinski et al. 2001) than non-PPI inhibitors.

This is reflected in the average LE\textsuperscript{12} of known PPI inhibitors (~1.0 kJ/atom) (Wells et al. 2007) compared to a higher average of around 1.5 kJ/atom (or more) for traditional small molecule compounds (Hajduk et al. 2005; Buchwald 2010). The organic dyes identified in the present study show significant improvement in both CD40–CD154 inhibitory activity and LE since the discovery of suramin (MW 1297 Da; LE = 0.36) (Margolles-Clark et al. 2009) or the recently reported BIO8898 (1, MW 909 Da; LE = 0.44) (Silvian et al. 2011). Progressive improvement in LE was observed starting from the suramin analog

\[ \text{LE} = \text{Ligand efficiency (see sections 1.1 and 2.1.ii)} \]
DR80 (2, MW 1300 Da; LE = 0.40), its substructure CS7B (4, MW 461 Da; LE = 0.83) and the dye PDB (3, MW 878 Da; LE = 0.69). Our group has also recently discovered a naphthalene sulfonic acid derivative, MB1 (5, MW 507 Da, LE = 1.11) that has the highest reported LE for CD40–CD154 inhibition (Table 3-1) (Margolles-Clark et al., 2010) approaching those of the best-known small molecule PPI inhibitors.

Evidence of specificity is particularly encouraging for the present compounds since a number of them are likely to show a good degree of nonspecific protein binding. I tested for specificity using a panel of interactions closest to CD40–CD154 PPI within the TNFSF (Figure 3-2) and an unrelated PPI from the growth factor receptor superfamily (EGF-R–EGF). Compounds that showed the greatest activity for CD40–CD154 inhibition relative to any other (DR80 (2), PDB (3), and MB1 (5)) were chosen for further characterization (Figure 3-2; Table 3-1). It was noted that among these compounds CD40–CD154 inhibition was closely followed by that of OX40–OX40L, 41BB–41BBL and RANK–RANKL and they consistently fell short of inhibiting BAFFR–BAFF and TNFR–TNFα interactions. Although this order does not correlate with the overall similarities of these TNFSF receptors and ligands, knowledge of the binding site may provide some insight to assess whether local similarities can explain this observation.
Among these compounds, ErB (6) was a notable exception due to its promiscuous nonspecific binding /inhibitory activity at ~10 µM levels not only in all test performed here, but also in inhibiting insulin–insulin receptor interaction (Schlein et al. 2002) as well as in other tests, e.g., in inhibiting galactokinase activity (Wierenga et al. 2008) (Table 1-2). Biochemical screening assays are often confounded by the presence of compounds that are known ‘promiscuous inhibitors’ (McGovern et al. 2002), ‘frequent hitters’ (Roche et al. 2002), or contain chemically reactive functional groups such as protein-reactive electrophilic false positives as well as chelators and polyionic ‘warheads’ (Rishton 2003).

ErB (6), together with several other dye compounds, have been found to act as promiscuous inhibitors in high-throughput screening (HTS) assays. Polymolecular conglomeration (Roterman et al. 1993; Stopa et al. 1998) or aggregation (McGovern et al. 2002; Shoichet 2006) have been suggested as possible mechanisms. To investigate the possibility of aggregation effects, we repeated the CD40–CD154 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 et al. 2006), but observed no significant effect on IC₅₀s for any of the above dyes including ErB (6) (data not shown). Xanthene dyes can aggregate in aqueous solutions, but at higher concentrations; for example, with K_dS around 1 mM for dimerization of ErB (6) and its structural analogs (Valdes-Aguilera et al. 1989).
In the hope of deriving compounds devoid of the structural attributes that might contribute to any observed non-specificity, while retaining those features necessary for sufficient binding affinity to the target protein, I performed shape similarity searches using the prepared three dimensional structures of our best compounds (CS7B (4), MB1 (5)) as query against the conformers prepared from the PubChem Compounds repository, a database of ~340,000 compounds. Top 200 compounds were selected based on computed Tanimoto coefficients (S2) to obtain for use in our ELISA-based screening assay (Figure 3-3, Figure 3-4, and Figure 3-5). However, as pointed out earlier (section 1.1), a limitation of screening using libraries that are largely populated with the compounds that are customized to target traditional drug pockets is that they are skewed towards smaller and flatter molecules, while most PPI inhibitors tend to be larger, more rigid, and three-dimensional (Buchwald 2010; Mullard 2012). Therefore, an alternative approach would be to use libraries that are more tailored to PPI inhibition as discussed before (section 1.1) or to use medicinal chemistry to refine the structures of our existing compounds to systematically derive ones with better activity and specificity.

In order to investigate if the inhibition of binding extended to that of CD40–CD154 PPI’s function, I performed various in-vitro cell-based biochemical assays using the best compounds. The representative compound PDB (3) concentration-dependently inhibited the soluble CD154-induced up-regulation of two of the three tested cell-surface markers (MHC-II, CD40, and CD54) (Figure 3-7). DR80 (2), CS7B (4), and MB1 (5) were also shown to inhibit the proliferation of human
B cells, consequent to CD154 stimulation at non-cytotoxic levels (Buchwald et al. 2010) - a clear indication of their ability to interfere with the CD40–CD154 co-stimulatory PPI. Additionally, to assess the potential of these compounds in an auto-immunity setting, I used the BDC2.5 mouse model of type I diabetes that has T-lymphocytes carrying a transgenic T-cell receptor (TCR) capable of recognizing only the BDC 2.5 auto-antigen. Our tested compounds DR80 (2) and PDB (3) dose-dependently inhibited the antigen-mediated T-cell proliferation (Figure 3-8). Although such inhibition happened at concentration levels well above that causing inhibition of binding, they were also well below predetermined toxic levels (Figure 3-6) and correlated more or less with the activities of these compounds in a binding assay performed in the presence of protein-containing media. Despite the observed CD40–CD154 inhibitory activity in cell-based assays being consistent with that seen in cell-free biochemical assays, there does exist a window of non-specificity requiring an order of magnitude of higher dye concentrations in a cellular context. We hope to circumvent this barrier through a better understanding of the structural basis of inhibition to thence dissect out determinants of non-specificity, while refining the useful part of the dyes’ structures.

6.2 SPECIFIC INHIBITION

An accepted path to traverse this challenging area of PPI drug discovery is to systematically advance the initial discovery of a sufficiently active and specific binder (having micromolar affinity) to one that has desired (nanomolar) activity, specificity and ligand efficiency. This can be best done using the
knowledge of their inhibitory mechanisms (binding partner, nature of binding, possible binding sites and interactions involved) obtained from biophysical approaches and structure elucidation (Mullard 2012). To identify the binding partner, I exploited an inherent property of these dyes, which causes a shift in the absorbance spectra upon protein binding (metachromasy). DR80’s (2), spectrum shifted only upon the addition of CD154, but not an equimolar amount of CD40, indicating preferential binding to CD154 (Figure 4-1). This result is in agreement with what was seen earlier in competition assays by our laboratory (Buchwald et al. 2010). Following this, I purified the extracellular domain of CD154 with a hexa-histidine tag for use in further mechanistic studies and to possibly obtain a co-crystal structure with our best small molecule compound (Figure 4-2). I also verified the trimeric status (Figure 4-3) of the purified protein and confirmed its ability to bind CD40 (Figure 4-4A) and cause NF-κB transactivation in cells expressing CD40 receptor (Figure 4-4B).

Inhibition can occur either through binding at the interface of PPI (competitive or orthosteric inhibition) or at an alternate site which may cause conformational change that can interfere with the PPI (non-competitive or allosteric inhibition). In case of the former, addition of excess of the binding partner can displace the small-molecule from the binding site and therefore, such inhibition is reversible (or) surmountable, while allosteric inhibition which results in a conformational change is generally considered irreversible (or) insurmountable. Using the Schild analysis, I tested the ability of CD154 to bind to CD40 in the presence of the inhibitors suramin, DR80 (2), and ErB (6). At
concentrations of CD40 and CD154 used in the binding assays, suramin, DR80 (2) and ErB (6) inhibited the CD40–CD154 PPI. However, greater CD154 concentrations were able to circumvent the inhibitory effect of the small molecules and restore binding to maximum in the case of DR80 (2) and ErB (6), but not with suramin (data not shown). Since we know that the DR80 binds CD154 and not CD40, this suggests that at higher concentrations of added CD154, the protein may be present in excess of what can be bound by DR80 (2) and is therefore available to bind to CD40. Whereas binding by ErB (6), which has been shown to occur on both CD40 and CD154, can probably be outcompeted at the PPI interface in the presence of excess CD154 (Figure 4-5).

In order to determine potential binding pockets for the identified small molecules on CD154, I used a computational algorithm that identified pockets that are hit mainly by chemically diverse probe molecules with a relatively high binding affinity (Hajduk et al. 2005). As described earlier (section 1.1) this FFT-mapping (Fast Fourier Transform correlation) algorithm (Brenke et al. 2009) is based on Multiple Solvent Crystal Structures (MSCS), an X-ray method that identifies consensus sites based on the clusters of overlapping solvent probes across structures of the same protein solved using various organic solvents (Dennis et al. 2002). The method identified consensus sites on both CD40 and CD154, the largest clusters being found on CD154 indicating it to be more druggable than CD40 that had several, albeit smaller clusters. This is in agreement with our experimental observations that most of our small molecules preferentially bind to CD154 (Margolles-Clark et al. 2009; Buchwald et al. 2010).
Glide docking scores of CS7B (4), MB1 (5), and ErB (6) observed at the competitive site (CS) were only modest (Table 4-1), which is likely due to the relatively small size and ill-defined nature of this pocket. Furthermore, the competitive site is composed of a large number of charged residues that do not represent a suitable environment for the interaction with the lipophilic atoms of the docked small molecule ligands. The docking scores generated by both standard (SP) and extra precision (XP) mode of Glide as well as induced fit docking (which allows for greater flexibility) at the competitive site was very similar for both binders and non-binders and, therefore, not in agreement with the observed experimental specificities (Table 4-1). The seemingly better scores for BIO8898 (1) may be due to its large size that allows for extensive van der Waals contacts and some hydrophobic interactions with accessible non-polar amino acids.

Experimental binding data correlated better with the docking results at the allosteric site than the docking scores of the competitive site (no correlation at all). The better Glide XP docking scores for CS7B (4) and MB1 (5) resulted mainly from the hydrophobic enclosure of their substituted benzene and naphthalene moieties by aromatic residues (Tyr 170, Tyr 172, Val 228, Leu259, Leu261) at the interface of the A and C subunits of CD154. These interactions were considerably smaller for TZ (7), which has less bulky aromatic groups. Hydrogen bond formation between the acid moieties of the PPI inhibitors and the backbone amides of accessible residues also contributed to the better binding. The docked pose of TZ (7) suggested an unfavorable interaction of its charged
carboxylate group on its central pyrazole ring with the surrounding hydrophobic pocket (Figure 4-7). This interaction may be a primary reason for a poor docking score and, therefore, poor binding to the allosteric site (as we anticipated in this docking experiment). Therefore, our docking study suggests that the tested compounds may bind to the allosteric site. In addition, the co-crystal structure of CD154 with BIO8898 and SP307 with TNFα also supports small molecule binding at this site on the ligands of TNFSF. Together, these results raise the possibility that the site at the hydrophobic trimeric core may be druggable. Accordingly, an allosteric inhibitory mechanism that involves intercalation between monomeric subunits seems feasible for our small molecules as well, making the constitutively trimeric CD154 a possible druggable target. Considering the surmountable nature of our compounds’ inhibition upon simultaneous addition of CD40 and CD154 in the presence of the inhibitor DR80 (2) (the parent structure of CS7B (4) as demonstrated earlier (section 4.3)), the possibility of initial weak inhibitor binding at the subunit interface followed by its subsequent access to the critical residues in the hydrophobic core of trimeric CD154 remains a possibility worth investigating.

6.3 NON-SPECIFIC INHIBITION

Promiscuous inhibitors as well as false positive hits greatly confound the results of high-throughput screens and must be identified in the earliest steps to divert developmental time and effort to advance real as well as specific inhibitors. Our group had identified ErB (6) as a relatively potent and non-specific inhibitor of a number of important receptor-ligand–type PPIs within the TNFSF. This was
in agreement with its long-known inhibitory activity on dopamine uptake (IC₅₀ ≈ 50 μM) (Lafferman et al. 1979; Logan et al. 1979; Mailman et al. 1980; Silbergeld et al. 1982), acetylcholine release (Augustine et al. 1980), or other proteins’ interactions or activities within a consistent concentration range (Table 1-2). This unique nature of ErB (6) makes it a useful tool to understand the mechanisms of non-specificity, the knowledge of which can be used to develop criteria to eliminate the same.

Since ErB (6) is an FDA-approved food colorant (FD&C Red No. 3, erythrosine), I also investigated whether any other approved food colors possess such inhibitory activity. Among the six FDA approved food colorants (6, 7, 9, 13, 14, and 18) (Glória 2006), this inhibitory activity seems to be unique for ErB (6) (Figure 5-2). ErB’s inhibition of large variety of PPIs within the TNF receptor-ligand superfamily (TNFRSF–TNFSF) as well as outside of it (EGF-R–EGF) was with a remarkably consistent IC₅₀ in the 2–20 μM range (Table 5-1). Toxicity is an obvious concern related to the promiscuous inhibitory activity, and such effects are a particularly controversial issue for synthetic food colorants. Toxicity was observed starting at ~30 μM in the cell lines tested (THP-1, Jurkat and HEK-293T (Figure 5-6) using ErB (6) and with even lower concentrations of RB (20), an analog of ErB (6) approved for use as food colorant in Japan, but not with other food colorants (ALR (9), TZ (7)) in the concentrations tested. However, the ADI¹³ for ErB is 0–0.1 mg/kg/day, which is about a hundred-fold lower than its inhibitory

¹³ ADI – Accepted daily intake
concentrations (~2–20 mg/L) or even lower compared to the observed toxic concentrations to cells (~30 mg/ml). Hence, it can be considered to be relatively non-toxic at levels consumed, but this is certainly a contentious subject. Various adverse reactions, mainly possible behavioral alterations and food allergies, have been often and controversially linked to food colorants (MacGibbon 1983). ErB (6) itself has been associated with the controversial Feingold hypothesis that food colorants caused hyperkinetic behavior in children (Feingold 1975; Silbergeld et al. 1982; Schab et al. 2004). For the iodine-containing ErB (6), thyroid toxicity was also a major concern (Poulsen 1993). One relatively recent study even found a number of food colors, including ErB (6), TZ (7), and ALR (9) as well as RB (20) and EO (21) to be genotoxic causing dose-related DNA damage in the gastrointestinal organs at doses (10 or 100 mg/kg) that are not very far from their ADIs (Table 5-1) (Sasaki et al. 2002). It is important to note that the current ADI for ErB (6) is already considerably lower than that of any other FDA-approved food color, e.g., 7 mg/kg body weight/day for ALR (9) or 7.5 for TZ (7) (Table 5-1) (Glória 2006). For ErB (6), this value is based on the no observed effect level (NOEL) of 1 mg/kg body weight/day (60 mg per person per day) for effects on thyroid function in humans observed at the next highest dose of 3.3 mg/kg BW/day and obtained using a 10-fold safety factor (Joint 1991). Considering the relatively potent promiscuous inhibitory activity of ErB (6), which is unique among food colorants, such a low ADI seems to be well justified. On the other hand, since the nonspecific inhibitory activity of ErB (6) as well as RB (20) seems to become a concern at concentrations starting around a 2–20 μM
(approx. 2–20 mg/L) range, related effects should not be an issue if the ADI guidelines (0.1 mg/kg body weight/day) are followed. Nevertheless, because ErB (6) is a quite hydrophobic (log $P_{o/w} > 5$) (Figure 5-9) and BBB$^{14}$-permeable molecule, considerable tissue accumulation might take place (e.g., biological toxicity seemed log$P$-related in xanthenes dyes (Levitan 1977)); hence, adherence to the ADI guidelines would seem a reasonable precaution, and it should be extended to RB (20) as well where it is approved for food use.

To identify the structural basis of the promiscuity of ErB (6), I tested some of its structural analogs (20–24) (Figure 5-8, Table 5-2). Among them, RB (20), a poly-chlorinated analog of ErB (6) and a food colorant approved in Japan, showed similar, maybe even more pronounced promiscuous inhibitory activity. The non-specific inhibitory activity was somewhat diminished in the poly-brominated analogs (21 and 22) and essentially lacking in the non-iodinated FL (23) (Figure 5-8). The non-iodinated, but hydroxyl-substituted gallein (24) showed inhibitory activity in a few selected interactions. Gallein, and its close structural analog M119 (NSC119910), but not fluorescein have also been shown to inhibit the interaction of the G-protein subunit $G_{\beta\gamma}$ with effectors such as phosphoinositide 3-kinase $\gamma$ (PI3-kinase $\gamma$) (Lehmann et al. 2008). The exact mechanism of this promiscuous inhibitory activity is somewhat unclear at this point; the poly-iodinated ErB (6) and RB (20) bind with low micromolar affinity to all proteins I tested so far. Since inhibition of protein-protein interactions with

$^{14}$ BBB – Blood brain barrier
small molecules is a challenging task (Wilson 2009; Buchwald et al. 2010), the existence of such low-micromolar promiscuous inhibitors is intriguing. The Schild analyses (Figure 4-5) and its reversed version with plate-coated CD154 (data not shown) seem to indicate at least a partially reversible (surmountable) mechanism as inhibition by ErB (6) could be overcome with increasing CD40 or CD154 concentrations.

Xanthene dyes such as ErB (6) and RB (20) are well-known phosphorescent probes, which can cause the photosensitized oxidation of amino acids resulting in photo-damage to proteins (Mignaco et al. 1997; Davies 2003; Zhang et al. 2009). We repeated the CD40–CD154 PPI inhibition assay with dark and light conditions in parallel and found the activity of ErB (6) and RB (20) to diminish in dark about 5 to 10-fold whereas those of DR80 (2) and anti-CD154 mAb were virtually unchanged (Figure 5-7). Hence, photosensitivity accounts for some of the inhibiting activity seen under in vitro conditions, where light is a factor, but most of the activity is still retained in dark. Light showed about a similar enhancing effect on the inhibitory effect of ErB (6) on the binding of ouabain to the digitalis receptor (Hnatowich et al. 1982). For comparison, a set of compounds identified as possible PPI inhibitors for TNF-R–TNFα, ultimately turned out to modify the receptor covalently by a photochemical reaction due to light exposure, causing the activity to be 50 to >1000-fold diminished in dark (Carter et al. 2001). The presence of bromine or iodine atoms is known to enhance the yield of intersystem crossing to the reactive triplet state of these dyes. Transfer of energy from this state to molecular oxygen results in the
formation of singlet oxygen, which can oxidize accessible amino acids in proteins causing photo-damage. Consistent with this, the tetraiodo xanthene derivatives such as ErB (6) and RB (20) showed the greatest photosensitizing activity in our experiments as well as others’ (Figure 5-7), (Ben Amor et al. 2000). Because of their photo-oxidative ability, xanthene dyes, and in particular ErB (6), RB (20), and EO (21), have also been explored as possible light-activated insecticides (Ben Amor et al. 2000) or antimicrobials (Waite et al. 2009). Intralesionally applied RB (20) is being investigated as a possible chemoablation agent in metastatic melanoma (Thompson et al. 2008).

There also have been suggestions that some of these xanthene dyes, and in particular ErB (6), might be capable of specific interactions with the nucleotide binding sites of membrane energy-transducing enzymes (such as ATPases) because of the structural overlap between ErB and AMP (adenosine monophosphate) (Neslund et al. 1984) or ADP (Majima et al. 1998), and such binding sites are present on many different proteins (Saito et al. 2006). However, CPK structures clearly reveal that even if stick structures might allow some overlap, true structural analogy is lacking due to the large size of the iodine substituents (Figure 5-10), and molecular size is, of course, a major determinant of binding ability (Buchwald 2005; Buchwald 2008). In fact, better overlap might be possible with the flavine part of FAD (flavine adenine dinucleotide). Similarly, the observations of competitive binding to mono-deiodinase enzymes, and estrogen receptor have been explained on the basis of structural analogy of ErB (6) with thyroxine and estradiol respectively. ErB also showed reversible
competitive behavior in the Schild analysis with CD40–CD154 as described above (Figure 4-5). However, the structure of ErB is clearly distinguishable from the natural ligands of the many proteins that it inhibits or from the structural scaffold identified as specific inhibitors by our group.

A mechanism with reversible binding and multiple possible binding sites on the protein surface, at least some of which overlap with the binding site of the protein partner, as it has been suggested on the basis of spectroscopic experimental evidence in the case of insulin–insulin receptor inhibition by ErB (6) (Schlein et al. 2002) and in the case of ErB (6) binding to BSA (Zhang et al. 2009), seems more likely here as well. I confirmed this using Jobs analysis, which showed ErB (6) to bind with a stoichiometry of 5-6 to BSA (Figure 5-15) and 8-9 to CD154 (Figure 5-16). Compounds ErB (6), as well as RB (20), especially in their spiro isomeric forms, are not only relatively large, very flat structures, but also particularly rigid ones having no rotatable bonds that can relatively easily bind to flat, hydrophobic pockets (Figure 1-8). Entropy and more specifically loss of entropy due to binding plays a critical role in modulating binding efficiency, but predicting its contribution is non-trivial and controversial (Murray et al. 2002; Tirado-Rives et al. 2006; Reynolds et al. 2008). The barrier to binding due to the loss of rigid–body entropy varies across a considerable range (Reynolds et al. 2007), its value being somewhere around 15–20 kJ/mol, which corresponds to about three orders of magnitude in affinity, according to a more recent estimate (Murray et al. 2002). This might explain at least part, the promiscuous binding activity of ErB (6) and RB (20): a relatively large, flat
molecule with a rigid structure capable of van der Waals interactions (including aromatic stacking) with no entropy loss due to blocking of rotational freedoms. Presence of the large iodine substituents also seems important for the non-specific inhibitory activity; the smaller the size the less prominent the promiscuous inhibitory activity found in the assays performed here (Figure 5-9A). Hydrophobic interactions will also account for the binding of ErB (6) to its many targets as inhibitory activity among the tested xanthenes correlates well with the octanol-water partition coefficient (log P), a well-known index of hydrophobicity (Figure 5-9B).

Identification of binding sites on proteins for targeting by PPI modulators is an essential and a challenging task. NMR-based studies using representative fragments from larger chemical libraries have observed that such sites are consistently susceptible for attack by a diverse group of chemical structures and can be identified thus (Hajduk et al. 2000; Hajduk et al. 2005). The best-known algorithm for prediction of druggable hot spots on protein surfaces uses a probes set of 15 solvent molecules to tag pockets on which they cluster (or interact with) the most (Dennis et al. 2002; Landon et al. 2007; Brenke et al. 2009). Promiscuous compounds like the ones identified and characterized in the present study (ErB (6) and RB (20)), have been consistently shown to bind many proteins and either possibly bind to several sites on the proteins’ surface or form clusters on one or more such pockets. These interactions have also been shown in our case among others to coincide also with the protein-interaction interface despite lack of obvious structural similarity to any of their known natural or synthetic
ligands. Therefore, we hypothesize that the pockets that are first targets of promiscuous inhibitors like ErB (6) and RB (20) are likely to be druggable. Thus, these compounds may also have potential as tools/probes to assess protein druggability and to predict druggable hot-spots which are non-trivial, yet essential preliminary steps to PPI drug-discovery.

6.4 SUMMARY

Our group had discovered the first small-molecule organic dye inhibitors of the CD40–CD154 PPI, a well-established target for immunosuppressant drug development within the TNFSF. Among these inhibitors, some showed specificity for CD40–CD154 relative to other PPI in the TNFSF, whereas others were non-specific or promiscuous. Following this, I have identified further compounds having improved ligand efficiencies using iterative structure similarity search algorithm and confirmed their activities using in-vitro cell based biochemical assays. Although, the cellular effects on CD40–CD154 were consistent with the inhibitory effects in the binding experiments, they were seen to require higher dye concentrations than the cell-free assays indicated due to possible non-specific effects. In the hope of eliminating the determinants of non-specificity, I performed shape similarity searches, using the best compounds as query and have obtained several new compounds whose CD40–CD154 inhibitory potential can be tested.
Identification of the mechanism of inhibition is important to provide knowledge to help advance the initial hits to ones having desired nanomolar inhibitory activities as well as adequate specificities and ligand efficiencies. Following expression, preparation, and purification of adequate amounts of CD154, I exploited the property of metachromasy to spectrophotometrically identify the preferential binding of our prototypical compound DR80 (2) to CD154. Using Schild analysis, I was also able to show this to be a partially reversible and surmountable inhibition upon simultaneous addition of CD154 and DR80 (2) to CD40.

I further performed computational docking experiments using Glide (Schrödinger, LLC) to score the binding of CS7B (4, a DR80 sub-structure) and MB1 (5, our most active compound) at the two distinguishable druggable sites picked using the FTMAP druggable-pocket finding algorithm. The Glide docking scores in the XP mode distinguished binders from non-binders at the allosteric site at the interior core of the trimer (an established druggable site in the TNFSF) better than at the competitive site at the PPI interface. Combining the results of the Schild analysis and the predictions from the docking experiments, a mechanism that involves initial weak binding at the interface followed by the intercalation between the CD154 subunits and subsequent access to the druggable hydrophobic core seems a likely hypothesis to be tested experimentally.
Since non-specific inhibitors can appear among useful hits from biochemical screening assays creating confusing results, their early identification and elimination can increase the efficiency of the drug discovery process. Therefore, I used as tool the promiscuous compound ErB (6) that was identified from our screens to study the mechanisms that can give rise to non-specificity. I showed that ErB (6), as well as its closely related structural analog RB (20), can inhibit multiple PPIs within the TNFSF as well as outside of it in concentration ranges that agreed well with that observed by several groups for various protein interactions. In a cellular context, this promiscuity readily translated to toxic effects in various cell-lines tested in concentration ranges (~30 mg/L) not very far from that causing inhibition (2 - 20 mg/L). However, such cytotoxicity is observed only at 100-fold greater concentrations than the ADI (0.1 mg/kg body weight/day) for ErB (6). Among the xanthene analogs tested, such activity correlated well with molecular size and hydrophobicity indicating the role of hydrophobic and van der Waals forces in its interactions with its many targets. Photo-enhanced oxidation of accessible amino acids also accounts for part of the inhibition, despite lacking physiological relevance. Stoichiometry analyses (Job’s plot) showed that proteins could bind multiple ErB molecules possibly at one or at multiple sites, and in many instances; this can coincide with the ligand binding sites despite lack of obvious similarity of ErB (6) to the known ligand structures. Thus, the potential of ErB (6) and its close analog RB (20) to cluster on druggable pockets on protein targets and thereby serve as useful probes to assess protein druggability and to tag druggable ‘hot-spots’ remains to be investigated.
REFERENCES


