EXPLOITING STRUCTURAL DISORDER TO ENHANCE SMALL MOLECULE INHIBITION OF THE ONCOPROTEIN c-MYC DIMERIZATION WITH ITS PARTNER MAX

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By
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ABSTRACT

The transcription factor c-Myc, in its normal function, is involved in cell cycle regulation. The uncontrolled cell proliferation consequent to c-Myc deregulation is typical of cancer and neoplastic diseases. Most known functions of c-Myc depend upon the dimerization between its basic-helix-loop-helix-leucine zipper (bHLHZip) domain and a similar domain within the partner protein Max. Small molecules capable of specifically and selectively disrupting the c-Myc-Max interaction have been reported. Because of c-Myc’s involvement in cancer, the inhibition of its dimer formation with Max is a promising way to down-regulate its activity for therapeutic purposes. Small molecules interfering with c-Myc-Max dimer formation must directly interact with one or both protein monomers. These monomers are intrinsically disordered and lack a stable structure, as they undergo coupled folding and binding and they assume a defined structure only upon dimerization. The possibility of directly targeting disordered proteins with small molecules has not yet been broadly considered. In the studies described here, recombinant c-Myc, Max and derived peptides were employed in purified component assays based on several biophysical techniques, including fluorescence polarization, circular dichroism, and gel electrophoresis, to elucidate the mechanism of action of small molecule inhibitors of c-Myc-Max dimer formation.
originally described by the Prochownik lab at the Children’s Hospital, Pittsburgh. The synthesis of several modified small molecules further allowed for structure-activity studies. It was found that these compounds bind in a plastic mode to short segments of the c-Myc bHLHZip domain. NMR spectroscopy was employed to collect structural information about the studied complexes, confirming their dynamic nature. It is hypothesized that the combined presence of hydrophobic residues and low sequence conservation make such sites prone to specific small molecule binding. The different location of binding sites on c-Myc was found to result in different mechanisms of disruption of c-Myc-Max dimers. The presence of HLZip dimerization equilibria competing with c-Myc-Max dimerization was found to facilitate the inhibition of the latter. The multiple binding sites on c-Myc were further exploited in the design of bivalent inhibitors which interact with two such sites and display enhanced affinity for c-Myc.
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Chapter 1

Background

1.1. *Intrinsically disordered proteins*

Proteins constitute one of the main structural and functional components of living organisms [1]. Their polymeric nature allows for a wide range of compositions and functions to be attained from the linear linkage of 20 unique L α-amino acid building blocks [2]. A relevant aspect of such versatility is their ability to fold into complex three-dimensional structures predominantly composed of specific arrangements of repeated secondary structure elements [3-5]. These are natural outcomes of the few sterically favored orientations of the polymers’ peptide backbones, where clashes between side chains are avoided [6]. As little as three secondary structure elements represent the vast majority of protein structured components in which the backbone arrangement is oriented over a single axis: α-helix, parallel and anti-parallel β-strand [7-9] (Figure 1). A few more structural motifs, also widely conserved, allow for changes in direction of the backbone, thus giving place to unique three-dimensional folds [10].

The folding of linear polymers into diverse and complex structures as a sole consequence of the distribution of different side chains over their homogeneous backbone is a fascinating and still not fully understood molecular event [11]. Its self-evident complication, underscored by the very tight enthalpy-entropy balance underlying it [12], makes unsurprising the fact that some proteins do not,
Fig. 1 Representation of L-amino acid backbone dihedral angles, Ramachandran plot highlighting populated dihedral regions and models of corresponding secondary structure arrangements [6]. The displayed Ramachandran plot represents average allowed regions, which may differ based on amino acid identity, especially for glycine and proline.
in their functional state, attain a stable three-dimensional fold. It has been repeatedly demonstrated, in recent years, that proteins that are folded only locally or partially (collapsed state), or even fully disordered and flexible often play crucial roles in certain cell functions [13-18]. Rather than focusing only on the subtle combinations of local and long range interactions and enthalpy–entropy tradeoffs that give place to folded states [19], a new paradigm for protein function ought to be introduced, which accounts for different, and often orthogonal functional roles to different levels of proteins’ structural organization or lack thereof, as evolution appears to exploit, with different purposes, the full available range of physico-chemical properties of these polymers (Figure 2).

The strict correlation between a protein’s (folded) structure and function has nonetheless been a paradigm for decades within the fields of biochemistry and molecular biology [20], since the century old, visionary at the time, ‘schloss und schlussel’ (lock and key) definition of the interaction between an enzyme and its substrate provided by Emil Fischer [21]. This perspective, even when applied to the function of proteins that are indeed natively folded, tends to bias the intuitive understanding of such function towards that of a rigid body that performs its tasks by means of shape complementary interactions with other rigid bodies (substrates, ligands, or protein partners). The relevance of conformational rearrangements and flexibility [22, 23], allosteric events [24, 25] and induced fit interactions [26, 27] within folded proteins has surely been long understood. It has not been considered, however, possibly because of the lack of interest in disordered proteins (or their being
Fig. 2 ‘Old’ and ‘New’ structure function paradigms. **a.** ‘Old’ paradigm: a polypeptide sequence may exploit a cellular function only upon folding into a stable three-dimensional structure. **b.** ‘New’ paradigm: depending on its specific function, a polypeptide may remain flexible and unstructured, fold transiently or locally, or fold into a stable three-dimensional structure.
disordered) until recent years, as a bridging aspect between the old ‘structure’ and the newer ‘disorder’ paradigms for protein function. A minor shift of perspective in assessing the functional characteristics of a protein can lead to their evaluation based on the extent of backbone flexibility, where ‘disorder’ can be redefined as extensive residual flexibility, a feature that can still be observed, to lower and changing extents, depending on specific functional requirements, within folded proteins as well. Such flexibility standpoint reconstitutes a single protein function paradigm where different extents of backbone plasticity are associated with different functional goals.

While the definitions of folded or disordered proteins can be convenient for practical purposes and cutoff parameters can be established to discriminate between the two [28]. All proteins are peptide chains with similar physical-chemical properties: these have been modulated by evolution over a continuous range of functional and structural outcomes. Even the backbones of disordered proteins that lack rigid tertiary folds have to comply, in their arrangement, to steric requirements to avoid side chain clashes. They may therefore display, in a transient fashion, the same secondary structure features found within their folded counterparts [6], or other allowed backbone arrangements (such as polyproline type II helixes [29-33]), rather than completely random and truly ‘disordered’ ones. The focus of this thesis will be on a biological system involving proteins that are currently generally defined as intrinsically disordered or natively unstructured, lacking stable secondary and tertiary structure over sequence segments of at least forty consecutive amino acids.
1.1.1. **Mechanisms of interaction and function of disordered proteins.**

Extensive backbone flexibility can be advantageously exploited in biological contexts in a number of ways. These have been widely analyzed in recent years by research groups investigating the structure and function of intrinsically disordered proteins with several approaches. It is worthwhile mentioning the most relevant of such experimental tools that have proved themselves useful in the study of disordered proteins [34]:

- *Proteolytic mapping.*
- *Circular dichroism.*
- *NMR spectroscopy.*
- *Hydrodynamic measurements.*

**a) Proteolytic mapping**

For peptides to be hosted within the active site of a proteolytic enzyme, they have to be in an extended conformation [35-37]. The analysis of the fragment products of proteolytic digestion for a protein substrate provides information about which specific recognition sites for a given protease are available for cleavage, therefore most likely in a flexible conformation and exposed to the solvent, over the substrate protein sequence [38-40] (Figure 3).
b) Circular dichroism

This technique is based on the differential adsorption of circularly polarized light by chiral chromophores [41, 42]; the wavelength spectrum of this phenomenon over the region of peptide electronic absorption (~160-260 nm) provides information about the relative content of secondary structure elements within a protein [43, 44]. Random coil conformations, typical of disordered proteins, display characteristic spectra [41, 42] (Figure 4).

c) NMR spectroscopy

This technique can provide detailed information about protein structure and dynamics [45-50]. When applied to intrinsically disordered proteins, the most indicative data are backbone chemical shift trends, that distribute over the field according to their secondary structure conformation [51-54] (Figure 5a); relaxation

Fig. 3 Partial proteolysis as a tool to study protein disorder: only cleavage sites found within unfolded and flexible protein segments may access the enzymatic active site and be digested in the absence of prior denaturation. Peptide fragments may be analyzed by SDS or 2D gel electrophoresis, or by mass spectroscopy.
time measurements [55, 56] (that can also be obtained in an indirect fashion upon analysis of peaks shape [57, 58] or sign [59], and can elucidate local and segmental dynamic properties within a peptide chain, Figure 5b), and diffusion measurements (that can be performed through the use of pulsed field techniques and are informative of the overall shape and volume of a peptide) [60, 61]. NOE distance constraints, the main source of information employed for the production of solution structures of folded proteins, are, in the case of disordered peptides, few and little informative, due to their non-linear convolution over a conformational ensemble [54], and involve practical difficulties in their assignment due to the high degeneracy of side chain resonances of disordered proteins [50].
Fig. 5 Schematic representation of NMR approaches that are most useful in studying disordered proteins. **a.** Sequence mapping of backbone chemical shifts represented as difference of the observed shift from a reference random coil value. The presence of secondary structure induces shifts from such values in a consistent direction for each studied nucleus, resulting in a widespread distribution of backbone resonances. Smaller shifts, more erratic in sign are observed for disordered protein segments. Particularly useful to this analysis are $^1\text{H}$-α and $^{13}\text{C}$-α signals, as their shift tends to be more exclusively affected by their secondary structure rather than perturbation effects induced, for example, by ligand binding, as in the case of $^1\text{HN}$ or $^{15}\text{N}$ amide chemical shifts [54]. **b.** Spin-spin (or horizontal) relaxation measurements: the faster motions of disordered protein segments reduce the magnetization exchange between nuclei (mainly protons) resulting in its slower loss of coherence and longer relaxation times compared to folded domains [34].
d) *Hydrodynamic measurements*

Disordered proteins display characteristic behaviors in a series of commonly employed biochemical techniques, and their disorder can be inferred from their behavior in such experimental settings. In gel filtration chromatography, disordered proteins occupy a considerably larger volume than folded ones of similar molecular weight (larger Stokes radius), thus displaying a larger apparent molecular weight than folded proteins when studied with this technique [60, 62]. Similarly, the increased hydrodynamic volume of disordered proteins can be detected by other techniques, including small angle X-ray scattering [63, 64] and dynamic light scattering [60].

The features of intrinsic disorder are implicated in the following mechanistic and functional outcomes, which will be discussed in some depth.

a) Coupled binding and folding transitions

b) Simultaneous interaction with multiple binding partners

c) Alternative interaction with multiple, different binding partners (‘Moonlighting’)  
d) Post-translational modification

e) Splicing events
a) Coupled folding and binding

This molecular event involves the formation of a rigid tertiary structure only upon a protein’s interaction with a partner protein or nucleic acid [65-69]. The former can either be folded or also intrinsically disordered, thus undergoing a coupled folding and binding transition itself. In the case of interaction with a folded partner, a network of specific hydrogen bonding, hydrophobic and electrostatic interactions between the two molecules provides a suitable scaffold to stabilize in a rigid conformation, often characterized by distinct secondary structure elements, the segment of the disordered protein involved in binding [70, 71]. In the case of a coupled folding and binding transition involving both protein partners, the interaction resembles the folding transition for a natively structured peptide chain, with the significant difference that the necessary sequence segments forming a hydrophobic core and driving the folding event are distributed over two (or more) independent peptide chains, which can therefore assume a stable tertiary structure only upon encounter with each other [72] (Figure 6).

Coupled binding and folding can be exploited in regulation strategies, where tight balance and fine tuning of interdependent molecular events is required, as it produces interactions characterized by high specificity but relatively low affinity [17]. This outcome can be ascribed to a considerable entropy loss, a consequence of the induction of rigid structure on proteins involved in a coupled binding and folding event. Such entropy loss has to be accounted for within the free energy balance of the binding event, it therefore limits the overall free energy released upon the
Fig.6 Coupled folding and binding function of intrinsically disordered proteins: the entropy loss associated with the folding transition results in highly specific interactions with relatively low affinity. At the bottom is shown the bZip domain homodimer of the transcription factor c-Jun complexed with CRE double stranded DNA (PDB: 1jnm [72]). The protein monomers are flexible and disordered and form the observed coiled coil α-helical structure only upon interaction with each other (in the leucine zipper segment) and DNA (in the basic region).
establishment of the network of inter-molecular interactions responsible for both
specificity and affinity of binding. As a consequence, such interactions, while
providing the complementarity between two (or more) partners needed for specific
binding, do not contribute to its affinity as much as they would were they to happen
between two (or more) previously folded partners [73].

b) Simultaneous interaction with multiple partners

Intrinsic disorder over an entire protein sequence or portions of it can be
exploited to flexibly tether multiple interaction sites, which themselves may be
intrinsically disordered, and thus involved in coupled folding and binding as mentioned
above, or natively folded [74-78]. The flexible linkage provided by intrinsically
disordered segments may allow for the ideal scaffolding of large protein complexes,
such as those involved in the transcription machinery [79], by means of
accommodating incoming components of a complex and successively letting them
rearrange to reach the correct relative orientations and interactions. Similarly, flexible
components of multi-enzyme complexes, such as the fatty acid synthase, make it
possible to shuttle the substrate from one active site to the other [80]. The flexibility of
disordered proteins may be further exploited in the stabilization of multiprotein
complexes, as these would be more suitable than their native counterparts for adapting
their features to irregular exposed surfaces of a pre-formed complex, or interact
sequentially with any of its components and help recruit the others through a ‘fly
casting’ mechanism [81-84] (Figure 7).
Simultaneous binding to multiple partners. As an example of this function the intrinsically disordered p27Kip1 cyclin-dependent-kinase inhibitor is shown bound to the cyclin A-Cdk2 complex, involved in cell cycle regulation (PDB: 1jsu [84]).
c) Alternative interaction with different binding partners

Another unique feature of proteins characterized by intrinsic disorder is their capacity of employing the same recognition site to interact with different protein partners [74, 85]. These are generally coupled binding and folding transitions with natively folded partner proteins, where the structural outcome for the intrinsically disordered component varies upon interaction with different partners. This feature may also be exploited in regulatory mechanisms, where the possibility of alternating or switching the outcome of binding events can be particularly useful in the functioning of feedback loops or response to several types of signals and stimuli [86, 87] (Figure 8).

d) Post-translational modification

Other molecular events that are made possible by features of intrinsic disorder involve several types of enzymatic post-translational modification [88, 89]. As previously described in the case of proteolytic cleavage, post-translational modifications such as phosphorylation [90, 91] require that the substrate protein backbone be extended and solvent accessible for it to fit within an enzymatic active site [92] (Figure 9). Once again these events, in particular phosphorylation, are commonly involved in regulation mechanisms, especially with the role of traducting extra-cellular signals inside the cellular milieu.
Fig. 8 Alternative binding to different partners: the same segment of the p53 tumor suppressor protein (SRHKKLMF) can bind to the S100B calcium binding protein (left - red, PDB: 1dt7 [86]) in a helical conformation and to the NAD-dependent deacetylase Sirtuin (right - green, PDB: 2h4f [87]) in an extended strand conformation, as well as to other protein partners, assuming yet different structural arrangements.
**Fig.9** Intrinsically disordered proteins as enzymatic substrates: pCdk2-Cyclin A complex (yellow) bound to a HHASPRK peptide substrate (PDB: 1gy3 [92]). The peptide substrate must be solvent-exposed and in an extended conformation in order to access the enzyme’s active site.
e) **Splicing events**

These final examples of roles and functions for intrinsic disorder in proteins’ sequence involve the mechanism of alternative splicing of a gene’s pre-mRNA to encoding mRNA as a consequence of the differential removal of introns from the nucleotide sequence, giving place to different isoforms of one protein [93, 94], and a protein analog of this process known as protein splicing [95, 96]. In the first case it may be observed that the protein segments that may or may not be present in different isoforms depending on the mRNA splicing event are generally intrinsically disordered [97]. It is intuitive that alternative splicing events that were to affect the sequence of folded protein domains would disrupt their native contacts in an irreversible way, thus preventing the folding from taking place and disrupting the protein’s function. If, on the other hand, such events were to involve the expression of flexible protein segments that may link multiple folded domains to each other, or interact with sites on such folded domains in a self-inhibitory or self-activating manner, the presence or absence of the alternative splicing regions might result in effectively modulating the protein’s function. In the case of protein splicing, the peptide segments subject to removal have been defined inteins, and the flanking ones exeins, by analogy with the terminology employed for nucleic acids processing [95].

The involvement of intrinsic disorder in protein splicing summarizes some of the functions of such protein characteristic: most splicing mechanisms involve the autocalalytic cleavage and reconnection of a protein sequence sequence by enzymatic domains within the inteins, which therefore operate on disordered and solvent exposed
target segments of the sequence, as reported above, and are also often connected to each other by disordered and flexible segments within the intein itself, which make it possible for the interaction between the active site and substrate site to occur inside the same protein sequence without excessive orientation and spatial constraints derived from the very presence of both such sites on the same sequence. Once again, protein splicing can be useful in regulatory mechanisms in that it may result in conversion of precursor proteins to active proteins with faster response time to appropriate stimuli than in the case of newly expressed protein [96, 98].

1.1.2. Relevance of disordered proteins in eukaryotic cell signaling

The nature of the various advantages intrinsic disorder grants towards the occurrence of some types of molecular events or interactions, makes these advantages particularly relevant to the establishment of regulatory networks [74, 99]. It is therefore not surprising that intrinsic disorder is more frequently found in higher organisms, and eukaryotes in general, than in bacteria; bioinformatics studies where the prediction of protein disorder based on primary sequence properties has been applied to genomic databases, indicate that an average of less than 10% of bacterial proteins contain disorder regions of 50 residues or longer, while this fraction rises to 25-30% of the proteins of several eukaryotes [13]. The interdependence between different specialized cells, and the importance of interpreting and processing external inputs makes tight and strict cellular regulation indispensable in higher organisms [76]. The evolutionary processes that have led to the development of such sophisticated signaling and
regulation networks have likewise led to the evolution and specialization of disordered proteins, in spite of the slight counter-intuitiveness of the idea that flexible, seemingly unorganized proteins have evolved after and not before ones which display complex folding patterns. Recent studies suggest that disordered proteins may in fact have evolved before ordered ones as nucleic acid interacting peptides (such as the ribosome protein component), followed by the appearance of folded enzymes needed for complex metabolic pathways, and eventually a second emergence of protein disorder in eukaryotic signaling and regulation (Dunker, A.K., unpublished results). Bioinformatics analyses have estimated that roughly 40% of all proteins involved in signal transduction and cellular transformation are either fully disordered or contain substantial disordered segments of at least 50 consecutive amino acids [15, 100]. One common aspect of many of the intrinsic disorder properties discussed above is their aptitude in hub interactions, either in situations where the disordered protein itself is a hub protein that can interact with multiple partners simultaneously or alternatively, or ones where disordered proteins interact with folded hub proteins through coupled binding and folding transitions, thus enhancing the binding versatility of the latter thanks to the relatively low affinity of their specific interactions (Figure 10). Furthermore, the aptness of disordered proteins for post-translational modification events often makes them play relevant roles within cell signaling networks such as phosphorylation cascades [91].

The relevance of intrinsic disorder to eukaryotic cell signaling and regulation leads to a correspondingly unusually high number of disordered proteins being
involved in disease states where components of such regulation networks are compromised (Figure 11), such as cancer. The percentage of proteins with disordered segments of 50 amino acids or longer rises to roughly 60% amongst cancer associated proteins \[15, 100\]. It is also connected to diseases that depend upon protein misfolding or aggregation, especially neurological disorders such as Alzheimer’s and Parkinson’s diseases \[100, 101\]. In such cases the presence of a high number of hydrophobic residues, typically found within the core of folded proteins, in solvent exposed, disordered regions, promotes their reciprocal interaction, through the formation of inter-molecular \(\beta\) sheets, where individual strands are provided by individual proteins, that leads to the formation of high molecular weight aggregates and fibrils with noxious consequences to neural signaling.

**Fig.10** Simplified example of a scale free network, where a small number of ‘hubs’ are linked to a large number of elements with low connectivity. Networks of this type are found in cell signaling and regulation.
1.1.3. Targeting intrinsically disordered proteins with chemical tools

The roles that intrinsic disorder plays in relevant cellular functions, which have been outlined above, make evident the appeal of interfering with their activity by means of chemical interference tools. Because these cell signaling and regulation roles of intrinsic disorder have been recognized only in recent years, and, in part, because of the still common acknowledgment of the ‘structure-function’ paradigm, intrinsically disordered proteins are not generally considered as suitable targets for modulation with small molecules. Small molecule pharmaceutical agents are generally expected to

![Fraction of proteins involved in signaling functions and specific classes of diseases which contain consecutive segments of disorder of the specified lengths (reproduced with permission from Uversky, et al., [100]).]
interact with rigid and defined clefts or ‘pockets’ over the surface of their protein target: the existence of such sites is unlikely within intrinsically disordered protein segments.

Small molecular weight organic compounds, having sizes of 500 or less Daltons, are universally recognized as ideal pharmaceutical tools due to their generally relatively low costs, ease of delivery, good cell permeability, relative metabolic stability (that can be often optimized by removing undesired features which make a small molecule a suitable substrate for enzymatic processing and degradation) [102, 103]. A majority of marketed pharmaceuticals contains small molecule active agents; correspondingly, a vast majority of drugs, estimated as roughly 80% of the total marketed pharmaceuticals, is targeted at relatively rigid binding pockets within folded proteins, such as receptors and, to a lesser extent, enzyme active sites [104-106] (Figure 12). The optimization of a drug’s potency can be generally achieved, in these cases, by optimization of the shape complementarity between binding site and ligand [107]. This can be attained either by structure activity relationship analysis of series of derivatives of a lead molecule bearing modifications at crucial positions [108], or, in recent years, by means of in-silico based molecular docking methods that rely on the availability of a crystallographic structure for a protein target [109, 110]. Naturally occurring agonists or antagonists for a receptor or enzyme substrates often provide useful templates for the design of pharmaceutical agents targeted at their protein partner. Historically, natural products or natural product derivatives have indeed represented a substantial fraction of the marketed drugs. Even over the last 15 years,
Fig. 12 ‘Classical’ interaction between a rigid and well defined binding pocket cavity and a small molecule drug ligand: aspirin bound to phospholipase A2, represented as ribbon (top) and surface (bottom, PDB: 1tgm [106]).
during which the pharmaceutical industry arena has seen a widespread diffusion of combinatorial libraries and high-throughput screening approaches to identify new leads, up to 60% of newly marketed drugs are still natural products, natural product derivatives or inspired by natural products [111, 112].

The observed abundance of natural products can be correlated, as suggested above, with the nature of the most frequent protein targets for small molecule drugs, the binding pockets of which have evolved together with natural ligands that fit inside them with optimized interactions, excellent shape complementarity, and, in several cases, in a mainly rigid fashion (it is indeed commonly preferred to limit the number of rotatable bonds within a drug molecule in order to decrease the entropic cost of its shape-complementary interaction with a binding pocket on a target protein [107]).

Several cell signaling and regulation pathways, which can lead to disease states when unbalanced, involve protein-protein interactions, often disordered proteins. The chemical modulation of protein-protein interactions, albeit appealing, has hardly been considered a realistic goal for several years, even in the case of interactions between two folded protein partners, which approaches more closely the established, widespread, structure function paradigm, because of the intrinsic nature of such interactions, involving relatively extended and flat contact surfaces instead of binding pockets with defined shapes within cavities on a protein’s surface. In recent years, however, James Wells demonstrated that in several cases a large portion of the free energy contribution to the interaction between folded proteins with extended contact surface areas is provided by only a small number of amino acids, often involving some
extent of plasticity and structural rearrangement upon binding, which he defined ‘hot spots’ [113]. This finding has unlocked the opportunity of designing and employing small molecules to interfere with protein-protein interactions, and several examples have been reported over the past few years with promising implications for new pharmaceutical approaches to the treatment of a number of diseases [114-117] (Figure 13). Furthermore, examples have been reported of small molecules interfering with the interaction between folded proteins and disordered protein partners, such as that between apoptosis inhibitors of the Bcl2 family and the intrinsically disordered Bak BH3 peptide [118-120] (Figure 14), or that between the intrinsically disordered cancer suppressor p53 and its silencer HDM2/MDM2 [121, 122]. Generally, low emphasis has been placed on the fact that the folding transition for the disordered protein partners involves a substantial loss of entropy, making therefore these interactions relatively easy to inhibit. Furthermore, while lacking the deep cavity features and solvent isolation of binding pockets for receptors’ ligands or enzymes’ substrates, interactions between one folded and one intrinsically disordered protein partner generally occur within pronounced clefts over the surface of the folded protein, thus, at least in part, reintroducing those shape complementarity considerations that are crucial in the design of enzyme inhibitors and receptor agonists or antagonists and that cannot be straightforwardly accounted for (i.e. by means of in-silico based molecular docking screenings) when targeting plastic ‘hot spots’ over the ‘flat’ interaction surface between two folded proteins [123].
Fig. 13 Small molecule interaction with a protein-protein interface ‘hot spot’ [113]: the interaction between Interleukin-2 and its receptor α-chain (displayed on the left, IL-2 as surface, interacting segment of the receptor as ribbon, showing interacting side chains, PDB: 2b5i [116]) is inhibited by a small molecule interacting with part of the protein recognition interface on IL-2 (shown on the right PDB: 1py2 [117]). Notice the absence of deep cavities on IL-2 surface and the conformational rearrangements within this protein from its receptor bound state to the complex with the inhibitor.
Fig.14 Inhibition of protein-protein interactions characterized by coupled folding and binding events through small molecules binding to the folded protein partner: the interaction between the apoptosis regulator of the Bcl2 family Bcl_{XL} and disordered protein segments that assume an α-helical conformation upon binding (shown in the left panels as ribbon and surface is Bcl_{XL} – white – bound to the Bim BH3 peptide – black –PDB: 3fdl [118]) may be inhibited by small molecules binding to Bcl_{XL}, like the ‘fragment’ ligands from an ‘SAR by NMR’ screening shown on the right (PDB: 1ysg [119]). Notice that, unlike the small molecule inhibitor shown in figure 13, these molecules bind inside a fairly pronounced cavity on the Bcl_{XL} surface, naturally occupied by interacting peptides in a α-helical conformation, which display a convex interaction surface.
In spite of the evident and increasing progress in targeting protein-protein interactions with small molecules, intrinsically disordered proteins are still not generally considered druggable targets, or, when protein disorder plays a role in the mechanism of action of a small molecule, its relevance and implications are hardly acknowledged. Nonetheless, several examples have been reported of small molecules interacting with isolated short stretches of amino acids, which are too small to produce a stable tertiary fold, and in several cases have been found to correspond to disordered segments within full length proteins in their biologically active state [124-127]. Phage display technology [128] and surface plasmon resonance [127] have been especially useful techniques in detecting such interactions. Furthermore, some of these binding events involve biologically active agents or even marketed drugs. An early example of interaction between a small molecule and a disordered peptide was the finding of a disordered region of α-tubulin binding to Taxol [124], followed by the discovery of the interaction of this small molecule with a disordered loop of Bcl2 [128]. Later studies suggested the specific interaction of the topoisomerase I inhibitor Camphothecin [126] and that of the chemotherapeutic agent NK109 with short peptide sequences characterized by conserved sites (Figure 15), which lead the authors to introduce the definition of ‘drug target motif’ [127]. More recently, it has been shown that a substrate targeted enzymatic inhibition approach [129], which grants the advantage of highly specific and controlled effects compared to active site inhibition, and had been originally hypothesized to be attainable through the use of complementary peptides interacting with enzymatic substrate protein segments, can be achieved with small
**Fig.15** Computational model of the experimentally proven interaction between the anti-cancer drug candidate NK109 and a short peptide segment (LGDPNSSRIP) the residues crucial for binding are PNxxxxP, defined by the authors as a ‘drug target motif’ (Adapted without permission from Morohashi, et al. [127]).
molecules interacting with short stretches of amino acids, known to lack a stable tertiary fold. Also in this example the studied system, involving the human amyloid precursor protein (APP) is of great therapeutic interest [130]. In conclusion, while the amount of information regarding direct interactions between small molecules and disordered proteins is small, it clearly suggests the occurrence of such binding events, with some structural insight in a few cases, and underscores the promise of such chemical approach for the treatment of different types of diseases.

1.2. c-Myc

The protein product of the MYC oncogene, c-Myc is a transcription factor of the basic helix-loop-helix leucine zipper family (bHLHZip) whose deregulated function is implicated in a number of human malignancies [131-133]. Such deregulation can be the outcome of a number of genetic aberration phenomena, including insertional mutagenesis [134, 135], chromosomal translocation [136, 137] and gene amplification [138, 139]. It may also depend on deregulated protein expression, functional regulation, and degradation profiles [140-143]. The c-Myc protein, which normally has a very short half-life and is subject to tight regulation, is a potent proliferating agent, promoting or silencing the expression of an extremely large number of genes [144, 145], leading to a series of cellular processes that result in G0/G1 phase to S phase cell cycle progression [146] and cell proliferation [147-149]. It also participates in a negative feedback regulation system in which it can act as a pro-apoptotic factor [150]. It is unsurprising that deregulation of c-Myc leads to
uncontrolled cell proliferation typical of cancer and neoplastic diseases [151, 152], where apoptotic signals, including those generated by c-Myc, are also often bypassed and silenced through a number of mechanisms [153].

1.2.1. c-Myc structure and function

The c-Myc protein product is a 439 amino acid long single peptide chain, intrinsically disordered throughout its sequence in the absence of protein partners. It is characterized by a transcriptional activation domain at its N terminus [154, 155], comprised of residues 1-143 and a bHLHZip DNA binding – heterodimerization domain at the C terminus comprised of residues 350-439 [156, 157]. Most biological functions of c-Myc, including the binding of target E-box DNA sequences (having the recognition epitope CACGTG) require the heterodimerization of its HLHZip domain with a similar domain found within the partner protein Max [158-160] (Figure 16). Such interaction promotes the specific DNA binding operated by the basic regions found in each protein, stabilized by electrostatic interactions with the negatively charged backbone of the double stranded nucleotide sequence. It further leads to a correct structural scaffolding for the further interactions with other protein partners.

**Fig.16** Schematic representation of the c-Myc sequence with location of functional domains aligned to that of its partner protein Max.
occurring over other regions of the c-Myc sequence.

1.2.1.1. bHLHZip and DNA binding

The 85 amino acids long bHLHZip domain of c-Myc undergoes a coupled folding and binding transition which generates appropriate, structured contact surfaces with the homologous domain found in Max and consequently with target E-box DNA sequences [157] (Figure 17). The HLHZip domains of the two proteins may form heterodimers even in the absence of DNA binding by their N terminal basic regions [161]. These complexes display the features of a four-helix bundle, which extends at its C terminal end in a left handed coiled-coil formed by the leucine zipper regions of each protein. This arrangement of α-helixes is oriented along a two-fold rotation pseudo-symmetry axis. The α-helixes formed by the two proteins interact with each other over a series of predominantly hydrophobic contacts. The N terminal helix, or helix1, which extends without interruption to the basic region when the heterodimer is bound to DNA, is linked to helix2 by a loop segment of roughly 10 amino acids lacking defined secondary structure. This linking creates a core segment of the dimerization interface in which the helix1 of each protein contacts a portion of the helix2 of the same peptide, as well as both helix1 and helix2 of the partner protein. This portion of the HLH dimerization interface resembles, unlike the extended and linear leucine zipper dimerization interface, a small and relatively simple hydrophobic core similar to that found in natively folded peptides with complex three-dimensional structures. Upon binding of target E-box DNA, the N terminal basic regions also undergo a structural
Fig.17 Crystal structure of the c-Myc-Max heterodimer bound to target E-box DNA, containing the palindromic recognition sequence CACGTG (PDB: 1nkp [157]).
induction to α-helical conformation to clamp the major groove of the nucleotide duplex, the negatively charged backbone of which forms a series of specific electrostatic contacts with the positively charged side chains of basic residues found on both proteins. The DNA binding by c-Myc-Max heterodimers has been shown to be cooperative and may not only be attained by pre-formed dimers, but also in a sequential mode where the basic region of one protein monomer interacts with E-box DNA to template the successive binding of the second protein monomer [68]. The c-Myc-Max heterodimeric complex, besides providing a structural scaffold for DNA binding, is crucial for the c-Myc activity as a repressor of transcription, as it binds to and silences DNA bound transcriptional activators of genes targeted for down-regulation within the cell transformation framework of c-Myc function [162].

1.2.1.2. transactivation domain and other functional domains

The segment of c-Myc not implicated in Max dimerization or DNA binding, constituting the outstanding majority of c-Myc sequence, is also characterized by extensive structural disorder. The one most relevant continuous segment of it with known functional purpose is the N terminal transactivation domain, spanning residues 1-143. This domain is responsible for recruiting several protein partners of c-Myc and finally provides a template for the formation of large supramolecular complexes, which organize and facilitate the assembly of the transcriptional machinery [163-167]. Among the functions of such complexes is the modification and remodeling of chromatin, with the purpose of making target genes available for transcription [168,
This is attained to a large extent through the acetylation and deacetylation of basic residues on histones, an event that modulates the affinity of these positively charged proteins for the negatively charged DNA backbone. The binding interaction between the c-Myc transactivation domain and TRRAP (transactivation/transformation domain associated protein) mediates the indirect association of histone acetylation complexes with c-Myc [164]. The loss of positive charge from basic histone side chains as a consequence of acetylation results in the loosening of the histone-DNA interaction, making the latter available for the transcriptional machinery. Myc can also facilitate chromatin remodeling upon interaction with its protein partner INI1 [163]. It further directly promotes transcription by recruiting RNA Polymerase II and PTEFb (positive transcription elongation factor) complex [165, 170]. Other functional segments of c-Myc outside its bHLHZip domain are five c-Myc homology box domains, characterized by sequence similarity between each other [154, 166]. The first two of these domains are found within the transactivation domain (residues 44-63 and 128-143 respectively), the latter encompass residues 188-199 (IIIa), 259-270 (IIIb), and 304-324 (IV). These domains are responsible for the regulation of c-Myc activity, in particular through the phosphorylation of the Thr48 and Ser62 residues found within the c-Myc homology box I [142, 143]. The last short domain with a known specific function is known as primary nuclear localization signal (NLS) and it spans residues 320-328.
1.2.1.3. c-Myc and bHLHZip network of interactions

The dimer formation between c-Myc and Max is a molecular event that has to be considered within the context of a network of interactions it is part of, rather than as a standalone-binding occurrence. The bHLHZip domain architecture, an evolution of simpler dimeric transcription factor DNA binding motifs, such as basic leucine zipper (bZip) [171] or basic helix loop helix (bHLH) [172], is found over a series of transcription factors or transcriptional repressors, involved in homo and heterodimerization events [173]. Besides c-Myc and Max, the bHLHZip family of transcription factors includes transcriptional repressors of the Mad family [174, 175]: Mad1, Mad3, Mad4, and Mxi are the most representative examples of this group. Mad proteins have a biological function orthogonal to that of c-Myc, as they are involved in cell differentiation processes, which occur during the G0 phase of the cell cycle. Mad proteins, similarly to c-Myc, cannot homodimerize and they also need Max as an obligate heterodimerization partner in order to bind DNA and exploit their function [157, 176]. Mad proteins can compete with c-Myc for Max binding [177] (Figure 18), although the different cell-cycle expression profile of c-Myc and proteins of the Mad family may reduce the likelihood of their simultaneous presence in the cellular nucleus.

Furthermore, p22 Max, one of two known Max isoforms (p21 and p22), can form homodimers with a binding affinity close to that of c-Myc-Max or Mad-Max heterodimer formation (≤ 1µM), while p21 Max displays very low affinity for homodimer formation (> 10 µM) [178, 179]. The affinity of the two Max isoforms for heterodimeric binding partners is virtually identical. The two Max isoforms only differ
Fig. 18 Schematic representation of the network of interactions between transcription factors of the bHLHZip family.

Fig. 19 Schematic representation of the two Max isoforms: p21, with low affinity for homodimer formation, and p22, bearing a 9 amino acids acidic insertion at the N terminus of the basic region, with high homodimerization affinity.
in the presence, for p22 Max, of a 9 amino acids acidic insert at the N terminus of the basic region (figure 19). While p22 Max homodimers do not display transcriptional activity, they can ‘silently’ bind target DNA sequences with the possible purpose of ‘buffering’ the transcriptional activity of the several heterodimeric binding partners of Max. The affinity for homodimer formation of p22 Max is also dependent on phosphorylation events that occur at serine residues found within or in proximity to the 9 amino acids insert described above [180]. Although a complete and detailed understanding of the implications of the possible competitions between bHLHZip dimerization interactions within the cell nucleus milieu is still lacking, the general information available suggests that they contribute to a tight and precise regulation of transcriptional events. Such regulation is extremely important due to the dramatic consequences on the cell fate caused by the downstream events that follow the activation and/or repression of transcription by c-Myc and Mad proteins [146]. This is made particularly clear when considering the dangerous outcome of c-Myc deregulated function [131, 133].

1.2.2. Approaches to c-Myc inhibition

The pharmaceutical inhibition of c-Myc function is an appealing therapeutic target [181-183], and several research groups have undertaken this challenge using a number of different methods. While it would be beyond the scope of the present thesis to provide a comprehensive report of all the published studies aimed at c-Myc
inhibition, it will be useful to provide an overview of the main approaches that have been experimented:

a) RNA interference and antisense oligo-nucleotide approaches

b) Stabilization of quadruplex DNA

c) Compounds interfering with c-Myc phenotype

d) Inhibitors of c-Myc-Max dimer formation

1.2.2.1. RNA interference and antisense oligo-nucleotide approaches

Two main nucleotide-based approaches have been attempted so far: one involves RNA interference [184-186], the other the use of antisense oligo-nucleotides [187]. Both of these approaches are aimed at interfering with c-Myc function at the translation stage, that is, preventing the mRNA from being translated into protein sequence. The first approach, which has achieved widespread popularity in recent years, is based on exploiting a naturally occurring cascade of interactions, triggered by small double stranded RNA molecules, micro RNA (miRNA) or small interfering RNA (siRNA) [188] that, upon cleavage by the enzyme Dicer and incorporation into RISC, lead to the degradation of target mRNA [189, 190]. This is achieved as portions of the digested product, or guide strand, hybridize with the target mRNA while the rest of the molecule is involved in recruiting a RNA-induced silencing complex (RISC) with the purpose of cleaving the mRNA molecule. The use of artificial siRNA molecules targeted against the mRNA encoding for c-Myc, meant to trigger the
described silencing cascade, has been experimented in vitro with some success [184, 185].

The second similar, yet conceptually simpler, approach is based on the use of oligo-nucleotides complementary to segments of the mRNA strand encoding for c-Myc. The consequent double strand formation over portions of the mRNA molecule leads to a breakdown of the ribosomal peptide synthesis process and degradation of the ‘blocked’ mRNA. Also this approach has shown some promise at inhibiting the proliferation of cultured cancer cell lines [187]. Both these approaches are however limited in their efficacy by inherent difficulties in the delivery and cellular uptake of nucleotide based agents, which are characterized by low cell permeability and are subject to undesired enzymatic cleavage processes.

1.2.2.2. Stabilization of quadruplex DNA

Another nucleic-acid targeted approach to c-Myc inhibition is the stabilization of quadruplex DNA structures formed within the c-myc gene promoter element [191]. Such stabilization prevents the single stranded DNA from assuming the extended conformation required for the initiation of the transcriptional process; it therefore ultimately impedes the expression of c-Myc protein. A conspicuous advantage of this approach is the possibility of targeting the quadruplex structure with low molecular weight agents, which may possess the several advantages of cost, ease of delivery and stability relative to biologicals. Small molecules stabilizing the c-myc promoter
quadruplex structure have been shown to inhibit c-Myc dependent transcription in a luciferase reporter gene assay [191, 192] (Figure 20).

**Fig.20** *c-myc* inhibitors that act by stabilizing a G-quadruplex structure in its early promoter DNA segment. The substituted porphyrin TMPvP4 is shown in complex with the G-quadruplex structure (PDB: 2a5r [191]). The expanded seleno-porphyrin Se2SAP was developed from the first compound [192].
1.2.2.3. Compounds interfereing with c-Myc phenotype

Several groups have undertaken the approach of screening collections of compounds for their ability to inhibit the proliferation of mammalian cell lines characterized by abnormal c-Myc activity. While this approach has the advantage of simultaneously assessing some physico-chemical parameters of the tested compounds, in particular cell permeability, it does not provide direct insight about the mechanism of action of hit molecules. It is further complicated by the inherent difficulties in designing control experiments, because of the complexity of mammalian cells and the multiple aberrations generally found in cancerous cell lines. The extent, relevance and mechanism of c-Myc deregulation in different cell lines is hard to assess, as well as the actual interference of hit molecules with molecular events and pathways connected with c-Myc activity rather than other cellular processes that may be altered in the screened cell line. A third negative aspect of this approach is the difficulty in adapting it for structure activity relationship analysis and lead optimization, due to the lack of any structural or mechanistic understanding of the hit molecule’s mode of action and specific biological targets. In spite of these caveats, some small molecules have been reported that have also been reasonably confirmed to interfere with c-Myc related cellular events, and display moderate inhibitory activity against c-Myc dependent cancer cell lines [193, 194] (Figure 21a).
Fig. 21 Other classes of c-Myc inhibitors. a. Compounds capable of interfering with c-myc phenotype: ‘Myra’ compounds [193]; EP2175, EP58269 [194]. b. Inhibitors of c-Myc-Max heterodimer formation reported by various groups: IIA6B17, IIA6B20 [195]; NY2279 [197]; ‘Mycro’ compounds [198].
1.2.2.4. Inhibitors of c-Myc-Max dimer formation

Due to the crucial role played by the heterodimerization with Max in relation to the functioning of c-Myc, a widely explored approach to the inhibition of the latter protein consists in inhibiting the formation of this complex. Several groups have devised screening methods aimed at monitoring the extent of c-Myc-Max heterodimerization both in purified component and cell-based assays in the presence of candidate inhibitor molecules [195-199] (Figure 21b). These assays have been performed to screen combinatorial or diversity oriented libraries of low molecular weight compounds, with sizes ranging between a few hundred and a few thousands molecules (which are considered small for current high throughput screening standards), and they in all cases resulted in encouraging hit rates, although the activity of hit compounds were generally modest, well into the micro molar range. Generally, hit compounds found in these studies display adequate drug-like properties. Purified component screens have relied on fluorescence based techniques. In one such assay, the fluorescence resonance energy transfer (FRET) effect between fluorescently tagged recombinant c-Myc and Max bHLHZip domains occurring upon heterodimer formation was monitored [195]. Genetically encoded fluorescent tags, derived from mutations of green fluorescent protein (GFP), have been employed in these studies, through the cloning and expression of fusion versions of c-Myc and Max bHLHZip peptides with cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) respectively (MycCFP, MaxYFP). The overlap between absorption and emission spectra of these two fluorescent proteins leads to the FRET effect when they are within
several angstroms of each other, as in the case of heterodimer formation. A second, fluorescent-based assay consisted in monitoring the fluorescence polarization of fluorescently tagged target DNA sequences (E-Box) of c-Myc-Max heterodimers in the presence of purified recombinant c-Myc and Max bHLHZip domains and screened inhibitors [198]. When bound to the large protein complex, the tumbling rate in solution of the relatively small oligo-nucleotide is slower than in its unbound state. The slower motion of the linked fluorescent tag therefore causes a lower extent of depolarization of the emitted light after excitation with a polarized beam. Relative changes in the residual fluorescence polarization of the fluorophore are therefore informative of the extent of E-box binding by c-Myc-Max heterodimers, and ultimately of any inhibitory effect induced by screened compounds on the formation of this complex.

Using a different approach, Yin et al., in a cell-based yeast two-hybrid assay, monitored the activity of a reporter gene, the expression of which was dependent upon heterodimerization between c-Myc and Max [196]. In this assay, fusion proteins between the c-Myc HLHZip domain and the DNA binding domain of yeast Gal4 transcription factor and between Max HLHZip and the transcriptional activation domain of the same protein were expressed in yeast cells. Only upon heterodimer formation between c-Myc and Max HLHZip was the Gal4 activity restored. The extent of Gal4 activation, dependent on c-Myc-Max heterodimer formation can therefore be monitored by means of a colorimetric assay were a colored substitute for the galactose substrate of the galactosidase enzyme, controlled by Gal4 induction, is employed. The
enzymatic digestion of the substrate, dependent on Gal4 activity and ultimately c-Myc-Max heterodimer formation, can be observed by a change of color of the screened cell cultures.

All the described approaches have the quality of being easily performed in high throughput settings, using multiple well plate readers, and they can be relatively simply adapted for control and validation purposes, by means of generating FRET pairs, measuring DNA binding, or designing yeast two-hybrid systems where the dimerization between different bHLHZip, HLH or bZip protein pairs is monitored. Interestingly, hit compounds from different studies have very low structural similarity, and the screening of a diversity-oriented library by Yin et al. produced seven hit molecules structurally unrelated to each other (Figure 22), furthermore the yeast two hybrid approach employed in this screening allowed for a thorough validation of these compounds’ specificity, confirmed from their ability to selectively inhibit the heterodimerization between c-Myc and Max and not other bHLH, bHLHZip and bZip homo and heterodimer pairs expressed in yeast two hybrid constructs.

While these studies have relied on random screens, without preliminary knowledge of molecular features that might be desirable for hypothetical inhibitors of c-Myc-Max heterodimer formation, it is worthwhile mentioning one case of rational design approach to the inhibition of c-Myc-Max heterodimerization, in which peptides or peptido-mimetic compounds have been prepared aimed at binding to the helix1 segment of the c-Myc bHLHZip domain [200, 201]. These peptides have been designed based on molecular modeling studies performed on the crystallographic
**Fig. 22** Inhibitors of c-Myc-Max heterodimer formation first reported by Yin et al. [196].
structure of c-Myc in its heterodimeric complex with Max, and can bind to their target c-Myc segment in a helical conformation. Some of these peptides have displayed growth inhibition of c-Myc dependent cancer cell lines, but, similarly to oligo-nucleotide based c-Myc inhibitors, they are limited in their utility by difficulty of delivery, low cell permeability, and susceptibility to enzymatic cleavage typical of peptides.
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Chapter 2

SAR Studies on one inhibitor’s structural scaffold

2.1. Introduction

The general goal of the studies presented in this thesis has been the structural and mechanistic understanding of the mode of action of small molecule inhibitors of c-Myc-Max dimer formation, with a focus on exploiting such understanding for the design of compounds with improved potency. These studies were started on known inhibitor molecules, which were selected for their structural simplicity (including lack of chiral centers), ease of synthesis, or, alternatively, commercial availability and optimal drug-like properties. Of all the low molecular weight inhibitors of c-Myc-Max heterodimer formation known at the time, those reported by Yin et al. best complied with these requirements [1]. Furthermore, these compounds, selected from a commercial, diversity oriented, library of drug like molecules, displayed reduced similarities to each other, compared to hit molecules from ad hoc combinatorial libraries screened in other studies, which, being based on a common synthetic strategy, generally show only modest chemical diversity [2, 3]. Such low similarity between molecules showing equivalent inhibitory activities of c-Myc-Max heterodimerization was intriguing because of its contrast with the usual structure activity correlation trends observed for small molecule ligands of structured and relatively rigid binding pockets, where active compounds commonly display, if not actual chemical similarity, steric and shape overlap of analogous moieties and functional groups [4-6].
The first step taken for the described study was the choice of one structure out of the seven hit molecules reported by Yin et al., which displayed ease of synthesis and versatility for chemical modification at different positions, with the goal of synthesizing a series of derivatives of such compound and assessing structure activity relationships for this particular binding scaffold. The chosen compound, \((5Z)-5-[(4-ethylphenyl)methylidene]-2-sulfanylidene-1,3-thiazolidin-4-one\) (figure 1, 1), named 10058-F4 in the original study, can be easily prepared by Knoevenagel condensation of rhodanine with 4-ethylbenzaldehyde [7], and the replacement of the latter with other aromatic or aliphatic aldehydes can generate a fair number of derivatives with equal ease of synthesis. This compound offers other possibilities for modification, such as introducing substitutions on the imide nitrogen. Furthermore, 10058-F4 and derivative compounds containing aromatic moieties conjugated to the rhodanine ring through a 5-exo double bond display fluorescence with an emission maximum centered at ~470 nm when excited at 380 nm. A brief description of each approach to the modification of the 10058-F4 structure follows below (illustrated in figures 1 and 2).

2.2. Synthetic strategies

\begin{itemize}
  \item[a)] Knoevenagel condensation
  \item[b)] Mannich Reaction
  \item[c)] Nucleophilic substitution: S nucleophile
  \item[d)] Nucleophilic substitution: N nucleophile
  \item[e)] Reduction of 5-exo double bond
\end{itemize}
Fig. 1 Synthetic strategies for the modification of 10058-F4 (1-Rh). a. Knoevenagel condensation of aldehydes and ketones with rhodanine in acidic conditions and obtained compounds. b. Mannich reaction with nucleophilic center at the rhodanine ring amide nitrogen, the secondary amine substrate is piperidine. c. Two synthetic approaches to the methylation of the sulfur atom in position 3 of the rhodanine ring. d. Nucleophilic substitution at the amide nitrogen position e. Reduction of the 5-exo double bond.
2.2.1. Knoevenagel condensation

The carbon in position 5 of the rhodanine ring bears two protons made relatively acidic by the simultaneous presence in the two α positions of a carbonyl group and a sulfur atom. The first acts as an electron-withdrawing group both by inductive and resonance effect, and both moieties may contribute to delocalize the negative charge resulting from the basic extraction of an acidic proton; for the same reasons, under acidic conditions, they promote the establishment of a keto-enol equilibrium. Several procedures have been reported where this property is exploited for the condensation of rhodanine with aldehydes or ketones, which can be performed both under basic or acidic conditions [7-10] (Figure 1a). Noticeably, in both cases the condensation products are nearly quantitatively Z isomers at the 5-exo double bond position, due to the steric hindrance of the carbonyl oxygen bound to the carbon in position 4 of the ring, possibly increased by its solvation sphere in protic solvents. The characterization of this structural feature can be easily achieved by means of NMR spectroscopy, as the vinylic proton in the E position of the 5-exo double bond is considerably deshielded because of several neighboring π systems. In the present study, a procedure where the substrates were heated to reflux in neat acetic acid with added acetate salts to promote the dissociation of the acidic proton was used [7]. This procedure, which will be reported in detail in the methods section, besides providing excellent yields in short times, presented the noticeable advantage of allowing for the isolation of highly pure products by simple dilution of the reaction mixture with water. This treatment causes a nearly quantitative precipitation of product molecules, while
the water soluble rhodanine and (in most cases) liquid aldehyde or ketone substrate can be washed away from the precipitate. The same procedures for condensation reactions of rhodanine may be performed with the related compound 2,4-thiazolidinedione, in which an oxygen atom replaces the sulfur atom bound to carbon 2 of the rhodanine ring [10, 11].

Fig.2 Compounds obtained by combined modifications of phenyl ring substitents and 5-member ring from the 10058-F4 structural scaffold (a) and compounds purchased from ChemBridge corporation (b).
2.2.2. Mannich reaction

A second approach to the structural modification of 10058-F4 takes advantage of the relatively high acidity of the proton bound to the imide nitrogen in position 3 of the rhodanine ring. Reference literature pKa values suggest that this value depends upon the presence of the thio-carbonyl moiety in position 2 of the rhodanine ring, which can better stabilize a delocalized negative charge compared to an oxy-carbonyl [12]. This property also results in a nucleophilic reactivity upon deprotonation of the rhodanine imide nitrogen: the extensive delocalization of negative charge on the ‘soft’ thio-carbonyl sulfur atom grants the latter with good nucleophilic properties, and nucleophilic substitution reactions on halogen substituted substrates performed upon deprotonation of the rhodanine imide nitrogen tend to occur with the sulfur acting as a nucleophile as will be discussed below, or without discrimination between sulfur and nitrogen reactivity, leading to mixtures of products. One reported method to specifically insert substitutions on the imidic nitrogen consists in a modified version of the Mannich reaction [13], where the attack on an iminium cation generated by condensation between formaldehyde and a secondary amine, is performed by this heteroatom instead of a carbon in the α-position of a carbonyl moiety. Due to the specific characteristics of the iminium electrophylc substrate, possibly related to the presence of a positive charge that may direct it towards the attack by the harder nitrogen nucleophile rather than the softer sulfur, this reaction displays complete selectivity towards substitution at the imide nitrogen in position 3 of the rhodanine ring. It was performed in this study using piperidine as the substrate secondary amine,
in order to test the effect of introducing a relatively bulky hydrophobic substituent on the more polar end of the 10058-F4 structural scaffold (Figure 1b).

2.2.3. Nucleophilic substitution: S nucleophile

Proton extraction at the imide nitrogen position of the rhodanine ring leads to the formation of a delocalized negative charge, which can grant nucleophilic properties to both the imide nitrogen itself, and the sulfur atom of the thio-carbonyl moiety in position 2 of the ring. Several substrates can undergo nucleophilic attack from this last reactive center in a selective manner. A reported procedure describes the reaction of rhodanine with methyl iodide under basic conditions to yield a thioenol ether product [7]. We performed this reaction to assess the relevance to the inhibitory activity of 10058-F4 of the hydrogen bond donor properties of the imide nitrogen and of the soft thio-carbonyl acceptor. As the thioenol ether product is considerably labile under acid conditions, we exploited a modified basic procedure for the successive Knoevenagel condensation of aldehyde and ketone substrates with the modified rhodanine ring instead of the acidic one described above (Figure 1c) [7].

2.2.4. Nucleophilic substitution: N nucleophile

Due to the aforementioned difficulties in orienting nucleophilic substitution reactions towards the attack by the rhodanine ring imide nitrogen, which is in competition with the nucleophilic reactivity of the thio-carbonyl sulfur atom, an effective way to obtain selective reaction at the nitrogen atom was achieved by
substituting the rhodanine ring with the closely related 2,4-thiazolidinedione moiety. The absence of the thio-carbonyl sulfur atom in the latter allows for the selective reactivity of the imide nitrogen towards the attack of such substrates as aliphatic or allylic halide [14]. Also in this case, this reaction was performed to assess the effect of hydrophobic substituents on the imide nitrogen atom, without the presence, as in the case of the described products of Mannich reactions, of a basic tertiary amine within such substituents. The substrate of choice for this purpose was allyl bromide (Figure 1d).

2.2.5. Reduction

The last modification of the 10058-F4 structural scaffold explored in this study involved the reduction of the 5-exo double bond in order to evaluate the effect of removing the molecules’ symmetry plane. The products of such reaction are chiral and were tested as racemic mixtures, as it is known that pure enantiomers tend to racemize due to the relative acidity of the proton in position 5 of the rhodanine ring [15]. This modification was of further interest because a structure very similar to its products is found in the PPARγ inhibitor anti-diabetic drug Rosiglitazone. In order to achieve selective reduction of the 5-exo double bond, α conjugated to the carbonyl moiety in position 4 of the rhodanine ring, a reported procedure was attempted which exploits a reduction approach based on the vinilogy principle modified from chemistry for the reduction of carbonyl groups. Lithium borohydride was employed [8, 16], formed in situ upon cation exchange of sodium borohydride with lithium salts, in the presence of
pyridine [17] (figure 1e). While the detailed mechanism for the selective reduction of the conjugated double bond by this reagent is not fully understood, it is possible that the coordination of the cation by pyridine might prevent its coordination by the carbonyl oxygen and direct the hydride attack towards the conjugated double bond. This procedure has also been reported for similar substrates with the use of sodium borohydride, the lower reactivity of this reducing agent compared to lithium borohydride results however in relatively longer reaction times and lower yields [8, 18]. Both reducing agents are mild enough to avoid reducing carbon-oxygen double bonds within carboxyl or amide moieties. Nonetheless, the number of possible reduction events competing with that of the 5-exo double bond in the substrate compounds employed here, including that of both oxy-carbonyl and the (most likely more reactive) thio-carbonyl moiety forming the imide group in the rhodanine ring, resulted in the formation of consistent amounts of side products, and the purification of the desired products was attained by reversed phase HPLC. Consistent with literature reports, the reaction yields were relatively poor. The product compounds were noticeable for the appearance, in their NMR spectrum, of a coupling pattern between geminal protons bound to the reduced methylene carbon, due to its constrained rotation.

2.3. Activity profile

Several tests were performed on the series of synthesized derivatives of 10058-F4 to assess their potency as inhibitors of heterodimer formation between c-Myc and
Max both in purified component and cell-based assays. Taking advantage of the intrinsic fluorescence of 10058-F4 and related compounds a fluorescence polarization assay was used to assess the direct binding between these molecules and isolated c-Myc or Max protein monomers. This assay constitutes a significant novelty in the study of c-Myc-Max inhibitors, as it is the first experimental setting aimed at detecting the direct specific binding interactions of these molecules, rather than their functional effect (dimer disruption). The synthesized compounds were also tested for their ability to disrupt c-Myc-Max dimer binding of target E-box DNA sequences in an electrophoretic mobility shifts assay (EMSA). In this assay, the relative amounts of free and protein-bound fluorescently tagged oligo-nucleotides are detected based on the differential mobility of the two species in polyacrylamide gels [19, 20]. The synthesized inhibitors were also tested by the Prochownik lab at the University of Pittsburgh, where the original screening by Yin et al. had been performed, in cell based assays similar to those used previously, including the c-Myc-Max yeast two-hybrid system described in Chapter 1 [1], and growth inhibition of c-Myc overexpressing human cancer cell lines (HL60 [21] and Daudi Burkitt’s lymphoma [22]). While these studies do not strictly pertain to this thesis, an overview of their results will be reported in the context of a comprehensive analysis of corresponding or differential behavior observed for various compounds over these multiple assays.
2.3.1. Direct binding to c-Myc monomer: fluorescence polarization

The ability of the studied small molecules to disrupt the c-Myc Max heterodimeric complex suggests the capacity of these compounds to interact with either or both monomeric protein species. Furthermore, control experiments described in the original report of these molecules by Yin et al. [1], aimed at assessing the selectivity for c-Myc-Max over other hetero and homodimeric complexes, suggested a likely preferential interaction between the inhibitors and c-Myc. The compounds were tested for their ability to disrupt different HLH, HLHZip or leucine zipper transcription factors dimeric complexes. This assay was performed, similarly to the initial screening of a library of compounds for disruption of c-Myc-Max heterodimers, with a series of yeast two-hybrid constructs. The c-Myc-Max inhibitor 10058-F4, and six more compounds reported as selective, displayed very low disruption of any other complexes except for c-Myc-Max heterodimers. These compounds displayed very low disruption of Max homodimers, supporting their selective interaction with c-Myc as suggested above.

In order to directly test this hypothesis in the case of the compound 10058-F4, a fluorescence polarization assay was designed in which the intrinsic fluorescence of this inhibitor was exploited. When a relatively small fluorescent molecule interacts with a larger one, its tumbling rate in solution decreases, thus increasing the amount of residual polarization of the emitted light when a polarized excitation beam is employed [23]. This assay was effectively optimized to assess the binding between 10058-F4 and c-Myc or p21 Max monomers.
The p21 Max isoform was chosen in order to avoid a convolution of the experimental results due to p22 Max homodimer formation [24]. In the presence of the latter complex, if binding was detected, it would not be possible to discriminate, by monitoring the fluorescence polarization of the small molecule ligand, whether this compound is interacting with p22 Max monomers freed upon disruption of the homodimeric complex, or with this complex itself. Furthermore, if the small molecule interacted with monomeric Max, when using p22 Max, the experiment would be biased by the competition of the p22 Max - 10058-F4 complex with the homodimer formation of p22 Max. The binding interaction between p22 monomers and small molecules would be therefore observed only in the presence of a large excess of the latter, required to compete the p22 homodimerization by mass action. The specific fluorescence polarization experimental setting would nonetheless be unsuitable to detect a binding interaction under such conditions, as the fluorescence polarization increase of the fraction of small molecules bound to Max monomers would be overcome by the larger fraction of excess unbound molecules. The fluorescence polarization experimental setting employed to assess any direct interaction between small molecule inhibitors of dimerization and either c-Myc or p21 Max bHLHZip monomers therefore requires that the small molecule fluorescent component be present in a limiting amount, or in an equimolar mixture with the protein component employed.

The results of this fluorescence polarization assay for 10058-F4 confirmed that this inhibitor is involved in a direct binding interaction with c-Myc and not with p21
Max bHLHZip monomers (Figure 3a). The measurement of 10058-F4 fluorescence polarization in samples containing different molar ratios of this compound and c-Myc bHLHZip further showed that this interaction occurs with a 1:1 stoichiometry, and therefore likely involves one specific binding site on the c-Myc bHLHZip protein monomer (Figure 3b). A titration of this binding interaction was performed upon serial dilution of equimolar mixtures of 10058-F4 and c-Myc bHLHZip. The experimental data were fit to a quadratic equation derived from the thermodynamic balance equation for the studied interaction, simplified by the use of an equimolar ratio of the two species involved in binding. A background correction of the fluorescence polarization values was made necessary by the relatively low fluorescence intensity of 10058-F4, which led to a relatively large contribution from highly polarized scattered light. Such correction was simply performed by means of subtracting from each data point the background polarization value of the samples minus the inhibitor multiplied by the fraction of its light intensity. The detected binding affinity displayed a dissociation constant (K_D) of 5.3 ± 0.7 µM (Figure 5), considerably higher than common drug–protein interactions occurring within folded and relatively rigid binding pockets [25], possibly because of the nature of an intrinsically disordered protein binding site, characterized by inherent flexibility and lacking the three-dimensional complexity required to define an extensive pattern of interactions between ligand and protein site. Nonetheless, the measured interaction affinity seems reasonably high when compared to affinity values for c-Myc and Max HLHZip heterodimer formation. This interaction involves the entire studied domain rather than a small portion of it, as is likely the case
Fig. 3 Study of general features of 10058-F4 binding interaction by fluorescence polarization. **a.** The inhibitor and its derivatives do not interact with p21 Max monomers (light blue bars: 25 µM pure compound; yellow bars: 25 µM compound + p21 Max; dark blue bars: 50 µM pure compound; red bars: 50 µM compound + p21 Max; representative of three trials, error bars indicate the standard error of the mean). **b.** The binding between 10058-F4 and monomeric c-Myc bHLHZip occurs with a 1:1 stoichiometry, indicating the presence of a specific binding site.
for a small molecule inhibitor binding. The dimer binding affinity has been reported in the high nanomolar range [26], therefore only one order of magnitude tighter than that measured for the interaction between c-Myc monomers and 10058-F4, in spite of the obvious dramatic difference between the surface areas involved. A possible explanation for this thermodynamic behavior is the lower entropic cost involved in the binding interaction between c-Myc monomers and 10058-F4, in which large fractions of the protein’s bHLHZip domain are likely to remain flexible, compared to the complete folding and loss of mobility of this domain upon heterodimerization with Max.

A preliminary screening of the synthesized derivatives of 10058-F4 and some other related compounds bearing similar modifications which had been purchased from ChemBridge corporation, San Diego, CA (Figures 1a, 2), was performed, exploiting the fluorescence polarization technique, where the binding between each compound and c-Myc bHLHZip was tested at two equimolar concentrations of each component: 25 and 50 µM. No background correction of the fluorescence polarization values was deemed necessary for such preliminary screening due to its simplified nature, as the data were qualitatively interpreted in terms of presence or absence of binding over two concentrations only. The differential extent of background scattering components within the fluorescence polarization of different compounds varying in fluorescence intensity, as well as intrinsic differences in the fluorescence lifetimes of several, slightly different, fluorophores, therefore lead to some extent of variation from an ideally constant baseline fluorescence polarization value for the free compounds in the
absence of c-Myc bHLHZip. The data were therefore plotted for each screened molecule as relative polarization with reference to the polarization value for the free compound at 25 µM (Figure 4). A subset of 9 of compounds, characterized by enhanced c-Myc activity based on this preliminary screening or the other performed assays discussed below, was further analyzed by performing a full titration of their binding interaction with c-Myc over a broader range of concentrations, as previously done for 10058-F4 (Figure 5). Background corrections were performed for these experiments. These compounds displayed affinities ranging from 2.3 to 11 µM, relatively close to that of the parent compound 10058-F4, in spite of the variety of modifications represented within this subset of derivatives. More generally, the screening results for the full set of synthesized derivatives, also resulted in a fairly relaxed structure activity relationship profile, with most compounds displaying binding to c-Myc at least at the highest tested concentration of 50 µM. This unusual behavior might be related to the particular structural features of binding complexes involving an intrinsically disordered protein segment lacking a stable and rigid tertiary fold; the extensive flexibility of the protein binding site might allow for its structural rearrangement to accommodate small molecule ligands with different functional groups and structural features. This result is consistent with the observed structural diversity within the group of seven inhibitors of c-Myc-Max heterodimer formation first reported by Yin et al. [1]. Some structure activity trends were recorded nonetheless for the series of synthesized 10058-F4 derivatives, which will be discussed at the end of this chapter (section 2.4).
Fig. 4 Fluorescence polarization screening of the synthesized library of 10058-F4 derivatives. Polarization values for each studied compound have been normalized to the polarization observed for the free molecule at 25 µM concentration. Light and dark blue bars represent the polarization of free compounds at 25 and 50 µM respectively. These data have been displayed to confirm that no increase in polarization was observed due to an increase in scattering at higher compound concentrations. Yellow and red bars represent the polarization of equimolar mixtures of each compound and c-Myc bHLHZip at 25 and 50 µM respectively. Data represent the average of three independent trials, error bars indicate the standard error of the mean.

Fig. 5 Titration of the binding interaction between c-Myc bHLHZip and selected compounds monitored by fluorescence polarization. Compounds were selected based on their overall performance over a number of essays, including yeast-two-hybrid disruption of c-Myc-Max heterodimers, disruption of E-box DNA binding by c-Myc-Max heterodimers, monitored by EMSAs and growth inhibition of the c-Myc-dependent HL60 human cancer cell line. Data represent the average of three to five independent trials, error bars indicate the standard error of the mean.
2.3.2. Disruption of c-Myc-Max DNA binding: EMSA

A subset of 10058-F4 derivatives displaying good c-Myc binding affinity was tested for their ability to disrupt the binding of target E-box DNA sequences by c-Myc-Max heterodimers. These experiments were performed taking advantage of the electrophoretic mobility shifts assay (EMSA) technique [19]. First, the mobility on 8% polyacrylamide gels of a limiting amount of double stranded oligonucleotides, 22 base pairs long, containing the E-box palindromic recognition sequence CACGTG at their center and a hexachlorofluorescein (Hex) fluorescent label on one strand, was monitored in the absence and presence of varying concentrations of c-Myc-p21 Max mixtures. These preliminary titrations, also performed for E-box binding by p22 Max homodimers, allowed for the optimization of experimental conditions before testing the inhibitors’ ability to disrupt the c-Myc-Max-DNA ternary complex. The binding of target DNA by bHLHZip homo or heterodimers occurs in a cooperative fashion, and the observed dissociation constant values for the binary interaction between homo or heterodimers and DNA are nearly two orders of magnitude lower than the dissociation constants for the HLHZip dimerization events [26, 27]. For this reason higher inhibitor concentrations are needed than those required for the disruption of c-Myc-Max heterodimers. Furthermore, the excess of inhibitor required to induce disruption of E-box DNA binding by c-Myc-Max heterodimers will depend upon the concentration of the latter, as the transcription factors – DNA complex will be harder to disrupt with increasing protein concentrations. Lastly, the slow dissociation rate of this complex has to be taken into account when testing its disruption by small molecule inhibitors, as no
Fig. 6 Disruptive effect of selected inhibitors, representative of modifications of the phenyl ring substituents, 5-member ring and both from the 10058-F4 structural scaffold, on E-box DNA binding by c-Myc-Max heterodimers monitored by EMSA.
disruption would be detected if samples containing pre-formed c-Myc-Max-DNA ternary complexes were incubated for too short periods of time. Due to these considerations, experimental conditions to test the small molecules inhibitory effect on DNA binding by c-Myc-Max heterodimers were carefully chosen with particular attention to the following aspects:

1. The employed c-Myc-p21 Max dimer concentration (50 nM) was narrowly above the observed pseudo-dissociation constant for E-box DNA binding ($K_{obs} = 22 \pm 3$ nM, refer to Chapter 5 for further details).

2. The inhibitors were pre-incubated with c-Myc before the addition of p21 Max and E-box DNA, as the faster dissociation rate of the c-Myc inhibitor complex would allow for faster thermodynamic equilibration of the samples.

The inhibitors were tested over a range of concentrations (from 200 µM to 12.5 µM). Generally they displayed only partial disruption of E-box DNA binding even at the highest tested concentrations, which approached the saturation solubility of these compounds (Figure 6). The observed inhibition correlated qualitatively with the c-Myc binding affinities, when these values had been measured, but were considerably higher than the latter, as expected from the thermodynamic considerations about the system of equilibria involved in c-Myc-Max-DNA ternary complex formation discussed above. The compounds that showed the strongest inhibition of E-box DNA binding by c-Myc-Max heterodimers were 10058-F4 derivatives bearing hydrophobic or halogen substituents in the para position of the phenyl ring or products of the Mannich reaction modification, possibly also bearing the same favorable substituents on the phenyl ring.
A similar experiment was performed with the same inhibitors and monitoring the E-box DNA binding by p22 Max homodimers, in order to further assess the compounds specificity towards disruption of c-Myc-Max heterodimers. The inhibitors were incubated with 100 nM p22 Max before addition of E-Box to the reaction mixtures, however no disruption of DNA binding was detected in this case for any of the tested compounds (Figure 7b).

2.3.3. Cell based assays

The full series of 10058-F4 derivatives were tested for disruption of c-Myc-Max heterodimers in the same cell based yeast two-hybrid assay originally employed by Yin et al. [1] to discover the parent c-Myc-Max inhibitor and six other, structurally diverse, selective inhibitors. This assay, similarly to the previously described fluorescence polarization one, was first performed as a general screening at a single inhibitor concentration (20 µM), then the inhibitory effect of the best studied compounds was tested over a range of concentrations. This experiment was particularly useful as it allowed assessment of the potency of synthesized compounds that lacked fluorescence properties, including all those obtained from condensation of rhodanine with aliphatic aldehydes and ketones. The results of this assay were well correlated with the general fluorescence polarization screening of the compounds’ ability to bind monomeric c-Myc bHLHZip, albeit in a qualitative fashion, as both assays were not performed over a sufficiently broad range of concentrations to allow for the full titration of their inhibitory potencies or binding affinities (Figure 8a).
Fig. 7 Quantitation of E-box DNA binding disruption by 10058-F4 and the two compounds that performed best in this assay, 12-Rh-NCN1 and 28-Rh-NCN1 (representative of three trials, error bars indicate the standard error of the mean, the line is present solely to guide the eye) (a) and control experiment confirming that the screened inhibitors are unable to disrupt E-box DNA binding by p22 Max homodimers (b), adapted with permission from Wang et al. [28].
The compounds were also tested for their ability to inhibit the growth of c-Myc overexpressing HL60 human cancer cells [21]. Several 10058-F4 derivatives displayed activity in this assay, some of them considerably higher than the parent compound: the 10058-F4 measured IC$_{50}$ was in fact 49 µM, the best measured value was 4.6 µM for compound 764, a commercially available derivative bearing a hydrophobic allylic substituent on the imide nitrogen position. Compounds that displayed improved growth inhibition of HL60 cells compared to 10058-F4 were re-evaluated in more depth in the previously described assays, and full titrations of their c-Myc binding affinities were performed. The correlation between c-Myc binding affinity, disruption of c-Myc-Max heterodimers measured by their E-box DNA binding in EMSA or by yeast-two-hybrid experiments, and growth inhibition of HL60 cells for these compounds was however relatively poor: the Mannich reaction product derivatives, which showed the best c-Myc binding affinities and strongest disruption of c-Myc-Max E-box DNA binding were indeed only marginally better than 10058-F4 at inhibiting HL60 growth (Table 1). The best HL60 inhibiting compounds, however, carried hydrophobic substituents at the imide nitrogen position similar, albeit less bulky, to those found in the 10058-F4 derivatives obtained by means of Mannich reaction. The failure of the latter compounds to express equivalent potencies in cell growth inhibition assays to those observed in purified component assays may be due to poor cell permeability properties dependent upon their larger size, the presence of a basic moiety (and positive charge at physiological pH) and, possibly, low metabolic stability of the β amino substituted group bound to the imide nitrogen. In spite of such partial lack of correlation, all the
Fig. 8 Results of cell based assays performed by the Prochownik lab, Children’s Hospital, Pittsburgh (adapted with permission from Wang et al. [28].

a. Screening of the synthesized 10058-F4 derivatives (bearing phenyl ring modifications) for disruption of c-Myc-Max heterodimerization in a yeast-two-hybrid assay, similar to the one employed for the original library screening that produced the compounds studied in this work. Compounds were tested at a single concentration of 20 μM. Compounds indicated by an asterisk and grey bars were tested at 10 μM concentration.

b. Co-immuno-precipitation assay from HL60 cells treated with indicated inhibitors. An anti-Max antibody was used for the pull-down and anti-Myc for detection. Decreased band intensities indicate disruption of c-Myc-Max heterodimers in cells.

c. Growth inhibition of c-Myc expressing (TGR1) and c-Myc depleted (KO, KO-HMG) cell lines. The studied inhibitors affect exclusively c-Myc dependent cell lines.
performed assays contributed to define a series of features of 10058-F4 derivatives beneficial to their inhibition of c-Myc activity.

Assays were also performed aimed at confirming the selective inhibition of c-Myc-Max heterodimers in a cellular setting by the screened inhibitors: the extent of c-Myc-Max dimerization was confirmed to be reduced in the presence of inhibitors by means of antibody based co-immunoprecipitation (CoIP) pull down assays [1, 24, 28] (Fig. 8b). These compounds were also shown not to significantly inhibit the growth of c-Myc independent cell cultures. A c-Myc KO-HMG cell line was employed, in which the high expression of the HMGA1b transcription factor compensates for the inherently slower cell proliferation consequence of the permanent inactivation of c-Myc [28-30] (Figure 8c).

Table 1 Summary of measured activity values over a range of assays for the best tested compounds. Values are micromolar.

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>$K_D$</th>
<th>HL60 $IC_{50}$</th>
<th>Y2H</th>
</tr>
</thead>
<tbody>
<tr>
<td>10058-F4 (1-Rh)</td>
<td>5.3 (0.7)</td>
<td>49</td>
<td>9.1 (0.7)</td>
</tr>
<tr>
<td>12-Rh</td>
<td>6 (1)</td>
<td>41</td>
<td>4.5 (0.7)</td>
</tr>
<tr>
<td>22-Rh</td>
<td>N.D.</td>
<td>51</td>
<td>3.4 (0.2)</td>
</tr>
<tr>
<td>27-Rh</td>
<td>11 (2)</td>
<td>23</td>
<td>inactive</td>
</tr>
<tr>
<td>28-Rh</td>
<td>3.8 (0.4)</td>
<td>36</td>
<td>6 (1)</td>
</tr>
<tr>
<td>1-Rh-Sme</td>
<td>6.7 (0.6)</td>
<td>18</td>
<td>---</td>
</tr>
<tr>
<td>#015</td>
<td>N.D.</td>
<td>6.5</td>
<td>---</td>
</tr>
<tr>
<td>#474</td>
<td>3.2 (0.3)</td>
<td>10</td>
<td>---</td>
</tr>
<tr>
<td>#764</td>
<td>7.0 (0.9)</td>
<td>4.6</td>
<td>---</td>
</tr>
<tr>
<td>1-Rh-NCN1</td>
<td>9.2 (0.8)</td>
<td>42</td>
<td>7.5 (0.3)</td>
</tr>
<tr>
<td>12-Rh-NCN1</td>
<td>2.3 (0.3)</td>
<td>38</td>
<td>---</td>
</tr>
<tr>
<td>28-Rh-NCN1</td>
<td>4.5 (0.8)</td>
<td>29</td>
<td>5.5 (0.4)</td>
</tr>
</tbody>
</table>
2.4. Conclusion

The first meaningful result of the experiments on the inhibitor of c-Myc-Max heterodimer formation 10058-F4 and related compounds is the evidence of their direct interaction with the c-Myc bHLHZip monomer and lack of binding to the same domain of the c-Myc dimerization partner Max. This result was predicted, as the compounds ability to disrupt the bHLHZip heterodimeric complex implies their interaction with one or both protein monomers, and their demonstrated selectivity towards disruption of c-Myc-Max heterodimers and not Max homodimers suggests that such interaction involves the c-Myc bHLHZip monomer only. The fluorescence polarization assay exploiting the intrinsic fluorescence of 10058-F4 constitutes the first experimental confirmation of this prediction.

The synthesis and screening of a series of molecules derived from 10058-F4, accompanied by the testing of a smaller set of commercially available related compounds, provided further insights about the structural features of these molecules responsible for their binding interaction with c-Myc bHLHZip monomers. Some structure activity relationship information could be inferred from the fluorescence polarization screening of the compounds’ binding to purified c-Myc bHLHZip and the other performed assays, aimed at evaluating the inhibition of c-Myc activity at a number of levels, including its target DNA binding, (as heterodimer with Max), and c-Myc dependent proliferation of cancerous cells. These observations can be summarized as follows:
a) Substitutions on the E position of the 5-exo double bond strongly diminish the c-Myc binding affinity.

b) Aliphatic and halogen substitutions on the benzene ring generally increase the c-Myc binding affinity.

c) Polar substitutions on the benzene ring generally decrease the c-Myc binding affinity.

d) The effect of the substitution position on the benzene ring generally displays an o < p < m trend.

e) Non-aromatic, bulky hydrophobic groups replacing the benzene ring with comparable shape and size (i.e. cyclohexane ring) do not cause a noticeable reduction in c-Myc binding affinity.

f) Hydrophobic substitutions on the rhodanine ring imide nitrogen generally increase the c-Myc binding affinity; removal of the relatively acidic proton from this atom by formation of a methyl thioenol ether does not noticeably affect it.

g) Reduction of the 5-exo double bond and loss of the molecule’s symmetry plane causes a loss in c-Myc binding affinity.

The reported observations have been recently used by a collaborating group in a pharmacophore model derived from the series of synthesized 10058-F4 derivatives [31]. Active and inactive compounds have been structurally aligned to generate a fingerprint of molecular features likely to favor or disfavor the binding interaction with c-Myc. The obtained model has been employed for the in silico screening of the
Fig. 9 Employment of the collected SAR information for the 10058-F4 binding scaffold with a ‘lead hopping’ purpose. a. Pharmacophore model generated with the Sybil Galahad and Tuplets algorithms (adapted with permission from Mustata et al. [31]). b. Structure of the two most promising, experimentally validated, hits obtained from screening the ZINC database with the described pharmacophore model. Notice the low extent of observable structural similarity between these molecules and 10058-F4.
publicly available ZINC database [32], generating hits displaying low structural similarity to the parent compound and its derivatives (Figure 9). Based on considerations of the hits’ predicted absorption, distribution and metabolism properties, 9 commercially available hit molecules were purchased from ChemBridge Corporation and screened for their ability to disrupt the c-Myc-Max dimerization in a circular dichroism assay. It was found that 7 out of 9 tested compounds were able to at least partially disrupt the c-Myc-Max interaction. The affinity of the 4 best inhibitors for c-Myc monomers was assessed to be close, albeit generally lower, than that of the parent compound 10058-F4. Two of these compounds also displayed promising inhibition of HL60 cells growth. The ability of a pharmacophore model to identify c-Myc inhibitors with different structural scaffolds, a practice dubbed ‘lead hopping’ [33], may be exploited in the attempt to overcome the cell permeability and metabolic stability issues that likely hinder the utility of some of the studied inhibitors. Furthermore, the experimental confirmation of this model’s predictions underscores the reliability of the structure activity correlations described here. In spite of the overall consistency of these observed trends, the most striking result of this structure activity relationship study is, however, the generally small effect of structural modifications on the inhibitors’ activity, suggesting a strong structural allowance for the interaction of diverse small molecule ligands with a binding site on c-Myc. This observation is also suggested by the structural diversity observed between hit molecules from original screens aimed at the inhibition of c-Myc-Max heterodimerization. A possible rationale for this unusual behavior can be provided by the intrinsic disorder features of the target
protein binding site: its inherent structural flexibility may allow for conformational rearrangements of its side chains and, unlike most folded binding sites, the backbone as well, to optimize their shape complementarity and network of interactions with the small molecule ligand. This explanation of the described experimental results provides a first insight about the nature and features of interactions between small molecules and intrinsically disordered proteins, underscoring favorable and previously unreported characteristics of this specific type of interaction, in particular the high structural versatility of ligand molecules capable of specific binding. While the studied binding events do not display high affinities, if the relative surface areas of interaction are taken into account, they are moderately favored compared to coupled binding and folding protein-protein interaction events.

2.5. Methods

**Compound synthesis.**

Preparation of RH and TZD compounds. All reagents and solvents were > 99% purity and were used as purchased (Sigma-Aldrich, St. Louis, MO; Fisher Scientific, Pittsburgh, PA). Spectroscopic information for all the described compounds is reported in the Appendix section. rhodanine or 2,4-thiazolidinedione (3 mmol) and 0.69 g ammonium acetate were dissolved with heating in 4.5 ml glacial acetic acid. The carbonylic substrate (1.1 equivalents) was slowly added. The mixture was refluxed for 1 to 12 hr, cooled to room temperature and diluted with 50 ml water to precipitate the product.
Compounds were characterized by 1H and 13C NMR. The reaction is stereoselective and only Z product is detectable by NMR.

Preparation of RH-NCN-1 compounds. rhodanine condensation product (0.5 mmol) was dissolved in 2 ml hot ethanol. Formaldehyde (37% w/V in water, 1.2 equivalents) and piperidine (1.1 equivalents) were added. Product started precipitating in ~5 minutes. The mixture was then cooled and further stirred at room temperature for 2 hours. The precipitate was collected, washed with water and characterized as above.

Preparation of 1RH-S-Me. rhodanine (10 mmol) was dissolved at room temperature in 25 ml 0.5 M aqueous NaOH. CH\textsubscript{3}I (1.1 equivalents) was then added dropwise. The mixture was stirred at room temperature for 5.5 hr, extracted with three 15 ml portions of dichloromethane, and washed with water. The product was crystallized from methanol.

Condensation of 1RH-S-Me with aldehydes. RH-S-Me (0.83 mmol) and 1.1 equivalents of aldehyde substrate were dissolved at room temperature in 2.5 ml anhydrous ethanol with triethylamine (115 \textmu l). The mixture was stirred at room temperature until precipitate formed (15 - 90 min), then diluted with 20 ml water and acidified to pH 4-5 with 1N HCl. The precipitate was collected, washed with water and characterized as above.
Nucleophilic substitution at 2,4-thiazolidinedione imide nitrogen. TZD condensation product (0.5 mmol) was dissolved in 5 mL acetone in the presence of 60 mg K$_2$CO$_3$. Allyl bromide (1.2 eq.) was added and the reaction heated to reflux for 3 hours; after cooling to room temperature, precipitate salts were removed by filtration and the solvent evaporated to yield > 85% product.

Reduction of 5-exo double bond. rhodamine condensation product (1 mmol) was dissolved in DMF at 0°C. LiCl (50 mg) was added, followed by 1.2 equivalents NaBH$_4$. The reaction mixture was gradually heated to reflux, refluxed for 10 min, then cooled to room temperature., quenched with diluted HCl. Crude product was extracted with dichloromethane and purified by reversed phase HPLC.

**Expression and purification of recombinant c-Myc353-437 and Max.**

A construct encoding the hexahistidine (His6)-tagged bHLHZip region of human c-Myc residues 353-437, was generated by Dalia Hammoudeh using QuickChange site directed mutagenesis (Stratagene, La Jolla, CA) to exclude a GGCD extension at the C terminus of the coding region from the c-Myc/pET SKB3 plasmid, kindly supplied by Dr. S. K. Nair (University of Illinois, Urbana-Champaign) and over-expressed in *E.coli* strain BL21DE3(plysS). His6 -tagged human Max isoforms, p22 Max (160 amino acids) and p21 Max (151 amino acids), both in the pQE-10 vector (Qiagen, Chatsworth, CA) (19,20), were ove-rexpressed in *E.coli* strain M15(pRep4). Briefly, bacterial cultures were grown at 37°C in LB to an OD$_{600}$~ 0.8 and then induced with
0.5 mM IPTG for 5 hours. Cultures were harvested and lysed in a buffer containing: 8 M urea; 100 mM NaH2PO4; 10 mM Tris; pH 8.0. Proteins were purified on an NTA-Ni column with a pH gradient elution. Max proteins were further purified by reversed-phase HPLC. The His6 tag of c- Myc353-437 was cleaved using TEV protease (previously expressed in a pET24 vector [from S.K. Nair] and purified on NTA-Ni-agarose under native conditions - 0.3 M NaCl; 50 mM NaH2PO4; pH 8.0; 10 mM to 250 mM imidazole gradient). The cleavage was performed in 50 mM Tris; 1 mM DTT; 1mM EDTA; pH 8.0 buffer, for 72-96 hours. The final c-Myc bHLHZip product was then further purified by HPLC and lyophilized.

**Fluorescence polarization assays.**

Samples of inhibitor at 25 μM and 50 μM concentration, in the absence and presence of an equimolar concentration of purified c-Myc353-437 peptide, were prepared in 1X PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.4 mM KH2PO4, pH 7.4); 1 mM DTT; 5% DMSO. The samples were analyzed in a Photon Technology International QuantaMaster fluorimeter (Birmingham, NJ) equipped with polymer sheet polarizers at an excitation wavelength of 380 nm and an emission wavelength of 468 nm. Alternative settings (ex: 470 nm; em: 600 nm), were employed for the compounds 7RH and 8RH, which have longer wavelength absorption and emission spectra. Each sample was analyzed in triplicate at 25°C with sample specific G-factor determination. Titration experiments were performed with the same instrumental settings, temperature, and buffer conditions upon two-fold serial dilution.
of equimolar mixtures of inhibitor and c-Myc353-437. Reported data represent the average of three to five independent experiments. Data were background-corrected and fit to a quadratic equation derived from the thermodynamic expression of binding equilibrium:

\[
\text{Eq. 1} \quad \frac{[\text{complex}]}{[C]_0} = \frac{2 + K_D/[C]_0 - \sqrt{(-2 - K_D/[C]_0)^2 - 4}}{2},
\]

where \([C]_0\) represents the total concentration of inhibitor and of c-Myc353-437. The value of \(K_D\) was determined from the experimental polarization data by fitting to Eq. 2 using KaleidaGraph (SynergySoftware, Reading, PA) where \(\text{pol}_0\) is the polarization in the absence of binding and \(\Delta\text{pol}\) is the total change in polarization [23].

\[
\text{Eq. 2} \quad \text{polarization} = \text{pol}_0 + \Delta\text{pol} \cdot \left( \frac{[\text{complex}]}{[C]_0} \right)
\]

**Electrophoretic mobility shift Assays (EMSAs).**

Experiments were performed on 8% polyacrylamide:bis-acrylamide (80:1) gels in 0.5 X TBE (44.4 mM Tris base, 44.4 mM boric acid, 10 mM EDTA, pH 8.0). Binding reactions were prepared in a buffer consisting of 1X PBS (pH 7.3); 1 mM EDTA; 0.1% NP40; 5% glycerol; 1 mM dithiothreitol; and 0.4 mg/mL BSA. A 22 base-pair E-box-containing double-stranded DNA oligonucleotide labeled on one strand with hexachlorofluoresceine (HEX) consisted of the following sequence: 5’-HEX-CACCCGGTCACGTGGCCTACAC-3’ and was synthesized by Integrated DNA Technologies Inc (Coralville, IA). The oligonucleotide was used at 10 nM
concentration in all reactions, which also contained 60 nM each of purified c-Myc bHLHZip, p21 Max, and the indicated amount of each compound. Samples containing proteins and inhibitor were first incubated for 90 min at 25°C, followed by addition of the oligonucleotide and an additional 15 minutes incubation before loading on a running gel. Similar control experiments were performed employing 100 nM p22 Max instead than a c-Myc bHLHZip – p21 Max mixture. Gels were run at 20°C and scanned on a BioRad FX molecular imager (BioRad, Hercules, CA). Data were analyzed with BioRad Quantity One software.

The results described in this chapter were published in part in:

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31. Mustata, G., A.V. Follis, D.I. Hammoudeh, S.J. Metallo, H. Wang, E. Prochownik, J.S. Lazo and I. Bahar (2009) Discovery of Novel Myc-Max Heterodimer Disruptors with a 3-Dimensional Pharmacophore Model. Journal of Medicinal Chemistry, 52(5), 1247-1250. Figure 9 has been adapted from this article with permission from ACS.


Chapter 3

Structural studies on the binding interaction between monomeric c-Myc bHLHZip domain and inhibitors of its dimerization with Max

3.1. Introduction

In the previous chapter structural plasticity and the flexibility of complexes between protein and small molecule ligands was suggested to explain the structural diversity of ligands and loose structure activity relationships inferred for interactions between the monomeric c-Myc bHLHZip domain and low molecular weight inhibitors of its dimerization with the partner protein Max. It has been further demonstrated, through experiments performed in this laboratory, that the structural diversity observed between such compounds depends, in some cases, on their interactions with different sites within the c-Myc bHLHZip sequence, each one comprising a short stretch of amino acids. Three such binding sites have been found: all seven specific inhibitors of c-Myc-Max heterodimerization and one c-Myc-Max/Id2-E47 dual specific compound originally described by Yin et al, interact with one of these sites [1, 2]. Two of these binding sites have been located by means of screening the interaction of the fluorescent inhibitors 10058-F4 and 10074-G5 with a series of truncated or mutated variants of c-Myc bHLHZip. These compounds had been demonstrated, by means of a fluorescence polarization experiment, to bind simultaneously to the full-length wild-type version of c-Myc bHLHZip, suggesting that they interact with two different sites on this protein. Fluorescence polarization was also exploited to monitor their binding to truncated and
mutated protein variants. The exact location of these sites has been confirmed by assessing the binding of 10058-F4 and 10074-G5 to two synthetic peptides narrowly encompassing each binding region: a c-Myc$_{402-412}$ segment was shown to interact with 10058-F4 with a binding affinity ($K_D = 13 \pm 1 \mu M$) close to that of the complex formation between 10058-F4 and the full length c-Myc bHLHZip domain ($K_D = 5.3 \pm 0.7 \mu M$), and a similar result was obtained for the binding between 10074-G5 and a c-Myc$_{363-381}$ segment ($K_D = 4.4 \pm 0.8 \mu M$, $K_D = 2.8 \pm 0.7 \mu M$) [1]. Several non-fluorescent inhibitors have been shown to bind to either of these sites based on their ability to compete with 10058-F4 or 10074-G5 and displace either fluorescent compound from c-Myc binding [2]. A third binding site, adjacent but C-terminal to the 10074-G5 site, has been located by means of screening the effect of the non-fluorescent inhibitor 10074-A4 on the circular dichroism spectrum of truncated variants of c-Myc bHLHZip. This inhibitor had been shown not to displace either fluorescent inhibitor from their respective binding sites on c-Myc [2]. The three binding sites found on the bHLHZip domain of c-Myc are located, moving from the N terminus to the C terminus, at the interface between basic region and helix 1, residues 366-375 interaction site of 10074-G5 and 10050-C10; between helix 1 and loop region, residues 374-385, interaction site of 10074-A4 and between helix 2 and leucine zipper, residues 400-409, interaction site of 10058-F4, 10031-B8, 10075-G5, 10009-G9 and of the dual c-Myc-Max / Id2-E47 inhibitor 10019-D3 (Figure 1a) [2]. These results indicate that, in spite of the distribution of structurally different molecules over multiple interaction sites within the c-Myc bHLHZip domain, several unrelated
compounds may also interact with the same site. Similarly, the series of derivatives of 10058-F4 described in the previous chapter showed loose structure activity relationships, and compounds bearing largely different modifications were still able to interact with the c-Myc bHLHZip domain with affinities which were not significantly higher or lower than that of the parent molecule, and likely bind to the same site as the latter.

The sequence analysis of the c-Myc bHLHZip domain, isolated or in the context of its alignment with other bHLHZip proteins, such as Max or proteins of the Mad family, provides clues to the preference for specific small-molecule binding to these specific sites. The binding sites lie at the border between wide regions of predicted disorder and two narrower ones of predicted order within the intrinsically disordered domain [3]. These sites with lower disorder probability, centered at the C termini of helix 1 and helix 2, are characterized by a higher content of hydrophobic residues compared to the rest of the c-Myc bHLHZip sequence (Figure 1b). Interestingly, only one, less pronounced, such region of low predicted disorder, located in correspondence of either helix1 or helix2, is observed in each of the other analyzed bHLHZip sequences, and the hydrophobic character of the latter is also reduced. Furthermore, the sequence alignment between multiple bHLHZip transcription factors makes clear the presence of residues that are uniquely non-conserved in the c-Myc sequence, but relatively conserved within the remaining sequences, especially within the small-molecule binding sites (Figure 1b, c). The results of such sequence analysis may be further generalized to suggest that reduced disorder probability and relatively
Fig. 1 Location and sequence characteristics of inhibitors binding sites on c-Myc. (continued on next page)
Summary of inhibitors binding sites locations found from direct binding studies between fluorescent inhibitors and mutated or truncated variants of c-Myc bHLHZip domain and from competition studies between fluorescent and non-fluorescent inhibitors. The sequence segments employed for the structural studies described in this chapter are outlined at the bottom of the panel. Disorder probability for the c-Myc bHLHZip domain assessed with the PONDR VSL2B algorithm [3]. Regions containing hydrophobic clusters and non-conserved residues are overlaid to the plot. Inhibitors binding sites are located in segments characterized by high hydrophobic content, reduced disorder probability and presence of non-conserved residues. Sequence alignment between c-Myc, Max and Mad proteins. Arrows indicate residues that are non-conserved specifically in c-Myc. Black arrows indicate such residues located within one inhibitors’ binding site.

High hydrophobicity, possibly correlated with some function in the natural network of interactions of a disordered protein [4], may generate suitable binding sites for small molecules, and specific sequence conservation patterns within such sites may be responsible for the specificity of such an interaction. With the aim of achieving a more detailed understanding of the mechanisms underlying such binding events, structural analyses were performed on some of the studied interactions between c-Myc and small molecules and will be discussed in this chapter.

3.2. Structural studies of independent binding sites

The localized nature of the studied binding interactions was exploited in their structural analysis by means of assessing the consequences of binding to synthetic peptides narrowly encompassing each binding site. This approach made it possible to identify unambiguously specific amino acids involved in each binding interaction. Circular dichroism and NMR spectroscopy were employed in these structural studies. The first technique provides loose information about the secondary structure
characteristics of each peptide under investigation. It was observed that changes induced upon binding of a small molecule ligand on the circular dichroism spectrum of short peptides narrowly encompassing its interaction site were more pronounced than those observed when the binding interaction occurred on the full length c-Myc bHLHZip peptide, as the spectrum of the latter remained practically unchanged after each binding event. This result strengthens the evidence of interactions occurring over short segments of the c-Myc bHLHZip domain sequence: as the overall CD signal is produced by the averaged signal of each residue over a protein’s sequence, conformational changes will be more noticeable when they involve a relatively high fraction of the overall sequence, as in the case of short synthetic peptides, than when they occur over a small portion of residues of a complete protein domain (Figures 3a, 7a, 10a,b).

The second technique employed for the described structural studies, NMR spectroscopy, is widely exploited in structural studies of biomolecules. Techniques have been developed to produce structural models of polysaccarides, nucleic acids, and proteins based on information extracted from their NMR spectra with computational aid [5-14]. A noticeable advantage of this approach over X-ray crystallography is the possibility of studying the structure and dynamics of biomolecules in solution rather than in tightly packed arrays with periodic repeats [8, 12, 15-17]. Traditionally, most structural information from NMR spectroscopy has been obtained from Nuclear Overhauser Enhancement (NOE) experiments [13, 18, 19]. These are indicative of magnetization transfer effects between NMR active nuclei that are in spatial proximity
to each other. NOE data can therefore be converted into distance constraints under the form of pseudo bonds to be employed during an annealing protocol in a molecular mechanics simulation [20-22]. For a structure determination, several such simulations are run to generate a cluster of solutions. Structural constraints from other experimental sources are also becoming commonly employed in such structure determination routines, in particular chemical shift and residual dipolar coupling information [23-28].

It has been empirically observed that the chemical shifts of a protein’s backbone NMR signals follow trends consistent with their secondary structure, in particular $\alpha$ proton signals move upfield from empirically determined reference random coil values when involved in $\alpha$ helical structure and downfield when they are located within $\beta$ sheets. A similar but reversed trend is observed for $\alpha$ carbon signals [29]. The dependence of backbone chemical shifts on the secondary structure of a protein was first exploited in the chemical shift indexing (CSI) approach [30, 31], as a way to characterize a protein’s secondary structure based on NMR data, and later developed in algorithms to generate dihedral constraints for the backbone $\Phi$ and $\Psi$ angles to be employed together with NOE constraints in solution structure determination [32, 33]. The correlation between chemical shifts and protein structure can be mathematically implemented based on two approaches. One consists in adapting standard ab initio computational techniques to the prediction of chemical shifts, by means of developing algorithms that relate a NMR active nucleus within a specific structural milieu to a local shielding environment, easily related to a predicted chemical shift, like other dedicated algorithms that predict the potential energies of chemical groups or atoms [24, 25, 34].
The correspondence between experimental data and chemical shift predictions from structural models may therefore be assessed in an iterative fashion to help advance the latter. A second, more empirical, approach, consists in relating specific chemical shifts patterns to a peptide’s expected backbone conformation based on chemical shifts databases of proteins with known structures [29, 32, 33]. In both cases the computational burden is highly reduced by calculating, when predicting the chemical shift of one residue from its structure or when predicting its backbone conformation from chemical shift data, contributions from the two amino acids adjacent to it, as it has been found that less proximal residues have insignificant effects [34].

A crucial aspect of NMR based structural studies of biomolecules with polymeric compositions of repeated units is the correct sequence assignment of resonance signals produced by each unit, amino acids in the case of proteins. In the NMR study of proteins several methods have been developed to achieve this goal, that take advantage of the multi-dimensional resolution of highly crowded spectra through magnetization transfer phenomena [35]. A straightforward way to generate sequence assignments for small proteins is based on the strong NOE effect observed between the \( \alpha \) proton of one amino acid and the amide proton of the next residue in a peptide sequence. The \( \alpha \) proton – amide proton region of a NOESY spectrum can be conveniently overlaid to the corresponding region of a COSY or TOCSY spectrum, which display cross peaks between protons coupled through bonds, therefore between \( \alpha \) proton and amide proton within one same amino acid. The overlaid spectra generate a pattern of cross peaks where NOE signals interconnect the COSY or TOCSY cross
peaks of adjacent residues by appearing at the $\alpha$ proton frequency of one residue and amide proton frequency of the following.

Other, complementary methods to obtain sequence assignments, often employed for the study of large proteins which may yield very crowded spectra even when resonances are distributed over two dimensions, exploit magnetization transfer between different nuclei of the peptide backbone in higher dimensionality experiments, thus relating backbone nuclei of one residue to those of an adjacent one. Due to the low gyromagnetic ratios and poor isotopic abundances of the required magnetically active nuclei, $^{13}$C and $^{15}$N, isotopic labeling is often required in order to perform such experiments [35-37].

The NMR studies of interactions between c-Myc segments and small molecule ligands were performed by means of comparing the spectral features of free peptides to those of bound ones, in particular $^{1}$H and $^{13}$C chemical shift indexing was performed. Sequential assignments for backbone signals were obtained by means of analyzing NOE cross peaks between $\alpha$ proton and amide proton of the successive residue, the proton assignments were then exploited in the successive assignment of $\alpha$ carbon signals in HMQC spectra. NOESY spectra were also collected in order to assess specific interactions between small molecules and peptides, or relative positioning of residues within the latter indicative of specific structural induction. These experiments are, however, poor in information when performed on short peptides inherently lacking a very rigid structure [23, 38], possibly even after a small-molecule binding event, and characterized by intermediate relaxation times, that may
lead to weak NOE effects. More reliance was therefore placed on chemical shift information, which was further exploited, besides the mentioned chemical shift indexing approach, to predict backbone and side chain dihedral orientations to be implemented in a molecular modeling routine. Models were generated for each studied peptide in its free and bound state with the PREDICTOR program [33], accompanied in part by the side chains rotamer library based scwrl3 program for modeling of side chain orientations [39]. Raw models were energy minimized in a water box using CHARMM parameters [40]. Molecular docking, performed with AutoDock4 software [41], was employed to generate models of the complexes by means of generating poses of small molecule ligands on models of the peptides in their bound conformation (Figure 2). The obtained models represent an average conformation of highly dynamic molecular species, free peptides and complexes alike, and do not capture such dynamic aspects of the studied systems, which may however be inferred from several features of the NMR spectra as well as other data.

3.2.1. Binding interaction between c-Myc and 10058-F4

The structural consequences of 10058-F4 binding on the conformation of the monomeric c-Myc bHLHZip domain were assessed by means of recording the protein’s CD spectrum in the absence and presence of inhibitor. It was noticed that little change was induced by the inhibitor’s binding on the CD spectrum of the 85 amino acid long full length c-Myc bHLHZip, while a noticeable effect was observed
Fig. 2 Flow-scheme of the procedure employed to generate structural models of the studied peptides, free and in complex with interacting inhibitors, based on chemical shift NMR information.
on the spectrum of the c-Myc402-412 peptide, narrowly encompassing the binding site of the compound (Figure 3a). A strong minimum at 207 nm, typical of random coil features disappeared from the spectrum [42], while the negative signal intensity slightly increased at longer wavelengths. These results, as mentioned above, are consistent with the localized nature of the studied binding interaction: the inhibitor’s binding to the short peptide induces conformational changes in a higher fraction of residues than when it occurs on the full length c-Myc bHLHZip domain, resulting in more pronounced changes of the circular dichroism spectrum of the shorter peptide. Furthermore, in the c-Myc402-412 peptide several conformational constraints and residual structure induced by amino acids flanking this sequence, would be absent, which might have effects on its conformation in the full length bHLHZip domain. These constraints might effectively bias the average backbone conformation of the target sequence within the full length bHLHZip domain in a similar fashion to that observed upon complex formation with the inhibitor, resulting in little conformational changes being induced by this event. More changes would be observed when the inhibitor binds c-Myc402-412, actively promoting structural induction in the fully disordered short peptide.

The binding interaction between c-Myc and 10058-F4 was studied in deeper detail by means of NMR spectroscopy. The spectra of c-Myc402-412 displayed differences in $^1$H and $^{13}$C chemical shifts for 4 backbone and several side chain signals upon addition of 10058-F4 (Figure 3b, c, Table 1). The splitting observed for Tyr$^{402}$ $\beta$ proton peaks indicated that complex formation induced these protons to become
diastereotopic. The $^1$H aromatic signals of Tyr$_{402}$ shifted upfield upon complex formation in both c-Myc402-412 and full-length c-Myc353-437, yet this ring, and the 10058-F4 aromatic moiety, were rotationally unconstrained (Figure 3d, e). Strong quenching (80%) and a blue shift (302 to 296 nm) in the emission maximum of the Tyr$_{402}$ fluorescence in the 10058-F4 complex with either c-Myc402-412 or c-Myc353-437 indicated proximity between the aromatic moieties of the peptide and inhibitor and a change in the surroundings of the tyrosine [43] (Figure 4). The NOESY spectrum of the c-Myc402-412-10058-F4 complex displayed 16 inter-molecular and 7 inter-residue cross-peaks (none of which are present in the NOESY spectrum of the free peptide), involving residues located at the N terminus of the peptide. These cross-peaks indicated the formation of a hydrophobic cluster comprised of side chains from Tyr$_{402}$, Ile$_{403}$, Leu$_{404}$, Val$_{406}$, Ala$_{408}$ and the aromatic ring and ethyl moiety of the inhibitor (Figure 5). The weak intensity and low number of cross-peaks are due to the intermediate relaxation time range of the small peptide. The paucity of NOESY signals from the C terminus suggested higher mobility in solution than the N terminal residues.

The collected chemical shift information was exploited to assess secondary structure trends of c-Myc402-412 in its free and bound states by means of $^1$Hα and $^{13}$Cα chemical shift indexing [30, 31]: peaks affected by complex formation moved further from random coil values in the same direction of the field as observed for their shift in the unbound state (Figure 6a, b). Such observations suggested a correspondence between the average conformation of the highly dynamic free peptide
Fig. 3 Conformational rearrangement of c-Myc402-412 peptide upon binding to 10058-F4. **a.** Effect on the circular dichroism spectrum of 20 μM peptide upon addition of 100 μM inhibitor (white: free, black: bound; inset shows the effect of the inhibitor’s binding to full length c-Myc bHLHZip – residues 353-437). **b.** Effect on the $^1$Hα – side chain region of the COSY spectrum of the peptide (blue: free, red: bound). **c.** Effect on the $^1$Hα - $^{13}$Cα region of the HMQC spectrum of the peptide. **d.** Effect on the aromatic region of the COSY spectrum (10058-F4 resonances are noticeable in the bound spectrum). **e.** Effect on the aromatic region of the COSY spectrum of full length c-Myc bHLHZip (adapted with permission from [1]).
Fig. 4 Quenching of Tyr402 fluorescence upon 10058-F4 binding. a. Effect on 50 μM c-Myc402-412 peptide (white: free, black: bound). b. Effect on 50 μM full length c-Myc bHLHZip.
Fig. 5 NOE information about the c-Myc402-412-10058-F4 interaction. a. Full NOESY spectrum of the peptide bound to the inhibitor. b. Close up on the aromatic region displaying cross peaks between Tyr402 and 10058-F4 aromatic protons. c. Sequence mapping of the detected cross peaks.
Fig. 6 Models of free and bound c-Myc402-412 based on backbone chemical shift information. a. Chemical shift indexing of $^1$Hα resonances (blue: free, red: bound). b. Chemical shift indexing of $^{13}$Cα resonances. c. Overlaid backbone representations of the free and bound models of the peptide. d. Docking model of the complex between 10058-F4 and c-Myc402-412 (adapted with permission from [1]).
and the more rigid bound state, possibly related to the presence of local conformational constraints in the free peptide [44, 45]. This observation is consistent with the hypothesis that, in the full-length c-Myc bHLHZip domain, such residual structural features ought to be more pronounced, therefore resulting in limited structural induction when the small molecule binds to this protein compared to the c-Myc402-412 peptide. Due to the limited NOESY information it was not possible to effectively employ distance constraints to generate NMR structures; instead, models of the peptide in its free and bound states were obtained using chemical shift-based dihedral constraints [33]. A docking simulation was then performed between the bound structure and 10058-F4, with the resulting model intended to represent one reasonable conformation out of the likely dynamic ensemble constituting the complex. In the complex model the peptide conformation did not correspond to that found in the c-Myc-Max crystal structure, thus clearly demonstrating that the product of a coupled folding and binding reaction may not be useful in predicting potential small-molecule binding sites or their conformations. Furthermore, the conformation of the peptide in the bound form appears incompatible with formation of the HLHZip interface and provides a rationale for dimer inhibition (Figure 6c). In the c-Myc402-412–10058-F4 docking, the inhibitor was located at the center of a C shaped cavity, in an orientation that allowed for hydrophobic interactions to take place between its aromatic ring and ethyl tail and the peptide’s N-terminal hydrophobic side chains. The carbonyl oxygen of 10058-F4 was within hydrogen bonding range with Ser_{406} and Gln_{407} side chains.
(Figure 6d). Although generated independently, this model matched the NOESY indication of hydrophobic interactions.

3.2.2. Binding interaction between c-Myc and 10074-G5

This interaction was first studied, as in the case of 10058-F4, by means of circular dichroism. Also in this case it was observed that more pronounced changes were induced upon ligand binding on the spectrum of the c-Myc363-381 peptide, narrowly encompassing the binding site of 10074-G5, than on that of the full-length c-Myc bHLHZip domain. The observed changes in the peptide’s spectrum were similar to those observed on the spectrum of c-Myc402-412 upon binding of 10058-F4: a minimum at 207 nm, indicative of the random coil state of the free peptide, was strongly reduced in intensity (Figure 7a). NMR analysis of complex formation between 10074-G5 and c-Myc363-381 showed changes in the chemical shift of 10 Hα, 11 Cα signals and the terminal side chain resonance of two Arg residues (Figure 7b, c, Table 2). The larger number of backbone chemical shift differences in the 10074-G5 complex indicated that more residues underwent a conformational change in this complex. A convergence to ~ 7.4 ppm of the ⁱH shifts of the inhibitors’ hetero-aromatic moiety, observed at ~7.9 and ~6.2 ppm in the free compound, showed an altered chemical environment for this ring (Figure 7d). The NOESY spectrum of the complex showed 25 inter-residue cross-peaks, which were absent in the free peptide, including a pattern of signals between residues three positions away indicating an α-helical conformation within Leu₃₇₀-Leu₃₇₇ (Figure 8). No NOESY cross-peaks between nonadjacent
Fig. 7 Conformational rearrangement of c-Myc363-381 peptide upon binding to 10074-G5. **a.** Effect on the circular dichroism spectrum of 20 µM peptide upon addition of 100 µM inhibitor (white: free, black: bound; inset shows the effect of the inhibitor’s binding to full length c-Myc bHLHZip – residues 353-437). **b.** Effect on the $^1$Hα – side chain region of the COSY spectrum of the peptide (blue: free, red: bound). **c.** Effect on the $^1$Hα - $^{13}$Cα region of the HMQC spectrum of the peptide. **d.** Effect on the aromatic region of the COSY spectrum of the peptide (10074-G5 resonances in the bound spectrum are overlapped with the aromatic phenylalanine signals; adapted with permission from [1]).
Fig. 8 NOE information about the c-Myc363-381-10074-G5 interaction. **a.** Full NOESY spectrum of the peptide bound to the inhibitor. **b.** Sequence mapping of the detected cross peaks. Ambiguously assigned cross peaks are displayed at any possible position with fractional values determined by the total number of possible assignments.
**Fig. 9** Models of free and bound c-Myc363-381 based on backbone chemical shift information. **a.** Chemical shift indexing of $^1$Hα resonances (blue: free, red: bound). **b.** Chemical shift indexing of $^{13}$Cα resonances. **c.** Overlaid backbone representations of the free and bound models of the peptide. **d.** Docking model of the complex between 10074-G5 and c-Myc363-381 (adapted with permission from [1]).
residues were observed in the free peptide. The signal overlap between the aromatic signals of phenylalanine residues and inhibitor prevented the unambiguous assignment of inter-molecular cross-peaks. The NMR data was analyzed, as was done for the c-Myc402-412-10058-F4 interaction, by means of chemical shift indexing and further employed to generate dihedral constraints for molecular modeling. Again $^1$Hα and $^{13}$Cα chemical shift indexing showed that peaks affected by complex formation generally moved further from random coil values in the same direction of the field as observed for their resonances in the free state, suggesting the possibility of a preferred average conformation of the dynamic free peptide that roughly matches that observed in its more rigid bound state (Figure 9a, b). Molecular models of the c-Myc363-381 peptide in its free and bound state also supported this indication (Figure 9c). The docking between c-Myc363-381 and 10074-G5 displayed the inhibitor enclosed in a cavity generated by a kink at the N-terminus of a helical segment spanning from Leu$_{370}$ to Arg$_{378}$, its biphenyl moiety close to the aromatic ring of Phe$_{375}$ and the electron-rich hetero-aromatic and nitro moieties interacting with the positively charged Arg$_{366-367}$ (Figure 9d). This model also agreed with the independent NOESY results, which indicated the induction of a helical segment between Ser$_{373}$ and Leu$_{377}$ upon complex formation.

3.2.3. Binding interaction between c-Myc and 10074-A4

The binding interaction of 10074-A4 with monomeric c-Myc bHLHZip was of interest as this was the only compound out of five non-fluorescent specific inhibitors of
c-Myc-Max heterodimer formation first reported by Yin et al. that failed to compete either fluorescent inhibitor 10058-F4 or 10074-G5 from their respective binding site on c-Myc. Circular dichroism was therefore exploited to locate the interaction site of this compound on c-Myc, based on the observation, inferred from the previously studied interactions, that noticeable changes can be observed in the circular dichroism spectrum of a peptide upon binding of a small-molecule ligand, provided that this event affects a large enough fraction of the residues represented in the peptide sequence. The analysis of circular dichroism spectra of several truncated segments of the c-Myc bHLHZip domain in the absence and presence of 10074-A4 indicated the c-Myc370-409 peptide as the segment more narrowly encompassing this inhibitor’s binding site (Figure 10a, b). Interestingly, a strong induced circular dichroism effect was observed on the small molecule upon binding, possibly due to the diastereoselective interaction of one enantiomer of this molecule, characterized by one stereogenic center, with the chiral peptide [46]. In order to characterize more specifically the structural features of the binding interaction between 10074-A4 and its deduced binding site, NMR spectroscopy was employed on c-Myc370-409. As before, the backbone $^1$H assignments for the pure peptide were obtained from $^5$H$_i$-NH$_{(i+1)}$ NOESY cross peaks; proton information was then mapped on to a $^1$H-$^{13}$C HMQC spectrum to obtain $^{13}$C$_\alpha$ assignments. Addition of an excess racemic mixture of 10074-A4 to the peptide caused changes in backbone chemical shifts of residues predominantly in the helix-1 and loop regions (Figure 10c, d, Table 3), suggesting that the exact location of the interaction site for this compound is near but C-terminal to
**Fig. 10** Conformational rearrangement of c-Myc370-409 peptide upon binding to 10074-A4. **a.** Effect of the addition of 100 µM inhibitor on the circular dichroism spectrum of the peptide (20 µM). Notice the induced circular dichroism band generated by the small molecule in the bound spectrum (white: free, black: bound). **b.** Effect of the inhibitor’s (100 µM) binding on the circular dichroism spectrum of full length c-Myc bHLHZip – residues 353-437 (20 µM). **c.** Effect on the $^1$Hα – side chain region of the TOCSY spectrum of the peptide (blue: free, red: bound). **d.** Effect on the $^1$Hα - $^{13}$Cα region of the HMQC spectrum of the peptide (adapted with permission from Hammoudeh et al. [2]).
that of 10074-G5. NOESY spectra of the free and bound peptide indicated strong structural induction upon complex formation with 10074-A4 as shown by the increased number of crosspeaks in the $^1$H$_\alpha$ region. The NOESY spectrum of the complex confirmed the presence of hydrophobic interactions between the inhibitor and mainly hydrophobic groups on the peptide (residues Leu377, Ile381, and Leu384; interactions are also observed with Arg378 and Asp 379 residues) located in this region (Figure 11). As with the other two binding sites, the backbone chemical shift information was used to assess secondary structure trends in the peptide by means of chemical shift indexing, and was further employed to generate dihedral constraints for molecular modeling with the PREDITOR program [33]. Also in this case the obtained models represent a likely average conformation of the peptide from an ensemble of peptide structures in its bound state and more dynamic free-state. The $^1$H CSI [30] of the free peptide reveals a pattern of mixed downfield (typical of $\beta$-sheet structures) and upfield (typical of $\alpha$-helices) shifts with respect to random coil values alternating with segments of residual helical content, as also indicated by the $^{13}$C CSI [31] (Figure 12a, b). Such a pattern, considered typical of coil conformations, could be associated with regions displaying residual structure in the presence of local conformational constraints, as opposed to a more dynamic random coil state, where the backbone chemical shifts would more consistently match the expected random coil values [23, 38, 44, 45, 47]. The models of the peptide in its ‘free’ and ‘bound’ state generated from dihedral constraints suggest the formation of a cavity at the N terminus of the loop region, flanked by Phe374/375, Ala376 and Leu377 in a helical conformation at the N terminus of helix-1.
Fig. 11 NOE information about the c-Myc370-409-10074-A4 interaction. 

a. Full NOESY spectrum of the peptide bound to the inhibitor. 


c. Sequence mapping of the detected cross peaks (light grey: i-i+3, dark grey: i-i+1, hatched: inter molecular; adapted with permission from Hammoudeh et al. [2]).
Fig. 12 Chemical shift analysis of free and bound c-Myc370-409 peptide. 

a. Chemical shift indexing of $^1$H$\alpha$ resonances (blue: free, red: bound). 
b. Chemical shift indexing of $^{13}$C$\alpha$ resonances. 
c. Chemical shift indexing of $^{13}$C$\beta$ resonances.
Fig. 13 Models of free and bound c-Myc370-409 based on backbone chemical shift information. **a.** Overlaid backbone representations of the free and bound models of the peptide. **b.** Docking model of the complex between 10074-A4 and c-Myc370-409 (adapted with permission from Hammoudeh et al. [2]).
sequence (Figure 13a, b). Although determined independently, the model of the bound state is highly consistent with the indication of α-helical segments from NOE cross peaks. A comparison of the free and bound models indicates that the relative repositioning of two segments, roughly corresponding to residues from the helix-1 and loop regions, generates a conformation favorable to binding. Molecular docking of the inhibitor to the bound model suggests a possible mode of binding of the compound to the described site favored mainly by a series of hydrophobic interactions. There are an unusually high number of hydrophobic residues in the helix1-loop segment of the c-Myc bHLHZip compared to the entire domain (7 out of 11 amino acids, or 64% in this segment, versus 31 out of 85, or 36% in the entire domain). The docking of both enantiomers displayed a similar mode of binding and similar docking scores (0.3 kcal mol\(^{-1}\) in favor of the S enantiomer). Docking poses for both enantiomers are generally consistent with the independently generated NOE data. This simulation provided a general understanding of the binding interaction but cannot generate precise binding information or identification of a favored binding enantiomer.

3.3. NMR binding studies on full-length c-Myc bHLHZip

The structural studies performed on synthetic or recombinant (as in the case of c-Myc370-409) peptides encompassing segments of the c-Myc bHLHZip domain sequence within which the binding sites of three different small molecule ligands were located were further validated by analyzing the effect of the same binding interactions on full length c-Myc bHLHZip. Partial \(^1\)H backbone assignments for c-Myc353-437
were obtained, taking advantage of the low redundancy of some amino acids found within or in proximity of each binding site (Ser373, Phe374, Phe375, Asp379, Pro382, Thr400, Tyr402, and Ser405). The assigned resonances allowed identification of changes in the c-Myc353-437 peptide corresponding to those observed in c-Myc402-412 upon complex formation with 10058-F4 (Figure 14a). A change in shift and shape of the aromatic signals of 10058-F4 was also observed in this case, possibly due to its increased segregation from the solution environment (Figure 3d). The similar behaviors of the minimal c-Myc402-412 peptide and the larger c-Myc353-437 bHLHZip domain confirmed that the binding interaction with 10058-F4 caused only local perturbations around residues 402-412 and that flanking residues remained largely unaffected. The NMR study of 10074-A4 binding had previously indicated that its interaction site on c-Myc was adjacent to that of the compound 10074-G5. We therefore tested whether the binding of one of these compounds to c-Myc would alter the affinity of the other. The binding between the fluorescent compound 10074-G5 and the c-Myc bHLHZip domain was monitored by FP upon serial dilution of a 1:1 protein-inhibitor mixture in the presence of a constant excess (100 µM) of the nonfluorescent compound 10074-A4. The affinity value observed (K_{obs} = 3 ± 1 µM) for 10074-G5 under these conditions was the same as its c-Myc affinity in isolation (K_D = 2.8 ± 0.7 µM), demonstrating that the binding of this compound was not affected by the presence of 10074-A4. This result indicated that the binding sites on c-Myc are truly independent and that even two adjacent and closely spaced binding sites could independently interact with their target inhibitor, most likely because of the scarcity of
Fig. 14 $^1$Hα – side chain region of COSY spectra of full length c-Myc bHLHZip (residues 353-437) free and bound to different inhibitors. a. Overlaid spectra of the free protein (blue) and bound to 10058-F4 (red). Assigned resonance shifts are indicated with arrows. Black arrows indicate resonance shifts also observed in c-Myc402-412 upon binding to 10058-F4. b. Overlaid spectra of the free protein (grey), bound to 10058-F4 (red), 10058-F4 and 10074-G5 (green), 10058-F4, 10074-G5 and 10074-A4 (dark blue, adapted with permission from Hammoudeh et al. [2]).
medium or long distance conformational constraints in the disordered state of the protein. This conclusion was supported by NMR studies on c-Myc353-437 in its free form and upon the sequential addition of the three inhibitors 10058-F4, 10074-G5 and 10074-A4 (Figure 14b). Samples were also analyzed containing c-Myc353-437 and 10074-G5 with further addition of 10074-A4. Chemical shift changes similar to those observed upon complex formation with peptide segments spanning the isolated binding sites (c-Myc402-412 for 10058-F4, c-Myc363-381 for 10074-G5, and c-Myc370-409 for 10074-A4) were observed in the full c-Myc bHLHZip domain upon binding of each compound. The changes in chemical shifts induced by each binding event appeared to be independent from each other, and only the residues Phe374, Phe375, Asp379, and Ile381 were affected by both 10074-G5 and 10074-A4 binding (Figure 15) although, as determined by affinity measurements, there is no competition for binding between the two compounds. These experiments provided a partial sequence mapping of the conformational rearrangements caused by each compounds’ binding to the full-length c-Myc bHLHZip, and support the idea of local and independent binding interactions.

3.4. Conclusion

In the present chapter results have been reported elucidating several structural aspects of the interaction between multiple segments of the monomeric c-Myc bHLHZip domain and diverse small molecules interacting with them. The indication of binding events occurring over local segments of sequence, encompassing narrow stretches of adjacent amino acids has been thoroughly confirmed in all the studied
Fig. 15 NMR evidence of simultaneous binding of multiple inhibitors to the full length c-Myc bHLHZip domain (continued on next page).
Fig. 15 a. Sequence aligned chemical shift indexing of $^{1}$H$_{\alpha}$ resonances for the three studied peptides free and in complex with their respective inhibitors (c-Myc363-381-10074-G5; c-Myc402-412-10058-F4; c-Myc370-409-10074-A4) and for the partially assigned c-Myc353-437 resonances. Black bars are used for the free peptides, colored ones for residues that display changes in chemical shift (>0.02 ppm) upon binding of each inhibitor. Values for residues that remain unchanged upon complex formation are indicated as white bars. The bound values in the c-Myc353-437 plot are deduced from the protein spectrum in the presence of all three inhibitors; the color-coding for this plot is deduced from the observed changes in chemical shift upon sequential addition of these compounds. Residues that displayed changes in chemical shift upon addition of both 10074-G5 and 10074-A4 (Phe374/375, Asp379, Ile381) are indicated in shaded blue-green. b. Model of the simultaneous binding of 10074-F4 (red), 10074-G5 (green), and 10074-A4 (blue; adapted with permission from Hammoudeh et al. [2]).

cases by means of sequence mapping of chemical shift changes and appearance of NOESY cross peaks upon formation of each complex. General information about the nature of the interactions responsible for binding has been collected, and further developed through the use of molecular modeling of the studied complexes. The recorded data suggest binding events largely driven by hydrophobic interactions, which make possible the desolvation of solvent exposed apolar moieties. These interactions might be particularly relevant in binding interactions involving intrinsically disordered proteins, as the solvent exposure of hydrophobic moieties tends to be more pronounced in this case than in folded proteins. Several polar, or hydrogen bonding, interactions, that may play a more relevant role than hydrophobic ones in determining the ligands’ positioning and orientation, appear to further contribute to the inhibitors’ binding affinity and specificity. Strong indications were obtained of the consistent presence of residual conformational flexibility in peptides and small-molecule ligands alike, upon complex formation. The reported data represent the first experimental structural
characterizations of interactions between small molecules and intrinsically disordered protein segments, and contribute to elucidate several atypical aspects of these binding events, which are consistent throughout the studied cases, and are in sharp contrast with structural features of binding interactions involving stably folded proteins.

3.5. Methods

**Synthesis and purification of c-Myc402-412 and c-Myc363-381 peptide.**

The c-Myc402-412 peptide was synthesized at the University of Delaware in the laboratory of Prof. Neal Zondlo using standard Fmoc solid phase synthesis on a Rainin PS3 automated peptide synthesizer. The peptide was synthesized as the C-terminal amide (Rink amide resin) and was acetylated at the N-terminus while still attached to the resin (via removal of the terminal Fmoc and treatment with acetic anhydride). The peptide was cleaved from the resin using 92.5% trifluoroacetic acid (TFA), 2.5% triisopropyl silane, and 5% water. The c-Myc363-381 peptide was synthesized by the University of Vermont Protein Core Facility and was delivered as the acetylated and amidated lyophilized crude peptide. Each peptide was redissolved in water, filtered, and purified to homogeneity by reversed phase HPLC (Vydac C18) using a water/acetonitrile gradient containing 0.1% TFA. The concentration of c-Myc402-412 was determined by its absorbance at 280 nm. The concentration of c-Myc363-381, which lacks appreciable absorbance at 280 nm, was assessed by integration of the HPLC 215 nm peak against a bovine serum albumin standard after normalization of the extinction coefficients of the two peptides.
Expression and purification of c-Myc370-409 peptide

The coding region of c-Myc370-409 was generated by Dr. Prochownik, University of Pittsburgh, via PCR amplification from the sequence encoding for eMyc353-439, followed by cloning into pET151D-TOPO vector (Invitrogen). This vector codes for a hexahistidine (6xHis)-tag which is separated by a TEV protease digestion site from the N terminus of the insert. The protein was over-expressed in *E. coli* BL21DE3(pLysS). Bacterial cultures were grown at 37 °C in LB medium to OD₆₀₀≈ 0.8, then induced with 0.5 mM IPTG for 5 hours. Cultures were harvested and lysed in a buffer containing: 8 M urea; 100 mM NaH₂PO₄; 10 mM Tris; pH 8.0. The protein was purified on a Ni-NTA agarose (Qiagen) column with a pH gradient elution and desalted with a Amicon Ultra centrifuge membrane (Millipore). The 6xHis tag was cleaved using TEV protease. The digested protein was further purified by reverse phase HPLC (Vydac C18) using a water/acetonitrile gradient containing 0.1% TFA and then lyophilized. The protein concentration was determined by measurement of OD₂₈₀.

Fluorescence Measurements.

Emission spectra (275 nm – 500 nm, excitation= 270 nm) of tyrosine fluorescence of triplicate samples containing 50 µM c-Myc402-412 or c-Myc353-437 in the absence and presence of equimolar 10058-F4 in 1X PBS (pH 7.4) were recorded on a Photon Technology International Quanta Master fluorimeter at 25°C. The inhibitor was added from a 3.3 mM stock solution in DMSO. Blank spectra recorded from pure 1X PBS and 50 µM 10058-F4 in 1X PBS were subtracted from the spectra of corresponding c-Myc-containing samples.
Circular Dichroism.

Samples of c-Myc402-412 or c-Myc363-381 (20 µM) in the absence and presence of 10058-F4 or 10074-G5 respectively were prepared in 5 mM sodium phosphate, pH 7.4; 50 mM KF. Samples of c-Myc370-409 (20 µM) in the absence and presence of 10074-A4 were prepared in 1X PBS (pH 7.4). The inhibitors were added from 10 mM stock solutions in ethanol. Spectra were recorded at 25 °C in a 1 mm path-length quartz cuvette on a Jasco J710 spectropolarimeter. Shown spectra are averaged from five independent samples. The effect of the inhibitors was also monitored on 10 µM samples of c-Myc353-437.

NMR spectroscopy c-Myc363-381, Myc402-412.

Experiments were performed on a 500 MHz magnet Varian INOVA instrument equipped with a 5 mm double resonance indirect detection probe. TOCSY and NOESY spectra of the pure c-Myc402-412 peptide in 90% H2O – 10% D2O were recorded by Prof. Neal Zondlo, University of Delaware, on a 600 MHz magnet Bruker AVC instrument equipped with a 5 mm TXI triple-resonance indirect detection probe. Peptide samples in the absence or presence of inhibitor were prepared in 100% D2O, 5 mM sodium phosphate buffer, pH 7.5 or 90% H2O – 10% D2O, 5 mM sodium phosphate buffer, pH 6.3 (for H_i(i) – H_N(i+1) NOE sequential assignments). All samples used for NOESY experiments were degassed by sonication and purged with nitrogen. The final peptide concentration was ~0.2 mM for c-Myc402-412 samples, ~0.5 mM for c-Myc363-381. 10058-F4 or 10074-G5 were added to peptide solutions
from a 0.1 M stock in DMSO-d$_6$ to a final concentration of ~0.5 mM. Complete backbone $^1$H resonance assignments for c-Myc402-412 (Table 1) and c-Myc363-381 (Table 2) were obtained from TOCSY, COSY and $H_{\alpha}(i)$ – $H_N(i+1)$ NOEs of low pH, 90% H$_2$O – 10% D$_2$O samples of the pure peptides (Figure 16a, b). $^{13}$C resonance assignments for the first two peptides were obtained from HMQC of 100% D$_2$O samples, pH 7.5. Spectra of the pure peptides were not affected beyond the disappearance of amide and amine signals within the explored pH range. Two-dimensional $^1$H homonuclear and $^1$H-$^{13}$C HMQC spectra were recorded at 25°C over sweep widths of ~10X10 ppm (~140X10 $^{13}$C) with 16-64 scans/t$_1$ increment, 1.5-2 s relaxation delay and sizes of 512-1024X2048 complex points. NOE mixing times of 300 and 250 ms were employed for c-Myc402-412 and c-Myc363-381 respectively. All spectra were acquired with gradients or selective presaturation water suppression. A TOCSY mixing time of 60 ms was used in all cases. A C-H 1-bond coupling constant of 140 Hz was used for HMQC spectra, acquired on natural $^{13}$C abundance samples. Spectra were processed using MestReC software (MestreLab Research, Santiago de Compostela, Spain). Data were filled by linear prediction to a final Fourier transform size of 2048x2048 points and weighted by sine square and sine bell apodization over $t_1$ and $t_2$ respectively before Fourier transformation. A covariance NMR algorithm [48] was used in addition to the standard weighting functions in processing the NOESY spectra. Chemical shifts were referenced to a trimethylsilylpropionate standard.

Experiments were performed on a 500 MHz magnet Varian INOVA instrument equipped with a 5 mm double-resonance indirect detection probe. All experiments were performed at 25 °C. Samples containing c-Myc370-409, in the absence and presence of 10074-A4, or c-Myc353-437 in the absence and presence of 10058-F4 or 10074-G5 only; 10058-F4 and 10074-G5; 10074-G5 and 10074-A4; 10058-F4, 10074-G5 and 10074-A4, were prepared in 100% D₂O, 5 mM sodium phosphate buffer, pH 7.5. Samples of the pure proteins were also prepared in 90% H₂O – 10% D₂O, 5 mM sodium phosphate buffer, pH 6.3. All samples used for NOESY experiments were degassed by sonication and purged with nitrogen. The final peptide concentration was ~0.5 mM. Each small molecule was added to peptide solutions from a 0.1 M stock in DMSO-d₆ to a final concentration of ~1 mM. Complete backbone ¹H resonance assignments for c-Myc370-409 (Table 3) and partial assignments for c-Myc353-437 were obtained from TOCSY and Hα(i) – HN(i+1) NOEs of low pH, 90% H₂O – 10% D₂O samples (Figure 16c-f). ¹³C resonance assignments for c-Myc370-409 were obtained from HMQC of 100% D₂O samples, pH 7.5. Spectra of the pure peptides were not affected beyond the disappearance of amide and amine signals within the explored pH range. All spectra were acquired over similar sweep widths of ~10 ppm in t₁ and t₂ for homonuclear experiments, or ~140 ppm in t₁ for HMQC experiments, and sizes of 512-1024x2048 complex points. COSY spectra were acquired with water suppression using selective presaturation and 16-32 scans per t₁ increment and a relaxation delay of 1.5 to 2.5 s. TOCSY spectra were acquired, with water suppression
using TNTOCSY on resonance presaturation, 64 scans per $t_1$ increment, a relaxation delay of 1.5 to 2.5 s and a TOCSY mixing time of 60 ms. NOESY spectra were acquired with TNNOESY presaturation, 64 scans per $t_1$ increment, a relaxation delay of 1.5 or 2.5 s and a NOESY mixing time of 150 ms. HMQC spectra were acquired on natural 13C abundance samples using on resonance water pre-saturation, 128 scans per $t_1$ increment, a abundance samples using on resonance water pre-saturation, 128 scans per $t_1$ increment, a relaxation delay of 2.0 seconds, with a C-H 1-bond coupling constant of 140 Hz. Spectra were processed using MestReC software (MestreLab Research, Santiago de Compostela, Spain). Data were filled by linear prediction to a final Fourier transform size of 2048x2048 points and weighted by sine square and sine bell apodization over $t_1$ and $t_2$ respectively before Fourier transformation. Chemical shifts were referenced to a trimethylsilylpropionate standard.

Molecular Modelling of c-Myc402-412, c-Myc363-381 and c-Myc370-409 peptides in their free and bound state, and in complex with respective inhibitors.

Scwrl3 [39] software was employed for assessment of side chains orientation in the free and bound average conformation models of c-Myc402-412, c-Myc363-381 and c-Myc370-409, generated from approximate $\Phi - \Psi$ backbone and $\chi_1$ side-chain angles obtained from $^1$H$\alpha$, $^{13}$C$\alpha$, $^{13}$C$\beta$ (and $^1$HN for the free peptides) chemical shift values using the web server PREDITOR [33] (Tables 4, 5, 6). Energy minimization was carried on for 10000 time-steps in an automatically generated cubic water box (PSFgen) using CHARMM27 [40] parameters implemented in NAMD2 [49] software.
Three glycine residues were appended at the N and C termini of c-Myc\textsubscript{402-412} models in order to reduce their electrostatic attraction during energy minimization. Due to the lack of PREDITOR constraints for terminal residues a beta sheet starting conformation of Tyr\textsubscript{402}, shown to have a role in 10058-F4 binding, was estimated from the lack of cross peaks between its aromatic signals and terminal methyl groups of Ile\textsubscript{403} or Leu\textsubscript{404}. The minimized conformer structures of both peptides displayed no bad parameters after a PROCHECK validation test [50]. The inhibitors were flexibly docked to the bound conformation model of their respective binding site using AutoDock LGA algorithm [41]. Docking between 10074-A4 and c-Myc\textsubscript{370-409} will be discussed in detail in the next section. In the docking between the complex conformer structure of each peptide and 10058-F4 or 10074-G5, selected side chain rotamers, chosen upon experimental indications and results of preliminary rigid docking, and all the rotating bonds of 10058-F4 or 10074-G5 were kept flexible during docking. The following side chains were unconstrained except for \( \chi_1 \) rotation: for c-Myc\textsubscript{402-412}- Tyr\textsubscript{402}, Ser\textsubscript{405}, Gln\textsubscript{407}, Gln\textsubscript{411}; for c-Myc\textsubscript{363-381}- Arg\textsubscript{366} Glu\textsubscript{369}, Arg\textsubscript{372}, Phe\textsubscript{375}. The best poses were geometrically optimized using UFF parameters [51] to 0.1 kcal/molÅ convergence. The final complex models were validated with PROCHECK analysis. Structural representations were prepared using PyMol software (DeLano Scientific LLC, South San Francisco, CA).

**Docking between c-Myc\textsubscript{370-409} segment in the bound state and 10074-A4.**

Docking between the complex conformer structure of the peptide and both enantiomers
of 10074-A4 was performed using the AutoDock LGA algorithm [41]. A test docking of 25 runs was performed using a 50 point side cubic energy grid with 1 Å/point resolution to assess pose clustering. A 60 point side cubic energy grid with 0.375 Å/point resolution, centered on the expected binding site (based on NMR information), was then used for energy scoring in the final docking. A total of 10 docking runs with an initial population of 150 random conformations were performed with 2500000 energy evaluations each. Selected side chain rotamers, chosen upon experimental indications and results of preliminary rigid docking, and all the rotating bonds of 10074-A4 were kept flexible during docking. The following side chains were unconstrained except for χ1 rotation: Phe374, Leu377, Arg378, Gln380, Ile381, Glu383, Leu384, Glu385. The best poses were geometrically optimized using UFF parameters [51] to 0.1 kcal mol⁻¹ Å⁻¹ convergence. The final complex models were validated with PROCHECK analysis [50].

**Sequence Analysis of the Binding Sites.**

An alignment of the bHLHZip region of human c-Myc was performed using the ClustalW algorithm [52] with default settings to the bHLHZip of Max (NCBI accession code NP66092.1), Mad1 (NP002348.1), Mad3 (NP112590.1), Mxi (NP569157.2), Mad4 (NP06445.1). Nonconserved residues were identified according to the following criteria: i) ≥60% conservation within aligned bHLHZip excluding c-Myc, ii) c-Myc residue dissimilar from any other sequence in the corresponding position. Out of 22 nonconserved residues found in the 84 residues long bHLHZip
sequence (26% rate), 12 were located in one of the three inhibitors' binding sites (47% rate). Highly conserved residues (≥80%) were found in 28 positions (33%).

The bHLHZip sequences of c-Myc, Max, Mad1, Mad3, Mxi and Mad4 were searched for clusters of hydrophobic residues (aliphatic or aromatic side chain): three such clusters of 4 amino acids were found in c-Myc: F_{374}FAL, V_{393}VIL, A_{401}YIL, one of 3 amino acids in the zipper region of Max (ALL) and one in the loop of Mad1,3,4 (LV/IPL). The first cluster in c-Myc is located within the binding regions of 10074-G5 and 10074-A4, the last within that of 10058-F4. The same bHLHZip sequences were analyzed for disorder prediction with the PONDR® VSL2B algorithm [3]. Two regions of predicted low disorder were found in c-Myc but not in the other bHLHZip proteins in correspondence of the binding sites. Mutations of c-Myc residue at the interface between helix2 and leucine zipper, which reduced 10058-F4 binding affinity, also resulted in increased disorder prediction in the binding segment. Mutations at the interface between basic region and helix-1, which increased the binding affinity of 10074-G5 [1], did not increase the disorder probability.

Synthesis of 10058-F4, Expression and purification of c-Myc353-437 are described in Section 2.5. Compounds 10074-G5 and 10074-A4 were purchased from ChemBridge corporation (San Diego, CA).
Fig. 16 $^{1}HN -^{1}Ha$ region of NOESY and TOCSY spectra of the studied peptides, collected from 90% H$_2$O-10% D$_2$O samples, pH 6.5, employed for sequential backbone assignments. a. NOESY spectrum of c-Myc402-412. b. NOESY spectrum of c-Myc363-381. c. TOCSY spectrum of c-Myc363-381. d. NOESY spectrum of c-Myc370-409. e. TOCSY spectrum of c-Myc370-409. f. NOESY spectrum of c-Myc353-437.
Table 1 Resonance assignments for c-Myc402-412 peptide free and in complex with 10058-F4. Chemical shifts were referenced to a trimethylsilylpropionate standard.

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Table 2 Resonance assignments for c-Myc363-381 peptide free and in complex with 10074-G5. Chemical shifts were referenced to a trimethylsilylpropionate standard.

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Table 3 Resonance assignments for c-Myc370-409 peptide free and in complex with 10074-A4. Chemical shifts were referenced to a trimethylsilylpropionate standard.

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The results described in this chapter were published in part in:
Structural rationale for the coupled binding and unfolding of the c-Myc oncoprotein by
small molecules. Chemistry & Biology, 15(11), 1149-1155.
independent binding sites for small molecule inhibitors on the c-Myc oncoprotein.
Journal of the American Chemical Society, 131, 7390-7401.

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References

1. Follis, A.V., D.I. Hammoudeh, H. Wang, E. Prochownik and S.J. Metallo (2008) Structural rationale for the coupled binding and unfolding of the c-Myc oncoprotein by small molecules. *Chemistry & Biology*, 15(11), 1149-1155. Figures 3, 6, 7, 9 were adapted from this article with permission from Elsevier publishing.

2. Hammoudeh, D.I., A.V. Follis, E.V. Prochownik and S.J. Metallo (2009) Multiple independent binding sites for small molecule inhibitors on the c-Myc oncoprotein. *Journal of the American Chemical Society*, 131, 7390-7401. Figures 10, 11, 13, 14, 15 were adapted from this article with permission from ACS.


Chapter 4

Insights into inhibitors’ mechanism of action from thermodynamic and kinetic studies

4.1. Introduction

The previous chapter dealt with the elucidation of several structural aspects of the interaction between small molecules and multiple, independent segments of the intrinsically disordered c-Myc bHLHZip monomeric domain. These studies shed light on some potentially general features of interactions between small molecules and intrinsically disordered proteins. None of the reported studies, however, expressly investigated the thermodynamic attributes of these molecules’ inhibition of c-Myc-Max heterodimerization, besides the basic measurement of their binding affinity for monomeric c-Myc bHLHZip. In this chapter experiments aimed at assessing in a quantitative way thermodynamic and kinetic parameters involved in the inhibitors’ mechanism of action are described. These include titrations of the competition between p21 Max and different inhibitors for binding to monomeric c-Myc bHLHZip, measurements of the kinetics of c-Myc-Max heterodimers disruption induced by inhibitors, and assessment of the enthalpy and entropy contributions to their free energy of binding to monomeric c-Myc bHLHZip. The most interesting result of these assays was the observation of considerably different mechanistic consequences on the disruption of c-Myc-Max heterodimers depending on the different locations of the inhibitors’ binding sites over the c-Myc bHLHZip sequence.
4.2. *Competition studies*

With the goal of assessing the inhibitors’ ability to disrupt c-Myc-Max heterodimers in a simple system of equilibria, in which thermodynamic parameters could be relatively easily extrapolated from experimental data, heterodimers were formed between the c-Myc bHLHZip and the p21 Max isoform, with low homodimer affinity but strong heterodimer affinity [1] ($K_{D,\text{dimer}} = 0.43\pm0.02$ µM). The heterodimer complex displayed a characteristic α-helical CD curve, with a strong minimum at 222 nm, expected from the complete folding of the HLHZip domain. The transition from heterodimer to disordered monomers involves a significant reduction in helical content and therefore a reduced ellipticity signal at 222 nm. The titration of the CD signal at 222 nm upon serial dilution of equimolar c-Myc-Max mixtures was performed as a preliminary experiment to assess the dissociation constant for heterodimerization reported above (Figure 1a). Addition of an excess concentration of the inhibitors 10058-F4, 10074-G5 or 10074-A4 to 1.5 µM equimolar solutions of c-Myc and p21 Max, a sufficiently high concentration to observe nearly complete heterodimer formation, confirmed their ability to disrupt such complex and induce unfolding of the complex into freed monomers (Figure 1b). The binding sites of these inhibitors were previously found to be located at the interface between basic region and helix1 (10074-G5), between helix1 and the loop (10074-A4), and between helix2 and the leucine zipper (10058-F4) as reported in the previous chapter. The binding site of the first inhibitor is located at the very edge of the domain involved in the HLHZip heterodimer
formation between c-Myc and Max, while those of the other two inhibitors are found within such dimerization interface.

The addition of 10058-F4 to the heterodimer led to complete disordering of c-Myc and Max. A titration of this effect was performed by serial dilutions of the inhibitor’s concentration (Figure 1c). The competition constant parameter \( K_{\text{comp}} \) employed to fit these data, which corresponds, in thermodynamic terms, to the ratio of c-Myc-inhibitor \( K_D \) versus c-Myc-Max \( K_D \), was 12.4±0.4 for the competition between 10058-F4 and p21 Max. This value is very close to the ratio (12.3) between the independently determined c-Myc binding affinities. The addition of 10074-G5 also strongly disrupted the complex, although not to the same extent as 10058-F4. The higher than predicted competition constant between 10074-G5 and p21 Max (37±2 versus a ratio of 6.7 between the independently measured \( K_D \)s) and an increased helical content at the titration endpoint, indicated lower efficacy of this inhibitor in disrupting c-Myc-Max complexes. This observation may be explained by the position of 10074-G5 interaction site, which lies at the extreme edge of the c-Myc dimerization interface. Some residual associations between the leucine zipper regions, located at the opposite end of the domain, may still be possible in the presence of 10074-G5. In the case of 10074-A4 the experimental trend matched that observed for 10058-F4, but with a higher competition constant value (32±3), as expected from the lower affinity for direct binding to monomeric c-Myc bHLHZip of this inhibitor (as measured by titrating the induced circular dichroism effect observed for the compound upon interaction with this protein target) compared to 10058-F4. The simultaneous titration of both 10058-F4 and
10074-G5 resulted in a slightly lower $K_{\text{comp}}$ (10.0±0.4) value than observed for 10058-F4 alone, but did not show significant cooperative effect, as expected in a thermodynamic system of independent binding equilibria, where the tightest interaction contributes the most to its outcome.

In the competition titrations performed at 25°C, the observed $K_{\text{comp}}$ correlated well with the independently determined $K_D$ values for the species in competition with each other in the case of 10058-F4 and 10074-A4. The results for 10074-G5 were, however, less coherent with other experiments, as the ellipticity value at the titration endpoint indicated a higher residual helical content than that observed for 10058-F4, and the corresponding $K_{\text{comp}}$ was considerably higher than expected. With the aim of understanding such unexpected behaviour for this inhibitor, the same experiment was repeated at 37°C for the inhibitors 10058-F4 and 10074-G5. The results in this case indicated that the $K_{\text{comp}}$ for 10058-F4 remains nearly unchanged ($K_{\text{comp}_{25}}= 12.4±0.4$, $K_{\text{comp}_{37}}= 14±2$), while this parameter strongly decreases for 10074-G5 to a value more consistent with that expected from the direct binding affinity between c-Myc and this compound ($K_{\text{comp}_{25}}= 37±2$, $K_{\text{comp}_{37}}= 10±3$, figure 1d). The endpoint ellipticity value at 37°C also shifted to match that observed for 10058-F4. Taken together, these results suggest the formation of a relatively unstable ternary complex between c-Myc, Max and 10074-G5 in the presence of a large excess of inhibitor when the experiment is performed at 25°C, but which becomes unstable at the higher temperature of 37°C, with complete dissociation of Max occurring.
**Fig. 1** Disruption of c-Myc-p21 Max heterodimers by small molecule inhibitors studied by circular dichroism. **a.** Control titrations of homo or heterodimerization affinities of p21 Max (black triangles), p22 Max (blue diamonds), c-Myc-p21 Max (red circles) monitored by measuring the samples ellipticity at 222 nm. **b.** UV circular dichroism spectra of the weighted average of 10 μM c-Myc and p21 Max monomers (white), 10 μM c-Myc-p21 Max mixture (black), and the same mixture in the presence of 200 μM 10058-F4 (blue) 10074-A4 (green) or 10074-G5 (red). **c.** Titrations of the disruption of 1.5 μM c-Myc-p21 Max heterodimers upon addition of increasing concentrations of the studied inhibitors monitored by measuring the ellipticity at 222 nm. Blue data are for 10058-F4, green for 10074-A4, red for 10074-G5, purple for 1:1 10058-F4:10074-G5 mixture. **d.** The same experiment shown in panel c, with titrations performed at 25°C (full circles) and 37°C (open circles) for 10058-F4 (blue) and 10074-G5 (red).
4.3. Kinetic studies

Circular dichroism experiments, similar to those employed to perform the competition experiments described above, were exploited to study the rate of disruption of c-Myc-Max HLHZip heterodimers promoted by the same 10058-F4, 10074-G5 and 10074-A4 inhibitors, binding to different sites over the c-Myc bHLHZip domain. Circular dichroism was employed to monitor the ellipticity value at 222 nm, indicative of the helical content of the protein component [2], of equimolar mixtures of c-Myc and p21 Max (1.25 µM) after addition of excess concentrations of inhibitors. It was observed that both 10058-F4 and 10074-A4 give place to similar rates of c-Myc-Max heterodimer disruption, which do not show significant concentration dependence. The fitting of disruption kinetics to a first order constant provides similar values for both compounds at two tested concentrations (k\textsubscript{10058-F4(200µM)}= 4.2±0.1•10\textsuperscript{-4} s\textsuperscript{-1}; k\textsubscript{10058-F4(100µM)}= 4.7±0.1•10\textsuperscript{-4} s\textsuperscript{-1}; k\textsubscript{10074-A4(200µM)}= 4.9±0.3•10\textsuperscript{-4} s\textsuperscript{-1}; k\textsubscript{10074-A4(100µM)}= 5.6±0.4•10\textsuperscript{-4} s\textsuperscript{-1}). The inhibitor 10074-G5 disrupted the c-Myc-Max heterodimer much more rapidly and with a strong concentration dependence compared to both 10074-A4 and 10058-F4 (k\textsubscript{10074-G5(100µM)}= 8±2•10\textsuperscript{-3} s\textsuperscript{-1} at 100 µM, figure 2). The pseudo first order constants of c-Myc-Max disruption observed at different excess concentrations of 10074-G5 provide a good fit to a second order constant (k\textsubscript{10074-G5}= 62 M\textsuperscript{-1}s\textsuperscript{-1}, R\textsuperscript{2}= 0.954). The extent of heterodimer disruption once equilibrium is reached depends on the overall inhibitor concentration in all cases.

These results are consistent with the hypothesis that both 10074-A4 and 10058-F4 need to capture c-Myc monomers freed upon dissociation of the c-Myc-Max
Fig. 2 Kinetics of disruption of c-Myc-p21 Max heterodimers by small molecule inhibitors studied by monitoring the samples ellipticity at 222 nm. a. Kinetics of disruption of 1.25 µM c-Myc-p21 Max by 10074-G5 at 200 µM (circles), 100 µM (crosses), 50 µM (diamonds) and 25 µM (triangles) concentration. b. Linear fitting of the pseudo first order constants obtained from curves in panel a to extrapolate a second order constant for disruption kinetics. Error bars represent the standard error of the mean. c. Kinetics of disruption of 1.25 µM c-Myc-p21 Max by 10058-F4 at 200 µM (circles) and 100 µM (crosses). d. Kinetics of disruption of 1.25 µM c-Myc-p21 Max by 10074-A4 at 200 µM (circles), 100 µM (crosses). e. Relaxation kinetics of c-Myc-p21 Max complexes upon dilution of 1.25 µM samples to 0.42 µM.
heterodimer in order to inhibit the latter complex, their rate of disruption should therefore correlate with the intrinsic dissociation rate of c-Myc-Max heterodimers. This hypothesis was tested by studying the relaxation kinetics of c-Myc-Max heterodimers upon dilution (Figure 2e). It was observed that the first order treatment of these kinetic data provided a rate constant \( k = 5.3 \pm 0.3 \times 10^{-4} \text{ s}^{-1} \) comparable to that reported for Max homodimer dissociation \((<2 \times 10^{-3} \text{ s}^{-1})\) [3], and close to those observed for the c-Myc-Max heterodimer disruption promoted by both 10074-A4 and 10058-F4. This supports the likelihood that these two inhibitors indeed act by capturing c-Myc monomers produced by the intrinsic dissociation of c-Myc-Max heterodimers at equilibrium. Conversely 10074-G5 might be more actively promoting the heterodimer disruption, as its binding site on c-Myc is located at the edge of the c-Myc-Max interface, and its binding to c-Myc might still occur without full disassociation of the latter from Max. The interaction between c-Myc and Max within the resulting trimeric complex would be distorted and destabilized compared to the c-Myc-Max heterodimer.

4.4. Evidence of formation of an intermediate c-Myc-Max-10074-G5 complex

Further experiments were designed to elucidate the faster c-Myc-Max disruption rate observed for 10074-G5 and overall different kinetic and thermodynamic behavior observed for the disruption of c-Myc-Max complexes by 10074-G5 compared to the inhibitors 10058-F4 and 10074-A4. The faster disruption induced by this inhibitor compared to both 10074-A4 and 10058-F4 might be explained by the binding of the inhibitor at the interface between basic region and helix-1 of c-Myc prior to its
dissociation from Max. It is indeed known that the basic regions remain unfolded and
dynamic in bHLHzip dimers in the absence of target DNA, and it is likely that
transient unfolding occurs at the edges of the heterodimerization interface, including
the N terminus of helix-1, without a complete dissociation of the protein dimers, as
suggested by NMR structural studies of Max bHLHzip homodimers in solution [4].

In this mechanism of inhibition, a fourth thermodynamic state would be
introduced as the previously hypothesized ternary complex between c-Myc, Max and
inhibitor, generating a thermodynamic cycle which leads, through either possible
associative (or, in reverse, dissociative) pathway, from the three monomeric species,
through c-Myc-Max or c-Myc-10074-G5 dimeric species, to the ternary complex
(Figure 3a). Conversely, in the inhibitory model proposed for the compounds 10074-
A4 and 10058-F4, the two binary complexes between c-Myc and Max and between c-
Myc and inhibitor can interconvert with each other only through dissociation to their
individual components (Figure 3b). For the suggested 10074-G5 mechanism of
inhibition, in the presence of a sufficient excess of any component of the ternary
complex (either c-Myc, Max, or inhibitor) the equilibrium may be shifted towards its
formation.

Such thermodynamic reasoning was exploited to assess the formation of the
proposed ternary complex between c-Myc, Max and 10074-G5 by means of a
competition experiment, different from those described previously, where inhibitors
had been titrated against constant concentrations of c-Myc and p21 Max. In this new
experiment, increasing concentrations of Max were added to a constant concentration
Fig. 3 Schematic representation of two possible mechanisms of small molecule inhibition or interference with c-Myc-Max heterodimer formation based on the reported experimental data. a. ‘Ternary complex’ mechanism, hypothesized for 10074-G5. b. ‘Direct competition’ mechanism, hypothesized for 10058-F4 and 10074-A4.
(10 µM) of an equimolar mixture of c-Myc and 10074-G5. The c-Myc component was fluorescently tagged with fluorescein, upon reaction of a C terminal cystein tag with iodoacetamido-fluorescein (c-MycFLU), and the intrinsic fluorescence of the nitrobenzofurazan moiety of 10074-G5 was also exploited. Preliminary experiments were performed to verify the sufficient resolution of the absorption and emission spectra of these two fluorophores (Figure 4a, b).

At any Max concentration, the fluorescence polarization of both the c-Myc bound fluorescein and 10074-G5 was recorded. The binding between c-Myc and Max results in a decreased tumbling rate in solution of the c-Myc fluorescein label, therefore in an increase of its fluorescence polarization (binding titrations are described in Chapter 5); the binding between 10074-G5 and c-Myc results in increased fluorescence polarization of the small molecule ligand [5]. It was observed that, in the absence of Max, but in the presence of c-Myc, the polarization of 10074-G5 was high (~0.35) compared to that of the pure compound (~0.2). However, in the same samples the fluorescein polarization was low (~0.12), as the small size of 10074-G5 does not cause a noticeable decrease in tumbling rate of c-Myc upon complex formation. Upon addition of Max, the fluorescence polarization of 10074-G5 decreased to ~0.28 but never reached the value observed for the free compound, while that of the c-Myc bound fluorescein rapidly increased to a plateau at a value (~0.22) previously determined, from stoichiometric titrations, to represent saturated binding between the two protein species, as the working concentrations were kept above the dissociation constants of both binary complexes throughout the experiment (Figure 4c, d). The
failure of 10074-G5 polarization to decrease down to the value observed for the free compound indicates residual association between this compound and the protein component even after saturated binding between c-Myc and Max had been attained. The experimental data were analyzed by means of numerical integration using KinTecSim simulation software. The polarization data for 10074-G5 and c-Myc-FLU were fitted to a ‘thermodynamic cycle’ model (Figure 3a), involving formation of ternary complex (Figure 4e), and a ‘direct competition’ one (Figure 3b), where, as observed for the disruption of c-Myc-Max dimers by 10074-A4 or 10058-F4, the interconversion between c-Myc-Max and c-Myc-inhibitor may occur only through the dissociation of either complex (Figure 4f). The independently determined dissociation constants between c-Myc and Max and that between c-Myc and 10074-G5 were employed in both cases, and experimental polarization endpoints were employed in the fitting. In the ‘thermodynamic cycle’ model, one single parameter was optimized representing the dissociation constants of either Max or 10074-G5 from the ternary complex. The relative values of these two parameters correspond to the ratio between the dissociation constants of c-Myc-Max and c-Myc-10074-G5 binary complexes, because of the need for energy conservation throughout a system of equilibria that generates a thermodynamic cycle. It was observed that this model provided an excellent fit to the experimental polarization data with dissociation parameters, relative to the ternary complex formation, of 3.5 µM for Max and 22.8 µM for 10074-G5: these values are higher than the dissociation constants of either c-Myc-Max or c-Myc-10074-G5 bimolecular complex (0.43±0.02 µM and 2.8±0.7 µM respectively). The alternative
Fig. 4 Experimental assessment of formation of a ternary complex between c-Myc, p21 Max and 10074-G5 (continued on next page).
Fluorescence excitation and emission spectra of 10074-G5 (red) and c-Myc353-439-conjugated fluorescein (c-MycFLU, blue). b. Relative fluorescent intensities of each fluorophore at wavelength settings for 10074-G5 (560 nm) and fluorescein (520 nm). The relative concentration of the two fluorophores matches that employed in subsequent experiments. c. Stoichiometric titration of p21 Max into 10 µM c-MycFLU causes a linear increase of the fluorescence polarization of the latter fluorophore. d. Stoichiometric titration of c-Myc FLU into 10 µM 10074-G5 causes a linear increase of the fluorescence polarization of the latter fluorophore. e. Addition of p21 Max to a 1:1, 10 µM mixture of c-MycFLU and 10074-G5. While the c-MycFLU fluorescence polarization (open circles) increases to its fully bound value, as indicated by the stoichiometric titration in panel c, that of 10074-G5 (filled circles) does not decrease to its fully free value, as estimated from the titration in panel d. Experimental data are fit by numerical integration using a model reflecting the ‘ternary complex’ system of equilibria shown in figure 3a; experimental endpoints and values for the dissociation constants between c-Myc and p21 Max and that between c-Myc and 10074-G5 are employed. The full line represents the overall trend of 10074-G5 fluorescence polarization, obtained from the summation of the decreasing values generated by the c-Myc-10074-G5 species (short dashes) and increasing ones generated by the c-Myc-p21 Max-10074-G5 ternary complex (alternating dots and dashes). The composite polarization trend for c-MycFLU is shown as long dashes. The arrow on the y-axis indicates the polarization value of free 10074-G5. f. Fitting the same titration to a ‘direct competition’ model, represented in figure 3b results in a poor match to the experimental data.

‘direct competition’ model generated a good fit for the c-MycFLU polarization values, as expected by the occurrence of an interaction between c-Myc and Max upon increase of Max concentration in either model, but did not fit the experimental trend for 10074-G5 polarization, as the fitting curve rapidly decreased to the polarization value observed for the free compound. The same models were employed to similarly fit the circular dichroism competition data at 25°C, using ellipticity endpoints for each of the thermodynamic species involved in each model, which had been independently determined from previous measurements. Also in this case the ‘thermodynamic cycle’ model, involving the formation of a ternary complex (Figure 5a), and not the ‘direct
competition’ model (Figure 5b), fit the data for c-Myc-Max disruption by 10074-G5 when the same thermodynamic parameters optimized for the fitting of the polarization data were employed. The ‘direct competition’ model, on the other hand, employed with the specific, independently determined, dissociation constant values for each c-Myc-inhibitor complex, fit reasonably well the circular dichroism titrations of c-Myc-Max disruption by 10074-A4 or 10058-F4. In summary, the described experiments support the formation of a ternary complex between c-Myc-Max and the inhibitor 10074-G5 but not in the presence of either 10058-F4 or 10074-A4: both these inhibitors are in direct competition with c-Myc for Max binding.

The observed formation of a ternary complex between c-Myc, Max and 10074-G5, can therefore explain the fast kinetics of disruption of c-Myc-Max dimers by inhibitors binding to c-Myc at the edge of its segment involved in dimer formation with Max compared to ones having binding sites comprised within it; the lower than expected $K_{\text{comp}}$ value in competition experiments between 10074-G5 and Max; and the high helical content at the endpoint of this competition titration. The fitting of this titration, performed at 25°C, to a direct competition equation is deprived of thermodynamic meaning, giving place to seemingly unreasonable values, as this mathematical treatment does not correctly represent the studied system of equilibria. When the data were collected at 37°C, a temperature at which the ternary complex formation is prevented, such significance is restored.
Fig. 5 Numerical integration fitting of the disruption titration of c-Myc-p21 Max dimers by 10074-G5 at 25°C shown in figure 1c and 1d. a. Fitting to a ‘ternary complex’ model (figure 3a) using the same parameters optimized for the titration shown in figure 4e. The solid line indicates the trend resulting from the summation of each individual component: c-Myc-Max dimer (alternated dots and dashes), ternary complex (long dashes), free c-Myc (dots), c-Myc-10074-G5 and free Max (short dashes, partially overlapped) b. Fitting to a direct competition model (figure 3b).
4.5. Thermodynamics of inhibitors binding to c-Myc

Comprehending the way by which the studied inhibitors disrupt c-Myc-Max dimers is enhanced by an understanding of the thermodynamic aspects of their direct binding to c-Myc monomers. A complex involving a small molecule and a short stretch of adjacent amino acids lacking a stable fold, is rather unusual, although examples of similar interactions can be found in the recent literature regarding substrate targeted kinase inhibitors [6] or drug target motifs [7]. Overall, the localized nature of these inhibitors binding compared to the dimer formation between c-Myc and Max is consistent with an entropic advantage in the first case, as the number of residues undergoing structural induction and strong conformational constraints would be much reduced compared to the $\alpha$-helix formation and loop organization over about 70 amino acids in each protein necessary for the heterodimer formation. It was of interest, however, to investigate what the entropy – enthalpy balance would be within the localized binding sites themselves for the direct binding event between c-Myc and the studied inhibitors, regardless of any relative entropic advantage involved in their competition with Max for c-Myc binding. In earlier efforts to elucidate structural aspects of the binding between such inhibitors and c-Myc, modeling had been employed, aided by NMR studies of the complexes in solution (see Chapter 3) [8, 9]. The results of those experiments indicated a high extent of residual flexibility in these complexes, both in peptide and inhibitor bond rotations, compatible with a reduced loss of entropy upon complex formation. Once again fluorescence polarization was exploited to study the thermodynamic aspects of the interaction between the
intrinsically disordered c-Myc bHLHZip monomer and small molecules inhibitors of its hetero-dimerization with Max, exploiting the intrinsic fluorescence of both 10074-G5 and 10058-F4 to titrate their binding affinity for c-Myc monomers over a range of temperatures. The obtained dissociation constant values ($K_D^T$, where T indicates the temperature at which a given titration was performed) were converted to their free energy equivalent at the corresponding temperature ($\Delta G^\circ$), the data were then plotted against temperature to be fitted to the basic thermodynamic Gibbs equation $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$. The observed changes in entropy upon complex formation were close to zero in the case of 10074-G5 (0±3 cal•mol\(^{-1}\)•K\(^{-1}\)) and actually positive in the case of 10058-F4 (+17±3 cal•mol\(^{-1}\)•K\(^{-1}\), Figure 6). This result is in agreement with the

![Graph](image)

**Fig. 6** Temperature dependence of 10058-F4 (diamonds) and 10074-G5 (triangles) binding affinity for c-Myc bHLHZip provides information regarding the relative enthalpy and entropy contributions to each binding event. Error bars represent the standard error of the mean.
indication of residual flexibility within the complexes from NMR experiments, also the
different values observed for the two inhibitors are consistent with the stronger
structural induction observed for 10074-G5 binding compared to 10058-F4. Overall the
relatively favorable entropic balance for the binding of these inhibitors to c-Myc
suggests that the complex formation may be largely driven by desolvation of
hydrophobic residues within the binding sites, which are particularly exposed to the
solvent as they are found over an unfolded stretch of flexible amino acids rather than
within the enclosed cavity of a folded binding pocket. The importance of desolvation
of hydrophobic residues in ligand binding to sites of the latter kind has been
thoroughly proven [10, 11]. It seems likely that desolvation plays a key role in the
complexes studied here as well, where a negative entropy component, albeit limited
compared to typical protein folding, must also be present as a consequence of the
structural constraints induced on the binding segments upon complex formation with a
small molecule.

4.6. Conclusion
A series of techniques were employed to elucidate the thermodynamic and kinetic
aspects of the disruption of c-Myc-Max complexes by small molecule inhibitors, and
the mechanistic consequences of the different location of these compounds’ binding
sites on c-Myc monomers. The described results enhance the understanding of
interactions between small molecules and intrinsically disordered proteins, especially
regarding their competition with natural binding partners. It was shown that the studied
binding events display rather different thermodynamic features from those observed in the interactions between intrinsically disordered proteins and their natural protein partners. A favorable entropy tradeoff [12], a feature commonly observed for small molecule binding to folded pockets, was observed for binding events between small molecules and disordered protein segments, in spite of the dynamic, flexible nature of the latter. Binding interactions between multiple disordered proteins or disordered proteins and folded protein partners tend to be characterized instead by a net entropy loss [13], and this thermodynamic feature has been hypothesized to be involved in a tight regulation of such interactions within large protein networks [14].

The consequences of the localized nature of the studied binding events, involving short stretches of adjacent amino acids, were further investigated and it was possible to demonstrate that differences in the location of inhibitors binding sites on the c-Myc bHLHZip monomer can lead to dramatic differences in the mechanism by which they abrogate its dimer formation with Max. The formation of a low affinity ternary complex between c-Myc-Max and one such inhibitor, which is ultimately responsible for the fast action of this compound, further supports the overall flexible and localized nature of such novel complexes between biological and synthetic molecules. A thorough understanding of these thermodynamic and mechanistic aspects might find applications in the design of small molecules as tools of finely targeted or multivalent chemical interference with intrinsically disordered proteins functions.
**4.7. Methods**

**Expression and purification of cys tagged c-Myc353-437.**

A form of the c-Myc bHLHZip domain (residues 353-437), encoding a hexahistidine tag separated by a TEV protease digestion site from the N terminus of the insert, and including a GGCD tag at the C terminus “c-Myc-cys” (c-Myc/pET SKB3 construct kindly supplied by Dr. S. K. Nair, University of Illinois, Urbana-Champaign) was over-expressed in *E.coli* BL21DE3(plysS) cells. Bacterial cultures were grown at 37 °C in LB medium to OD\textsubscript{600} \approx 0.8, then induced with 0.5 mM IPTG for 5 hours. Cultures were harvested and lysed in a buffer containing: 8 M urea; 100 mM NaH\textsubscript{2}PO\textsubscript{4}; 10 mM Tris; pH 8.0. The protein was purified on a NTA-Ni column with a pH gradient elution. The 6xHis tag of c-Myc-cys was cleaved using TEV protease in order to improve its solubility. The protein was further purified by C18 reversed phase HPLC and lyophilized. The Cys tag in the c-Myc-cys protein was kept in its reduced state by adding 5 mM dithiothreitol (DTT) during the buffer exchange procedure prior to TEV cleavage of the 6xHis tag. The acidic conditions of the HPLC purification and immediate lyophilization of the purified c-Myc-cys fractions further insured that the protein was kept reduced until the fluorescein tagging reaction was performed. Protein concentrations were determined by measurement of OD\textsubscript{280}.

**Fluorescence tagging of c-Myc-cys** (performed by Dalia Hammoudeh).

To a solution of TEV cleaved and HPLC purified Myc-cys (~200 µM) in 1X PBS (pH 7.4) were added 10 equivalents of 5-iodoacetamido fluorescein (5-IAF) and stirred for
3 hours in the dark at room temperature. The reaction mixture was quenched with excess acetic acid and purified by HPLC. The product (“c-MycFLU”) was quantified with a bicinchoninic acid assay. A ~17% fraction labeling of the product was assessed from comparison of this assay with the concentration of fluorescein based on OD_{490} measurement at pH 7.4. Reaction conditions near neutral pH and with low fraction labeling reduce the possibility of undesired labeling of amine groups.

**Circular Dichroism Titrations.**

Samples were prepared containing varying concentrations of protein component, ranging between 0.1 µM and 25 µM in 1X PBS (pH 7.4) and incubated for 90 minutes. Samples were analyzed at 25°C in a 1 cm cuvette by monitoring their ellipticity at 222 nm using a JASCO J710 spectropolarimeter. Data were converted to mean residual ellipticity and fit to the following equation, which is derived from the definition of thermodynamic equilibrium and assumes the presence of equimolar concentrations of c-Myc and Max when applied to heterodimer formation.

\[
\frac{[\text{complex}]}{[C_0]} = 4 + \frac{K_D}{[C_0]} - \sqrt{\left(\frac{-4 - K_D}{[C_0]}\right)^2 - 16} \div 4
\]

where \(C_0\) represents the maximum concentration of homo or heterodimer that may be formed, equating that of each monomer in the case of c-Myc-p21 Max heterodimers, or half that of p21 Max or p22 Max monomers in the study of homodimer formation. The 0-1 scale of the equation was fit to the ellipticity values experimental endpoints.
Circular Dichroism monitoring of c-Myc-Max heterodimer disruption.

All experiments were performed in triplicate with buffer background subtraction on a Jasco J710 spectropolarimeter. Samples for competition experiments between inhibitors and p21 Max for c-Myc binding were prepared in 1X PBS (pH 7.4); 1.5 µM solutions of c-Myc353-437 were incubated for 1 hour with varying concentrations of 10058-F4 or 10074-G5, obtained from 1:1 serial dilutions of 1mM compound stock solution in ethanol; 1.5 µM MaxS was then added and reactions were further incubated for 20 minutes. Readings for the competition experiment were performed at 25 °C and 37 °C in a 1 cm path-length cuvette by monitoring ellipticity at 222 nm. Data were converted to MRW and plotted versus 10058-F4 concentration. Experimental data were fitted using the following equation, which is derived from thermodynamic considerations by combining the two competing equilibria in which c-Myc is implicated, that is binding to Max or inhibitor:

\[ \Theta = \Theta_0 + \Delta \Theta \left\{ \left[ \frac{[I]}{[M]} - 1 \right] + \sqrt{\left[ \frac{[I]}{[M]} - 1 \right]^2 + 4 \cdot \frac{[I]}{[M]} \cdot (K_{comp} - 1)} \right\} \frac{2 \cdot K_{comp} - 2}{K_{comp} - 2} \]

Where \( \Theta_0 \) and \( \Delta \Theta \) are ellipticity in the absence of inhibitor and total change in ellipticity respectively; \( [I] \) is the concentration of inhibitor (variable); \( [M] \) is the concentration of c-Myc and Max (fixed) and \( K_{comp} \) represents the ratio between the c-Myc-inhibitor and c-Myc-Max dissociation constants (\( K_{comp} = k_{d, inhibitor} / k_{d, Max} \)). Such treatment is made possible by the use of equal concentration of Myc and Max in the samples. Kinetics experiments were performed at 25 °C in a 1 cm path-length cuvette.
by monitoring the ellipticity at 222 nm of equimolar 1.25 µM solutions of Myc353-437-p21 Max in 1X PBS (pH 7.4) after addition of the indicated concentrations of inhibitors or after 3 fold dilution (for relaxation kinetics experiments). Data were fit to a single exponential equation, as experiments were performed in pseudo-first order conditions, with a large excess of inhibitor compared to the protein component.

Fluorescence and Fluorescence Polarization Measurements

All experiments were performed in triplicate with a Photon Technology International QuantaMaster fluorimeter (Birmingham, NJ) equipped with polymer sheet polarizers. Experiments aimed at demonstrating the formation of a ternary complex between 10074-G5, c-MycFLU and p21 Max were performed by monitoring in the same sample the fluorescence polarization of two different fluorophores, one being the inhibitor itself, the second the fluorescein tag on c-MycFLU. Preliminary excitation and emission scans were performed in order to assess the resolution of absorption and emission bands of the two fluorophores: excitation and emission maxima were observed to be 470 nm and 550 nm for 10074-G5 and 495 nm and 520 nm for the fluorescein tag. Due to the higher fluorescence intensity of the fluorescein fluorophore compared to the nitrobenzofurazan fluorophore of 10074-G5, samples were prepared where the c-Myc protein component contained mainly untagged c-Myc353-437 peptide and was doped with 1% c-MycFLU. The residual intensity observed in these conditions when monitoring the emission wavelength of one fluorophore while exciting at the maximum absorption wavelength of the other was in both cases low.
enough to warrant an independent measurement of the polarization of either fluorophore (<25% fluorescein contribution to 10074-G5 signal, < 2% 10074-G5 contribution to fluorescein signal). The ternary complex formation was assessed in a competition titration experiment, where to 10 µM equimolar mixtures of c-MycFLU and 10074-G5 were added increasing concentrations of p21 Max, ranging between 5 µM and 100 µM. The fluorescence polarization of both 10074-G5 and c-MycFLU was monitored at each Max concentration with sample specific G-factor determination. The resulting polarization data were fit by means of numerical integration as described in section 4.4.

Temperature dependent measurements of the binding affinity of 10058-F4 or 10074-G5 for c-Myc monomers were performed by monitoring the fluorescence polarization of either compound (for 10058-F4, excitation: 380 nm, emission: 468 nm, for 10074-G5, excitation: 470 nm, emission: 550 nm) upon serial dilution of 1:1 mixtures of c-Myc_{353-437} and inhibitor. Concentrations ranged between 200 µM and 400 nM. Full titrations were performed for each compound at 5, 10, 15, 20, 25, 30 and 37°C. Sample specific G-factor determination and background correction were applied. Data were fit to a quadratic equation derived from the thermodynamic expression of binding equilibrium:

\[
\text{Eq.2 } \frac{[\text{complex}]}{[C]_0} = \frac{2 + K_D/[C]_0}{2} - \sqrt{\frac{(-2 - K_D/[C]_0)^2 - 4}{2}},
\]
where $[C]_0$ represents the total concentration of 10058-F4 or 10074-G5 and of c-Myc353-437. The value of $K_D$ was determined from the experimental polarization data by fitting to Eq. 3 using KaleidaGraph (Synergy Software, Reading, PA) where $pol_0$ is the polarization in the absence of binding and $\Delta pol$ is the total change in polarization [5].

**Eq.3**  
\[
polarization = pol_0 + \Delta pol \cdot \left( \frac{[\text{complex}]}{[C]_0} \right)
\]

**Synthesis of 10058-F4, Expression and purification of c-Myc353-437, p21 Max** are described in Section 2.5. Compounds 10074-G5 and 10074-A4 were purchased from ChemBridge corporation (San Diego, CA).
References


Chapter 5

Effect of competing bHLHZip interactions on the inhibition of c-Myc-Max heterodimer formation

5.1. Introduction

As previously reported, c-Myc does not homodimerize and its heterodimerization interaction with Max is necessary to most of its known cellular functions, and definitely to its role as transcriptional activator [1, 2]. Max serves as an obligate binding partner for c-Myc as well as the bHLHZip transcriptional repressors of the Mad family and is characterized by high constitutive expression [3]. Max exists in two alternatively spliced isoforms, p21 and p22, the latter having a 9 amino acid acidic insertion at the N terminus of the basic region. The p22 Max isoform can form homodimers and silently bind E-box target DNA sequences with affinities close to those observed for its heterodimer formation with c-Myc. The p21 Max isoform displays considerably lower homodimerization and E-box binding affinities [4]. The heterodimer formation with c-Myc has similar affinity for both isomers. Both p21 and p22 Max have been simultaneously isolated from cell lysates [5], and are capable of heterodimerizing with each other with reduced E-box affinity compared to p22 Max homodimers [3]. While c-Myc and Mad proteins can potentially compete for Max binding [6], giving place to opposite outcomes – proliferation or differentiation – in the cell-cycle fate, the different cell cycle expression profile of c-Myc and Mad makes their simultaneous in vivo presence improbable. On the other hand the homodimer
forming ability of p22 Max, further regulated by its phosphorylation [5], could function to finely tune the activity of its heterodimerization partners. While the specific function of the two Max isoforms is yet unclear, the current evidence suggests that the tunable silent DNA binding by these proteins is necessary for a stringent dynamic regulation of transcriptional activation by bHLHZip TFs [7]. A recently published article supports this hypothesis by providing a quantitative thermodynamic analysis of the relative amounts of c-Myc and Max bHLHZip monomers (the use of the bHLHZip segment only of Max cancels the distinction between its two isoforms), and different heterodimeric and homodimeric species at different protein concentrations and temperatures [8]. Homo or heterodimerization is a common mechanism for DNA recognition by TFs [9], competition for a protein partner by different protein monomers has been described in several TF families, including bHLHZip [6, 10], basic-leucine zipper (bZip) [11] and basic-helix-loop-helix (bHLH) [12]. It might therefore be generally exploited in transcriptional regulation strategies [7]. It was investigated here whether the presence of competing bHLHZip dimerization equilibria, likely to occur in the cellular nucleus, could influence the inhibitors’ effect on c-Myc-Max dimers [13]. More specifically, it was tested whether the efficacy of the specific inhibitors of c-Myc-Max heterodimerization, the overall object of the current study, might be enhanced by the presence of the competing p22 Max homodimer formation. One would predict that the disruption of c-Myc-p22 Max heterodimers might be facilitated by the free energy returned upon homodimerization of the released p22 Max. Since p21 Max dimerizes only weakly, there would be a negligible free energy
contribution from the released p21 Max, making c-Myc-p21 Max more difficult to inhibit (Figure 1). Such perturbation of competing equilibria might be particularly relevant to the inhibition of TFs since they tend to form very stable complexes with DNA. More generally, the shifting of equilibria rather than the outright inhibition of one interaction may make difficult systems (such as disordered TFs that dimerize over an extensive surface area [14] without obvious sites for inhibition) into useful targets for modulating transcriptional activity or other cell signaling functions.

**Fig.1** Schematic free energy diagram of the competing equilibria of bHLHZip dimer formation in the absence and presence of the c-Myc inhibitor 10058-F4. The ability of p22 Max to form stable homodimers facilitates disruption by 10058-F4 since both p22 Max and c-Myc can simultaneously form favorable complexes, Max with itself and c-Myc with 10058-F4 (reproduced with permission from [13]).
5.2. *p22 Max homodimer formation.*

In order to accurately assess the relative inhibition of heterodimer formation between c-Myc and Max by small molecules in the presence of the two different Max isoforms p22 and p21, preliminary measurements were performed of thermodynamic parameters for the p22 Max and p21 Max homodimerization interactions. The proteins’ homodimer formation affinity was measured at 25°C by circular dichroism by means of monitoring the ellipticity at 222 nm of samples containing different concentrations of each Max isoform. The observed dissociation constants (K_D) for homodimerization were comparable to published values: 1.24±0.04 μM for p22 Max and 14±1 μM for p21 Max. The observed dissociation constant for c-Myc-p21 Max heterodimerization, similarly measured and previously employed to convert competition constant parameters from competition experiments between inhibitors and Max for c-Myc binding into c-Myc affinities of the inhibitors, was 0.43±0.02 μM (refer to Chapter 4 Figure 1a). The affinity value could not be determined by circular dichroism for the c-Myc-p22 Max complex due to the convolution of the ellipticity at 222 nm between helical content generated by c-Myc-p22 Max heterodimers and p22-Max homodimers. E-box binding by c-Myc-Max heterodimer and Max homodimer pairs was then monitored by means of electrophoretic mobility shift assays (EMSAs) [15] (Figure 2a). Titrations of E-box binding by the various proteins indicated that c-Myc-p22 Max heterodimer bound DNA slightly better than c-Myc-p21 Max (K_{obs}= 14±3 nM and 22±3 nM for c-Myc-p22 Max and c-Myc-p21 Max respectively), and p22 Max homodimer affinity for E-box (K_{obs}= 46±7 nM) was weaker than that of either
heterodimer. Homodimers of p21 Max were confirmed to bind E-box very poorly
($K_{\text{obs}} > 1 \, \mu M$). A curve fitting equation was developed for these protein titration
experiments, based on the thermodynamic cycle generated by the ‘monomer’ and
‘dimer’ DNA binding pathways. In the first pathway, the interaction of one peptide
with DNA is the first event of the complex formation; in the latter the pre-formed
protein dimer binds the DNA. The possible occurrence of these two microscopic
binding pathways results in the observed macroscopic cooperative effect for DNA
binding by bHLHZip proteins [16, 17] (Figure 2b). A correction for the effect of
complex formation on the free protein concentration can be obtained by means of
numerical iterations, where the concentration of protein bound to DNA obtained at one
iteration is subtracted from the total protein concentration value in the next. The same
fitting can be applied to Max homo-dimer titrations, with the difference of introducing
a factor of two in accounting for dimer formation and DNA binding ($X_0 = X + 2X_2 + 2C$
according to the formalism used in figure 2b). This mathematical treatment is
thermodynamically accurate and results in excellent fits of the experimental data likely
because of a correct accounting of the studied system of equilibria (Figure 2c), it
presents however the shortcoming of lacking one single fitted parameter related to the
observed dissociation constant of the c-Myc-Max-E-box complexes in terms of c-Myc-
Max heterodimer concentration. A second, simpler fitting was performed (Figure 2d),
where a Langmuir isotherm was adapted with an exponential Hill coefficient to the
$K_{\text{obs}}$ term to account for cooperative binding. The obtained Hill coefficient power root
of the fitted $K_{\text{obs}}$ parameter provides a $K_{\text{obs}}$ value represented in terms of c-Myc-Max
Fig. 2 Preliminary study of the binding affinity for E-box DNA by the studied bHLHZip hetero and homodimers (continued on next page).
Fig. 2 a. Typical appearance of a free to bound band-shift on a polyacrylamide gel. b. Derivation of an analytical equation to analyze the experimental binding data that takes into account both E-box DNA binding by pre-formed bHLHZip dimers as well as sequential binding of two monomers to one double stranded E-box sequence. c. Experimental binding data fit to the equation described in panel b. Shown are curves for E-box DNA binding by c-Myc-p22 Max heterodimers (black circles), c-Myc-p21 Max heterodimers (black triangles), p22 homodimers (white circles) and p21 homodimers (white triangles). Concentrations on the x axis indicate the total concentration of each monomer for heterodimeric complexes, total monomer concentration for homodimeric complexes. Shown data are representative of five or more independent experiments, error bars represent the standard error of the mean. d. Same data shown in panel c fit to a cooperative binding equation involving a Hill coefficient. While this treatment is less accurate in thermodynamic terms than the fitting shown in panel c it has the advantage of easily providing an observed dissociation constant values (Kobs). Data are plotted as a function of the maximum dimer concentration in each sample, this results in a different position in this plot compared to panel c of the Max homodimer binding titrations.

heterodimer concentration. These experiments confirmed, for the protein components specifically employed in this study, the occurrence of p22 Max homodimerization events with affinities only slightly lower than the corresponding c-Myc-Max heterodimerization events, the relative insensitivity of the latter to the employed Max isoform, and the poor homodimer forming affinity of the p21 Max isoform. After establishing these thermodynamic relationships, measurements of relative inhibition of c-Myc-Max heterodimerization in the presence of either Max isoform were performed.

5.3. **Differential inhibition of c-Myc-p21 Max and c-Myc-p22 Max complexes**

The differential effect on heterodimer formation between c-Myc and Max in the presence of the two Max isoforms p21 and p22 was studied employing the inhibitor 10058-F4. The relative inhibition was studied in vitro by means of purified component
assays for binding events representing successive steps of the c-Myc functional process 
*in vivo*: heterodimer formation with Max and binding of E-box target DNA sequences. Of particular interest was the relative inhibition in the presence of different Max isoforms of the latter event, as it is characterized by high cooperativity and binding affinity, and the inhibitors previously tested for inhibition of E-box binding by c-Myc-Max heterodimers performed relatively poorly at disrupting this binding interaction compared to their ability to inhibit the heterodimerization between the HLHZip regions of c-Myc and Max [18].

5.3.1. Inhibition of c-Myc-Max dimer formation

In order to assess in vitro the hypothesis that the small-molecule inhibition of c-Myc-Max heterodimer formation is favored by the presence of competing bHLHZip dimerization equilibria, the c-Myc bHLHZip domain was fluorescently tagged with fluorescein (c-MycFLU), using a unique Cys residue added at its C-terminus, as described in Chapter 4. Using fluorescence polarization (FP) titrations the affinities of p21 Max and p22 Max for c MycFLU were measured. This experimental approach made it possible to measure the c-Myc-p22 Max affinity, which could not be conveniently assessed by means of circular dichroism due to the interference of p22 Max homodimer formation on the ellipticity at 222 nm. As a limiting amount of c-MycFLU was employed in the described fluorescence polarization experiment, the p22 Max homodimerization, concurrent with the heterodimer formation would cause only minor interference with the binding titration. The obtained dissociation constant ($K_D$)
values were within error of each other, 1.0±0.1 µM for c MycFLU-p21 Max and 0.9±0.2 µM for c MycFLU-p22 Max (Figure 3a). This value is, in the case of the c-MycFLU-p21 Max heterodimerization, in fair agreement with the one previously determined by circular dichroism, albeit slightly lower than it, the difference in detected affinities may be attributed however to inherent differences between the employed experimental techniques. The extent of heterodimer formation between 1.5 µM mixtures of c-MycFLU-p21 Max or c MycFLU-p22 Max was then monitored in the presence of varying concentrations of the c-Myc inhibitor 10058-F4 (Figure 3b). The resulting data were analyzed in terms of competition for c Myc binding, in a similar way to what was previously done to assess the disruption of c-Myc-Max heterodimers by small-molecule inhibitors in circular dichroism experiments, to yield a competition constant (K_{comp}) parameter, which represents, in thermodynamic terms, the ratio between the K_D of c-Myc-inhibitor and the K_D of the c-Myc-Max complexes. It was observed that 10058-F4 competition against c-MycFLU-p21 Max provides a K_{comp} of 12±2, this value correlates well with the ratio expected from the independently measured affinities of inhibitor binding to c-Myc and c-Myc-Max heterodimer formation. When p21 Max is replaced by the p22 isoform, the ability of the inhibitor to disrupt the c-MycFLU-p22 Max heterodimers is enhanced more than four fold (K_{comp}= 2.6±0.4). This enhanced disruption of the c-Myc-p22 Max heterodimer may be attributed to the additional free energy generated by the homodimer formation between p22 Max molecules when displaced from the heterodimer. This first result is therefore in agreement with the stated hypothesis that competing interactions between
Fig. 3 Employment of fluorescently tagged c-MycFLU to measure heterodimer formation between c-Myc and p22 Max, while avoiding convolution of the helical signal detected by circular dichroism due to p22 Max homodimer formation. a. Heterodimerization affinity measured by titration of fluorescently tagged c-MycFLU and p21 Max (black circles) or p22 Max (white circles). Values represent the average of three or more trials and error bars indicate standard error of the mean. b. Disruption of c-Myc-p21 Max (black circles) and c-Myc-p22 Max (white circles) heterodimers by 10058-F4. Error bars represent the standard error of three or more trials (adapted with permission from [13]).
different pairs of bHLHZip proteins may enhance the specific chemical inhibition of one such complex.

5.3.2. Inhibition of c-Myc-Max DNA binding

The thermodynamic rationale for the hypothesized perturbation of competing equilibria introduced by the inhibitor was then tested in the more complex thermodynamic setting of DNA binding events. First E-box binding by c-Myc-p21 Max heterodimers was titrated in the presence of a fixed concentration of 10058-F4 (50 µM, Figure 4a). The inhibitor caused a two-fold decrease in the c-Myc p21 Max affinity for E-box ($K_{obs} = 48\pm16$ nM). It should therefore be expected that, in the presence of the same inhibitor concentration, upon replacement of p21 Max with p22 Max a considerable fraction of E-Box would be bound to p22 Max homodimers rather than c-Myc p22 Max heterodimers.

As predicted, with the c-Myc-p22 Max heterodimer a 50 µM concentration of 10058-F4 shifted the fraction of E-box bound to Max homodimers to nearly half despite the higher affinity of the c-Myc-p22 Max heterodimer compared to the c-Myc-p21 heterodimer. It was therefore not possible to make a direct comparison with c-Myc-p21 Max heterodimers based on a protein titration. Instead the concentration of heterodimers was held constant while the concentration of 10058-F4 was varied. At 150 nM each of c-Myc and p22 Max, E-box was completely bound by the heterodimer. When 10058-F4 was included at 100 µM there was a dramatic shift and nearly all of the E-box DNA was bound to p22 Max homodimer instead of heterodimer. There was
no significant increase in the fraction of free E-box, and there was a partial shift
towards homodimer binding down to a 10058-F4 concentration of 12.5 µM (Figure 4b).  In contrast, a 200 µM concentration of 10058-F4 was not sufficient to completely disrupt the binding of E-box by 150 nM c-Myc-p21 Max heterodimers and a 100 µM concentration caused little disruption (Figure 4c). A quantitative comparison between the detectable inhibition of E-box binding by c-Myc p21 Max heterodimers and the described shift towards binding by p22 Max homodimers was performed by evaluating the relative decrease of c-Myc-bound E-box at different 10058-F4 concentrations. This comparison accounts for the different complexes present in the two experiments, as well as any difference in the extent of E-box binding in the absence of inhibitor. It was found that the same inhibitory effect is achieved in the presence of p22 Max by inhibitor concentrations four fold lower than those required in the presence of p21 Max, in spite of the higher affinity for E-box of c-Myc p22 Max (Figure 4d).

5.3.3. Rationale of differential inhibition effects from simulation of the system of equilibria

A mathematical analysis of this system of equilibria based on mass action of each species involved in the thermodynamic system was performed by means of assumption free numerical integration (KinTekSim, KinTek Corp.), which requires no assumptions beyond the definition of the mechanism for the interacting species. The results indicated that the effectiveness of an inhibitor increases as the affinity of the interaction involving the uninhibited component increases. In this case as Max
Fig. 4 Differential inhibition of c-Myc-Max binding of target E-box DNA in the presence of p21 or p22 Max isoforms. (continued on next page)
homodimer affinity increases (p22 versus p21 Max) Max more effectively competes with itself for binding to c-Myc facilitating the formation of c-Myc-10058-F4 complexes. Further, if the concentration of Max were increased, the relative advantage to the inhibitor in a system containing p22 Max would increase. The absolute amount of inhibition (that is c-Myc-10058-F4 complex) would, however, always decrease as the concentration of either Max protein increased. Such analysis supports the intuitive conclusion that uninhibited interactions in competition with inhibited ones may enhance inhibitory effects on the latter by external agents, this being true even in the more subtle case where a component may be shared between uninhibited and inhibited complex, such as the Max protein in the studied case.

5.4. Conclusion

These results provide insight into the mode of action of one c-Myc inhibitor, likely shared by the other studied inhibitors, which may involve the perturbation of competing biological equilibria rather than simple inhibition. Combining this
observation with the established existence of competing protein-protein interactions within transcriptional regulation and other cellular pathways [19-21], especially involving intrinsically disordered proteins [22, 23], suggests the possibility of exploiting the perturbation of these competing equilibria for the purposes of chemical interference. In order to develop such chemical modulators, it would be useful to examine what the liberated binding partner of a protein-protein interaction would do (to determine if a free energy gain can result) when selecting a partner to target. In the case of TFs, perturbation compared to simple disruption may allow even strongly bound, heteromeric TF-DNA complexes to be disrupted if the uninhibited protein partner is in excess and can participate in binding interactions. The achievement of chemical tools capable of modulating hub proteins would benefit the study of their functions and may bestow the possibility of their direct targeting for pharmaceutical applications [24]. The natural occurrence of multiple equilibria for proteins implicated in a given system might make small-molecules with relatively low affinity, granted sufficient target specificity, viable tools to selectively affect one single protein in that system.

5.5. Methods

Fluorescence Polarization Titrations and Competition Assay

To determine the c-Myc heterodimer affinity for p21 Max and p22 Max, samples were prepared containing 100 nM c-MycFLU and serial dilutions of p21 Max or p22 Max spanning a concentration range between 10 µM and 20 nM in 1X PBS (pH 7.4). The
samples were analyzed in a Photon Technology International QuantaMaster fluorimeter (Birmingham, NJ) equipped with polymer sheet polarizers at an excitation wavelength of 495 nm and an emission wavelength of 520 nm. Triplicate samples were analyzed for each concentration at 25 °C with sample specific G-factor determination and background correction. Data were fit to a Langmuir binding isotherm equation, neglecting the effect of c-Myc bound Max on the overall Max concentration. For a binding competition assay, triplicate samples were prepared in 1X PBS buffer (pH 7.4) containing 1.5 µM each of c-MycFLU and p21 Max or p22 Max, in the absence and presence of 10058-F4 at concentrations ranging between 1.65 and 100 µM. The samples were analyzed as above. The experimental data from the competition experiments were fit using the following equation, which is derived from thermodynamic considerations, relying on the presence of an equimolar ratio between c-MycFLU and p21 Max or p22 Max

\[ \text{Eq.2} \quad \text{pol} = \text{pol}_0 + \Delta \text{pol} \left\{ \frac{-[I]/[M] - 1 + \sqrt{([I]/[M] + 1)^2 + 4[I]/[M] \cdot (K_{comp} - 1)}}{2 \cdot K_{comp} - 2} \right\} \]

where \( \text{pol}_0 \) is the polarization in the absence of inhibitor and \( \Delta \text{pol} \) is the total change in polarization after complete disruption of c-MycFLU-Max heterodimers, \([I]\) is the concentration of 10058-F4 (variable), \([M]\) is the concentration of c-MycFLU and Max (fixed) and \( K_{comp} \) represents the ratio between the c-MycFLU-inhibitor and c-MycFLU-Max dissociation constants (\( K_{comp} = K_{D_{inhibitor}}/K_{D_{Max}} \)).
Electrophoretic Mobility Shift Assays (EMSAs)

Experiments were performed on 8% polyacrylamide:bis-acrylamide (80:1) gels in 0.5 X TBE. Binding reactions were prepared in a buffer consisting of 1X PBS (pH 7.4); 1 mM EDTA; 0.1% NP40; 5% glycerol; 1 mM dithiothreitol; and 0.4 mg/mL BSA. A 22 base-pair E-box-containing double stranded DNA oligonucleotide labeled on one strand with hexachlorofluorescein (HEX) consisted of the following sequence: 5’-HEX-CACCCGTCACGTGGCCTACAC-3’ and its unlabeled complement (Integrated DNA Technologies Inc., Coralville, IA). The duplex oligonucleotide was used at 10 nM in all reactions. Homo and heterodimer DNA binding affinities were determined by titrating increasing equimolar concentrations of proteins (at least five independent experiments each). Mechanistic aspects of DNA binding by bHLHZip and bZip transcription factors, involving multiple binding pathways between protein monomers, dimers, and target DNA, leading to cooperative binding, have been described [16, 17]. In this work we do not define a binding mechanism. The binding titrations were fit to a Langmuir binding isotherm with a cooperativity coefficient (Hill coefficient) to take into account the cooperative binding and yield an observed dissociation constant, $K_{obs}$, for E-box binding. Fitting the data to a fully cooperative model or a noncooperative model led to a worse fit, however, the $K_{obs}$ values for both models were within <5% (a fraction of the error) of that for the partially cooperative model used. The effect of DNA binding on protein concentration was neglected in the fit but was determined to have a similarly negligible effect on the reported affinity. DNA binding disruption experiments were performed at 25±1°C by incubating 150 nM
c-Myc(353-437) with 10058-F4 in concentrations ranging between 12.5 and 200 µM for 40 minutes, followed by addition of 150 nM p21 Max and E-box and a further 15 minute incubation. Equilibrium perturbation experiments were performed similarly with p21 Max replaced by p22 Max. All gels were run at 20°C and scanned on a BioRad FX molecular imager (BioRad, Hercules, CA). Data were analyzed with BioRad Quantity One software. Andrew T. Daab contributed to these experiments.

**Synthesis of 10058-F4, Expression and purification of c-Myc353-437, p21 and p22 Max isoforms** are described in Section 2.5. Compounds 10074-G5 and 10074-A4 were purchased from ChemBridge corporation (San Diego, CA).

The results described in this chapter were published in part in:

References


Chapter 6

Design of inhibitors with improved potency through multivalent binding

6.1. Introduction

All of the inhibitors of heterodimer formation between c-Myc and Max studied within this project share a common mechanism of action, which involves binding to distinct, short and intrinsically disordered regions of the monomeric c-Myc bHLHZip domain, distorting its structure, and preventing or reversing its interaction with Max [1]. In Chapter 2, derivatives of one such compound, 10058-F4, have been reported to be up to 6-fold more active when tested in a variety of biophysical and biological assays [2]. Despite these improvements, the IC$_{50}$’s of all c-Myc compounds identified to date remain high in cell-based assays (in the 5-50 µM range) [2-6]. Furthermore, only weak and incomplete structure activity relationship correlations have been observed for the studied derivatives of 10058-F4: this prevents the rational design of derivatives with greatly improved potencies. Thus, while demonstrating proof of principle that the inhibition of c-Myc-Max heterodimerization is a viable therapeutic option, the immediate clinical application of these compounds remains elusive. Analogs with even better binding properties, or methods for improving the activity of pre-existing analogs, are therefore clearly needed. However, given the lack of defined binding pockets on these compounds’ protein target it is not certain that even an extensive medicinal chemistry optimization could generate tight binders.
The knowledge that these compounds bind to multiple sites on the relatively unstructured c-Myc bHLHZip monomer may suggest an alternative approach to improving their efficacy. It should in fact be possible to link together two inhibitors of c-Myc-Max heterodimer formation with different binding sites on c-Myc and achieve bivalent binding with enhanced affinity. This would occur as a result of the local concentration of one moiety being increased near its binding site following binding of its companion moiety. Additionally, the off-rate of each moiety should be reduced as a result of tethering by its companion. Multivalent binding is of increasing interest in drug discovery studies, both in the context of fragment based drug design and that of independent binding moieties connected by an extended, flexible or rigid, un-functionalized linker [7-11]. This latter approach seemed a promising means of improving affinity and specificity of compounds binding c-Myc, and might be more generally applied to small molecules interacting with highly flexible protein segments similar to those found in the c-Myc monomer. It has been shown within this laboratory that all seven compounds previously identified by Yin et al. [6] bind to the bHLHZip domain of monomeric c-Myc at one of three distinct sites [1, 12]. Molecular models, based on NMR information, of the compounds 10074-G5 and 10058-F4 in association with synthetic peptides comprising their cognate binding sites have been described in Chapter 3. Each of the reported sites comprises a short stretch of predominantly hydrophobic amino acids, located at the interface between the basic region and helix1 and that between helix 2 and leucine zipper of the monomeric c-Myc bHLHZip respectively [1].
**Fig.1** Schematic representation of the cross-linking between c-Myc inhibitors 10074-G5 (red) and 10058-F4 (blue) based on the structural alignment of their docked poses to the backbone model of the c-Myc370-409 peptide, partially encompassing the binding site of both compounds.
Bivalent inhibitors of c-Myc-Max dimerization were designed by means of linking derivative structures of inhibitors 10074-G5 and 10058-F4 with flexible aliphatic chains varying in length and connection site, in order to generate different orientations of the two individual binding moieties. The intended target consisted of the two independent inhibitors 10074-G5 and 10058-F4 binding sites on the c-Myc bHLHZip domain, which are separated from one another by a stretch of approximately 25 amino acids, characterized by extensive backbone flexibility (Figure 1). This last feature was expected to allow, at the cost of some entropy loss, for the two binding sites to assume a relative orientation and distance from each other compatible with that imposed by the linker on the two binding moieties.

6.2. SAR studies on 10074-G5

A comprehensive series of derivatives of 10058-F4, described in Chapter 2, had been previously synthesized and structure-activity relationship (SAR) studies had been performed. Weak SAR correlations were observed for this series of related compounds, likely because of the inherent flexibility of the peptide – small-molecule complexes involving a disordered, dynamic protein segment, which might allow for accommodating different moieties and functional group substituents within the ligand. These studies were nonetheless useful in generating an adequate understanding of the pharmacophoric features of the 10058-F4 molecular scaffold, as confirmed by their satisfactory exploitation in computational models aimed at virtual screening of drug-like molecule libraries and lead hopping [13]. NMR studies of the binding segment
(amino acids 402-412) and the full c-Myc bHLHZip domain (amino acids 353-437) in complex with 10058-F4 had further contributed to the understanding of the interactions between this compound and its protein target.

In the case of 10074-G5 only NMR studies had been performed, both on the complex between this compound and the c-Myc segment spanning residues 363-381, and that involving the full-length c-Myc bHLHZip. While these experiments provided a general understanding of the mode of binding of 10074-G5 and its features crucial to the interaction, no information about the consequences on binding affinity of structural modifications of this compound was available. A limited series of derivatives of the 10074-G5 core structure was therefore synthesized, aimed at a targeted SAR study, with the explicit goal of identifying structural modifications resulting in lower molecular weight and improved atom economy compared to the parent structure (Figure 2, compounds 3-7). A simple synthetic strategy to introduce different substitutions on the amine in position 4 of the condensed heteroaromatic nitrobenzofurazan moiety was envisioned by performing nucleophilic aromatic substitutions on 4-chloro-7-nitrobenzofurazan with aromatic and aliphatic primary and secondary amines. Reductions in size and molecular weight of the biphenyl substituent found in the parent compound were explored in particular. It was found that the downsizing of the latter to a single phenyl ring (3) resulted in a mild reduction of binding affinity (7±1 µM, compared to 2.8±1 µM for the parent structure), while the complete removal of the aromatic ring, substituted by a dimethylamine group (4), resulted in a complete loss of affinity. It was further observed that reduction of the
nitro group in position 7 of the condensed heteroaromatic core to an amine, achieved by treatment with sodium dithionite, also resulted in a complete loss of binding affinity. The reduced binding is consistent with the hypothesized interaction of the partial negative charge of this strong dipolar group with positively charged arginine side chains. Compounds with functional groups apt for connection with a linker moiety were lastly tested; the set of 2, 3 and 4 aminobenzoic acids was therefore reacted with 4-chloro-7-nitrobenzofurazan. The binding affinity for c-Myc bHLHZip of these three compounds was reasonable in all cases, with a slight advantage of the 4-substituted derivative 7 (3±1 µM) over the remaining two (13±5 µM 2-aminobenzoate derivative 5, 16±6 µM 3-aminobenzoate derivative 6).

6.3. Synthetic Strategies

It was planned to connect the obtained derivative structures of the parent compound 10074-G5 to a linking aliphatic chain by means of amide formation between their carboxylic moiety and a terminal amine on the linker. Two different strategies to connect the linker chain to the second, 10058-F4-derived, binding scaffold were designed as well. One introduced the linker on the molecule’s aromatic ring, the other on its 5 member ring. With the goal of assessing the effect of the linker only on the binding moiety derived from 10074-G5, N-(5-aminopenty1)acetamide was condensed with its 3 and 4 amino benzoic acid derivatives to yield compounds 8* (3 substituted, not shown) and 8 (4 substituted). Similar derivatives, bearing the
acetylated linker chain only were also prepared according to each connecting strategy envisioned for the 10058-F4 binding moiety (compounds 10 and 15).

6.3.1. 6-member ring linker chain introduction

Three bivalent inhibitors were synthesized by connecting a mono Boc protected pentane-1,5-diamine linker via amide bond formation to a carboxylic derivative of 10058-F4 (Figure 2, compound 9) [14]. Condensation of 9 with N-(5-aminopentyl)acetamide was also performed (compound 10) in order to assess the effect of the linker only on the binding affinity for c-Myc of the parent 10058-F4 structure. The deprotected terminal amine on the linker chain bound to compound 9 was coupled via amide formation with the described carboxylic derivatives of 10074-G5 to yield bivalent compounds 11, 12, 13 (Figures 2, 3).

6.3.2. 5-member ring linker chain introduction

An alternative linking approach was explored, meant to test the modification of the connection between the linker chain and the binding moiety derived from 10058-F4, similarly to what was done for 10074-G5 by connecting the linker chain to 5, 6 or 7. In this strategy, Boc protected 6-bromo N-hexan-1-amine was connected by nucleophilic substitution to the imide nitrogen of a 2,4-thiazolidinedione derivative of 10058-F4 (Figure 2, compound 14) [15]. The obtained product, after deprotection, was acetylated to obtain compound 15, or connected by amide formation to 5, 6 or 7 to yield a second set of bivalent compounds (16,17,18, Figures 2, 4).
Fig. 2 Structures of synthesized compounds. Structures 1 and 2 correspond to compounds 10074-G5 and 10058-F4 respectively.
Fig. 3 Synthetic scheme for bivalent compounds 11, 12, 13 and control compound 10, obtained by connecting a linker chain to the carboxylic moiety found in compound 9 by means of amide formation.
Fig. 4. Synthetic scheme for bivalent and control compounds obtained by nucleophilic attack of 2,4-thiazolidinedione amide nitrogen to aliphatic bromides.
6.3.3. Anthranilic acid derivatives

The synthetic step involving the amide formation between 9 or 14 and the 2-aminobenzoic acid (anthranilic acid) derivative of 10074-G5 consistently generated products other than the desired ones in a number of reaction conditions. NMR analysis of organic extracted fractions of the reaction mixtures indicated the formation of an amine linked nitrobenzofurazan dimer. The observed behavior was potentially explained with a hypothetical reaction mechanism which implies a strong tendency towards the formation of dimeric complexes, with a symmetric distribution around an inversion center, for 2-aminobenzoic acid nitrobenzofurazan derivatives compared to their 3 and 4 substituted counterparts. Quantum mechanical molecular modeling suggests that the complex formation may be driven by hydrogen bonding between the amine donor of one molecule and carboxylic acceptor of the other, an interaction observed in a crystal form of anthranilic acid [16]. Upon activation of a carboxylate moiety towards nucleophilic attack with 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), such attack may be performed by the aromatic substituted secondary amine of the complementary molecule for each complex, rather than by the expected (and generally more reactive) aliphatic primary amine at the terminus of a linker chain. This event may be contextualized within a cascade of nucleophilic attacks, determined by the geometry of the dimeric complex, which result in a pericyclic-like reaction with the consequent formation of a symmetric, covalent nitrobenzofurazan dimer linked by an amine, and an asymmetric one between the two anthranilic acid moieties of the original complex (Figure 5).
Fig. 5 Hypothetical mechanism for side reactions observed when attempting amide formation between nitrobenzofurazan-conjugated anthranilic acid and primary amines found in the employed linker chains. **a.** Representation of HOMO (full surfaces) and LUMO (mesh) of a model dimeric complex of nitrobenzofurazan-conjugated anthranilic acid bearing EDC-activated acidic moieties calculated using the SCF method. The distribution of HOMO and LUMO over the two molecules in the complex suggests the possibility of reactions involving transfer of electron pairs between them. **b.** Highlighting of the proposed path of electron motions during the pericyclic-like reaction (in green). The electron movement would follow a spiral path. **c.** Schematic representation of the proposed reaction mechanism.
This problem in the synthesis of anthranilic acid derivative linked inhibitors was successfully overcome by means of following a linear synthesis approach instead of a converging one. This was achieved by linking, through amide formation, the amine terminus of the linker chain, which had been previously connected to the 10058-F4 derived binding moiety to form compounds 9 and 14, with Boc-protected anthranilic acid, followed by deprotection and nucleophilic aromatic substitution with 4-chloro-7-nitrobenzofurazan (Figures 3,4). Some degradation of these anthranilic acid derived bivalent inhibitors was however observed after long term storage (>6 months), especially when the compounds were stored as dry powder, suggesting that reactivity linked to intermolecular interactions like those hypothesized in this section might occur in the solid phase.

6.3.4. Variations on linker chain length

Lastly, a series of bivalent inhibitors was prepared with longer or shorter linker moieties in order to explore how the length of the linker chain would affect their c-Myc affinity. The length of the linker chain previously introduced on 14 was reduced by reacting this compound with Boc protected 4-bromobutan-1-amine to yield compound 35, or the linker previously introduced on compounds 9 and 14 was extended by means of amide formation with Boc protected linear amino acids (Boc-glycine or Boc-aminocaproic acid). Further deprotection and amide formation with 7 produced bivalent compounds 19, 20, 21, 22, 23 (Figure 6).
Fig. 6 Synthesized bivalent compounds with varying linker length.
6.4. Activity Profile

The ability of the synthesized compounds aimed at bivalent binding to c-Myc monomers to inhibit c-Myc function by means of disrupting its dimer formation with Max was tested in a number of assays as previously done for derivatives of 10058-F4. These assays were aimed at assessing the compounds’ efficacy at various stages of their inhibition mechanism, from their ability to bind c-Myc monomers to that of preventing E-box DNA binding by c-Myc-Max heterodimers, to the growth inhibition of c-Myc dependent cancer cell lines.

6.4.1. Direct binding to c-Myc monomer: fluorescence polarization

The binding affinity of the monovalent binding derivatives of the parent compounds 10074-G5 and 10058-F4 for recombinant monomeric c Myc bHLHZip (amino acids 353-437) was first measured in a fluorescence polarization (FP) assay [17] as similarly done for previously studied inhibitors. The observed affinities were generally close to those of the parent structures; in the series of amino benzoic acid derivatives of 10074-G5, namely 5, 6, 7, the para substituted compound 7 bound c-Myc ~5 times tighter than 5 and 6. The effect of the acetylated linker in compound 8 only mildly improved the binding affinity of 7, and, in the case of compound 9, addition of the linker to yield 10 was actually detrimental. In the context of the second explored connection between the linker chain and a derivative of 10058-F4, due to the low fluorescence of the 2,4-thiazolidinedione derivative compounds compared to the rhodanine derivatives, the c-Myc affinity of 15 was assessed in a competition assay
where the displacement of the fluorescent 10058-F4 from c-Myc binding upon addition of increasing concentrations of 15 was measured. Also in this case the c-Myc binding affinity was little affected by the introduction of the linker chain (Table 1, Figure 7).

Table 1 Tabulated binding affinities between studied compounds and purified c-Myc bHLHZip (residues 353-437) measured by fluorescence polarization.

<table>
<thead>
<tr>
<th>monovalent</th>
<th>$K_D$ ($\mu$M)</th>
<th>bivalent</th>
<th>$K_D$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (10074-G5)</td>
<td>2.8 ± 0.7</td>
<td>11</td>
<td>24 ± 4</td>
</tr>
<tr>
<td>2 (10058-F4)</td>
<td>5.7 ± 0.7</td>
<td>12</td>
<td>400 ± 100</td>
</tr>
<tr>
<td>3</td>
<td>7 ± 1</td>
<td>13</td>
<td>4.7 ± 0.8</td>
</tr>
<tr>
<td>4</td>
<td>&gt; 200</td>
<td>16 (linkN1)</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>5</td>
<td>13 ± 5</td>
<td>17</td>
<td>110 ± 20</td>
</tr>
<tr>
<td>6</td>
<td>16 ± 6</td>
<td>18</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>7</td>
<td>3 ± 1</td>
<td>19</td>
<td>320 ± 50</td>
</tr>
<tr>
<td>8</td>
<td>1.7 ± 0.9</td>
<td>20</td>
<td>55 ± 8</td>
</tr>
<tr>
<td>9</td>
<td>26 ± 7</td>
<td>21</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>10</td>
<td>90 ± 40</td>
<td>22</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td>15</td>
<td>7 ± 2</td>
<td>23</td>
<td>5.0 ± 0.8</td>
</tr>
</tbody>
</table>

The affinity of the synthesized bivalent inhibitors was then measured, by monitoring the fluorescence polarization of the compounds’ nitrobenzofurazan moiety. The compounds affinities were enhanced by more than 3 orders of magnitude compared to the corresponding monovalent unlinked structures, and were better than that of c-Myc-Max dimer formation. It was found that the linker connection by amide formation with the carboxylic group substituting the benzene ring of 9 (compounds 11, 12, 13) resulted in lower affinities than substitution of the 2,4-thiazolidinedione imide nitrogen (compounds 16, 17, 18). With respect to the variations on the 10074-G5
Fig. 7 Fluorescence polarization binding titrations between the studied compounds and c-Myc353-437 Data represent the average of three to five independent trials, error bars represent the standard error of the mean.
binding scaffold, the para substituted derivatives displayed the highest affinities and the meta the lowest (Table 1, Figure 7). The compounds’ specificities were confirmed by their lack of binding to Max p21 at a concentration of 10 µM (Figure 8).

![Figure 8](image)

**Fig. 8** Control experiments showing lack of binding between the synthesized bivalent inhibitors and p21 Max isoform. For each compound polarization values are shown without (-) and with (+) protein present.

As previously reported, unlike the Max p22 isoform, the p21 Max isoform shows low homodimerization affinity, thus making its disordered monomeric bHLHZip domain an excellent negative control for non-specific binding. The estimated free energy of binding of the bivalent compounds was roughly proportional to the sum of those of their monovalent components. In the thermodynamic analysis of multivalent binding a $\Delta G^S$ term is used to relate the free energy of binding of a multivalent compound to the sum of those of its monovalent components. This term implies the diverse energy contributions resulting from the linking of independent binding components [18, 19].
The $\Delta G^S$ term generally constitutes an unfavorable contribution to the binding affinity of distinct binding components connected by a flexible linker to a given target. The estimated $\Delta G^S$ for the studied bivalent compounds binding to c-Myc bHLHZip, based on the sum of the affinities of their unlinked components 5, 6 or 7 and 9 or 2, is relatively low, in spite of the lack of optimization of length, chemical nature, and connection site of the linking moiety, suggesting that the flexible nature of the target protein might facilitate the binding of bivalent ligands compared to more rigidly structured binding sites. The low $\Delta G^S$ further indicates that no major loss in conformational entropy of the target protein occurs as a consequence of binding, which seems to involve mainly the localized interaction sites of the parent compounds and only to a minor extent the flexible protein segment connecting them.

The observed binding affinities of the series of bivalent inhibitors with different linker lengths further supported this mode of binding: variations of the linker length resulted in minor changes in binding affinity – generally a mild loss (with the only exception being compound 19, which displayed considerably reduced binding affinity) – compared to the original series of bivalent compounds with 6 heavy atoms in the linker chain (Figure 9). These results suggest that extension of the linker length may allow for an optimal interaction of each binding moiety with its c-Myc site with only limited rotational constraints on the flexible chain connecting them. Correspondingly, the backbone plasticity of the target protein may allow for the correct binding of such moieties when connected by shorter linker chains.
6.4.2. Kinetics of disruption of c-Myc-Max interaction

The kinetics of disruption of c-Myc-Max heterodimers by bivalent inhibitors were studied in circular dichroism experiments similar to those described in Chapter 4 for the monitoring of disruption kinetics of c-Myc-Max heterodimers by the inhibitors 10074-G5, 10058-F4 and 10074-A4. It was found that full disruption of c-Myc-Max heterodimers was obtained at considerably lower concentrations than those required in the case of monovalent inhibitors. The kinetics of disruption were considerably faster than those previously observed for the fastest monovalent disruptor, 10074-G5, and unlike the latter, displayed no concentration dependence (Figure 10). While the described experiments do not make it possible to completely rule out the possibility that this latter effect is an artifact due to some limiting rate external to that
Fig. 10 Kinetic of disruption of c-Myc-Max heterodimers by bivalent inhibitors measured by monitoring the helical content of the protein component by means of circular dichroism. a. Representative time course measurement for each studied compound. b. Logarithmic plot of the observed rates compared to those previously recorded for monovalent compounds 10074-G5 and 10058-F4.
of the phenomenon of interest, such as mixing effects and instrumental response times, the obtained data suggest that the fast disruption of c-Myc-Max heterodimers by bivalent inhibitors may be dictated by some dynamic process of the protein dimer itself, in a similar way (only much faster) to what was observed for the inhibitors 10058-F4 and 10074-A4 that acted by capturing freed monomers upon spontaneous dissociation of the c-Myc-Max complex. In the case of bivalent inhibitors, the observed fast rates of disruption suggest that the rate of partial unfolding of helix1, which may make the 10074-G5 site available for binding, may limit the inhibitors access to this site. The binding of the bivalent inhibitors’ 10074-G5 derived moiety to this segment would then result in a strong enough destabilization of the c-Myc-Max complex in its HLH portion to allow for the tethered 10058-F4 derived binding moiety to promptly interact with its own target site and promote the full disruption of the c-Myc-Max heterodimer. In the presence of an adequate concentration of a bivalent inhibitor, each partial unfolding of the helix1 N terminus of the c-Myc-Max HLHZip complex, which likely happens at a considerably higher rate than full dissociation of the heterodimers, would result in the inhibitors capture of this partially unfolded state and, consequently, extremely fast full disruption of the c-Myc-Max heterodimer.

6.4.3. Disruption of c-Myc-Max DNA binding: EMSA

The efficacy of the linked compounds 11, 12, 13, 16, 17, 18 in inhibiting the c-Myc-Max interaction was evaluated by measuring the binding of a target DNA sequence (E-box) by the protein heterodimers in an electrophoretic mobility shift assay
Strong disruption of DNA binding was observed in all cases at higher protein concentrations and lower inhibitor concentrations than those required in similar experiments performed with the parent compounds 10074-G5 and 10058-F4, demonstrating the appeal of the bivalent binding approach to enhance the inhibition of c-Myc function by small molecules (Figure 11). The bivalent inhibitors were actually so effective at disrupting the DNA binding that it was not possible to completely titrate the effect by dilution of the inhibitor.

6.4.4. Cell based assays

It has been reported in Chapter 2 and elsewhere that 10074-G5, 10058-F4 and numerous analogs of 10058-F4 can specifically inhibit the growth of c-Myc-dependent human cancer cell lines [2, 6] but exert substantially less effect on the growth of c-Myc knockout cells [21]. Several analogs of 10058-F4 have also been previously identified as being 5-8-fold more potent than the parental compound in both HL60 promyelocytic cells [22] and Burkitt lymphoma (BL) cells [23]. The bivalent compounds were therefore tested for growth inhibition of HL60 and BL cells in the Prochownik laboratory at the University of Pittsburgh, where it was found that most such inhibitors outperform the parent compounds in this assay. Several of the compounds were found to be quite potent and significantly better than 10074-G5, 10058-F4 or any of the former compound’s previously tested analogs [2]. Of these, the best was compound 16 (LINK N1), with an IC₅₀ assessed to be between 0.5-1.0 µM in both of the above cell lines (Table 2). In contrast, higher concentrations of the bivalent compounds were
Fig. 11 Inhibition of c-Myc-Max DNA binding measured by electrophoretic mobility shift assay (EMSA). a. Inhibition by bivalent compounds 11, 12, 13. b. Inhibition by bivalent compounds 16, 17, 18. c. Inhibition by monovalent compounds 10074-G5 and 10058-F4. Inhibitor concentrations are micromolar. The c-Myc-Max dimer concentration was 1 µM in a and b, 50 nM in c.
required to inhibit the growth of the non-transformed rat fibroblast line TGR1 or of TGR1-derived c-Myc null rat fibroblasts reconstituted with either of the c-Myc target genes HMGA1b or MT-MC1, which restore a normal growth rate [2, 24]. These results indicate that the studied bivalent compounds are specifically growth inhibitory for cells expressing the highest levels of c-Myc (Figure 12).

**Table 2** Growth inhibition of HL60 and Burkitt’s Lymphoma c-Myc dependent cancer cell lines by bivalent compounds. Values represent IC\(_{50}\) in µM.

<table>
<thead>
<tr>
<th></th>
<th>HL60</th>
<th>BL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (10074-G5)</td>
<td>25</td>
<td>20</td>
</tr>
<tr>
<td>2 (10058-F4)</td>
<td>50</td>
<td>40</td>
</tr>
<tr>
<td>11</td>
<td>&gt;10</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>&gt;30</td>
<td>20-35</td>
</tr>
<tr>
<td>13</td>
<td>6</td>
<td>5-10</td>
</tr>
<tr>
<td>16 (linkN1)</td>
<td>0.5-0.7</td>
<td>0.7-0.9</td>
</tr>
<tr>
<td>17</td>
<td>&gt;30</td>
<td>&gt;10</td>
</tr>
<tr>
<td>18</td>
<td>7-9</td>
<td>2-4</td>
</tr>
<tr>
<td>19</td>
<td>7-13</td>
<td>7-11</td>
</tr>
<tr>
<td>21</td>
<td>7-9</td>
<td>14-16</td>
</tr>
<tr>
<td>22</td>
<td>2-4</td>
<td>4-6</td>
</tr>
</tbody>
</table>

The lower, albeit promising, potency of the bivalent inhibitors in cell based assays compared to ones involving purified components, and the partial lack of correlation between the assays (Figure 13), may reflect a generally poor cell permeability of these compounds, which are relatively bulky and display higher than optimal polar surface areas [25]. The effects of the linker chain connectivity on the compounds conformation and shape may also affect their cell permeability in a
Fig. 12 Compound 16 (linkN1) cell growth inhibition for HL60 (a), Burkitt’s lymphoma (b), TGR1 (c) and c-Myc knockout (d) cells. Black: cell growth in the absence of inhibitor, blue: + 40 µM 10058-F4 (2); red: + 4 µM (d), 2 µM (a, c) or 1 µM (b) linkN1; orange: + 2 µM (d), 1 µM (a, c) or 500 nM (b) linkN1; yellow + 500 nM (a) or 200 nM (b) linkN1 (data from Prochownik lab., University of Pittsburgh).
different manner from what is observed for their c-Myc binding affinity, resulting in the aforementioned incomplete correlation between their performance in cell based and purified components assays. Five additional bivalent inhibitors were synthesized with the goal of providing an initial, partial evaluation of the dependence of the molecules’ poor activity against cancer cell growth on their cell permeability and metabolic stability. A fluorinated substitute of 14, compound 24, the choice of which was based upon the results of structure activity relationship studies described in Chapter 2, was employed for this purpose [26, 27]. Upon linking, the obtained compounds, 25, 26, 27, 28, 29 (Figure 14) display, on average, lower molecular weights and polar surface areas than the first group of bivalent compounds (589 vs. 657 Da and 188.6 vs. 226.6 Å² respectively – compound 21 was included in the new set of inhibitors in these
statistics), and the metabolic stability of their binding moiety derived from 10058-F4, previously shown to be low [28], may be improved as well [26, 27]. The c-Myc affinities of compounds 25, 26, 27, 28, 29, as measured by fluorescence polarization, were slightly lower than those of corresponding bivalent compounds from the first group, their inhibitory potencies against the growth of HL60 and BL cells were, however, on average twice as high as those of the previously tested compounds, although none of the new molecules outperformed compound 16-LINK N1 (Table 3, Figure 15). The consistently promising activity of the last group of bivalent compounds suggests that the scaffold of molecule 24 might be chosen as a starting point for further optimization of bivalent inhibitors.

Fig.14 Fluorinated building block derivative of 10058-F4 and second group of bivalent inhibitors.
Table 3 c-Myc affinities and cell line growth inhibition of the second group of bivalent compounds.

<table>
<thead>
<tr>
<th></th>
<th>KD (nM)</th>
<th>HL60 (μM)</th>
<th>BL (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>3.0 ± 0.5</td>
<td>2-6</td>
<td>2-2.5</td>
</tr>
<tr>
<td>26</td>
<td>4.6 ± 0.8</td>
<td>3-3.5</td>
<td>6-8</td>
</tr>
<tr>
<td>27</td>
<td>1.7 ± 0.4</td>
<td>3-4</td>
<td>1.2</td>
</tr>
<tr>
<td>28</td>
<td>11 ± 2</td>
<td>1.5-1.7</td>
<td>1-1.2</td>
</tr>
<tr>
<td>29</td>
<td>3.6 ± 0.6</td>
<td>6-10</td>
<td>6-7</td>
</tr>
</tbody>
</table>

To determine whether the disruption of c-Myc-Max heterodimers by bivalent compounds observed with recombinant proteins could be reproduced in cultured cells in a manner that correlated with growth inhibition Dr. Huabo Wang in the Prochownik lab performed co-immunoprecipitation experiments: HL60 cells were incubated for 8 hours with serial dilutions of the four most effective bivalent compounds. Equivalent numbers of cells were then lysed and subjected to immunoprecipitation with an anti-Max antibody [29] followed by SDS-PAGE and immunoblotting with an anti-c-Myc mAb. All four bivalent compounds were highly effective in preventing and/or disrupting the c-Myc-Max association in a concentration-dependent manner. In general the concentrations of compounds required to achieve a >50% dissociation between c-Myc and Max were in good agreement with the concentrations needed to inhibit cell growth (Figure 16).
Fig. 15 Activity data for the second group of bivalent inhibitors (25-29, continued on next page).
Fig. 15 a. Fluorescence polarization titrations of c-Myc binding, data represent the average of three to five independent trials, error bars represent the standard error of the mean. Inhibition of HL60 (b) and BL (c) cell growth by compound 27. Inhibition of HL60 (d) and BL (e) cell growth by compound 28 (red: + 2 µM inhibitor; orange: + 1 µM inhibitor; yellow: + 0.5 µM inhibitor; black: DMSO only, cell inhibition data from the Prochownik lab, University of Pittsburgh).

Fig. 16 Co-immunoprecipitation assays with the four most potent bivalent compounds. HL60 cells were incubated for 6 hr with the indicated concentration of each compound. 10058-F4 at the indicated concentration was used as a control. Total cell lysates were then prepared and precipitated with an anti-Max antibody. The immunoprecipitate was then subjected to SDS-PAGE and immunolotting with an anti-c-Myc mAb as previously described (data from Prochownik lab., University of Pittsburgh).
6.5. Structural studies of the interaction between bivalent inhibitors and c-Myc bHLHZip

To confirm the occurrence of simultaneous bivalent binding at the expected protein sites, the affinity of bivalent compounds \textbf{11} and \textbf{13} for peptides encompassing segments of c-Myc bHLHZip containing the binding site of either 10074-G5 (amino acids 363-381) or 10058-F4 (amino acids 390-439) was measured by fluorescence polarization. The observed affinities correlated well with those of the corresponding unlinked derivatives of 10074-G5 and 10058-F4 to the full-length cMyc bHLHZip, confirming that the binding of each component of the linked compounds still occurs at the expected site and that the high binding affinity of these molecules actually depends on the presence of both binding sites on the target (Figure 17, Table 4).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig17.png}
\caption{Schematic representation of the recombinant proteins and synthetic peptides encompassing one or both monovalent inhibitors binding sites.}
\end{figure}

\textbf{Table 4} Binding affinities of bivalent compounds to truncated c-Myc bHLHZip segments. Values are μM.

\begin{table}
\centering
\begin{tabular}{ccc}
\hline
 & c-Myc 363-381 & c-Myc 390-439 \\
\hline
11 & 10 ± 3 & 122 ± 9 \\
13 & 0.8 ± 0.6 & 53 ± 16 \\
\hline
\end{tabular}
\end{table}
The overall effect of bivalent ligands binding to c-Myc bHLHZip was examined by monitoring the circular dichroism (CD) spectrum of this protein in the absence and presence of compounds 11, 12, 13, 16, 17, 18. As has been described in Chapter 3, the effect of either monovalent compound 10074-G5 or 10058-F4 on the c-Myc bHLHZip CD spectrum was rather limited: the typical random coil features of this peptide, characterized by low signal intensity and a minimum at ~208 nm [30], were conserved upon binding of either of these compounds. It was previously demonstrated that this behavior is related to the localized nature of the inhibitors’ binding sites, as conformational rearrangements of the peptide upon ligand binding are limited to a narrow set of amino acids and are hard to observe within the CD spectrum of the full length target protein domain, which remains mainly flexible and unstructured [1]. In the present case of bivalent compounds, it is unclear whether the introduced distance constraint between the binding moieties interacting with each protein site would result in more consistent conformational rearrangements over the entire target protein domain. It was observed, however, that binding of any bivalent inhibitor did not result in major changes in shape or intensity of the c-Myc bHLHZip CD spectrum (Figure 18). This result further confirms that the flexible nature of the protein segment between the two binding sites, which seems to facilitate optimal bivalent binding of the studied ligands, is preserved to a large extent when this binding occurs.

Further structural insight on the c-Myc interaction of 18, the compound with the highest affinity for this protein target, was obtained by means of analyzing the effects of binding on c-Myc backbone $\alpha^{1}H$ NMR signals. As discussed in Chapters 1
Fig. 18 Circular dichroism spectra of c-Myc bHLHZip in the absence (white circles) and presence (black circles) of bivalent inhibitors.
and 3, the magnetic field distribution of these signals is coherently associated with the backbone dihedral angles of each amino acid [31], and their change in shift upon a molecular event such as ligand binding can be related to conformational rearrangements associated with it. Other effects on backbone chemical shift signals, unrelated to conformational changes, might be induced by shielding effects dependent on the different chemical environment in proximity of an amino acid upon ligand binding. In both cases changes in backbone chemical shift can help identify residues directly involved in a binding interaction [32]. In the case of the unbound ID c-Myc bHLHZip monomer, the $^1$H backbone resonances were only partially assigned, but to a satisfactory extent for the sequence regions spanning the binding sites of 10074-G5 and 10058-F4, thanks to the low redundancy of several amino acids found within these segments. The chemical shift pattern of the assigned resonances is typical of ID protein regions [33], and does not display extended segments with a consistent secondary structure trend, suggesting instead the presence of residual structure at a local level. Upon addition of 18, changes in the $^1$H signals of several residues within the binding sites of both 10074-G5 and 10058-F4, including respectively Phe$_{374-375}$ and Tyr$_{402}$, were observed (Figure 19a). Similar changes in the Tyr$_{402}$ aromatic signals to those induced upon binding of 10058-F4 were also observed (Figure 19b). Comparison of COSY spectra of the $^1$H region of c-Myc in the presence of 18 or both 10074-G5 and 10058-F4 simultaneously further suggested that the overall interaction of the first molecule with the target protein results in a pattern of chemical shift changes that closely resemble that induced by the independent and simultaneous
binding of the latter two (Figure 19c). The NOESY spectrum of c-Myc bHLHZip in the presence of 18 shows a series of intermolecular cross-peaks that involve the same residues or proximal ones to those whose backbone resonances are affected by this compound’s binding (figure 20).

Fig.19 NMR study of the interaction between c-Myc bHLHZip and compound 18 (continued on next page).
Fig. 19 a. Chemical Shift Indexing analysis of assigned backbone $^1$H α resonances of c-Myc353-437 in the absence (black) and presence (red) of 18. b. Overlaid $^1$H α region of the COSY spectra of pure c-Myc353-437 (grey), the same protein in the presence of both 10074-G5 (1) and 10058-F4 (2) (blue), or compound 18 (red). c. Overlaid aromatic region of the COSY spectra of c-Myc353-437 in the absence (grey) and presence (red) of 18.

6.6. Conclusion

It has been repeatedly shown that certain small molecules can effectively inhibit or reverse c-Myc-Max heterodimerization and suppress the proliferation of a variety of c-Myc-over-expressing cell types in a highly specific manner [2, 3, 6]. The work described here demonstrates that these molecules bind independently to distinct sites in the bHLHZip domain of the relatively unstructured c-Myc monomer and nuclear magnetic resonance-based studies using synthetic peptides have provided structural models for the bound compounds [1, 2].

A new generation of compounds has been designed with the intent of achieving bivalent binding to the intrinsically disordered monomeric bHLHZip domain of c-Myc. The underlying strategy consisted in connecting two scaffold structures previously found to interact with different regions of the c-Myc bHLHZip domain with a flexible linker, here an aliphatic chain moiety. One such scaffold was derived from the inhibitor 10074-G5, binding at the interface between basic region and helix1 (amino acids 365-375), the second binding moiety was derived from 10058-F4, binding to the c-Myc segment at the interface between helix2 and leucine zipper (amino acids 402-409) [1].
Fig.20 NOE study of the complex between compound 18 and c-Myc353-437 (continued on next page).
The analysis of the observed c-Myc binding affinities of the bivalent compounds described here shows that derivatives with the linker chain connected to the 5 member ring of the binding moiety derived from 10058-F4 have higher affinities than derivatives where the linker is connected to the phenyl ring found within this ligand scaffold. As for modifications of the binding moiety derived from 10074-G5, 4-aminobenzoic acid derivatives have the highest affinities within the series of different aminobenzoic acid stereoisomers while the 3-substituted compounds were the lowest. These observations are, as indicated, in fair agreement with the relative binding affinities of the non-linked scaffold components of each linked compound. Changes in the linker length lead to minor changes in affinity, with the optimal linker size estimated as 5-6 heavy atoms.

The increased affinity for c-Myc binding of the synthesized inhibitors was confirmed to be a consequence of their bivalent binding to the expected target sites on this protein. NMR and CD experiments provided structural information about the mode of interaction of these compounds with c-Myc and confirmed that upon their binding the flexible nature of the ID target protein is mainly preserved, as previously shown for monovalent binding inhibitors.

The ability of the described bivalent compounds to interfere with c-Myc biological functions was confirmed in vitro in an EMSA assay. The bivalent inhibitors
were all superior by orders of magnitude to the monovalent ones in disrupting target DNA binding by c-Myc-Max heterodimers. These compounds were finally tested for growth inhibition of c-Myc over expressing human cancer cell lines HL60 and Burkitt’s lymphoma. The observed potencies were strongly encouraging, albeit lower than the direct c-Myc binding affinities of these compounds would have suggested, and their correlation with the latter was incomplete. This difference likely reflects a combination of factors such as uptake, subcellular distribution and metabolism that have not been studied here. For example, it is noticeable that the molecular weight of each bivalent compound, including its linker, is more than twice that of each parental compound. Moreover, the polar surface of bivalent compounds is quite high. Both of these factors are known to substantially affect a compound’s uptake [25]. The results obtained upon screening of a second group of bivalent compounds with reduced polar surface areas and lower molecular weights support such indication. Julie Eiseman in the Department of Pharmacology, University of Pittsburgh has begun initial experiments to assess cellular uptake of the inhibitors. The bivalent compounds reported here did not incorporate any of the previously reported analogs of 10058-F4 [2] and “optimized” analogs of 10074-G5 have not yet been identified. Rather, they relied on derivatives that were chosen based on the simplicity of the proposed linking strategy and consideration of yields. It is therefore reasonable to suppose that further improvements might be achievable when such optimized individual analogs are used for the synthesis of subsequent generations of bivalent compounds.
Despite the above-cited disparities, it was possible to observe that HL60 cells and BL cells, both of which express extremely high levels of c-Myc, were more sensitive to bivalent compounds than were fibroblasts with normal levels of the protein. This difference might reflect any one of a number of non-mutually exclusive differences between transformed and non-transformed cells such as differential compound uptake, distribution and metabolism as well as the possibility that both tumor cell lines are “addicted” to c-Myc or have developed “oncogene amnesia” [34, 35] and are therefore unable to tolerate as severe a reduction as are non-transformed cells. HL60 and BL cells might also be formally analogous to those that over-express topoisomerase I or II, which tend to be more sensitive, rather than less sensitive, to chemotherapeutic agents that target these enzymes [36].

Overall, the described bivalent compounds displayed strongly enhanced activities compared to the monovalent parent structures, and are promising for further development with therapeutic purposes. The reported results indicate that multivalent binding is effective in improving the potency of c-Myc targeting agents; they also suggest that it could be an appealing approach to the targeting of ID proteins in a more general sense. ID proteins are increasingly of interest because of their frequent involvement in eukaryotic cell signaling and regulation [37]. The sequence inspection of biologically relevant ID proteins indicates that multiple short hydrophobic segments, similar to the interaction site on c-Myc for 10074-G5 and 10058-F4, might be found on several single flexible protein domains (Figure 21). When targeting ID proteins, bivalent binding might make it possible to overcome the low binding affinity of
Fig. 21 Prediction of disordered regions with the PONDR VSL2 algorithm for the full sequence of three, biologically relevant, intrinsically disordered proteins: c-Myc, MDM2 and Amyloid beta (Aβ) precursor protein (APP). The pattern of narrow regions with low disorder probability, observed within the c-Myc bHLHZip domain in correspondence of the sites exploited here for bivalent binding, is consistently found over the sequence of these three proteins. Shaded areas indicate natively folded domains.
monovalent binding scaffolds, without the need of an extensive development of the linker component. The protein flexibility could facilitate bivalent binding by allowing for the involved target sites to come at an optimal reciprocal orientation and distance from each other at a relatively low energy cost, thus reducing constraints on the linker size, and, due to the absence of deep, rigid clefts on disordered interaction sites, by reducing restrictions on the positioning and direction of the linker in the connection between the latter and binding moieties.

6.7. Methods

All reagents and solvents were > 98% purity and were purchased from Sigma-Aldrich. Mono Boc-protected 1,5 N-pentyldiamine was purchased from Novabiochem (Läufelfingen, CH). Dichloromethane (DCM) and dimethylformamide (DMF) were dried on molecular sieves. Glassware was dried by flaming before usage. NMR, recorded on 400 MHz or 300 MHz Varian INOVA instruments, and LC-MS, performed with a Varian 500-MS Ion Trap spectrometer, were employed for product characterization. HPLC, with a 30 minutes gradient 100%A:0%B to 0%A:100%B (where A=water, 0.1% V/V trifluoroacetic acid [TFA], B= acetonitrile, 0.1% V/V TFA) on a Varian ProStar instrument equipped with a C18 reversed phase Alltech Econosphere column was employed for product purification, when needed, and purity assessment. Detection wavelengths were set at 350 and 450 nm.
Condensation of benzaldehydes with rhodanine or 2,4-thiazolidinedione.

Rhodanine or 2,4-thiazolidinedione (3 mmol) and 0.69 g ammonium acetate were dissolved with heating in 4.5 mL glacial acetic acid. The aldehyde substrate (1.1 equivalents) was slowly added. The mixture was refluxed for 2 hours, cooled to room temperature and diluted with 50 mL water to precipitate the product. The reaction is stereoselective and only Z product is detectable by NMR. Yields were 99% for 1, 95% for 9, 91% for 14 and 60% for 24.

Nucleophilic aromatic substitution of 4-chloro-7-nitrobenzofurazan with aromatic amines or dimethylamine.

To 300 mg 4-Chloro-7-nitrobenzofurazan (1.5 mmol), dissolved in 4 mL anhydrous methanol, 10 µL triethylamine were added, followed by dropwise addition of 1.05 equivalents of reactant primary or secondary amine, dissolved in 6 mL anhydrous methanol. The reactions were stirred at room temperature for three hours, diluted with water, precipitate was collected and recrystallized from water-DMF or water-methanol mixtures. Yields ranged between 73% and 94%. Due to extensive formation of byproducts when attempting to react 31, 33, 35, 37 or 39 with 5, a small-scale reaction (20 µmol) was similarly performed in the final step of preparation of the bivalent compounds 11, 16, 25, 26, 28 using 32, 34, 36, 38 or 40 respectively as the amine reactant, with yields ranging between 58% and 86%.
Amide formation.
The carboxylic substrate (0.4 mmol to 40 µmol, 1 equivalent) was dissolved in the presence of 1.2 equivalents 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) in 5 mL to 1.5 mL anhydrous 2:1 DCM-DMF mixture. A catalytic amount of triethylamine was then added, followed by the amine (1.1 equivalents), and the reaction stirred at room temperature for 24 hours. The reaction mixture was then diluted with DCM if necessary to a final volume of 5 mL, quenched with basic water (to remove unreacted acid); it was then washed with diluted aqueous HCl (to remove unreacted amine), followed by solvent evaporation. Yields ranged between 55% and 95%.

Nucleophilic substitution at 2,4-thiazolidinedione imide nitrogen.
Compound 14 or 24 (0.4 mmol) was dissolved in 5 mL acetone in the presence of 60 mg K$_2$CO$_3$. Tert-butyl N-(6-bromohexyl)carbamate or tert-butyl N-(4-bromobutyl)carbamate (1.05 eq.) was added and the reaction heated to reflux for 3 hours; after cooling to room temperature, precipitate salts were removed by filtration and the solvent evaporated to yield 83% - 98% product.

Acetylation of tert-butyl N-(5-aminopentyl)carbamate and 33.
Tert-butyl N-(5-aminopentyl)carbamate (0.75 mmol, 1 equivalent) or 33 (60 µmol, 1 equivalent) were dissolved in 5 mL or 1 mL respectively anhydrous DCM, cooled to 0°C. Acetyl chloride (1.5 equivalents) was then added and the reaction allowed to
warm to room temperature over a 20 minutes period, followed by quenching with water. The solvent was the evaporated to yield 99% N-(5-acetamidopentyl)carbamate or 97% 15.

**Boc deprotection.**

Substrates were dissolved in anhydrous DCM, 5% TFA (1 to 2 mL) and stirred at room temperature for 2 hours, followed by a wash with aqueous K$_2$CO$_3$ and solvent evaporation. Yields were nearly quantitative in all cases.

**Protein cloning, expression and purification.**

A c-Myc390-439 truncated version of the c-Myc bHLHZip was produced from the c-Myc/pET SKB3 construct, kindly supplied by Dr. S. K. Nair (University of Illinois, Urbana-Champaign), by insertion into the pET151D vector, encoding an N-terminal hexahistidine (6xHis), separated by a TEV protease digestion site, with the TOPO® ligation system, and over-expressed in *E.coli* BL21(DE3) (pLysS). Bacterial cultures were grown at 37°C in LB medium to OD$_{600}$≈ 0.8, then induced with 0.5 mM IPTG for 5 hours. The protein was purified by Ni-agarose chromatography with a pH gradient elution. The 6xHis was cleaved using TEV protease. The cleaved product was further purified by HPLC and lyophilized. Protein concentration was determined by measurement of OD$_{280}$. 

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**Fluorescence Polarization Titrations.**

Samples were analyzed in a Photon Technology International QuantaMaster fluorimeter (Birmingham, NJ) equipped with polymer sheet polarizers. Titration experiments were performed upon serial dilution of 1:1 equimolar solution of inhibitor and either c-Myc353-437, c-Myc390-439 or c-Myc363-381 in a 1X PBS buffer (pH 7.4), 5% DMSO, over concentrations ranging between 200 µM and 1.5 nM. Excitation and emission wavelengths were independently determined for each inhibitor. Reported data represent the average of at least three independent experiments. Data were fit to a quadratic equation derived from the thermodynamic expression of binding equilibrium:

\[
\frac{[\text{complex}]}{[C]_0} = \frac{2 + K_D/[C]_0 - \sqrt{(-2 - K_D/[C]_0)^2 - 4}}{2},
\]

where \([C]_0\) represents the total concentration of inhibitor and of c-Myc peptide. The value of \(K_D\) was determined from the experimental polarization data by fitting to Eq. 2 using KaleidaGraph (Synergy Software, Reading, PA) where \(\text{pol}_0\) is the polarization in the absence of binding and \(\Delta\text{pol}\) is the total change in polarization[17].

\[
\text{polation} = \text{pol}_0 + \Delta\text{pol} \cdot \left( \frac{[\text{complex}]}{[C]_0} \right)
\]

Due to the very tight binding of some of the tested compounds, it was impossible to perform a full binding titration with an acceptable fluorescence intensity, therefore the \(\text{pol}_0\) parameter was experimentally determined from samples of each compound in the absence of c-Myc353-437 at a concentration of 25 µM. Binding specificity experiments were performed by monitoring the FP of 10 µM inhibitors solutions in the above-
described buffer in the presence of equimolar p21 Max. This protein has low affinity for homodimer formation [29] and no inhibitor showed a change in polarization in its presence.

**Circular Dichroism.**

Samples of c-Myc353-437 (5 µM) in the absence and presence of an equimolar concentration of each tested inhibitor were prepared in 1X PBS buffer (pH 7.4). The inhibitors were added from 10 mM stock solutions in ethanol. Spectra were recorded at 25 °C in a 1 mm path-length quartz cuvette on a Jasco J710 spectro-polarimeter. Shown spectra are averaged from three independent samples.

**NMR spectroscopy.**

Experiments were performed on a 500 MHz magnet Varian INOVA instrument equipped with a 5 mm double-resonance indirect detection probe. Solutions of c-Myc353-437 (~500 µM) in the absence or presence of equimolar inhibitor 18, or 1 and 2 simultaneously, added from a 0.1 M stock in DMSO-d₆, were prepared in 100% D₂O, 5 mM sodium phosphate buffer, pH 7.5. Samples were also prepared of c-Myc353-437 alone in 90% H₂O – 10% D₂O, 5 mM sodium phosphate buffer, pH 6.3 (for Hₐ(i) – Hₓ(i+1) NOE sequential assignments). 2D ¹H homo-nuclear spectra were recorded at 25°C over sweep widths of ~10X10 ppm with 32-64 scans/t₁ increment, 1.5-2 s relaxation delay and sizes of 512-1024X2048 complex points. TOCSY and NOE mixing times of respectively 60 and 150 milliseconds were employed. All spectra
were acquired with selective presaturation water suppression. Partial backbone assignments for c-Myc353-437 were obtained from TOCSY, COSY and $H_a(i) – H_N(i+1)$ NOEs of low pH, 90% H$_2$O – 10% D$_2$O samples of the pure peptide (refer to Chapter 3). Spectra were processed using MestReC software (MestreLab Research, Santiago de Compostela, Spain). Data were filled by linear prediction to a final Fourier transform size of 2048x2048 points and weighted by sine square and sine bell apodization over $t_1$ and $t_2$ respectively before Fourier transformation.

**Electrophoretic Mobility Shift Assays (EMSA).**

Reactions containing c-Myc$_{353-437}$ and varying concentrations of each tested inhibitor in a buffer containing 1X PBS, 1 mM EDTA; 0.1% NP40; 5% Glycerol; 1 mM DTT; 0.4 mg/mL BSA were incubated for 30 minutes, followed by addition of pre-mixed p21 Max and a synthetic double-stranded oligonucleotide containing a consensus c-Myc-binding “E-Box” element (CACGTG). The final protein concentration was 1 µM for both c-Myc$_{353-437}$ and p21 Max, 10 nM E-Box DNA. The binding reaction was then allowed to proceed for an additional 15 minutes before loading on an 8% running gel (80:1 poly acrylamide:bis-acrylamide). Control experiments with the monovalent parent inhibitors 1 and 2 were similarly performed using a lower (50 nM) concentration of each protein component. Gels were run at 20°C in 0.5 X TBE and scanned on a BioRad FX molecular imager. Data were analyzed with BioRad Quantity One software.
Expression and purification of c-Myc353-437, p21 Max is described in Section 2.5.

Purification of c-Myc363-381 synthetic peptide is described in section 3.5.
References


APPENDIX

Appendix A
protein sequences

c-Myc353-437
N\textsubscript{353}VKRRTHN\textsubscript{360}VLERQR N E L\textsubscript{370}KRSFFALRDQ\textsubscript{380}IPELEN NEKA\textsubscript{390}PKVVILKKA
T\textsubscript{400}AYILSVQAE\textsubscript{410}414QKLISEEDDLL\textsubscript{420}RKRREQLKH\textsubscript{430}437LEQLRN S

T400AYILSVQAE\textsubscript{410}414QKLISEEDDLL\textsubscript{420}RKRREQLK KH\textsubscript{430}437LEQLRN S\textsubscript{437}GGCD

p21 Max
M\textsubscript{11}SDNDDIEVE\textsubscript{10}SDADKR AHHN\textsubscript{20}ALERK RDHI\textsubscript{30}KDSFHLRDS\textsubscript{40}VPSLQG KEKA
S\textsubscript{50}RAQILD KATE\textsubscript{60}YIQYMRK KNH\textsubscript{70}THQQDDDLLK\textsubscript{80}RQNALLEQQV\textsubscript{90} RALEKARS
SA\textsubscript{100}QLQTNYP SSD\textsubscript{110}NSLYTNAKG\textsubscript{120}S130TISA FDGGSD\textsubscript{140}SSSEEPEEP\textsubscript{150}QS RKKL
RMEA\textsubscript{150}151\textsubscript{151}S151

p22 Max
M\textsubscript{11}SDNDDIEVE\textsubscript{10}SDADKR AHHN\textsubscript{20}ALERK RDHIK\textsubscript{30}DSFHLRDS
V\textsubscript{50}PSLQGKEKASR\textsubscript{60}60QQLDKATE\textsubscript{70}YIQYMRK NH\textsubscript{80}THQQDDL LKR\textsubscript{90}RQNALLEQQ
V\textsubscript{100}R1\textsubscript{110}ALEKAR SSAQ\textsubscript{110}LQTNYP SSD\textsubscript{120}NSLYTNAKG\textsubscript{130}ST\textsubscript{1301}30TISA FDGGSDS\textsubscript{140}SSSEEPEEP
EEFQ\textsubscript{150}150SRK KL RMEAS\textsubscript{160}160

Appendix B
Spectroscopic characterization of compounds described in Chapter 2

1-Rh
(5Z)-5-[(4-ethylphenyl)methylidene]-2-sulfanylidene-1,3-thiazolidin-4-one (2)
UV/Vis (solvent: ethanol): \(\lambda_{\text{max}}\) 237 nm, 267 nm, 380 nm; Emission \(\lambda_{\text{max}}\) 468 nm (ex. 380 nm);
\(^1\)H NMR (300 MHz, DMSO \(d_6\)): \(\delta\) (ppm) 7.57 (s, 1H), 7.47 (d, \(J = 8.1\) Hz, 2H), 7.35 (d, \(J = 8.1\) Hz, 2H), 2.63 (q, \(J = 7.5\) Hz, 2H), 1.18 (t, \(J = 7.5\) Hz, 3H);
\(^13\)C NMR – \(^1\)H decoupled (75 MHz, DMSO \(d_6\)): \(\delta\) (ppm) 195.8, 169.6, 147.4, 132.0, 130.9, 130.6, 129.1, 124.5, 28.4, 15.3.

2-Rh
(5Z)-5-(phenylmethylidene)-2-sulfanylidene-1,3-thiazolidin-4-one
\(^1\)H NMR (300 MHz, DMSO \(d_6\)): \(\delta\) (ppm) 7.75 (s, 1H), 7.72-7.62 (m, 5H);
\(^13\)C NMR – \(^1\)H decoupled (75 MHz, DMSO \(d_6\)): \(\delta\) (ppm) 195.9, 169.7, 133.0, 131.5, 130.7, 130.5, 129.4, 125.7.

3-Rh
(5Z)-5-[(4-hydroxyphenyl)methylidene]-2-sulfanylidene-1,3-thiazolidin-4-one
UV/Vis (solvent: ethanol): \(\lambda_{\text{max}}\) 400 nm; Emission \(\lambda_{\text{max}}\) 468 nm (ex. 400 nm);
$^1$H NMR (300 MHz, DMSO $d_6$): $\delta$ (ppm) 10.51 (s, 1H), 7.64 (s, 1H), 7.53 (dd, $J^1 = 6.9$ Hz, $J^2 = 2.0$ Hz, 2H), 7.02 (dd, $J^1 = 6.9$ Hz, $J^2 = 2.0$ Hz, 2H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO $d_6$): $\delta$ (ppm) 195.5, 169.5, 160.4, 133.1, 132.4, 124.0, 120.9, 116.5.

4-Rh
(5Z)-5-[(4-methylphenyl)methylidene]-2-sulfanylidene-1,3-thiazolidin-4-one
$^1$H NMR (300 MHz, DMSO $d_6$): $\delta$ (ppm) 7.71 (s, 1H), 7.59 (d, $J = 7.5$, 2H), 7.46 (d, $J = 7.5$, 2H), 2.46 (s, 3H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO $d_6$): $\delta$ (ppm) 195.9, 169.7, 141.2, 131.7, 130.5, 130.2, 130.1, 124.3, 21.1.

5-Rh
(5Z)-5-[(3-methylphenyl)methylidene]-2-sulfanylidene-1,3-thiazolidin-4-one
$^1$H NMR (300 MHz, DMSO $d_6$): $\delta$ (ppm) 7.56 (s, 1H), 7.39 (t, $J = 7.2$ Hz, 1H), 7.36 (d, $J = 7.2$ Hz, 1H), 7.35 (s, 1H), 7.29 (d, $J = 7.2$ Hz, 1H), 2.36 (s, 3H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO $d_6$): $\delta$ (ppm) 195.6, 169.3, 138.8, 132.9, 131.7, 131.4, 130.9, 129.3, 127.6, 125.2, 20.9.

6-Rh
(5Z)-5-[(2-methylphenyl)methylidene]-2-sulfanylidene-1,3-thiazolidin-4-one
$^1$H NMR (300 MHz, DMSO $d_6$): $\delta$ (ppm) 7.59 (s, 1H), 7.50 (d, $J = 9.0$ Hz, 2H), 6.86 (d, $J = 9.0$ Hz, 2H), 3.51 (q, $J = 6.9$ Hz, 4H), 1.21 (t, $J = 6.9$ Hz, 6H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO $d_6$): $\delta$ (ppm) 194.9, 189.4, 169.4, 149.4, 133.3, 119.1, 116.6, 111.7, 110.5, 43.9, 12.4.

7-Rh
(5Z)-5-[(4-diethylamino)phenyl)methylidene]-2-sulfanylidene-1,3-thiazolidin-4-one
UV/Vis (solvent: ethanol): $\lambda_{\text{max}}$ 470 nm; Emission $\lambda_{\text{max}}$ 630 nm (ex. 470 nm);
$^1$H NMR (300 MHz, DMSO $d_6$): $\delta$ (ppm) 7.59 (s, 1H), 7.48 (d, $J = 9.0$ Hz, 2H), 6.86 (d, $J = 9.0$ Hz, 2H), 3.51 (q, $J = 6.9$ Hz, 4H), 1.21 (t, $J = 6.9$ Hz, 6H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO $d_6$): $\delta$ (ppm) 194.9, 189.4, 169.4, 149.4, 133.3, 119.1, 116.6, 111.7, 110.5, 43.9, 12.4.

8-Rh
(5Z)-5-[(4-dimethylamino)phenyl)methylidene]-2-sulfanylidene-1,3-thiazolidin-4-one
UV/Vis (solvent: ethanol): $\lambda_{\text{max}}$ 470 nm; Emission $\lambda_{\text{max}}$ 630 nm (ex. 470 nm);
$^1$H NMR (300 MHz, DMSO $d_6$): $\delta$ (ppm) 7.59 (s, 1H), 7.50 (d, $J = 9.0$ Hz, 2H), 6.90 (d, $J = 9.0$ Hz, 2H), 3.12 (s, 6H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO $d_6$): $\delta$ (ppm) 195.2, 169.7, 151.8, 133.3, 133.0, 119.8, 117.5, 112.2, 39.7.

9-Rh
(5Z)-5-[(4-methoxyphenyl)methylidene]-2-sulfanylidene-1,3-thiazolidin-4-one
$^1$H NMR (300 MHz, DMSO $d_6$): $\delta$ (ppm) 7.54 (s, 1H), 7.50 (d, $J = 8.7$ Hz, 2H), 7.06 (d, $J = 8.7$ Hz, 2H), 3.81 (s, 3H);
13C NMR – 1H decoupled (75 MHz, DMSO d₆): δ (ppm) 195.5, 169.5, 161.4, 132.7, 131.9, 125.5, 122.3, 115.1, 55.6.

10-Rh
(5Z)-5-[(3-methoxyphenyl)methylidene]-2-sulfanylidene-1,3-thiazolidin-4-one
1H NMR (300 MHz, DMSO d₆): δ (ppm) 7.65 (s, 1H), 7.51 (t, J = 8.0 Hz, 1H), 7.20 (d, J = 8.0 Hz, 1H), 7.18 (s, 1H), 7.13 (d, J = 8.0 Hz, 1H), 3.89 (s, 3H);
13C NMR – 1H decoupled (75 MHz, DMSO d₆): δ (ppm) 195.6, 169.3, 159.6, 134.3, 131.5, 130.4, 125.8, 122.4, 116.6, 115.5, 55.3.

11-Rh
(5Z)-5-[(2-methoxyphenyl)methylidene]-2-sulfanylidene-1,3-thiazolidin-4-one
1H NMR (300 MHz, DMSO d₆): δ (ppm) 7.86 (s, 1H), 7.64 – 7.52 (bd, 1H), 7.44 (d, J = 8.1 Hz, 1H), 7.20 (t, J = 8.1 Hz, 2H), 3.97 (s, 3H);
13C NMR – 1H decoupled (75 MHz, DMSO d₆): δ (ppm) 196.1, 169.5, 158.2, 133.0, 129.7, 126.8, 125.3, 121.4, 121.2, 112.0, 55.8.

12-Rh
(5Z)-5-[(4-chlorophenyl)methylidene]-2-sulfanylidene-1,3-thiazolidin-4-one
1H NMR (300 MHz, DMSO d₆): δ (ppm) 7.67 (s, 1H), 7.65 – 7.62 (m, 4H);
13C NMR – 1H decoupled (75 MHz, DMSO d₆): δ (ppm) 195.3, 169.2, 153.5, 132.0, 131.8, 130.1, 129.4, 126.2.

13-Rh
(5Z)-5-[(3-chlorophenyl)methylidene]-2-sulfanylidene-1,3-thiazolidin-4-one
1H NMR (300 MHz, DMSO d₆): δ (ppm) 7.69 (s, 1H), 7.65 (s, 1H), 7.62-7.52 (m, 3H);
13C NMR – 1H decoupled (75 MHz, DMSO d₆): δ (ppm) 195.2, 169.2, 153.0, 134.0, 131.1, 130.2, 130.1, 129.7, 128.1, 127.2.

14-Rh
(5Z)-5-[(2-chlorophenyl)methylidene]-2-sulfanylidene-1,3-thiazolidin-4-one
1H NMR (300 MHz, DMSO d₆): δ (ppm) 7.82 (s, 1H), 7.73-7.68 (m, 1H), 7.59-7.57 (m, 3H);
13C NMR – 1H decoupled (75 MHz, DMSO d₆): δ (ppm) 195.5, 169.1, 134.8, 132.1, 130.8, 130.4, 129.3, 129.0, 128.1, 126.1.

15-Rh
(5Z)-5-[(1-(4-bromophenyl)ethylidene]-2-sulfanylidene-1,3-thiazolidin-4-one
UV/Vis (solvent: ethanol): λₘₐₓ 445 nm;
1H NMR (300 MHz, DMSO d₆): δ (ppm) 7.87 (d, J = 8.1 Hz, 2H), 7.71 (d, J = 8.1 Hz, 2H), 2.57 (s, 3H);
13C NMR – 1H decoupled (75 MHz, DMSO d₆): δ (ppm) 197.2, 167.6, 135.8, 132.0, 131.7, 130.2, 129.0, 127.3, 26.7.

16-Rh
(5Z)-5-(naphthalen-1-ylmethylidene)-2-sulfanylidene-1,3-thiazolidin-4-one
$^1$H NMR (300 MHz, DMSO d$_6$): $\delta$ (ppm) 8.18 (s, 1H), 8.06 (d, J = 7.5 Hz, 1H), 7.96 (t, J = 7.8 Hz, 2H), 7.58-7.50 (m, 4H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO d$_6$): $\delta$ (ppm) 196.1, 168.8, 133.3, 131.2, 131.1, 130.0, 128.9, 128.6, 128.0, 127.5, 126.9, 126.8, 125.6, 123.2.

17-Rh
5-(propan-2-ylidene)-2-sulfanylidene-1,3-thiazolidin-4-one
UV/Vis (solvent: ethanol): $\lambda_{max}$ 340 nm;
$^1$H NMR (300 MHz, DMSO d$_6$): $\delta$ (ppm) 2.42 (s, 3H), 2.05 (s, 3H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO d$_6$): $\delta$ (ppm) 194.5, 167.0, 150.2, 122.4, 26.9, 20.7.

18-Rh, 19-Rh
(5E,Z)-5-(butan-2-ylidene)-2-sulfanylidene-1,3-thiazolidin-4-one
UV/Vis (solvent: ethanol): $\lambda_{max}$ 340 nm;
$^1$H NMR (300 MHz, DMSO d$_6$): $\delta$ (ppm) 7.71 (m, 3H), 7.45 (m, 2H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO d$_6$): $\delta$ (ppm) 196.2, 168.2, 141.5, 127.5, 41.0, 30.5, 25.2, 24.7.

20-Rh
5-cyclohexylidene-2-sulfanylidene-1,3-thiazolidin-4-one
UV/Vis (solvent: ethanol): $\lambda_{max}$ 340 nm;
$^1$H NMR (300 MHz, DMSO d$_6$): $\delta$ (ppm) 6.75 (d, J = 9.0 Hz, 1H), 2.22-2.11 (bd, J = 9.0 Hz, 1H), 1.38–1.20 (m, 4H), 1.92-1.60 (m, 6H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO d$_6$): $\delta$ (ppm) 196.2, 168.2, 141.5, 127.5, 41.0, 30.5, 25.2, 24.7.

21-Rh
(5Z)-5-(2,2-dimethylpropylidene)-2-sulfanylidene-1,3-thiazolidin-4-one
$^1$H NMR (300 MHz, DMSO d$_6$): $\delta$ (ppm) 6.78 (s, 1H), 1.16 (s, 9H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO d$_6$): $\delta$ (ppm) 196.5, 169.4, 146.8, 124.8, 34.4, 28.6.

22-Rh
(5Z)-5-(cyclohexylmethylidene)-2-sulfanylidene-1,3-thiazolidin-4-one
$^1$H NMR (300 MHz, DMSO d$_6$): $\delta$ (ppm) 7.71 (m, 3H), 7.45 (m, 2H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO d$_6$): $\delta$ (ppm) 195.5, 169.3, 163.0, 132.9, 130.5, 129.6, 125.2, 116.5.

23-Rh
(5Z)-5-[4-fluorophenyl)methylidene]-2-sulfanylidene-1,3-thiazolidin-4-one
$^1$H NMR (300 MHz, DMSO d$_6$): $\delta$ (ppm) 7.71 (m, 3H), 7.45 (m, 2H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO d$_6$): $\delta$ (ppm) 195.5, 169.3, 163.0, 132.9, 130.5, 129.6, 125.2, 116.5.
24-Rh
(5Z)-5-[(3-fluorophenyl)methylidene]-2-sulfanylidene-1,3-thiazolidin-4-one
$^1$H NMR (300 MHz, DMSO $d_6$): $\delta$ (ppm) 7.69 (s, 1H), 7.64 (t, $J = 7.2$ Hz, 1H), 7.46 (m, 3H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO $d_6$): $\delta$ (ppm) 195.4, 169.2, 162.3, 135.2, 131.4, 130.0, 127.1, 125.9, 117.6, 117.1.

25-Rh
(5Z)-5-[(2-fluorophenyl)methylidene]-2-sulfanylidene-1,3-thiazolidin-4-one
$^1$H NMR (300 MHz, DMSO $d_6$): $\delta$ (ppm) 7.67 (s, 1H), 7.65 (m, 1H), 7.57 (t, $J = 7.2$ Hz, 1H), 7.45 (m, 2H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO $d_6$): $\delta$ (ppm) 195.4, 169.2, 160.3, 133.0, 129.3, 128.0, 125.5, 122.2, 120.8, 116.2.

26-Rh
(5Z)-5-[(2,4-dihydroxyphenyl)methylidene]-2-sulfanylidene-1,3-thiazolidin-4-one
$^1$H NMR (300 MHz, DMSO $d_6$): $\delta$ (ppm) 10.8-10.4 (bs, 2H), 7.90 (s, 1H), 7.23 (d, $J = 6.6$ Hz, 1H), 6.52 (s, 1H), 6.51 (d, $J = 6.6$ Hz, 1H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO $d_6$): $\delta$ (ppm) 195.8, 169.8, 162.4, 159.9, 131.2, 128.1, 118.9, 112.0, 108.9, 102.6.

27-Rh
(5Z)-5-[(3,4-dihydroxyphenyl)methylidene]-2-sulfanylidene-1,3-thiazolidin-4-one
$^1$H NMR (300 MHz, DMSO $d_6$): $\delta$ (ppm) 10.1-9.9 (bs, 1H), 9.7-9.5 (bs, 1H), 7.55 (s, 1H), 7.09 (s, 1H), 7.07 (d, $J = 8.1$ Hz, 1H), 6.97 (d, $J = 8.1$ Hz, 1H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO $d_6$): $\delta$ (ppm) 195.6, 169.5, 149.3, 146.1, 132.9, 125.0, 124.5, 120.8, 116.7, 116.5.

28-Rh
(5Z)-5-[(4-(propan-2-yl)phenyl)methylidene]-2-sulfanylidene-1,3-thiazolidin-4-one
$^1$H NMR (300 MHz, DMSO $d_6$): $\delta$ (ppm) 7.68 (s, 1H), 7.58 (d, $J = 8.4$ Hz, 2H), 7.47 (d, $J = 8.4$ Hz, 2H), 3.00 (q, $J = 6.9$ Hz, 1H), 1.29 (d, $J = 6.9$ Hz, 6H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO $d_6$): $\delta$ (ppm) 195.6, 169.4, 151.7, 131.7, 130.7, 130.6, 127.4, 124.3, 33.4, 23.4.

29-Rh
(5Z)-5-[(3-hydroxyphenyl)methylidene]-2-sulfanylidene-1,3-thiazolidin-4-one
$^1$H NMR (300 MHz, DMSO $d_6$): $\delta$ (ppm) 7.58 (t, $J = 8.4$ Hz, 1H), 7.08 (d, $J = 8.4$ Hz, 1H), 7.04 (s, 1H), 6.97 (d, $J = 8.4$ Hz, 1H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO $d_6$): $\delta$ (ppm) = 116.3, 118.2, 121.9, 125.4, 130.5, 131.9, 134.2, 158.0, 169.4, 195.8.

30-Rh
(5Z)-5-[(2-ethoxyphenyl)methylidene]-2-sulfanylidene-1,3-thiazolidin-4-one
1H NMR (300 MHz, DMSO d6): δ (ppm) 7.91 (s, 1H), 7.54 (t, J = 7.5 Hz, 1H), 7.43 (d, J = 7.5 Hz, 1H), 7.20 (d, J = 8.7 Hz, 1H), 7.16 (t, J = 8.1 Hz, 1H), 4.23 (q, J = 6.6 Hz, 2H), 1.46 (t, J = 6.6 Hz, 3H);

13C NMR – 1H decoupled (75 MHz, DMSO d6): δ (ppm) 195.8, 169.4, 157.6, 132.8, 129.2, 126.4, 125.1, 121.4, 121.0, 112.7, 64.1, 14.5.

31-Rh

(SZ)-5-[(2-hydroxyphenyl)methylidene]-2-sulfanyliden-1,3-thiazolidin-4-one

1H NMR (300 MHz, DMSO d6): δ (ppm) 7.95 (s, 1H), 7.39 (dd, J1 = 12.3 Hz, J2 = 8.1 Hz, 2H), 7.03 (dd, J1 = 12.3 Hz, J2 = 8.1 Hz, 2H), 1.39 (t, J = 6.9 Hz, 3H);

13C NMR – 1H decoupled (75 MHz, DMSO d6): δ (ppm) 196.0, 169.5, 160.5, 131.7, 127.3, 123.8, 120.0, 116.3.

32-Rh

(SZ)-5-[(4-ethoxyphenyl)methylidene]-2-sulfanyliden-1,3-thiazolidin-4-one

1H NMR (300 MHz, DMSO d6): δ (ppm) 7.54 (s, 1H), 7.47 (d, J = 9 Hz, 2H), 7.04 (d, J = 9 Hz, 2H), 4.09 (q, J = 6.9 Hz, 2H), 1.39 (t, J = 6.9 Hz, 3H);

13C NMR – 1H decoupled (75 MHz, DMSO d6): δ (ppm) 195.3, 169.3, 160.5, 132.5, 131.7, 125.2, 121.9, 115.2, 114.7, 63.5, 14.5.

33-Rh

4-[(SZ)-4-oxo-2-sulfanyliden-1,3-thiazolidin-5-ylidene]methyl]benzaldehyde

1H NMR (300 MHz, DMSO d6): δ (ppm) 10.15 (s, 1H), 8.12 (d, J = 8.1 Hz, 2H), 7.90 (d, J = 8.1 Hz, 2H), 7.79 (s, 1H);

13C NMR – 1H decoupled (75 MHz, DMSO d6): δ (ppm) 195.5, 194.9, 192.6, 169.4, 138.3, 136.5, 130.9, 130.2, 129.8, 128.8.

34-Rh

4-[(SZ)-4-oxo-2-sulfanyliden-1,3-thiazolidin-5-ylidene]methyl]benzoic acid

1H NMR (300 MHz, DMSO d6): δ (ppm) 8.12 (d, J = 7.8 Hz, 2H), 7.74 (d, J = 7.8 Hz, 2H), 7.71 (s, 1H);

13C NMR – 1H decoupled (75 MHz, DMSO d6): δ (ppm) 195.6, 169.5, 166.6, 136.9, 131.8, 130.4, 130.1, 130.0, 128.0.

35-Rh

(SZ)-5-[(1-phenylethylidene)-2-sulfanyliden-1,3-thiazolidin-4-one

1H NMR (300 MHz, DMSO d6): δ (ppm) 7.58-7.55 (m, 5H), 2.75 (s, 3H);

13C NMR – 1H decoupled (75 MHz, DMSO d6): δ (ppm) 195.5, 167.7, 149.4, 141.8, 129.7, 129.0, 126.7, 124.6, 20.7.

36-Rh

4-[(SZ)-4-oxo-2-sulfanyliden-1,3-thiazolidin-5-ylidene]methyl]benzonitrile

1H NMR (300 MHz, DMSO d6): δ (ppm) 8.05 (d, J = 8.4 Hz, 2H), 7.83 (d, J = 8.4Hz, 2H), 7.76 (s, 1H);

13C NMR – 1H decoupled (75 MHz, DMSO d6): δ (ppm) 195.2, 169.2, 137.3, 133.1, 130.8, 129.2, 118.4, 112.2.
37-Rh
5-cyclopentylidene-2-sulfanylidene-1,3-thiazolidin-4-one
$^1$H NMR (300 MHz, DMSO $d_6$): $\delta$ (ppm) 2.91 (td, $J^1 = 5.7$ Hz, $J^2 = 1.2$ Hz, 2H), 2.45 (td, $J^1 = 5.7$ Hz, $J^2 = 1.2$ Hz, 2H), 1.90-1.80 (m, 4H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO $d_6$): $\delta$ (ppm) 195.5, 167.1, 160.7, 118.4, 24.9, 36.3, 33.7, 26.4.

38-Rh
(5Z)-5-[(3-nitrophenyl)methylidene]-2-sulfanylidene-1,3-thiazolidin-4-one
UV/Vis (solvent: ethanol): $\lambda_{max}$ 374 nm;
$^1$H NMR (300 MHz, DMSO $d_6$): $\delta$ (ppm) 8.41 (s, 1H), 8.29 (d, $J = 7.8$ Hz, 1H), 7.98 (d, $J = 7.8$ Hz, 1H), 7.80 (t, $J = 7.8$ Hz, 1H), 7.76 (s, 1H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO $d_6$): $\delta$ (ppm) 195.2, 169.5, 148.2, 135.7, 134.6, 130.9, 128.7, 124.7, 124.5.

39-Rh
(5Z)-5-[(4-nitrophenyl)methylidene]-2-sulfanylidene-1,3-thiazolidin-4-one
UV/Vis (solvent: ethanol): $\lambda_{max}$ 374 nm;
$^1$H NMR (300 MHz, DMSO $d_6$): $\delta$ (ppm) 8.40 (d, $J = 8.7$ Hz, 2H), 7.91 (d, $J = 8.7$ Hz, 2H), 7.79 (s, 1H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO $d_6$): $\delta$ (ppm) 195.2, 169.2, 147.4, 139.1, 131.3, 129.9, 128.5, 124.3.

40-Rh
(5Z)-5-[(3,4-dimethoxyphenyl)methylidene]-2-sulfanylidene-1,3-thiazolidin-4-one
$^1$H NMR (300 MHz, DMSO $d_6$): $\delta$ (ppm) 7.66 (s, 1H), 7.27-7.18 (m, 3H), 3.92 (d, $J = 0.9$ Hz, 3H), 3.90 (d, $J = 0.9$ Hz, 3H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO $d_6$): $\delta$ (ppm) 195.4, 169.4, 151.2, 149.0, 132.3, 125.6, 124.6, 122.3, 113.4, 112.1, 55.7, 55.5.

Rh-S-Me
2-(methylsulfanyl)-4,5-dihydro-1,3-thiazol-4-one
$^1$H NMR (300 MHz, CDCl$_3$): $\delta$ (ppm) 4.00 (s, 2H), 2.75 (s, 3H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, CDCl$_3$): $\delta$ (ppm) 203.4, 188.1, 40.4, 17.1.

1-Rh-S-Me
(5Z)-5-[(4-ethylphenyl)methylidene]-2-(methylsulfanyl)-4,5-dihydro-1,3-thiazol-4-one
UV/Vis (solvent: ethanol): $\lambda_{max}$ 385 nm;
$^1$H NMR (300 MHz, DMSO $d_6$): $\delta$ (ppm) 7.91 (s, 1H), 7.67 (d, $J = 8.1$ Hz, 2H), 7.48 (d, $J = 8.1$ Hz, 2H), 2.92 (s, 3H), 2.75 (q, $J = 7.5$ Hz, 2H), 1.29 (t, $J = 7.5$ Hz, 3H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO $d_6$): $\delta$ (ppm) 193.0, 179.1, 147.5, 135.2, 130.7, 130.6, 128.9, 125.0, 28.1, 15.4, 15.2.
4-Rh-S-Me
(5Z)-5-[(4-methylphenyl)methylidene]-2-(methylsulfanyl)-4,5-dihydro-1,3-thiazol-4-one
$^1$H NMR (300 MHz, DMSO d$_6$): $\delta$ (ppm) 7.90 (s, 1H), 7.65 (d, $J$ = 8.1 Hz, 2H), 7.45 (d, $J$ = 8.1 Hz, 2H), 2.92 (s, 3H), 2.46 (s, 3H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO d$_6$): $\delta$ (ppm) 192.9, 179.1, 141.4, 135.2, 130.5, 130.4, 130.0, 124.9, 21.1, 15.4.

12-Rh-S-Me
(5Z)-5-[(4-chlorophenyl)methylidene]-2-(methylsulfanyl)-4,5-dihydro-1,3-thiazol-4-one
$^1$H NMR (300 MHz, DMSO d$_6$): $\delta$ (ppm) 7.93 (s, 1H), 7.77 (d, $J$ = 8.4 Hz, 2H), 7.70 (d, $J$ = 8.4 Hz, 2H), 2.93 (s, 3H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO d$_6$): $\delta$ (ppm) 193.2, 178.9, 135.5, 133.6, 132.1, 132.0, 129.5, 126.8, 15.6.

22-Rh-S-Me
(5Z)-5-(cyclohexylmethylidene)-2-(methylsulfanyl)-4,5-dihydro-1,3-thiazol-4-one
$^1$H NMR (300 MHz CDCl$_3$): $\delta$ (ppm) 6.95 (d, $J$ = 9.3 Hz, 1H), 7.70 (d, $J$ = 8.4 Hz, 2H), 2.77 (s, 3H), 2.2-2.0 (m, 1H), 1.8-1.7 (m, 5H), 1.4-1.2 (m, 5H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, CDCl$_3$): $\delta$ (ppm) 193.5, 179.1, 145.9, 129.6, 43.7, 31.8, 26.0, 25.7, 16.3.

27-Rh-S-Me
(5Z)-5-[(3,4-dihydroxyphenyl)methylidene]-2-(methylsulfanyl)-4,5-dihydro-1,3-thiazol-4-one
$^1$H NMR (300 MHz, DMSO d$_6$): $\delta$ (ppm) 10.2-9.9 (bs, 1H), 9.7-9.5 (bs, 1H), 7.76 (s, 1H), 7.16 (s, 1H), 7.14 (d, $J$ = 6.8 Hz, 1H), 6.98 (d, $J$ = 6.8 Hz, 1H), 2.91 (s, 3H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO d$_6$): $\delta$ (ppm) 191.9, 179.3, 149.4, 146.1, 136.2, 124.7, 124.5, 121.6, 116.7, 116.4, 15.3.

28-Rh-S-Me
(5Z)-2-(methylsulfanyl)-5-[(4-(propan-2-yl)phenyl)methylidene]-4,5-dihydro-1,3-thiazol-4-one
$^1$H NMR (300 MHz, DMSO d$_6$): $\delta$ (ppm) 7.90 (s, 1H), 7.67 (d, $J$ = 8.1 Hz, 2H), 7.50 (d, $J$ = 8.1 Hz, 2H), 3.04 (qt, $J$ = 6.6 Hz, 1H), 2.91 (s, 3H), 1.30 (d, $J$ = 6.6 Hz, 6H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO d$_6$): $\delta$ (ppm) 193.0, 179.1, 152.0, 135.2, 130.8, 130.7, 127.4, 125.1, 33.5, 23.5, 15.5.

30-Rh-S-Me
(5Z)-5-[(2-ethoxyphenyl)methylidene]-2-(methylsulfanyl)-4,5-dihydro-1,3-thiazol-4-one
$^1$H NMR (300 MHz, DMSO d$_6$): $\delta$ (ppm) 8.17 (s, 1H), 7.59 (t, $J$ = 7.5 Hz, 1H), 7.58 (d, $J$ = 7.5 Hz, 1H), 7.26 (d, $J$ = 9.3 Hz, 1H), 7.20 (t, $J$ = 7.8 Hz, 1H), 4.27 (q, $J$ = 6.9 Hz, 2H), 2.92 (s, 3H), 1.50 (t, $J$ = 6.9 Hz, 3H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO $d_6$): δ (ppm) 193.3, 179.1, 157.8, 133.0, 129.6, 129.1, 126.0, 121.7, 121.0, 112.8, 64.1, 15.4, 14.5.

1-Rh-5-H
(+/-) 5-[(4-ethylphenyl)methyl]-2-(methylsulfanyl)-4,5-dihydro-1,3-thiazol-4-one
$^1$H NMR (300 MHz, CDC$_3$): δ (ppm) 9.1-8.85 (bs, 1H), 7.17 (d, J = 8.4 Hz, 2H), 7.13 (d, J = 8.4 Hz, 2H), 4.59 (dd, $J^1$ = 9.9 Hz, $J^2$ = 4.2 Hz, 1H), 3.52 (dd, $J^1$ = 14.1 Hz, $J^2$ = 4.2 Hz, 1H), 3.11 (dd, $J^1$ = 14.1 Hz, $J^2$ = 9.9 Hz, 1H), 2.63 (q, J = 7.5 Hz, 2H), 1.23 (t, J = 7.5 Hz, 3H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, CDCl$_3$): δ (ppm) 202.0, 177.9, 136.7, 130.6, 129.6, 129.1, 57.5, 36.7, 29.1, 16.1.

2-Rh-5-H
(+/-) 5-benzyl-2-(methylsulfanyl)-4,5-dihydro-1,3-thiazol-4-one
$^1$H NMR (300 MHz, CDCl$_3$): δ (ppm) 9.1-8.85 (bs, 1H), 7.17 (d, J = 8.4 Hz, 2H), 7.34-7.31 (m, 3H), 5.10 (dd, $J^1$ = 8.7 Hz, $J^2$ = 4.2 Hz, 1H), 3.47 (dd, $J^1$ = 13.2 Hz, $J^2$ = 4.2 Hz, 1H), 3.25 (dd, $J^1$ = 13.2 Hz, $J^2$ = 8.7 Hz, 1H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, CDCl$_3$): δ (ppm) 203.6, 178.4, 136.7, 129.2, 128.5, 127.1, 55.8, 36.6.

4-Rh-5-H
(+/-) 5-[(4-methylphenyl)methyl]-2-(methylsulfanyl)-4,5-dihydro-1,3-thiazol-4-one
$^1$H NMR (300 MHz, CDCl$_3$): δ (ppm) 9.92-9.81 (bs, 1H), 7.16-7.15 (m, 4H), 4.55 (dd, $J^1$ = 9.9 Hz, $J^2$ = 4.2 Hz, 1H), 3.47 (dd, $J^1$ = 14.1 Hz, $J^2$ = 4.2 Hz, 1H), 3.16 (dd, $J^1$ = 14.1 Hz, $J^2$ = 9.9 Hz, 1H), 2.36 (s, 3H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, CDCl$_3$): δ (ppm) 202.0, 176.9, 137.4, 132.5, 129.6, 128.8, 56.9, 37.9, 21.0.

1-RhNCN1
(5Z)-5-[(4-ethylphenyl)methylidene]-3-(piperidin-1-ylmethyl)-2-sulfanylidene-1,3-thiazolidin-4-one
$^1$H NMR (300 MHz, CDCl$_3$): δ (ppm) 7.68 (s, 1H), 7.42 (d, J = 8.4 Hz, 2H), 7.30 (d, J = 8.4 Hz, 2H), 5.06 (s, 2H), 2.73 (t, J = 5.4 Hz, 4H), 2.70 (q, J = 7.8 Hz, 2H), 1.54 (qt, J = 5.4 Hz, 4H), 1.37 (qt, J = 5.4 Hz, 2H), 1.26 (t, J 7.8 Hz, 3H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, CDCl$_3$): δ (ppm) 196.4, 170.0, 148.4, 133.5, 131.6, 131.5, 129.5, 122.6, 66.9, 53.4, 29.6, 26.7, 24.4, 15.8.

12-RhNCN1
(5Z)-5-[(4-chlorophenyl)methylidene]-3-(piperidin-1-ylmethyl)-2-sulfanylidene-1,3-thiazolidin-4-one
$^1$H NMR (300 MHz, CDCl$_3$): δ (ppm) 7.62 (s, 1H), 7.45 (d, J = 9.0 Hz, 2H), 7.41 (d, J = 9.0 Hz, 2H), 5.06 (s, 2H), 2.73 (t, J = 5.7 Hz, 4H), 1.54 (qt, J = 5.7 Hz, 4H), 1.37 (qt, J= 5.7 Hz, 2H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, CDCl$_3$): δ (ppm) 195.7, 169.8, 137.4, 132.6, 132.3, 131.6, 130.3, 124.5, 67.1, 53.4, 26.7, 24.4.
28-RhNCN1
(5Z)-3-(piperidin-1-ylmethyl)-5-[(4-(propan-2-yl)phenyl)methylidene]-2-sulfanylidene-1,3-thiazolidin-4-one
$^1H$ NMR (300 MHz, CDCl$_3$): $\delta$ (ppm) 7.66 (s, 1H), 7.43 (d, J = 8.4 Hz, 2H), 7.34 (d, J = 8.4 Hz, 2H), 5.06 (s, 2H), 2.96 (qt, J = 6.9 Hz, 1H), 2.74 (t, J = 5.4 Hz, 4H), 1.54 (qt, J = 5.4 Hz, 4H), 1.38 (qt, J = 5.4 Hz, 2H), 1.27 (d, J = 6.9 Hz, 6H);

$^{13}$C NMR – $^1H$ decoupled (75 MHz, CDCl$_3$): $\delta$ (ppm) 196.4, 152.9, 133.5, 131.7, 131.5, 128.1, 122.6, 66.9, 53.4, 34.8, 26.7, 24.4, 24.3.

40-RhNCN1
(5Z)-5-[(3,4-dimethoxyphenyl)methylidene]-3-(piperidin-1-ylmethyl)-2-sulfanylidene-1,3-thiazolidin-4-one
$^1H$ NMR (300 MHz, CDCl$_3$): $\delta$ (ppm) 7.64 (s, 1H), 7.13 (dd, J$_1$ = 8.7 Hz, J$_2$ = 2.4 Hz, 1H), 6.97 (d, J = 2.4 Hz, 1H), 6.96 (d, J = 8.7 Hz, 1H), 5.06 (s, 2H), 2.96 (qt, J = 6.9 Hz, 1H), 2.74 (t, J = 5.4 Hz, 4H), 1.54 (qt, J = 5.4 Hz, 4H), 1.38 (qt, J = 5.4 Hz, 2H);

$^{13}$C NMR – $^1H$ decoupled (75 MHz, CDCl$_3$): $\delta$ (ppm) 192.5, 169.9, 152.1, 150.1, 133.7, 127.1, 126.2, 113.0, 112.1, 100.6, 66.9, 56.7, 53.4, 26.7, 24.4.

1-TZDD
(5Z)-5-[(4-ethylphenyl)methylidene]-1,3-thiazolidine-2,4-dione
$^1H$ NMR (300 MHz, CDCl$_3$): $\delta$ (ppm) 7.86 (s, 1H), 7.43 (d, J = 8.4 Hz, 2H), 7.31 (d, J = 8.4 Hz, 2H), 2.71 (q, J = 7.5 Hz, 2H), 1.26 (t, J = 7.5 Hz, 3H);

$^{13}$C NMR – $^1H$ decoupled (75 MHz, CDCl$_3$): $\delta$ (ppm) 167.5, 166.9, 147.9, 134.8, 130.5, 130.4, 128.8, 121.0, 28.9, 15.2.

2-TZDD
(5Z)-5-(phenylmethylidene)-1,3-thiazolidine-2,4-dione
$^1H$ NMR (300 MHz, DMSO d$_6$): $\delta$ (ppm) 7.89 (s, 1H), 7.57 (d, J = 6.3 Hz, 2H), 7.44 (d, J = 6.3 Hz, 2H), 2.45 (s, 3H);

$^{13}$C NMR – $^1H$ decoupled (75 MHz, DMSO d$_6$): $\delta$ (ppm) 167.9, 167.3, 133.0, 131.8, 130.4, 130.0, 129.3, 123.5.

4-TZDD
(5Z)-5-[(4-methylphenyl)methylidene]-1,3-thiazolidine-2,4-dione
$^1H$ NMR (300 MHz, DMSO d$_6$): $\delta$ (ppm) 7.84 (s, 1H), 7.57 (d, J = 6.3 Hz, 2H), 7.44 (d, J = 6.3 Hz, 2H), 2.45 (s, 3H);

$^{13}$C NMR– $^1H$ decoupled (75 MHz, DMSO d$_6$): $\delta$ (ppm) 167.9, 167.3, 140.7, 131.8, 130.6, 130.1, 129.9, 122.3, 21.1.

6-TZDD
(5Z)-5-[(2-methylphenyl)methylidene]-1,3-thiazolidine-2,4-dione
$^1H$ NMR (300 MHz, DMSO d$_6$): $\delta$ (ppm) 7.95 (s, 1H), 7.46 (s, 1H), 7.43-7.37 (m, 3H), 2.45 (s, 3H);

$^{13}$C NMR– $^1H$ decoupled (75 MHz, DMSO d$_6$): $\delta$ (ppm) 168.2, 167.1, 138.7, 132.1, 130.9, 130.2, 129.5, 127.1, 126.6, 125.1, 19.4.
12-TZDD
(5Z)-5-[(4-chlorophenyl)methylidene]-1,3-thiazolidine-2,4-dione
$^1$H NMR (300 MHz, DMSO d$_6$): $\delta$ (ppm) 7.85 (s, 1H), 7.69-7.66 (m, 4H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO d$_6$): $\delta$ (ppm) 167.6, 167.2, 135.0, 131.9, 131.6, 130.4, 129.3, 124.3.

14-TZDD
(5Z)-5-[(2-chlorophenyl)methylidene]-1,3-thiazolidine-2,4-dione
$^1$H NMR (300 MHz, CDCl$_3$): $\delta$ (ppm) 8.22 (s, 1H), 7.54-7.48 (m, 2H), 7.41-7.35 (m, 2H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, CDCl$_3$): $\delta$ (ppm) 168.0, 167.0, 138.0, 132.3, 131.4, 131.2, 129.6, 128.0, 124.1.

22-TZDD
(5Z)-5-(cyclohexylmethylidene)-1,3-thiazolidine-2,4-dione
$^1$H NMR (300 MHz, CDCl$_3$): $\delta$ (ppm) 6.93 (d, J = 9.3 Hz, 1H), 2.1-2.0 (m, 1H), 1.9-1.6 (m, 5H), 1.1-1.4 (m, 5H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, CDCl$_3$): $\delta$ (ppm) 167.6, 167.2, 135.0, 131.9, 131.6, 130.4, 129.3, 124.3.

27-TZDD
(5Z)-5-[(3,4-dihydroxyphenyl)methylidene]-1,3-thiazolidine-2,4-dione
$^1$H NMR (300 MHz, DMSO d$_6$): $\delta$ (ppm) 10.1-9.8 (bs, 1H), 9.7-9.5 (bs, 1H), 7.70 (s, 1H), 7.09 (d, J = 1.5 Hz, 1H), 7.05 (dd, J$^1$ = 8.1 Hz, J$^2$ = 1.5 Hz, 1H), 6.96 (d, J = 8.1 Hz, 1H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO d$_6$): $\delta$ (ppm) 168.2, 167.6, 148.7, 145.9, 132.8, 124.4, 124.0, 118.8, 116.5, 116.3.

28-TZDD
(5Z)-5-[(4-(propan-2-yl)phenyl)methylidene]-1,3-thiazolidine-2,4-dione
$^1$H NMR (300 MHz, DMSO d$_6$): $\delta$ (ppm) 7.84 (s, 1H), 7.59 (d, J = 8.4 Hz, 2H), 7.48 (d, J = 8.4 Hz, 2H), 3.01 (qt, J = 6.9 Hz, 1H), 1.29 (d, J = 6.9 Hz, 6H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO d$_6$): $\delta$ (ppm) 168.4, 167.0, 144.8, 124.9, 42.0, 31.7, 26.2, 25.8.

30-TZDD
(5Z)-5-[(2-ethoxyphenyl)methylidene]-1,3-thiazolidine-2,4-dione
$^1$H NMR (300 MHz, DMSO d$_6$): $\delta$ (ppm) 7.53 (td, J$^1$ = 8.4 Hz, J$^2$ = 1.5 Hz, 2H), 7.48 (dd, J$^1$ = 7.8 Hz, J$^2$ = 1.5 Hz, 1H), 7.21 (d, J = 8.4 Hz, 1H), 7.16 (t, J = 7.8 Hz, 1H), 4.23 (q, J = 6.9 Hz, 2H), 1.47 (t, J = 6.9 Hz, 3H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO d$_6$): $\delta$ (ppm) 168.8, 168.1, 158.1, 133.0, 129.1, 127.2, 124.0, 122.2, 121.5, 113.3, 64.7, 15.2.

34-TZDD
4-[[5Z]-2,4-dioxo-1,3-thiazolidin-5-ylidene]methyl]benzoic acid
$^1$H NMR (300 MHz, DMSO d6): $\delta$ (ppm) 8.12 (d, J = 8.1Hz, 2H), 7.87 (s, 1H), 7.76 (d, J = 8.1 Hz, 2H);
$\text{^1}C\text{NMR} - \text{^1}H\text{ decoupled (75 MHz, DMSO d6):}\ \delta\text{ (ppm) 168.1, 166.7, 137.2, 131.6,}
130.0, 129.9, 129.8, 129.5, 126.8. $

1-TZDD-NC-1

(5Z)-5-[(4-ethylphenyl)methylidene]-3-(prop-2-en-1-yl)-1,3-thiazolidine-2,4-dione

$\text{^1}H\text{ NMR (300 MHz, CDCl}_3):\ \delta\text{ (ppm) 7.83 (s, 1H), 7.39 (d, J = 8.4 Hz, 2H), 7.30 (d, J = 8.4 Hz, 2H), 5.85 (ddd, J}_1^1 = 18.6 Hz, J}_2 = 11.4 Hz, J}_3 = 6.0 Hz, 1H), 5.25 (dd, J}_1 = 18.6 Hz, J}_2 = 11.4 Hz, 2H), 4.30 (d, J = 6.0 Hz, 2H), 2.91 (qt, J = 6.9 Hz, 1H), 1.24 (t, J = 6.9 Hz, 6H);$

$\text{^13}C\text{NMR - ^1}H\text{ decoupled (75 MHz, CDCl}_3):\ \delta\text{ (ppm) 167.5, 166.1, 152.1, 134.0, 131.1, 130.7, 130.6, 127.6, 120.5, 119.0, 44.0, 34.4, 23.9.}$

28-TZDD-NC-1

(5Z)-3-(prop-2-en-1-yl)-5-[(4-(propan-2-yl)phenyl)methylidene]-1,3-thiazolidine-2,4-dione

$\text{^1}H\text{ NMR (300 MHz, CDCl}_3):\ \delta\text{ (ppm) 7.83 (s, 1H), 7.39 (d, J = 8.4 Hz, 2H), 7.30 (d, J = 8.4 Hz, 2H), 5.85 (ddd, J}_1 = 18.6 Hz, J}_2 = 11.4 Hz, J}_3 = 6.0 Hz, 1H), 5.25 (dd, J}_1 = 18.6 Hz, J}_2 = 11.4 Hz, 2H), 4.30 (d, J = 6.0 Hz, 2H), 2.91 (qt, J = 6.9 Hz, 1H), 1.24 (d, J = 6.9 Hz, 6H);$

$\text{^13}C\text{NMR - ^1}H\text{ decoupled (75 MHz, CDCl}_3):\ \delta\text{ (ppm) 167.5, 166.1, 152.1, 134.0, 131.1, 130.7, 130.6, 127.6, 120.5, 119.0, 44.0, 34.4, 23.9.}$

Appendix C

Spectroscopic characterization of compounds described in Chapter 6

**nitro-N-(2-phenylphenyl)-2,1,3-benzoxadiazol-4-amine (1)**

UV/Vis (solvent: ethanol): $\lambda_{\text{max}}$ 335 nm, 471 nm; Emission $\lambda_{\text{max}}$ 550 nm (ex. 470 nm);

$\text{^1}H\text{ NMR (300 MHz, CDCl}_3):\ \delta\text{ (ppm) 8.42 (d, J = 8.7 Hz, 1H), 7.58 (bs, 1H), 7.62-7.42 (m, 4H), 7.41-7.31 (m, 5H), 6.65 (d, J = 8.7 Hz, 1H);}$

$\text{^13}C\text{NMR - ^1}H\text{ decoupled (75 MHz, CDCl}_3):\ \delta\text{ (ppm) 183.3, 145.3, 141.8, 138.1, 137.9, 136.5, 134.3, 132.4, 129.7, 129.6, 129.5, 129.3, 129.1, 128.5, 125.2, 101.7.}$

**(5Z)-5-[(4-ethylphenyl)methylidene]-2-sulfanylidene-1,3-thiazolidin-4-one (2)**

UV/Vis (solvent: ethanol): $\lambda_{\text{max}}$ 237 nm, 267 nm, 380 nm; Emission $\lambda_{\text{max}}$ 468 nm (ex. 380 nm);

$\text{^1}H\text{ NMR (300 MHz, DMSO d6):}\ \delta\text{ (ppm) 7.57 (s, 1H), 7.47 (d, J = 8.1 Hz, 2H), 7.35 (d, J = 8.1 Hz, 2H), 2.63 (q, J = 7.5 Hz, 2H), 1.18 (t, J = 7.5 Hz, 3H);}$

$\text{^13}C\text{NMR - ^1}H\text{ decoupled (75 MHz, DMSO d6):}\ \delta\text{ (ppm) 195.8, 169.6, 147.4, 132.0, 130.9, 130.6, 129.1, 124.5, 28.4, 15.3.}$

**7-nitro-N-phenyl-2,1,3-benzoxadiazol-4-amine (3)**

UV/Vis (solvent: ethanol): $\lambda_{\text{max}}$ 335 nm, 473 nm;

$\text{^1}H\text{ NMR (300 MHz, DMSO d6):}\ \delta\text{ (ppm) 11.09 (bs, 1H), 8.53 (d, J = 8.7 Hz, 1H), 7.55-7.50 (m, 4H), 7.37 (m, 1H), 6.74 (d, J = 8.7 Hz, 1H);}$

$\text{^13}C\text{NMR - ^1}H\text{ decoupled (75 MHz, DMSO d6):}\ \delta\text{ (ppm) 144.9, 144.1, 142.3, 137.7, 130.5, 129.6, 126.4, 123.9, 123.0, 101.6.}$

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N,N-dimethyl-7-nitro-2,1,3-benzoxadiazol-4-amine (4)
UV/Vis (solvent: ethanol): \( \lambda_{\text{max}} \) 335 nm, 475 nm;
\(^1\)H NMR (300 MHz, DMSO d6): \( \delta \) (ppm) 8.50 (d, J = 9.3 Hz, 1H), 6.40 (d, J = 9.3 Hz, 1H), 3.39 (s, 6H).

2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]benzoic acid (5)
UV/Vis (solvent: ethanol): \( \lambda_{\text{max}} \) 483 nm; Emission \( \lambda_{\text{max}} \) 570 nm (ex. 470 nm);
\(^1\)H NMR (400 MHz, DMSO d6): \( \delta \) (ppm) 11.12-11.03 (bs, 1H), 8.56 (d, J = 9.0 Hz, 1H), 8.05 (s, 1H), 7.89 (d, J = 7.5 Hz, 1H), 7.76 (d, J = 7.5 Hz, 1H), 7.66 (t, J = 7.5 Hz, 1H), 6.79 (d, J = 9.0 Hz, 1H);
\(^{13}\)C NMR – \(^1\)H decoupled (75 MHz, DMSO d6): \( \delta \) (ppm) 166.4, 144.8, 143.9, 141.8, 138.0, 137.3, 132.3, 132.0, 130.3, 129.8, 126.7, 124.1, 123.5.

3-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]benzoic acid (6)
UV/Vis (solvent: ethanol): \( \lambda_{\text{max}} \) 468 nm; Emission \( \lambda_{\text{max}} \) 560 nm (ex. 470 nm);
\(^1\)H NMR (300 MHz, DMSO d6): \( \delta \) (ppm) 11.12-11.03 (bs, 1H), 8.56 (d, J = 9.0 Hz, 1H), 8.05 (s, 1H), 7.89 (d, J = 7.5 Hz, 1H), 7.76 (d, J = 7.5 Hz, 1H), 7.66 (t, J = 7.5 Hz, 1H), 6.79 (d, J = 9.0 Hz, 1H);
\(^{13}\)C NMR – \(^1\)H decoupled (75 MHz, DMSO d6): \( \delta \) (ppm) 167.6, 152.7, 149.6, 143.4, 132.6, 131.3, 130.6, 128.9, 117.4, 113.0, 110.8.

4-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]benzoic acid (7)
UV/Vis (solvent: ethanol): \( \lambda_{\text{max}} \) 468 nm; Emission \( \lambda_{\text{max}} \) 560 nm (ex. 470 nm);
\(^1\)H NMR (300 MHz, DMSO d6): \( \delta \) (ppm) 8.69 (d, J = 7.8 Hz, 1H), 8.03 (t, J = 7.8 Hz, 2H), 7.63 (t, J = 7.5 Hz, 2H), 6.58 (d, J = 7.5 Hz, 2H);
\(^{13}\)C NMR – \(^1\)H decoupled (75 MHz, DMSO d6): \( \delta \) (ppm) 195.6, 169.5, 166.6, 157.9, 156.4, 149.9, 134.5, 131.2, 129.2, 116.6, 116.2, 100.6, 39.5, 31.0, 28.3, 27.3, 24.0, 14.8.

N-[5-(4-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]phenyl)formamido]pentyl]acetamide (8)
UV/Vis (solvent: ethanol): \( \lambda_{\text{max}} \) 470 nm; Emission \( \lambda_{\text{max}} \) 545 nm (ex. 470 nm);
\(^1\)H NMR (300 MHz, CDCl3): \( \delta \) (ppm) 8.49 (d, J = 8.1 Hz, 1H), 7.68 (d, J = 8.1 Hz, 2H), 7.27 (d, J = 8.1 Hz, 2H), 6.64 (d, J = 8.1 Hz, 1H), 3.35 (t, J = 6.9 Hz, 2H), 1.98 (s, 3H), 1.62 (qt, J = 6.9Hz, 2H), 1.53 (qt, J = 6.9Hz, 2H), 1.38 (qt, J = 6.9Hz, 2H);
\(^{13}\)C NMR – \(^1\)H decoupled (75 MHz, CDCl3): \( \delta \) (ppm) 190.6, 187.3, 161.6, 157.9, 156.4, 149.9, 134.5, 131.2, 129.2, 116.6, 116.2, 100.6, 39.5, 31.0, 28.3, 27.3, 24.0, 14.8.

7-4-[(5Z)-4-oxo-2-sulfanylidene-1,3-thiazolidin-5-ylidene]methyl]benzoic acid (9)
\(^1\)H NMR (300 MHz, DMSO d6): \( \delta \) (ppm) 8.12 (d, J = 7.8 Hz, 2H), 7.74 (d, J = 7.8 Hz, 2H), 7.71 (s, 1H);
\(^{13}\)C NMR – \(^1\)H decoupled (75 MHz, DMSO d6): \( \delta \) (ppm) 195.6, 169.5, 166.6, 136.9, 131.8, 130.4, 130.1, 130.0, 128.0.

N-[5-{4-[(5Z)-4-oxo-2-sulfanylidene-1,3-thiazolidin-5-ylidene]methyl}phenyl]formamido]pentyl]acetamide (10)
$^1$H NMR (300 MHz, CDCl$_3$): δ (ppm) 8.98 (bt, J = 2.5 Hz, 1H), 7.52 (d, J = 3.6 Hz, 2H), 7.48 (d, J = 3.6 Hz, 2H), 7.17 (s, 1H), 3.84 (t, J = 7.2 Hz, 2H), 3.37 (t, J = 7.2 Hz, 2H), 2.72 (s, 3H), 2.07 (m, 2H), 1.68 (m, 1H), 1.55 (dqt, J$^1$ = 6.6 Hz, J$^2$ = 7.2 Hz, 1H), 1.41 (dqt, J$^1$ = 8.5 Hz, J$^2$ = 7.2 Hz, 1H);

$^{13}$C NMR – $^1$H decoupled (75 MHz, CDCl$_3$): δ (ppm) 191.6, 178.0, 163.2, 159.2, 155.3, 137.3, 127.3, 117.8, 113.0, 44.2, 43.2, 33.5, 32.1, 17.4, 15.4.

N-[5-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino][phenyl]formamido]pentyl]-4-[[5Z]-4-oxo-2-sulfanyliden-1,3-thiazolidin-5-ylidene[methyl]benzamide (11)

UV/Vis (solvent: ethanol): $\lambda_{\text{max}}$ 319 nm, 470 nm; Emission $\lambda_{\text{max}}$ 398 nm (ex. 319 nm), 588 nm (ex. 470 nm);

$^1$H NMR (300 MHz, CDCl$_3$): δ (ppm) 8.62 (d, J = 7.8 Hz, 1H), 8.07 (dd, J$^1$ = 7.8 Hz, J$^2$ = 7.5 Hz, 4H), 7.67 (t, J = 7.5 Hz, 1H), 7.63 (d, J = 7.5 Hz, 2H), 7.61 (s, 1H), 7.58 (t, J = 7.5 Hz, 1H), 7.23 (d, J = 7.5 Hz, 1H), 4.17 (dt, J$^1$ = 7.2 Hz, J$^2$ = 4.8 Hz, 2H), 4.05-3.95 (bs, 1H), 3.76 (qt, J = 7.2 Hz, 2H), 3.18-3.08 (bs, 1H), 2.38 (t, J = 7.2 Hz, 1H), 2.35-2.25 (bs, 1H), 1.36-1.32 (m, 2H), 0.91 (qt, J = 7.2 Hz, 2H), 0.88 (qt, J = 7.2 Hz, 2H);

$^{13}$C NMR – $^1$H decoupled (75 MHz, CDCl$_3$): δ (ppm) 194.2, 174.3, 173.4, 162.9, 152.4, 144.6, 144.2, 143.4, 139.0, 134.2, 130.6, 130.2, 128.1, 126.7, 123.3, 122.2, 117.1, 115.7, 113.4, 108.1, 107.7, 105.6, 101.4, 92.3, 60.3, 56.2, 35.6, 26.7, 25.0;

ESI-MS (m/z), solvent H$_2$O: 325.1 ([m+1*H$_2$O]$^{2+}$).

N-[5-[[3-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino][phenyl]formamido]pentyl]-4-[[5Z]-4-oxo-2-sulfanyliden-1,3-thiazolidin-5-ylidene[methyl]benzamide (12)

UV/Vis (solvent: ethanol): $\lambda_{\text{max}}$ 466 nm; Emission $\lambda_{\text{max}}$ 560 nm (ex. 466 nm);

$^1$H NMR (300 MHz, CDCl$_3$): δ (ppm) 8.43 (d, J = 8.7 Hz, 1H), 8.03 (s, 1H), 8.00 (d, J = 7.8 Hz, 1H), 7.86 (d, J = 8.1 Hz, 2H), 7.60 (d, J = 7.8 Hz, 1H), 7.56 (s, 1H), 7.53 (d, J = 7.8 Hz, 2H), 7.48 (t, J = 7.8 Hz, 1H), 6.73 (d, J = 8.7 Hz, 1H), 6.68 (bs, 1H), 4.82-4.65 (bs, 1H), 4.65-4.58 (bs, 1H), 3.47 (dt, J$^1$ = 6.6 Hz, J$^2$ = 7.2 Hz, 1H), 3.37 (dt, J$^1$ = 6.6 Hz, J$^2$ = 7.2 Hz, 1H), 3.13 (t, J = 7.2 Hz, 2H), 1.65 (qt, J = 7.2 Hz, 2H), 1.52 (qt, J = 7.2 Hz, 2H), 1.18 (qt, J = 7.2 Hz, 2H);

$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO d$_6$): δ (ppm) 198.0, 184.9, 182.9, 175.1, 166.7, 165.3, 158.0, 155.6, 153.6, 151.8, 148.6, 145.5, 145.0, 144.4, 142.9, 138.3, 134.1, 132.3, 127.8, 124.0, 122.4, 77.1, 57.2, 42.5, 34.8, 25.2, 23.5;

ESI-MS (m/z), solvent H$_2$O: 325.1 ([m+1*H$_2$O]$^{2+}$).

N-[5-[[4-(7-nitro-2,1,3-benzoxadiazol-4-yl)amino][phenyl]formamido]pentyl]-4-[[5Z]-4-oxo-2-sulfanyliden-1,3-thiazolidin-5-ylidene[methyl]benzamide (13)

UV/Vis (solvent: ethanol): $\lambda_{\text{max}}$ 466 nm; Emission $\lambda_{\text{max}}$ 550 nm (ex. 466 nm);

$^1$H NMR (300 MHz, CDCl$_3$): δ (ppm) 8.48 (d, J = 7.8 Hz, 1H), 7.88 (dd, J$^1$ =1.8 Hz, J$^2$ = 7.5 Hz, 2H), 7.80 (dd, J$^1$ = 1.8 Hz, J$^2$ = 7.5 Hz, 2H), 7.67 (dd, J$^1$ = 2.1 Hz, J$^2$ = 7.8 Hz, 1H), 7.60-7.39 (m, 4H), 6.64 (t, J = 8.1 Hz, 1H), 4.9-4.75 (bs, 1H), 4.8-4.6 (bs, 1H), 3.46 (dt, J$^1$ = 2.5 Hz, J$^2$ = 7.2 Hz, 1H), 3.35 (dt, J$^1$ = 6.9 Hz, J$^2$ = 7.8 Hz, 1H), 3.15-3.05 (m, 2H), 1.64 (qt, J = 7.2 Hz, 2H), 1.54 (qt, J = 7.2 Hz, 2H), 1.31-1.21 (m, 2H);

$^{13}$C NMR – $^1$H decoupled (75 MHz, CDCl$_3$): δ (ppm) 197.2, 190.4, 188.2, 174.8, 174.3, 167.7, 152.5, 136.6, 132.3, 131.2, 130.7, 124.6, 124.3, 120.7, 116.3, 111.9, 100.6, 93.6, 91.9, 88.3, 67.9, 63.9, 32.1, 30.3, 24.7;
ESI-MS (m/z), solvent H2O: 325.1 ([m+1*H2O]2+).

(5Z)-5-[(4-ethylphenyl)methylidene]-1,3-thiazolidine-2,4-dione (14)
1H NMR (300 MHz, CDCl3): δ (ppm) 7.86 (s, 1H), 7.43 (d, J = 8.4 Hz, 2H), 7.31 (d, J = 8.4 Hz, 2H), 7.21 (q, J = 7.5 Hz, 2H), 1.26 (t, J = 7.5 Hz, 3H);
13C NMR – 1H decoupled (75 MHz, CDCl3): δ (ppm) 167.5, 166.9, 147.9, 134.8, 130.5, 130.4, 128.8, 121.0, 28.9, 15.2.

N-6-[(5Z)-5-[(4-ethylphenyl)methylidene]-2,4-dioxo-1,3-thiazolidin-3-yl]hexyl]acetamide (15)
1H NMR (300 MHz, CDCl3): δ (ppm) 7.87 (s, 1H), 7.43 (d, J = 7.8 Hz, 2H), 7.30 (d, J = 7.8 Hz, 2H), 7.12-7.04 (bs, 1H), 3.74 (t, J = 6.9 Hz, 2H), 3.28 (dt, J1 = 1.5 Hz, J2 = 6.9 Hz, 2H), 2.70 (qt, J = 6.9 Hz, 2H), 2.68 (q, J = 7.5 Hz, 2H), 2.15 (s, 3H), 1.68 qt, J = 6.9 Hz, 2H), 1.58 (qt, J = 6.9 Hz, 2H), 1.38 (m, 2H), 1.26 (t, J = 7.5 Hz, 3H);
13C NMR – 1H decoupled (75 MHz, CDCl3): δ (ppm) 172.7, 167.2, 162.2, 148.2, 134.7, 131.1, 129.5, 120.8, 116.8, 59.6, 40.7, 34.0, 30.2, 29.5, 28.2, 26.7, 22.9, 15.7.

N-6-[(5Z)-5-[(4-ethylphenyl)methylidene]-2,4-dioxo-1,3-thiazolidin-3-yl]hexyl]-2-[7-nitro-2,1,3-benzoxadiazol-4-yl]amino]benzamide amine (16, LinkN1)
UV/Vis (solvent: ethanol): λmax 466 nm; Emission λmax × 550 nm (ex. 466 nm);
1H NMR (300 MHz, CDCl3): δ (ppm) 8.61 (d, J = 8.4 Hz, 1H), 8.48 (d, J = 7.5 Hz, 1H), 7.67 (d, J = 7.8 Hz, 2H), 7.62 (t, J = 7.5 Hz, 2H), 7.60 (s, 1H), 7.52 (d, J = 8.4 Hz, 1H), 7.42 (d, J = 7.8 Hz, 2H), 7.31 (d, J = 7.5 Hz, 1H), 3.49 (m, 1H), 3.41 (dt, J1 = 6.0 Hz, J2 = 6.9 Hz, 2H), 3.15 (m, 1H), 2.70 (q, J = 7.5 Hz, 2H), 2.27 (m, 1H), 1.87 (m, 1H), 1.62 (tq, J = 6.9 Hz, 2H), 1.39 (qt, J = 6.9 Hz, 2H), 1.28 (m, 2H), 1.25 (t, J = 7.5 Hz, 3H);
13C NMR – 1H decoupled (75 MHz, CDCl3): δ (ppm) 183.2, 175.5, 166.6, 158.7, 154.2, 153.3, 151.4, 145.2, 141.4, 137.6, 137.1, 133.4, 130.3, 129.5, 128.4, 120.3, 116.3, 116.0, 109.1, 97.4, 80.9, 40.9, 35.2, 29.5, 29.2, 26.3, 25.3, 17.4, 15.8.
ESI-MS (m/z), solvent H2O: 615.8 ([m+1]+).

N-6-[(5Z)-5-[(4-ethylphenyl)methylidene]-2,4-dioxo-1,3-thiazolidin-3-yl]hexyl]-3-[7-nitro-2,1,3-benzoxadiazol-4-yl]amino]benzamide amine (17)
UV/Vis (solvent: ethanol): λmax 468 nm; Emission λmax × 550 nm (ex. 468 nm);
1H NMR (300 MHz, CDCl3): δ (ppm) 8.36 (d, J = 9.0 Hz, 1H), 7.54 (d, J = 6.9 Hz, 2H), 7.51 (s, 1H), 7.29 (d, J = 1.5 Hz, 1H), 7.38-7.24 (m, 3H), 7.23 (d, J = 6.9 Hz, 2H), 6.72 (d, J = 9.0 Hz, 1H), 3.80 (t, J = 6.0 Hz, 1H), 3.70 (dtt, J1 = 1.5 Hz, J2 = 1.5 Hz, J3 = 6.9 Hz, 2H), 3.30 (t, J = 6.9 Hz, 2H), 2.67 (q, J = 7.5 Hz, 2H), 2.04 (m, 1H), 1.97 (m, 1H), 1.85 (tq, J = 6.9 Hz, 2H), 1.58 (qt, J = 6.9 Hz, 2H), 1.35 (qt, J = 6.9 Hz, 2H), 1.16 (t, J = 7.5 Hz, 3H);
13C NMR – 1H decoupled (75 MHz, CDCl3): δ (ppm) 179.3, 172.8, 172.3, 168.6, 165.0, 160.2, 156.5, 152.4, 145.7, 144.6, 142.5, 142.2, 138.7, 135.7, 130.6, 129.2, 125.7, 122.3, 119.3, 106.2, 100.6, 36.2, 34.3, 33.1, 30.3, 29.4, 28.3, 26.5, 15.9.
ESI-MS (m/z), solvent H2O: 615.6 ([m+1]+).

N-6-[(5Z)-5-[(4-ethylphenyl)methylidene]-2,4-dioxo-1,3-thiazolidin-3-yl]hexyl]-4-[7-nitro-2,1,3-benzoxadiazol-4-yl]amino]benzamide amine (18)
UV/Vis (solvent: ethanol): $\lambda_{\text{max}}$ 470 nm; Emission $\lambda_{\text{max}}$ 550 nm (ex. 470 nm);

$^1$H NMR (300 MHz, CDCl$_3$): $\delta$ (ppm) 8.37 (d, J = 7.8 Hz, 1H), 7.83 (d, J = 8.1 Hz, 1H), 7.77 (s, 1H), 7.44 (d, J = 8.1 Hz, 1H), 7.41 (d, J = 7.8 Hz, 2H), 7.35 (d, J = 8.1 Hz, 2H), 7.29 (d, J = 7.8 Hz, 2H), 7.07 (d, J = 7.8 Hz, 1H), 4.37 (t, J = 6.6 Hz, 1H), 3.45-3.25 (m, 4H), 2.69 (q, J = 7.5 Hz, 2H), 1.99 (m, 1H), 1.86 (m, 1H), 1.68 (qt, J = 6.9 Hz, 2H), 1.62 (qt, J = 6.9 Hz, 2H), 1.38 (qt, J = 6.9 Hz, 2H), 1.14 (t, J = 7.5 Hz, 3H);

$^{13}$C NMR – $^1$H decoupled (75 MHz, CDCl$_3$): $\delta$ (ppm) 177.6, 176.0, 170.6, 158.2, 148.0, 145.8, 139.6, 136.4, 134.9, 131.3, 131.0, 129.4, 125.7, 116.7, 115.2, 112.5, 100.1, 98.4, 90.9, 43.9, 37.2, 32.1, 31.1, 29.5, 28.6, 26.5, 15.7;

ESI-MS (m/z), solvent H$_2$O: 651.1 ([m+1*2H$_2$O]$^+$).


UV/Vis (solvent: ethanol): $\lambda_{\text{max}}$ 467 nm; Emission $\lambda_{\text{max}}$ 550 nm (ex. 467 nm);

$^1$H NMR (300 MHz, CDCl$_3$): $\delta$ (ppm) 8.55 (d, J = 8.4 Hz, 1H), 7.93 (d, J = 8.4 Hz, 2H), 7.74 (d, J = 7.5 Hz, 2H), 7.64 (d, J = 7.5 Hz, 2H), 7.60 (s, 1H), 7.28 (d, J = 8.4 Hz, 2H), 6.67 (d, J = 8.4 Hz, 1H), 4.05 (d, J = 15.0 Hz, 1H), 3.93 (d, J = 15.0 Hz, 1H), 3.73 (t, J = 7.5 Hz, 2H), 3.23 (t, J = 7.5 Hz, 2H), 1.56 (m, 2H), 1.37 (qt, J = 7.5 Hz, 2H), 1.22 (qt, J = 7.5 Hz, 2H);

$^{13}$C NMR – $^1$H decoupled (75 MHz, CDCl$_3$): $\delta$ (ppm) 192.5, 188.0, 180.8, 172.1, 170.4, 163.0, 159.4, 148.9, 133.9, 130.8, 124.1, 122.5, 116.2, 112.0, 107.5, 105.1, 100.2, 99.1, 90.7, 79.6, 79.1, 56.4, 36.4, 31.7, 26.3, 24.7, 16.6;

ESI-MS (m/z), solvent H$_2$O: 345.0 ([m+Na]$^+$).


UV/Vis (solvent: ethanol): $\lambda_{\text{max}}$ 468 nm; Emission $\lambda_{\text{max}}$ 545 nm (ex. 468 nm);

$^1$H NMR (300 MHz, CDCl$_3$): $\delta$ (ppm) 7.94 (d, J = 8.4 Hz, 1H), 7.79 (dd, J$^1$ = 4.2 Hz, J$^2$ = 7.5 Hz, 1H), 7.55 (dd, J$^1$ = 4.2 Hz, J$^2$ = 7.5 Hz, 1H), 7.53 (d, J = 7.5 Hz, 1H), 7.48 (s, 1H), 7.46 (d, J = 8.1 Hz, 1H), 7.39 (d, J = 7.5 Hz, 1H), 7.34 (d, J = 8.1 Hz, 1H), 6.96 (d, J = 8.4 Hz, 1H), 6.67 (d, J = 8.1 Hz, 1H), 6.63 (d, J = 8.1 Hz, 1H), 3.81 (dt, J$^1$ = 8.1 Hz, J$^2$ = 6.5 Hz, 1H), 3.47 (dt, J$^1$ = 8.1 Hz, J$^2$ = 6.5 Hz, 1H), 3.37 (dt, J$^1$ = 14.4 Hz, J$^2$ = 6.5 Hz, 1H), 3.27 (dt, J$^1$ = 14.4 Hz, J$^2$ = 6.5 Hz, 1H), 3.18 (m, 1H), 3.08 (m, 1H), 2.62 (qt, J = 6.5 Hz, 2H), 2.47 (m, 2H), 2.37 (t, J = 7.5 Hz, 2H), 1.83 (dqt, J$^1$ = 5.7 Hz, J$^2$ = 6.5 Hz, 1H), 1.67 (qt, J = 7.5 Hz, 2H), 1.42 (m, 1H), 1.28 (qt, J = 6.5 Hz, 2H), 1.17 (qt, J = 7.5 Hz, 2H);

$^{13}$C NMR – $^1$H decoupled (75 MHz, CDCl$_3$): $\delta$ (ppm) 188.1, 186.5, 174.0, 171.5, 164.5, 149.7, 149.4, 148.5, 147.2, 141.9, 136.2, 134.6, 130.0, 128.5, 126.2, 124.2, 116.6, 115.9, 104.6, 100.6, 79.1, 61.7, 52.2, 49.3, 37.3, 32.5, 32.0, 25.7, 24.8, 16.8, 15.4;

ESI-MS (m/z), solvent H$_2$O: 383.1 ([m+Na]$^{2+}$).

N-[[4-[[5Z]-5-[[4-ethylphenyl]methylidene]-2,4-dioxo-1,3-thiazolidin-3-yl]butyl]-4-[[7-nitro-2,1,3-benzoxadiazol-4-yl]amino]benzamide (21)

UV/Vis (solvent: ethanol): $\lambda_{\text{max}}$ 494 nm; Emission $\lambda_{\text{max}}$ 552 nm (ex. 494 nm);
**N-{6-[(5Z)-5-[(4-ethylphenyl)methylidene]-2,4-dioxo-1,3-thiazolidin-3-yl]hexyl}-2-((4-[7-nitro-2,1,3-benzodiazol-4-yl]amino)phenyl)formamido)acetamide (22)**

UV/Vis (solvent: ethanol): \( \lambda_{\text{max}} \) 470 nm; Emission \( \lambda_{\text{max}} \) 550 nm (ex. 470 nm).

**N-{6-[(5Z)-5-[(4-ethylphenyl)methylidene]-2,4-dioxo-1,3-thiazolidin-3-yl]hexyl}-6-((4-[7-nitro-2,1,3-benzodiazol-4-yl]amino)phenyl)formamido)hexanamide (23)**

UV/Vis (solvent: ethanol): \( \lambda_{\text{max}} \) 470 nm; Emission \( \lambda_{\text{max}} \) 545 nm (ex. 470 nm).

**N-(5Z)-5-[(4-fluorophenyl)methylidene]-1,3-thiazolidine-2,4-dione (24)**

UV/Vis (solvent: ethanol): \( \lambda_{\text{max}} \) 470 nm; Emission \( \lambda_{\text{max}} \) 545 nm (ex. 470 nm).
N-{(5Z)-5-[(4-ethylphenyl)methylenem]-2,4-dioxo-1,3-thiazolidin-3-yl}butyl]-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]benzamide (25)

UV/Vis (solvent: ethanol): $\lambda_{max}$ 493 nm; Emission $\lambda_{max}$ 550 nm (ex. 493 nm);

$^1$H NMR (300 MHz, DMSO d$_6$): $\delta$ (ppm) 8.81 (dd, $J^1 = 1.2$ Hz, $J^2 = 8.4$ Hz, 1H), 8.07 (d, $J = 8.4$ Hz, 2H), 7.79 (dd, $J^1 = 1.2$ Hz, $J^2 = 8.4$ Hz, 1H), 7.77 (s, 1H), 7.69 (d, $J = 8.4$ Hz, 2H), 7.55 (dt, $J^1 = 2.4$ Hz, $J^2 = 8.1$ Hz, 1H), 7.51 (d, $J = 8.1$ Hz, 1H), 7.41 (d, $J = 8.1$ Hz, 1H), 7.39 (t, $J = 8.1$ Hz, 1H), 4.25-4.15 (bs, 1H), 3.70 (dt, $J^1 = 6.0$ Hz, $J^2 = 6.5$ Hz, 2H), 3.23 (m, 2H), 2.68 (q, $J = 7.5$ Hz, 2H), 1.61 (dqt, $J^1 = 9.0$ Hz, $J^2 = 6.5$ Hz, 1H), 1.54 (m, 1H), 1.26 (qt, $J = 6.5$ Hz, 2H), 1.21 (t, $J = 7.5$ Hz, 3H);

$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO d$_6$): $\delta$ (ppm) 175.7, 174.0, 162.9, 151.0, 144.5, 143.8, 143.6, 142.3, 140.0, 138.4, 135.2, 131.9, 130.0, 129.7, 127.9, 127.4, 123.4, 111.2, 109.1, 108.8, 89.9, 38.1, 29.4, 27.9, 23.1, 15.6;

ESI-MS (m/z), solvent H$_2$O: 587.7 ([m+1]$^+$).

N-{(5Z)-5-[(4-fluorophenyl)methylenem]-2,4-dioxo-1,3-thiazolidin-3-yl}hexyl]-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]benzamide (26)

UV/Vis (solvent: ethanol): $\lambda_{max}$ 470 nm; Emission $\lambda_{max}$ 545 nm (ex. 470 nm);

$^1$H NMR (300 MHz, DMSO d$_6$): $\delta$ (ppm) 8.51 (d, $J = 9.0$ Hz, 1H), 7.94 (d, $J = 7.5$ Hz, 1H), 7.65 (s, 1H), 7.90-7.55 (m, 3H), 7.48 (t, $J = 7.5$ Hz, 1H), 7.42 (dt, $J^1 = 2.1$ Hz, $J^2 = 9.0$ Hz 1H), 7.34 (t, $J = 7.5$ Hz, 1H), 6.41 (d, $J = 9.0$ Hz, 1H), 3.67 (t, $J = 6.6$ Hz, 2H), 3.58 (t, $J = 6.6$ Hz, 2H), 1.69 (tt, $J^1 = 6.6$ Hz, $J^2 = 7.2$ Hz, 2H), 1.62 (t, $J = 6.6$ Hz, $J^2 = 7.2$ Hz, 2H), 1.44 (qt, $J = 7.2$ Hz, 2H), 1.33 (qt, $J = 7.2$ Hz, 2H);

$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO d$_6$): $\delta$ (ppm) 177.6, 174.4, 170.5, 167.5, 166.2, 165.9, 145.7, 143.0, 139.8, 132.9, 129.9, 128.7, 127.1, 125.7, 121.3, 117.0, 113.6, 109.6, 99.8, 99.4, 91.0, 77.8, 46.0, 43.3, 34.8, 33.7, 28.9, 26.2;

ESI-MS (m/z), solvent H$_2$O: 605.1 ([m+1]$^+$).

N-{(5Z)-5-[(4-fluorophenyl)methylenem]-2,4-dioxo-1,3-thiazolidin-3-yl}hexyl]-4-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]benzamide (27)

UV/Vis (solvent: ethanol): $\lambda_{max}$ 470 nm; Emission $\lambda_{max}$ 545 nm (ex. 470 nm);

$^1$H NMR (300 MHz, DMSO d$_6$): $\delta$ (ppm) 8.70 (d, $J = 8.1$ Hz, 1H), 7.81 (s, 1H), 7.74 (d, $J = 8.4$ Hz, 2H), 7.69 (dt, $J^1 = 3.0$ Hz, $J^2 = 8.7$ Hz, 2H), 7.40 (dt, $J^1 = 2.7$ Hz, $J^2 = 8.7$ Hz, 2H), 7.20 (d, $J = 8.1$ Hz, 1H), 6.79 (bt, 1H), 6.65 (d, $J = 8.4$ Hz, 2H), 3.38 (dt, $J^1 = 9.6$ Hz, $J^2 = 7.2$ Hz, 1H), 3.34 (t, $J = 7.2$ Hz, 2H), 3.15 (dt, $J^1 = 9.6$ Hz, $J^2 = 7.2$ Hz, 1H), 1.81 (qt, $J = 7.2$ Hz, 2H), 1.44 (qt, $J = 7.2$ Hz, 2H), 1.33 (qt, $J = 7.2$ Hz, 2H), 1.29 (qt, $J = 7.2$ Hz, 2H);

$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO d$_6$): $\delta$ (ppm) 189.4, 187.1, 179.5, 162.9, 162.6, 161.5, 155.9, 143.2, 140.3, 135.9, 133.6, 133.4, 140.7, 120.1, 116.9, 116.1, 114.3, 113.2, 90.7, 77.6, 55.1, 35.3, 32.5, 29.6, 27.5, 25.7;

ESI-MS (m/z), solvent H$_2$O: 605.3 ([m+1]$^+$).

N-{(5Z)-5-[(4-fluorophenyl)methylenem]-2,4-dioxo-1,3-thiazolidin-3-yl}butyl]-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]benzamide (28)

UV/Vis (solvent: ethanol): $\lambda_{max}$ 469 nm; Emission $\lambda_{max}$ 550 nm (ex. 469 nm);

$^1$H NMR (300 MHz, DMSO d$_6$): $\delta$ (ppm) 8.82 (dd, $J^1 = 2.7$ Hz, $J^2 = 8.7$ Hz, 1H), 8.08 (dd, $J^1 = 1.5$ Hz, $J^2 = 8.1$ Hz, 2H), 7.79 (dt, $J^1 = 1.2$ Hz, $J^2 = 8.4$ Hz, 2H), 7.74 (d, $J = 8.7$
Hz, 1H), 7.72 (dt, J^1 = 1.5 Hz, J^2 = 8.4 Hz, 2H), 7.71 (s, 1H), 7.42 t, J = 8.1 Hz, 1H), 7.32 (t, J = 8.1 Hz, 1H), 3.85 (dt, J^1 = 1.5 Hz, J^2 = 7.0 Hz, 1H), 3.70 (dt, J^1 = 1.5, Hz, J^2 = 7.0 Hz, 1H), 1.55 (m, 1H), 3.22 (ddt, J^1 = 6.3 Hz, J^2 = 12.6 Hz, J^3 = 7.0 Hz, 1H), 2.79 (ddt, J^1 = 6.3 Hz, J^2 = 12.6 Hz, J^3 = 7.0 Hz, 1H), 1.62 (m, 1H), 1.25 (qt, J = 7.0 Hz, 2H);

\(^{13}\)C NMR – \(^1\)H decoupled (75 MHz, DMSO d_6): \(\delta (ppm) 182.3, 171.8, 168.6, 164.8, 158.0, 151.0, 147.6, 140.1, 138.2, 135.2, 134.0, 132.0, 130.0, 128.0, 127.5, 116.7, 116.0, 115.4, 110.2, 108.9, 91.2, 71.5, 34.9, 33.6, 28.8, 21.5; ESI-MS (m/z), solvent H_2O: 576.7 ([m+1]^+).

N-[4-[(5Z)-5-[4-fluorophenyl]methylidene]-2,4-dioxo-1,3-thiazolidin-3-yl]butyl]-4-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]benzamide (29)

UV/Vis (solvent: ethanol): \(\lambda_{\text{max}}\) 485 nm; Emission \(\lambda_{\text{max}}\) 547 nm (ex. 485 nm);

\(^1\)H NMR (300 MHz, DMSO d_6): \(\delta (ppm) 8.48 (d, J = 9.0 Hz, 1H), 7.89 (d, J = 8.7 Hz, 1H), 7.75 (dt, J^1 = 1.8 Hz, J^2 = 7.5 Hz, 2H), 7.72 (s, 1H), 7.43 (dt, J^1 = 3.0 Hz, J^2 = 7.5 Hz, 2H), 7.28 (d, J = 8.7 Hz, 1H), 6.65 (d, J = 8.7 Hz, 2H), 6.39 (d, J = 9.0 Hz, 1H), 3.70 (t, J = 6.6 Hz, 2H), 3.15 (m, 2H), 1.86 (qt, J = 6.6 Hz, 2H), 1.21 (qt, J = 6.6 Hz, 2H); \n
\(^{13}\)C NMR – \(^1\)H decoupled (75 MHz, DMSO d_6): \(\delta (ppm) 179.5, 155.1, 154.4, 153.0, 148.2, 144.4, 142.0, 141.6, 141.1, 122.9, 113.3, 112.8, 112.0, 111.0, 109.3, 101.5, 99.8, 96.3, 79.7, 36.1, 26.1, 23.8, 15.2; ESI-MS (m/z), solvent H_2O: 577.0 ([m+1]^+).

tert butyl N-(5-acetamidopentyl)carbamate

\(^1\)H NMR (300 MHz, CDCl_3): \(\delta (ppm) 6.46 (bs, 1H), 4.78 (bs, 1H), 3.21 (dt, J^1 = 6.0 Hz, J^2 = 6.6 Hz, 2H), 3.04 (bt, J = 6.6 Hz, 2H), 2.00 (s, 3H), 1.54 (m, 4H), 1.44 (s, 9H), 1.36 (qt, J = 6.3 Hz, 2H); \n
\(^{13}\)C NMR – \(^1\)H decoupled (75 MHz, CDCl_3): \(\delta (ppm) 171.3, 156.9, 54.1, 40.1, 30.8, 30.3, 29.5, 29.0, 24.4, 23.5.

N-(5-aminopentyl)acetamide (30)

\(^1\)H NMR (300 MHz, CDCl_3): \(\delta (ppm) 5.9-5.7 (bs, 1H), 3.24 (bt, 2H), 2.69 (t, J = 6.9 Hz, 2H), 1.97 (s, 3H), 1.49 (qt, J = 7.2 Hz, 4H), 1.38 (qt, J = 6.9 Hz, 2H); \n
\(^{13}\)C NMR – \(^1\)H decoupled (75 MHz, CDCl_3): \(\delta (ppm) 176.8, 42.3, 40.0, 33.6, 29.8, 24.6, 23.7.

tert-butyl N-[5-[[4-[5Z]-4-oxo-2-sulfanylidene-1,3-thiazolidin-5-ylidene][methyl]phenyl]formamidopentyl]carbamate

\(^1\)H NMR (400 MHz, CDCl_3): \(\delta (ppm) 7.90 (d, J = 8.8 Hz, 1H), 7.54 (s, 1H), 7.48 (d, J = 8.8 Hz, 2H), 7.08 (t, J = 6.0 Hz, 1H), 4.84 (bs, 1H), 3.47 (dt, J^1 = 7.2 Hz, J^2 = 6.0 Hz, 2H), 3.13 (dt, J^1 = 7.2 Hz, J^2 = 6.0 Hz, 2H), 1.69 (qt, J = 7.2 Hz, 2H), 1.54 (qt, J = 7.2 Hz, 2H), 1.50-1.43 (m, 2H), 1.42 (s, 9H); \n
\(^{13}\)C NMR – \(^1\)H decoupled (100 MHz, CDCl_3): \(\delta (ppm) 195.7, 170.7, 168.7, 166.7, 135.9, 130.5, 130.4, 130.3, 130.2, 130.1, 127.9, 127.0, 79.1, 42.6, 40.0, 31.5, 29.7, 29.0, 23.9.

N-(5-aminopentyl)-4-[[5Z]-4-oxo-2-sulfanylidene-1,3-thiazolidin-5-ylidene][methyl]benzamide (31)
$^1$H NMR (400 MHz, CDCl$_3$): $\delta$ (ppm) 10.3 (bs, 1H), 8.3 (bs, 2H), 7.87 (d, $J = 8.0$ Hz, 2H), 7.54 (s, 1H), 7.51 (d, $J = 8.0$ Hz, 2H), 6.64 (bs, 1H), 3.50 (dt, $J_1 = 7.5$ Hz, $J_2 = 6.0$ Hz, 2H), 3.15 (t, $J = 7.5$ Hz, 2H), 1.68 (qt, $J = 7.5$ Hz, 2H), 1.54 (qt, $J = 7.5$ Hz, 2H), 1.50-1.43 (m, 2H).

N-[(2-aminophenyl)formamido]pentyl]-4-[[[(5Z)-4-oxo-2-sulfanylidene-1,3-thiazolidin-5-ylidene]methyl]benzamide (32)

$^1$H NMR (300 MHz, CDCl$_3$): $\delta$ (ppm) 8.37 (d, $J = 8.1$ Hz, 1H), 8.10 (d, $J = 7.5$ Hz, 2H), 7.73 (s, 1H), 7.67 (t, $J = 8.1$ Hz, 1H), 7.65 (d, $J = 7.5$ Hz, 2H), 7.57 (t, $J = 8.1$ Hz, 1H), 7.16 (d, $J = 8.1$ Hz, 1H), 3.75-3.60 (m, 2H), 3.60-3.45 (m, 2H), 1.10 (qt, $J = 7.2$ Hz, 2H), 0.92 (qt, $J = 7.2$ Hz, 4H);

$^{13}$C NMR – $^1$H decoupled (75 MHz, CDCl$_3$): $\delta$ (ppm) 193.9, 171.7, 152.7, 135.2, 133.2, 130.7, 126.8, 125.9, 121.5, 121.1, 119.0, 118.7, 109.0, 53.1, 46.0, 29.3, 27.9, 27.2.

tert-butyl N-[[6-[[[(5Z)-5-[[4-ethylphenyl)methylidene]-2,4-dioxo-1,3-thiazolidin-3-yl]hexyl]carbamoyl]phenyl]carbamate

$^1$H NMR (300 MHz, DMSO d$_6$): $\delta$ (ppm) 7.50 (d, $J = 8.1$ Hz, 2H), 7.39 (d, $J = 7.5$ Hz, 2H), 7.28 (d, $J = 7.5$ Hz, 2H), 3.38 (tt, $J_1 = 7.2$ Hz, $J_2 = 6.9$ Hz, 2H), 2.99 (m, 1H), 3.10 (m, 1H), 2.68 (q, $J = 6.9$ Hz, 2H), 1.83 (qt, $J = 6.9$ Hz, 2H), 1.69 (m, 1H), 1.38 (qt, $J = 6.9$ Hz, 4H), 1.24 (t, $J = 7.5$ Hz, 3H);

$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO d$_6$): $\delta$ (ppm) 183.0, 175.8, 143.6, 135.3, 133.4, 129.0, 128.1, 121.1, 110.6, 77.3, 35.1, 32.8, 32.2, 28.2, 28.0, 27.2, 25.4, 22.2, 15.4.

(5Z)-3-[6-aminohexyl]-5-[[4-ethylphenyl)methylidene]-1,3-thiazolidine-2,4-dione (33)

$^1$H NMR (300 MHz, CDCl$_3$): $\delta$ (ppm) 7.80 (s, 1H), 7.39 (d, $J = 7.5$ Hz, 2H), 7.28 (d, $J = 7.5$ Hz, 2H), 7.25 (d, $J = 8.1$ Hz, 2H), 2.99 (s, 1H), 3.10 (m, 1H), 2.68 (q, $J = 6.9$ Hz, 2H), 1.83 (qt, $J = 6.9$ Hz, 2H), 1.69 (m, 1H), 1.38 (qt, $J = 6.9$ Hz, 4H), 1.24 (t, $J = 7.5$ Hz, 3H);

$^{13}$C NMR – $^1$H decoupled (75 MHz, CDCl$_3$): $\delta$ (ppm) 170.0, 168.3, 150.9, 148.4, 135.1, 131.1, 129.5, 121.9, 34.1, 32.9, 30.4, 29.6, 28.4, 27.9, 26.1, 15.8.

tert-butyl N-[[6-[[[(5Z)-5-[[4-ethylphenyl)methylidene]-2,4-dioxo-1,3-thiazolidin-3-yl]hexyl]carbamoyl]phenyl]carbamate

$^1$H NMR (300 MHz, CDCl$_3$): $\delta$ (ppm) 7.94 (d, $J = 6.3$ Hz, 2H), 7.56 (dd, $J_1 = 1.5$ Hz, $J_2 = 8.1$Hz, 1H), 7.50 (d, $J = 6.3$ Hz, 2H), 7.48 (s, 1H), 7.46 (dt, $J_1 = 1.5$ Hz, $J_2 = 8.1$ Hz, 1H), 7.40 (t, $J = 8.1$ Hz, 1H), 7.25 (d, $J = 8.1$ Hz, 1H), 3.38 (dt, $J_1 = 7.8$ Hz, $J_2 = 6.9$ Hz, 2H), 3.18 (dt, $J_1 = 8.4$ Hz, $J_2 = 6.9$ Hz, 1H), 3.04 (dt, $J_1 = 8.4$ Hz, $J_2 = 6.9$ Hz, 1H), 2.65 (q, $J = 7.5$ Hz, 2H), 2.15 (m, 1H), 1.83 (m, 1H), 1.70-1.60 (m, 2H), 1.56 (s, 1H), 1.51 (qt, $J = 6.9$ Hz, 2H), 1.40 (qt, $J = 6.9$ Hz, 2H), 1.24 (t, $J = 7.5$ Hz, 3H);

$^{13}$C NMR – $^1$H decoupled (75 MHz, CDCl$_3$): $\delta$ (ppm) 189.0, 175.8, 172.6, 150.7, 138.2, 133.6, 131.9, 130.8, 129.9, 129.2, 128.5, 128.1, 112.7, 109.1, 100.6, 86.6, 54.1, 43.6, 34.3, 33.1, 29.5, 29.1, 28.9, 28.3, 26.5, 15.9.

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2-amino-N-(6-[(5Z)-5-[(4-ethylphenyl)methylidene]-2,4-dioxo-1,3-thiazolidin-3-yl]hexyl)benzamide amine (34)

$^1$H NMR (300 MHz, CDCl$_3$): $\delta$ (ppm) 7.76 (d, J = 6.3 Hz, 2H), 7.44 (s, 1H), 7.48-7.40 (m, 5H), 7.31 (d, J = 8.1 Hz, 1H), 3.74 (dt, $J^1$ = 7.5 Hz, $J^2$ = 6.9 Hz, 1H), 3.37 (dt, $J^1$ = 6.3 Hz, $J^2$ = 6.9 Hz, 2H), 3.17 (dt, $J^1$ = 7.5 Hz, $J^2$ = 6.9 Hz, 1H), 2.87 (m, 2H), 2.70 (q, J = 7.5 Hz, 2H), 1.85 (dq, $J^1$ = 14.4 Hz, $J^2$ = 6.9 Hz, 1H), 1.64 (dq, $J^1$ = 14.4 Hz, $J^2$ = 6.9 Hz, 1H), 1.37 (qt, J = 6.9 Hz, 2H), 1.32 (m, 2H), 1.26 (t, J = 7.5 Hz, 3H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, CDCl$_3$): $\delta$ (ppm) 170.7, 168.7, 165.1, 151.0, 148.0, 136.0, 131.8, 131.2, 130.0, 129.7, 126.8, 123.6, 110.4, 101.9, 49.4, 46.4, 43.8, 32.2, 28.3, 22.3, 18.4, 15.8.


$^1$H NMR (300 MHz, CDCl$_3$): $\delta$ (ppm) 8.4-8.2 (bs 1H), 8.04 (s, 1H), 7.87 (d, J = 8.1 Hz, 1H), 7.54 (d, J = 8.1 Hz, 2H), 7.46 (d, J = 8.1 Hz, 1H), 5.4-5.2 (bd, J = 16.8 Hz, 1H), 3.91 (d, J = 6.0 Hz, 1H), 3.79 (d, J = 6.0 Hz, 1H), 3.46 (dt, $J^1$ = 8.1 Hz, $J^2$ = 6.6 Hz, 1H), 3.36 (dt, $J^1$ = 6.6 Hz, $J^2$ = 7.2 Hz, 1H), 3.10 (m, 2H), 2.46 (t, J = 7.2 Hz, 1H), 2.33 (t, J = 7.2 Hz, 1H), 2.17 (t, J = 7.2 Hz, 1H), 1.63 (qt, J = 6.6 Hz, 2H), 1.48 (d, J = 16.8 Hz, 9H), 1.26 (qt, J = 6.6 Hz, 2H), 1.08 (qt, J = 7.2 Hz, 2H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, CDCl$_3$): $\delta$ (ppm) 191.9, 174.7, 172.2, 164.0, 156.7, 152.2, 149.0, 131.2, 128.7, 85.5, 54.1, 45.9, 43.7, 37.5, 32.4, 28.9, 28.4, 21.8, 14.9.


$^1$H NMR (300 MHz, CDCl$_3$): $\delta$ (ppm) 7.87 (s, 1H), 7.43 (d, J = 7.8 Hz, 2H), 7.42 (d, J = 7.8 Hz, 2H), 7.41 (d, J = 7.8 Hz, 2H), 7.30 (s, 1H), 7.43 (d, J = 7.8 Hz, 2H), 7.36 (t, J = 6.0 Hz, 1H), 3.42 (t, J = 7.0 Hz, 2H), 3.14 (dt, $J^1$ = 6.0 Hz, $J^2$ = 7.0 Hz, 2H), 2.69 (q, J = 7.5 Hz, 2H), 1.89 (qt, J = 7.0 Hz, 2H), 1.64 (qt, J = 7.0 Hz, 2H), 1.44 (s, 9H), 1.26 (t, J = 7.5 Hz, 3H);
$^{13}$C NMR – $^1$H decoupled (75 MHz, CDCl$_3$): $\delta$ (ppm) 168.8, 167.1, 148.2, 134.7, 131.4, 131.1, 129.5, 120.9, 90.0, 69.2, 34.7, 31.4, 30.2, 29.9, 28.7, 26.6, 15.8.

(5Z)-3-(4-aminobutyl)-5-[(4-ethylphenyl)methylidene]-1,3-thiazolidine-2,4-dione (35)

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\[ ^1\text{H NMR (300 MHz, CDCl}_3\text{)}: \delta \text{ (ppm) 7.81 (s, 1H), 7.68-7.52 (bs, 2H), 7.40 (d, J = 8.4 Hz, 2H), 7.28 (d, J = 8.4 Hz, 2H), 3.42 (dt, J^1 = 5.7, J^2 = 6.6 Hz, 1H), 3.39 (dt, J^1 = 5.7, J^2 = 6.6 Hz, 1H), 3.12 (dt, J^1 = 4.8 Hz, J^2 = 6.9 Hz, 2H), 2.69 (q, J = 7.5 Hz, 2H), 1.88 (qt, J = 6.6 Hz, 2H), 1.76 (m, 1H), 1.66 (m, 1H), 1.25 (t, J = 7.5 Hz, 3H); } ^{13}\text{C NMR – }^1\text{H decoupled (75 MHz, CDCl}_3\text{): } \delta \text{ (ppm) 169.4, 168.5, 148.6, 135.6, 131.2, 130.9, 129.5, 121.6, 40.3, 32.7, 30.4, 29.5, 26.6, 15.8.} \]

tert-butyl N-[(6Z)-5-[(4-ethylphenyl)methylidene]-2,4-dioxo-1,3-thiazolidin-3-yl]hexyl)carbamoyl)methyl]carbamate
\[ ^1\text{H NMR (300 MHz, CDCl}_3\text{)}: \delta \text{ (ppm) 7.61 (s, 1H), 7.39 (d, J = 8.1 Hz, 2H), 7.24 (d, J = 8.1 Hz, 2H), 7.20-7.05 (bs, 1H), 5.35-5.20 (bd, J = 12.0 Hz, 1H), 4.49 (d, J = 5.1 Hz, 1H), 3.74 (dt, J^1 = 1.8 Hz, J^2 = 6.5 Hz, 2H), 3.64 (d, J = 5.1 Hz, 1H), 3.38 (dt, J^1 = 2.7 Hz, J^2 = 6.5 Hz, 2H), 3.27 (m, 2H), 2.68 (q, J = 7.5 Hz, 2H), 2.46 (m, 1H), 2.40 (m, 1H), 1.85 (qt, J = 6.5 Hz, 2H), 1.47 (d, J = 12.0 Hz, 9H), 1.26 (m, 2H), 1.17 (dt, J^1 = 1.8 Hz, J^2 = 7.5 Hz, 3H); } ^{13}\text{C NMR – }^1\text{H decoupled (75 MHz, CDCl}_3\text{): } \delta \text{ (ppm) 171.4, 169.0, 151.3, 146.7, 132.4, 130.7, 129.6, 129.1, 127.7, 83.9, 57.1, 45.0, 43.1, 39.9, 36.2, 35.4, 33.2, 29.4, 28.6, 17.9, 15.9.} \]

2-amino-N-[(6Z)-5-[(4-ethylphenyl)methylidene]-2,4-dioxo-1,3-thiazolidin-3-yl]hexyl]acetamide
\[ ^1\text{H NMR (300 MHz, CDCl}_3\text{)}: \delta \text{ (ppm) 9.4-9.15 (bs, 1H), 9.1-9.0 (bs, 1H), 7.86 (s, 1H), 7.44 (d, J = 7.5 Hz, 2H), 7.32 (d, J = 7.5 Hz, 2H), 7.15-7.05 (bs, 1H), 4.26 (d, J = 6.9 Hz, 1H), 3.81 (m, 1H), 3.72 (d, J = 6.9 Hz, 1H), 3.39 (m, 1H), 3.25 (m, 2H), 2.81 (m, 2H), 2.70 (q, J = 7.5 Hz, 2H), 2.30-2.00 (m, 2H), 1.65 (m, 1H), 1.45 (m, 1H), 1.32 (qt, J = 6.9 Hz, 4H), 1.27 (t, J = 7.5 Hz, 3H); } ^{13}\text{C NMR – }^1\text{H decoupled (75 MHz, CDCl}_3\text{): } \delta \text{ (ppm) 175.5, 167.8, 161.8, 148.7, 135.8, 131.2, 129.6, 116.9, 94.6, 54.1, 47.2, 40.2, 34.7, 30.9, 29.6, 26.3, 21.2, 15.8.} \]

tert-butyl N-[(5Z)-5-[(4-ethylphenyl)methylidene]-2,4-dioxo-1,3-thiazolidin-3-yl]hexyl]carbamoyl)pentyl]carbamate
\[ ^1\text{H NMR (300 MHz, CDCl}_3\text{)}: \delta \text{ (ppm) 7.83 (s, 1H), 7.44 (d, J = 8.1 Hz, 2H), 7.30 (d, J = 8.1 Hz, 2H), 6.55-6.45 (bs, 1H), 5.70-5.60 (bs, 1H), 3.39 (ddt, J^1 = 0.6 Hz, J^2 = 6.0 Hz, J^3 = 6.6 Hz, 2H), 3.25 (q, J = 6.6 Hz, 1H), 3.11 (dt, J^1 = 5.7 Hz, J^2 = 6.6 Hz, 2H), 3.02 (m, 1H), 2.69 (q, J = 7.5 Hz, 2H), 2.35 (t, J = 7.2 Hz, 2H), 1.87 (qt, J = 7.2 Hz, 2H), 1.65 (qt, J = 7.2 Hz, 4H), 1.44 (s, 9H), 1.36 (qt, J = 6.6 Hz, 4H), 1.26 (dt, J^1 = 0.6 Hz, J^2 = 7.5 Hz, 3H); } ^{13}\text{C NMR – }^1\text{H decoupled (75 MHz, CDCl}_3\text{): } \delta \text{ (ppm) 178.8, 168.4, 153.5, 148.4, 135.0, 131.1, 129.5, 122.0, 116.5, 99.6, 55.2, 44.7, 40.5, 34.4, 33.2, 33.1, 30.6, 30.3, 29.5, 29.1, 28.3, 26.5, 25.0, 19.0, 15.8.} \]

1TZD-C4-2BnNHBoc
tert-butyl N-[(5Z)-5-[(4-ethylphenyl)methylidene]-2,4-dioxo-1,3-thiazolidin-3-yl]butyl]carbamoyl)phenyl]carbamate
\[ ^1\text{H NMR (300 MHz, CDCl}_3\text{)}: \delta \text{ (ppm) 7.96 (dd, J^1 = 1.5 Hz, J^2 = 7.5 Hz, 2H), 7.57 (d, J = 8.1 Hz, 1H), 7.52 (d, J = 7.5 Hz, 2H), 7.50 (s, 1H), 7.48 (d, J = 8.1 Hz, 1H), 7.44 (t, J =}
8.1 Hz, 1H), 7.28 (t, J = 8.1 Hz, 1H), 3.43 (dt, J^1 = 3.9 Hz, J^2 = 7.0 Hz, 2H), 3.32 (dt, J^1 = 2.5 Hz, J^2 = 7.0 Hz, 2H), 2.70 (q, J = 7.5 Hz, 2H), 2.13 (dqt, J^1 = 12.0 Hz, J^2 = 7.0 Hz, 1H), 2.04 (m, 1H), 1.84 (m, 2H), 1.61 (s, 9H), 1.27 (t, J = 7.5 Hz, 3H);

\^13C NMR \(- ^1H\) decoupled (75 MHz, CDCl_3): \(\delta\) (ppm) 171.5, 147.3, 144.6, 138.8, 133.6, 131.1, 130.8, 129.9, 129.5, 128.3, 128.1, 125.2, 109.1, 100.6, 86.6, 46.6, 43.8, 29.2, 28.9, 28.3, 17.2, 16.6, 15.8.

**2-amino-N-[(4-[5Z]-5-([(4-ethylphenyl)methylidene]-2,4-dioxo-1,3-thiazolidin-3-yl]butyl)benzamide (36)**

\(^1H\) NMR (300 MHz, CDCl_3): \(\delta\) (ppm) 7.78 (dd, J^1 = 1.2 Hz, J^2 = 8.1 Hz, 2H), 7.48 (d, J = 7.5 Hz, 1H), 7.46 (s, 1H), 7.45 (d, J = 8.1 Hz, 2H), 7.44 (d, J = 7.5 Hz, 1H), 7.42 (t, J = 7.5 Hz, 1H), 7.32 (t, J = 7.5 Hz, 1H), 6.4-5.5 (bs, 2H), 3.77 (dt, J^1 = 6.0 Hz, J^2 = 7.0 Hz, 1H), 3.43 (dt, J^1 = 6.0 Hz, J^2 = 7.0 Hz, 1H), 3.25 (m, 2H), 2.87 (qt, J = 7.0 Hz, 2H), 2.69 (q, J = 7.5 Hz, 2H), 2.03 (m, 1H), 1.75 (m, 1H), 1.26 (t, J = 7.5 Hz, 3H);

\(^13C\) NMR \(- ^1H\) decoupled (75 MHz, CDCl_3): \(\delta\) (ppm) 183.6, 179.6, 171.8, 148.2, 135.1, 131.8, 129.7, 129.2, 127.6, 126.8, 120.0, 115.9, 112.9, 96.6, 83.3, 60.6, 51.5, 47.3, 43.1, 35.8, 15.8.

**tert-butyl N-[(6-[5Z]-5-([(4-fluorophenyl)methylidene]-2,4-dioxo-1,3-thiazolidin-3-yl]hexyl)carbamate (36)**

\(^1H\) NMR (300 MHz, DMSO d_6): \(\delta\) (ppm) 7.52 (dt, J^1 = 2.1 Hz, J^2 = 8.7 Hz, 2H), 7.25 (d, J^1 = 2.4 Hz, 1H), 7.21 (dt, J^1 = 2.4 Hz, J^2 = 8.7 Hz, 2H), 6.71 (bs, 1H), 3.46 (t, J = 6.5 Hz, 2H), 3.30 (m, 2H), 2.84 (q, J = 6.5 Hz, 2H), 1.72 (qt, J = 6.5 Hz, 2H), 1.37 (m, 2H), 1.32 (s, 9H), 1.20 (qt, J = 6.5 Hz, 2H);

\(^13C\) NMR \(- ^1H\) decoupled (75 MHz, DMSO d_6): \(\delta\) (ppm) 183.1, 163.3, 155.9, 141.2, 136.5, 132.8, 131.3, 121.3, 116.1, 115.8, 77.6, 49.2, 35.4, 32.5, 29.6, 28.6, 27.5, 25.7.

**[5Z]-3-([6-aminohexyl]-5-[(4-fluorophenyl)methylidene]-1,3-thiazolidine-2,4-dione (37)**

\(^1H\) NMR (300 MHz, DMSO d_6): \(\delta\) (ppm) 7.83 (s, 1H), 7.69 (dt, J^1 = 1.5 Hz, J^2 = 8.7 Hz, 2H), 7.40 (dt, J^1 = 1.8 Hz, J^2 = 8.7 Hz, 2H), 3.55 (t, J = 7.2 Hz, 2H), 2.70 (qt, J = 7.2 Hz, 2H), 1.82 (qt, J = 7.2 Hz, 2H), 1.57 (qt, J = 7.2 Hz, 2H), 1.38 (qt, J = 7.2 Hz, 4H);

\(^13C\) NMR \(- ^1H\) decoupled (75 MHz, DMSO d_6): \(\delta\) (ppm) 168.1, 167.6, 158.9, 158.5, 132.8, 132.7, 131.0, 130.0, 123.5, 118.7, 116.9, 116.6, 99.8, 35.2, 32.3, 27.3, 27.1, 25.1.

**tert-butyl N-[2-[(6-[5Z]-5-([4-fluorophenyl)methylidene]-2,4-dioxo-1,3-thiazolidin-3-yl]hexyl]carbamoyl]phenylcarbamate (Boc-38)**

\(^1H\) NMR (300 MHz, CDCl_3): \(\delta\) (ppm) 7.92 (d, J = 7.2 Hz, 1H), 7.54 (d, J = 8.4 Hz, 1H), 7.5-7.4 (m, 5H), 7.15 (dt, J^1 = 1.5 Hz, J^2 = 8.1 Hz, 2H), 4.68-4.53 (bs, 1H), 4.05 (dt, J^1 = 8.4 Hz, J^2 = 6.6 Hz, 1H), 3.38 (t, J = 6.6 Hz, 2H), 3.18 (dt, J^1 = 8.4 Hz, J^2 = 6.6 Hz, 1H), 2.34-2.04 (m, 1H), 1.83 (dqt, J^1 = 10.5 Hz, J^2 = 6.6 Hz, 1H), 1.65 (m, 2H), 1.58 (s, 9H), 1.26 (qt, J = 6.6 Hz, 2H);

\(^13C\) NMR \(- ^1H\) decoupled (75 MHz, CDCl_3): \(\delta\) (ppm) 166.8, 171.3, 150.5, 136.5, 132.8, 132.7, 132.5, 130.1, 129.8, 128.3, 128.2, 127.9, 121.7, 117.2, 116.9, 109.0, 100.5, 86.5, 43.5, 42.4, 41.0, 34.3, 33.2, 30.4, 29.0, 26.7.
tert-butyl N-[4-[(5Z)-5-[(4-fluorophenyl)methylidene]-2,4-dioxo-1,3-thiazolidin-3-yl]butyl]carbamate

$^1$H NMR (300 MHz, DMSO $d_6$): $\delta$ (ppm) 7.60 (dt, $J^1 = 2.1$ Hz, $J^2 = 8.1$ Hz, 2H), 7.33 (d, $J = 2.4$ Hz, 1H), 7.29 (dt, $J^1 = 2.4$ Hz, $J^2 = 9.0$ Hz, 2H), 6.89-6.85 (bs, 1H), 3.56 (dt, $J^1 = 2.4$ Hz, $J^2 = 6.6$ Hz, 2H), 2.96 (dt, $J^1 = 6.0$ Hz, $J^2 = 6.6$ Hz, 2H), 1.81 (qt, $J = 6.6$ Hz, 2H), 1.52 (qt, $J = 6.6$ Hz, 2H), 1.41 (s, 9H);

$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO $d_6$): $\delta$ (ppm) 183.1, 175.8, 164.2, 155.9, 136.4, 132.8, 131.3, 131.2, 121.3, 116.1, 115.9, 77.8, 42.5, 35.2, 29.9, 29.5, 23.7, 14.5.

(5Z)-3-[(4-aminobutyl)-5-[(4-fluorophenyl)methylidene]-1,3-thiazolidine-2,4-dione (39)

$^1$H NMR (300 MHz, DMSO $d_6$): $\delta$ (ppm) 7.9-7.8 (bs, 2H), 7.83 (s, 1H), 7.68 (dt, $J^1 = 1.2$ Hz, $J^2 = 8.7$ Hz, 2H), 7.38 (dt, $J^1 = 1.2$ Hz, $J^2 = 8.7$ Hz, 2H), 3.58 (t, $J = 7.2$ Hz, 2H), 2.86 (dt, $J^1 = 6.3$ Hz, $J^2 = 7.2$ Hz, 2H), 1.87 (qt, $J = 7.2$ Hz, 2H), 1.71 (qt, $J = 7.2$ Hz, 2H);

$^{13}$C NMR – $^1$H decoupled (75 MHz, DMSO $d_6$): $\delta$ (ppm) 167.9, 167.5, 159.5, 159.0, 158.5, 132.5, 130.9, 123.4, 121.7, 117.8, 116.5, 114.0, 38.2, 34.4, 29.2, 25.9.

tert-butyl N-[2-[(4-[(5Z)-5-[(4-fluorophenyl)methylidene]-2,4-dioxo-1,3-thiazolidin-3-yl]butyl]carbamoyl)phenyl]carbamate

$^1$H NMR (300 MHz, CDCl$_3$): $\delta$ (ppm) 7.93 (dd, $J^1 = 1.5$ Hz, $J^2 = 8.4$ Hz, 2H), 7.55 (dt, $J^1 = 1.2$ Hz, $J^2 = 6.9$ Hz, 2H), 7.51 (s, 1H), 7.49 (dt, $J^1 = 1.5$ Hz, $J^2 = 6.9$ Hz, 2H), 7.16 (t, $J = 8.4$ Hz, 1H), 7.10 (t, $J = 8.4$ Hz, 1H), 4.11 (t, $J = 6.6$ Hz, 1H), 3.41 (ddt, $J^1 = 2.1$ Hz, $J^2 = 6.6$ Hz, $J^3 = 6.9$ Hz, 2H), 3.36 (dt, $J^1 = 5.7$ Hz, $J^2 = 6.9$ Hz, 1H), 3.28 (dt, $J^1 = 5.7$ Hz, $J^2 = 6.9$ Hz, 1H), 1.98 (m, 1H), 1.75 (m, 1H), 1.60 (s, 9H), 1.24 (dqt, $J^1 = 7.8$ Hz, $J^2 = 6.9$ Hz, 1H), 1.21 (dqt, $J^1 = 7.8$ Hz, $J^2 = 6.9$ Hz, 1H);

$^{13}$C NMR – $^1$H decoupled (75 MHz, CDCl$_3$): $\delta$ (ppm) 186.7, 170.1, 163.1, 150.5, 138.1, 133.5, 132.0, 129.8, 129.4, 128.3, 128.2, 127.9, 122.5, 116.6, 109.0, 86.6, 54.0, 44.8, 40.2, 37.1, 30.2, 28.1.

2-amino-N-[4-[(5Z)-5-[(4-fluorophenyl)methylidene]-2,4-dioxo-1,3-thiazolidin-3-yl]butyl]benzamide (40)

$^1$H NMR (300 MHz, CDCl$_3$): $\delta$ (ppm) 7.72 (dd, $J^1 = 1.8$ Hz, $J^2 = 8.7$ Hz, 2H), 7.57 (t, $J = 8.7$ Hz, 1H), 7.46 (dt, $J^1 = 3.3$ Hz, $J^2 = 8.7$ Hz, 2H), 7.42 (s, 1H), 7.38 (dt, $J^1 = 1.8$ Hz, $J^2 = 8.4$ Hz, 2H), 7.16 (dt, $J^1 = 2.7$ Hz, $J^2 = 8.7$ Hz, 1H), 3.41 (dt, $J^1 = 13.8$ Hz, $J^2 = 7.8$ Hz, 2H), 2.85 (dt, $J^1 = 4.5$ Hz, $J^2 = 7.0$ Hz, 2H), 2.24 (m, 1H), 1.92 (dqt, $J^1 = 11.1$ Hz, $J^2 = 7.0$ Hz, 1H), 1.72 (dqt, $J^1 = 13.2$ Hz, $J^2 = 7.0$ Hz, 1H), 1.26 (dqt, $J^1 = 11.1$ Hz, $J^2 = 7.0$ Hz, 1H);

$^{13}$C NMR – $^1$H decoupled (75 MHz, CDCl$_3$): $\delta$ (ppm) 167.7, 166.2, 162.1, 161.5, 142.2, 133.5, 131.7, 130.0, 129.6, 128.2, 126.7, 121.6, 116.1, 114.4, 113.6, 110.5, 90.3, 60.7, 51.1, 30.2, 21.6.
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