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Small-molecule perturbation of competing interactions between c-Myc and Max

Ariele Viacava Follis, Dalia I. Hammoudeh, Andrew T. Daab, Steven J. Metallo *

Department of Chemistry, Georgetown University, 37th & O Streets, NW, Reiss Science Center, Washington, DC 20057, USA

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ABSTRACT

The oncogenic transcription factor c-Myc undergoes coupled binding and folding of its basic-helix-loop-helix-leucine zipper domain (bHLHZip) upon heterodimerization with its partner protein Max. The latter exists in two isoforms: p21, which homodimerizes poorly, and p22, which homodimerizes well. We show that the effect of 10058-F4 (a small-molecule that binds disordered c-Myc monomers and disrupts the c-Myc–Max complex) on both c-Myc–Max heterodimerization and DNA binding is dependent on the nature of the Max isoform. In the presence of p22 Max the effective inhibitor concentration is lower than in the presence of p21 Max, as the p22 Max homodimer formation affects the thermodynamics by competing against the c-Myc–Max heterodimerization event.

Intrinsically disordered (ID) proteins are characterized by a lack of stable structure and extensive backbone flexibility.[1,2] These properties are often useful in the role of hubs in protein interaction networks involved in cell signaling and regulation in eukaryotes.[3,4] ID proteins can interact with multiple binding partners by means of coupled folding and binding transitions,[5] and may assume different structures when binding to different partners.[1,6] The entropic cost associated with ID folding grants their interactions high specificities yet low affinities.[1] These phenomena combined (multiple protein partners, specific low affinity binding) give rise to finely balanced networks of competing interactions. Deregulation of such ID protein functions often leads to disease states.[3]

The oncoprotein c-Myc is an ID transcription factor that binds target DNA sequences through coupled folding and binding of its bHLHZip domain with a similar domain on the partner protein Max.[7] c-Myc does not homodimerize and this interaction is necessary to its cellular functions.[8,9] 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.[10] 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, but not p21 Max, can form homodimers and silently bind DNA.[11] While c-Myc and Mad proteins can compete for Max binding,[12] 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 property of p22 Max, further regulated by its phosphorylation,[13] could function to finely tune the activity of its heterodimerization partners. Both p21 and p22 Max have been simultaneously isolated from cell lysates,[13] and are capable of heterodimerizing with each other with reduced E-box affinity compared to p22 Max homodimers.[10] While the specific function of these 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.[14] Homo or heterodimerization is a common mechanism for DNA recognition by TFs.[15] Competition for a protein partner by different protein monomers has been described in several TF families, including bHLHZip,[12,16] basic-leucine zipper (bZip)[17] and basic-helix-loop-helix (bHLH),[18] and may be generally exploited in transcriptional regulation strategies.[14]

Several small-molecules ('c-Myc inhibitors') capable of disrupting the heterodimer formation between c-Myc and Max have been reported.[19–22] We have shown that some c-Myc inhibitors function by specifically binding to segments of the monomeric c-Myc bHLHZip, thus preventing its dimerization with Max.[23] We 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. More specifically, we tested whether the efficacy of specific inhibitors of c-Myc–Max heterodimerization 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 (Fig. 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

* Corresponding author. Tel.: +1 202 687 2065; fax: +1 202 687 6209.
E-mail address: sjm24@georgetown.edu (S.J. Metallo).

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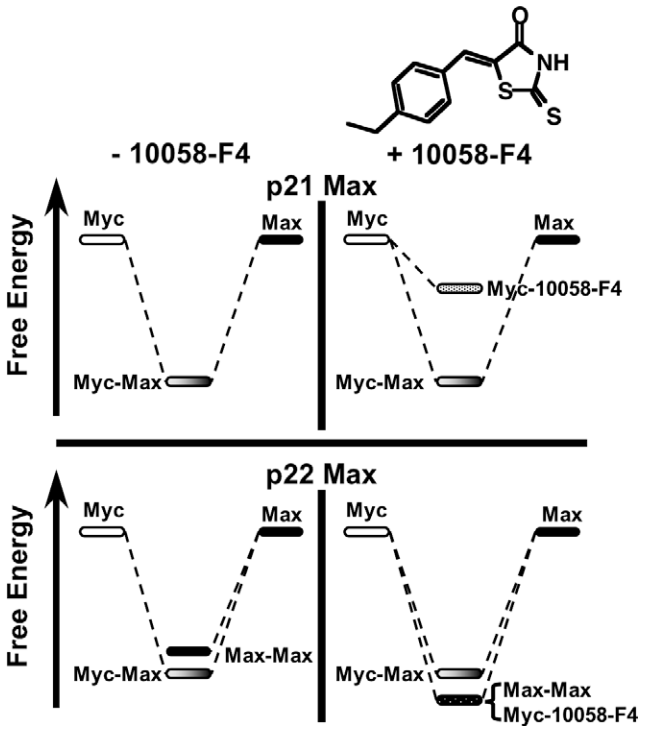

Figure 1. Schematic free energy diagram of the competing equilibria of bHLHZip dimer formation in the absence and presence of the c-Myc inhibitor 10058-F4 (structure shown). The ability of p22 Max to form stable homodimers facilitates disruption by 10058-F4 since both p22 Max and c-Myc can simultaneously form favorable complexes, Max with itself and c-Myc with 10058-F4.

difficult systems (such as disordered TFs that dimerize over an extensive surface[24] without obvious sites for inhibition) into useful targets for modulating transcriptional activity or other cell signaling functions.

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, we fluorescently tagged the c-Myc bHLHZip domain with fluorescein (c-MycFLU), using a unique Cys residue added at its C-terminus. Using fluorescence polarization (FP) titrations the affinities of p21 Max and p22 Max for c-MycFLU were measured. 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 (Fig. S1).[25] We then monitored the extent of heterodimer formation between 1.5 μM mixtures of c-MycFLU–p21 Max or c-MycFLU–p22 Max in the presence of varying concentrations of the c-Myc inhibitor 10058-F4.[20] The resulting data were analyzed in terms of competition for c-Myc binding 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 (Fig. 2A).[26] We observed that the inhibitors 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[23] and 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 fourfold (K_{comp} = 2.6 ± 0.4). We attribute this enhancement to the additional free energy generated by the homodimer formation between p22 Max molecules when displaced from the heterodimer.

Next we evaluated whether a similar phenomenon would occur when the bHLHZip dimers bound their specific DNA target sequence (E-box). E-box binding by c-Myc–Max heterodimer and Max homodimer pairs was monitored by means of electrophoretic mobility shift assays (EMSAs). Titrations of E-box binding by the various proteins indicated that c-Myc–p22 Max 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 lower than that of either heterodimer (Fig. 2B). Homodimers of p21 Max were confirmed to bind E-box very poorly (K_{obs} > 1 μM, Fig. S2).[10] We wished to test the thermodynamic rationale for the hypothesized perturbation of competing DNA binding equilibria introduced by the inhibitor. First we titrated E-box binding by c-Myc–p21 Max heterodimers in the presence of a fixed concentration of 10058-F4 (50 μM). The inhibitor caused a twofold decrease in the c-Myc–p21 Max affinity for E-box (K_{obs} = 48 ± 16 nM, Fig. 2B). We would therefore expect 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 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 p22 Max heterodimer compared to the p21 heterodimer. It was therefore not possible to make a direct comparison with 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 (Fig. 2C). 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 fraction of free E-box, and there was a partial shift towards homodimer binding down to a 10058-F4 concentration of 12.5 μM (Fig. 2C). In contrast, a 200 μM concentration of 10058-F4 was not sufficient to completely disrupt the binding of E-box by c-Myc–p21 Max heterodimers and a 100 μM concentration caused little disruption (Fig. 2D). 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 fourfold lower than those required in the presence of p21 Max, in spite of the higher affinity for E-box of c-Myc–p22 Max (Fig. 3). Analysis of this system of equilibria (based on mass action and assumption free numerical integration [KinTekSim, KinTek Corp.]) indicates that the effectiveness of an inhibitor increases as the affinity of the interaction involving the uninhibited component increases. In this case as Max homodimer affinity increases (p22 vs 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.

These results provide insight into the mode of action of a c-Myc inhibitor, which may involve the perturbation of competing biological equilibria rather than simple inhibition. Combining this observation with the existence of competing protein–protein interactions within transcriptional regulation and other cellular pathways,[27–29] especially involving ID proteins,[3] suggests the possibility of exploiting the perturbation of these competing equilibria for the purposes of chemical interference. In order to develop such

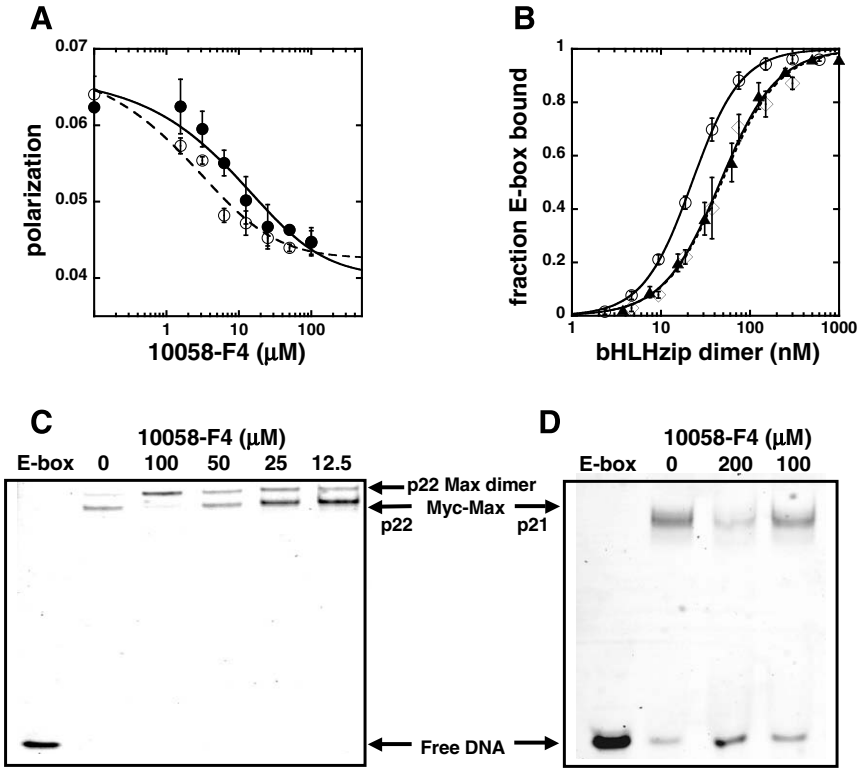

Figure 2. (A) Disruption of c-Myc–p21 Max (black circles) and c-Myc–p22 Max (white circles) heterodimers by 10058-F4; error bars represent the standard error of three or more trials. The values on the *y*-axis indicate the polarization values for the complexes in the absence of inhibitor. (B) E-box DNA binding titrations by c-Myc–p21 Max (white circles) and p22 Max (black triangles). Titration of c-Myc–p21 Max in the presence of 50 μM 10058-F4 (white diamonds). Error bars represent the standard error of five or more trials. (C) Perturbation effect of 10058-F4 on E-box DNA binding (10 nM) by 150 nM c-Myc–p22 Max. The inhibitor causes a shift of the bound DNA from c-Myc–p22 Max heterodimers to p22 Max homodimers. (D) Effect of 10058-F4 on E-box DNA binding by 150 nM c-Myc–p21 Max monitored by EMSA.

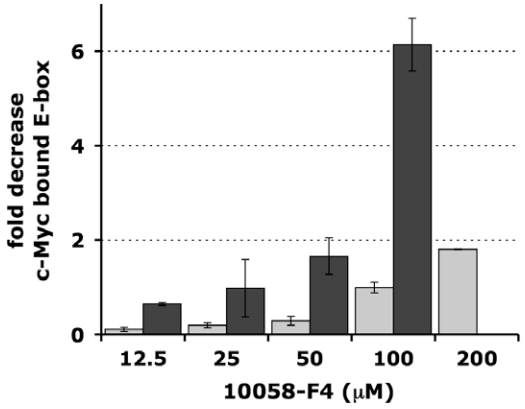

Figure 3. Comparison between the measured inhibition of E-Box binding by c-Myc–p21 Max[23] and c-Myc–p22 Max upon addition of 10058-F4 at varying concentrations. The data are plotted as relative decrease of c-Myc-bound E-box to account for the different E-box complexes present in the two experiments. Values represent the average of three trials and error bars indicate the standard error.

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.[30]

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.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.12.025.

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