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Synergistic Enhancement of the Potency and Selectivity of Small Molecule Transcriptional Inhibitors

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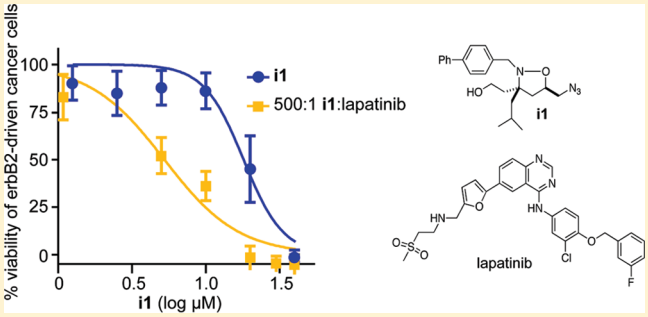
Supporting Information

ABSTRACT: In spite of their considerable therapeutic potential, the development of highly potent and selective transcriptional inhibitors has proven elusive. We demonstrate that combinations of transcriptional inhibitors of erbB2 expression and existing therapeutic agents that target erbB2 activity and lifetime lead to a synergistic increase in activity, with dose reductions as high as 30-fold as compared to individual agents. The synergy is selective for erbB2 overexpressing cancer cells. These results highlight the potential of a generalizable approach that will improve the utility of transcriptional inhibitors as both biochemical tools and potential therapeutics.



KEYWORDS: *Antitumor agents, gene expression, protein−protein interactions, synergy, transcription*

The aberrant function of a growing number of transcriptional activators is associated with the development and progression of human diseases such as cancer.[1−3] Molecules that interfere with the ability of transcriptional activators to control expression of their target genes thus have great promise as biochemical tools and therapeutics.[4−7] Activators regulate transcription through a complex network of interactions with transcriptional machinery proteins;[8,9] blocking these interactions inhibits transcription. However, the affinities of activators for their transcriptional machinery binding partners are modest (high nanomolar to low micromolar K_D values), and correspondingly, small and large molecule inhibitors of these interactions typically require micromolar concentrations to exert their effects, ultimately limiting their utility.[7,10−12] Additionally, the selectivity of molecules that interact with shared coactivators is a recurring cause for concern.[6] We previously[13−16] described the development of a new class of small molecule transcriptional inhibitors that mimic transcriptional activation domains; this includes **i1** (Figure 1), an isoxazolidine that mimics the activation domain of the transcription factor ESX and interferes with the transcription of the ESX-regulated oncogene erbB2 at micromolar concentrations. Here, we present a strategy that mitigates the potency and selectivity concerns of transcriptional inhibitors through a multipronged intervention against the ErbB2 regulatory pathway. The use of **i1** in tandem with other agents that target the activity and lifetime of the erbB2 oncoprotein leads to simultaneous dose reductions of greater than 15-fold for both agents and increased selectivity for erbB2+ cells up to 30-fold. This strategy should be readily applicable to other agents that disrupt the protein−protein interactions responsible for oncogene transcription.

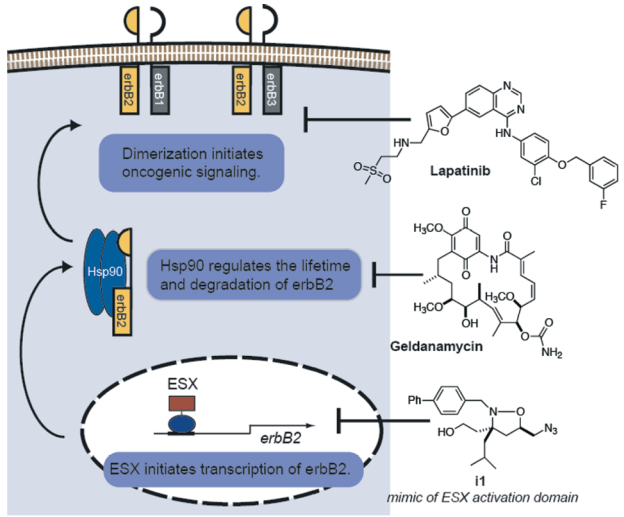


Figure 1. Schematic of the erbB2 pathway and points of small molecule intervention.[28,29,32] Combinations that inhibit both the transcription of erbB2 and the lifetime or activity of the mature protein have a synergistic increase in activity against erbB2-driven cancer cells. Drug combinations to synergistically target disorders ranging from cancer to inflammation have been identified in recent years. These combinations often have the benefit of reduced toxicity in addition to increased selectivity relative to the single agents. However, applications to inhibitors of protein−protein interactions and, in particular, the transient and poorly characterized protein−protein interactions that regulate transcription have not been reported.[25−27,30]

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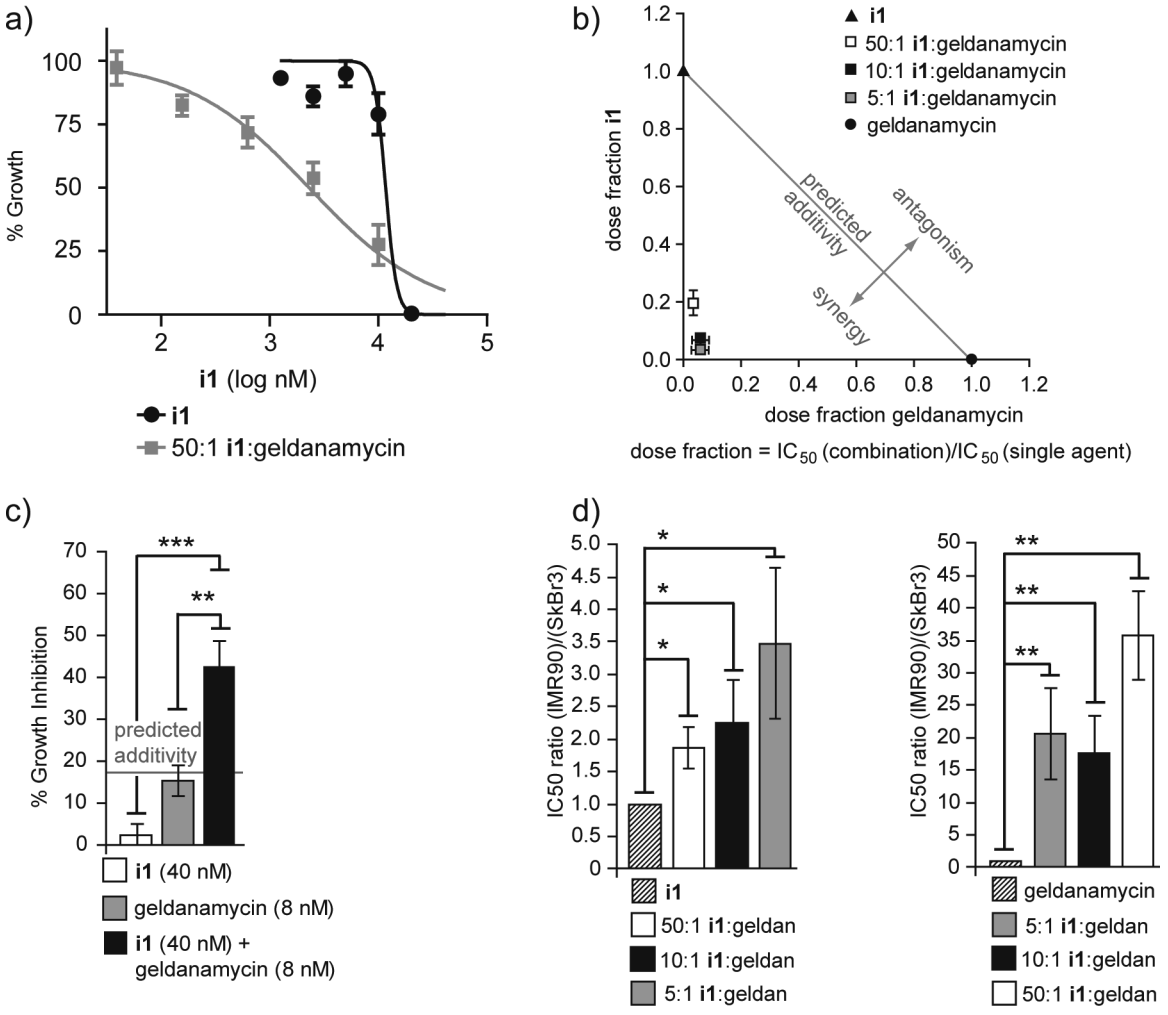

Figure 2. (a) Dose−effect curves for **i1** as a single agent and a 50:1 combination of **i1**:geldanamcyin after 3 days of dosing. (b) IC_{50} values of fixed dose ratios of **i1** and geldanamycin were measured in SKBR3 cells after 3 days of dosing and plotted on an isobologram. (c) The % effect (100 − % growth) for the indicated doses for the 3 day dosing period of a growth time course (Figures S1c,d in the Supporting Information). Predicted additivity was calculated as indicated in the Supporting Information. (d) The IC_{50} values from panel b were compared to IC_{50} values for the same combinations in IMR90 cells (Figure S2a−c in the Supporting Information), and the resulting ratios were plotted as shown, normalized to the effects of **i1** and geldanamycin as single agents. The difference between the ratios for 5:1 and 50:1 **i1**:geldanamcyin is not statistically significant ($p = 0.056$). Error bars indicate error compounded from one standard deviation of experiments performed in triplicate. For all other experiments, error bars indicate one standard deviation from experiments performed in triplicate unless noted otherwise. *P* values: * < 0.05, ** < 0.01, and *** < 0.001.

We chose erbB2 for this investigation because of its clinical relevance and because the complexities of its regulatory network make effective treatment with individual agents a challenge. The erbB2 protein is a trans-membrane tyrosine kinase that is overexpressed in approximately one-quarter of breast cancers,[17] where it has been shown to drive an aggressive phenotype marked by more rapid metastasis and shorter life expectancy than breast cancers that do not overexpress erbB2.[18,19] Furthermore, erbB2 overexpressing (erbB2+) cancer cells are known to undergo growth arrest and cell death if erbB2 expression is suppressed.[20] The clinical significance of erbB2 overexpression can be seen in the variety of existing treatments designed to suppress erbB2 signaling, including antibodies that target the protein's extracellular domain[21] and tyrosine kinase inhibitors, which target the protein's ability to trans-phosphorylate other members of the erbB family and initiate cell survival and proliferation programs (Figure 1).[22] These approaches have met with difficulty in clinical practice, but an increasing body of evidence suggests that although erbB2-driven cancers are adept at compensating for partial inhibition of erbB2 activity, they are still vulnerable to interventions that reduce erbB2 levels.[23,24]

The discovery of multicomponent therapeutics has emerged in recent years as an effective strategy for increasing efficacy and decreasing off-target effects relative to single agents in a number of cases.[25−27] We reasoned that it might be possible to obtain similar benefits by combining **i1** with other agents that target erbB2. One point of intervention along the erbB2 pathway is Hsp90, part of a chaperone complex that maintains erbB2 stability and assists in membrane localization.[28,29] The natural product geldanamycin reduces cellular erbB2 levels by binding to Hsp90 and inhibiting its function (Figure 1), but its toxicity prevents its use as a therapeutic agent.[28,29] Thus, our initial efforts tested the hypothesis that dual targeting of the erbB2 pathway with the two PPI inhibitors geldanamycin and **i1** would synergistically increase potency and specificity relative to the individual agents. As shown in Figure 2a, a 50:1 combination of **i1**:geldanamycin resulted in an IC_{50} in SkBr3 (erbB2+) cells that is >10-fold lower than **i1** alone. To test if the potency increase is truly synergistic, both the isobologram

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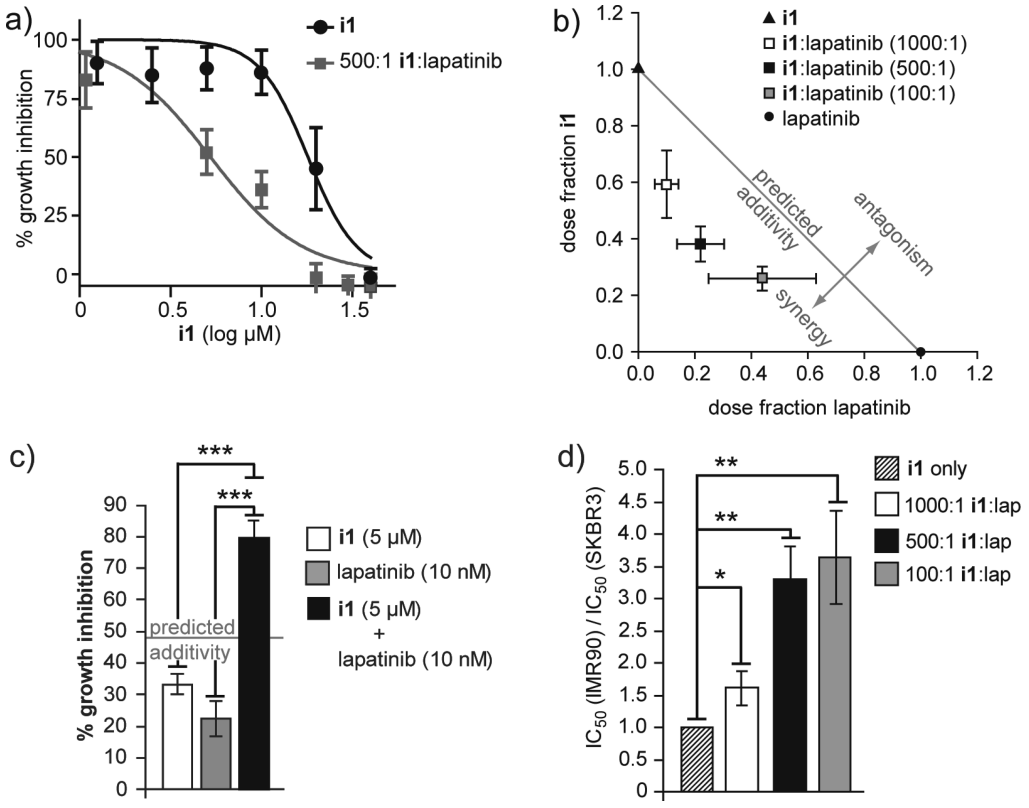

Figure 3. (a) Dose−effect curves for **i1** as a single agent, and a 500:1 combination of **i1**:lapatinib after 2 days of dosing. (b) IC_{50} values of fixed dose ratios of **i1** and lapatinib were measured in SKBR3 cells after 2 days of dosing and plotted on an isobologram. (c) The % effect (100 − % growth) for the indicated doses for the 3 day dosing period of a growth time course (Figures S3f,g in the Supporting Information). Predicted additivity was calculated as indicated in the Supporting Information. (d) The IC_{50} values from panel b were compared to the IC_{50} values for the same combinations in IMR90 cells (Figure S3e in the Supporting Information), and the resulting ratios were plotted as shown, normalized to the effects of **i1** and lapatinib as single agents. Error bars indicate the error compounded from one standard deviation of experiments performed in triplicate. For all other experiments, error bars indicate one standard deviation from experiments performed in triplicate unless noted otherwise. *P* values: * < 0.05, ** < 0.01, and *** < 0.001.

and the multiplicative additivity (Bliss) models were employed. For the former, the IC_{50} values of fixed ratios of **i1**:geldanamycin were measured and compared to a hypothetical case representing additivity, in which both components act as though they are the same agent (Figure 2b).[25,26,30] IC_{50} ratios (combination:single agent) that fall below the additivity line are indicative of *positive* synergy, and by this measure, the combinations of the two PPI inhibitors exhibit an impressive degree of synergy. The 5:1 **i1**:geldanamycin combination is synergistic as defined by the multiplicative additivity or Bliss model (Figure 2c).[25,26,30] This degree of synergy increased over longer growth times, indicating robust inhibition of proliferation from combination treatment (Figure S1b,c in the Supporting Information). In addition, the combination of geldanamycin and **i1** concomitantly produced an 85% drop in erbB2 levels (Figure S1a in the Supporting Information).

As outlined earlier, **i1** displays modest selectivity for erbB2+ cancer cell lines, and geldanamycin is broadly toxic.[29] However, combinations of **i1** and geldanamycin show increased selectivity for erbB2+ cancer cells when compared to nontumorigenic IMR90 cells, cells whose growth is not driven by erbB2 (Figure 2d). This is most notable in comparison with geldanamycin alone, where the combinations produce a 20−35-fold selectivity improvement. These results indicate that the synergy is erbB2-dependent and not a result of general toxicity. These data further suggest that transcriptional inhibitors can be used in combinations with agents that have broad activity to selectively effect specific shared targets.

We next examined the potential for synergy between **i1** and lapatinib, a reversible erbB2/erbB1 kinase inhibitor that is used clinically in the treatment of erbB2+ cancers (Figure 1).[31] An initial trial of a 500:1 ratio of **i1**:lapatanib produced a >10-fold decrease in the IC_{50} relative to **i1** alone in SkBr3 (Figure 3a). That this decrease was due to synergy was tested as before with via both the isobologram and the multiplicative additivity (Bliss) methods in SkBr3 cells (Figure 3b). The IC_{50} ratios of the **i1**:lapatinib combinations fell significantly below the additivity line, demonstrating a synergistic effect. Consistent with the impact on viability, **i1** and lapatinib had moderate effects on erbB2 and phosphorylated erbB2 levels as single agents. However, the **i1**:lapatinib combination was significantly ($p < 0.05$) more effective at reducing the total amount of active (phosphorylated) erbB2 than equivalent amounts of either **i1** or lapatinib (Figure S3a in the Supporting Information).

In addition to the increased potency, combinations of **i1** and lapatinib are less toxic to erbB2-negative, nontumorigenic IMR90 cells, leading to greater selectivity than the use of **i1** in isolation (Figure 3d). As an additional readout for synergy, we dosed colonies of SkBr3 cells with compound (5 μM **i1**, 10 nM lapatinib, or a combination of the two) for 9 days (Figure S3f in the Supporting Information). The combination treatment was much more effective than either **i1** or lapatinib in isolation or

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the multiplicative sum of the individual effects (Figure S3g in the Supporting Information). In contrast, combinations of **i1** and the erbB1 selective kinase inhibitor erlotinib[32] did not display significant synergy (Figure S4 in the Supporting Information). This result is consistent with models that implicate the erbB2/erbB3 dimer as the primary driver of oncogenesis.[23,24]

In conclusion, by using a small molecule combination that simultaneously curbs expression of the genetic driving force behind a diseased state while also targeting related cellular processes, we achieve a synergistic increase in effect that is specific to the target cell population. Although synergistic combinations of well-established drugs have recently emerged for improving efficacy,[27,30] this is the first time that synergistic interactions between two molecules, which target protein−protein interactions, have been used to overcome the activity and selectivity issues common to this class of molecules. In doing so, these data support a generalizable approach that will improve the utility of transcriptional inhibitors as both biochemical tools and potential therapeutics. As outlined earlier, **i1** displays modest selectivity for erbB2+ cancer cell lines, and geldanamycin is broadly toxic.[29] However, combinations of **i1** and geldanamycin show increased selectivity for erbB2+ cancer cells when compared to nontumorigenic IMR90 cells, cells whose growth is not driven by erbB2 (Figure 2d). This is most notable in comparison with geldanamycin alone, where the combinations produce a 20−35-fold selectivity improvement. These results indicate that the synergy is erbB2-dependent and not a result of general toxicity. These data further suggest that transcriptional inhibitors can be used in combinations with agents that have broad activity to selectively effect specific shared targets.

■ ASSOCIATED CONTENT

Supporting Information

Complete experimental details, description of the methods used to calculate synergy, and supporting Figures S1−S4. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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