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TITLE: Novel Anticancer Agents that Block Dissociation of Hsp90 from Estrogen Receptors in Breast Cancers

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14. ABSTRACT Heat shock protein 90 (Hsp90) is a chaperone protein that facilitates the folding of estrogen receptors (ERs) and other proteins involved in breast cancer proliferation. This protein is under investigation as a target of anticancer drugs because breast cancer cells contain Hsp90 in an activated high affinity conformation that is particularly susceptible to Hsp90 inhibitors. These inhibitors include the anticancer agent geldanamycin (GDM), which is thought to inhibit the proliferation of cancer cells in part by blocking agonist-induced release of steroid hormone receptors such as ERs from Hsp90. To further stabilize this interaction, we synthesized estrogen receptor ligands coupled to GDM to enforce heterodimerization of ER and Hsp90 in mammalian cells. We demonstrated that at a concentration of 10 micromolar, an estrone (E1)-GDM chimera can heterodimerize recombinant ER and Hsp90 proteins in vitro. Moreover, competition experiments established that this compound stabilizes interactions between ERs and Hsp90 in human cells. These studies demonstrated that bifunctional small molecules can affect ERs by heterodimerization with Hsp90, providing a new strategy for inhibiting ERs involved in breast cancer proliferation.					
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Introduction

Heat shock protein 90 (Hsp90) is an abundant ATP-dependent cellular chaperone that facilitates the folding of estrogen receptors (ERs) and other key oncogenic proteins involved in breast cancer proliferation.¹ This protein is under intense investigation as a target of anticancer drugs because breast cancer cells contain Hsp90 in an activated high affinity conformation that is particularly susceptible to Hsp90 inhibitors.¹ These inhibitors include the anticancer agents geldanamycin (GDM) and 17-allylaminogeldanamycin (17AAG), the latter of which is currently in clinical trials for the treatment of breast cancer. These drugs are thought to inhibit the proliferation of cancer cells in part by blocking agonist-induced release of steroid hormone receptors such as ERs from Hsp90. In an effort to specifically affect interactions between ERs and Hsp90, chimeric compounds comprising GDM linked to β -estradiol via short tethers containing seven carbon atoms were reported in 1999.² These chimeric compounds selectively induce degradation of ERs and the HER2 oncoprotein in MCF7 breast cancer cells.² However, due to the short tether used to link the protein ligands, these compounds do not stabilize the heterodimerization of ER and Hsp90 proteins.

My laboratory has been working on the design and synthesis of small molecules that heterodimerize ERs with target proteins in living cells. As a proof of concept, we reported in 2003³ and 2004⁴ the synthesis of small molecules that heterodimerize ERs and streptavidin (SA) in living yeast cells. Examination of an x-ray crystal structure of GDM bound to the ATP-binding pocket of Hsp90,⁵ enabled us to design similar compounds with the potential to actively stabilize the association of ER and Hsp90 proteins. We report here the synthesis and biological evaluation of these chimeric compounds as a new strategy for controlling the function of estrogen receptors involved in breast cancer proliferation.

Body

Based on X-ray crystal structures of ER β bound to the antiestrogen ICI164384⁶ and Hsp90 bound to geldanamycin,⁵ the chemical inducer of dimerization (CID) **1** was designed as shown in Figure 1. Molecular modeling indicated that the 18-atom linker between the ER ligand (an estrone oxime) and the Hsp90 ligand (geldanamycin) should enable compound-mediated heterodimerization of the two proteins without promoting unfavorable steric interactions. Compounds **2** and **3** were designed as negative controls designed to bind only Hsp90 (compound **2**) or ER (compound **3**) as a result of structural modifications known to decrease affinity for these proteins.^{7, 8}

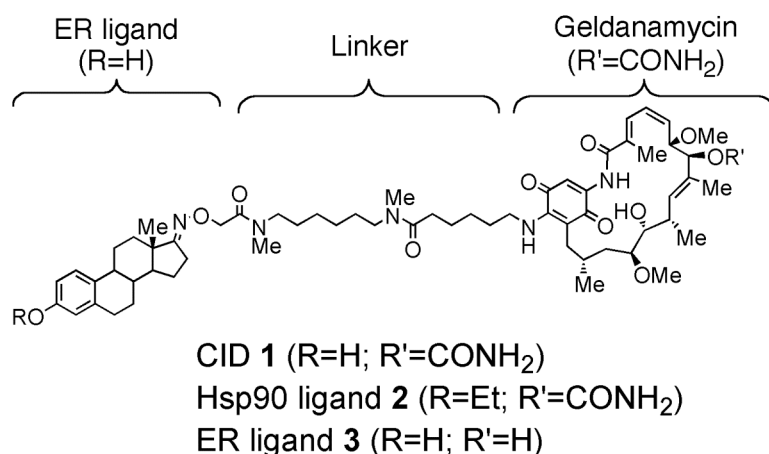


Figure 1. Structures of chimeric compounds. The chemical inducer of dimerization (CID) **1** comprises an ER ligand linked to geldanamycin. The control compound **2** was designed to bind Hsp90 but not ER. The control compound **3** was designed to bind ER but not Hsp90.

Compounds **1-3** were synthesized as shown in Figure 2. This route is similar to our previously reported synthesis of biotinylated estrone oximes.⁴ The geldanamycin used for synthesis was generously provided by the National Cancer Institute.

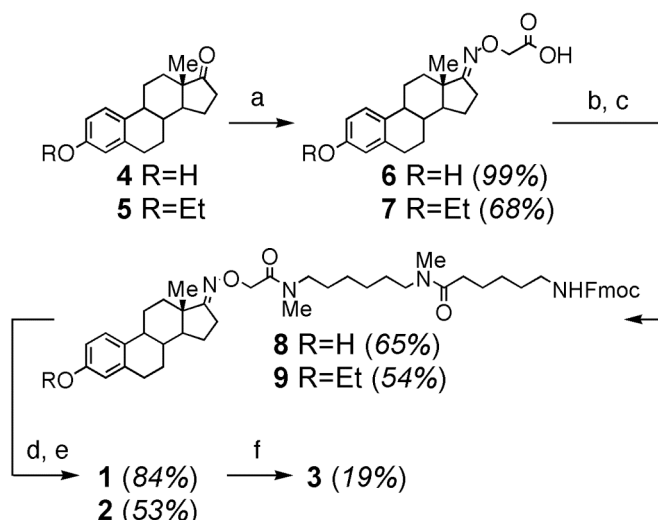


Figure 2. Synthesis of the estrone – geldanamycin CID **1** and related control compounds. Reagents and conditions: a) $\text{H}_2\text{NOCH}_2\text{COOH}$, pyridine. b) DCC, NHS, *N,N*-dimethyl-1,6-hexanediamine. c) DCC, NHS, *N*-Fmoc-6-aminohexanoic acid. d) Piperidine, DMF. e) Geldanamycin, DMSO. f) *t*-BuOK, DMSO.

The effects of compounds **1-3** on heterodimerization of ER α and Hsp90 β proteins were initially assessed in vitro using co-immunoprecipitation experiments. As shown in Figure 3, addition of a rabbit polyclonal IgG against Hsp90 β strongly immunoprecipitated this protein and ER α only in the presence of compound **1** (10 μM). Heterodimerization of the two proteins mediated by compound **1** was blocked upon addition of β -estradiol or geldanamycin, demonstrating specificity of molecular recognition.

To assess the effect of compounds on protein subcellular localization, mammalian expression vectors were constructed encoding green fluorescent ER α -AcGFP red fluorescent mRFP-Hsp90 β . These plasmids were cotransfected into human SKN cells and cellular fluorescence was imaged by confocal laser scanning microscopy. As shown in Figure 4, these experiments revealed that cells overexpressing Hsp90 β showed nuclear-localized ER α that did not colocalize with Hsp90 β . In contrast, treatment with compound **1** resulted in cytoplasmic ER α that colocalized with cytoplasmic Hsp90 β . This pattern of colocalization was also observed upon treatment with geldanamycin (data not shown). Addition of β -estradiol to cells treated with **1** resulted in relocalization of ER α to the nucleus of cells overexpressing Hsp90. This relocalization was not observed in cells treated with geldanamycin alone (data not shown). These results provide strong evidence that compound **1** is capable of heterodimerizing ER α and Hsp90 β through a novel mechanism in living human cells.

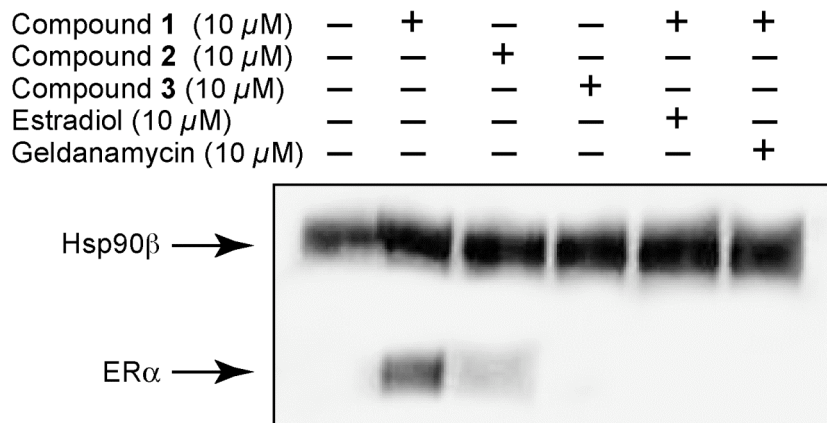


Figure 3. Analysis of heterodimerization of ER and Hsp90 proteins mediated by **1** in vitro. ER α (100 nM, 1.3 μ g), Hsp90 β (100 nM, 1.8 μ g), anti-Hsp90 IgG (0.95 μ g), and bovine serum albumin (2 μ g) were equilibrated with other compounds shown in a total volume of 20 μ L for 4 h at 4 $^{\circ}$ C. Protein A-conjugated agarose was added to immunoprecipitate the Hsp90-bound ER. The agarose was washed to remove unbound protein, bound proteins were eluted by thermal denaturation, and immunoprecipitated proteins were analyzed by SDS PAGE.

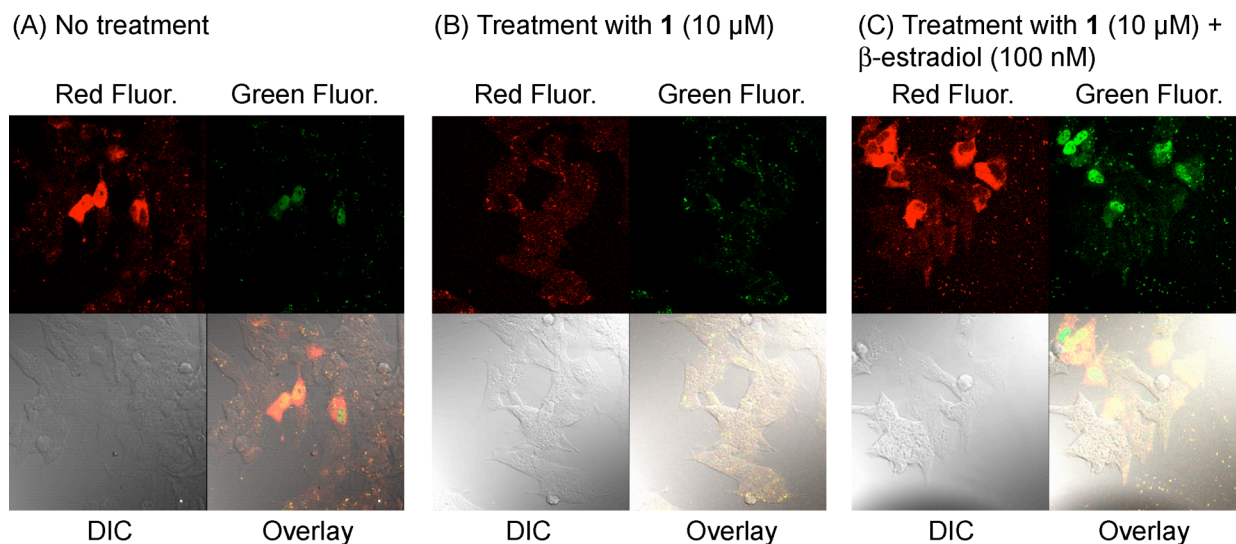


Figure 4. Effects of compounds on the subcellular localization of green fluorescent ER α -AcGFP and red fluorescent mRFP-Hsp90 β in transiently transfected human SKN cells.

To assess effects of compound **1** on ER-mediated reporter gene expression, SKN cells were cotransfected with ER α and a luciferase reporter gene. As shown in Figure 5, these experiments revealed that compound **1** is a partial agonist capable of both partially activating and partially inhibiting ER-mediated gene expression.

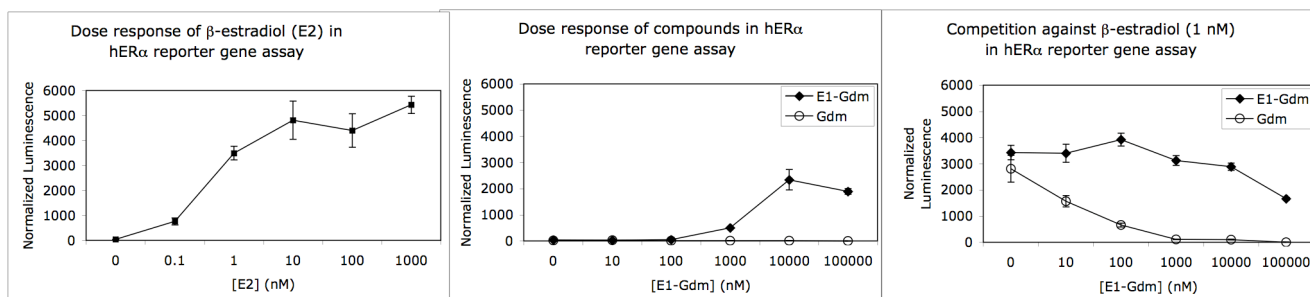


Figure 5. Effects of compounds on ER-mediated reporter gene expression. Left: Activation of ER-mediated reporter gene expression by β -estradiol. Middle: Activation of ER-mediated reporter gene expression by geldanamycin (Gdm) and CID **1** (E1-Gdm). Right: Inhibition of ER-mediated reporter gene expression by CID **1** (E1-Gdm) and geldanamycin (Gdm).

Key Research Accomplishments

Key research accomplishments include: (1) synthesis of a small molecule that heterodimerizes ERs and Hsp90 proteins in vitro and in living cells. (2) Demonstration that this compound partially inhibits ER-mediated reporter gene expression.

Reportable Outcomes

These results were presented as a poster:

Mottram, L. F, MacBride M. M., and Peterson, B. R. "Novel Anticancer Agents that Block Dissociation of Hsp90 from Estrogen Receptors in Breast Cancers" *Era of Hope, Department of Defense Breast Cancer Research Meeting - Philadelphia, PA (6/8/05-6/11/05)*

These results will be submitted for publication in the near future.

Conclusions

We proved the concept that small molecules can heterodimerize estrogen receptors and Hsp90 proteins in living cells. Future studies using higher affinity ER antagonists linked to geldanamycin have the potential to provide to improved anti-breast cancer agents.

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Appendices - None