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Synthesis and preliminary evaluation steroidal antiestrogen–geldanamycin conjugates

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ABSTRACT

Three novel steroidal antiestrogen–geldanamycin conjugates were prepared using a convergent strategy. The antiestrogenic component utilized the 11β-(4-functionalized-oxyphenyl) estradiol scaffold, while the geldanamycin component was derived by replacement of the 17-methoxy group with an appropriately functionalized amine. Ligation was achieved in high yield using azide alkyne cyclization reactions. Evaluation of the products against two breast cancer cell lines indicated that the conjugates retained significant antiproliferative activity.

Introduction

Breast cancer is the most prevalent form of cancer in women and the well-established association between the human estrogen receptor (ER) and cell proliferation provided the basis for endocrine (antihormonal) therapy.[1,2] However, prolonged treatment with antiestrogens often results in the development of hormonal resistance, leading to recurrence of the disease and the use of more potent, but nonselective, therapeutic agents.[3–5] One strategy that attempts to circumvent the effects of resistance is the use of drug conjugates in which two therapeutic agents are combined into a single entity.[6–8]

As part of our program in breast cancer research, we have focused on using ER as a targeting mechanism for which the steroidal anti-estrogenic component may also provide a beneficial therapeutic response. The choice of the therapeutic component is also critical as it should not only be active within the same concentration range as the hormonal component but exert a complementary or synergistic effect. The ER-targeting component was developed in our initial work with the 11β-(4-substituted-oxyphenyl) estradiols.[9,10] Based on the affinity of the steroids for the ER and their antiestrogenic activity, we prepared a steroidal antiestrogen–mitomycin C conjugate to test our concept.[11] Although the compound retained high ER affinity and antiestrogenic properties, it was no more active than mitomycin C and displayed no selectivity toward ER-expressing breast cancer cells. One possible explanation for the lack of synergy may have involved the properties of the linker. Unfortunately, issues regarding the availability mitomycin C precluded further studies with this conjugate. Therefore we elected to evaluate the effect of linker length and conformational flexibility using the Hsp90 N-terminal inhibitor, geldanamycin (GDA), as the therapeutic component (Fig. 1).

Heat shock proteins (HSP) are molecular chaperones that are critical for the maintenance of cellular homeostasis through regulation of protein transport, conformational folding and maturation.[12] Hsp90 is a 90 kDa protein that is often overexpressed in breast cancer, as well as other cancers, and, as a result of these increased levels, is responsible for maintaining high levels of active oncogenic proteins.[13–15] One of these proteins is ERα which, when dormant, is confined to the nucleus in an Hsp90 complex.[16] Disruption of the Hsp90-ERα complex leads to improper folding of ERα and its subsequent degradation, resulting in down-regulation of its corresponding pathways, such as transcription. Therefore, disruption of Hsp90-mediated responses provides an alternative target for breast cancer therapy, and has led to the use of geldanamycin (GDA) and its derivatives as therapeutic agents.

The geldanamycin component was developed based upon our work with chaperone inhibiting agents. Structure–activity relationship studies demonstrated that modification at the 17-position not only generates GDA derivatives that exhibit reduced toxicity, but this position is also substituent tolerant as groups at this position of GDA exit the Hsp90 binding pocket and thus do not significantly affect inhibitory activity.[17] Other 17-GDA derivatives have been synthesized that exhibit improved solubility and lower toxicity than GDA, but are still hepatotoxic.[18,19] Therefore we planned to

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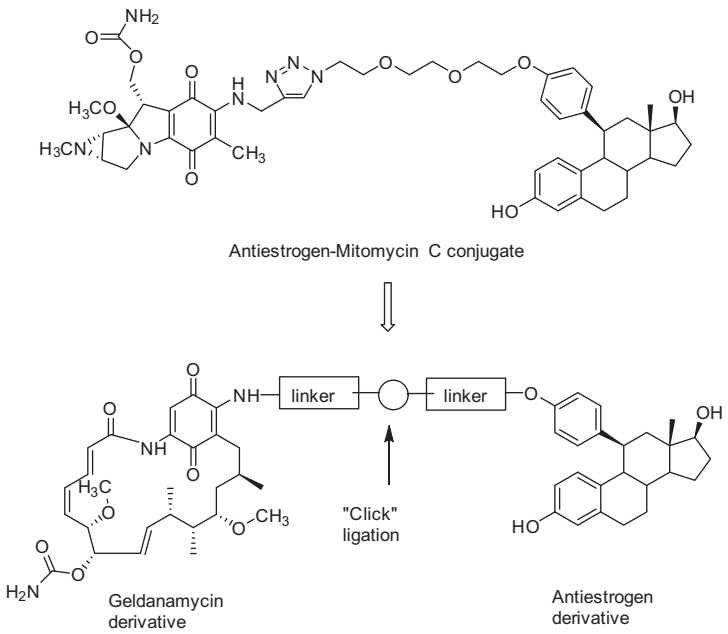


Figure 1. Proposed extension of research from the antiestrogen–mitomycin C conjugate to the antiestrogen–geldanamycin conjugates.

introduce modifications at the 17-postion that will permit conjugation to the steroidal derivatives.

We chose a convergent approach in which each component contained a side chain that is terminally substituted with a reactive functionality. The final step then involves a ligation reaction under mild conditions. The reaction selected for this study was the Huisgen [3+2] cycloaddition reaction between a terminal azide and a terminal alkyne to generate a chemically stable triazole moiety.[20–22] The reaction has the advantage of being chemoselective and allowing the reactive groups to reside on either component. In this study we chose to use different lengths of the linker to investigate what effect, if any, it exerts on the biological activity of the final conjugate. The overall synthetic strategy for our conjugates is shown in Figure 2.

Results

The synthesis of the steroidal antiestrogen component was accomplished using a strategy similar to one described for our 11β-(4-substituted oxyphenyl) estradiols.[9,10] Scheme 1 deltenone 3-ethylene ketal **1** was converted initially to the 11β-(4-hydroxyphenyl) estra-4,9-diene-3,17-dione **2**. This compound then served as the intermediate for the preparation of the requisite 11β-(4-azidoethoxyphenyl)estradiol **4a** and 11β-(4-*N*-propargyl-*N*-methylaminoethoxyphenyl) estradiol components **4b**. For the propargyl derivative, we prepared the 2-(*N*-propargyl-*N*-methylamino)ethanol which was then coupled to the 11β-(4-hydroxyphenyl) estra-4,9-diene-3,17-dione **2** using the Mitsunobu reaction to give **3b**. Aromatization with acetic anhydride-acetyl bromide followed by reduction–saponification gave the desired product **4b**. Overall yields for the two compounds were 28% (eight steps) and 19% (seven steps), respectively. We had previously characterized the azido derivative **4a**, determined its binding affinity (RBA = 39%) and showed that it was a full antagonist of ERα. The *N*-propargyl-*N*-methyl derivative **4b** is a close analog of the RU39411 for which we had determined ER affinity (RBA = 39%) and efficacy (full antagonism). Having demonstrated that additional substituents distal to the nitrogen in the side chain did not adversely affect either binding

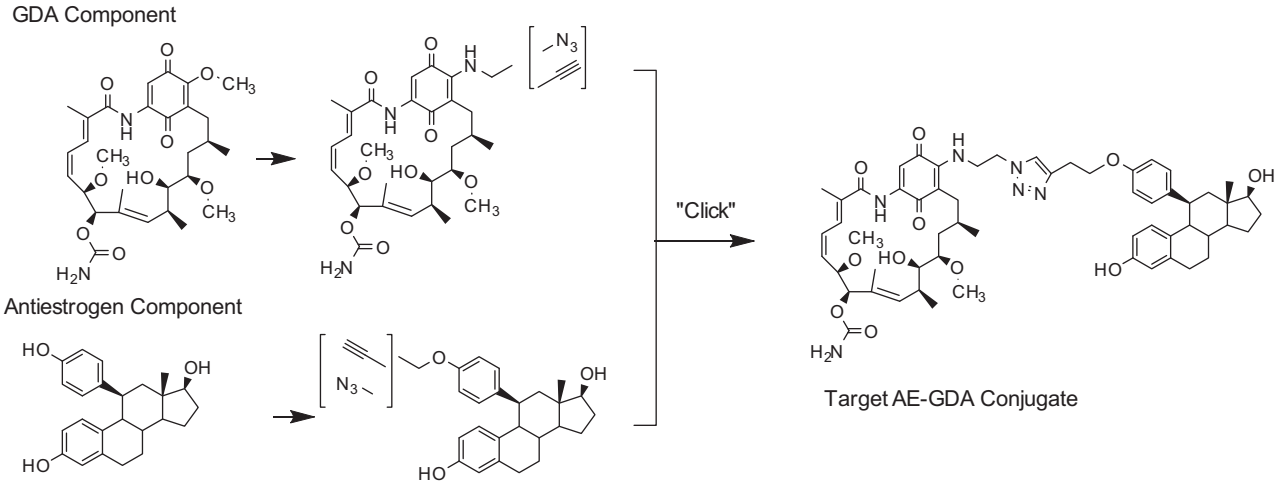


Figure 2. Approach for synthesis of individual components and assembly as AE–GDA conjugates.

or efficacy, we felt that the steroidal components were appropriate substrates for subsequent ligation reactions[10] (see Schemes 1–3).

The geldanamycin components were prepared using variations of methods previously described for 17-amino derivatives.[15,23] Scheme 2 treatment of geldanamycin **5** with either propargyl amine or ώ-azido pentaethylene glycol amine in dichloromethane gave the corresponding 17-amino geldanamycin components **6a** and **6b** in 80% and 68% yields, respectively. For the third geldanamycin component, a two step procedure was used, similar to that employed in our previous preparation of the biotinylated derivative. Geldanamycin **5** was initially treated with a fivefold excess of 1,5-pentanediamine in dichloromethane. Purification by column chromatography gave the 17-(5-aminopentyl)amino geldanamycin **6c** in a 95% yield. Bertozzi's difluoro-cyclooctyne carboxylic acid[24] was converted to the corresponding acyl chloride and immediately reacted with 17-(5-aminopentyl)amino geldanamycin **6c** to form the corresponding amide **6d**. The product was isolated in a 42% yield following column chromatography.

Ligation to form the final antiestrogen–geldanamycin conjugates used two versions of the "click" reaction. Scheme 3 in the conventional version, we used the 17-propargylamino geldanamycin **6a** and the 11β-(4-azidoethoxyphenyl) estradiol **4a** as coupling partners to give the 1,2,3-triazole conjugate **7a** with a short linker in a 46% isolated yield. Coupling the 17-(azidopentaethylene glycolamino) geldanamycin **6b** with 11β-(4-*N*-propargyl-*N*-methylaminoethoxyphenyl) estradiol **4b** under the same conditions gave the triazole conjugate **7b** with a longer linker in 47% isolated yield. The third conjugate was prepared from the cyclooctynylated amino geldanamycin **6d** and 11β-(4-azidoethoxyphenyl) estradiol **4a** in which the copperless-method gave the corresponding annulated triazole **7c** in a 73% isolated yield.

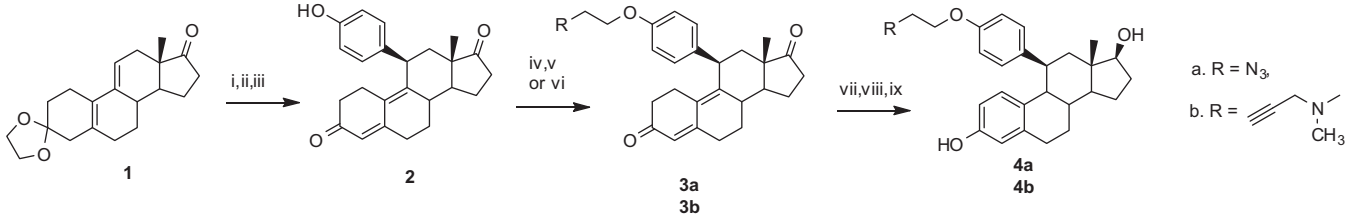
The three new conjugates and geldanamycin were evaluated for antiproliferative activity against MCF-7 and SKBr3 breast cancer cell lines (Table 1). In this assay, the antiproliferative activity of geldanamycin **5** in the two cell lines was determined to be 9.8 and 8.5 nM, respectively. Conjugate **7a** with the shortest linker group manifested an IC_{50} of 1150 ± 90 nM in MCF-7 and 710 ± 160 nM in SKBr3 cells. Conjugate **7b** with the longer linker was more potent with IC_{50} values of 102 ± 4.6 nM and 41 ± 4.6 in the respective cell lines. Conjugate **7c** that incorporated the bulkier Bertozzi linker had an IC_{50} value of 15200 ± 3000 nM in MCF-7 cells and was not therefore evaluated in the SKBr3 cell line. The results indicated that while all of the new conjugates retained significant antiproliferative activity, however, the potency was clearly modulated by the additional linker and antiestrogen components.

The objectives of this study were to evaluate the effects of the linker on the antiproliferative activity of the antiestrogen-drug conjugate. We had observed in our initial study with a antiestrogen–mitomycin C conjugate that a long, linear oligoethylene glycol linker retained high ER binding affinity (RBA = 7%), similar to the effects observed previously by Essigmann and co-workers with their 7α-derivatives.[25] In that study, the antiproliferative activity of the conjugate was comparable to that of the parent mitomycin C.[11] In this study, the two conjugates **7a** and **7b** having the least sterically constrained linkers were also the most potent compounds. The conjugate **7c**, having the cyclooctyl triazole closest to the 11β position of estradiol was most likely to produce significant steric interactions with the estrogen receptor which would compromise the targeting toward ER-expressing cells. The results suggest that the accessibility of the antiestrogenic component for the target membrane ER may influence the overall potency. The least sterically demanding conjugate **7b** is an order of magnitude more potent than the conjugate with the shorter linker **7a** which is an order of magnitude more potent than the sterically compromised conjugate **7c**.

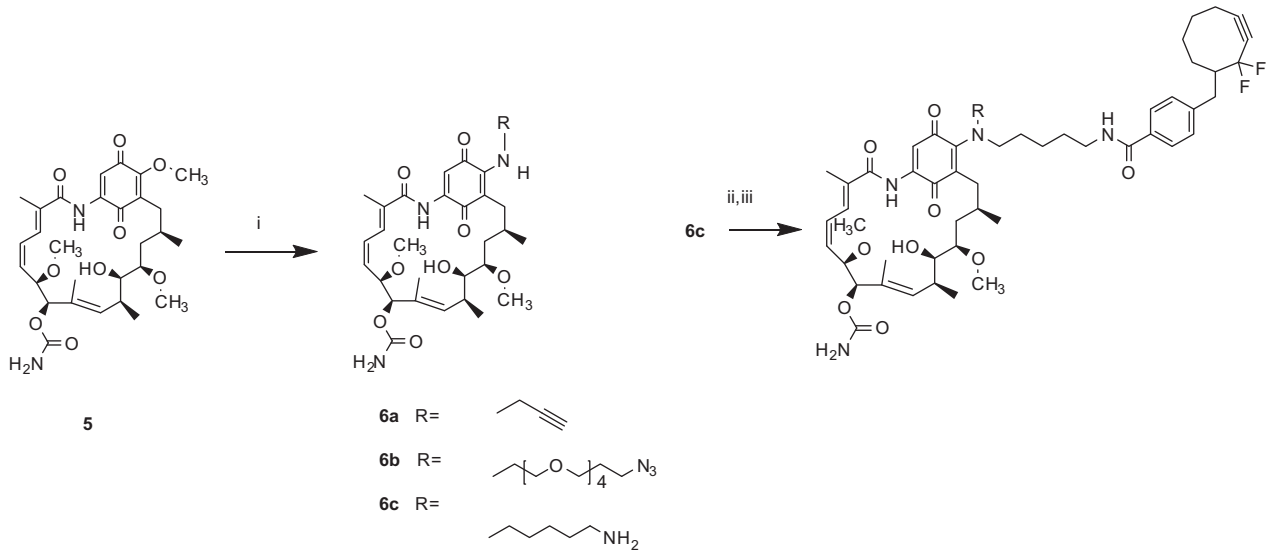
The linker component may also affect the therapeutic activity. Previous studies indicated that geldanamycin forms a stable complex with Hsp90 via a complex set of interactions that are modulated by substituents at the 17-position. With the 17-amino-17-desmethoxy derivatives, the exit site for this group corresponds to the heteroatom and therefore the length of the group would be expected to affect the biological response. In this study, two conjugates **7a** and **7b** display sub-micromolar activity against ER-expressing cells, although, both compounds are more active against the SKBr3 breast cancer cells that do not express ER. In those cells, conjugate **7b**, having the longer linker, while less potent than geldanamycin alone, is more than an order of magnitude more potent than **7a**, the conjugate with the shorter linker. Activity of the more complex conjugate **7c** was not determined, but the results suggest that the longer, more conformationally flexible linkers are favored at the 17-position.

The results suggest that ER-targeting was not the major factor underlying the biological effectiveness of the conjugates. If ER-targeting were the major component, one would expect thet cytotoxicity to be greater in MCF-7 cells as opposed to the SKBr3 cells. This response pattern was observed with our steroidal antiestrogen–mitomycin C conjugate in which ER-based selectivity was not achieved, even though the ER binding affinity for the conjugate was relatively high.[25] For the two most active conjugates **7a** and **7b**, activity was greater in the SKBr3 cells than in the MCF-7, a pattern that was similar to geldanamycin alone. Therefore it appears that the overall antiproliferative responses were modulated by the presence of the steroidal components, but did not enhance the overall effect compared to geldanamycin. It should be noted that the desired response pattern was observed for our doxorubicin–antiestrogen conjugate that we recently described in which MCF-7 antiproliferative activity was enhanced compared to doxorubicin alone and almost sevenfold greater than that observed in MDA-MB-231 cells which are ER-negative.[26]

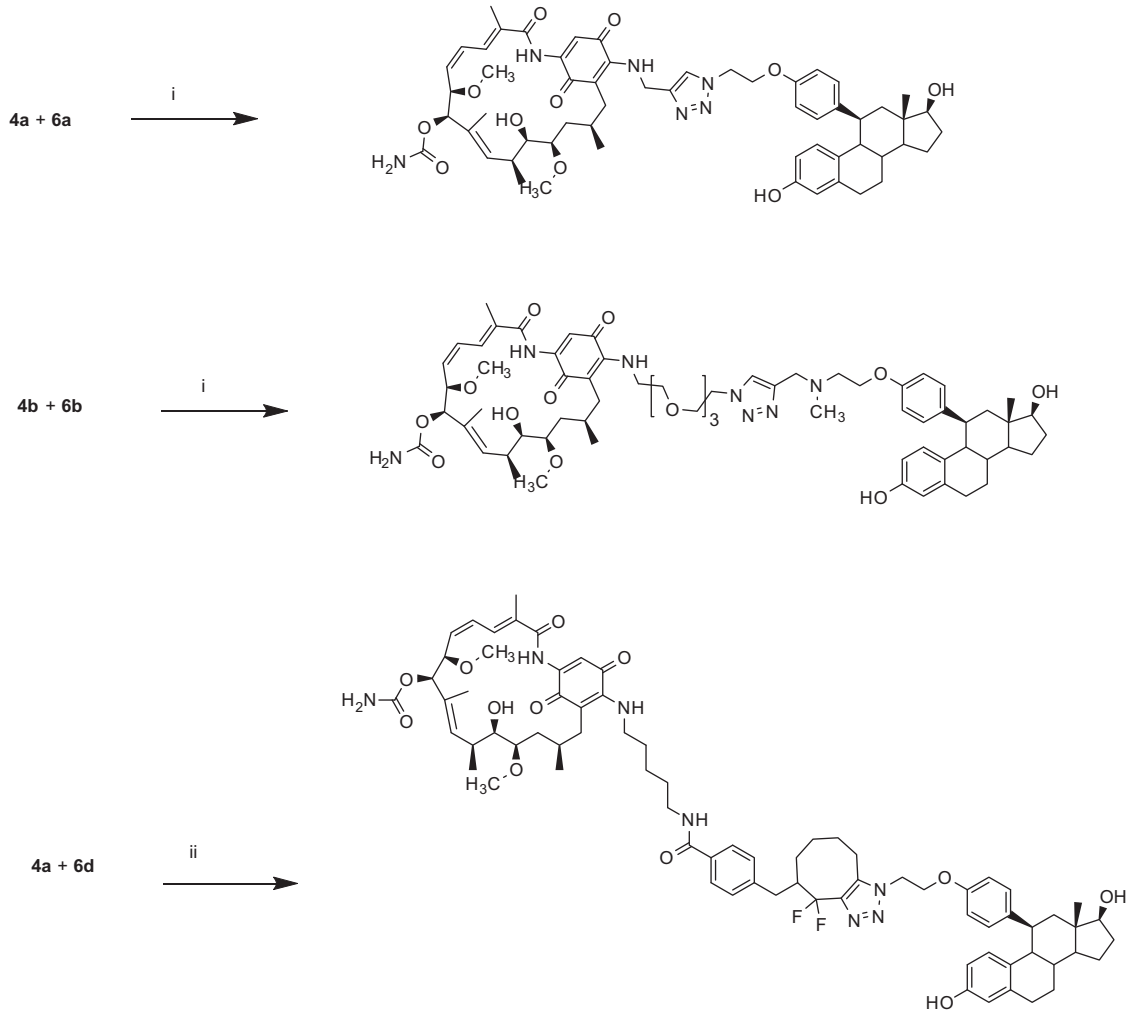
One of the significant differences between our doxorubicin–antiestrogen conjugate and the current series of geldamycin conjugates is that the former contain a component that allows the drug to dissociate within cancer cells. As with the mitomycin C conjugate, the synthetic strategy used in this study did not incorporate



Scheme 1. Synthesis of steroidal antiestrogen component. Reagents and conditions: (a) $CF_3COCF_3{\cdot}H_2O$, H_2O_2 (50%), C_5H_5N, CH_2Cl_2, 0 °C, 18 h; (b) $TMSiOC_6H_4MgBr$, CuI, THF; 16 h; (c) HOAc–H_2O (7:3), 1.5 h; (d) $TsOCH_2CH_2OTs$, Cs_2CO_3, CH_3CN, 13 h; (e) NaN_3, EtOH, 4 h; (f) $(HCCHCH_2)(CH_3)NCH_2CH_2OH$, DEAD, PS-$PPh_3$, CH_3CN, 16 h; (g) Ac_2O, AcBr, CH_2Cl_2, 16 h; (h) $NaBH_4$, MeOH, 1 h; (i) NaOH, MeOH, 16 h.



Scheme 2. Synthesis of geldanamycin components. Reagents and conditions: (a) amine, CH_2Cl_2, rt, 24 h; (b) substituted benzoic acid, $SOCl_2$, toluene, 70 °C, 2 h; (c) CH_2Cl_2, TEA, 0 °C–rt, 2 h.



Scheme 3. Ligation of steroidal antiestrogen and geldanamycin components using 'click' chemistry. Reagents and conditions: (a) $CuSO_4$–5·H_2O, sodium ascorbate, *t*-BuOH–H_2O, rt, 18–70 h; (b) *t*-BuOH–H_2O, rt, 24 h.

Table 1
Anti-proliferation activity of ateroidal antiestrogen–geldanamycin (AE–GDA) conjugates **7a–7c**

Compd	MCF-7 (IC_{50})	SKBr3 (IC_{50})
5 (**GDA**)	9.8 ± 0.1[a] nM	8.5 ± 1.1[a] nM
7a	1150 ± 90 nM	710 ± 160 nM
7b	102 ± 4.6 nM	41 ± 4.6 nM
7c	15200 ± 3000 nM	N.D.

IC_{50} = concentration needed to produce 50% inhibition.
[a] Ref. 15. ND = not determined.

that property. It is possible that for these conjugates that cellular uptake may be mediated via the membrane estrogen receptor but that effective intracellular distribution requires dissociation of the therapeutic component from the antiestrogen targeting group. Continued association with the antiestrogen component may reduce the effectiveness of the drug from accessing its site of action, even if elevated intracellular concentrations are obtained. Oligoethylene glycol linkes, such as those used in **7b** and the doxorubicin–anitestrogen conjugate, may also contribute physicochemical properties that enhance cellular uptake. Because of the potent antiproliferative activity observed for **7b,** incorpoaration of a linker that can impart both properties may generate the desired biological effect.

In conclusion, we have described a convergent strategy for the preparation of a novel series of novel steroidal antiestrogen-drug conjugates. This approach has distinct advantages in preparing and evaluating combinations of targeting groups, therapeutic drugs and linkers. The conjugates in this study were obtained in good overall yields and demonstrated significant activity against two breast cancer cell lines. Although one of the compounds (**7b**) demonstrated significant antiproliferative activity, it did not, however, demonstrate enhanced potency compared to the parent drug or selectivity for ER-expressing cells as compared to non-expressing cells. The results suggest that further modifications in both ER-targeting strategies and linking groups are needed in order to achieve greater potency and selectivity in therapeutic drug delivery. The effects of different linkers on both ER binding and Hsp90 warrant further evaluation as well. Those studies are in progress and will be described in future publications.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.03.116.

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