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Biotinylated Geldanamycin

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Inhibition of the 90 kDa heat shock proteins (Hsp90) represents a promising new chemotherapeutic approach for the treatment of several cancers. Hsp90 is essential to the survival of cancer cells and is inhibited by members of the ansamycin family of antibiotics. In particular, the quinone-containing antibiotics geldanamycin (GDA) and herbimycin A inhibit Hsp90 function in vitro at low micromolar concentrations via interaction with an ATP binding domain. Many proteins bind ATP, and the discovery of selective Hsp90 inhibitors requires the identification of other proteins that bind GDA and may cause undesired effects. Biotinylated analogues of GDA with varying tether lengths have been synthesized to elucidate other proteins that competitively bind GDA. Analogues containing a photolabile tether have also been prepared as a complementary method for the removal of GDA-bound proteins from neutravidin-containing resin. Preliminary studies indicate several proteins other than Hsp90 are isolated with biotinylated GDA.

Introduction

Heat shock proteins (Hsp's) are molecular chaperones responsible for protein transport, conformational activation, disaggregation, and maturation of nascent polypeptides.[1] Recent studies have shown that a large number of disease states occur as a consequence of Hsp function.[2] The 90 kDa heat shock proteins (Hsp90) are overexpressed in cancer cells, and these increased levels are essential for maintaining high intracellular concentrations of active oncogenic proteins, including Raf-1, v-Src, steroid hormone receptors, and many others.[3] In fact, proteins involved in all six hallmarks of cancer are dependent upon Hsp90 for their conformational maturation.[4] Consequently, Hsp90 inhibition represents a promising new approach to cancer chemotherapeutic development by the simultaneous inhibition of multiple oncogenic targets.[5]

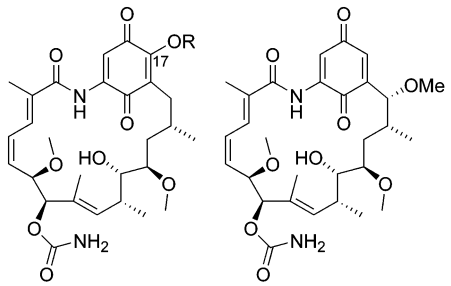

FIGURE 1. Inhibitors of Hsp90.

Originally geldanamycin (GDA, **1**, Figure 1) was believed to be an inhibitor of Src kinase,[6] but later studies by Whitesell and Neckers demonstrated that GDA binds to Hsp90.[7] In their studies, they prepared affigel-10 covalently bound to GDA and were successfully able to isolate Hsp90 by affinity purification. At the time, other proteins were also observed, but the identity of those proteins remained unknown. Subsequent studies have shown Src kinase to be an Hsp90-dependent client protein and that inhibition of Hsp90 leads to drastic reduction in Src kinase activity.[8] GDA and herbimycin A (**3**, Figure 1) inhibit Hsp90's inherent ATPase activity

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in vitro at low micromolar concentrations.[9] However, in vivo GDA is a 100 nM inhibitor of the Hsp90 multiprotein complex.[10] The poor bioavailability and high cytotoxicity of GDA has led to the development of 17-allylamino geldanamycin (17-AAG, **2**), which recently entered Phase I clinical trials for the treatment of several cancers.[9a,11] Although better tolerated, 17-AAG exhibits toxicity unrelated to Hsp90 inhibition and has formulation difficulties.[4,12]

GDA and 17-AAG inhibit Hsp90 by competing with ATP binding to a highly conserved nucleotide binding site located near the N-terminus of the homodimeric protein.[13] Unlike most ATP binding sites, the N-terminal ATP binding site has a unique bent conformation, requiring both ATP and GDA to adopt a folded or puckered shape upon binding to Hsp90, as determined by cocrystal structures.[13,14] Although this bent shape may lead to some selectivity of GDA for Hsp90 versus other ATP binding proteins, it is likely that GDA shares a high affinity for proteins other than Hsp90, as a consequence of normal evolutionary processes.[15] Furthermore, the bent conformation of GDA differs significantly from its native crystallographic form.[14] It has been suggested that this change in conformation results in an affinity of GDA for Hsp90 lower than that of other Hsp90 inhibitors, which have a predisposed bent conformation.[14,16] The flexibility observed in the GDA macrocycle is likely to allow GDA to bind other ATP-dependent proteins in a similar fashion. The toxicity of GDA and analogues unrelated to Hsp90 inhibition may be the result of binding to similarly shaped ATP binding motifs. The design of analogues with selective affinity for Hsp90 requires the identification of other proteins that bind GDA as a control for the design of future Hsp90 inhibitors.

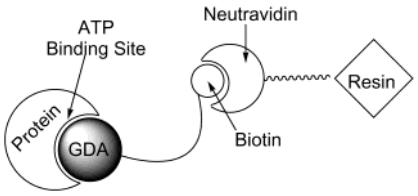
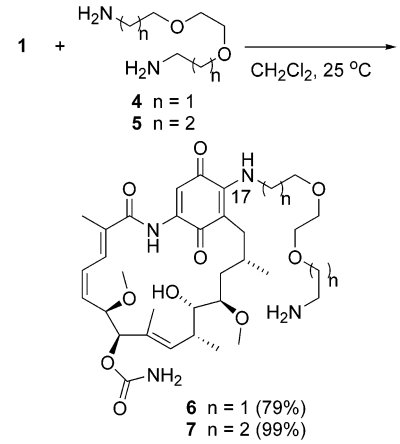


FIGURE 2. Affinity purification of GDA-binding proteins.

In an effort to identify GDA-binding proteins, biotinylated derivatives of GDA have been prepared for affinity purification of these proteins. Addition of neutravidin-containing resin enables the isolation of GDA binding proteins (Figure 2).

Examination of the cocrystal structure of GDA bound to both bovine[13a] and yeast[14a] Hsp90 revealed that both the free hydroxyl and carbamate group reside deep within the ATP binding site and thus were not suitable moieties for the incorporation of biotinylated linkers. Methoxy quinones undergo nucleophilic substitution reactions,[9c,d,18] and provide an alternative method for biotin introduction. The cocrystal structure of GDA bound to Hsp90 shows the quinone moiety to reside near the protein−solution interface of the nucleotide binding domain, with the methoxy group directed away from the interior of the protein. Replacement of the methoxy group with an appropriate tether[19] provides a solvent exposed biotin

SCHEME 1. Synthesis of GDA Amines



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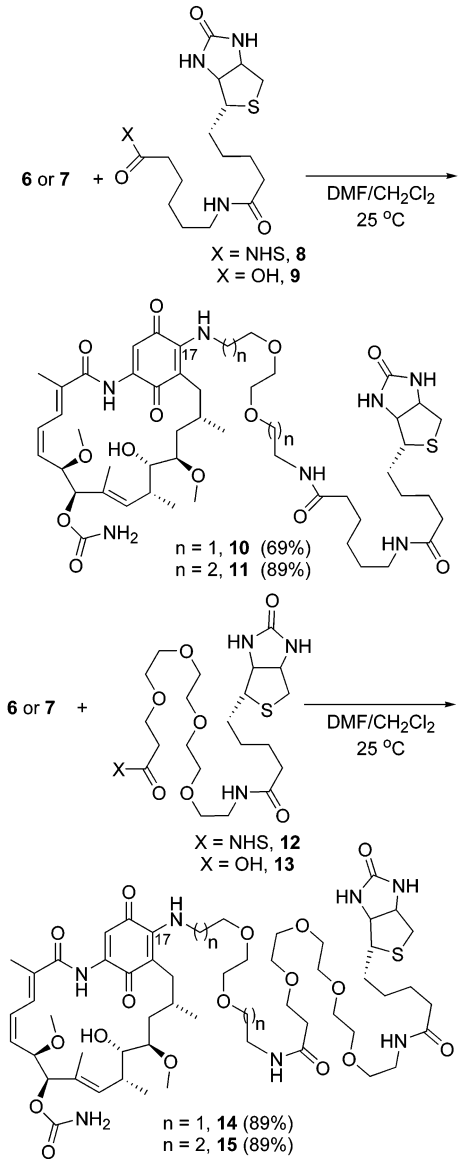
SCHEME 2. Synthesis of Biotinylated GDA

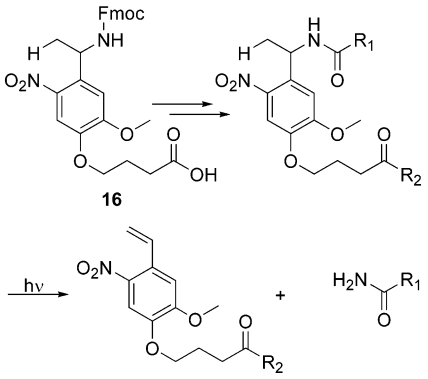


handle capable of interacting with neutravidin for affinity purification. The incorporation of a photolabile group in the GDA-biotin tether provided an alternative method for removal of GDA binding proteins from the immobilized complex. SDS−PAGE followed by peptide sequencing serves to isolate and identify proteins other than Hsp90 that bind GDA. Herein we describe the synthesis of four photolabile and four nonphotolabile derivatives of biotinylated GDA along with evidence supporting the isolation of proteins other than Hsp90 with biotinylated GDA.

Results and Discussion

Synthesis of Biotinylated GDA Derivatives. Methoxy quinones undergo rapid Michael addition and β-elimination with primary amines to furnish the corresponding vinylagous amide products.[18] Consequently, GDA was treated with bis(alkylamino) ethyleneglycols **4** and **5** to afford **6** and **7**, respectively (Scheme 1). Ten equivalents of the diamine were added to a yellow solution of GDA. The methoxy quinone was converted to

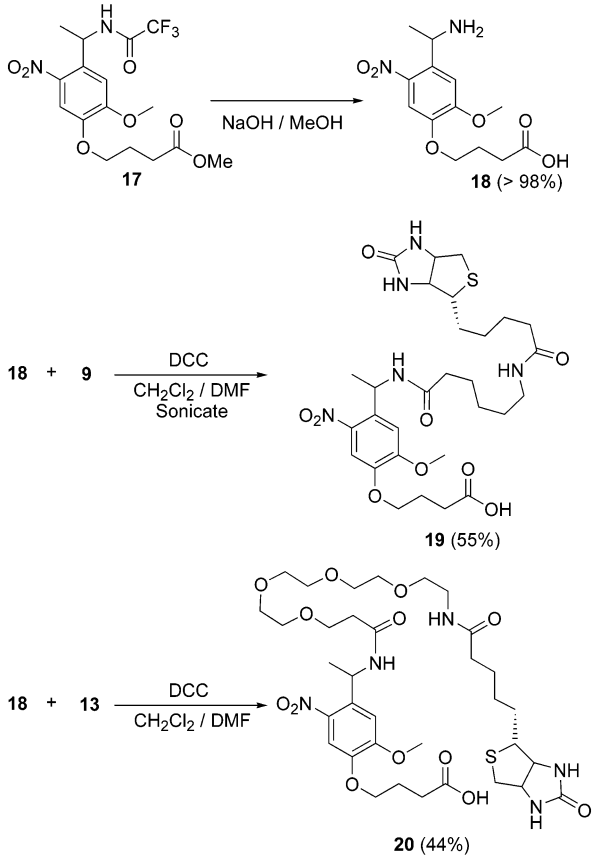
SCHEME 3. Photolabile Linker



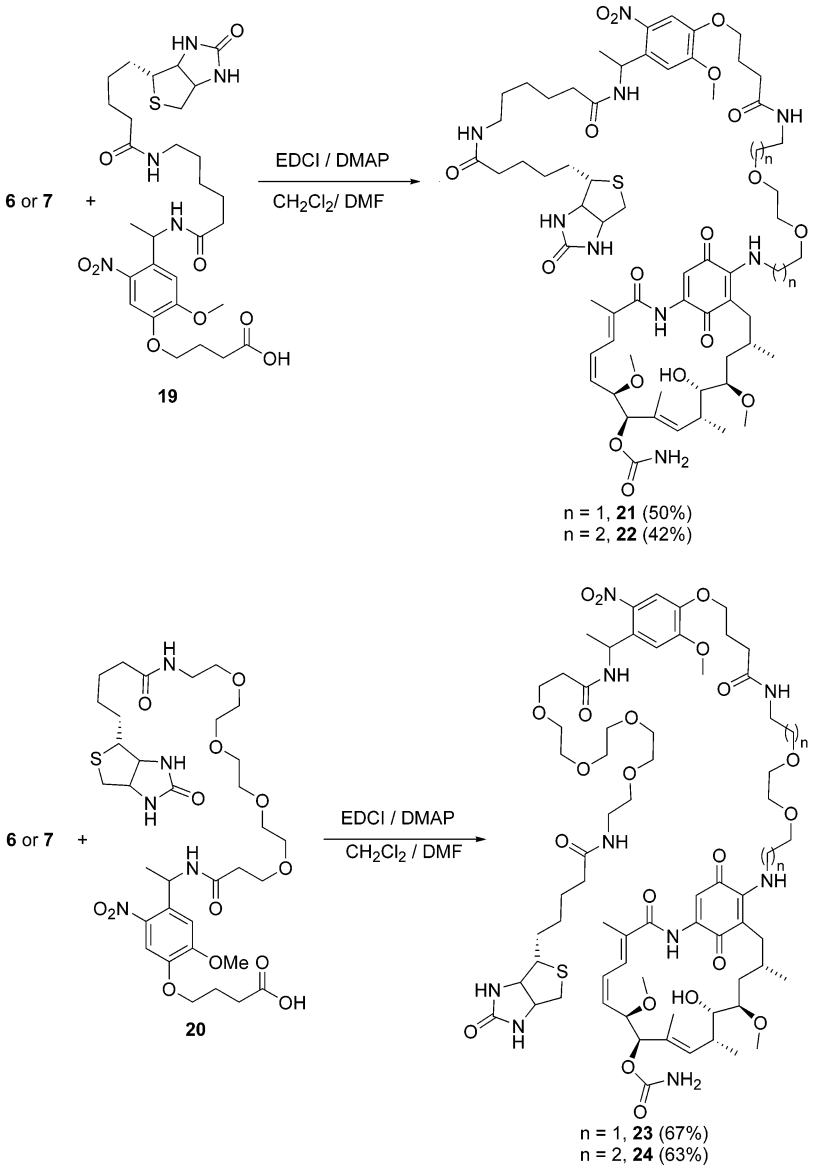
the amino quinone as evidenced by the appearance of a purple solution resulting from vinylagous amide formation. The reaction was complete within 30 min as demonstrated by reverse-phase HPLC to provide the desired products in >96% purity. Extended reaction times resulted in the formation of a complex mixture of products and low yields of the desired vinylagous amides. In solution, these compounds decompose at −20 °C and were either stored as a purple solid or used immediately in subsequent reactions.

Coupling of the *N*-hydroxysuccinimide (NHS)-activated ester **8**[20] with **6** and **7** provided the biotinylated GDA analogues **10** and **11**, respectively (Scheme 2). The reaction was complete within 20 min and provided the easily separable purple product **10**, in 89% yield. Likewise, **11** was prepared in an analogous fashion, providing the desired product in 69% yield. A more hydrophilic

SCHEME 4. Construction of Photolabile Linkers



SCHEME 5. Synthesis of Photolabile Biotinylated GDA



PEG-derivative, **12**,[20] was used for the preparation of two additional GDA analogues, **14** and **15** (Scheme 2). These reactions required the addition of **6** or **7** to a solution of **11** and provided both **14** and **15** in 89% yields, respectively.

Synthesis of Photolabile Biotinylated GDA Derivatives. Incorporation of a photolabile group between the biotin and GDA portions of the affinity purification handle provided an alternative method for removal of GDA-bound proteins from the immobilized complex. Alternatively, this linker may prove useful for isolation of Hsp90 multiprotein complexes that are formed between the chaperone, client proteins, immunophilins, partner proteins, and cochaperones. Holmes previously reported the use of **16** as a photolabile linker for solid-phase synthesis (Scheme 3).[21] The half-life of the benzylic C−N bond is only 40 s when exposed to 365 nm ultraviolet light in buffered (pH 7.4) aqueous solution. Photolysis of such functionality results in the cleavage of the linker to form the corresponding amide and styrene products (Scheme 3). Holmes has previously prepared **16** in six steps, and minor modification of this procedure gave the unprotected amino acid **18**, which was subsequently used to prepare photolabile analogues of biotinylated GDA.

The biotinylated photolabile linker **19** was assembled by coupling the unprotected amino acid **18** with **9**.[20] Treatment of LC-biotinic acid (**9**) with dicyclohexyl carbodiimide (DCC) in methylene chloride and dimethyl formamide, followed by addition of **18** led to poor yields of **19**. Acceptable yields were obtained when the reaction was sonicated for 5 min after the addition of DCC and for an additional hour upon addition of **18**. Photoinduced cleavage of **19** occurred when the sample was exposed to light for long periods of time. Consequently, purification of photolabile compounds was accomplished using reverse-phase HPLC to provide **19** in 55% yield (Scheme 4). In contrast to the alkyl-derived product, **20** was prepared by the treatment of **13**[20] with DCC, followed by addition

(20) Biotin-tethered compounds were provided as both the free acid and activated esters by Quantabiodesign.
(21) Holmes, C. P. *J. Org. Chem.* **1997**, *62*, 2370−2380.

of **18**. The desired product was purified via reverse phase HPLC to give **20** in 44% yield.

The photolabile biotin intermediate **19** was coupled with **6** and **7** using 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDCI) and dimethyl amino pyridine (DMAP, Scheme 5). Flash chromatography in the absence of significant light provided the desired compounds **21** and **22** in moderate yields. Likewise, compounds **23** and **24** were synthesized by an analogous procedure using **20** and either GDA-amine **6** or **7**.

Isolation of GDA-Bound Proteins. To verify biotinylated-GDA derivatives were capable of the isolation of Hsp90, recombinant Hsp90 from yeast (Hsp82) was overexpressed and purified to homogeneity following the procedure of Buchner and co-workers.[22] Purified Hsp82 was incubated with **14** for 1 h in a buffered solution at 4 °C. Neutravidin-containing resin was added to the mixture and further incubated at 4 °C for 30 min. After centrifugation and removal of the supernatant, the resin was successively washed with buffer and resuspended in a minimal amount of buffer. Increasing concentrations of GDA were added to the mixture to competitively displace Hsp82 from the neutravidin resin. SDS−PAGE of the GDA competition experiments determined both that Hsp82 was bound to the neutravidin resin and that the protein could be displaced with unmodified GDA (Figure 3).

Affinity Purification of GDA-Binding Protiens. Jurkat A3 cells are known to be sensitive to inhibition with GDA (IC_{90} = 100 nM).[23] Lysate containing the Jurkat A3 proteome[24] was incubated with **14** at 4 °C in buffer for 1 h. Neutravidin resin was added to the

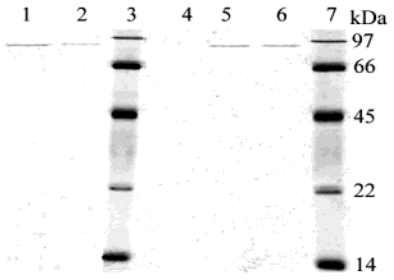


FIGURE 3. Binding of biotinylated GDA with purified Hsp 82. Purified Hsp82 (300 μg) was exposed to **14** (200 μg), incubated with neutravidin resin, and centrifuged, and the supernatant was removed. The resin/**14**/protein complex was washed with buffer. The second wash (lane 1) shows band intensities higher than those of the third wash (lane 2), indicating the removal of excess Hsp82. The resin was exposed to **1** (10 μg) for 15 min at 4 °C with shaking and centrifuged. The supernatant was removed and denatured in SDS sample buffer before separation by SDS−PAGE (lane 4). The experiment was repeated with the same resin using 50 μg (lane 5) and 100 μg (lane 6) of **1**. Molecular weight markers are in lanes 3 and 7. Increasing band intensity from lanes 4 to 6 show that as increasing concentrations of **1** were added, more Hsp82 was released from the neutravidin resin. The gel was visualized with silver stain.

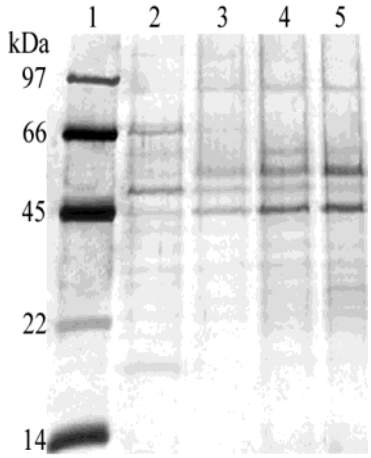


FIGURE 4. Affinity isolation of GDA-binding proteins. Jurkat A3 lysate exposed to **14** (200 μg) was incubated with neutravidin resin and centrifuged, and the supernatant was removed. The washed resin was exposed to **1** (10 μg) for 15 min at 4 °C with shaking and centrifuged. The supernatant was removed, denatured in SDS sample buffer, and separated by SDS−PAGE (lane 2). The experiment was repeated with the same resin using 50 μg (lane 3), 100 μg (lane 4), and 300 μg (lane 5) of **1**. Lane 1 represents the molecular weight markers. Visualization of the separated proteins with silver stain showed increasing intensities of proteins with elevated concentrations of **1**.

mixture and incubated for an additional 35 min at 4 °C. The heterogeneous mixture was centrifuged, and the nonbinding proteins were removed by successive washing with an appropriate buffer. The washed resin was resuspended in buffer and incubated with increasing concentrations of GDA. As can be seen from Figure 4, Hsp90 was competitively displaced with increasing amounts of GDA. However, proteins other than Hsp90 were also competitively displaced with unmodified GDA. These data indicate that proteins other than Hsp90 are also isolable with biotinylated GDA and may play a significant role in GDA's affect on tumor cells. Studies are now underway to determine the identity of these proteins, as well as their affinity for GDA or Hsp90. The results of these studies will be presented in due course.

Conclusion

The syntheses of eight biotinylated analogues of GDA containing photolabile and nonphotolabile tethers have been accomplished, incorporating both hydrophobic and hydrophilic tethers. These molecules were prepared by the treatment of diamines with GDA to provide amino modified GDA products, which could be easily coupled to a number of biotin-containing carboxylic acids. Incubation of **14** with purified recombinant Hsp90 from yeast and affinity purification using neutravidin resin resulted in the capture and release of Hsp90. Incubation of **14** with the Jurkat A3 proteome resulted in the isolation of several proteins, including Hsp90. Proteins isolated from these experiments were sufficient for mass spectrometric identification. Studies are now underway to determine the identity of these proteins, their K_d's for GDA, and determination of a conserved ATP binding pocket to which these molecules bind.

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Supporting Information Available: Spectra and experimental procedures for all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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