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Synthesis of Hsp90 dimerization modulators

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Abstract—The synthesis and evaluation of several chemical modulators of heat shock protein 90 (Hsp90) dimerization is presented. These agents may represent useful tools to study the importance of N-terminal dimerization and also to determine subunit interface(s) in Hsp90.

The molecular chaperone Hsp90 plays a key, but poorly understood, role in the folding, assembly, and activation of a large number of signal transduction molecules, in particular kinases, transcription factors, and steroid hormone receptors.[1] In carrying out these functions Hsp90 hydrolyzes ATP as it cycles between ADP- and ATP-bound forms, and this ATPase activity is regulated by the transient association with a variety of co-chaperones.[2] Current models of the Hsp90 chaperone cycle propose that client protein capture occurs via an N-terminal 'molecular clamp,' in a manner analogous to the DNA binding functions of GyrB and MutL.[3] In this model, ATP binding to the N-terminal domain promotes an intermonomeric N-terminal dimerization reaction (molecular clamp) that favors client protein binding (Fig. 1).

The role of ATP-driven N-terminal dimerization in the Hsp90 chaperone cycle has, however, recently been questioned.[4] To help resolve this controversy, we set to develop several chemical modulators of Hsp90 dimerization (Fig. 2).

Protein modulation through use of small-molecule chemical inducers or disruptors of dimerization has been successfully utilized for other proteins.[5] Chemical modulation of dimerization may be used to manipulate cellular regulatory pathways from signal transduction to transcription, and to create model systems for study of the specific interactions between proteins and small-molecule chemical ligands. This strategy utilizes a homo- or hetero-dimer to induce or inhibit proximity of two proteins.

The ansamycin antibiotic geldanamycin (GM) binds with high affinity to the Hsp90 N-terminal ATP-pocket[6] and, synthetically, is a convenient choice to create chemical modulators of Hsp90 dimerization. To better explore the spatial characteristics of Hsp90 N-termini dimerization, we chose to enter two variables in our

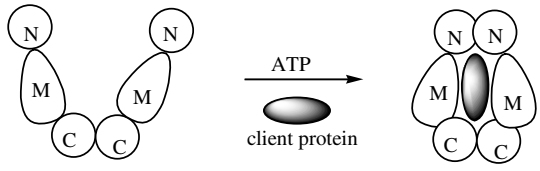

Figure 1. Schematic representation of cycling between the open and closed conformations of Hsp90. N, N-terminus; M, middle domain; and C, C-terminus.

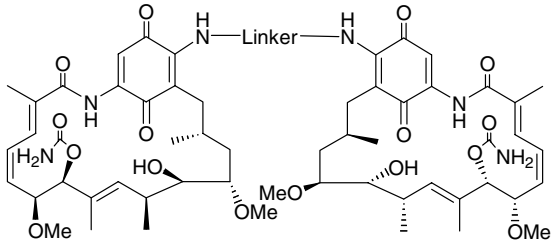

Figure 2. Hsp90 dimerization modulators.

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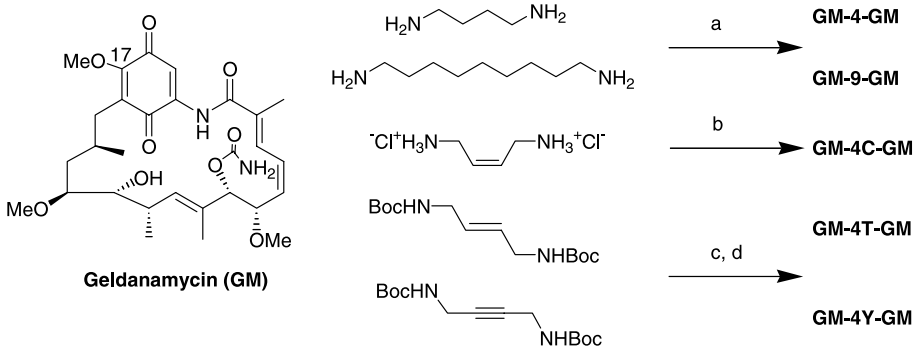
dimer synthesis. One variable pertains to linker length, while the other to linker rigidity and ability to spatially orient the two adjoined GM molecules. If the model proposed in Figure 1 is correct, these dimeric GM compounds should specifically modulate N–N domain dimerization reactions, without altering the C-terminal dimerization site. The synthesis of these compounds and their affinity for the Hsp90 ATP-pocket are reported here; detailed analyses of their effects on quaternary interactions of Grp94, the endoplasmic reticulum Hsp90 member, summarized below, will be detailed elsewhere.[7] In brief, the intermolecular subunit interface in native Grp94 was analyzed by a combined chemical crosslinking/tandem mass spectrometry approach. Two primary subunit interactions were identified, the expected C-terminal homodimerization domain and a novel intermolecular N+M interaction site.[7] Neither interface was altered upon addition of ATP, ADP, radicicol or GM. In contrast, the binding of **GM-4-GM** and, to a lesser degree, **GM-9-GM** selectively disrupted the intermolecular N+M interactions without disrupting the C-terminal homodimerization interactions. These data are consistent with either a 'spacing' function for the GM dimers, with the two GM moieties constraining the intermolecular dynamics of the N-terminal domains and/or a 'blocking' function, where binding of the GM dimer to one subunit imposes a steric block to interactions with the paired subunit. Experiments are currently underway to distinguish between these two alternatives.

Synthesis of geldanamycin dimers. When bound to Hsp90, GM is buried inside the protein cavity leaving the C17 methoxy functionality exposed to the solvent. This group easily undergoes a Michael reaction when in the presence of primary amines, resulting, in general, in compounds with retained Hsp90 binding ability, and thus, much chemistry on GM has used this modification.[8] Here, Hsp90-dimerization modulators were created by reacting GM with the corresponding α,ε-diamines (Scheme 1).[9] For the synthesis of GM dimers coupled via a saturated 4- or 9-carbon linker, respectively, the corresponding diamine was stirred with 2 equiv of GM at room temperature in dry DMF. Reaction initiation was easily observed as the solution instantly turned from deep yellow (color of GM) to dark purple (color of C17-alkylamino-desmethoxy-GMs) upon diamine addition. Complete conversion was observed after 30–60 min. For dimers bearing an unsaturated linker, the diamine was not commercially available and had to be first synthesized. We chose to start their synthesis from the corresponding α,ε-dichlorides. These were reacted with NaN_3 to result in diazides, which were further reduced to the diamine via a Staudinger reaction, and later isolated as dihydrochlorate salts.[10] Dimers containing a double bond in the linker were successfully created by the reaction of these diaminoalkene hydrochlorates with GM in dry DMF using DIEA as base. Coupling of these diaminoalkenes with GM required 24 h to complete, likely due to steric constraints imposed on the addition of a second GM to the GM-linker-NH_2 intermediate. Surprisingly, 1,4-diamino-2-butyne hydrochlorate failed to satisfactorily react with GM under these conditions and a large amount of derivatives with hydrolyzed ansa ring was observed. Our previous experience in the synthesis of GM-heterodimers suggested that this impediment may be overcome by generating the amine from a Boc-protected amine via TFA treatment.[8] Boc-protected 1,4-diamino-2-butyne was generated from the corresponding amine upon treatment with BocON in the presence of TEA in CH_2Cl_2.[10a] In this fashion, the synthesis of GM dimer containing the butyne linker was achieved in 50% yield.

Competitive binding to Hsp90. We next proceeded to assess whether these dimers retain binding affinity for the Hsp90 ATP-pocket (Fig. 3).[11] An assay that measures the ability of compounds to compete BODIPY-labeled GM binding to Hsp90 was used.[12] Competitive displacement studies were performed with these dimers and the Hsp90 inhibitors GM and its C17-allylamino derivative, 17AAG.[13] EC_{50} values were determined as the competitor concentrations were 50% of the fluorescent GM-BODIPY displaced. As seen in Figure 3, all dimers retained good binding to Hsp90. Recorded EC_{50}s ranged from 45 ± 3 nM for **GM-4T-GM**, to 91 ± 4 nM for **GM-4GM** and 345 ± 5 nM and 465 ± 7 nM for **GM-4C-GM** and **GM-9-GM**, respectively. In the same assay, recorded EC_{50}s for GM and 17AAG were 35 ± 2 nM and 45 ± 3 nM, respectively.

In summary, these agents have the characteristics that allow for their use as chemical modulators of Hsp90 dimerization and thus, may represent useful tools to study the importance of N-terminal



Scheme 1. Synthesis of GM dimers. Reagents and conditions: (a) DMF, rt, 30–60 min; (b) DIEA, DMF, rt, 24 h; (c) TFA/CH_2Cl_2 (1:4), 1 h, rt; (d) DIEA, DMF, rt, 24 h.

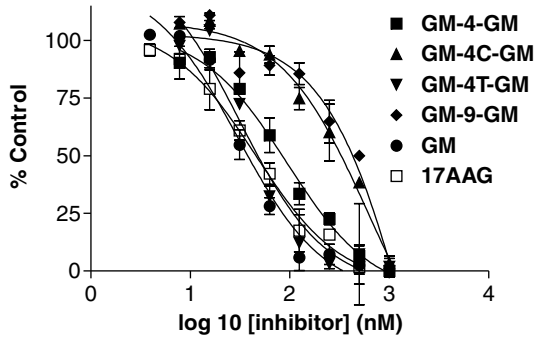


Figure 3. Competitive binding of synthesized dimers to Hsp90 alpha.

dimerization and also to determine subunit interface(s) in Hsp90.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.03.092.

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9. **GM-4-GM**: A solution of GM (28 mg, 0.05 mmol) and 1,4-diaminobutane (2.2 mg, 0.025 mmol) in dry DMF (0.5 mL) was stirred for 1 h at room temperature (rt) under inert atmosphere. The solvent was quickly removed under high vacuum, and the mixture applied to a silica gel column. CH_2Cl_2/acetone (4:1) was first applied to remove any unreacted GM (yellow), followed by CH_2Cl_2/acetone (2:1) to afford **GM-4-GM** (27.0 mg, 90%) as a purple solid. R_f (CH_2Cl_2/acetone 3:1) 0.1; ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.16 (s, 2H), 7.25 (s, 2H), 6.95 (d, J = 11 Hz, 2H), 6.58 (dd, J = 11 Hz, 2H), 6.27 (m, 2H), 5.89–5.84 (m, 4H), 5.18 (s, 2H), 4.96 (br s, 4H), 4.31 (d, J = 10 Hz, 2H), 4.16 (br s, 2H), 3.59–3.44 (m, 8H), 3.36 (s, 6H), 3.27 (s, 6H), 2.76–2.68 (m, 4H), 2.37 (t, J = 11 Hz, 4H), 2.03 (s, 6H), 1.79–1.64 (m, 16H), 1.00–0.96 (m, 12H). MS *m*/*z* 1167.6 (M+Na). **GM-9-GM**: A solution of GM (21 mg, 0.037 mmol) and 1,9-diaminononane (2.9 mg, 0.018 mmol) in dry DMF (0.5 mL) was stirred for 0.5 h at rt under inert atmosphere. The solvent was quickly removed under high vacuum, and the mixture applied to a silica gel column. CH_2Cl_2/acetone (4:1) was first applied to remove any unreacted GM (yellow), followed by CH_2Cl_2/acetone (2:1) to afford **GM-9-GM** (20.0 mg, 91%) as a purple solid. R_f (CH_2Cl_2/acetone 3:1) 0.15; ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.18 (s, 2H), 7.27 (s, 2H), 6.95 (d, J = 11 Hz, 2H), 6.57 (dd, J = 11 Hz, 2H), 6.28 (m, 2H), 5.91–5.82 (m, 4H), 5.18 (s, 2H), 4.76 (br s, 4H), 4.34-4.28 (m, 4H), 3.58–3.40 (m, 8H), 3.35 (s, 6H), 3.26 (s, 6H), 2.75–2.60 (m, 4H), 2.41 (t, J = 11 Hz, 2H), 2.01 (s, 6H), 1.84 (s, 6H), 1.70–1.63 (m, 4H), 1.40–1.29 (m, 8H), 1.00–0.95 (m, 12H). MS *m*/*z* 1215.9 (M+H). **GM-4C-GM**: A solution of GM (11.8 mg, 0.021 mmol), (*Z*)-1,4-diamino-2-butene, HCl salt (1.4 mg, 0.009 mmol), and 0.03 mL *N*,*N*-diisopropylethylamine (DIEA) in dry DMF (0.25 mL) was stirred for 2 h at rt under inert atmosphere. The solvent was quickly removed under high vacuum, and the mixture applied to a silica gel column. CH_2Cl_2/acetone (4:1) was first applied to remove any unreacted GM (yellow), followed by CH_2Cl_2/acetone (2:1) to afford **GM-4C-GM** (7.4 mg, 75%) as a purple solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.13 (s, 2H), 7.30 (s, 2H), 6.95 (d, J = 11 Hz, 2H), 6.58 (dd, J = 11 Hz, 2H), 6.16 (m, 2H), 5.89–5.80 (m, 6H), 5.20 (s, 2H), 4.78 (br s, 4H), 4.34–4.14 (m, 6H), 3.58–3.40 (m, 4H), 3.36 (s, 6H), 3.28 (s, 6H), 2.75–2.63 (m, 4H), 2.35 (t, J = 11 Hz, 2H), 2.03 (s, 6H), 1.85(s, 6H), 1.85–1.67 (m, 6H), 1.00–0.95 (m, 12H). MS *m*/*z* 1165.7 (M+Na). **GM-4T-GM**: A solution of Boc-protected (*E*)-1,4-diamino-2-butene (2.2 mg, 0.0077 mmol) in 1 mL CH_2Cl_2/TFA (4:1) with added 10% thioanisole was stirred for 1 h at rt. Solvent was speedily removed, and GM (9.2 mg, 0.016 mmol) dissolved in dry DMF (0.6 mL) containing 0.02 mL DIEA was added to the crude material. The resulting solution was stirred for 24 h at rt under inert atmosphere. The solvent was quickly removed under high vacuum, and the mixture applied to a silica gel column. CH_2Cl_2/acetone (5:1) was first applied to remove any unreacted GM (yellow), followed by CH_2Cl_2/acetone (1:1) to afford **GM-4T-GM** (6.4 mg, 75%) as a purple solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.10 (s, 2H), 7.26 (s, 2H), 6.91 (d, J = 11 Hz, 2H), 6.54 (dd, J = 11 Hz, 2H), 6.30 (m, 2H), 5.86–5.72 (m, 6H), 5.15 (s, 2H), 4.75 (br s, 4H), 4.26 (d, J = 10 Hz, 2H), 4.15–3.04 (m, 4H), 4.01 (br s, 2H), 3.52–3.38 (m, 4H), 3.31 (s, 6H), 3.22 (s, 6H), 2.71–2.58 (m, 4H), 2.25 (t, J = 11 Hz, 2H), 1.97 (s, 6H), 1.75 (s, 6H), 1.75–1.60 (m, 6H), 0.95–0.92 (m, 12H). MS *m*/*z* 1165.7 (M+Na). **GM-4Y-GM**: A solution of Boc-protected 1,4-diamino-2-butyne (1.1 mg, 0.0039 mmol) in 1 mL CH_2Cl_2/TFA (4:1) with added 10% thioanisole was stirred for 1 h at rt.

Solvent was speedily removed, and GM (5.0 mg, 0.089 mmol) dissolved in dry DMF (0.5 mL) containing 0.01 mL DIEA was added to the crude material. The resulting solution was stirred for 24 h at rt under inert atmosphere. The solvent was quickly removed under high vacuum, and the mixture applied to a silica gel column. CH_2Cl_2/acetone (5:1) was first applied to remove any unreacted GM (yellow), followed by CH_2Cl_2/acetone (1:1) to afford **GM-4Y-GM** (2.2 mg, 50%) as a purple solid. ^1H NMR (400 MHz, $CDCl_3$): δ 9.10 (s, 2H), 7.32 (s, 2H), 6.95 (d, J = 11 Hz, 2H), 6.56 (dd, J = 11 Hz, 2H), 6.30 (m, 2H), 5.89–5.84 (m, 4H), 5.20 (s, 2H), 4.79 (br s, 4H), 4.35–4.30 (m, 6H), 3.94 (br s, 2H), 3.59–3.41 (m, 4H), 3.36 (s, 6H), 3.27 (s, 6H), 2.76–2.69 (m, 4H), 2.32 (t, J = 11 Hz, 2H), 2.06 (s, 6H), 1.79 (s, 6H), 1.79–1.60 (m, 6H), 0.875–0.85 (m, 12H). MS *m*/*z* 1163.7 (M+Na).

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11. **Competitive binding measurements**: A 400 μM stock of each dimer was prepared in DMSO. Binding studies were performed in a low binding black 96-well plate (Corning # 3650). The assay buffer contained 20 mM HEPES (K), pH 7.3, 50 mM KCl, 5 mM $MgCl_2$, 20 mM Na_2MoO_4, and 0.01% NP40. Before each use, 0.1 mg/mL bovine gamma globulin (BGG) (Panvera Corporation, Madison, WI) and 2 mM DTT (Fisher Biotech, Fair Lawn, NJ) were freshly added. Each well contained 30 nM Hsp90 alpha (Stressgen # SPP776), 5 nM GM-BODIPY, and varying concentrations of competitor in 100 μL assay buffer. Control wells containing GM-BODIPY alone (Tracer), GM-BODIPY with Hsp90 alpha (Control), and finally, buffer alone were also platted. The plate was left on a shaker at 4 °C for 24 h and fluorescence polarization values in mP were recorded using an Analyst AD (Molecular Devices) instrument. Data recorded in each well were calculated as %Control = (recorded value in well − recorded value tracer)/(recorded value in control well − recorded value tracer) × 100 and plotted against competitor concentrations.
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