Pergamon

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Bioorganic & Medicinal Chemistry Letters 13 (2003) 3975–3978

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Synthesis of Novel Fluorescent Probes for the Molecular Chaperone Hsp90

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Received 3 July 2003; revised 27 August 2003; accepted 27 August 2003

Abstract—Heat shock protein 90 (Hsp90) is a molecular chaperone necessary for maintaining oncogenic transformation. There is substantial interest in developing novel agents that bind to the N-terminal of the chaperone. Here we report the synthesis and characterization of two fluorescent Hsp90 inhibitors and probe their use in an Hsp90 fluorescent polarization assay.

Hsp90 is a molecular chaperone involved in many aspects of oncogenic transformation.[1–5] The function of this protein may be modulated with compounds that bind to a conserved pocket in its amino-terminus.[6,7] The endogenous ligands for the pocket are ATP and ADP and it is believed that these nucleotides function as a switch in determining the conformation of the chaperone.[8–10] Interference with the chaperone function induces the proteasomal degradation of a subset of client proteins involved in growth and survival pathways (i.e., steroid receptors, Raf1 kinase, Akt, certain transmembrane tyrosine kinases).[1–5]

An Hsp90 inhibitor, 17AAG, is now in Phase I clinical trial for cancer. Although certainly effective in many tumor models, in clinic the drug is faced with limitations. The drug is poorly soluble and necessitates intravenous administration. The presence of a benzoquinone moiety in its structure is thought to be cause of its liver toxicity. There is thus, substantial interest in developing novel agents that bind to the N-terminal of the chaperone and have more drug-like properties.

Existent in vitro assays probing for Hsp90 binding include displacement of Hsp90 from immobilized GM,[11–13] isothermal calorimetry,[14] circular dichroism,[15] measurements of ATPase activity[16–19] and a filter binding assay that characterizes the binding of Hsp90 alpha with [^{3}H]17AAG.[20] A more suitable assay for HTS is however, fluorescence polarization (FP). FP is a homogenous technology consisting of simple mix reagents and read format that can be easily automated.[21] The theory behind the method is that when a fluorescent sample is excited with a polarized light, the emission is polarized. Small dye molecules are rapidly rotating molecules and the initially photoselected orientational distribution is randomized prior to emission resulting in low fluorescence polarization. However, binding of the dye to a large, slowly rotating molecule results in high FP. The method gives therefore, a direct readout of the extent of dye binding to the macromolecule. Unfortunately, there are no known fluorescent-labeled Hsp90 inhibitors that could be used in an FP assay. Here we report the synthesis and characterization of two fluorescent derivatives of geldanamycin (GM), a naturally occurring Hsp90 inhibitor.[22] We show that these ligands tightly bind Hsp90 and that their interaction with the chaperone is competed by the known Hsp90 inhibitors 17AAG and PU3.

Synthesis of Hsp90 Fluorescent Probes[23]

The sole known chemical modification in the skeleton of GM allowing for activity is at C17.[24,25] The methoxy group found at this position easily undergoes a Michael reaction when in the presence of primary amines. Two fluorescent dyes primarily used in FP assays, FITC and BODIPY, were chosen to be linked to GM. The commercially available fluorescein-5-isothiocyanate was

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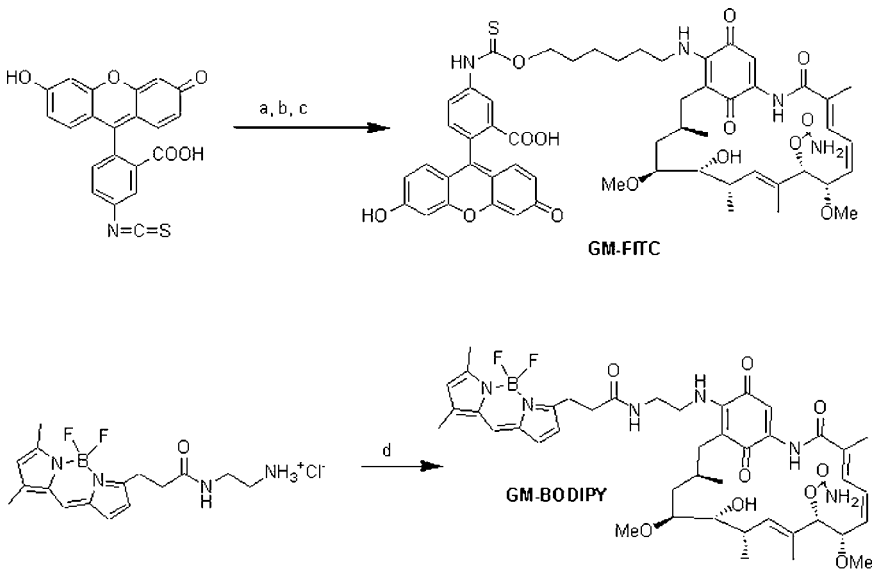
doi:10.1016/j.bmcl.2003.08.065



Scheme 1. Synthesis of the GM-fluorescent ligands. (a) 6-(Boc-amino-)-1-hexanol, TEA, DMF, 60°C, 24 h; (b) TFA/CH_2Cl_2 (1:4), 45 min, rt; (c) GM, TEA, DMF, rt, 24 h; (d) GM, DIEA, CH_2Cl_2, rt, 24 h.

reacted with 6-(Boc-amino)-1-hexanol by heating in DMF in the presence of triethylamine (TEA) as base to afford the corresponding thiocarbamate in 50% yield. Deprotection of Boc using trifluoroacetic acid (TFA) in CH_2Cl_2 occurred in 45 min and resulted in the corresponding TFA salt. This was reacted with GM in DMF in the presence of TEA for 24 h. The reaction afforded GM-FITC in 60% yield. For the construction of GM-BODIPY we used the commercially available 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionyl ethylenediamine hydrochloride (BODIPY® FL EDA) (Molecular Probes # D2390). The Michael reaction of this derivative with GM occurred smoothly in CH_2Cl_2 with diisopropylamine (DIEA) as base to afford GM-BODIPY. The linker between GM and dyes is longer than six atoms and should not considerably obstruct binding to the chaperone (Scheme 1).

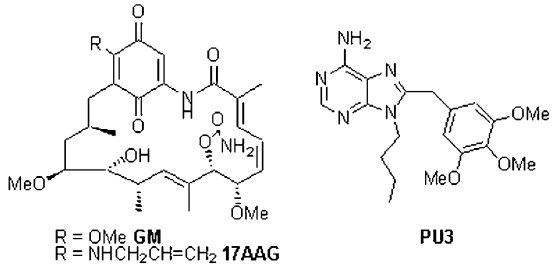


Figure 1. Structures of the known Hsp90 inhibitors GM, 17AAG and PU3.

Binding to Hsp90

We next proceeded to asses the suitability of these probes for Hsp90 in a homogenous FP assay format using an Analyst AD (Molecular Devices) instrument. A stock of 10 μM of each tracer was prepared in DMSO and diluted with HFB buffer [20 mM HEPES (K) pH 7.3, 50 mM KCl, 1 mM DTT, 5 mM $MgCl_2$, 20 mM Na_2MoO_4, 0.01% NP40 with 0.1 mg/mL BGG] to obtain 10 and 4 nM solutions for GM-BODIPY and GM-FITC, respectively. Different amounts of Hsp90 alpha (Stressgen # SPP776) dissolved in HFB were added

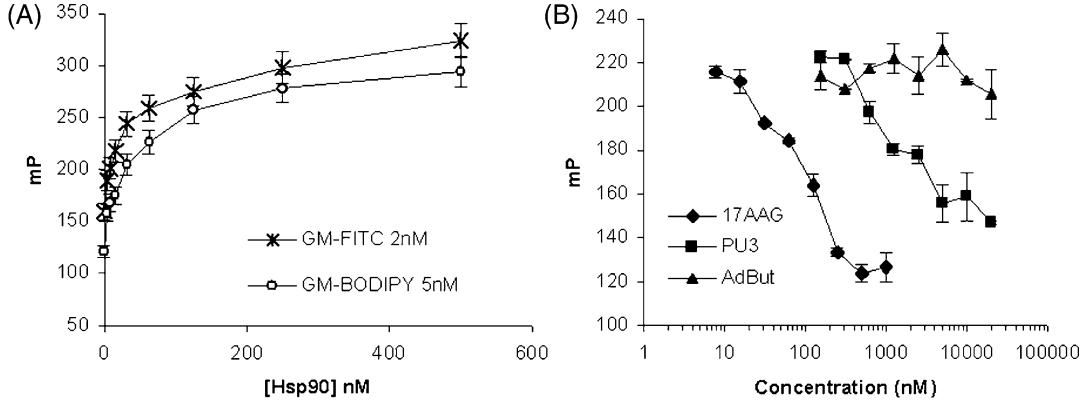


Figure 2. (A) Titration curves for binding of GM-BODIPY and GM-FITC to Hsp90 α. Protein was added in triplicate wells. (B) Competition assay using the Hsp90 inhibitors 17AAG and PU3 to displace GM-BODIPY binding to Hsp90 α. Ad-But is a derivative of PU3 that does not bind Hsp90. Drugs were added in duplicate wells. Each plot is the average of two experiments.

to a low binding black 96-well plate (Corning # 3650) in a 50 μL volume. To each well were added 50 μL of the tracer solution. Some wells were left with buffer or tracer alone to serve as controls. The plate was left on a shaker at 4 °C for 3 h and the FP values in mP were recorded. The measured FP value (mP) was plotted against the protein concentration (Fig. 1). Both tracers performed well in the assay. The titration curve showed that the probes bind tightly to Hsp90 α (K_ds, 33.8±1.2 nM and 23.3±0.9 for GM-BODIPY and GM-FITC, respectively). The dynamic range of FP was approximately 160 mP at 180 min.

Competitive Displacement of GM-BODIPY by Hsp90 Inhibitors

Competitive displacement studies were performed with the Hsp90 inhibitors 17AAG and PU3 and additionally as a control, with Ad-But, a PU3 derivative that does not bind Hsp90.[27,28] Stocks of these drugs were made in DMSO at concentrations of 200 μM for 17AAG and 4 mM for PU3 and Ad-But. The drugs were serially diluted in binding buffer and the GM-BODIPY tracer and Hsp90 α were added at 5 and 40 nM concentrations, respectively. Maximum concentration of used DMSO was 0.25% (v/v). The plate was left on a shaker at 4 °C for 5 h and the FP values in mP were recorded. A window of 100 mP was observed between wells containing protein and tracer and wells containing tracer only. The measured FP values (mP) were plotted against the competitor concentration (Fig. 2). EC_{50} values were determined as the competitor concentrations where 50% of the tracer was displaced. As previously recorded,[28] PU3 was found to be a 30-times weaker Hsp90 inhibitor than 17AAG (EC_{50} = 3.2 μM vs 110 nM). Ad-But could not displace Hsp90 bound GM-BODIPY even at the maximally measured concentration of 20 μM.

In summary, two novel Hsp90-binding fluorescent probes were prepared and their application in the development of a homogenous FP assay for the Hsp90 chaperone demonstrated.

Acknowledgements

The work was funded in part by NIH-NCI (1U01CA91178-03), AACR-Cancer Research and Prevention Foundation and a generous donation from the Taub Foundation.

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23. **GM-FITC**: To a solution of FITC-NCS (78 mg, 0.20 mmol) and 6-(Boc-amino)-1-hexanol (43 mg, 0.20 mmol) in DMF (0.75 mL) at 60 °C was added TEA (16 μL, 0.4 mmol). After it was heated with stirring for 24 h, the solution was cooled to room temperature, and the solvent was removed in vacuo. Silica gel column chromatography with DCM/hexanes/MeOH (6:4:1) afforded the corresponding thiocarbamate (60 mg, 50%) as an orange crystalline solid. ^{1}H NMR (400 MHz, MeOH-d_4): δ 7.33–7.25 (m, 6H), 7.21–7.16 (m, 4H), 5.76–5.68 (m, 1H), 5.05 (d, J = 17.1 Hz, 1H), 5.01 (d, J = 10.2 Hz, 1H), 4.51–4.48 (m, 1H), 4.05 (dd, J = 1.6, 9.0 Hz, 1H), 3.98 (t, J = 8.4 Hz, 1H), 3.44–3.36 (m, 2H), 3.25 (dd, J = 3.1, 14.0 Hz, 1H), 3.20 (dd, J = 3.3, 13.4 Hz, 1H), 2.66 (dd, J = 9.8, 13.4 Hz, 1H), 2.48–2.44 (m, 2H). MS m/z 608.1 (M + H). To the thiocarbamate (7 mg, 0.012 mmol) was added a mixture of CH_2Cl_2/TFA (0.3 mL/0.1 mL) and the resulting solution was stirred at room temperature for 45 min. The solvent was removed in vacuo. The crude was taken up in DMF (0.5 mL) and GM (6.4 mg, 0.012 mmol) and an excess triethylamine (50 μL, 36 mmol) were added to the solution. The mixture was stirred under inert gas atmosphere for 24 h. After solvent removal in vacuo, the product was purified on a silica gel column eluting with CH_2Cl_2/EtOAc/hexanes/MeOH (4:2:3:1) to afford GM-FITC (5.5 mg, 44%) as a yellow-orange solid. R_f (CH_2Cl_2/EtOAc/hexanes/MeOH 4:2:3:1) 0.23; ^{1}H NMR (400 MHz, MeOH-d_4): δ 7.15 (d, J = 8.0 Hz, 1H), 7.14–7.02 (m, 3H), 6.65–6.50 (m, 8H), 5.85 (t, J = 8.7 Hz, 1H), 5.58 (d, J = 9.6 Hz, 1H), 5.20 (s, 1H), 4.58–4.52 (m, 2H), 3.59–3.44 (m, 3H), 3.30 (s, 3H), 3.27 (s, 3H), 2.72–2.68 (m, 2H), 2.31–2.26 (m, 1H), 1.98 (s, 3H), 1.84–1.71 (m, 2H), 1.62 (s, 3H), 1.57–1.48 (m, 6H), 0.95 (d, J = 6.8, 6H). MS m/z 1057.4 (M + Na).

GM-BODIPY: A solution of BODIPY® FL EDA (1.1 mg, 0.003 mmol), GM (2.5 mg, 0.005 mmol) and DIEA (3 μL, 0.018 mmol) in CH_2Cl_2 (0.6 mL) was stirred for 24 h. The mixture was added to a silica gel column and eluted with DCM/acetone (3:1) to afford GM-BODIPY (1.5 mg, 60%) as an orange solid. R_f (CH_2Cl_2/acetone 3:1) 0.28; ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.12 (s, 1H), 7.19 (s, 1H), 7.04 (s, 1H), 6.95–6.93 (m, 1H), 6.81 (d, $J = 3.8$ Hz, 1H), 6.60–6.55 (m, 2H), 6.24–6.23 (m, 2H), 6.11 (s, 1H), 5.92–5.82 (m, 2H), 5.17 (s, 1H), 4.75 (bs, 2H), 4.37–4.28 (m, 2H), 3.57–3.44 (m, 5H), 3.34 (s, 3H), 3.28 (s, 3H), 3.27–3.22 (m, 2H), 2.75–2.69 (m, 2H), 2.54 (s, 3H), 2.32–2.30 (m, 1H), 2.29 (s, 3H), 2.03 (s, 3H), 1.86 (s, 3H), 1.72–1.69 (m, 1H), 1.56–1.52 (m, 1H), 0.98 (d, $J = 6.8$ Hz, 3H), 0.92 (d, $J = 6.6$ Hz, 3H). MS m/z 885.4 (M + Na).

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