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Synthesis of a red-shifted fluorescence polarization probe for Hsp90

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Abstract—The synthesis of a red-shifted cy3B-GM ligand and its evaluation as a fluorescence polarization probe for Hsp90 is presented.

Heat shock protein 90 (Hsp90) is a protein with important roles in maintaining the malignant phenotype.[1] Activation of signaling pathways mediated by Hsp90 protein clients is necessary for cell proliferation, regulation of cell cycle progression, and apoptosis. Additionally, gain-of-function mutations responsible for transformation often require Hsp90 for the maintenance of their folded, functionally active conformations. These characteristics suggest Hsp90 as an important target in cancer therapy and prompt for the identification, development, and clinical translation of small molecule inhibitors of the chaperone.[1]

The biological role of Hsp90 is mediated by one fundamental feature: its ability to interact with client substrates. Association of Hsp90 with client proteins is regulated by the activity of the N-terminal domain, which binds ATP to mediate ATP hydrolysis and a series of association–dissociation cycles between Hsp90 and client substrates.[2] The N-terminal region ATP pocket binders are the first to be identified as inhibitors of Hsp90 activity. Some of these are natural products and their derivatives such as the ansamycin geldanamycin (GM).[3]

Fluorescence polarization (FP) is an assay with wide applicability in the discovery of novel protein modulators. The principle of FP is based on the observation that when a relatively small, fast-tumbling fluorescent-labeled compound is excited with plane-polarized light, the emitted light is random with respect to the plane of polarization, resulting in a lower mP value. When the compound is bound to a bigger molecule (in this case Hsp90), the complex tumbles much slower and the emitted light is polarized, resulting in a higher mP value. Thus, the change of mP reflects the interaction between the fluorescent-labeled compound and the protein. The mP value is proportional to the fractions of bound ligand and the assay is very powerful in measuring real-time protein–inhibitor interactions in solution.[4]

We have previously reported the development of such assay for Hsp90 using BODIPY-labeled geldanamycin (GM) as tracer.[5,10d] While this ligand was a useful tool in evaluating the structure–activity relationship (SAR) in our purine-scaffold Hsp90 inhibitors,[6] its use in high-throughput screening (HTS) may be limited due to interference from test compounds. The two main factors that can modulate such interference are the concentration and the emission wavelength of the fluorophore. When the fluorophore is used at low concentrations, fluorescence contribution from library compounds may have an impact on the assay because the detection readout is a measure of the sum of all fluorescent species in the assay. The fluorophore concentration may be increased to reduce the contribution from other fluorescent compounds. However, this may also require the use of higher concentrations of library compounds in screens. Another shortcoming of such approach is that the assay window may decrease with higher fluorophore concentrations. A better alternative to resolving this issue is the use of red-shifted dyes in labeling of the

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FP ligand.[7] This may limit the interference of fluorescent compounds as fewer library members emit at such long wavelength. Introduction of a red-shifted dye may also help reduce false-positives resulted from light scattering caused by insoluble compounds.

Cy3B is a red-shifted dye that has been found to be particularly well suited for FP due to its increased fluorescence intensity, fluorescence lifetime of ~2.9 ns, and stability of its signal in a variety of aqueous solvent conditions.[8] We present here the use of this dye in the synthesis of a red-shifted cy3B-GM ligand. Further, we evaluate the applicability of this tracer as a FP probe for Hsp90.

Synthesis of the red-shifted Hsp90 ligand.[9] For the synthesis of the cy3B-GM tracer, an amino functionality was first tethered to GM (Scheme 1). 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 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.[10] Reaction of 1,4-diaminobutane with GM progressed rapidly in DMF to result in 17-(4-aminobutylamino)-17-demethoxy-geldanamycin. To limit the formation of possible GM dimers, 2 equiv of 1,4-diaminobutane was used in this transformation. The resulted intermediate was further reacted in DMF with cy3B-NHS using DMAP as catalyst to form the desired ligand as a pinkish-purple compound. Using this methodology we made from 1 mg cy3B-NHS an amount of cy3B-GM ligand sufficient for screening over 1 million library compounds in the 384-well microtiter format.

Evaluation of cy3B-GM as an Hsp90 FP tracer.[11] Next we determined the suitability of cy3B-GM for the Hsp90 FP assay (Fig. 1). To develop a fluorescence polarization assay, the binding affinity of the probe to the protein should be high and the binding range or the assay window (maximum mP at saturation – minimal mP at no protein) should be large. To determine the binding affinities of the fluorescent ligand, we chose to titrate 3 nM fluorescent GM with increasing concentrations of Hsp90 α (0–125 nM).

A stock of 10 μM cy3B-GM was prepared in DMSO and diluted with HFB buffer (20 mM Hepes (K), pH 7.3, 50 mM KCl, 2 mM DTT, 5 mM $MgCl_2$, 20 mM Na_2MoO_4, and 0.01% NP40 with 0.1 mg/mL BGG) to obtain a 6 nM solution. Different amounts of Hsp90 α (Stressgen # SPP776) dissolved in HFB were added to a low binding black 96-well plate (Corning # 3650) in a 50 μL volume. To each well was added 50 μL of the

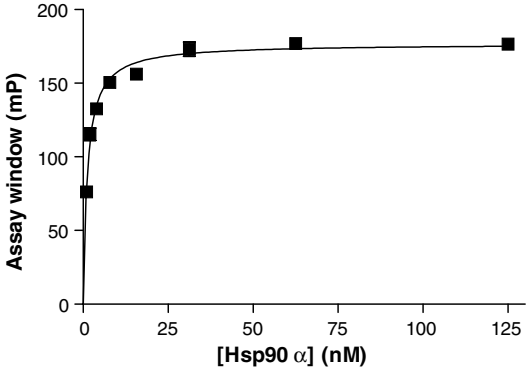
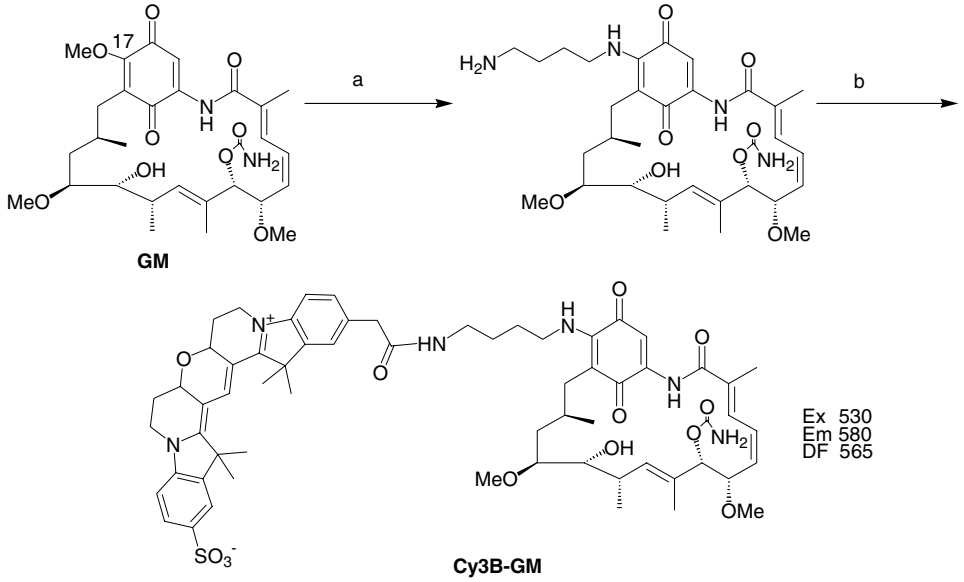


Figure 1. Dose–response curve for the binding of 3 nM cy3B-GM to recombinant Hsp90 α. Different amounts of protein (0–125 nM) were incubated with the ligand at 4 °C and the response measured at 7 h. Fluorescence polarization was read with an Analyst GT instrument. The assay window data were obtained by subtracting free tracer values from values recorded in the presence of specified protein concentrations. Each plot is the average of two experiments.



Scheme 1. Synthesis of the cy3B-GM ligand. Reagents and conditions: (a) 1,4-diaminobutane, DMF, rt, 10 min; (b) cy3B-NCS, DMAP, DMF, rt, 1.5 h.

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 7 h and the FP values in mP were measured (Fig. 1).[11]

As shown in Figure 1, at lower Hsp90 concentrations, a low mP value was obtained; as the concentration of Hsp90 increased, a greater fraction of fluorescent GM bound to the chaperone and polarization progressively increased to reach saturation. The titration curve showed that cy3B-GM bound to Hsp90 α with low nanomolar affinity. A good dynamic range of approximately 170 mP was also measured for the assay.

Validation of cy3B-GM as an Hsp90 FP tracer. Competitive displacement studies were performed with the Hsp90 inhibitors 17AAG,[12] PU-H71,[6b] PU24FCl,[13] and ADP.[2] Binding affinities to Hsp90 have been determined for these agents by independent methods and further, their cellular activity is known.[5,6b,13,14]

These Hsp90 inhibitors, initially dissolved in DMSO, were serially diluted in the HFB binding buffer. Cy3B-GM and Hsp90 α were added at 3 and 30 nM concentrations, respectively. At 30 nM Hsp90 α, the assay window is close to the maximum value (Fig. 1), making it more sensitive. The plate was left on a shaker at 4 °C for 7 h and the FP values in mP were recorded. EC_{50} values were determined as the competitor concentrations where 50% of the tracer was displaced (Fig. 2). EC_{50} for these compounds were determined to be as follows: 64.9 ± 3.8 nM for 17AAG, 59.6 ± 5.7 nM for PU-H71, 1200 ± 60 nM for PU24FCl, and 120 μM for ADP. These data correspond well with the cellular activity of these compounds. A nanomolar biological activity was determined for 17AAG in various cellular systems.[1a,12] PU24FCl and PU-H71 are both derivatives of the PU-scaffold class.[15] The low micromolar potency (1–6 μM) of PU24FCl[13b] and the 50–150 nM potency of PU-H71[5b] determined in several cancer cells correlate well

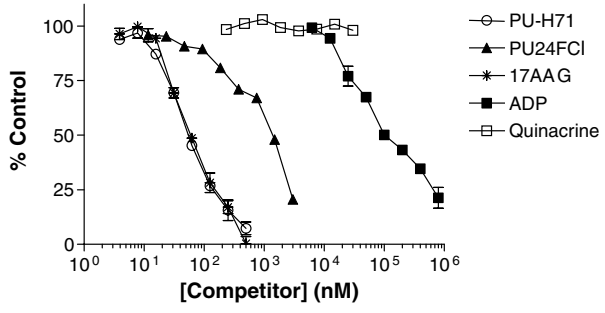


Figure 2. Binding of cy3B-GM to Hsp90 α is competed by known Hsp90 inhibitors. PU-H71 (open circles), PU24FCl (solid triangles), 17AAG (asterisk), ADP (solid square), and the fluorescent dye quinacrine (open squares) were serially diluted in buffer on a 96-well plate. A mixture of 30 nM Hsp90 α and 3 nM cy3B-GM was added and the plate incubated at 4 °C for 7 h. Values recorded in wells with added inhibitor were normalized to data in control wells and plotted against the concentration of tested inhibitor. Drugs were added in triplicate wells.

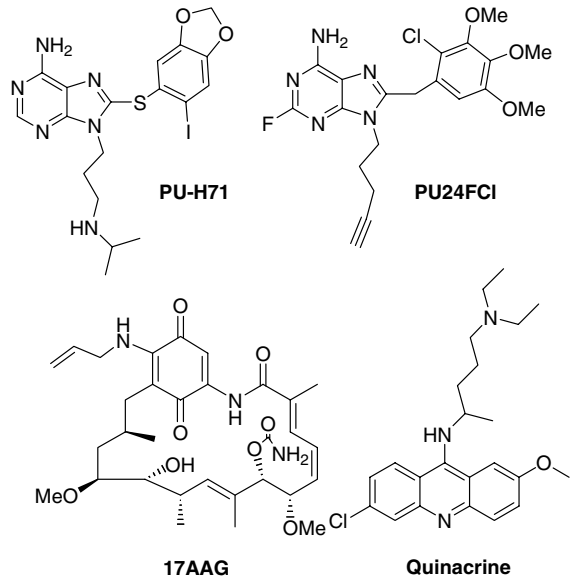


Figure 3. Chemical representation of known Hsp90 inhibitors PU-H71, PU24FCl, and 17AAG. The highly fluorescent LOPAC™ library member, quinacrine, is also depicted.

with the EC_{50}s of 1.2 μM and 60 nM, respectively, recorded with cy3B-GM in the Hsp90 FP assay (Fig. 3).

The LOPAC™ compound quinacrine, described by a Schering–Plough team as the highest autofluorescent compound of that library,[7] was used to test assay interference from autofluorescent compounds. Quinacrine at 3 μM interfered with readings at 5 nM BODIPY-GM concentrations.[5] However, the red-shifted cy3B-GM is free of interference from 30 μM quinacrine even at 3 nM (Fig. 2, open squares).

In summary, we present here the synthesis of a fluorescent tool for probing Hsp90 binding. This agent may be used both in HTS efforts aimed at identifying novel Hsp90 inhibitory compounds and in evaluating chemistry directed at improving the SAR around a known scaffold.

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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.2006.06.025.

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9. Cy3B-GM. To an Eppendorf vial containing 23 μL of 1 M solution of 1,4-diaminobutane (0.023 mmol) in dry DMF was added in 25 μL aliquots a solution of GM (6.4 mg, 0.011 mmol) in dry DMF (0.2 mL). The mixture was vortexed after each addition. The solvent was quickly removed under high vacuum, and the mixture was applied to a preparative TLC plate. Purification was obtained eluting with CH_2Cl_2–MeOH (2% TEA) at 2:1 to afford 6 mg of the product as a purple solid. R_f (CH_2Cl_2–MeOH (2% TEA)) 0.4. This intermediate (1.2 mg, 0.002 mmol) was added to the cy3B-NHS ester (GE Healthcare#25190114) (1 mg, 0.002 mmol) in dry DMF (50 μL) containing a catalytic amount of DMAP. The resulted mixture was stirred for 1.5 h and applied for purification to an HPLC system. The product was obtained as a pinkish-purple solid (1 mg, 72% yield). MS *m*/*z* 1181.3 (M+Na).
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11. FP measurements were performed on an Analyst GT instrument (Molecular Devices, Sunnyvale, CA). Measurements were taken in black 96-well microtiter plates (Corning # 3650) where both the excitation and the emission occur from the top of the wells. In the Analyst GT, a xenon arc lamp provides excitation light that passes through an excitation filter and then a polarizer filter. A beam-splitter filter directs the polarized excitation light into the well and emitted fluorescence transmits back through the same beam-splitter filter, through a polarizer filter, then through the emission filter for detection. In this study, read time was 0.1 s per well. The excitation polarizer filter is fixed in the parallel position, while the emission polarizer filter is changed for measuring parallel and perpendicular emission fluorescence intensity. All polarization values are expressed in millipolarization (mP) units. The mP values were calculated using the equation $mP = 1000 \times [(I_S - I_{SB}) - (I_P - I_{PB})]/[(I_S - I_{SB}) + (I_P - I_{PB})]$, where I_S is the parallel emission intensity measurement and I_P is the perpendicular emission intensity sample measurement, while I_{SB} and I_{SP} are the corresponding measurements for background (buffer). Total fluorescence was determined as $2 \times I_P + I_S$. For cy3B-labeled GM, measurements were made with excitation at 530 nm (25-nm bandwidth) and emission at 580 nm (10-nm bandwidth) using a 565 nm dichroic filter. Data were imported into SoftMaxPro4 and analyzed in GraphPad Prizm4.
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