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Research Paper

A small molecule designed to bind to the adenine nucleotide pocket of Hsp90 causes Her2 degradation and the growth arrest and differentiation of breast cancer cells

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Abstract

Background: The Hsp90s contain a conserved pocket that binds ATP/ADP and plays an important role in the regulation of chaperone function. Occupancy of this pocket by several natural products (geldanamycin (GM) and radicicol) alters Hsp90 function and results in the degradation of a subset of proteins (i.e. steroid receptors, Her2, Raf). We have used the structural features of this pocket to design a small molecule inhibitor of Hsp90.

Results: The designed small molecule PU3 competes with GM for Hsp90 binding with a relative affinity of 15–20 μM. PU3 induces degradation of proteins, including Her2, in a manner similar to GM. Furthermore, PU3 inhibits the growth of breast cancer cells causing retinoblastoma protein hypophosphorylation, G1 arrest and differentiation.

Conclusions: PU3 is representative of a novel class of synthetic compounds that binds to Hsp90 and inhibits the proliferation of cancer cells. These reagents could provide a new strategy for the treatment of cancers.

Keywords: Her2; Hsp90 inhibitor; Novel anti-tumor agent; Geldanamycin

1. Introduction

The Hsp90 family of chaperones is comprised of four known members: Hsp90 α, Hsp90 β, Grp94 and Trap-1. Hsp90 is an abundant cellular chaperone required for the ATP-dependent refolding of denatured or 'unfolded' proteins and for the conformational maturation of a variety of key proteins involved in the growth response of the cell to extracellular factors. These include the steroid receptors as well as the Raf serine kinase and some tyrosine kinases [1–7]. Hsp90 is also thought to play a role in maintaining the stability and function of mutated proteins. It seems to be required for the expression of mutated p53 and *v*-src, but is required to a much lesser extent or not at all for their wild-type counterparts [8,9].

Hsp90s have been shown to be overexpressed in multiple tumor types and as a function of oncogenic transformation [10]. Although mutational analysis suggests that Hsp90 is essential for eukaryotic cell survival, cancer cells seem to be especially sensitive to short-term pharmacological interference with the chaperone's activity suggesting that Hsp90s may be a novel target for anti-cancer drug development [11].

The Hsp90 family members possess a unique pocket in their N-terminal region that is specific to and conserved among all Hsp90s from bacteria to mammals, but is not present in other molecular chaperones [12]. The pocket binds ATP and ADP with low affinity and has weak ATPase activity [13]. The bent shape adopted by the nucleotides when inside this pocket is very peculiar and is not observed in other chaperones known to have ATP/ADP pockets. However, in the somewhat structurally homolo-

Abbreviations: GM, geldanamycin; RD, radicicol; 17AAG, 17-allylamino-17-demethoxygeldanamycin; MFGM, milk fat globule membrane protein; Rb, retinoblastoma protein; H&E, hematoxylin and eosin; TEA, triethylamine

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gous proteins DNA gyrase and MutL, the adenine nucleotides adopt a similar conformation.

The ansamycin antibiotics geldanamycin and herbimycin (GM, HA) and radicicol (RD) (Fig. 1) (originally isolated on the basis of their ability to cause the reversion of cells transformed by the *v*-src protooncogene) were also determined to bind to this Hsp90 pocket, with much higher affinities than the adenine nucleotides [14–16].

The last step in the refolding of a protein is its ATP-dependent dissociation from Hsp90 and other chaperones involved in the process. ADP inhibits the formation of the complex, locking the protein in a different conformation. It is therefore suggested that the nucleotide functions as a molecular switch regulating two opposing conformational states of Hsp90. The inhibitors seem to be mimicking the ADP-bound state. Occupancy of the pocket by the natural products prevents the dissociation of the proteins from the chaperone complex and as a consequence, the trapped proteins do not achieve their mature functional conformation. Instead they undergo ubiquitin-dependent degradation in the proteasome [1–7].

Addition of ansamycins to cells induces the proteasomal degradation of a small subset of proteins involved in signal transduction. These include the steroid receptors and Raf kinase, as well as certain transmembrane tyrosine kinases. Of these, the Met and Her2 tyrosine kinases are among the most sensitive targets described. In addition, GM causes the selective degradation of certain proteins that undergo mutation in cancer, such as *v*-src, bcr-abl and p53, but has little or no effect on their normal counterparts. Most cellular proteins are unaffected by the drugs [1–7].

Since the Hsp90 chaperones are the only proteins shown to directly interact with GM, HA and RD it is likely that their biologic effects are due to binding to these proteins. Drugs that target Hsp90 in cancer cells and therefore stimulate depletion of oncogenic proteins could be of clinical benefit. 17-Allylamino-GM (17AAG) is currently in Phase I clinical trial at several institutions including ours. However, its relative insolubility makes it difficult to formulate and administer. It is not easily synthesized and currently must be generated by fermentation. The dose limiting toxicity of ansamycins is hepatic and may be secondary to the nucleophilicity of the quinone and not to its binding to Hsp90. Additionally, Hsp90 is a family of at least four known members, which contain homologous but not identical N-terminal ATP/ADP binding pockets. These proteins are likely to have specific biological roles and it is possible that the effects of GM and RD are a sum of unselective binding to all the family members. Molecules that could differentiate among the four proteins would thus constitute important tools for the study of their individual functions and could have selective activity against specific cancers. In order to evaluate such possibilities we set out to develop a soluble and easily synthesizable small molecule that binds to the Hsp90 pocket and shares biological activity with GM and RD. Once this goal is achieved, the structure could be used for the development of selective Hsp90 inhibitors with specific cellular effects.

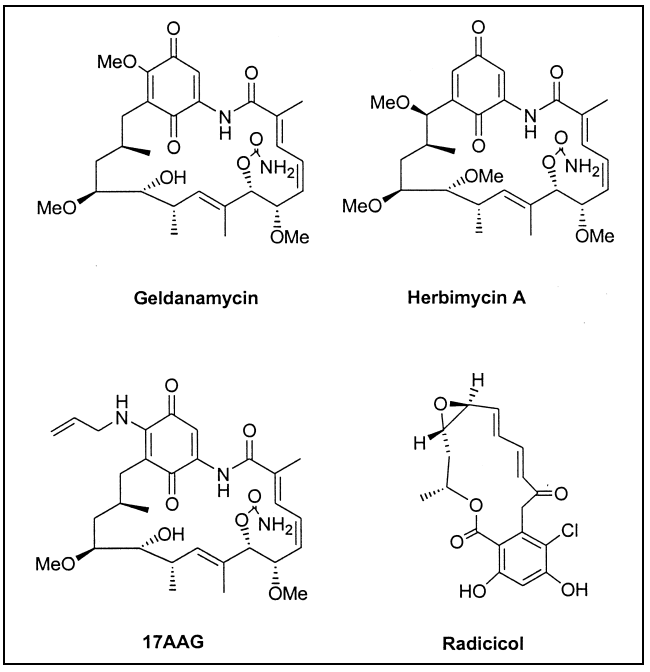

Fig. 1. Structural representation of the known Hsp90 inhibitors.

2. Results and discussion

2.1. Design of the Hsp90 inhibitor

A detailed study of the mode of binding of GM (Protein Data Bank code 1YET), RD (code 1BGQ) and ADP (code 1AMW) to the Hsp90 N-terminal ADP/ATP binding site revealed several important factors to be considered in designing a molecule that would interact with this pocket: (1) a key interaction between the inhibitor and the Asp93/Ser52 at the base of the pocket, (2) interactions at the top involving Lys112, Lys58 and hydrogen acceptor functionalities on the inhibitor, (3) a hydrophobic pocket that lies midway in the binding site and that is constituted by Met98, Val150, Leu107, Leu103, Phe138 and Val186 (the amino acid numbering as in human Hsp90 α). Additionally, the inhibitor should adopt a folded (C-shaped) conformation even in the free state. A structure with minimal structural changes from unbound to complexed state should not pay much entropic penalty and binding would be predominantly determined by enthalpic factors. Lastly, but not least important, the molecule should have a higher affinity than ADP for Hsp90.

More than 10% of the proteins encoded by the yeast genome depend on purine-containing ligands for their function. It is therefore expected that diverse purine libraries would yield bioactive compounds with good solubility

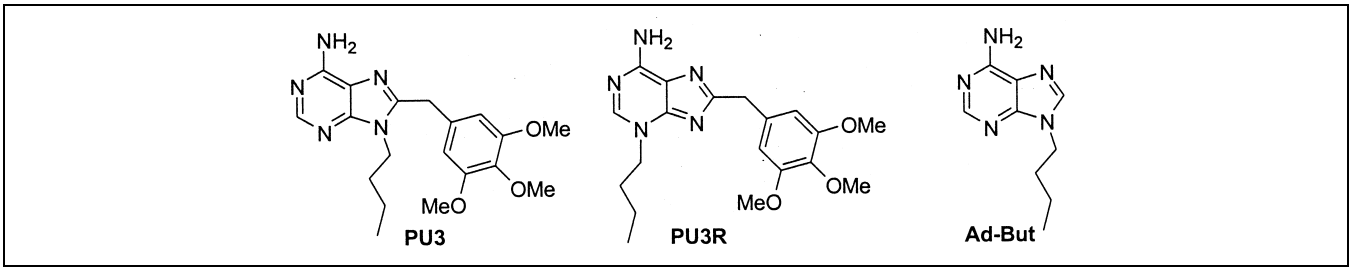

Fig. 2. Structures of the designed Hsp90 inhibitor PU3 and of two control compounds PU3R and Ad-But.

and cell permeability. Selectivity for one protein over another should be achievable by correspondingly functionalizing the skeleton, which is easily carried out in this class of molecules due to the significant possibilities of performing chemistry on the purine skeleton. Although the first and second sets of interactions mentioned above are conserved in the homologous pockets of DNA gyrase and MutL, the components of the pocket wall and hydrophobic pocket are entirely different. This fundamental difference makes it possible to design purine derivatives selec-

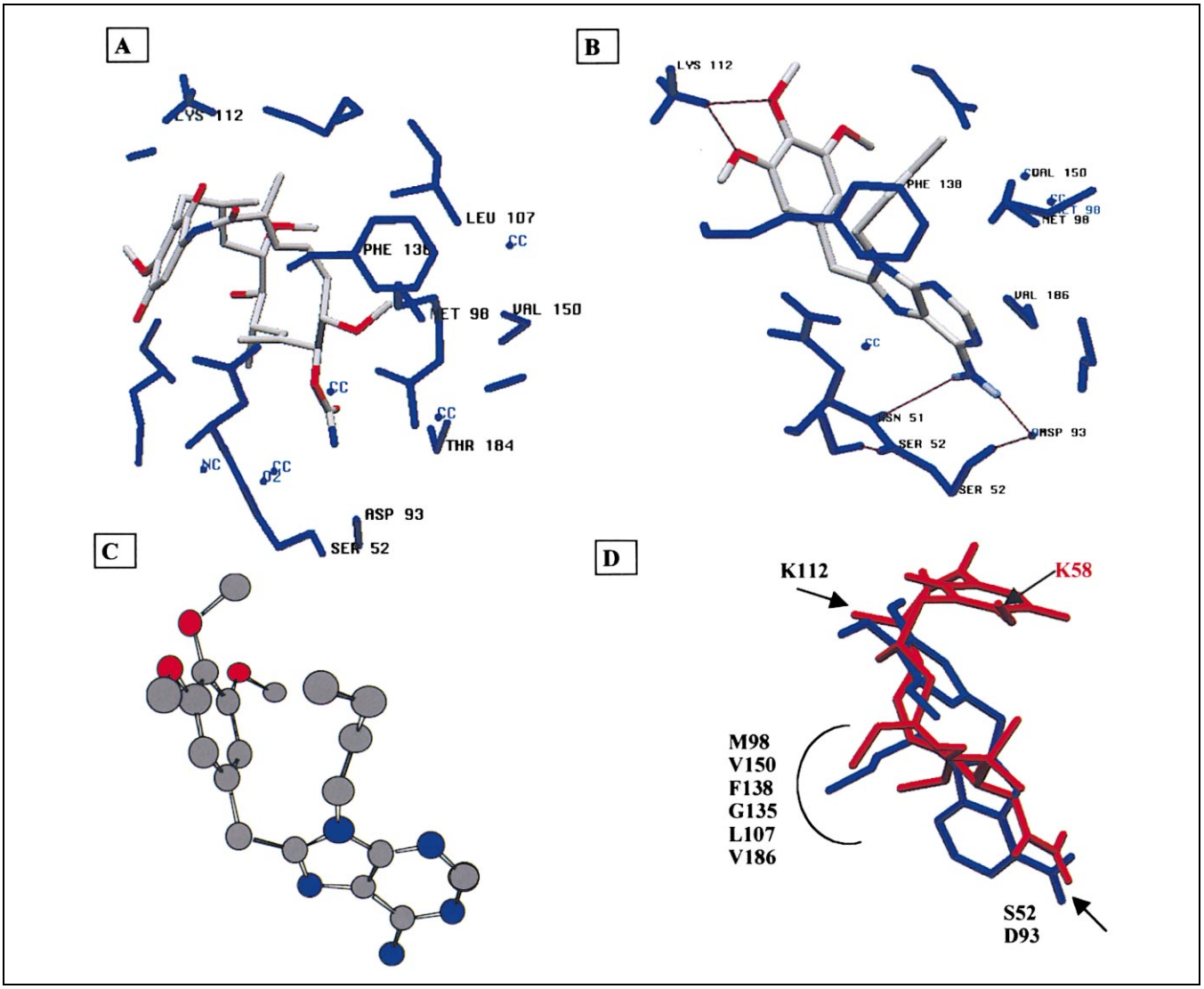

Fig. 3. (A) Crystal structure of GM bound to Hsp90 (ID code 1YET). (B) Structure of PU3 docked into the same pocket. (C) Crystal structure of PU3. The protein backbone is represented in blue, while for the inhibitors the carbon backbone is drawn in gray, N atoms in blue and O atoms in red. (D) Superimposed representation of GM and the modeled PU3 in the ADP binding pocket of Hsp90 α. Important interactions shared by the two molecules with the pocket are represented in black. GM = red, PU3 = blue.

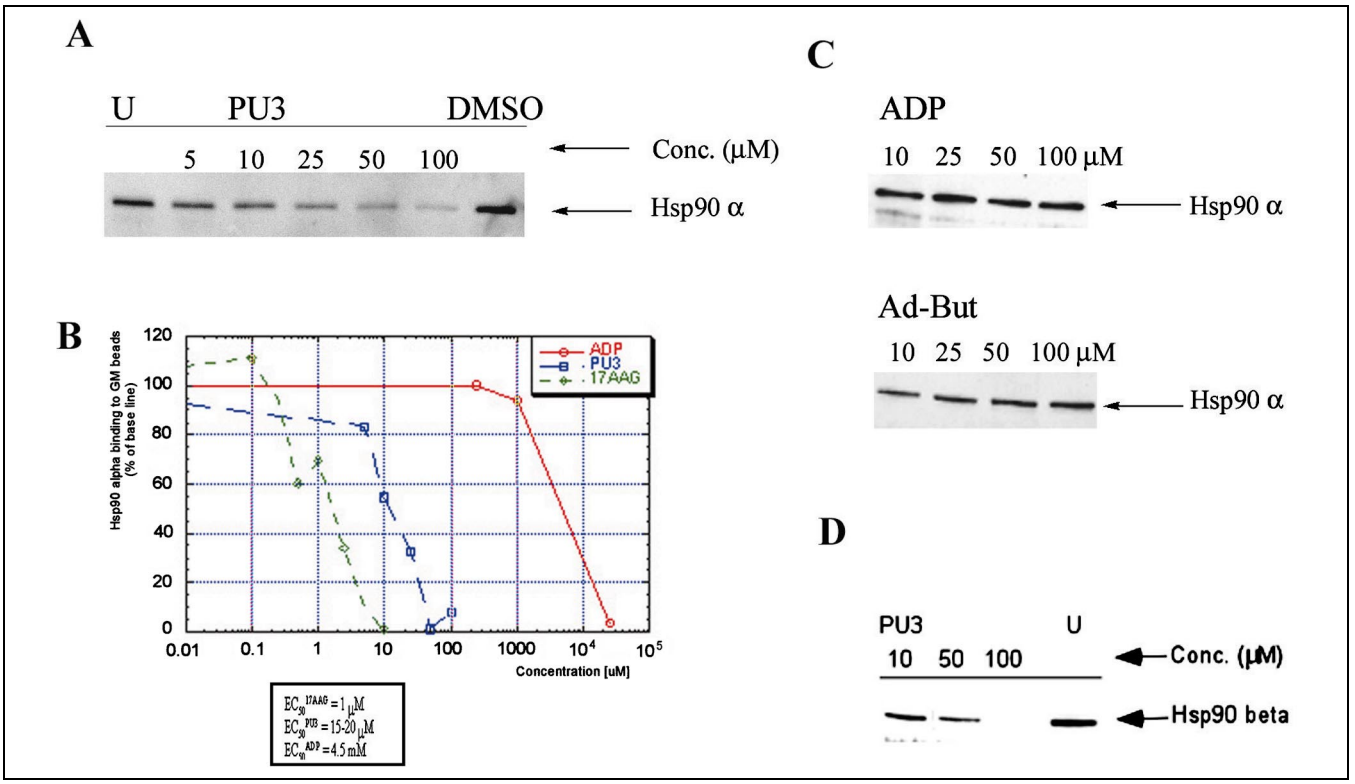

Fig. 4. PU3 competes with GM for Hsp90 binding. Hsp90 protein was incubated with GM affinity beads and its binding to the immobilized GM was competed with increased concentrations of PU3 (A), ADP and Ad-But (C). Data obtained from image analysis of the competition of Hsp90 α by PU3, 17AAG and ADP were graphed as shown in B. (D) Rabbit reticulocyte lysate was used as source of protein and Hsp90 β binding to the GM beads was assessed. U = base-line binding of Hsp90 to the GM beads.

tive for the several homologous pockets, since the size and nature of the purine 9-N-alkyl can be easily modified.

Based on all of the above observations we theoretically designed the molecule PU3 (Fig. 2) and docked the structure into the Hsp90 α ADP/ADP binding site. The molecule showed a good theoretical fit and fulfilled all of the important interactions with the protein pocket (Fig. 3B). At the top, the Lys112 interacts with two of the methoxy groups on the phenyl moiety, the butyl chain occupies the described hydrophobic pocket, while at the base, Asp93 and Ser52 are hydrogen-bound to the purine NH_2. The structure of the molecule was determined by X-ray crystallography and is very similar to the one determined by computer modeling, suggesting that PU3 adopts a folded shape in both the free and bound states (Fig. 3C). GM crystallizes from organic solvents in significantly more open, extended conformation [17] which according to Roe et al. [16] accounts for weaker binding affinity of GM compared to RD, a molecule that binds to Hsp90 in the conformation observed in the free crystal state.

PU3 occupies most of the pocket filled by GM and extends more deeply into the hydrophobic wall. However, the small molecule lacks the interaction with Lys58 that GM establishes through its methoxy group on the benzoquinone (Fig. 3D). This would probably result in a lower affinity of PU3 for Hsp90 and points for future improvements of the PU3 molecule.

The 'rule of 5' of Lipinsky [18] predicts that this molecule should have acceptable pharmacokinetics. PU3 has one hydrogen bond donor, the sum of Ns and Os is 8, has a molecular weight of 371 and a M log P value of 2.2. This suggests that PU3 is a reasonable starting point for the development of bioavailable drugs.

2.2. PU3 binds to Hsp90 in vitro

GM and RD were shown to bind full-length dimeric Hsp90 with K_ds of 1.2 μM and 19 nM, respectively [16]. We determined the concentration of PU3 required to compete with GM for Hsp90 binding in order to obtain a relative binding affinity for this compound. GM was immobilized on Affigel 10 resin (Bio-Rad) via a method slightly modified from described. [19]. Preincubation of purified Hsp90 with PU3 inhibited binding of the protein to immobilized GM with an EC_{50} of 15–20 μM, whereas only about 1 μM of 17AAG was required under similar conditions (Fig. 4B). These numbers suggest that our designed first generation inhibitor binds to Hsp90 α at an affinity approximately 20-fold weaker than does 17AAG. ADP and the control compound Ad-But, a molecule that lacks the trimethoxybenzyl moiety of PU3, exhibited no activity at similar concentrations (Fig. 4C). More than 4.5 mM ADP was necessary to compete with GM for 50% of Hsp90 binding. Rabbit reticulocyte lysates were addition-

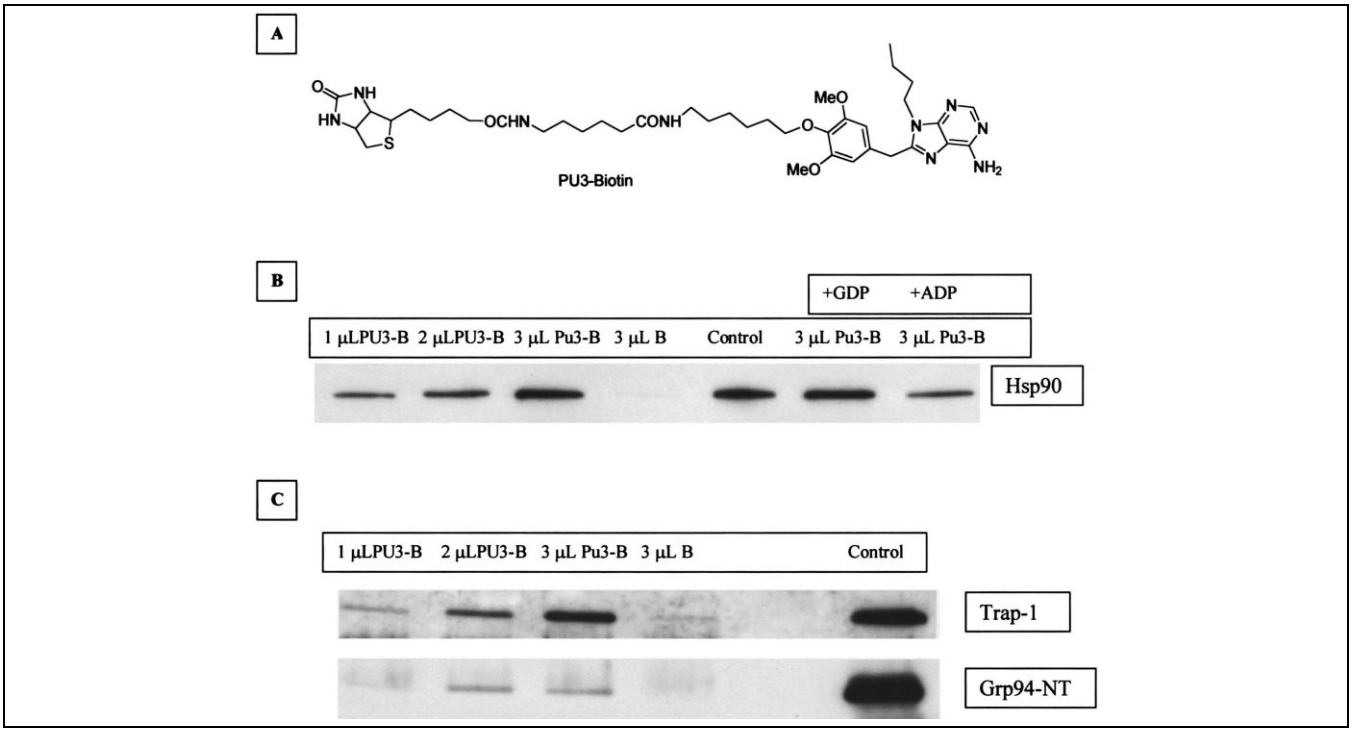

Fig. 5. Affinity immobilization of Hsp90, Trap-1 and Grp94 N-terminal (1–355) by PU3. (A) Structure of the PU3-biotin derivative. (B and C) An increasing amount of PU3-B was used with a constant amount of streptavidin beads and protein. Specificity of binding is demonstrated by the absence in binding of proteins to the streptavidin-biotin beads alone and competition of binding by ADP and not GDP. Control = specific protein loaded as marker (amounts differ). B = biotin.

ally employed as a source of Hsp90 proteins for competition assays (Fig. 4D) demonstrating that PU3 is a potential inhibitor of both Hsp90 α and β.

The 4-methoxy functionality of PU3 points outside the pocket when the molecule is bound to Hsp90 (Fig. 3). Molecular modeling suggests that a modification at that position will still allow binding to the pocket. Therefore, a biotinylated PU3 (PU3-B) was constructed (Fig. 5A) and attached to a streptavidin solid support to study whether this molecule is able to affinity immobilize the members of the Hsp90 family. The molecule specifically binds to Hsp90 and Trap-1 as well as the N-terminal fragment of Grp94 that contains the ADP/ATP binding pocket (Fig. 5B,C).

2.3. PU3 depletes cancer cells of Hsp90-dependent signaling proteins

GM binding to Hsp90 affects the function and stability of steroid receptors and certain signaling proteins including Raf1 and Her2. PU3 affects this subset of proteins in a similar fashion (Fig. 6). As expected, PU3 destabilizes the estrogen receptor complexes and induces dose-dependent degradation of the protein (Fig. 6A). The tyrosine kinase Her2 is one of the most sensitive targets of GM. PU3 causes a rapid decrease in Her2 levels; more than 50% of Her2 is degraded less than 3 h after drug treatment (Fig. 6B). Addition of PU3 causes degradation of Her2 in both MCF-7, a breast cancer cell line with moderate Her2 expression, as well as in SKBr3, a Her2 overexpressing line. The effects are seen at doses as low as 10 μM (Fig. 6A). Like GM, PU3 causes the accumulation of a lower migrating band that is thought to represent incompletely glycosylated Her2 sequestered in the endoplasmic reticulum (170 kDa vs. 180 kDa mature Her2). However, the levels of Raf1 and Her3 are less sensitive and more than 50 μM drug is necessary to bring these proteins to basal levels. The control derivatives Ad-But (Fig. 6C) and PU3R (data not shown) had no effect on Her2 or Raf1 levels at similar concentrations. The set of proteins degraded by PU3 is limited to those sensitive to GM. The compound has no effect on the levels of tubulin, PI3 kinase (PI3K) (p85 unit) and β-actin, proteins also unaffected by GM and RD.

2.4. PU3 induces the synthesis of Hsp90

It is known that ansamycins induce the synthesis of Hsp90 and Hsp70 [19,20] and we observe the same phenomena following treatment of MCF-7 cells with PU3. Treatment of cells with 17AAG induces a protein band that migrates more rapidly than Trap-1 and that is recognized by an anti-Trap-1 antibody (Fig. 7A). A similar effect is observed after PU3 addition, but not with the control PU3 isomer, PU3R (Fig. 7B). Although the identity of this protein band is unknown,

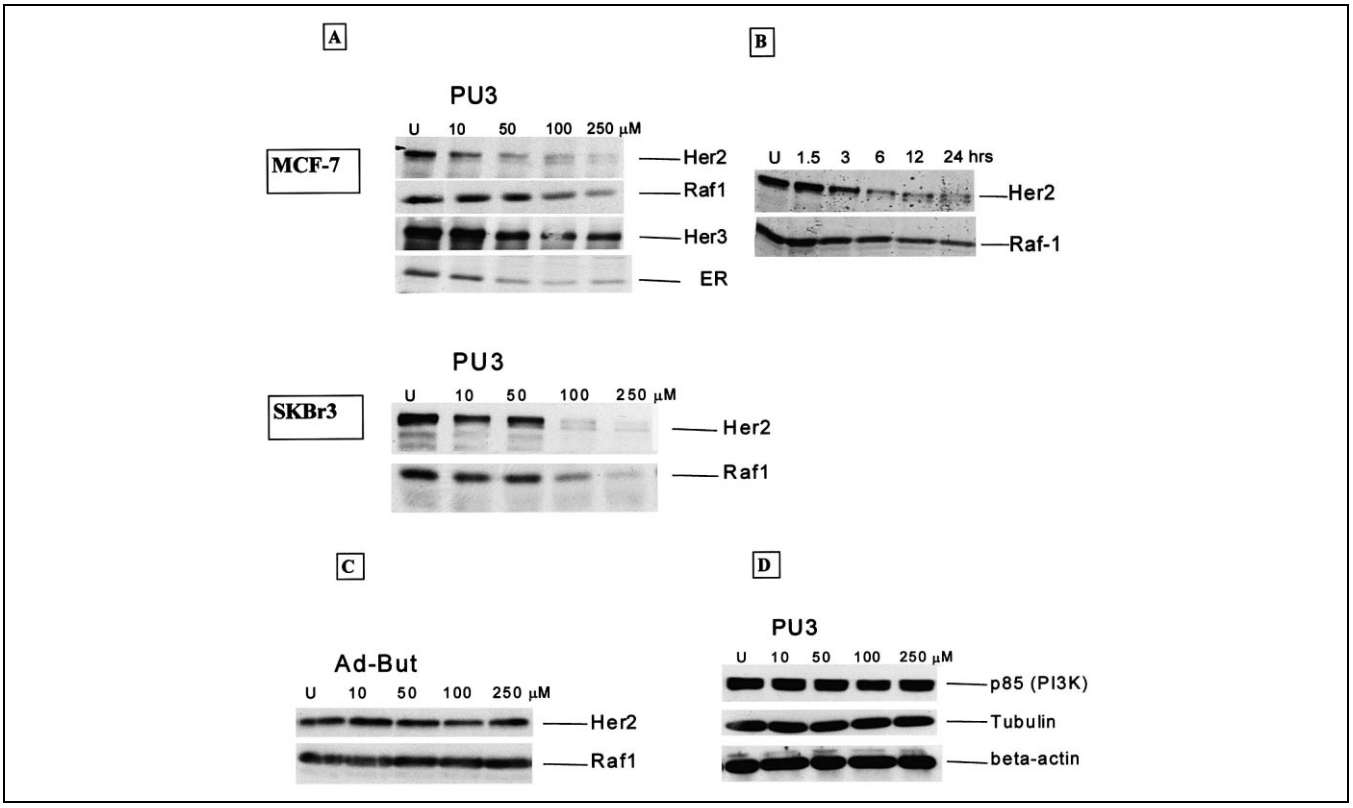

Fig. 6. PU3 induces the degradation of a subset of proteins in the MCF-7 and SKBr3 breast cancer cells in a fashion similar to GM. (A) MCF-7 and SKBr3 cells were treated with increasing concentrations of drug for 24 h. (B) Time-dependent degradation of Her2 and Raf1 in the MCF-7 cell line by addition of 100 μM PU3. (C) The control Ad-But has no effect on these proteins at similar doses. (D) PU3 has no effect on protein levels involved in different signaling pathways.

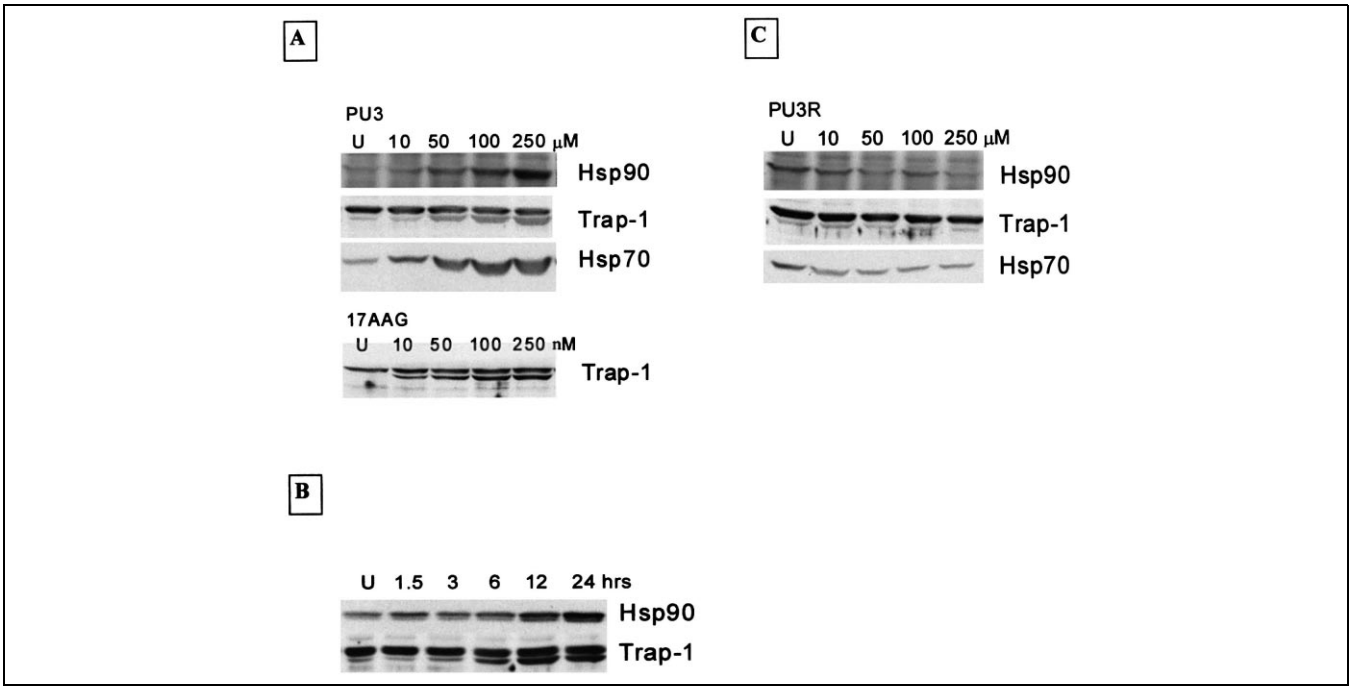

Fig. 7. PU3, like GM, increases the cellular levels of Hsp90 and Hsp70. (A) MCF-7 cells were treated with increasing concentrations of drug for 24 h. Protein levels were assessed by Western blotting. (B) Time-dependent effect of PU3 (100 μM) on protein levels in MCF-7 cell line. (C) The control purine PU3R, an isomer of PU3, has no effect on the studied protein levels at identical concentrations.

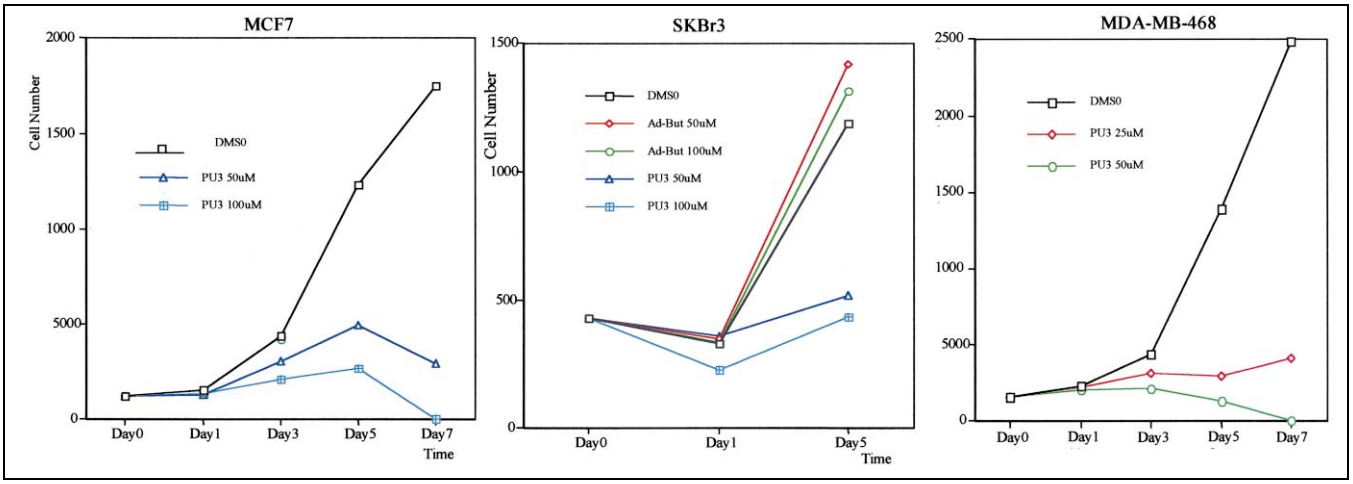

Fig. 8. Antiproliferative effects of PU3 and the control compound Ad-But on MCF-7, SKBr3 and MDA-MB-468 breast cancer cell lines.

its appearance seems to be a marker of cellular exposure to ansamycins.

2.5. *In vitro antiproliferative activity of PU3*

We examined the effect of PU3 on the growth of three breast cancer cell lines: MCF-7, SKBr3 and MDA-MB-468. The compound showed antiproliferative effects at low micromolar concentrations similar to those that compete for Hsp90 binding and induce protein degradation (Fig. 8).

2.6. *PU3 arrests breast cancer cells in G1*

Breast cancer cells treated with PU3 arrest in the G1

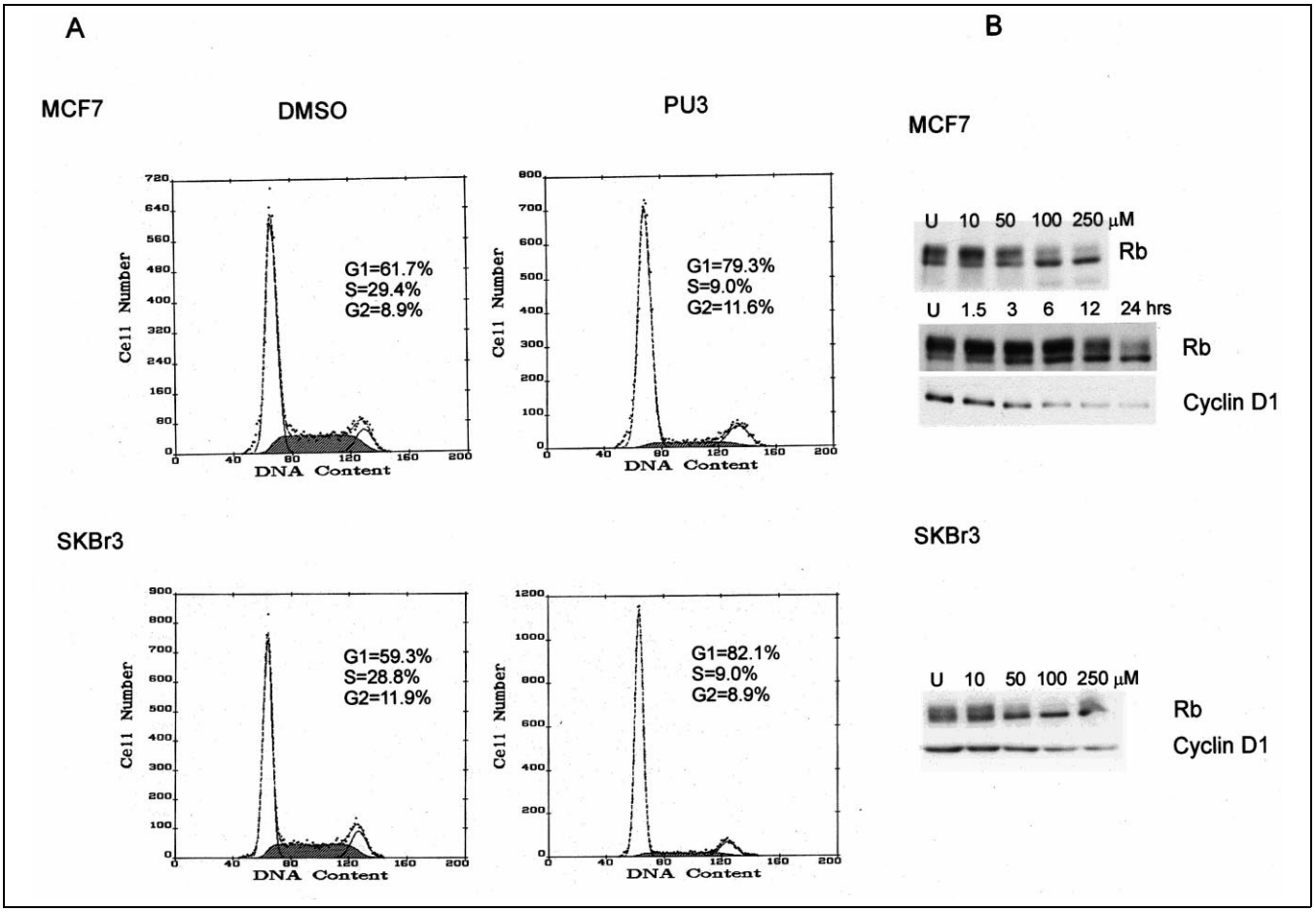

Fig. 9. (A) 100 μM PU3 causes predominantly G1 block associated with (B) reduction in cyclin D1 expression and Rb hypophosphorylation in the breast cancer cell lines MCF-7 and SKBr3.

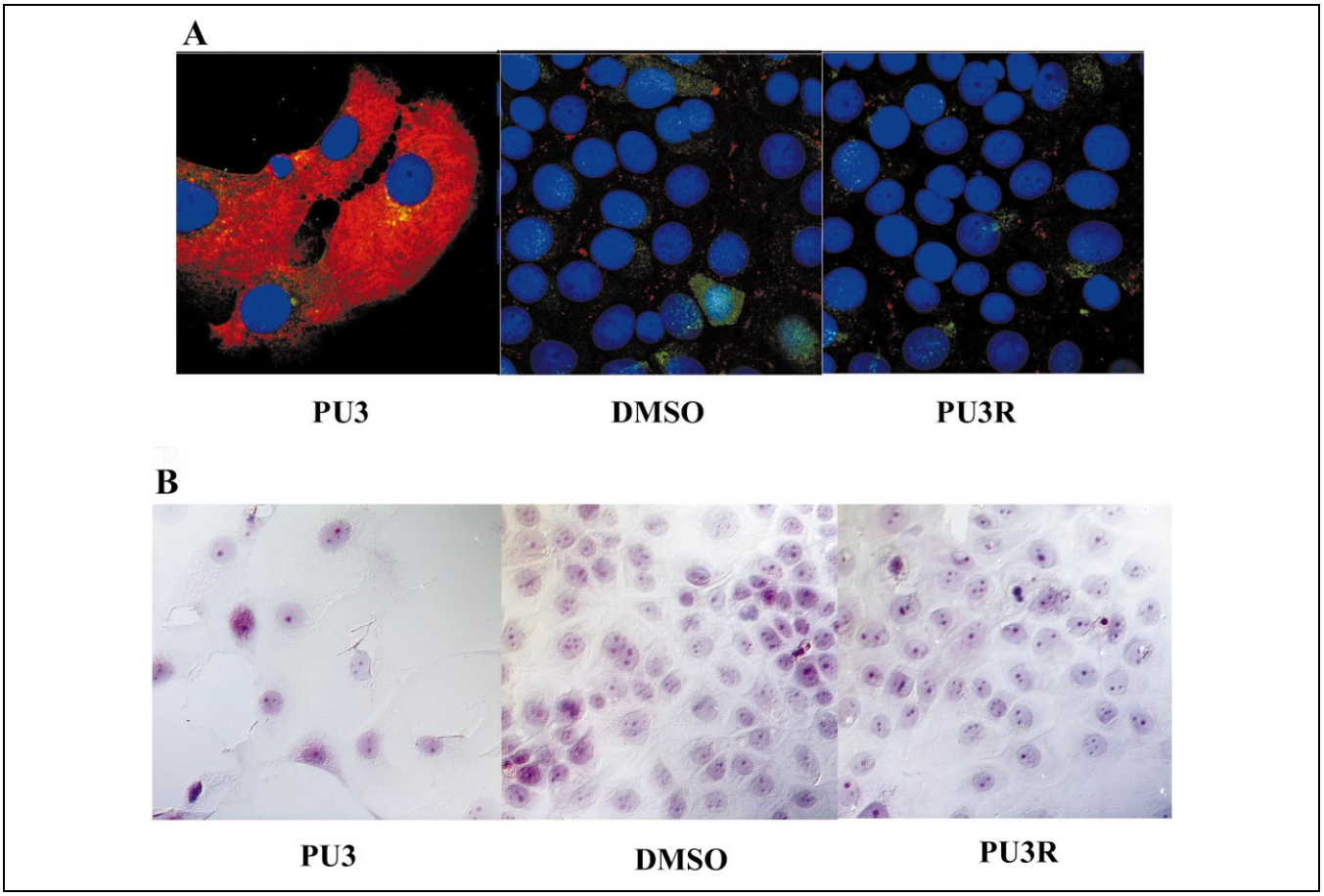

Fig. 10. (A) PU3 induces MFGM protein in MCF-7 cells. MFGM protein was detected with an anti-MFGM antibody and a rhodamine- (red) labeled secondary antibody. Nuclear DNA was stained with DAPI. (B) PU3 induces significant morphological differentiation in the MCF-7 line. Cells were stained with H&E.

phase of the cell cycle and the process is associated with hypophosphorylation of the retinoblastoma protein (Rb) and reduction in D-cyclin expression (Fig. 9). We have previously shown that ansamycins induce the Rb-dependent G1 arrest of epithelial cancer cells [21]. The mechanism of the G1 arrest in breast cancer cells that overexpress Her2 involves down regulation of a Her2-initiated, PI3K- and akt kinase-dependent pathway that is required for efficient expression of cyclin D. This results in rapid reduction of D-cyclin-associated kinase activity and G1 arrest. PU3 causes a similar phenotype (Fig. 9A). The compound causes predominantly G1 block associated with Rb hypophosphorylation at 12 h after treatment and a rapid decrease in cyclin D levels (3–6 h after treatment) (Fig. 9B).

2.7. Induction of morphologic and functional differentiation

In breast cancer cells treated with ansamycins, G1 block is followed by morphological and functional differentiation.

MCF-7 cells are round with scanty eosinophilic cytoplasm, indistinct cell margins and contain the prominent nucleoli characteristic of cancer cells. They express minimal levels of milk fat proteins and contain few fat-containing vacuoles or other features of mammary differentiation. Cells exposed to PU3 flatten, increase in size and have distinct cellular boundaries. These morphological changes are characteristic of mature epithelial differentiation and reversal of transformation (Fig. 10A).

The milk fat globule membrane protein (MFGM) is a component of the milk fat globule membrane, a structure that surrounds the milk fat in breast cells. Breast cancer cells express this protein at low levels, however, addition of 17AAG, a derivative of GM, induces the expression of the protein in the MCF-7 and SKBr3 cell lines [25]. The same phenomenon was observed upon addition of PU3 to the MCF-7 line (Fig. 10B).

The induction of a G1 arrest by a variety of manipulations is sufficient for induction of expression of some milk proteins. However, only ansamycins and the HDAC inhibitor SAHA cause extensive morphological and biochemical changes [25]. We found that the morphological phenotype induced by PU3 is very similar to 17AAG and thus, PU3 is likely to function by the same mechanism.

3. Conclusions

We have used the structure of the co-crystals of Hsp90 and its ligands (GM, RD and adenine nucleotides) to design a novel compound that interacts with the Hsp90–nucleotide binding pocket. The compound competes with GM for binding to Hsp90 α with a relative affinity of 15–20 μM, a potency that is proportional to its efficacy in inducing cellular effects. Addition of PU3 to cells causes the degradation of HER kinases, estrogen receptor and Raf kinase. Control proteins such as PI3K, tubulin and β-actin are unaffected. The proteins affected by PU3 are identical to those affected by ansamycins. Exposure of breast cancer cells to PU3 results in growth inhibition and profound morphologic changes. Growth inhibition results from arrest in the G1 phase of the cell cycle and is associated with reduction in D-cyclin expression and hypophosphorylation of the Rb protein. Cessation of proliferation is accompanied by induction of a more mature, epithelial morphology consistent with breast differentiation. The qualitative effects of PU3 on cellular protein expression, proliferation, and differentiation are very similar to those induced by GM and RD. We concluded that in PU3 we have successfully designed a compound that binds to the Hsp90 family pocket and elicits its biologic effects by regulating the function of these proteins. It is possible that PU3 also interacts with other proteins that contain a similar pocket, such as DNA gyrase or MutL. However, considering the weak homology these proteins have with the Hsp90s, this is unlikely to happen at low drug concentrations.

Taken together, the data suggest that the biologic effects of PU3 and ansamycins are overlapping and result from occupancy of the conserved pocket in Hsp90 family proteins.

4. Significance

The Hsp90 family of chaperones plays a key role in regulating the physiology of cells exposed to environmental stress and in maintaining the malignant phenotype in tumor cells. The function of these proteins may be modulated with compounds that bind to a conserved binding pocket in the amino-terminus of the protein. We have now designed and synthesized a compound, PU3, that binds to this pocket and shares the biologic properties of its natural product ligands. Development of a family of such compounds may allow the selection and identification of molecules that interact specifically with individual members of the Hsp90 family. Such reagents will be useful probes with which to study the individual members of the Hsp90 family and have potential utility as drugs.

Both ansamycins and PU3 cause the degradation of a subset of key signaling proteins and inhibit the proliferation of human cancer cells. The Her2 transmembrane tyrosine kinase is one of the most sensitive targets of these drugs. Her2 has oncogenic properties and is overexpressed in many human tumors, including approximately 30% of human breast cancers. Recently, an anti-Her2 antibody was shown to have limited therapeutic utility in these patients when given alone or in combination with cytotoxic chemotherapy. Breast cancer cells that express high levels of Her2 are quite sensitive to PU3 and 17AAG. The ansamycin derivative, 17AAG, is currently in early clinical trial in cancer patients. 17AAG is a potent anti-cancer drug, but it is poorly soluble, obtainable only by fermentation, and induces toxicities that are probably unrelated to Hsp90 binding. PU3 is less potent, but it is a first generation lead compound. It is likely that further medicinal chemistry will yield derivatives with increased binding affinity and activity. Moreover, PU3 is soluble, easily synthesized, and may be less toxic and adaptable to oral administration. The development of this novel class of small molecules that induces the destruction of Her2 could lead to a new method for the treatment of advanced breast cancer.

5. Materials and methods

PU3 and the control molecules were synthesized as described [22]. Trap-1 protein was a gift from Dr. Sara Felts and the N-terminal domain of Grp94 (1–355) was kindly provided by Dr. Shawn Vogen (Dr. Argon Yair's laboratory).

5.1. Computer modeling

The designed molecules were manually docked in the N-terminal Hsp90 α ADP/ATP binding pocket (Protein Data Bank code 1YET) and the complexes were minimized using the generalized born/surface area solvation method for water and AMBER* force field as implemented in version 6.5 Macromodel.

5.2. Immobilization of GM

To 2 ml of wet beads (Bio-Rad, Affigel 10) in 2 ml CH_2Cl_2 was added 50 mg of 1,4-diaminobutane and the mixture was shaken for 2 h at room temperature (RT). The beads were washed with 3×5 ml CH_2Cl_2/triethylamine (TEA) = 9:1, 3×5 ml DMF, 3×5 ml 2-PrOH and 1×5 ml DMF, followed by addition of 17 mg GM and 10 μl TEA in 1 ml DMF. The slurry was rotated overnight and then washed with 2×5 ml CH_2Cl_2, 2×5 ml 2-PrOH and again 2×5 ml CH_2Cl_2. The unreacted amines were capped by addition of excess Ac_2O–TEA in 1 ml CH_2Cl_2 (until no positive ninhydrin test, about 15–25 min). After extensive washes with 4×5 ml CH_2Cl_2 and 4×5 ml 2-PrOH, the GM beads were rotated overnight in 2-PrOH to allow for significant swelling and then washed and transferred to the appropriate buffer.

5.3. Hsp90 competition

The GM beads were blocked for 1 h at 4°C with 0.5% bovine serum albumin (BSA) in TEN buffer (50 mM Tris–HCl pH 7.4, 1 mM EDTA, 1% NP-40) prior to use. Hsp90 protein (Stressgen) (0.2 μg) or 2 μl of rabbit reticulocyte lysate (Promega) was incubated with or without drugs for 15 min on ice. To each sample were added 20 μl GM beads (wet volume) and the mixtures were rotated at 4°C for 1 h followed by two washes with 500 μl ice-cold TEN each. The GM beads-bound protein was eluted from the solid phase by heating in 35 μl 1×sodium dodecyl sulfate (SDS), analyzed by SDS–PAGE and visualized by immunoblotting with Hsp90 α (Stressgen #SPA-840) or Hsp90 β (Neomarkers) antibodies.

5.4. Preparation and use of the PU3-biotin molecule

PU3-biotin was assembled by selective deprotection of the 4-methoxy functionality of PU3 using β-bromo-9-BBN, followed by addition of an intermediate linker via a Mitsunobu reaction and finally, biotin was introduced by formation of an amide bond between the product and EZ-Link™ NHS-LC-Biotin (Pierce #21336). PU3-biotin was suspended in DMSO to yield a 100 mM solution. A volume of 1, 2 or 3 μl of this solution was added to 80 μl slurry (50% solution) of Sepharose-streptavidin (Zymed #43-4341) in phosphate-buffered saline (PBS) and incubated at RT for 20 min. The beads were washed extensively with 1×PBS–0.2% Tween, 1×PBS containing protease inhibitors, followed by 2×PBS–0.2% Tween. To the wet beads was added 0.3 μg protein in 40 μl RSBT buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.1% Tween) and the mix was incubated for 1 h at 4°C. The unbound protein was removed by washing with PBS–0.2% Tween. Proteins bound to the affinity matrix were eluted by boiling in 60 μl 1×SDS and separated by 10% SDS–PAGE. After transfer to nitrocellulose, the membranes were probed with anti-Trap-1 (MSK81), anti-Hsp90 α (Stressgen #SPA-840) and anti-Grp94 (Stressgen #SPA-850).

5.5. Cell culture

The human cancer cell lines MCF-7, SKBr3 and MDA-MB-468 were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in 1:1 mixture of DME:F12 supplemented with 2 mM glutamine, 50 U/ml penicillin, 50 U/ml streptomycin and 5% (for MCF-7 and MDA-MB-468) or 10% (for SKBr3) heat-inactivated fetal bovine serum (Gemini Bioproducts) and incubated at 37°C in 5% CO_2.

5.6. Protein assays

Cells were grown to 60–70% confluence and exposed to drugs or DMSO vehicle for the indicated time periods. Lysates were prepared using 50 mM Tris pH 7.4, 2% SDS and 10% glycerol lysis buffer. Protein concentration was determined using the BCA kit (Pierce Chemical Co.), according to the manufacturer's instructions. Clarified protein lysates (20–50 μg) were electrophoretically resolved on denaturing SDS–PAGE, transferred to nitrocellulose and probed with the following primary antibodies: anti-Her2 (C-18), -Her3 (C-17), -Raf1, -cyclin D1, -Rb (C-15) (Santa Cruz), anti-Hsp90, -Hsp70, -ER (Stressgen), anti-Trap-1 (MSK81), anti-β-actin, -tubulin (Sigma), anti-PI3K (p85) (Upstate Biotechnologies).

5.7. Antiproliferative index

Growth assays were performed by seeding 10 000 cells (MCF-7 and MDA-MB-468) and 20 000 cells (SKBr3) per well in 6-well dishes and incubating for 24 h before drug treatment. Drugs or vehicle were administered as outlined for each experiment, and cells were incubated for the time periods depicted and then the number quantified by a Coulter counter.

5.8. Flow cytometry

Cell cycle distribution was assayed according to Nusse et al. [23] with a Becton Dickinson fluorescence-activated cell sorter and analyzed by a Cell Cycle Multi-cycle system (Phoenix Flow System, San Diego, CA, USA).

5.9. Immunofluorescence (IF) and hematoxylin and eosin stain (H&E)

Cells were plated 5000 cells/chamber slide (fibronectin-coated Lab-Tek 2 well chamber slides, Fisher Scientific) and seeded for 24 h. Drugs or vehicle were added for 5 days after which, for IF, the slides were washed twice with ice-cold PBS and fixed with methanol and acetone solution (1:1) for 15 s. Fixed monolayers were washed with distilled water and blocked with 5% BSA in PBS solution. After blocking, cells were incubated with the primary antibody (anti-MFMG, Chemicon, 1:100 in 5% BSA in PBS) at 37°C and washed three times with 1% BSA in PBS, followed by incubation with a rhodamine-labeled secondary antibody for 1 h at 37°C. Nuclei were stained with DAPI at 1 μg/ml. For H&E, the cells were fixed with paraformaldehyde (4%) for 10 min at RT and stained according to standard H&E staining protocols [24].

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