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Antibiotic radicicol binds to the N-terminal domain of Hsp90 and shares important biologic activities with geldanamycin

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Abstract The molecular chaperone Hsp90 plays an essential role in the folding and function of important cellular proteins including steroid hormone receptors, protein kinases and proteins controlling the cell cycle and apoptosis. A 15 Å deep pocket region in the N-terminal domain of Hsp90 serves as an ATP/ADP-binding site and has also been shown to bind geldanamycin, the only specific inhibitor of Hsp90 function described to date. We now show that radicicol, a macrocyclic antifungal structurally unrelated to geldanamycin, also specifically binds to Hsp90. Moreover, radicicol competes with geldanamycin for binding to the N-terminal domain of the chaperone, expressed either by in vitro translation or as a purified protein, suggesting that radicicol shares the geldanamycin binding site. Radicicol, as does geldanamycin, also inhibits the binding of the accessory protein p23 to Hsp90, and interferes with assembly of the mature progesterone receptor complex. Radicicol does not deplete cells of Hsp90, but rather increases synthesis as well as the steady-state level of this protein, similar to a stress response. Finally, radicicol depletes SKBR3 cells of $p185^{erbB2}$, Raf-1 and mutant p53, similar to geldanamycin. Radicicol thus represents a structurally unique antibiotic, and the first non-benzoquinone ansamycin, capable of binding to Hsp90 and interfering with its function.

INTRODUCTION

The 90 kDa heat shock protein family (which includes Hsp90 and Grp94) comprises molecular chaperones that may have important functions in protein folding (Freeman and Morimoto 1996) and renaturation (Schneider et al 1996; Schumacher et al 1996; Thulasiraman and Matts 1996), and in transport of proteins through the endoplasmatic reticulum and the Golgi apparatus (Little et al 1994). Hsp90 binds to a number of specific proteins that require this interaction to execute their function. These proteins include steroid hormone receptors that require Hsp90 binding to acquire a mature conformation which allows for steroid binding (Nathan and Lindquist 1995; Johnson et al 1996; Pratt et al 1996), non-receptor tyrosine kinases such as v-src (Brugge et al 1983; Brugge 1986), serine/threonine kinases such as Raf-1 (Stancato et al 1993; Wartmann and Davis 1994) and Cdk4 (Stepanova et al 1996) and mutant p53 (Blagosklonny et al 1995). Stability and function of the receptor tyrosine kinase $p185^{erbB2}$ depends on the Hsp90 homologue Grp94 (Chavany et al 1996).

We recently identified benzoquinone ansamycins, a class of naturally occurring antibiotics represented by geldanamycin (GA) and herbimycin A, as the first specific inhibitors of Hsp90 function (Whitesell et al 1994). Structural analysis of the GA–Hsp90 interaction has demonstrated that the drug binds to a unique ATP/ADP binding site in the amino terminus of the protein which serves to regulate Hsp90 conformation (Grenert et al 1997; Prodromou et al 1997a, 1997b; Stebbins et al 1997).

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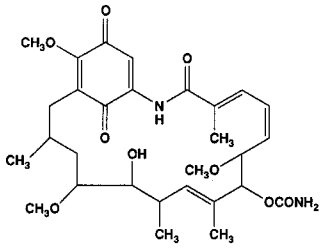
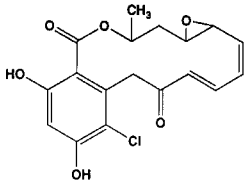


Geldanamycin



Radicicol

Fig. 1 Structures of radicicol and the benzoquinone ansamycin GA.

The benzoquinone ansamycins have proven very valuable for understanding the multiple activities of Hsp90 (Whitesell et al 1994; Blagosklonny et al 1995, 1996; Johnson and Toft 1995; Schulte et al 1995; Schneider et al 1996; Thulasiraman and Matts 1996; Whitesell and Cook 1996; Stancato et al 1997; Sullivan et al 1997). To date, only drugs containing the benzoquinone ansamycin moiety have been shown to bind to Hsp90.

Radicicol (Fig. 1) is a macrocyclic antifungal antibiotic that was originally isolated from *Monosporium bonorden* (Delmotte and Delmotte-Plaquee 1953). Although radicicol is structurally dissimilar from benzoquinone ansamycins, we now report that radicicol specifically competes with GA for binding to the N-terminal domain of Hsp90. Like GA, radicicol interferes with the assembly of the mature progesterone receptor complex, destabilizes the Hsp90-dependent proteins $p185^{erbB2}$, Raf-1 and mutant p53, and upregulates synthesis of Hsp90. Radicicol thus represents a novel non-benzoquinone ansamycin antibiotic which specifically targets Hsp90 function.

MATERIALS AND METHODS

Materials

Radicicol was supplied by the Pharmaceutical Research Institute, Kyowa Hakko Kogyo Co. Ltd, Shizuoka, Japan. Geldanamycin was obtained from the Developmental Therapeutics Program, NCI (Rockville, MD, USA). Drugs were dissolved in DMSO as 10 or 5 mM stock solutions. The mouse monoclonal antibodies JJ3 against p23 (Johnson et al 1994) and PR22 against the progesterone receptor (PR) (Sullivan et al 1986) have been described previously. We also used mouse monoclonal Hsp90 antibody (clone MA3–011, Affinity BioReagents, Neshanic Station, NJ, USA), mouse monoclonal antibody 3 for $p185^{erbB2}$ (clone 3B5, Oncogene Science, Uniondale, NY, USA), rabbit polyclonal Raf-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-p53 monoclonal antibody (PAb1801 Oncogene Science). [^{3}H]-progesterone was from NEN ([1,2-^{3}H(N)]progesterone, 1.9 TBq/mmol, NEN Dupont Co., Wilmington, DE, USA). All other chemicals were of highest analytical grade.

Cell culture

SKBR3 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum and 10 mM HEPES. For metabolic labeling, cells were washed with phosphate-buffered saline and kept in methionine-free medium for 30 min before adding 100 µCi/ml [^{35}S]methionine for 2 h.

Production of geldanamycin-affinity beads

GA was derivatized and immobilized as previously reported (Whitesell et al 1994). Briefly, 1,6-hexanediamine was added to GA (10 mM in $CHCl_3$) at a 10-fold molar excess and allowed to react for 2 h. After aqueous extraction, 17-hexamethylenediamine-17-demethoxygeldanamycin was dried, redissolved in DMSO and reacted with AffiGel 10 resin (Bio-Rad, Hercules, CA, USA). The resulting beads were washed in TNES buffer and blocked in 1% bovine serum albumin before use.

Use of geldanamycin-AffiGel beads

Full length chicken Hsp90, or the N-terminal fragment comprising the first 220 amino acids, were transcribed/translated in vitro using TNT rabbit reticulocyte lysate (Promega Corp.) in the presence of translation grade [^{35}S]methionine (1458 Ci/mmol, ICN) using SP6 polymerase and following manufacturer's instructions. Radicicol or GA, at varying concentrations, or DMSO solvent, were added at the end of the translation period and samples were incubated for an additional 1 h on ice. Following these incubations, 1 µL was removed for analysis of translation efficiency, and the remaining lysates were diluted 1:20 with TNES buffer. The solid phase GA binding assay was performed as previously described (Whitesell et al 1994).

In some experiments, 10 µg of the amino terminal fragment of Hsp90, expressed in bacteria and purified by column fractionation as previously described (Grenert et al 1997), was incubated directly with varying concentrations of soluble GA or radicicol (in the buffer described below but lacking molybdate and ATP), or DMSO solvent, prior to solid phase GA binding assay.

p23 Binding assay

Human p23 and Hsp90β were over-expressed and purified to > 98% purity by column fractionation as described earlier (Sullivan et al 1997). Five micrograms of p23 and 10 µg of Hsp90 were incubated in 200 µl of 10 mM Tris pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 5 mM ATP, 20 mM Na_2MoO_4 and 0.01% NP40. After mixing on ice, samples were incubated at 30°C for 30 min and subsequently chilled again. Samples were extracted using JJ3 antibody/protein A-sepharose beads for 1 h in ice and washed four times with 10 mM Tris pH 7.5, 1 mM EDTA. The sepharose beads were resuspended in SDS sample buffer and resolved on SDS-PAGE gels that were stained with Coomassie blue dye.

Reconstitution of progesterone receptor complexes

PR was prepared from oviduct cytosol of estradiol stimulated chicks as described (Johnson and Toft 1995). PR in cytosol was stripped of associated proteins by incubation in 500 mM KCl and then immunoprecipitated with 25 µl protein A Sepharose beads containing PR22 antibody. PR samples were incubated with 0.4 ml reticulocyte lysate (Green Hectares, Oregon, WI, USA) with or without an ATP-regenerating system (10 mM phosphocreatine, 3.5 U/100 µl lysate creatine phosphokinase, 2 mM ATP) and the inhibitors GA or radicicol, or DMSO solvent, at the concentrations indicated at 30°C for 30 min. After chilling and washing of the samples, they were suspended in 200 µl buffer containing 10 mM Tris, pH 7.5, 5 mM $MgCl_2$, 50 mM KCl, 2 mM DTT, 20 mM Na_2MoO_4, and a saturating level of [^{3}H]progesterone (20 nM [^{3}H]progesterone plus 200 nM unlabelled progesterone). The samples were incubated on ice for 1 h with frequent mixing. After four 1 ml washes, the pellets were suspended and 1/10th volume was measured for radioactivity (by liquid scintillation counting), while the remainder was analyzed by SDS-PAGE.

Western blotting and immunoprecipitations

Cells were lysed with TNES buffer (50 mM Tris pH 7.5, 1% NP40, 2 mM EDTA, 100 mM NaCl) containing 1 mM sodium orthovanadate, 20 µg/ml aprotinin, 20 µg/ml leupeptin and 1 mM PMSF. Twenty-five micrograms of total protein were separated on 8% SDS-polyacrylamide gels, transferred to nitrocellulose membrane by electroblotting and probed with the indicated primary antibodies as previously described (Schulte et al 1995). We used horseradish peroxidase (HRP)-conjugated secondary antibody to rabbit or mouse IgG (Amersham) in conjunction with Western blot chemiluminescence reagent (Renaissance, Du Pont). Films were scanned into a Macintosh computer using a Foto/Eclipse Gel Analysis System (Fotodyne) and processed using Adobe Photoshop and NIH image software. Immunoprecipitations were performed as described earlier (Schulte et al 1995) using 0.5 mg of total protein per condition.

Antiproliferative assay

SKBR3 cells were seeded in 96-well plates at a density of 3000 cells per well. Twenty-four hours later the cells were treated in quadruplicate with increasing concentrations of either GA or radicicol as indicated. Number of viable cells was assessed by MTT assay after 4 days, as described previously (An et al 1997).

RESULTS

Radicicol competes with geldanamycin for binding to Hsp90

GA binds in a saturable, hydrophobic manner to Hsp90 (Whitesell et al 1994; Grenert et al 1997). We have utilized this property of the drug to affinity purify Hsp90 from cell lysates by using GA covalently linked to a solid support (GA-affinity beads) (Whitesell et al 1994). Since pre-addition of excess soluble GA (or other benzoquinone ansamycins) to either intact cells or cell lysates specifically blocks subsequent Hsp90 binding to GA-affinity beads, this technique can be used to screen compounds for their ability to compete with GA for binding to the chaperone (Grenert et al 1997). In order to assess binding of radicicol to Hsp90, we used such a competition assay to examine whether soluble radicicol could block binding of Hsp90 to GA-affinity beads. We lysed SKBR3 cells in TNES buffer, pre-treated the lysates for 30 min on ice with varying concentrations of either radicicol or GA, and then incubated these lysates with GA-affinity beads. After washing the beads, we analyzed precipitated proteins both by silver staining (Fig. 2A), and immunoblotting with an Hsp90-specific antibody (Fig. 2B). Radicicol (and soluble GA) effectively competed with GA-affinity beads for binding to Hsp90. The displacement curves calculated from the data in Figure 2A suggest similar binding affinities for the two drugs (EC_{50} for competition was 38 nM for soluble GA and 17 nM for radicicol, see Fig. 2C).

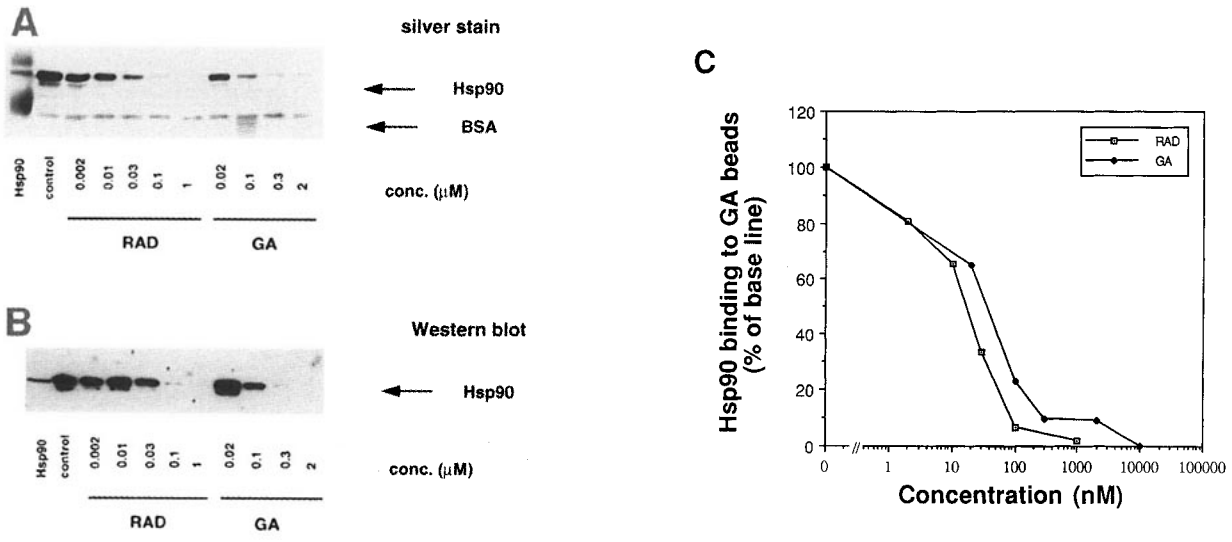


Fig. 2 Radicicol competes with GA for binding to Hsp90 in vitro. SKBR3 lysates were incubated with GA-affinity beads. Hsp90 binding to solid phase GA was competed by increasing concentrations of either soluble radicicol or GA. Hsp90 was detected by silver staining (A) or Western blot (B). Binding curves were compared after image analysis of the data shown in panel A (C). One of two similar experiments is shown.

Radicicol competes with geldanamycin for binding to the N-terminal domain of Hsp90

The binding site for GA has recently been shown to comprise an ATP/ADP binding site within the amino terminal 220 amino acids of Hsp90 (Grenert et al 1997; Prodromou et al 1997a, 1997b; Stebbins et al 1997). In order to determine if radicicol also binds to this region of the chaperone, we employed the GA-affinity bead binding assay with in vitro translated Hsp90 constructs. Wild-type Hsp90 or an N-terminal deletion mutant comprising the amino terminal 220 amino acids of Hsp90 (ΔC507) (Grenert et al 1997) were expressed by in vitro translation in reticulocyte lysate in the presence of [^{35}S]methionine, and translated proteins were specifically precipitated with GA-affinity beads. Purified proteins were separated on an 8% PAGE gel and analyzed by autoradiography (Fig. 3A). Binding of both the full-length protein and the N-terminal domain of Hsp90 to GA-affinity beads was blocked by pre-addition of radicicol (as well as by soluble GA) demonstrating that, like GA, radicicol binds Hsp90 in its N-terminal domain, likely at or near the GA-binding site.

Since in vitro translation in reticulocyte lysate is performed in an environment rich in various heat shock proteins and their complexes, the preceding observations could be a result of interference by radicicol with a heat shock protein other than Hsp90 that is a necessary component of the Hsp90 multimolecular complex. In order to determine whether radicicol interacts directly with the amino terminus of Hsp90, we expressed the N-terminal domain in bacteria and purified the peptide by column chromatography. The purified peptide bound to GA-affinity beads when incubated in TNES buffer (Fig. 3B). This interaction was markedly competed by excess soluble radicicol, thus demonstrating a direct interaction between the antibiotic and the amino terminus of Hsp90 independent of the presence of other heat shock or auxiliary proteins.

Radicicol prevents binding of Hsp90 to its co-chaperone p23

Hsp90 is a member of at least two distinct multimolecular complexes which variably include Hsp70, p60 (Hop), immunophilins such as FKBP52, FKBP54 and CyP40, the cdc37/p50 protein, and p23 (Johnson and Toft 1994; Pratt et al 1996; Pratt 1997; Sullivan et al 1997). The co-chaperone p23 is a ubiquitous protein in mammalian cells and is a component of those Hsp90 complexes which stabilize steroid receptors in their functional, ligand binding, 'mature' conformation (Johnson and Toft 1994, 1995; Johnson et al 1994). p23 is in ample supply in reticulocyte lysate, a standard source of Hsp90-containing chaperone complexes (Blagosklonny et al 1996; Johnson et al 1996). GA has previously been shown to block p23 binding to Hsp90, which is ATP-dependent, presumably by altering the conformation of Hsp90 as a result of binding to the ATP/ADP conformational switch region of the chaperone (Smith et al 1995; Grenert et al 1997; Prodromou et al 1997a; Sullivan et al 1997). In order to test whether radicicol also antagonizes p23 binding to Hsp90, we mixed highly purified preparations of Hsp90 and p23 under conditions defined in earlier studies

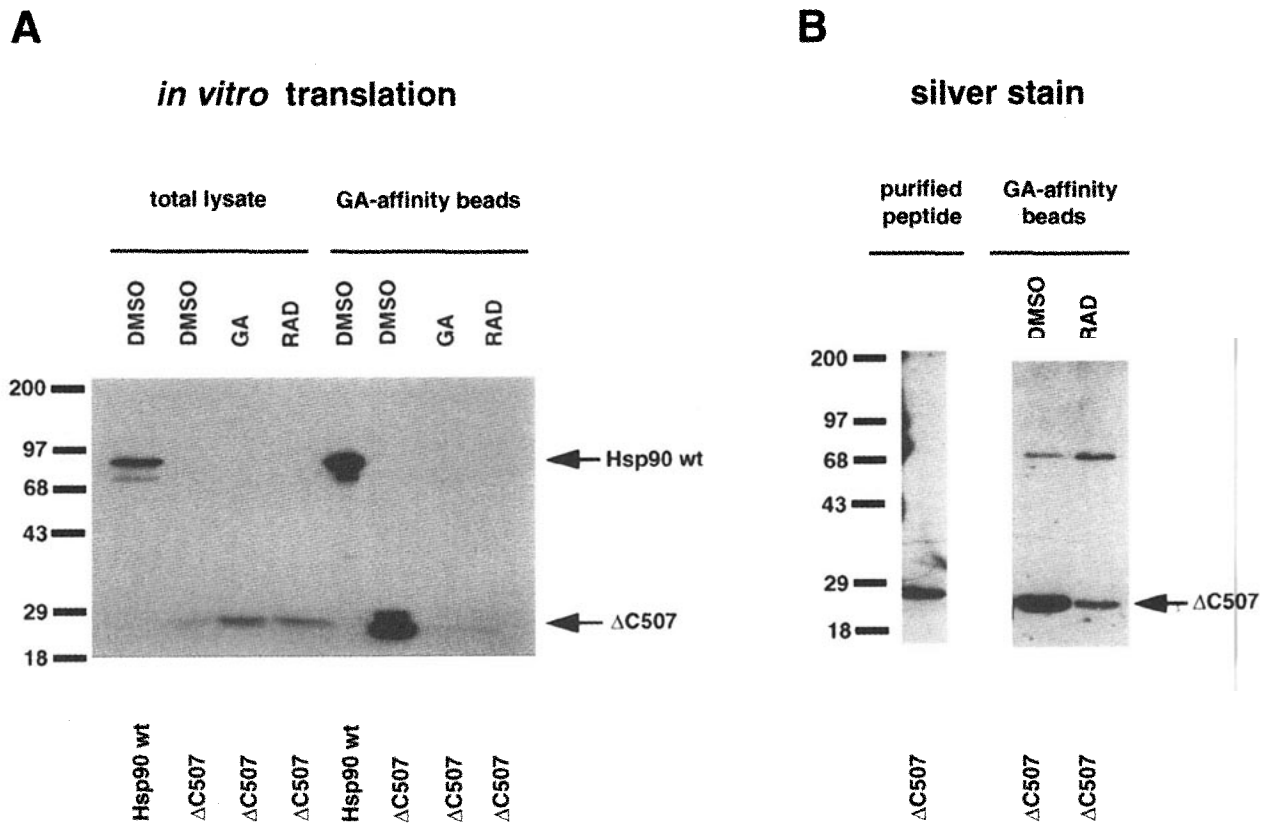


Fig. 3 Radicicol competes with GA for binding to the N-terminal domain of Hsp90. (A) Wild type Hsp90 (wt) or a deletion mutant (ΔC507) that encodes the N-terminal 220 amino acids of Hsp90 were in vitro translated in reticulocyte lysate in the presence of [^{35}S]methionine. At the end of the translation period radicicol, GA or DMSO solvent were added and the samples were incubated on ice for one hour. One microliter of total lysate was monitored for translation efficiency (left 4 lanes). The remainder (24 µL) was extracted with GA-affinity beads and analyzed concurrently by autoradiography in an 8% SDS-PAGE gel (right 4 lanes). (B) The Hsp90 deletion mutant ΔC507 was expressed in bacteria. The purified peptide was incubated with GA-affinity beads in TNES buffer. Affinity-purified protein was visualized by silver stain of an 8% SDS-PAGE gel. Radicicol, added in vitro, effectively competed with GA for binding to the purified peptide.

(Sullivan et al 1997) to favor the in vitro, ATP-dependent, association of these proteins. We added radicicol or GA (as a control) to the reaction at its initiation and assessed the effects on p23–Hsp90 association by immunoprecipitating p23 and visualizing precipitated proteins by Coomassie blue staining. The data in Figure 4 demonstrate that, like GA, radicicol effectively blocks p23 binding to Hsp90 in vitro at a concentration between 1 and 10 µM.

Radicicol affects the maturation of the progesterone receptor

In vitro experiments utilizing reticulocyte lysate have demonstrated that the PR binds sequentially to distinct Hsp90-containing multimolecular complexes which exist in dynamic equilibrium (Smith et al 1995; Johnson et al 1996). Functionally active (i.e. ligand binding) 'mature' receptors require association with Hsp90 multimolecular complexes which contain p23, and this conformational state is thus antagonized by GA (Smith et al 1995; Whitesell and Cook 1996). To test whether radicicol has a similar effect on PR conformation, we reconstituted the PR multimolecular complex in vitro by first immunoprecipitating PR prepared from chick oviduct cytosol which had been stripped of its associated proteins by high salt conditions. The multimolecular complex was then reconstituted by incubation with reticulocyte lysate and analyzed by Coomassie blue staining after re-precipitation. Hormone binding to the PR was assessed by incubation of the reconstituted PR multimolecular complex with [^{3}H]progesterone. Control conditions showed the completeness of stripping of the PR and the requirement of an ATP regenerating system for the reconstitution of the complex (Fig. 5A, lanes 1–3). Like GA, radicicol prevented reconstitution of the mature complex, as indicated by the absence of p23 and the presence of p60 (Hop) in the treated complexes (Fig. 5A, lanes 4–6), and by the lack of

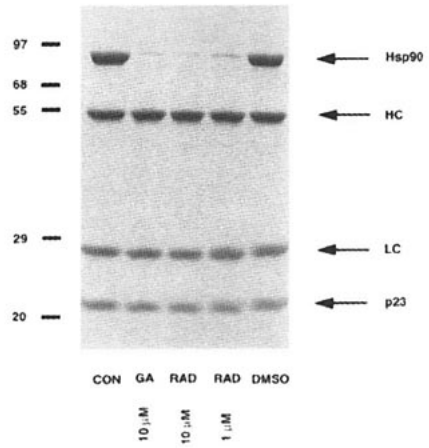


Fig. 4 Radicicol inhibits binding of p23 to Hsp90. Purified p23 and Hsp90 were incubated together in a buffer containing $MgCl_2$, ATP, molybdate and NP40 at 30°C. After 30 min, samples were extracted by immunoprecipitation with p23 antibody and analyzed by SDS-PAGE followed by Coomassie blue staining. GA, radicicol, or DMSO were added to the reactants at the beginning of the experiment in order to assess their ability to modify binding of Hsp90 to p23. HC = heavy chain of the anti-p23 antibody; LC = light chain of the antibody. Note that neither treatment affected the interaction of p23 with its antibody, but that Hsp90 was not co-precipitated in samples containing either GA or radicicol.

progesterone binding to these complexes (Fig. 5B). When PR is stripped of Hsp90 its hormone binding activity is quite stable at 4°C (lane 1), but is rapidly lost upon incubation at elevated temperatures (Smith 1993). When the PR is completely reconstituted with Hsp90 and p23 its hormone binding activity is stabilized (lanes 2 and 7), but formation of incomplete complexes due either to lack of ATP (lane 3), or the presence of GA or radicicol (lanes 4 and 6) results in loss of hormone binding.

Radicicol increases the synthesis of Hsp90

GA is known to increase the synthesis of Hsp90 (Whitesell et al 1994) and this is thought to occur as a direct result of interference in chaperone function (Hedge et al 1995). In order to see if radicicol has a similar effect, we treated SKBR3 cells with or without radicicol or GA overnight before labeling with [^{35}S]methionine for 2 h. Cells were lysed in TNESV buffer and newly synthesized Hsp90 was detected by immunoprecipitation and autoradiography of an 8% SDS-PAGE gel (Fig. 6A). At the same time, total Hsp90 was visualized by Western blotting of proteins in lysates of identically treated cells (Fig. 6B). Similar to what is seen following GA treatment, exposure of SKBR3 cells to radicicol resulted in an increase in both total and newly synthesized Hsp90, partly mimicking a cellular stress response.

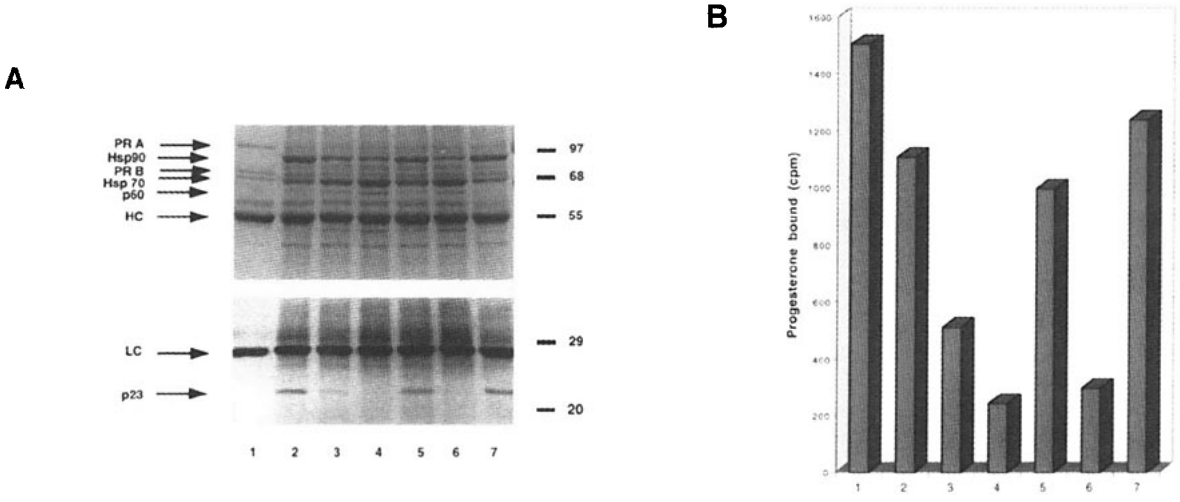


Fig. 5 Radicicol prevents the reconstitution of the p23-containing PR multimolecular complex. PR was immunoprecipitated from chick oviduct cytosol and stripped of its associated proteins by high salt wash. Following immobilization on protein A-sepharose beads, PR was incubated with reticulocyte lysate to allow for reconstitution of the multimolecular complexes that contain Hsp90. The complexes were separated on an 11% SDS-PAGE gel and analyzed by Coomassie blue staining (A). Reconstitution was allowed to occur at 30°C with or without an ATP regenerating system and in the presence of GA or radicicol. HC = anti-PR antibody heavy chain; LC = antibody light chain. Note the absence of p23 and the presence of p60 (Hop) in the Hsp90/PR complexes lacking an ATP regenerating system, or exposed to either radicicol or GA, while the presence of Hsp70 in these complexes was not altered by either treatment. The same samples were incubated with [^{3}H]progesterone and assayed for bound radioactivity as described in Methods (B). Lane 1 = stripped PR; lane 2 = reconstituted in presence of ATP regenerating system; lane 3 = reconstituted without ATP regenerating system; lane 4 = reconstituted with ATP regenerating system plus GA (10 μM); lane 5 = reconstituted with ATP regenerating system plus radicicol (1 μM); lane 6 = same as lane 5 but with 10 μM radicicol; lane 7 = same as lane 2 plus DMSO.B

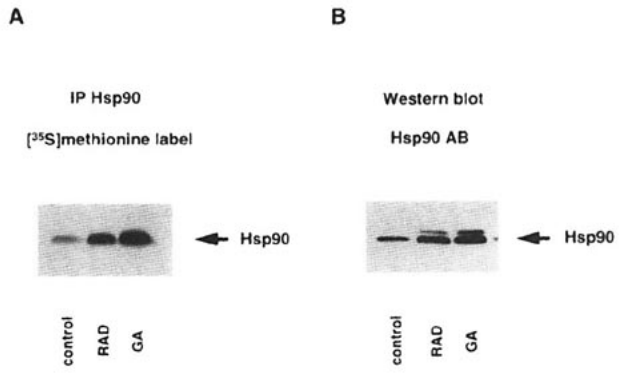


Fig. 6 Radicicol, like GA, increases the cellular level of Hsp90 by stimulating synthesis. SKBR3 cells were treated with radicicol or GA for 14 h before labeling newly synthesized protein with [^{35}S]methionine. Newly synthesized Hsp90 was detected by immunoprecipitation with Hsp90 antibody and autoradiography (A). Total Hsp90 levels were assayed by Western blotting (B). Hsp90 synthesis was markedly elevated by both drugs, leading to a moderate increase in steady-state level of the chaperone.

Radicicol depletes cells of Hsp90-dependent signaling proteins

A large number of signaling proteins other than steroid receptors has been described to exist in multimolecular complexes with Hsp90, and to depend on this association for stability and function. These proteins include Raf-1 (Stancato et al 1993; Wartmann and Davis 1994; Schulte et al 1995; Stancato et al 1997), mutant p53 (Blagosklonny et al 1995, 1996) and p185^{erbB2}, which depends on interaction with the Hsp90 homologue Grp94 (Chavany et al 1996). GA treatment results in destabilization and resultant depletion of these proteins from cells (Blagosklonny et al 1995; Schulte et al 1995; Chavany et al 1996). To test whether radicicol has similar effects on these Hsp90-dependent signaling proteins, we treated SKBR3 cells, a breast cancer cell line that expresses a high level of p185^{erbB2} and mutated p53, with increasing concentrations of the antibiotic for 6 or 20 h. We assayed the steady-state levels of Raf-1, mutant p53 and p185^{erbB2} by immunoblotting (Fig. 7A–C). The levels of Raf-1 were decreased by more than 90% after 20 h exposure to radicicol at a dose of 81 nM (EC_{50} = 32 nM). Mutant p53 and p185^{erbB2} were substantially depleted after 6 h exposure to the drug (EC_{50} = 42 nM and 18 nM, respectively).

Radicicol has similar antiproliferative activity to GA. We compared the antiproliferative activities of radicicol and GA on SKBR3 cells in a standard MTT assay. We observed IC_{50} values of 20 nM for radicicol and 5 nM for GA after 4 days of drug treatment (Fig. 8).

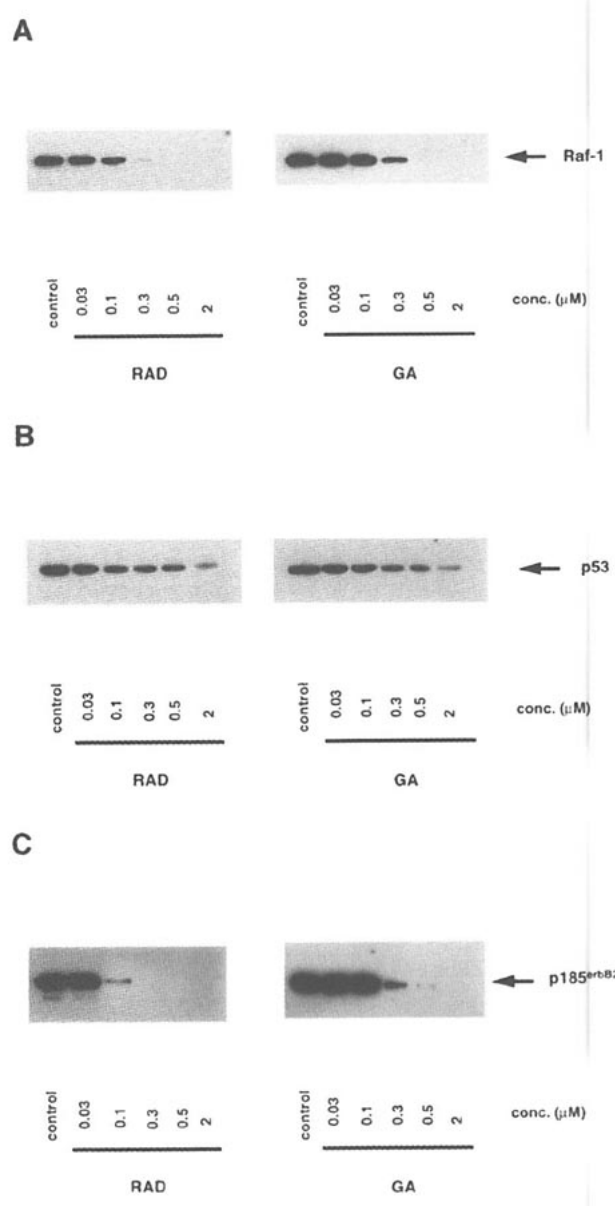


Fig. 7 Radicicol depletes cells of signaling proteins which interact with Hsp90. SKBR3 cells were treated with increasing concentrations of radicicol or GA for 20 h (A) or 6 h (B, C). Cells were lysed in TNES buffer and protein levels of Raf-1 (A), mutant p53 (B) and p185^{erbB2} (C) were assessed by Western blotting.

DISCUSSION

Even though the complete crystal structure of Hsp90 has not yet been solved, the structures of the N-terminal domains of human and yeast Hsp90 have recently been reported (Prodromou et al 1997a, 1997b; Stebbins et al 1997). The N-terminal domain contains a 15 Å deep pocket that binds to GA (Stebbins et al 1997). While it was initially suggested that the pocket region might serve as a peptide binding site (Stebbins et al 1997),

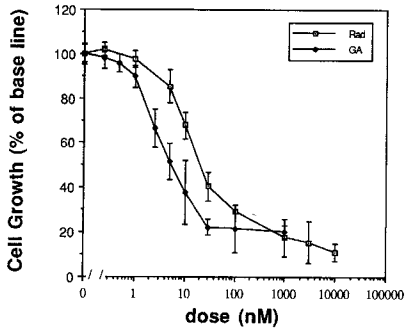


Fig. 8 Radicicol and GA have a similar antiproliferative effect on SKBR3 cells. SKBR3 cells growing in log phase were treated with increasing concentrations of radicicol or GA for 4 days. Cell growth was measured by MTT assay.

ample evidence now suggests that this site binds ATP or ADP and probably regulates the conformation of the Hsp90 molecule (Grenert et al 1997; Prodromou et al 1997a). The binding of GA to Hsp90 prevents binding of ATP and has profound effects on the composition of Hsp90-containing multimolecular chaperone complexes, and on signaling proteins which depend upon Hsp90 for their function (Whitesell et al 1994; Schulte et al 1995; Blagosklonny et al 1996; Sullivan et al 1997).

Radicicol is a macrocyclic antifungal antibiotic with no obvious structural similarity to the benzoquinone ansamycins (Mirrington et al 1964). It has been described as an inhibitor of v-src which reverts the transformed phenotype of v-src-expressing fibroblasts (Kwon et al 1992). Recently, it has also been reported to suppress transformation by the ras oncogene (Zhao et al 1995), yet the mechanism of action of this biologically active compound has remained obscure.

In this study, we show that radicicol, although structurally unrelated to benzoquinone ansamycins, acts as a specific inhibitor of Hsp90 function. Using a competition binding assay that utilizes GA-affinity beads, we have demonstrated that radicicol competes with GA for binding to Hsp90 with an apparent affinity similar to GA. Moreover, since radicicol competes with GA for binding to the purified N-terminal domain of Hsp90, it is likely that radicicol, like GA, binds to the ATP/ADP binding pocket of the chaperone.

We tested whether radicicol had similar biologic effects as GA by using several established models of Hsp90 function. The maturation of the PR depends upon its association with p23-containing Hsp90 complexes (Smith et al 1995; Sullivan et al 1997). Binding of GA to the pocket region of Hsp90 prevents ATP binding to this site and prevents the association of Hsp90 and p23 (Grenert et al 1997), even though p23 binding to Hsp90 appears to occur outside the N-terminal domain (J. P. Grenert and D. O. Toft, unpublished observations). This, in turn, results in accumulation of immature PR which are not capable of binding ligand (Smith et al 1995). We have shown in this study that radicicol also inhibits Hsp90–p23 association, leading to a similar accumulation of PR in an immature, non-ligand binding, conformation.

Finally, inhibition of Hsp90 function by GA has a detrimental effect on the function and stability of several Hsp90-dependent signaling proteins other than steroid receptors, including Raf-1, mutant p53 and $p185^{erbB2}$ (Blagosklonny et al 1995; Schulte et al 1995; Chavany et al 1996). The current data show that all three proteins were similarly depleted from SKBR3 cells upon treatment with radicicol at concentrations comparable to GA. Both GA and radicicol display similar anti-proliferative activity toward SKBR3 cells in vitro and have demonstrated significant but weak anti-tumor activity in animals, emphasizing the potential importance of the ATP-binding conformational switch region in the amino terminus of Hsp90 as a novel target for anti-cancer drug development. Thus, further investigation of the structural/functional relationships between GA and radicicol should provide clues for the development of new and more effective agents of this type.

In summary, we have demonstrated that radicicol competes with GA for binding to the N-terminal 25 kDa domain of Hsp90. Radicicol increases synthesis and steady-state level of Hsp90 as does GA, and it affects key features of Hsp90 function in a fashion identical to GA. Radicicol thus represents the first non-benzoquinone ansamycin inhibitor of Hsp90.

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