Novobiocin and Related Coumarins and Depletion of Heat Shock Protein 90-Dependent Signaling Proteins

Monica G. Marcu, Theodore W. Schulte, Leonard Neckers

Background: **Heat shock protein 90 (Hsp90) interacts with and stabilizes several oncogenic protein kinases (e.g., p185^{erbB2}, p60^{v-src}, and Raf-1) and is required for the stability and dominant–negative function of mutated p53 protein. Two unrelated antibiotics, geldanamycin and radicicol, bind specifically to an atypical nucleotide-binding pocket of Hsp90, a site that shares homology with the adenosine triphosphate (ATP)-binding domain of bacterial DNA gyrase B. This interaction leads to destabilization of proteins that interact with Hsp90. Since the nucleotide-binding site of gyrase B is targeted by coumarin antibiotics (e.g., novobiocin), we investigated whether these drugs can also interact with Hsp90 and affect its activity.** *Methods:* **We used immobilized novobiocin, geldanamycin, or radicicol to isolate either endogenous Hsp90 from cell lysates or Hsp90 deletion fragments translated *in vitro*. Effects of the coumarin antibiotics novobiocin, chlorobiocin, and coumermycin A1 on several proteins interacting with Hsp90 were assessed *in vitro* and *in vivo*.** *Results:* **Hsp90 binding to immobilized novobiocin was competed by soluble coumarins and ATP but not by geldanamycin or radicicol. A carboxy-terminal Hsp90 fragment bound immobilized novobiocin but not immobilized geldanamycin, while a geldanamycin-binding amino-terminal fragment did not bind novobiocin. All three coumarins markedly reduced cellular levels of p185^{erbB2}, p60^{v-src}, Raf-1, and mutated p53. Furthermore, novobiocin reduced Raf-1 levels in the spleens of mice treated with the drug.** *Conclusions:* **These coumarin antibiotics, particularly novobiocin, represent a first-generation alternative to other Hsp90-targeting drugs that are not as well tolerated. Novobiocin's unique interaction with Hsp90 identifies an additional site on this protein amenable to pharmacologic interference with small molecules. [J Natl Cancer Inst 2000;92:242–8]**

Heat shock protein 90 (Hsp90) is one of the most abundant heat shock proteins in eukaryotes and plays a key role in the stability and function of multiple cell-signaling molecules. A variety of transcription factors, including the dioxin receptor *(1),* steroid hormone receptors *(2–4),* and mutated p53 *(5,6),* as well as several oncogenic tyrosine and serine–threonine kinases, including p60^{v-src} *(7),* Raf-1*(8–10),* and p185^{erbB2} (Her2/Neu) *(11),* can be recovered as heterocomplexes containing Hsp90. Two structurally unrelated antibiotics, the benzoquinone ansamycins (e.g., geldanamycin) and radicicol, specifically interact with an Hsp90 amino-terminal nucleotide-binding domain, disrupting the chaperone's association with its client-signaling proteins and leading to their subsequent destabilization and proteolysis *(12–16).*

Since Hsp90 is considered to be a novel molecular target for anticancer therapeutics *(12),* we have focused our attention on discovering less toxic agents that are capable of inhibiting Hsp90 function in a manner similar to that seen with the ansamycins and radicicol. Both drugs bind to an atypical nucleotide-binding pocket in the amino terminus of Hsp90 that shares homology to the adenosine triphosphate (ATP)-binding domain of the bacterial DNA gyrase B protein *(16–21).* Since the nucleotide-binding site of gyrase B is the target of the coumarin antibiotics, including novobiocin, chlorobiocin, and coumermycin A1 (Fig. 1), we investigated whether these agents could also interact with Hsp90 and, if so, whether they could affect its chaperone activity. Since novobiocin, in particular, has been a clinically utilized antibiotic with tolerable toxicity, we were par-

Affiliation of authors: Department of Cell and Cancer Biology, Medicine Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD.

Correspondence to: Leonard Neckers, Ph.D., National Cancer Institute, 9610 Medical Center Dr., Suite 300, Rockville, MD 20850 (e-mail: len@helix.nih.gov).
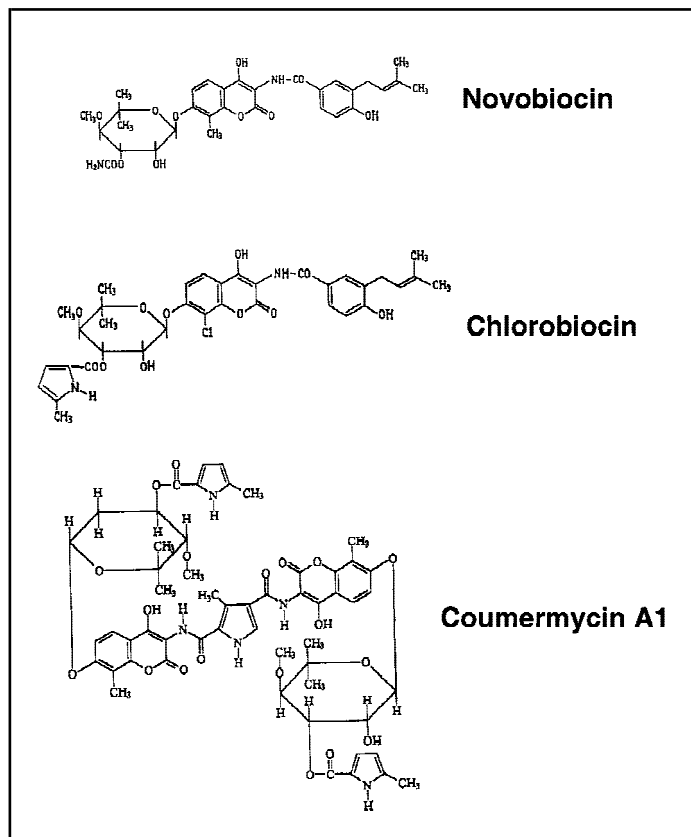
See "Note" following "References."

Fig. 1. Structures of novobiocin and the structurally related coumarins chlorobiocin and coumermycin A1.

ticularly interested in this compound's potential as an anti-Hsp90 agent.

MATERIALS AND METHODS

Cell Culture and Reagents

SKBR3 and MCF7 human breast carcinoma cells were purchased from the American Type Culture Collection (Manassas, VA), and v-src-transformed NIH 3T3 mouse fibroblasts were obtained from the National Cancer Institute, Bethesda, MD. The cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (Whittaker Bioproducts, Walkersville, MD), 1 mM glutamine, and 10 mM HEPES (pH 7.3) at 37 °C in an atmosphere of 5% carbon dioxide. When they were approximately 70% confluent, the cells were treated with various agents. Stock novobiocin, etoposide, and doxorubicin (Sigma Chemical Co., St. Louis, MO) solutions were prepared in distilled water. Chlorobiocin and geldanamycin (both from the Developmental Therapeutics Program, National Cancer Institute) and coumermycin A1 (Sigma Chemical Co.) were prepared in 100% dimethyl sulfoxide (DMSO).

Preparation of Novobiocin–Sepharose 6B and the Solid-Phase Novobiocin-Binding Assay

Novobiocin–Sepharose was prepared as follows: Three grams of epoxy-activated Sepharose 6B (Sigma Chemical Co.) was thoroughly washed and then swollen in 100 mL of distilled water for 1 hour at room temperature. The resin was washed further with coupling buffer (0.3 M sodium carbonate [pH 9.5]). The gel was mixed with 400 mg of novobiocin (sodium salt; Sigma Chemical Co.) in 10 mL of coupling buffer and incubated at 37 °C with gentle rotation for 20 hours. The excess ligand was washed away with coupling buffer, and the remaining epoxy-active groups were blocked with 1 M ethanolamine in coupling buffer for 12 hours at 30 °C with gentle shaking. The gel was thoroughly washed sequentially with coupling buffer, 0.5 M NaCl in coupling buffer, distilled water, 0.5 M NaCl in 0.1 M sodium acetate (pH 4), and again in distilled water; it was

then equilibrated in 25 mM HEPES (pH 8) containing 1 mM EDTA, 10% ethylene glycol, and 200 mM KCl and kept at 4 °C protected from light *(22)*.

Cell lysates were prepared in TNESV buffer (i.e., 50 mM Tris–HCl (pH 7.4), 1% Nonidet P-40, 2 mM EDTA, 100 mM NaCl, and 1 mM sodium orthovanadate), containing 1 mM phenylmethylsulfonyl fluoride, 20 μg of aprotinin, and 20 μg of leupeptin per milliliter. Total protein (300 μg per assay) was incubated with novobiocin-coupled resin (100 μL) in TNESV buffer, with or without previous addition of various drugs or ATP, for 1 hour at 4 °C with gentle rotation. The beads were then thoroughly washed with ice-cold TNESV buffer. Bound proteins were eluted by being boiled in reducing loading buffer and were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), followed by either silver staining (Bio-Rad Laboratories, Hercules, CA) or western blotting with appropriate antibodies.

Preparation of Geldanamycin–AffiGel Beads

Geldanamycin was derivatized and immobilized as previously reported *(12)*. Briefly, 1,6-hexanediamine was added to geldanamycin (10 mM in chloroform) at a 10-fold molar excess and allowed to react for 2 hours. After aqueous extraction, 17-hexamethylenediamine-17-demethoxygeldanamycin was dried, redissolved in DMSO, and reacted with AffiGel 10 resin (Bio-Rad Laboratories). Before use, the geldanamycin–AffiGel beads were washed in TNESV buffer and blocked with 1% bovine serum albumin.

Preparation of Radicicol–Sepharose Beads

Twenty-five milligrams of the radicicol derivative KF66658 (Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan) was dissolved in tetrahydrofuran and added to 1 mL of epoxy-activated hydroxyapatite–Sepharose beads (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). After 5 days of end-over-end mixing at room temperature, the beads were transferred into tetrahydrofuran/methanol (1 : 1, vol/vol) and then 20 mg of acetic anhydride was added. After end-over-end mixing at room temperature for 1 hour, two volumes of 1 M Tris–HCl (pH 7.5) were added for 15 minutes. Finally, the resin was washed three times in TNESV buffer lacking vanadate and, before use, was blocked in 1% bovine serum albumin.

Preparation of Hsp90 Constructs

Full-length chicken Hsp90 complementary DNA (cDNA) and the Δ380–728 carboxyl-terminal chicken Hsp90 deletion fragment, both subcloned in pGEM-7Z vector (Promega Corp., Madison, WI), were gifts of Dr. David Toft (Mayo Clinic, Rochester, MN), and their method of preparation was described previously *(23)*. The amino-terminal Δ1–222 fragment of chicken Hsp90 was obtained from full-length chicken Hsp90 cDNA by polymerase chain reaction with the use of two primers designed to contain *Bam*HI and *Eco*RI restriction sites, and the DNA fragment was then subcloned in pBluescript II SK vector (Stratagene Cloning Systems, La Jolla, CA).

In Vitro Transcription and Translation

Recombinant proteins were expressed from 1 μg of plasmids by *in vitro* transcription and translation with the use of the TnT™ Coupled Rabbit Reticulocyte Lysate Kit (Promega Corp.) in the presence of translation-grade [^{35}S]methionine (1458 Ci/mmol; ICN Pharmaceuticals, Inc., Costa Mesa, CA), using the appropriate DNA polymerase and following the manufacturer's instructions. The material from *in vitro* translation reactions (12–24 μL) was incubated with various concentrations of novobiocin or geldanamycin. After mixing at 4 °C for 30 minutes, 40 μL of resin with immobilized geldanamycin or 100 μL of novobiocin resin was added, and the mixture was incubated for 60 minutes at 4 °C while rotating. Resins were washed three times with TNESV buffer and boiled in sample buffer. After separation by SDS–PAGE, proteins were visualized by silver stain or autoradiography.

Western Blotting

Cells were lysed with TNESV buffer containing protease inhibitors. Total protein (50 μg) was separated on 10% polyacrylamide gels containing SDS, transferred to a nitrocellulose membrane by electroblotting, and blocked for 2 hours with a solution containing 5% nonfat dry milk, 10 mM Tris–HCl (pH 7.5), 2.5 mM EDTA (pH 8), 50 mM NaCl, and 0.05% Tween 20. The membranes were probed with the indicated primary antibodies and then by secondary antibodies conjugated to horseradish peroxidase, and the signal was detected with the use of chemiluminescence reagents (Pierce Chemical Co., Rockford, IL).

Mononuclear Cell Preparation

Thirty milliliters of human blood collected in a heparinized syringe was layered on a 20-mL Ficoll–Paque (Pharmacia LKB Biotechnology AB) cushion and centrifuged for 30 minutes at 1000*g* at 20 °C to separate the mononuclear cells. After isolation, the cells were washed twice with sterile phosphate-buffered saline (PBS), collected by centrifugation at 1000*g* for 10 minutes at 20 °C, and then incubated in DMEM supplemented with 10% fetal bovine serum. After 6 hours, the mononuclear cells were treated with novobiocin and further incubated at 37 °C overnight.

Treatment of Mice With Novobiocin

Mice were maintained in accordance with the guidelines of the National Institutes of Health. Healthy C57BL/6 mice weighing approximately 15 g were placed on a 5-day treatment schedule consisting of twice-daily intraperitoneal injections of novobiocin (100 mg/kg body weight) formulated as a 15-mg/mL solution in sterile water. The animals were killed 3 hours after the last injection. Their spleens were removed, minced, and briefly sonicated while suspended in cold PBS and centrifuged at 450*g* for 2 minutes at 4 °C. The supernatant (isolated cells) was then separated, and the cells were collected by centrifugation at 900*g* for 10 minutes at 4 °C. Cells were lysed with TNESV buffer containing protease inhibitors. The amount of total protein was assessed by the Bradford method (Bio-Rad Laboratories). Cell extracts were subjected to denaturing PAGE, electrotransferred, and blotted for Raf-1 protein. Films were scanned into a Power Macintosh 9500 computer, and the optical density of the Raf-1-specific bands was determined with the use of National Institutes of Health Image Software.

Statistical Methods

The means and standard deviations were determined and the statistical analysis was performed with the use of Microsoft Excel 98. *P* values reported are two-sided.

RESULTS

Binding of Hsp90 to Novobiocin–Sepharose

We immobilized novobiocin in a manner previously shown not to interfere with its ability to bind to topoisomerase II, the eukaryotic homologue of bacterial DNA gyrase B *(22)*. Immobilized novobiocin bound in a hydrophobic manner (i.e., binding was resistant to multiple washes in 0.6 *M* NaCl) to either pure Hsp90 (not shown) or Hsp90 present in cell lysate (Fig. 2, A and B). Preincubation of the lysate for 30 minutes with either excess soluble novobiocin, chlorobiocin, coumermycin A1, or ATP inhibited, in a dose-dependent manner, subsequent Hsp90 binding to novobiocin–Sepharose (Fig. 2, A and C). It is interesting that we failed to observe efficient competition between either soluble geldanamycin or radicicol and immobilized novobiocin for binding to Hsp90 (data not shown), although soluble novobiocin was able to compete with both geldanamycin- and radicicol-affinity beads for binding of Hsp90 (Fig. 3).

Binding Domains on Hsp90 for Novobiocin and Geldanamycin

Intrigued by the fact that neither geldanamycin nor radicicol could effectively compete Hsp90 binding to immobilized novobiocin, we investigated the possibility that novobiocin bound to a unique location on Hsp90. Both crystallographic and biochemical studies of the amino-terminal region of Hsp90 *(18–20)* have revealed the location of the geldanamycin-binding site to be uniquely contained within the first 221 amino acids. Thus, an amino-terminal fragment (designated Δ1), consisting of amino acids 1–221 of chicken Hsp90, binds immobilized geldanamycin [*see (18)* and Fig. 4]. To our surprise, however, this fragment did not bind to novobiocin–Sepharose beads (Fig. 4). In contrast, a COOH-terminal fragment of Hsp90 (designated Δ2, representing amino acids 380–728) that does not bind to immobilized geldanamycin does bind efficiently to novobiocin–Sepharose, and the binding is efficiently competed by excess soluble novobiocin (Fig. 4). These data indicate that the novobiocin-binding

Fig. 2. Novobiocin–Sepharose binding to heat shock protein 90 (Hsp90). Binding is competed by free novobiocin, chlorobiocin, coumermycin A1, and adenosine triphosphate (ATP). Cell lysates, prepared in lysis buffer as described in the "Materials and Methods" section, of human breast carcinoma SKBR3 cells were incubated with novobiocin–Sepharose alone or after preincubation on ice with increasing concentrations of soluble novobiocin, chlorobiocin, coumermycin A1, or ATP for 30 minutes. In **panels A** and **C,** bound Hsp90 was detected by silver staining. In **panel B,** novobiocin–Sepharose was added to cell lysate without competition, and bound Hsp90 was visualized by western blot analysis; **lane a, panel B:** novobiocin-bound Hsp90 affinity-purified from SKBR3 cell lysate; **lane b, panel B:** the Hsp90 signal obtained by blotting 50 μg of SKBR3 cell lysate directly.
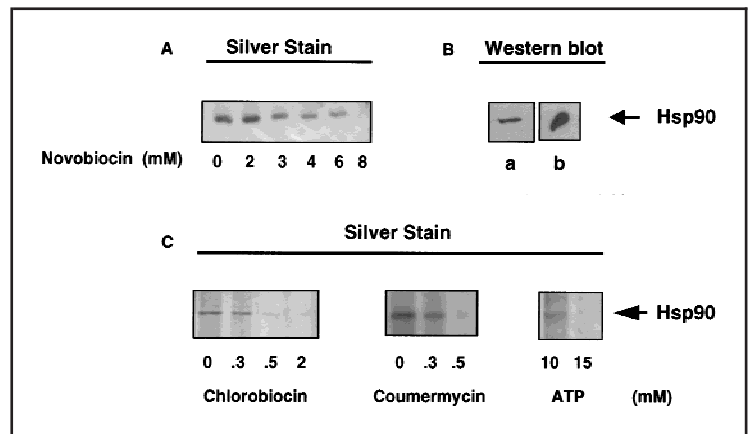

Fig. 3. Novobiocin competes with immobilized geldanamycin and immobilized radicicol for binding to heat shock protein 90 (Hsp90). Lysates were prepared from NIH 3T3 mouse fibroblasts, preincubated on ice with soluble novobiocin for 30 minutes, and then incubated with immobilized geldanamycin or immobilized radicicol. Affinity-purified proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and visualized by silver stain. Binding of Hsp90 to the resins was competitively blocked by novobiocin in a dose-dependent fashion. GA = geldanamycin; Rd = radicicol.
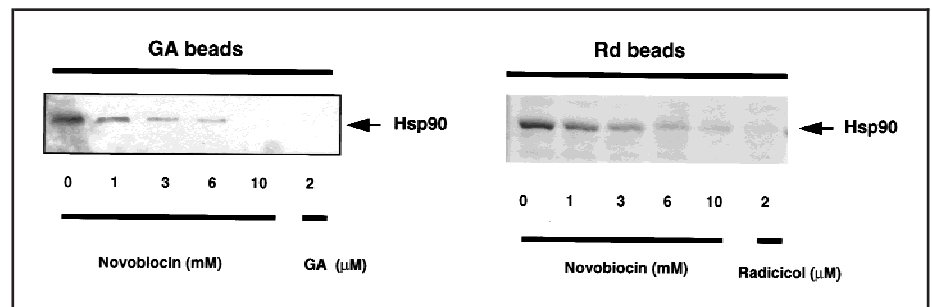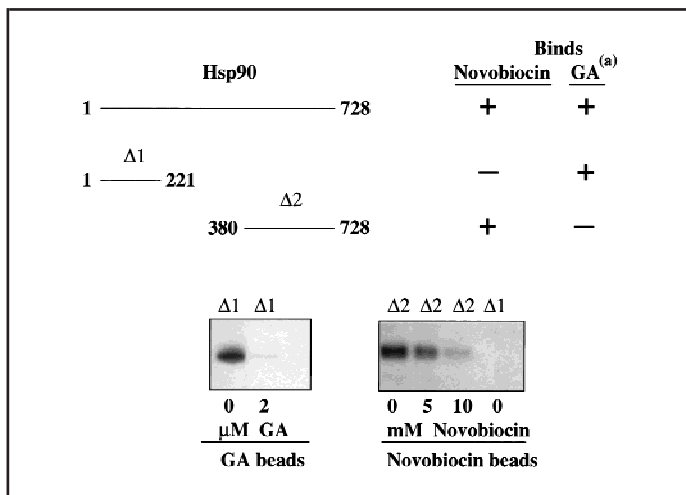
Fig. 4. Schematic representation of full-length heat shock protein 90 (Hsp90) and NH$_2$- and COOH-terminal deletion mutants and their ability to bind to immobilized novobiocin or immobilized geldanamycin. The two deletion mutants, Δ1 and Δ2, obtained as described in the "Materials and Methods" section, were translated *in vitro,* then incubated with geldanamycin (GA) or novobiocin beads in the presence of increasing concentrations of free GA or free novobiocin, respectively. Bound ^{35}S-labeled proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the gel was dried and visualized by autoradiography. (a) *See (18)* for the data describing the behavior of Δ2 and its binding to GA.

site on Hsp90 is different from the previously described amino-terminal geldanamycin/nucleotide-binding site.

Effect of Novobiocin on Hsp90-Dependent Signaling Proteins

A number of client protein kinases and transcription factors are recovered from cells associated with Hsp90. Both geldanamycin and radicicol dissociate these complexes and cause the destabilization and depletion of associated client proteins *(5,10,11).* Because novobiocin binds to a unique site on Hsp90, we wished to test its ability and the ability of structurally related coumarin antibiotics to manifest similar effects.

We treated SKBR3 cells and v-src-transformed NIH 3T3 fibroblasts with increasing concentrations of these drugs for 16 hours and then assessed the protein levels of Raf-1, p185^{erbB2}, mutant p53, and p60^{v-src}. The level of p185^{erbB2} was reduced by 80% relative to untreated controls after overnight treatment with 800 μ*M* novobiocin and by 40% compared with controls after overnight treatment with 300 μ*M* drug (Fig. 5, A). Raf-1, p60^{v-src},

and mutant p53 levels were also markedly reduced after 16 hours of novobiocin treatment. In v-src-transformed NIH 3T3 fibroblasts, the level of p60^{v-src} protein was reduced by 50% after exposure to 600 μ*M* novobiocin (Fig. 5, B). Similar to geldanamycin *(5),* novobiocin lowered mutant p53 protein levels in SKBR3 cells while depleting Raf protein to an undetectable level in the same cells (Fig. 5, A). As controls for protein loading and nonspecific toxicity, the steady-state levels of scinderin, an actin-associated protein, and glucose-regulated protein 78 (BiP or Grp78), an Hsp70 family chaperone localized to the endoplasmic reticulum, were monitored; they were found not to be altered by the doses/exposure times of novobiocin used in these experiments (Fig. 5, B). General interference with protein synthesis is not a likely explanation for these results, since overnight cycloheximide treatment of SKBR3 cells did not affect the steady-state levels of the proteins affected by novobiocin (data not shown). Finally, although novobiocin inhibits topoisomerase II at the concentrations used (100–1000 μ*M*), the drug had no effect on the stability of this protein (Fig. 5, A).

Effect of Other Topoisomerase II Inhibitors on Hsp90-Dependent Signaling Proteins

To determine whether the effects of novobiocin described above could be observed with structurally related coumarin antibiotics, we tested whether chlorobiocin and coumermycin A1 could deplete Hsp90 client proteins. Both p185^{erbB2} and Raf-1 were depleted by these drugs in a manner similar to novobiocin treatment, although at lower drug concentrations (Fig. 6, A and B). Maximal depletion of Raf-1 and p185^{erbB2} occurred with 500 μ*M* chlorobiocin and 100 μ*M* coumermycin A1, respectively. Scinderin, the related actin-binding protein gelsolin, and the chaperone Hsc70 all were unaffected by either of these drugs (Fig. 6, A and B).

Since the coumarin antibiotics are recognized inhibitors of topoisomerase II, we tested whether other topoisomerase II inhibitors, which do not act at the nucleotide-binding site, could similarly deplete Hsp90-dependent client proteins. In contrast to the results obtained with the coumarins, two structurally distinct topoisomerase II inhibitors, doxorubicin (i.e., Adriamycin) and etoposide (i.e., VP16), failed to affect p185^{erbB2} protein level in SKBR3 cells at concentrations that maximally inhibit topoisomerase II activity (Fig. 6, C). Therefore, it seems reasonable to conclude that the ability of the coumarins to deplete Hsp90-dependent proteins is related to their direct interaction with Hsp90 and not to their activity as topoisomerase II inhibitors.

Fig. 5. Novobiocin depletes cells of heat shock protein 90-dependent signaling molecules. SKBR3 human breast carcinoma cells or v-src-transformed mouse NIH 3T3 fibroblasts (the source of p60^{v-src}) were incubated for 16 hours with increasing concentrations of novobiocin. Cells were lysed in ice-cold lysis buffer as described in the "Materials and Methods" section, and protein levels were assessed by western blot analysis. **Panel A:** Protein levels of p185^{erbB2}, scinderin, p53, Raf-1, and topoisomerase II (Topo2) were measured in SKBR3 cell lysate after exposure of the cells to increasing concentrations of novobiocin. **Panel B:** p60^{v-src} and glucose-regulated protein 78 (Grp78 or BiP) levels were measured in v-src-transformed mouse NIH 3T3 fibroblast cell lysate after exposure of the cells to 0.6 m*M* novobiocin.
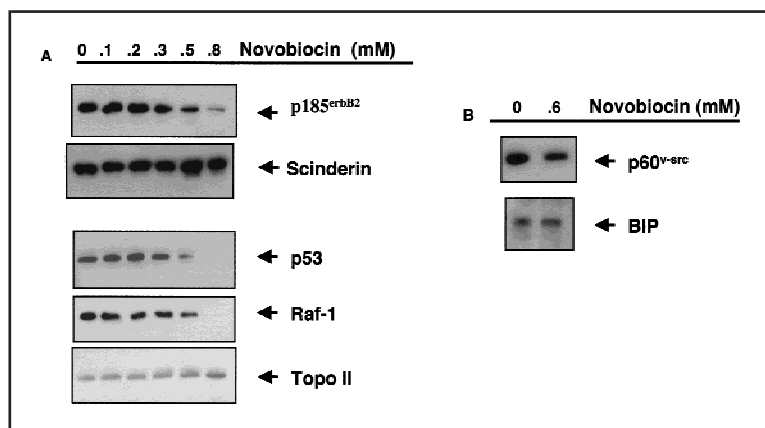
Fig. 6. Chlorobiocin and coumermycin A1, but not etoposide (i.e., VP16) or doxorubicin (i.e., Adriamycin) (structurally unrelated topoisomerase II inhibitors), deplete cells of heat shock protein 90 (Hsp90)-dependent signaling proteins. SKBR3 human breast carcinoma cells were treated with increasing concentrations of drugs for 16 hours and lysed in lysis buffer, and specific protein levels were measured by western blot analysis. In the case of VP16 and Adriamycin, concentrations known to inhibit topoisomerase II were used. **Panel A:** Raf-1, heat shock chaperone protein 70 (Hsc70), p185[erbB2], and scinderin protein levels after exposure of SKBR3 cells to increasing concentrations of chlorobiocin. **Panel B:** p185[erbB2], Raf-1, and gelsolin protein levels after exposure of SKBR3 cells to increasing concentrations of coumermycin A1. In **panels A** and **B,** the levels of scinderin and gelsolin, respectively, were measured to confirm equal protein loading of all lanes, since these proteins are not dependent on Hsp90 for stability. **Panel C:** p185[erbB2] protein level was measured after treatment of SKBR3 cells with VP16 and Adriamycin.
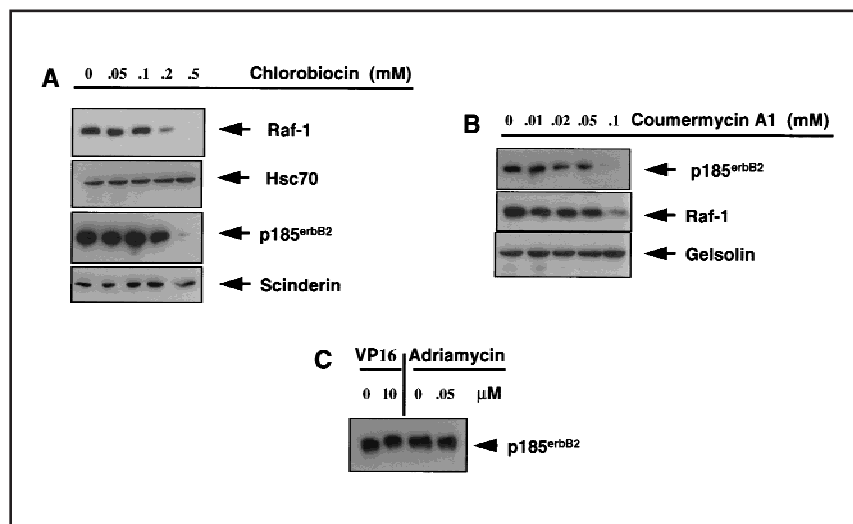
Effect of Novobiocin on Raf-1 in Normal Human Peripheral Blood Cells and in Mouse Splenocytes

To determine whether novobiocin could affect Raf-1 in human peripheral blood mononuclear (PBM) cells, we cultured freshly prepared, nonstimulated PBM cells with novobiocin for 14 hours and found Raf-1 protein to be depleted in a dose-dependent manner (Fig. 7, A). Gelsolin levels remained unaltered, even at the highest drug concentration tested, and treated cells remained viable as assessed by trypan blue dye exclusion.

Next, we examined whether *in vivo* administration of novobiocin could affect Raf-1 levels in murine splenocytes. Because Raf-1 depletion in PBM cells required several hours of exposure to the drug and because novobiocin is cleared relatively quickly from murine plasma (plasma clearance half-life = 80 minutes) *(24),* we chose to treat mice by intraperitoneal injection (100 mg/kg) for 5 days at 12-hour intervals. At this dose, the serum level of the drug has been reported to vary between 100 μg/mL and 450 μg/mL during the first hour *(24).* Our data show that spleens removed from mice receiving novobiocin contained statistically significantly less Raf-1 protein than did spleens from vehicle-treated controls (Fig. 7, B). On average, the Raf-1 protein in the novobiocin-treated group (n = 10) was reduced by 34% compared with vehicle-treated controls (n = 5; $P<.002$, Student's *t* test). In seven of 10 treated mice, the mean splenic Raf-1 protein level was reduced even further, to 29% of control. The splenic content of gelsolin remained unchanged in all mice.

DISCUSSION

Recently, it has become clear that heat shock proteins interact with a variety of proteins involved in cell proliferation [for review, *see (25)*]. It is interesting that Hsp90 is constitutively expressed at twofold to 10-fold higher levels in tumor cells than in their normal counterparts *(26).* The benzoquinone ansamycins geldanamycin and herbimycin A, together with an unrelated macrocyclic antibiotic, radicicol, have been shown to bind to an amino-terminal nucleotide-binding pocket in Hsp90, thus interfering with its chaperone function *(12,16,21,27,28).* Geldanamycin, in particular, has been very useful in identifying previously unrecognized client proteins that depend on Hsp90 for stability and function, and a reasonably well tolerated geldanamycin derivative, 17-allylaminogeldanamycin, has entered phase I clinical trial in patients with cancer.
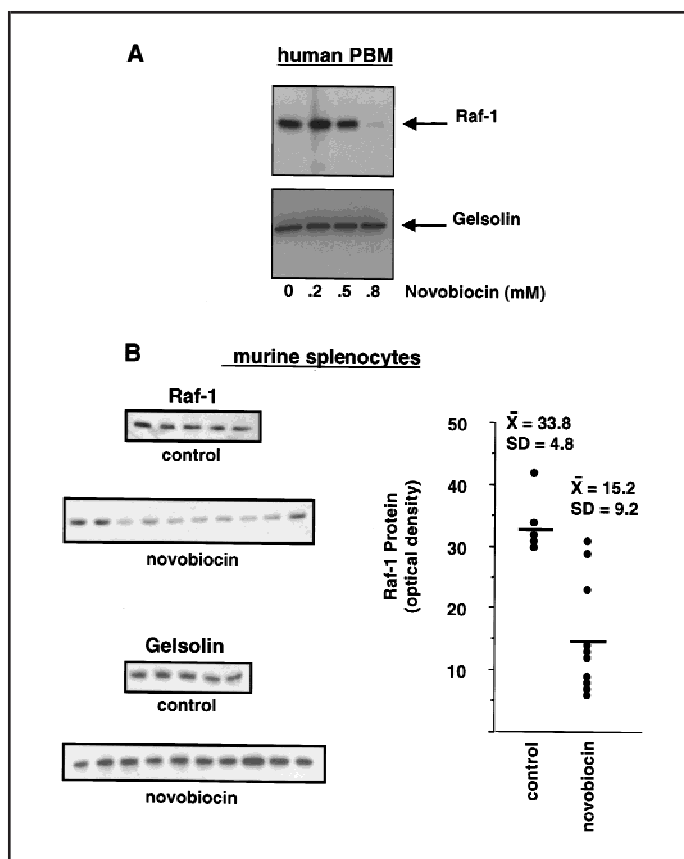

Fig. 7. Novobiocin depletes Raf-1 protein in human peripheral blood mononuclear (PBM) cells *in vitro* and in murine splenocytes *in vivo.* **Panel A:** Human PBM cells were incubated overnight with increasing concentrations of novobiocin and washed twice in cold phosphate-buffered saline, and Raf-1 and gelsolin protein levels were measured by western blot analysis. **Panel B:** Mice were given intraperitoneal injections of novobiocin (100 mg/kg body weight) twice daily at 12-hour intervals for 5 days. Mice were killed 3 hours after receiving the last injection, and their spleens were removed, washed in sterile phosphate-buffered saline, and minced. Splenocytes were collected as described in the "Materials and Methods" section and analyzed for Raf-1 and gelsolin proteins by western blot. The scatter graph depicts individual optical densities (arbitrary units) of the Raf-1 protein bands for each animal. The black bars (▬) represent the means (x̄) of each group. SD = standard deviation.

While the ansamycins and radicicol are exciting lead compounds with promising clinical utility, they may have a low therapeutic index, and these drugs display *in vivo* toxic effects that appear to be unrelated to their Hsp90 antagonism. Therefore, we have been searching for other compounds capable of interfering with Hsp90 function in a manner similar to that of the ansamycins. In 1997, Bergerat et al. *(17)* described a glycine-rich ATP-binding motif in bacterial DNA gyrase, and these investigators reported that Hsp90 contained a highly homologous region in its amino-terminal domain. This domain is fully contained within the ansamycin-binding pocket, and it has been shown to be the binding site for ATP, geldanamycin, and radicicol *(16,18–21)*. The homology between DNA gyrase and Hsp90 nucleotide-binding domains prompted us to investigate whether topoisomerase (gyrase) ATP-binding site inhibitors might also bind to and interfere with the function of Hsp90.

We show here that novobiocin and two related coumarin antibiotics, chlorobiocin and coumermycin A1 (*see* Fig. 1), bind to Hsp90. Thus, novobiocin–Sepharose is able to affinity precipitate Hsp90 from a cell lysate in a manner inhibited by excess ATP, and soluble novobiocin can compete with either immobilized geldanamycin or radicicol for binding to Hsp90. Since neither soluble geldanamycin nor radicicol is able to compete with novobiocin–Sepharose for Hsp90 binding, we thought it most likely that the novobiocin-binding site must be adjacent to or overlapping with, but not identical to, the ansamycin/radicicol-binding pocket. Such a hypothesis is consistent with a report *(29)* that the DNA gyrase B novobiocin-binding site overlaps, but is not identical to, the gyrase ATP-binding motif. It is intriguing, however, that the binding data that we have obtained with Hsp90 deletion mutants suggest that novobiocin specifically interacts with a previously unrecognized domain in the carboxy-terminal portion of Hsp90, quite distinct from the amino-terminal site where geldanamycin binds. The hypothesis that the binding sites for novobiocin and geldanamycin are spatially separated suggests a mechanism by which novobiocin binding to Hsp90 can interfere with the binding of geldanamycin or radicicol, since the amino and carboxy termini of the chaperone are thought to interact closely with each other in solution *(30)*. Indeed, a molybdate-influenced domain in the extreme carboxy terminus of Hsp90 has recently been suggested to regulate Hsp90 conformation and to affect geldanamycin binding *(31)*.

Although novobiocin appears to bind to a site on Hsp90 that is different from the geldanamycin/radicicol-binding site, it, like geldanamycin and radicicol, is able to interfere with the chaperone function of Hsp90 and to deplete tumor cells of a series of Hsp90-dependent signaling proteins. Thus, in SKBR3 breast cancer cells, a 16-hour exposure to novobiocin reduced p185^{erbB2}, mutated p53, and Raf-1 protein levels in a dose-dependent fashion, with maximal activity occurring at 500–800 μM novobiocin. The p60^{v-src} protein was also reduced in v-src-transformed NIH 3T3 cells after a 16-hour exposure to 600 μM novobiocin. Although the novobiocin analogues chlorobiocin and coumermycin A1 were similarly effective at depleting Hsp90-dependent signaling proteins, other topoisomerase inhibitors that do not bind topoisomerase at its nucleotide-binding domain, such as etoposide and doxorubicin, were inactive in this regard. The actin-associated proteins scinderin and gelsolin, the Hsp70 family member BiP (Grp78), and the constitutively expressed Hsp70 homologue Hsc70 were not affected by novobiocin or its analogues.

Novobiocin is a well-studied antibiotic, whose pharmacokinetics and toxicity profile are clearly understood. In humans, doses of 4 g/day (well below the maximum tolerated dose) yield a plasma level of 200–300 μg/mL or higher, 2 hours after oral administration, corresponding to a drug concentration of 300–500 μM *(32,33)*. Since our results demonstrated that novobiocin can deplete Raf-1 in human PBM cells exposed to the drug at these concentrations *in vitro,* we examined whether *in vivo* activity could also be documented. None of the mice receiving intraperitoneal bolus injections of novobiocin twice daily for 5 days displayed any visible signs of toxicity, and their motor activity or body weight was not affected (data not shown). However, seven of 10 treated mice displayed significantly reduced levels of splenic Raf-1 protein (29% of control) when compared with vehicle-treated animals.

Although the plasma clearance half-life of novobiocin in mice is only 80 minutes, its plasma clearance half-life in humans has been measured to be 6 hours *(32),* and high doses of drug (4–6 g) can be readily administered *(24)*. The importance of reducing the expression of particular signal transducers for improved response to chemotherapeutic agents is now appreciated. For example, recent clinical trials *(34)* have shown that HER-2-Neu antibody treatment nearly doubled the response rate to either paclitaxel or cyclophosphamide/doxorubicin regimens in patients with metastatic breast cancer. Our current findings suggest that novobiocin, or other coumarin antibiotics, should be further investigated for their *in vivo* anti-Hsp90 activity. These agents may represent a clinically better tolerated alternative to ansamycins or radicicol for depletion of Hsp90-dependent kinases and other signaling proteins, and their use might prove beneficial in combination with standard chemotherapy. Our data also suggest that pharmacologic interference with Hsp90 function at sites other than the chaperone's amino terminus is possible and should be further explored.

REFERENCES

(1) Wilhelmsson A, Cuthill S, Denis M, Wikstrom AC, Gustafsson JA, Poellinger L. The specific DNA binding activity of the dioxin receptor is modulated by the 90 kd heat shock protein. EMBO J 1990;9:69–76.

(2) Catelli MG, Binart N, Jung-Testas I, Renoir JM, Baulieu EE, Feramisco JR, et al. The common 90-kd protein component of non-transformed '8S' steroid receptors is a heat-shock protein. EMBO J 1985;4:3131–5.

(3) Sanchez ER, Toft DO, Schlesinger MJ, Pratt WB. Evidence that the 90-kDa phosphoprotein associated with the untransformed L-cell glucocorticoid receptor is a murine heat shock protein. J Biol Chem 1985;260:12398–401.

(4) Bohen SP, Yamamoto KR. Modulation of steroid receptor signal transduction by heat shock proteins. In: Morimoto RI, Tissieres A, Georgopoulos C, editors. The biology of heat shock proteins and molecular chaperones. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 1994. p. 313–34.

(5) Blagosklonny MV, Toretsky J, Neckers L. Geldanamycin selectively destabilizes and conformationally alters mutated p53. Oncogene 1995;11:933–9.

(6) Blagosklonny MV, Toretsky J, Bohen S, Neckers L. Mutant conformation of p53 translated *in vitro* or *in vivo* requires functional HSP90. Proc Natl Acad Sci U S A 1996;93:8379–83.

(7) Brugge JS. Interaction of the Rous sarcoma virus protein pp60^{src} with the cellular proteins pp50 and pp90. Curr Top Microbiol Immunol 1986;123:1–22.

(8) Stancato LF, Chow YH, Hutchison KA, Perdew GH, Jove R, Pratt WB. Raf exists in a native heterocomplex with hsp90 and p50 that can be reconstituted in a cell-free system. J Biol Chem 1993;268:21711–6.

(9) Wartmann M, Davis RJ. The native structure of the activated Raf protein kinase is a membrane-bound multi-subunit complex. J Biol Chem 1994; 269:6695–701.

(10) Schulte TW, Blagosklonny MV, Ingui C, Neckers L. Disruption of the Raf-1–Hsp90 molecular complex results in destabilization of Raf-1 and loss of Raf-1–Ras association. J Biol Chem 1995;270:24585–8.

(11) Chavany C, Mimnaugh E, Miller P, Bitton R, Nguyen P, Trepel J, et al. p185erbB2 binds to GRP94 *in vivo*. Dissociation of the p185erbB2/GRP94 heterocomplex by benzoquinone ansamycins precedes depletion of p185erbB2. J Biol Chem 1996;271:4974–7.

(12) Whitesell L, Mimnaugh EG, De Costa B, Myers CE, Neckers LM. Inhibition of heat shock protein HSP90–pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. Proc Natl Acad Sci U S A 1994;91:8324–8.

(13) Sepp-Lorenzino L, Ma Z, Lebwohl DE, Vinitsky A, Rosen N. Herbimycin A induces the 20 S proteasome- and ubiquitin-dependent degradation of receptor tyrosine kinases. J Biol Chem 1995;270:16580–7.

(14) Mimnaugh EG, Chavany C, Neckers L. Polyubiquitination and proteasomal degradation of the p185c-erbB-2 receptor protein-tyrosine kinase induced by geldanamycin. J Biol Chem 1996;271:22796–801.

(15) Sharma SV, Agatsuma T, Nakano H. Targeting of the protein chaperone, HSP90, by the transformation suppressing agent, radicicol. Oncogene 1998;16:2639–45.

(16) Schulte TW, Akinaga S, Soga S, Sullivan W, Stensgard B, Toft D, et al. Antibiotic radicicol binds to the N-terminal domain of Hsp90 and shares important biologic activities with geldanamycin. Cell Stress Chaperones 1998;3:100–8.

(17) Bergerat A, de Massy B, Gadelle D, Varoutas PC, Nicolas A, Forterre P. An atypical topoisomerase II from Archaea with implications for meiotic recombination. Nature 1997;386:414–7.

(18) Grenert JP, Sullivan WP, Fadden P, Haystead TA, Clark J, Mimnaugh E, et al. The amino-terminal domain of heat shock protein 90 (hsp90) that binds geldanamycin is an ATP/ADP switch domain that regulates hsp90 conformation. J Biol Chem 1997;272:23843–50.

(19) Prodromou C, Roe SM, O'Brien R, Ladbury JE, Piper PW, Pearl LH. Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. Cell 1997;90:65–75.

(20) Stebbins CE, Russo AA, Schneider C, Rosen N, Hartl FU, Pavletich NP. Crystal structure of an Hsp90–geldanamycin complex: targeting of a protein chaperone by an antitumor agent. Cell 1997;89:239–50.

(21) Schulte TW, Akinaga S, Murakata T, Agatsuma T, Sugimoto S, Nakano H, et al. Interaction of radicicol with members of the heat shock protein 90 family of molecular chaperones. Mol Endocrinol 1999;13:1435–48.

(22) Staudenbauer WL, Orr E. DNA gyrase: affinity chromatography on novobiocin–Sepharose and catalytic properties. Nucleic Acids Res 1981;9: 3589–603.

(23) Sullivan WP, Toft DO. Mutational analysis of Hsp90 binding to the progesterone receptor. J Biol Chem 1993;268:20373–9.

(24) Eder JP, Wheeler CA, Teicher BA, Schnipper LE. A phase I clinical trial of novobiocin, a modulator of alkylating agent cytotoxicity. Cancer Res 1991;51:510–3.

(25) Neckers LM, Mimnaugh E, Schulte TW. The Hsp90 chaperone family. In: Latchman DS, editor. Handbook of experimental pharmacology. Vol 126. Heidelberg (Germany): Springer-Verlag; 1998. p. 9–42.

(26) Ferrarini M, Heltai S, Zocchi MR, Rugarli C. Unusual expression and localization of heat-shock proteins in human tumor cells. Int J Cancer 1992;51:613–9.

(27) Johnson JL, Toft DO. Binding of p23 and hsp90 during assembly with the progesterone receptor. Mol Endocrinol 1995;9:670–8.

(28) Sullivan W, Stensgard B, Caucutt G, Bartha B, McMahon N, Alnemri ES, et al. Nucleotides and two functional states of hsp90. J Biol Chem 1997; 272:8007–12.

(29) Lewis RJ, Singh OM, Smith CV, Skarzynski T, Maxwell A, Wonacott AJ, et al. The nature of inhibition of DNA gyrase by the coumarins and the cyclothialidines revealed by X-ray crystallography. EMBO J 1996;15: 1412–20.

(30) Csermely P, Schnaider T, Soti C, Prohaszka Z, Nardai G. The 90-kDa molecular chaperone family: structure, function, and clinical applications. A comprehensive review. Pharmacol Ther 1998;79:129–68.

(31) Hartson SD, Thulasiraman V, Huang W, Whitesell L, Matts RL. Molybdate inhibits Hsp90, induces structural changes in its C-terminal domain, and alters its interactions with substrates. Biochemistry 1999;38:3837–49.

(32) Drusano GL, Townsend RJ, Walsh TJ, Forrest A, Antal EJ, Standiford HC. Steady-state serum pharmacokinetics of novobiocin and rifampin alone and in combination. Antimicrob Agents Chemother 1986;30:42–5.

(33) Eder JP, Teicher BA, Holden SA, Cathcart KN, Schnipper LE. Novobiocin enhances alkylating agent cytotoxicity and DNA interstrand crosslinks in a murine model. J Clin Invest 1987;79:1524–8.

(34) Cobleigh MA, Vogel CL, Tripathy D, Robert NJ, Scholl S, Fehrenbacher L, et al. Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. J Clin Oncol 1999;17:2639–48.

NOTE