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

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Background: Heat shock protein 90 (Hsp90) interacts with and stabilizes several oncopgenic protein kinases (e.g., p185\textsuperscript{erbB2}, p60\textsuperscript{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\textsuperscript{erbB2}, p60\textsuperscript{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]
Novobiocin
Chlorobiocin
Coumermycin A1

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

particularly 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). The cells were maintained in Dulbecco’s modified Eagle medium (DMEM) with 10% fetal bovine serum (Whittaker Bioproducts, Walkersville, MD). The cells were maintained in Dulbecco’s modified Eagle medium (DMEM) with 10% fetal bovine serum (Whittaker Bioproducts, Walkersville, MD). The cells were maintained in Dulbecco’s modified Eagle medium (DMEM) with 10% fetal bovine serum (Whittaker Bioproducts, Walkersville, MD).

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 2 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 TNESEV 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 TNESEV 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 TNESEV 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 TNESEV 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 TNESEV 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 carboxy-terminal chicken Hsp90 deletion fragment, both subcloned in pGEM-7Z vector (Promega Corp., Madison, WI), were gifts of Dr. David Toot (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 BamHI and EcoRI 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 an 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 TNESEV 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 TNESEV 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 (Phar- macia LKB Biotechnology AB) cushion and centrifuged for 30 minutes at 1000g at 20 °C to separate the mononuclear cells. After isolation, the cells were washed twice with sterile phosphate-buffered saline (PBS), centrifuged by centrifugation at 1000g 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 450g for 2 minutes at 4 °C. The supernatant (isolated cells) was then separated, and the cells were collected by centrifugation at 900g 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
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 assayed the protein levels of Raf-1, p185erbB2, mutant p53, and p60v-src. The level of p185erbB2 was reduced by 80% relative to untreated controls after overnight treatment with 800 μM novobiocin and by 40% compared with controls after overnight treatment with 300 μM drug (Fig. 5, A). Raf-1, p60v-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 p60v-src protein was reduced by 50% after exposure to 600 μM 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 μM), 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 p185erbB2 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 p185erbB2 occurred with 500 μM chlorobiocin and 100 μM 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 p185erbB2 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.
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 480 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 mg/mL and 450 mg/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 < 0.002, 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-allylamino-geldanamycin, has entered phase I clinical trial in patients with cancer.

![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), p185erbB2, and scinderin protein levels after exposure of SKBR3 cells to increasing concentrations of chlorobiocin. Panel B: p185erbB2, Raf-1, and gelsolin protein levels after exposure of SKBR3 cells to increasing concentrations of chlorobiocin. 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: p185erbB2 protein level was measured after treatment of SKBR3 cells with VP16 and Adriamycin.](https://academic.oup.com/jnci/article-abstract/92/3/242/2965049)
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 p185ErbB2, mutated p53, and Raf-1 protein levels in a dose-dependent fashion, with maximal activity occurring at 500–800 μM novobiocin. The p60v-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


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NOTE

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