Novobiocin Induces a Distinct Conformation of Hsp90 and Alters Hsp90−Cochaperone−Client Interactions†

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ABSTRACT: Hsp90 functions to facilitate the folding of newly synthesized and denatured proteins. Hsp90 function is modulated through its interactions with cochaperones and the binding and hydrolysis of ATP. Recently, novobiocin has been shown to bind to a second nucleotide binding site located within the C-terminal domain of Hsp90. In this report, we have examined the effect of novobiocin on Hsp90 function in reticulocyte lysate. Novobiocin specifically inhibited the maturation of the heme-regulated eIF2α kinase (HRI) in a concentration-dependent manner. Novobiocin induced the dissociation of Hsp90 and Cdc37 from immature HRI, while the Hsp90 cochaperones p23, FKBP52, and protein phosphatase 5 remained associated with immature HRI. Proteolytic fingerprinting of Hsp90 indicated that novobiocin had a distinct effect on the conformation of Hsp90, and molybdate lowered the concentration of novobiocin required to alter Hsp90's conformation by 10-fold. The recombinant C-terminal domain of Hsp90 adopted a proteolytic resistant conformation in the presence of novobiocin, indicating that alteration of Hsp90/cochaperone interactions was not the cause of the novobiocin-induced protease resistance within Hsp90's C-terminal domain. The concentration dependence of this novobiocin-induced conformation change correlated with the dissociation of Hsp90 and Cdc37 from immature HRI and novobiocin-induced inhibition of Hsp90/Cdc37-dependent activation of HRI's autokinase activity. The data suggest that binding of novobiocin to the C-terminal nucleotide binding site of Hsp90 induces a change in Hsp90's conformation leading to the dissociation of bound kinase. The unique structure and properties of novobocin-bound Hsp90 suggest that it may represent the "client−release" conformation of the Hsp90 machine.

Numerous proteins that are involved in the control of physiological processes within cells require the Hsp90[1] chaperone machine for their biogenesis and regulation [reviewed in (*1*−*4*)]. While Hsp90 can function to facilitate the renaturation of misfolded proteins (*5*−*9*), it is not required for the biogenesis of most proteins synthesized in a cell. Rather, Hsp90's specific clientele is primarily restricted to certain proteins involved in the regulation of signal transduction [reviewed in (*1*−*4*)]. Additionally, Hsp90 supports mutational drift in proteins (*10*, *11*) that may be essential to the microevolution of transformed cell populations. Thus, Hsp90's essential function as a chaperone, which is required for regulation of cellular signal transduction, makes it an attractive target for efforts to manipulate the growth and differentiation of normal and aberrant cell populations [reviewed in (*12*−*14*)].

Hsp90 functions through its interactions with numerous cochaperone partners [reviewed in (*1*−*4*)]. Some of Hsp90's cochaperone partners are thought to modulate the interaction of Hsp90 with specific families of clientele. For example, Hsp90's cochaperone Cdc37 is required for the biogenesis of numerous Hsp90-dependent protein kinases [reviewed in (*15*)]. Besides mediating the binding of Hsp90 to specific client targets, Hsp90-associated cochaperones also modulate Hsp90's ATP-driven reaction cycle and Hsp90's nucleotide-modulated conformation switching.

Hsp90 binds and hydrolyzes ATP via a Bergerat fold (*16*, *17*) within its N-terminal domain, which mediates global switching between at least two alternative Hsp90 conformations [reviewed in (*1*−*4*)]. The binding of ATP to Hsp90's N terminus induces the N-terminal domains within the Hsp90 dimer to associate, forming a "molecular clamp" about its client target (*18*−*22*). The Hsp90 specific inhibitor geldanamycin binds to this nucleotide binding site and prevents the formation of the closed conformation of Hsp90 (*23*, *24*), resulting in the destabilization of Hsp90−kinase complexes. Geldanamycin thus causes the accumulation of "intermediate" Hsp90 complexes (*25*, *26*) containing Hsc70 and the Hsp70−Hsp90 organizing protein p60−HOP (a homologue of the yeast protein, Sti1), as it prevents the progression of Hsp90 through its ATP-driven reaction cycle. Sti1 appears

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[1] Abbreviations: Hsp, heat shock protein; Hsp90, 90 kDa Hsp; Hsp70, used to generically refer to all members of the Hsp family; Hsc70, 70 kDa heat shock cognate protein; Cdc37, generically used to refer to the protein product of *CDC37* gene homologues regardless of source of organism; Hsp90-CT, C-terminal domain of Hsp90; HRI, heme-regulated eIF2α kinase; eIF, eukaryotic initiation factor; eIF2 α, α-subunit of eukaryotic initiation factor 2; TnT reticulocyte lysate, nuclease-treated rabbit reticulocyte lysate for coupled transcription/translation of protein; IgG, imunoglubulin G; IgM, immunoglobulin M; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PIPES, piperazine-N,N′-bis(2-ethanesulfonic acid); PP5, phosphoprotein phosphatase 5; TPR, tetratricopeptide repeat; FKBP, FK506 binding protein; DMSO, dimethyl sulfoxide; PVDF, poly(vinylidene difluoride); SD, standard deviation.

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to repress Hsp90's ability to consume ATP (*27*−*29*), as does Cdc37 (*30*, *31*).

The C-terminal region of Hsp90 mediates Hsp90's stable dimerization (*20*, *22*, *32*, *33*) and contains a second nucleotide binding site (*34*−*36*). Novobiocin binds to this nucleotide binding site and inhibits Hsp90 function (*34*, *35*, *37*). The C-terminal region together with Hsp90's middle domain mediates the interactions of Hsp90 with molybdate (*35*, *38*) and protein clients (*39*, *40*) and modulates Hsp90's ATPase activity (*20*, *41*). The binding of ATP to Hsp90's N-terminal domain is required for the second ATP site to become available for the binding of nucleotide (*34*−*36*) and for the ability of molybdate to stabilize Hsp90−client complexes (*42*). The ability of molybdate binding to "freeze" Hsp90's ATPase cycle causes the accumulation of "late" Hsp90 complexes containing the Hsp90 cochaperone p23 and an assortment of other cochaperones, such as Cdc37 and immunophilins (*38*, *42*−*46*). Mutagenesis studies indicate that the formation of stable complexes between p23, Hsp90, and client requires the N-terminal domain of Hsp90, the presence of hydrolyzable ATP, and sequences within the Hsp90-CT (*18*, *19*, *21*, *24*, *41*, *42*, *47*−*49*). The binding of p23 has also been proposed to stimulate the release of client substrate (*50*), and novobiocin appears to disrupt the interaction of p23 (*34*, *51*) and Hsp70 (*34*) with Hsp90. Thus, Hsp90 function is regulated by a complex series of reactions that are modulated by the binding, hydrolysis, and presumably exchange of ATP at two sites and the interactions of Hsp90 with cochaperones and client target proteins.

While reports indicate that novobiocin treatment of cultured cells leads to the loss of Hsp90-dependent protein kinases (*37*), mutant p53 (*37*), and HIF-1α (*52*) and that novobiocin disrupts the binding of Hsp90 and Hsp70 to the glucocorticoid receptor in vitro (*53*) and the AKT kinase in cells (*54*), the effect of novobiocin on the interaction of Hsp90 with other clients and Hsp90-associated cochaperones has yet to be studied in detail. In this report, we have examined the effect of novobiocin on Hsp90-dependent maturation of the HRI, Hsp90's conformational switching and its interactions with other cochaperone partners, and HRI under quasi-physiological conditions in rabbit reticulocyte lysate. The data indicate that novobiocin inhibits Hsp90 function by inhibiting Hsp90's ATP-driven chaperone cycle at a distinct stage. The properties and structure of novobiocin-bound Hsp90 distinguish this Hsp90 conformation from those it adopts when it is bound to geldanamycin or molybdate.

EXPERIMENTAL PROCEDURES

Analysis of the Effects of Novobiocin on the Maturation and Activation of HRI in Rabbit Reticulocyte Lysate. Untreated reticulocyte lysate was prepared by injecting New Zealand White rabbits with 1 mg/kg of 1% N-acetylphenylhydrazine (w/v) in sterile water for 5 days. After 4 days of recovery, blood was collected and washed four times with buffer containing 10 mM Hepes−KOH (pH 7.2), 134 mM NaCl, 5 mM KCl, 7.4 mM magnesium acetate, and 5 mM glucose. The reticulocytes were lysed in 1 volume of sterile deionized water, and the lysate was collected after centrifugation at 20000*g* for 10 min. Normal untreated reticulocyte lysate used in maturational incubations was incubated under conditions for protein synthesis with or without the addition of 20 μM hemin as described previously (*55*).

The [^{35}S]His-tagged HRI was synthesized for 30 min at 30 °C by coupled transcription/translation in nuclease-treated rabbit reticulocyte lysate (TnT, Promega) as previously described (*45*, *46*, *55*−*57*). Following inhibition of initiation by the addition of aurintricarboxylic acid (60 μM final) (*45*, *46*, *55*), HRI was then incubated with drug for the times indicated in the figures, or aliquots (3 μL) of the reaction mixtures were transferred to untreated heme deficient rabbit reticulocyte lysate protein synthesis mixtures (22 μL) containing drug or vehicle control (*45*, *46*, *55*). The reaction mixtures were then incubated at 30 °C for 65 min to determine the effects of the drugs on the maturation and activation ("transformation") of HRI.

Immunoadsorption of Complexes of HRI and Components of the Hsp90 Chaperone Machine from Rabbit Reticulocyte Lysate. The [^{35}S]His-tagged HRI was synthesized as described above and then mixed with 7 volumes of heme deficient rabbit reticulocyte lysate that was pretreated with drug or vehicle control for 5 min at 30 °C and further incubated for 60 min at 30 °C. Coadsorption of Hsp90 and Cdc37 in chaperone−HRI heterocomplexes was analyzed by adsorption with anti-(His_5) antibody (Qiagen) as described previously (*45*, *46*, *55*). To analyze the effect of novobiocin on the interaction of HRI with other cochaperones, HRI was synthesized and matured in the presence or absence of drug as described above. The reaction mixtures were divided in half and incubated with mouse anti-His_5 monoclonal antibody (Qiagen) for the adsorption of His-tagged HRI or with mouse JJ3 anti-p23 monoclonal antibody (provided from Dr. David Toft), mouse EC1 anti-FKBP52 monoclonal antibody (SRA-1400, Stressgen), or mouse M2 anti-FLAG monoclonal antibody (Sigma). The control reactions lacking template coding for His-tagged HRI (or FLAG-[^{35}S]PP5 for experiments examining PP5 interactions) were used as negative controls for nonspecific binding of chaperone and cochaperone components from reticulocyte lysate. Unless specified in the figure legends, the immune pellets were washed once with PIPES buffer (10 mM, pH 7.2) containing 150 mM NaCl with 0.5% Tween-20, followed by three washes with PIPES buffer lacking Tween-20. The samples were separated by SDS−PAGE and analyzed by electrotransfer to PVDF membrane (Bio-Rad) followed by autoradiography to visualize [^{35}S]His-tagged HRI or FLAG-[^{35}S]PP5. Western blotting with polyclonal anti-Hsp90 (*46*), polyclonal anti-Cdc37 (*44*), N27 anti-Hsp70 monoclonal (N27F3-4, StressGen), JJ3 anti-p23 monoclonal, F5 anti-HOP monoclonal, and EC1 anti-FKBP52 monoclonal antibodies was used to detect chaperone components coadsorbed with HRI or with basal Hsp90 complexes adsorbed from lysate using an anti-N terminus Hsp90 antibody (PA3-013 Affinity BioReagents).

Analysis of the Effect of Novobiocin on the Autokinase Activity of HRI. The [^{35}S]His-tagged HRI was synthesized and matured in heme deficient reticulocyte lysate as described above. After immunoadsorption of His-tagged HRI, its autokinase activity was assayed by incubation of the immune pellet in buffer containing 5 μCi of [γ-^{32}P]ATP (Perkin Elmer) and 2 mM unlabeled ATP for 5 min at 30 °C in the presence or absence of novobiocin, as described previously (*55*, *58*). Autophosphorylation of HRI was quantified by scanning densitometry of the autoradiogram screened to eliminate ^{35}S emissions. The band intensity of the [^{32}P]HRI was expressed as optical density (OD × mm^2).

Proteolytic Fingerprinting of Hsp90. TnT reticulocyte lysate was incubated under conditions for protein synthesis in the absence of plasmid at 30 °C in the presence of drug or vehicle control for 10 min. For sequential drug treatments, the second drug was applied after the first 5 min of incubation. The reaction mixtures were then chilled on ice and digested with the indicated amount of trypsin at the concentrations indicated in the figure legend as described previously (*38*). The samples were separated by SDS−PAGE and Western blotted using antibodies specific to the N terminus of Hsp90 (PA3-013 Affinity BioReagents) or the C terminus of Hsp90 (AC88: kindly provided by Dr. David Toft) (*38*).

To estimate the Hill coefficient for novobiocin binding, the intensity of the 78 kDa band generated upon incubation of untreated lysate with 24 μg/mL of trypsin and detected by Western blotting with the antibody specific for the N terminus of Hsp90 was assumed to represent 0% occupancy of Hsp90's novobiocin binding sites. The change in intensity of the 78 kDa band with varying concentrations of novobiocin was used to estimate the fraction of novobiocin-bound vs free Hsp90 from which the Hill plot was constructed.

Purification and Proteolytic Fingerprinting of the Hsp90-CT. To express Hsp90's C-terminal nucleotide binding domain, a PCR product encoding amino acids Q531 to D732 of human Hsp90α was amplified using primers that included exogenous sequences to facilitate subcloning, and this PCR product was ligated into pQE32 (Qiagen). The resultant gene product thus included human Hsp90α residues Q531-D732 fused to an N-terminal tag MRGSHHHHHHGIRM derived from the vector and linker sequences. This gene product was purified to apparent homogeneity from *Escherichia coli* lysates using metal−ion affinity chromatography on nickel affinity resin (Qiagen), wherein the recombinant Hsp90 gene product was eluted from this resin with imidazole. After elution, peak fractions were pooled and dialyzed against 100 mM ammonium bicarbonate and 1 mM DTT. After dialysis, the aliquots were lyophilized and stored in liquid nitrogen until further use.

Purified Hsp90-CT was dissolved with 100 μL (~0.4 μg/mL) of 20 mM Tris HCl, pH 7.4. Aliquots of Hsp90-CT (10 μL) were used for each fingerprint assay. Each sample was incubated on ice for 30 min with drug at the concentration indicated in the figure legend. Each sample was digested for 6 min on ice with 25 μL of assay buffer (10 mM Tris HCl, pH 7.4, 150 mM NaCl, 4 mM $CaCl_2$, and 0.1 mM EDTA) containing trypsin at the concentrations indicated in the figure legend. The reactions were terminated with boiling SDS sample buffer and analyzed by SDS−PAGE and Western blotting with the AC88 antibody, which detects an epitope in the C terminus of Hsp90 (AC88).

RESULTS

Effect of Novobiocin on the Interaction of Hsc70 and p23 with Hsp90. Hsp90 function is modulated through its interaction with other cochaperones. Consistent with results reported by Marcu and co-workers (*34*), novobiocin reduced the amount of Hsc70 and p23 coimmunoadsorbed with Hsp90 from reticulocyte lysate in a concentration-dependent manner (Figure 1A). While the concentration required to reduce the interaction of Hsc70 and p23 with Hsp90 was somewhat higher than those reported by Marcu et al. (*34*), these results confirm the ability of novobiocin to alter Hsp90's default interactions with its cochaperone partners.

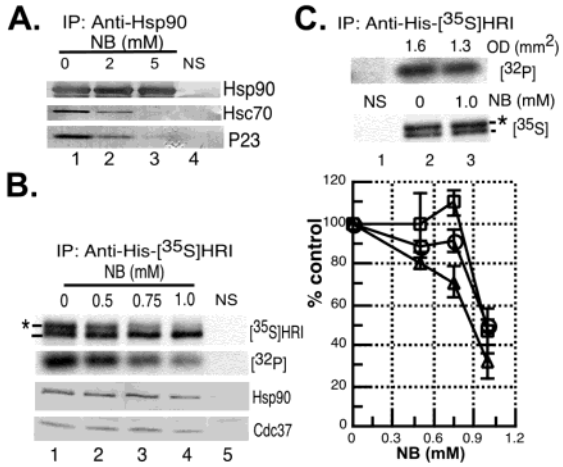

FIGURE 1: Effect of novobiocin on protein folding and Hsp90−cochaperone interactions in rabbit reticuocyte lysate. (A) Nuclease-treated reticulocyte lysate was incubated on ice in the absence of an ATP-regenerating system with 0, 2, or 5 mM novobiocin for 1 h. The samples were immunoadsorbed with anti-Hsp90 antibody and analyzed by SDS−PAGE and Western blotting for Hsp90 and coadsorbed Hsc70 and p23. (B) [^{35}S]His-tagged HRI was synthesized in TnT reticulocyte lysate and then matured in normal heme deficient lysate in the presence or absence (buffer control) of 0.5, 0.75, and 1.0 mM novobiocin as described under the Experimental Procedures. The His-tagged HRI was immunoadsorbed, and the samples were analyzed by SDS−PAGE and autoradiography ([^{35}S]HRI and [^{32}P]HRI, upper two panels) and Western blotting (Hsp90 and Cdc37). The His-tagged HRI was assayed for autokinase activity as described under the Experimental Procedures. NS: Analysis of sample lacking template coding for His-tagged HRI as a control for nonspecific binding of Hsp90 and Cdc37 to the immune resin (left-hand panels). Band densities of [^{32}P]HRI (open triangles) from the autoradiogram and Hsp90 (open circles) and Cdc37 (open squares) from the Western blots were quantified by densitometry (OD × mm^2) and plotted as percent of the buffer control vs novobiocin concentration (average of three experiments ± SD) (right-hand panel). (C) The His-tagged HRI was synthesized and matured in heme deficient reticulocyte lysate as described above. Immunoadsorbed HRI was assayed for autokinase activity in the presence (1 mM, lane 3) or absence (0 mM, lane 2) of novobiocin as described under the Experimental Procedures. The band intensity of [^{32}P]HRI was quantified by densitometry and expressed as OD × mm^2. NS: Analysis of sample lacking template coding for His-tagged HRI as a control for nonspecific binding of any endogenous HRI; autoradiogram of [^{35}S]HRI (lower panel) and autophosphorylated [^{32}P]HRI (upper panel).

Effect of Novobiocin on the Maturation and Activation of HRI. The maturation and activation of HRI in heme deficient reticulocyte lysate (HRI "transformation") require its interaction with Hsp90 and its cochaperone Cdc37 (*45*, *55*). Under normal conditions in reticulocyte lysate, Hsp90 and Cdc37 form a high affinity complex with protein kinases after their release from the ribosome that is stable in the presence of high salt (*44*, *45*). Geldanamycin inhibits Hsp90-facilitated transformation of newly synthesized HRI in reticulocyte lysate (*45*, *55*), prevents the formation of salt stabile high affinity complexes between Hsp90 and HRI, and blocks the incorporation of Cdc37 into complexes formed between Hsp90 and newly synthesized HRI (*44*, *45*). On the other hand, molybdate in the presence of ATP "freezes" Hsp90−client complexes, renders Hsp90−client complexes stable to washing with high salt, but also blocks the tranformation of

HRI (*44*, *45*). Therefore, we investigated whether novobiocin had the capacity to inhibit the ability of Hsp90 to facilitate the transformation of newly synthesized HRI, and whether it similarly altered the interactions of Hsp90 and Cdc37 with HRI.

The [^{35}S]His-tagged HRI was synthesized in reticulocyte lysate and subsequently matured in heme deficient lysate in the presence or absence of novobiocin. The Hsp90/Cdc37-dependent transformation of HRI resulted in the autophosphorylation of HRI and the appearance of a species of HRI with slower electrophoretic mobility during SDS−PAGE [Figure 1B and (*45*, *55*)]. Novobiocin inhibited the "transformation" of HRI in a dose-dependent fashion, as evidence by the decrease in the amount of [^{35}S]HRI that exhibited a slower electrophoretic mobility (Figure 1B).

The effect of novobiocin on the coimmunoadsorption of Hsp90 and Cdc37 with HRI was examined to determine whether novobiocin disrupted the interactions of Hsp90 and Cdc37 with HRI. Western blotting indicated that there was a dose-dependent decrease in the amounts of Hsp90 and Cdc37 that were coadsorbed with His-tagged HRI from heme deficient lysates (Figure 1B). Consistent with our previous work (*55*), reciprocal coimmunoadsorptions of HRI with anti-Hsp90 or anti-Cdc37 antibodies from novobiocin-treated lysate indicated that only the faster migrating species of HRI (e.g., untransformed HRI) was associated with Hsp90 and Cdc37 (not shown).

The Hsp90/Cdc37-dependent transformation of HRI in heme deficient lysate is accompanied by the activation of HRI's autokinase activity. Therefore, we measured the effect of novobiocin on the autophosposphorylation of HRI (Figure 1B, left panel). Again, novobiocin inhibited the activation of HRI's autokinase activity in a concentration-dependent manner. Quantification of the degree of novobiocin-induced inhibition of HRI's autophosphorylation indicated that it correlated closely with the extent of novobiocin-induced reduction of the interaction of Hsp90 and Cdc37 with HRI (Figure 1B): the concentrations of novobiocin that were required to inhibit the interaction of Hsp90 and Cdc37 with HRI and to inhibit HRI autokinase activity by 50% were both between 0.75 and 1 mM (Figure 1B).

Effect of Novobiocin on the Autophosphorylation of HRI. The autophosphorylation of HRI is required for its transformation into an active Hsp90-independent kinase (*45*, *55*). Because novobiocin inhibits DNA gyrase through its ability to bind at or near DNA gyrases's ATP binding pocket, we examined the effect of novobiocin on the autophosphorylation of transformed HRI in vitro at quasi-physiological ATP concentrations (e.g., 2 mM). The addition of 1 mM novobiocin to HRI autokinase assays had little direct effect on HRI's autokinase activity in vitro (Figure 1C), indicating that novobiocin did not compete with ATP for binding to HRI. Thus, there appears to be a good correlation between the ability of novobiocin to reduce the interaction of Hsp90 and Cdc37 with newly synthesized HRI and its ability to inhibit the Hsp90/Cdc37-dependent activation of HRI's autokinase activity.

Effect of Novobiocin on the Stability of the Interaction of Hsp90/Cdc37 with HRI. The strength of the interactions between Hsp90, Cdc37, and newly synthesized HRI is regulated through nucleotide-modulated conformational switching of Hsp90 (*44*, *45*). To further characterize the effect of novobiocin on the interactions of Hsp90 and Cdc37 with HRI, we examined the effect of novobiocin on the salt stability of complexes formed between Hsp90, Cdc37, and HRI. The newly synthesized His-tagged HRI was immunoadsorbed from control and drug-treated reticulocyte lysate, and immunoresins were washed with buffer containing a low or high concentration of NaCl (Figure 2). Western blot analysis indicated that in the absence of drug, high affinity complexes were formed between Hsp90 and Cdc37 and newly synthesized HRI, which were stable in the presence of high salt concentrations (Figure 2A, 0). Consistent with previous results (*44*, *45*), the presence of molybdate similarly led to the formation of salt stable Hsp90/Cdc37−HRI complexes (Figure 2A, MoO_4), while geldanamycin blocked the interaction of Cdc37 with Hsp90−kinase complexes and rendered the interaction of Hsp90 with HRI salt labile (Figure 2A, GA). In contrast, both Hsp90 and Cdc37 were coadsorbed with HRI from reticulocyte lysate treated with 1

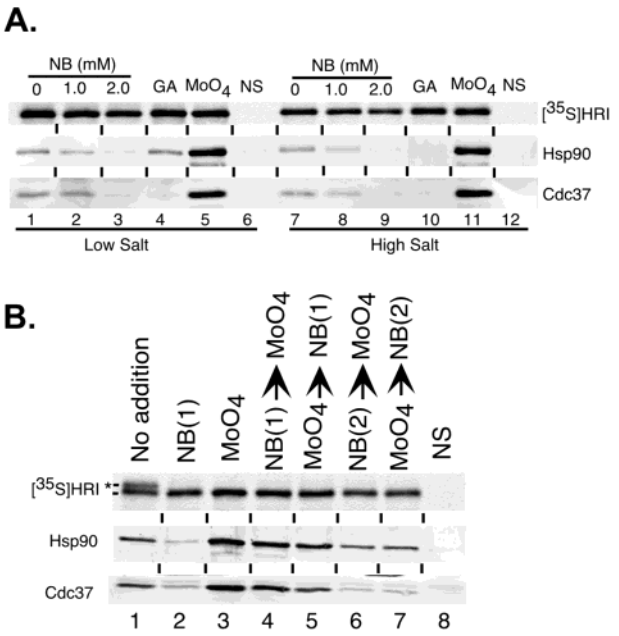

FIGURE 2: Effects of pharmacological agents on the salt stability of the interaction of Hsp90-Cdc37 with HRI. (A) [^{35}S]His-tagged HRI was synthesized in TnT reticulocyte lysate as described under the Experimental Procedues. Ten minutes after arresting initiation of translation, HRI was matured in the absence (0 mM, lanes 1 and 7) or presence of 1 (lanes 2 and 8) or 2.0 mM (lanes 3 and 9) novobiocin, 10 μg/mL geldanamycin (GA, lanes 4 and 10), or 20 mM sodium molybdate (MoO_4, lanes 5 and 11) for 1 h. The His-tagged HRI was immunoadsorbed, followed by washing of the immunoresins with 10 mM PIPES buffer (pH 7.2) containing no salt (lanes 1−6) or 500 mM NaCl (lanes 7−12). (B) The [^{35}S]His-tagged HRI was synthesized in TnT reticulocyte lysate and subsequently matured in normal heme deficient lysate that had been pretreated with buffer (lane 1), 1.0 mM novobiocin [NB(1), lanes 2 and 4], 2.0 mM novobiocin [NB(2), lane 6], or 20 mM sodium molybdate (MoO_4, lanes 3, 5, and 7) for 5 min. After 5 min of incubation at 30 °C, the samples were supplemented with buffer (lanes 1−3), 1.0 mM novobiocin [NB(1), lane 5], 2 mM novobiocin [NB(2), lane 7], or 20 mM sodium molybdate (MoO_4, lanes 4 and 6). After incubation for 60 min, the His-tagged HRI was immunoadsorbed, and the samples were washed as described under the Experimental Procedures. The samples were separated by SDS−PAGE and analyzed by autoradiography ([^{35}S]HRI) and Western blotting (Hsp90, middle panel; Cdc37, lower panel). NS: Analysis of sample lacking template coding for His-tagged HRI as a control for nonspecific binding of Hsp90 and Cdc37 to the immune resin (A, lanes 6 and 12; B, lane 8).

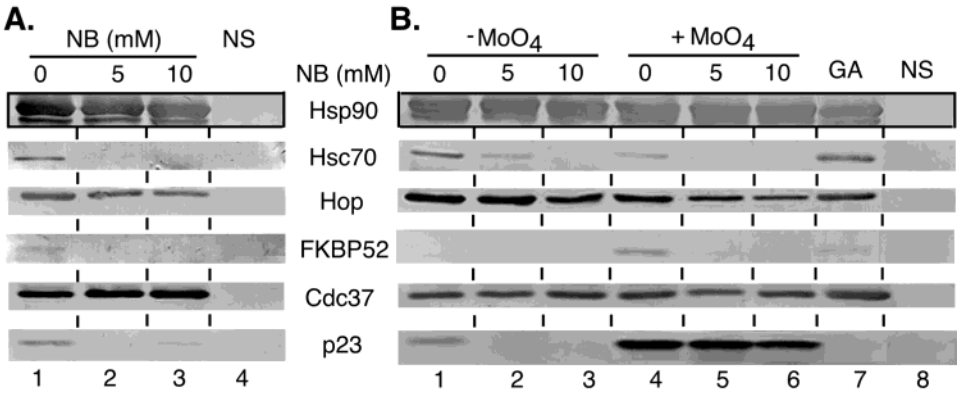

FIGURE 3: Effects of novobiocin on the basal interaction of Hsp90 with cochaperones. (A) The nuclease-treated TnT reticulocyte lysate was incubated without the addition of an ATP-regenerating system in the absence (lane 1) or the presence of 5 (lane 2) or 10 mM (lane 3) novobiocin on ice for 1 h. (B) The nuclease-treated TnT reticulocyte lysate was incubated under conditions for coupled transcription−translation for 10 min at 30 °C with no additions (lane 1), 5 (lane 2) or 10 mM (lane 3) novobiocin, 20 mM molybdate (lane 4), or 20 mM molybdate and 5 (lane 5) or 10 mM (lane 6) novobiocin, or 10 μg/mL geldanamycin (GA, lane 7). The Hsp90 was immunoadsorbed (boxed panel), and the samples were separated by SDS−PAGE and analyzed by Western blotting for Hsp90, Hsc70, HOP, FKBP52, Cdc37, and p23. NS: Sample prepared using a nonspecific IgG as a control for nonspecific binding of proteins to the immune resin (A, lane 4; B, lane 8). The boxed panels indicate the target protein for the immunoadsorption.

mM novobiocin, and their association with HRI was not affected upon washing with buffer containing high salt. However, neither Hsp90 nor Cdc37 were coadsorbed with HRI from lysate treated with 2 mM novobiocin, indicating that novobiocin can completely disrupt the interaction of Hsp90 with a client target at concentrations ≥ 2 mM. These results suggest that novobiocin has effects on Hsp90/Cdc37−kinase interactions that are distinct from those induced by molybdate and geldanamycin.

Effect of Novobiocin on Molybdate-Induced Stabilization of Hsp90−Cdc37−Kinase Complexes. As noted above, newly synthesized protein kinases form high affinity complexes with Hsp90 and Cdc37 that are stabile to washing in buffers containing high salt (e.g., 0.5 M NaCl) immediately after their release from the ribosome (*44*, *46*). Furthermore, the addition of molybdate in the presence of ATP "freezes" these high affinity complexes preventing kinase maturation and activation, while the addition of geldanamycin prevents the formation of these high affinity Hsp90−Cdc37−kinase complexes (*44*, *46*). Order-of-addition experiments have indicated that preincubation of reticulocyte lysate with either geldanamycin or molybdate blocks the pharmacological effect of the other upon its subsequent addition to lysate (*44*). The ability of novobiocin to induce dissociation of Hsp90−Cdc37 from immature HRI molecules suggests that the binding of novobiocin to Hsp90 induces Hsp90 to adopt a conformation distinct from the conformation that Hsp90 adopts in the presence of ATP and molybdate. Thus, we carried out order-of-addition experiments to determine whether molybdate or novobiocin had a dominant effect to the other upon its preincubation in reticulocyte lysate (Figure 2B). While the presence of molybdate stabilized the interaction of Hsp90 and Cdc37 with HRI, a reduction in the binding of Hsp90 and Cdc37 to HRI was apparent in the presence of 1 mM novobiocin and molybdate, and a marked reduction in the interaction between these proteins occurred in the presence of 2 mM novobiocin. Furthermore, this reduction in binding was observed whether molybdate was present in the initial preincubation (Figure 2B, lane 7) or whether it was added to lysate after preincubation with novobiocin (Figure 2B, lane 6). Thus, the binding of novobiocin and molybdate to Hsp90 does not appear to be mutually exclusive and may occur concurrently. In addition, it was quite clear that novobiocin inhibited the ability of molybdate to stabilize the binding of Hsp90 and Cdc37 to its client target, HRI.

Effect of Novobiocin on the Basal Interaction of Cochaperones with Hsp90. Marcu and co-workers have reported that Hsp70 and p23 are not coadsorbed with Hsp90 from novobiocin-treated reticulocyte lysate (*34*). However, their experiment was carried out on ice and it was not apparent from the description of the experimental protocol whether an ATP regenerating system was present. Therefore, we further characterized the effect of novobiocin on basal complexes formed between Hsp90 and its cochaperones in reticulocyte lysate. Anti-Hsp90 immunoadsorptions were carried out on ice, or at 30 °C in the presence of an ATP regenerating system, and analyzed for coadsorbing Hsc70, HOP, FKBP52, Cdc37, and p23. On ice, 4 and 10 mM novobiocin significantly reduced the interaction of Hsc70, FKBP52, and p23 with Hsp90, while it suppressed the interaction of HOP with Hsp90 to a small degree and had little effect on the amount of Cdc37 that was coadsorbed with Hsp90 (Figure 3A).

In the presence of an ATP regenerating system, Hsp90 is a mixture of molecules in their default conformation and those that are actively cycling after binding to cryptic client substrates. In the absence of molybdate, 5 mM novobiocin markedly reduced the binding of Hsc70 to Hsp90, with 10 mM novobiocin blocking their interaction completely (Figure 3B). Both concentrations of novobiocin inhibited the interaction of p23 and Hsp90. The interaction of Cdc37 with Hsp90 was affected very little in the presence of 5 mM novobiocin, while 10 mM novobiocin reproducibly caused a slight increase in the binding of Cdc37 to Hsp90. On the other hand, the interaction of HOP with Hsp90 was decreased by 13 ± 9 and 44 ± 19% (mean ± SD of three experiments) in the presence of 5 and 10 mM novobiocin, respectively, but was unaffected by 2 mM novobiocin (not shown).

The addition of molybdate to reticulocyte lysate in the presence of the ATP regenerating system caused a significant decrease in the amount of Hsc70 that bound Hsp90 and

caused a 27 ± 6% (mean ± SD of three experiments) decrease in the interaction of HOP with Hsp90 (Figure 3B, lane 4 vs lane 1). In contrast, molybdate increased in the amounts of FKBP52 and p23 bound to Hsp90 (Figure 3B). This is consistent with molybdate's ability to stabilize late Hsp90 heterocomplexes that lack Hsc70 and HOP but contain p23 and FKBP52. In the presence of molybdate, novobiocin eliminated the binding of both Hsc70 and FKBP52 to Hsp90. The 5 and 10 mM concentrations of novobiocin further decreased the interaction of HOP with Hsp90 by approximately 23 ± 14 and 55 ± 9%, respectively (mean ± SD relative to the molybdate control of three experiments). In contrast, novobiocin at concentrations of 5 and 10 mM had only a slight inhibitory effect on the interaction of Cdc37 with Hsp90 (Figure 3B). Furthermore, in the presence of molybdate, novobiocin had little effect on the interaction of p23 with Hsp90, with 10 mM novobiocin causing a slight but reproducible reduction in the interaction of p23 with Hsp90. Again, the effects of novobiocin on the basal interactions of Hsp90 with its cochaperones were distinct from those of geldanamycin: geldanamycin blocked the association of p23 with Hsp90, reduced the amount of HOP that was coadsorbed with Hsp90, but had little effect on the interactions of Hsc70, FKBP52, and Cdc37 with Hsp90 (Figure 3B, GA).

Effect of Novobiocin on the Interaction of p23, FKBP52, and PP5 with Newly Synthesized HRI. FKBP52, protein phosphatase 5 (PP5), and p23 are cochaperones that interact with Hsp90−client complexes that are formed "late" in the ATP-driven reaction cycle of Hsp90 [reviewed in (*1*, *2*, *4*)]. Recently, we have demonstrated that cochaperone components containing TPR motifs (i.e., FKBP52 and PP5), which interact with the C-terminal EEVD motif of Hsp90 (*59*−*65*), can be present as components of Hsp90−Cdc37 complexes containing bound kinase (*44*, *46*). Thus, to characterize further the step in the reaction cycle that is affected by novobiocin, we examined the effect of novobiocin on the interactions of the cochaperones p23, PP5, and FKBP52 with client, HRI, and the results were compared with the effects of molybdate and geldanamycin on these interactions.

Newly synthesized His-tagged HRI was immunoadsorbed from heme replete reticulocyte in the presence and absence of drug treatments (Figure 4A), and the coadsorption of Hsp90 and Cdc37. FKBP52 and p23 with HRI were assessed by Western blotting. Consistent with previous results (*44*, *46*), geldanamycin completely disrupted the interaction of Cdc37 with HRI, while a weak interaction of Hsp90 with HRI was maintained (Figure 4A, GA). Geldanamycin also inhibited the coadsorption of p23 and FKBP52 with His-tagged HRI. In contrast, 2 mM novobiocin completely disrupted the binding of both Hsp90 and Cdc37 to HRI (Figure 4A). Interestingly, Western blotting indicated that 2 mM novobiocin reproducibly increased the amount of FKBP52 that was coadsorbed with His-tagged HRI (Figure 4A). In addition, p23 (Figure 4A, lane 1) was detected to coadsorb with His-tagged HRI. In the presence of 2 mM novobiocin, however, the amount of p23 that was coadsorbed with HRI was reduced to a nearly imperceptible level (not readily visible on scanned data: Figure 4A, lane 2). Coadsorption of p23 with HRI was readily detectable in the presence of molybdate, and the level of coadsorbed p23 was decreased markedly in the presence of 2 mM novobiocin (Figure 4A, lane 3 vs lane 4). Currently, antibodies available to PP5 do not have sufficient sensitivity to detect PP5.

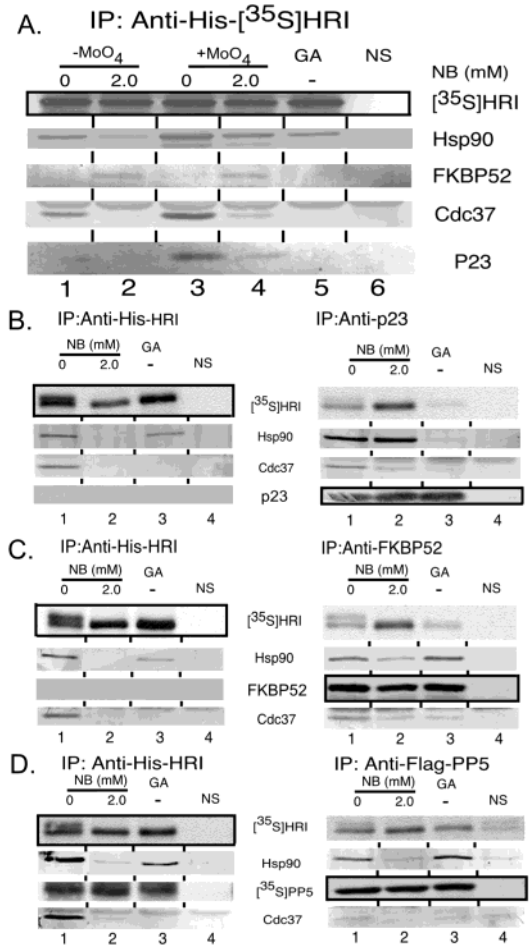

FIGURE 4: Effects of novobiocin on the interaction of HRI with Hsp90 cochaperones. (A) The [^{35}S]His-tagged HRI was synthesized in TnT reticulocyte lysate. After inhibition of initiation by the addition of aurintricarboxylic acid, the lysate was incubated for 1 h at 30 °C with no additions (lane 1), 2 mM novobiocin (lane 2), 20 mM molybdate (lane 3), 2 mM novobicin plus 20 mM molybdate (lane 4), or 10 μg/mL geldanamycin (GA, lane 5). (B) The [^{35}S]His-tagged HRI was synthesized in TnT reticulocyte lysate and then matured in normal heme deficient lysate in the absence (B−D, lane 1) or presence of 2 mM novobiocin (B−D, lane 2) or 10 μg/mL geldanamycin (B−D, GA, lane 3), as described under the Experimental Procedures. For the experiment shown in part D of the figure, the sample containing His-tagged HRI was mixed with an equal volume of lysate containing newly synthesized FLAG[^{35}S]PP5 (*46*) prior to dilution of the sample into heme deficient lysate. The samples for part A were immunoadasorbed with anti-His-tag antibody, while the samples for parts B−D were divided in half and immunoadsorbed with anti-His-tag (B−D, left panel), JJ3 anti-p23 (B, right panel), EC1 anti-FKBP52 (C, right panel), or M2 anti-FLAG (D, right panel) antibody. After they were washed, the samples were separated by SDS−PAGE and analyzed by autoradiography (A−D, [^{35}S]HRI, upper panel; D, [^{35}S]PP5) and Western blotting (Hsp90, FKBP52, Cdc37, and p23). The boxed panels indicate the target protein for the immunoadsorption. NS: Analysis of sample lacking template coding for His-tagged HRI as a control for nonspecific binding of Hsp90 and Cdc37 to the immune resin (A, lane 6; B−D, lane 4, all panels).

Immunoadsorptions of His-tagged HRI (Figure 4B−D) from heme deficient lysate were also carried out. Novobiocin and geldanamycin both inhibited the transformation of HRI in heme deficient lysate as evidenced by the lack of an

[^{35}S]HRI exhibiting slower electrophoretic mobility on SDS−PAGE (Figure 4B−D: left panels). The effects of novobiocin and geldanamycin on the coadsorption of Hsp90 and Cdc37 (Figure 4B−D: left panels) were the same as those observed for the experiments carried out in heme replete lysate (Figure 4A). However, the amount of HRI immunoadsorbed in these experiments was not sufficient to detect coadsorption of either p23 or FKBP52.

Immunoadsorptions of p23 (Figure 4B: right panel), FKBP52 (Figure 4C: right panel), and [^{35}S]FLAG-PP5 (Figure 4D: right panel) from heme deficient reaction mixes were carried out in parallel to overcome the poor detection sensitivity of antibodies directed against these proteins on Western blots. Geldanamycin markedly reduced the amount of Cdc37 that was coadsorbed with basal complexes containing p23 (Figure 4B: right panel), FKBP52 (Figure 4C: right panel), or PP5 (Figure 4D: right panel). While geldanamycin blocked the basal interaction of p23 with Hsp90 (Figure 4B: right panel), it had little effect on the basal interaction of FKBP52 (Figure 4C: right panel) and PP5 (Figure 4D: right panel) with Hsp90. Furthermore, while little HRI was coadsorbed with p23 from geldanamycin-treated lysate, geldanamycin only partially reduced the amount of HRI that was coadsorbed with FKBP52 and FLAG-PP5. The coadsorption of HRI with FLAG-PP5 in the presence of geldanamycin is consistent with our previous work (*46*, *66*).

In contrast to the effects of geldanamycin, HRI was coimmunoadsorbed in conjunction with p23, FKBP52, and FLAG-[^{35}S]PP5 from lysates treated with 2 mM novobiocin. Western blotting of samples from anti-cochaperone immunoadsorptions (Figure 4B−D: left panels) indicated that 2 mM novobiocin markedly reduced the amount of Cdc37 present in basal complexes with p23 and nearly completely blocked the interaction of Cdc37 with basal complexes containing FKBP52 or PP5. In addition, 2 mM novobiocin brought about a reduction in the amount of Hsp90 present in basal complexes with FKBP52 and PP5 but had only a marginal effect on the basal interaction of p23 with Hsp90. Data presented in Figure 1 suggest that novobiocin concentrations of greater than 2 mM may be required to quantitatively disrupt the basal interaction of p23 with Hsp90. Thus, the data suggest that the p23, FKBP52, and PP5 cochaperone components of the Hsp90 chaperone machine can maintain their interaction with HRI after novobiocin-induced dissociation of Hsp90 and Cdc37 from this client target. Because these cochaperones are components of "late" complexes formed between the Hsp90 chaperone machine and client targets, the data suggest that novobiocin may bind Hsp90 during or after the formation of these late complexes.

Effect of Novobiocin on the Conformation of Hsp90. Hsp90 gives different proteolytic fingerprints in its geldanamycin-bound and its molybdate-bound states (*44*). The proteolytic fingerprint of Hsp90 in the presence of geldanamycin is the same as the fingerprint of Hsp90 in its default conformation. Order-of-addition experiments have indicated that the geldanamycin-bound and molybdate-bound conformations are not freely interchangeable, such that Hsp90 adopts the conformation induced by the first agent added and does not convert to the alternate conformation upon the subsequent addition of either geldanamycin or molybdate (*44*). The biochemical properties of geldanamycin-bound Hsp90 suggest that Hsp90 is in its "open" conformation and poised to bind client, while the properties of molybdate-bound Hsp90 formed in the presence of ATP suggest that Hsp90 is in its "closed" conformation: a conformation equivalent to the salt stabile high affinity conformation that Hsp90 adopts with bound client [e.g., with newly synthesized HRI (*44*)] in the presence of hydrolyzable ATP (*24*, *38*, *42*, *49*).

Proteolytic fingerprinting of Hsp90 in situ in reticulocyte lysate was carried out to determine whether the binding of novobiocin induces Hsp90 to adopt a conformation that differs from the conformation it assumes upon binding of geldanamycin or molybdate (Figure 5). The membranes were blotted with antibodies directed against N-terminal residues of Hsp90 or C-terminal residues of Hsp90 (AC88) (*67*) to determine the position of the major cut sites (*44*). The trypsinolytic fingerprint of Hsp90 generated in the presence of 2 mM novobiocin was indistinguishable from the fingerprints of Hsp90 generated from untreated lysate (its default conformation, Figure 5A). The addition of geldanamycin either before or after the incubation of lysate with 2 mM novobicin also had no effect on Hsp90's fingerprint (data not shown). However, novobiocin concentrations of greater than 5 mM had significant effects on Hsp90's proteolytic fingerprint. At high novobiocin concentrations, a 50 kDa C-terminal fragment was detected by Western blotting with the AC88 antibody (Figure 5A, right panel). This fragment is also generated when molybdate alone is added to reticulocyte lysate (Figure 5B). In addition, the 30 kDa, the 50 kDa doublet, and the 78 kDa N-terminal fragments of Hsp90 were lost with increasing novobiocin concentrations and a new 73 kDa fragment appeared (Figure 5A, left panel).

Consistent with our previously published results (*44*), the addition of molybdate altered the fingerprint of Hsp90, with the binding of molybdate protecting Hsp90 from cleavage by trypsin at a site around amino acid 400 and decreasing the rate of cleavage at a site around amino acid 600, such that a major stable 50 kDa fragment representing the C-terminal half of Hsp90 was detected on Western blots with the AC88 anti-Hsp90 antibody (Figure 5B: right panel). The addition of 0.5−5 mM novobiocin in the presence of molybdate led to a concentration-dependent protection of Hsp90 from trypsinolytic cleavage at the site near amino acid 600, leading to the disappearance of the 78 kDa Hsp90 fragment detected by the anti-N-terminal Hsp90 antibody (Figure 5B: left panel) and an increase in the amount of 50 kDa C-terminal fragment detected by the AC88 anti-Hsp90 antibody (Figure 5B: right panel). Furthermore, a 30 kDa N-terminal fragment disappeared from Hsp90 fingerprints generated in the presence of molybdate and increasing concentrations of novobiocin (Figure 5B: left panel). Consistent with data presented in Figure 2, the order of addition of molybdate and novobiocin had no effect on the trypsinolytic fingerprints of Hsp90 (data not shown). Thus, in the presence of molybdate and novobiocin, only the major cleavage site near amino acid 280 and two minor sites around amino acid 230 of Hsp90 were cleaved, generating 40 kDa and 22/24 kDa bands detected by blotting with antibody specific to the N terminus of Hsp90 (Figure 5B: left panel). Cleavage near amino acids around 230 and 280 represents cutting of Hsp90 near the beginning and the end of the charged linker region that separates the N-terminal ATP binding domain of Hsp90 from its middle domain (*68*).

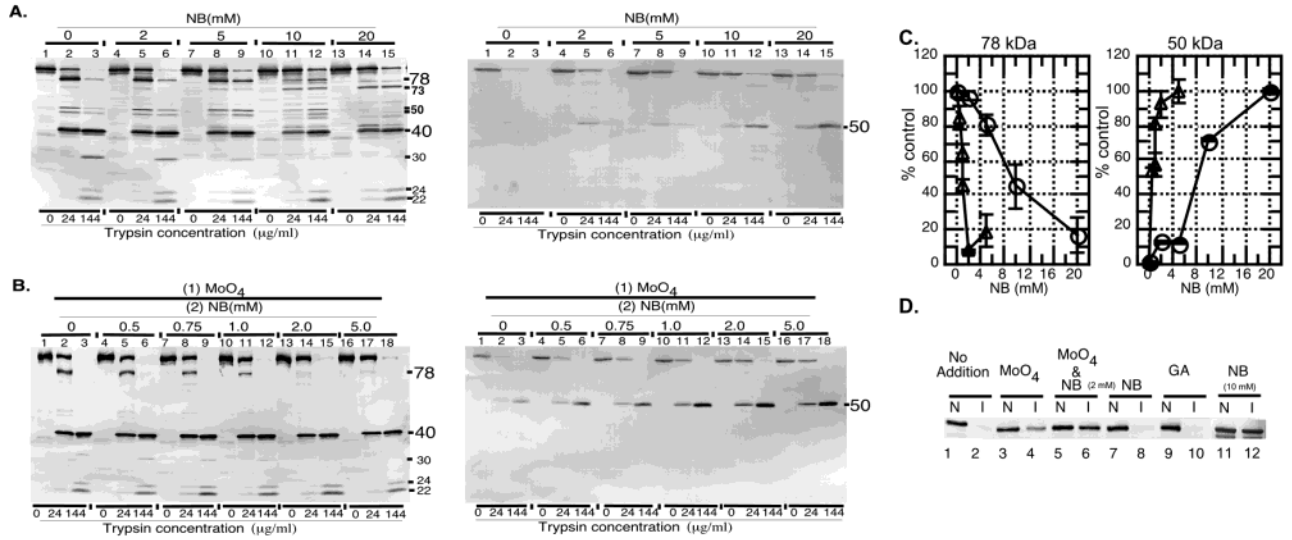

FIGURE 5: Proteolytic mapping of structural changes in Hsp90 induced by novobiocin. The TnT reticulocyte lysate was incubated under conditions for protein synthesis in the absence of plasmid for 10 min at 30 °C in the presence of the indicated concentrations of novobiocin (A, 0, 2, 5, 10, and 20 mM) or in the presence of 20 mM molybdate plus the indicated concentrations of novobiocin (B, 0, 0.5, 0.75, 1, 2, and 5 mM). The reactions were chilled on ice and incubated for 6 min in the presence of the indicated concentration of trypsin and analyzed as described under the Experimental Procedures. Western blot with AC88 anti-Hsp90 antibody, which recognizes an epitope within the C-terminal region of Hsp90 [AC88(α-CT), A and B, right panel]. Western blot with anti-Hsp90 antibody raised to sequence at the N terminus of Hsp90 [ABR(α-NT), A and B, left panel]. (C) The amounts of the 50 kDa band in the right panels and the 78 kDa band in the left panels from A (open circles) and B (open triangles) were quantified by scanning densitometry and plotted as a percent of the no novobiocin control (A and B, 78 kDa band, left panel, lane 2 = 100%) or percent of the maximum observed change (A and B, 50 kDa band, right panel, lane 15 = 100%). The values are the average of two experiments with the error bars indicating the range of the two points. (D) The ability of the AC88 anti-Hsp90 antibody (I) or nonimmune control antibody (N) to immunadsorb Hsp90 from rabbit reticulocyte lysate was determined as previously described (*38*). Briefly, the TnT reticulocyte lysate was incubated under conditions for protein synthesis in the absence of plasmid for 20 min at 30 °C, followed by a 10 min incubation in the presence of no additions (lanes 1 and 2), 20 mM sodium molybdate (lanes 3 and 4), 20 mM sodium molybdate and 2 mM novobiocin (lanes 5 and 6), 2 mM novobiocin (lanes 7 and 8), 10 μg/mL geldanamycin (lanes 9 and 10), or 10 mM novobiocin (lanes 11 and 12). After Hsp90 was immunoadsorbed, the amount of unadsorbed Hsp90 remaining in the unbound fraction was analyzed by SDS−PAGE and Western blotting as previously described (*38*).

The results presented in Figure 1 indicated that novobiocin-induced inhibition of HRI's Hsp90/Cdc37-dependent acquisition of autokinase activity correlated directly with disruption of the binding of Hsp90 and Cdc37 to HRI. Therefore, the decrease in the intensity of the 78 kDa N-terminal fragment and the increase in the intensity of the 50 kDa C-terminal fragment was quantified and used to estimate the change in Hsp90 conformation induced by increasing concentrations of novobiocin (Figure 5C). The concentration of novobiocin required to induce a 50% change in the cleavage of Hsp90 was approximately10 mM. A Hill plot, constructed assuming that the change in intensity of the 78 kDa band induced by novobiocin was proportional to the amount of novobiocin-bound Hsp90, yielded a Hill coefficient of 1.7 ($R = 0.98$) suggesting that the binding of novobiocin is highly cooperative.

However, in the presence of molybdate, only ~1 mM novobiocin was required to induce a 50% changed in the cleavage of Hsp90 (Figure 5C). Thus, the conformational change in Hsp90 that is induced by the binding of molybdate in the presence of ATP increased the binding affinity of Hsp90 for novobiocin by 10-fold. The change in Hsp90 conformation induced by novobiocin in the presence of molybdate correlated well with novobiocin-induced disruption of the interaction of Hsp90 and Cdc37 with HRI and inhibition of HRI's autokinase activity (Figure 1B). These results suggest that there may be a relationship between the conformational change induced in Hsp90 upon the binding of novobiocin to molybdate-bound Hsp90 and the dissociation of Hsp90−Cdc37 from complexes containing client kinase. It should be noted that in the presence of molybdate and 5 mM novobiocin, approximately 20% of the 78 kDa band remained relative to the molydate control, suggesting that there may be a population of Hsp90 molecules present in reticulocyte lysate that may not be capable of interacting with molybdate.

To further investigate the effect of novobiocin on the conformation of Hsp90, we examined the ability of the AC88 anti-Hsp90 antibody to immunadsorbed Hsp90 from reticulocyte lysate. AC88 does not appear to adsorb Hsp90 present in complexes containing bound client (*69*), and molybdate decreases the ability of the AC88 antibody to immunoadsorb Hsp90 from reticulocyte lysate (*38*). The AC88 anti-Hsp90 antibody quantitatively adsorbed Hsp90 from control, geldanamycin-treated, and novobiocin-treated (2 mM) lysate (Figure 5D). In the presence of molybdate, approximately 50% of the Hsp90 present in reticulocyte lysate was adsorbed by the AC88 antibody, while in the presence of both molybdate and novobiocin or high concentrations of novobiocin alone (10 mM) only about 5−15% of the Hsp90 was immunoadsorbed. Thus, overall, these results suggest that novobiocin interacts with Hsp90 complexes after Hsp90 adopts its molybdate-bound conformation: a conformation with properties similar to those exhibited by Hsp90 after it has formed salt stabile high affinity complexes with kinase client.

Novoboicin Directly Protects the Hsp90-CT from Cleavage by Trypsin. The effect of novobiocin on the sensitivity of Hsp90 to proteolytic cleavage could result from a direct effect of the binding of novobiocin to Hsp90, or it could be due to novobiocin altering the interaction of cochaperones with

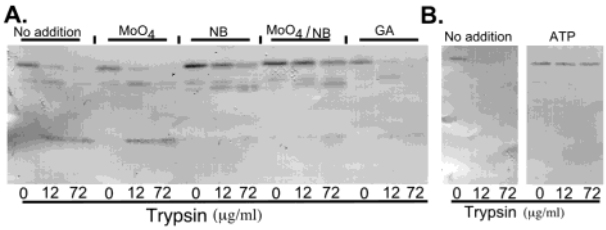

FIGURE 6: Proteolytic mapping of Hsp90-CT. The purified recombinant C-terminal domain was incubated for 10 min at 30 °C in the presence of (A) no additions, 20 mM sodium molybdate (MoO_4), 2 mM novobiocin (NB), 20 mM sodium molybdate and 2 mM novobiocin (MoO_4/NB), or 20 μg/mL geldanamycin (GA) or (B) no additions or 5 mM MgATP. The reactions were chilled on ice and incubated for 6 min in the presence of the indicated concentration of trypsin and analyzed by SDS−PAGE and Western blotting with AC88 anti-Hsp90 antibody.

Hsp90. To distinguish between these two possibilities, we examined the effect of novobiocin on the proteolysis of Hsp90's C-terminal domain (amino acids 584−730). The C-terminal domain of recombinant Hsp90 binds novobiocin in the absence of the N-terminal domain of Hsp90 (*34*). Thus, use of the His-tagged recombinant Hsp90-CT domain avoids potential problems caused by the allosteric regulation of the novobiocin binding site via the binding of nucleotide to the N-terminal domain of Hsp90 (*35*).

The recombinant Hsp90-CT was highly sensitive to digestion with trypsin in the absence of novobiocin (Figure 6A). As expected, geldanamycin, which binds to the N-terminal but not to the Hsp90-CT, had no effect on the protease sensitivity of the domain. Furthermore, molybdate did not protect the Hsp90-CT from cleavage by trypsin. However, novobiocin alone protected recombinant Hsp90-CT from trypsin cleavage, and molybdate had no additional effect on this protection. The ability of MgATP to similarly protect recombinant Hsp90-CT from trypsinolytic cleavage (Figure 6B) supports the hypothesis that novobiocin inhibits Hsp90 function by binding to a nucleotide binding site present in the Hsp90-CT and altering the structure near its binding site.

DISCUSSION

In this manuscript, we demonstrate that novobiocin inhibits Hsp90-dependent maturation of newly synthesized client target (e.g., the maturation and activation of the Hsp90/Cdc37-dependent kinase HRI) in a dose-dependent manner. In addition, novobiocin-induced inhibition of the Hsp90/Cdc37-dependent activation of HRI's autokinase activity correlated well with its ability to disrupt the interaction of Hsp90 and Cdc37 with newly synthesized HRI. The concentration range required to inhibit HRI's Hsp90-dependent transformation was similar to the range of novobiocin concentrations that Kanelakis and co-workers found were required to inhibit Hsp90-dependent restoration of steroid hormone binding to the glucocorticoid receptor in vitro using purified proteins (*53*).

The mechanism by which novobiocin inhibits Hsp90 function appears to be distinct from that of geldanamycin and molybdate, as novobiocin has effects distinct from those of geldanamycin and molybdate both on the basal interaction of cochaperones with Hsp90 and on the interactions of Hsp90/Cdc37 and cochaperones with the Hsp90 client kinase, HRI. Of further interest is the observation that the binding of novobiocin to Hsp90's C-terminal domain decreased the interaction of Hsp90 with cochaperones containing TPR domains, which interact with Hsp90's C-terminal EEVD motif. This suggests that novobiocin binding may alter the conformation of Hsp90's C terminus leading to the dissociation of TPR containing cochaperones. Of note is that while novobiocin markedly reduced or eliminated the interaction of Hsp90 with PP5 and FKBP52, it had less of an inhibitory effect on the basal interaction of HOP with Hsp90. This difference probably reflects the fact that HOP interacts with Hsp90 early in its ATP cycle at a point when the C-terminal nucleotide binding site may be inaccessible or more likely in a low affinity conformation. In contrast, FKBP52 and PP5 are present in late complexes in which the C-terminal nucleotide binding site would be open and readily accessible for novobiocin binding. These results are also consistent with the observations that the binding of HOP vs PP5 or FKBP52 to Hsp90 is mutually exclusive and that HOP interacts with Hsp90 when its N-terminal domain is in its "open", possibly nucleotide-free, conformation (*70*).

Novobiocin had effects on the interactions of Hsp90 with cochaperones and client kinase that were dominant to the effects of molybdate. Novobiocin altered the cochaperone composition of basal Hsp90 complexes, destabilized the binding of Hsp90/Cdc37 to HRI, and changed the interactions of cochaperones with HRI even when lysate was preincubated with molybdate. The dominance of the effect of novobiocin over molybdate and the ability of novobiocin to quantitatively dissociate Hsp90/Cdc37−kinase complexes suggests that novobiocin may bind to Hsp90 after molybdate and induce kinase release.

The notion that novobiocin may bind to Hsp90 after molybdate is supported by observations that suggest that the interaction of novobiocin with Hsp90 occurs after the formation of "late" Hsp90−client complexes. Geldanamycin induces accumulation of intermediate Hsp90 complexes that contain Hsc70 and weakly bound client, while molybdate stabilizes Hsp90/Cdc37−client complexes that contain "late" components of the Hsp90 machine (*25*, *26*). Nucleotide-modulated switching of Hsp90's conformation establishes Hsp90's and Cdc37's high affinity salt stabile binding to kinase (*44*, *45*). Molybdate and p23 stabilize Hsp90 binding to client, and the binding of molybdate and p23 to Hsp90 in the absence of client is thought to induce a similar Hsp90 conformation. Thus, the observation that p23, FKBP52, and PP5 ("late" cochaperone components of the Hsp90 machine) remain associated with HRI client after novobiocin-induced dissociation of Hsp90 and Cdc37 from HRI supports the hypothesis that novobiocin binds Hsp90 after the formation of late Hsp90−cochaperone client complexes. This notion would be consistent with the observed absence of Hsc70 (a component of intermediate Hsp90 heterocomplexes) and p23 (a component of late Hsp90 heterocomplexes) from basal Hsp90 complexes isolated from lysate incubated in the presence of novobiocin [Figure 1 and (*34*)]. Again, the dominance of the effects of novobiocin over molybdate-induced stabilization of Hsp90−client complexes suggests that the novobiocin-bound conformation of Hsp90 may be responsible for client release.

The observation that p23, FKBP52, and PP5 interact directly with HRI after novobiocin-induced dissociation of

Hsp90 and Cdc37 suggests that these cochaperones may play a role in chaperoning client folding or communicating changes in client conformation in some manner to Hsp90. While we had previously demonstrated a direct interaction of PP5 with HRI (*46*), past data had not allowed us to deduce whether FKBP52 or p23 had any direct interaction with HRI or whether they were present in Hsp90−kinase complexes simply due to their binding affinity for Hsp90 (*44*). The direct interaction of p23 and FKBP52 with HRI is consistent with the chaperone activity that these two proteins display in assays in vitro (*71*−*73*) and the observation that p23 is present in stable complexes with some client proteins, presumably after their dissociation from Hsp90 (*74*−*76*). This observation also invites the speculation that the interaction of these proteins with HRI may play a role in the reassembly of Hsp90 complexes with immature HRI, as reiterative cycles of Hsp90 action are required for kinase maturation (*44*, *77*). Furthermore, the interactions could play additional roles in regulating HRI function, such as the proposed role of FKBP52 in intracellular localization of Hsp90-dependent clients (*1*, *78*). This latter possibility is of interest, as novobiocin treatment appears to enhance the interaction of FKBP52 with HRI, and some "matured and activated" HRI, which exhibits a slower electrophoretic mobility, was coimmunoadsorbed with FKBP52 from heme deficient lysate (Figure 4C).

It has been suggested, however, that novobiocin might affect the function of the Hsp90 chaperone machine by affecting Hsc70 as well (*53*). While our data cannot conclusively rule out this possibility, the work presented here (which is discussed below) clearly demonstrates that novobiocin binds Hsp90 and directly alters its structure and function. As stressed in a recent review (*1*), Hsp90's ATP-driven reaction cycle is dynamic, such that at any given time basal heterocomplexes containing Hsp90 can be in the process of assembly or disassembly. Thus, we favor a model in which novobiocin compromises Hsc70's function indirectly through altering Hsp90's structure at a specific point in its reaction cycle and subsequently compromising Hsc70's ability to interact productively with the Hsp90 chaperone machine.

The notion that novobiocin can affect the function of cochaperone components "indirectly" through its effects on Hsp90 function is supported by additional observations. The addition of molybdate reproducibly caused an approximate 30% decrease in the interaction of HOP with Hsp90 and a significant decrease in the binding of Hsc70. The presence of molybdate would stabilize "late" complexes formed from intermediate Hsp90−HOP−Hsc70 heterocomplexes and cryptic clients present in the reticulocyte lysate. These complexes would lack Hsc70 and HOP but be sensitive to novobiocin-induced dissociation of cryptic substrate. On the other hand, Hsp90−HOP complexes are thought to be nucleotide-free prior to the association of Hsc70 containing bound client (*28*, *49*, *79*). As such, Hsp90's C-terminal nucleotide binding site in these complexes would be in its low affinity conformation and only be sensitive to novobiocin at very high concentrations, as we have observed. In addition, because nucleotide bound to Hsp90's N terminus appears to be required for the molybdate-induced effect on Hsp90's conformation (*24*, *42*), the C-terminal nucleotide binding site of nucleotide-free Hsp90−HOP would remain in its low affinity conformation, which is consistent with the observation that high concentrations of novobiocin are still required to cause a decrease Hsp90−HOP complexes in the presence of molybdate. The observation that the intensity of the 78 kDa N-terminal tryptic fragment generated in the presence of molybdate and 5 mM novobiocin is only reduced by approximately 80% (Figure 5B, discussed below) suggests that about 20% of the Hsp90 molecules present in reticulocyte lysate may be in a nucleotide-free form under our assay conditions. Approximately 30% of the Hsp90 in reticulocyte lysate appears to be bound to HOP (*1*).

Consistent with its unique effects on the interactions of Hsp90 with client and cochaperones, novobiocin also had distinct effects on the conformation of Hsp90 as assessed by proteolytic fingerprinting of Hsp90 in situ. Fingerprinting of Hsp90 indicated that the addition of novobiocin had little effect on the default conformation of Hsp90 at concentrations (1−2 mM) that significantly inhibited HRI transformation and induced the dissociation of Hsp90 and Cdc37 from newly synthesized HRI (Figure 1). Similarly, 1−2 mM novobiocin had little effect on the proteolytic fingerprint of Hsp90 generated in the presence of geldanamycin.[2] However, very high concentrations of novobiocin (10−20 mM) were found to alter the proteotlytic fingerprint of Hsp90 both in the presence[2] and in the absence of geldanamycin. Results from other laboratories indicate that occupancy of the N-terminal ATP binding site is required for the accessibility of the second C-terminal ATP binding site to novobiocin (*34*, *35*). Our results suggest that the default and geldanamycin-bound conformations of Hsp90 maintain the C-terminal nucleotide binding site in a low affinity conformation but that novobiocin-induced switching of Hsp90's conformation can occur in the presence of a sufficiently high concentration of novobiocin. The conformational change induced by the binding of novobiocin appears to be highly cooperative with a Hill coefficient of 1.7, suggesting that both novobiocin binding sites within an Hsp90 dimer must be occupied for the conformational switching to occur.

The molybdate-induced switching of Hsp90's conformation dramatically enhances the binding affinity of Hsp90's C-terminal nucleotide binding site for ligand. This hypothesis is supported by the observation that 2 mM novobiocin has a dramatic effect on the proteolytic fingerprint of Hsp90 when added to reticulocyte lysate in the presence but not in the absence of molybdate. The interaction of Hsp90−Cdc37 with newly synthesized HRI was also markedly reduced both in the presence or in the absence of molybdate when 2 mM novobiocin was added to reticulocyte lysate (Figures 1 and 2). Furthermore, 2 mM novobiocin blocked the Hsp90/Cdc37-dependent transformation of HRI in heme deficient lysate (Figures 1B and 5B−D). Binding of molybdate to Hsp90 stabilizes Hsp90 in a conformation that mimics the properties of salt stabile high affinity complexes formed between Hsp90/Cdc37 and bound client following nucleotide-modulated conformation switching of Hsp90 (*38*, *44*). Thus, ATP-induced "clamping" of Hsp90's N-terminal domain and the subsequent hydrolysis of this ATP may markedly enhance to accessibility or the affinity of Hsp90's C-terminal nucleotide binding site. Because molybdate markedly enhanced the binding affinity of Hsp90 for

[2] Yun, B.-G., and Matts, R. L. Unpublished observations.

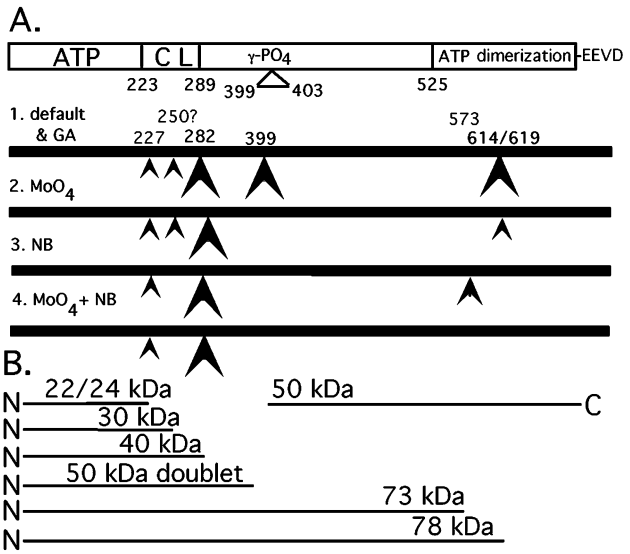

FIGURE 7: Summary of effects of pharmacological agents on the proteolytic fingerprint of Hsp90. (A) (1) Tyrpsin cleavage sites of Hsp90 in its default and geldanamycin-bound conformations (*67*, *68*). (2) Tyrpsin cleavage sites of Hsp90 in its molybdate-bound conformation. (3) Tyrpsin cleavage sites of Hsp90 in its novobiocin-bound conformation. (4) Tyrpsin cleavage sites of Hsp90 in the presence of molybdate and novobiocin. Large arrow, major cleavage site; small arrow, minor cleavage site; the presence or absence of an arrow or altered size indicates a change in susceptibility to cleavage by trypsin. (B) Origin of N- and C-terminal Hsp90 fragments. Note that the N-terminal fragments containing the charge linker (CL) region have abnormal electrophoretic mobility (*68*). ATP, nucleotide binding sites; γ-PO_4, putative site of interaction of the γ-phosphate of the ATP bound to Hsp90's N terminus (*35*, *80*).

novobiocin, it is likely that the release of bound phosphate [of which molybdate has been proposed to be a high affinity binding mimetic, (*42*)] is an important step kinetically in the regulation of the Hsp90 reaction cycle (*38*).

Past work identifying the location of the major sites in Hsp90 that are cleaved by trypsin (*67*, *68*) now allows us to accurately identify the sites within Hsp90 whose cleavage is altered upon the binding of molybdate and novobiocin (Figure 7). Relative to the amino acid sequence of human Hsp90α, Hsp90 has major trypsin cleavage sites at residues 288, 399, and 614/619 and minor cleavage sites near residues 228, 250, and 573 (*67*, *68*). The sites at residues 228, 250, and 288 represent cleavage of Hsp90 near the N terminus, the middle, and the C terminus of the highly charged linker region that connects the N-terminal nucleotide binding domain and the middle domain of Hsp90 (*67*, *68*). K573 is located at the C-terminal end of a highly charged sequence that is present between the middle and the C-terminal domains of Hsp90 (*67*). The site at 399 is within the region of Hsp90 that has been proposed to interact with (*35*) and catalyze the hydrolysis of (*80*) the γ-phosphate of ATP bound to the N-terminal domain of Hsp90, as well as being adjacent to the site that has been proposed to be the client binding pocket (*80*). The site at residue 614/619 is adjacent to the region that binds novobiocin (*34*, *35*), and the site at 573 is just upstream from this region.

Consistent with its ability to inhibit Hsp90 proteolysis at residue 399, molybdate is thought to act as a phosphate mimetic, which binds to the site occupied by the γ-phosphate of the ATP bound to the N-terminal domain of Hsp90 after its hydrolysis (*42*). Our previous work also indicates that molybdate reduces cleavage of Hsp90 at residue 614/619 (*38*). Our current work demonstrates that novobiocin (and MgATP) quantitatively protects Hsp90 from cleavage at the 614/619 site independent of molybdate. Thus, the ability of molybdate to retard cleavage at this site may represent molybdate's ability to enhance interaction of endogenous nucleotide with the C-terminal nucleotide binding site of Hsp90. In addition, the observation that novobiocin and MgATP protect Hsp90-CT from proteolytic cleavage indicates that the binding of novobiocin or nucleotide to the C-terminal site is directly responsible for the altered Hsp90 conformation and that the protection from proteolysis is not caused by altering the binding of a cochaperone to the site that would render the site inaccessible to protease. However, it cannot be excluded at this time that the changes in protease sensitivity of Hsp90 at other sites might be due to alterations in the interactions of cochaperones with Hsp90 as opposed to direct changes in structure.

Because the dissociation of client kinase from Hsp90−Cdc37 complexes in all likelihood requires the "unclamping" of the N-terminal nucleotide binding domains within the Hsp90 dimer, the data suggest that the binding of novobiocin to Hsp90 may induce this event. Because the N-terminal domain of Hsp90 does not bind novobiocin (*34*, *35*), occupancy of the C-terminal nucleotide binding site of Hsp90 by novobiocin would need to be communicated to Hsp90's N-terminal domain through its effects on the conformation of Hsp90 at its C terminus (*34*, *35*). This proposed communication between the N- and the C-terminal domains of Hsp90 is consistent with several observations: (i) occupancy of the N-terminal ATP binding site of Hsp90 is required for the C-terminal nucleotide binding site to become accessible (*35*, *36*); (ii) binding of novobiocin to the C-terminal nucleotide binding site disrupts nucleotide binding at the N terminus (*35*); (iii) sequences in the C terminus of Hsp90 regulate the hydrolysis of ATP bound to the N-terminal domain of Hsp90 (*20*, *41*), which is required for the ATP-dependent formation of stable Hsp90−client complexes containing p23 (*22*, *24*, *41*, *42*, *49*); and (iv) our proteolytic fingerprints indicate that novobiocin causes structural changes (and/or altered cochaperone−client interactions) in the C-terminal domain, the middle domain, and the linker region between the N-terminal and the middle domains of Hsp90.

In toto, the data presented in this paper suggest that the binding of novobiocin to the C-terminal nucleotide binding site of Hsp90 stimulates the dissociation of Hsp90 from client protein. Because the binding of novobiocin to Hsp90 inhibits binding of nucleotide to the N-terminal domain of Hsp90 (*34*, *35*) and N-terminally bound nucleotide is required to stabilize Hsp90 in its "closed", high affinity client binding conformation (*18*, *22*, *24*), we propose that novobiocin binding induces the dissociation of nucleotide from the N-terminal domain of Hsp90 and the destabilization of p23 binding and kinase release. The questions of whether this model will apply to other or all Hsp90 client targets, as well as what stimulates the recycling of Hsp90 from its novobiocin-bound state remain to be explored.

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