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Inhibition of Hsp70 by Methylene Blue Affects Signaling Protein Function and Ubiquitination and Modulates Polyglutamine Protein Degradation*

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The Hsp90/Hsp70-based chaperone machinery regulates the activity and degradation of many signaling proteins. Cycling with Hsp90 stabilizes client proteins, whereas Hsp70 interacts with chaperone-dependent E3 ubiquitin ligases to promote protein degradation. To probe these actions, small molecule inhibitors of Hsp70 would be extremely useful; however, few have been identified. Here we test the effects of methylene blue, a recently described inhibitor of Hsp70 ATPase activity, in three well established systems of increasing complexity. First, we demonstrate that methylene blue inhibits the ability of the purified Hsp90/Hsp70-based chaperone machinery to enable ligand binding by the glucocorticoid receptor and show that this effect is due to specific inhibition of Hsp70. Next, we establish that ubiquitination of neuronal nitric-oxide synthase by the native ubiquitinating system of reticulocyte lysate is dependent upon both Hsp70 and the E3 ubiquitin ligase CHIP and is blocked by methylene blue. Finally, we demonstrate that methylene blue impairs degradation of the polyglutamine expanded androgen receptor, an Hsp90 client mutated in spinal and bulbar muscular atrophy. In contrast, degradation of an amino-terminal fragment of the receptor, which lacks the ligand binding domain and, therefore, is not a client of the Hsp90/Hsp70-based chaperone machinery, is enhanced through homeostatic induction of autophagy that occurs when Hsp70-dependent proteasomal degradation is inhibited by methylene blue. Our data demonstrate the utility of methylene blue in defining Hsp70-dependent functions and reveal divergent effects on polyglutamine protein degradation depending on whether the substrate is an Hsp90 client.

The Hsp90/Hsp70-based chaperone machinery that regulates a wide variety of Hsp90 "client" proteins (for review, see Ref. 1) is also a part of the cellular defense against unfolded proteins (2). In this machinery, Hsp90 and Hsp70 have opposing effects on client protein stability. Hsp90 stabilizes client proteins, and when their cycling with Hsp90 is blocked by specific Hsp90 inhibitors, like geldanamycin and radicicol, the client proteins undergo rapid degradation through the ubiquitin/proteasome pathway (3). In contrast, Hsp70 along with its cochaperone Hsp40 is required for the degradation of many proteins (4, 5).

Similar opposing roles of Hsp90 and Hsp70 are seen with signaling proteins that are classic Hsp90 client proteins like the glucocorticoid receptor (GR)[3] and with signaling proteins that undergo very dynamic cycling with Hsp90-like neuronal nitric-oxide synthase (nNOS) (6). Opposing roles of Hsp90 and Hsp70 also regulate protein turnover in some of the polyglutamine expansion disorders. This group of neurodegenerative diseases is characterized by the accumulation of aberrant proteins and includes Huntington disease (HD), spinal and bulbar muscular atrophy (SBMA), and several autosomal-dominant spinocerebellar ataxias (*e.g.* SCA1, SCA3). Some of the mutant proteins that misfold and aggregate in these diseases, including huntingtin (7) in HD and the androgen receptor in SBMA (8), form heterocomplexes with Hsp90 and Hsp70. Inhibition of Hsp90 by geldanamycin prevents aggregation of these proteins in animal models of HD (9) and SBMA (10). Because Hsp90 binding to heat shock factor 1 (HSF1) maintains this transcription factor in an inactive state and treatment of cells with geldanamycin induces an HSF1-dependent stress response (11, 12), it is often proposed that geldanamycin alleviates the phenotype and accumulation of misfolded proteins in neurodegenerative disease models by inducing a stress response (9, 13, 14). However, this explanation cannot be correct because geldanamycin promotes proteasomal degradation of the polyglutamine-expanded androgen receptor (polyQ AR) in *Hsf1*$^{-/-}$ cells that cannot mount a stress response (8). Furthermore, overexpression of Hsp70 or Hsp40 decreases polyglutamine protein levels and improves viability in cellular models of HD (15) and SBMA (16), and overexpression ameliorates polyglutamine disease phenotypes in *Drosophila* and mouse models of neurodegenerative disease (Ref. 17–19; for review, see Ref.14). These observations raise the possibility that Hsp70 plays a critical role in diminishing polyglutamine toxicity when Hsp90 function is inhibited.

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[3] The abbreviations used are: GR, glucocorticoid receptor; CHIP, carboxyl terminus of Hsc70-interacting protein; HSF1, heat shock factor 1; HD, Huntington disease; nNOS, neuronal nitric-oxide synthase; polyQ AR, polyglutamine androgen receptor; SBMA, spinal and bulbar muscular atrophy; SCA, spinocerebellar ataxia; trAR112Q, amino-terminal fragment of the androgen receptor with 12 glutamines; GST, glutathione *S*-transferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid.

There is considerable evidence that Hsp70 promotes degradation of the polyglutamine expanded proteins by promoting ubiquitination mediated by chaperone-dependent E3 ubiquitin ligases. The most studied of these is CHIP (carboxyl terminus of Hsc70-interacting protein), a 35-kDa U-box E3 ubiquitin ligase (20). CHIP binds to Hsc/Hsp70 through its amino-terminal tetratricopeptide repeat domain (21, 22), and it binds to the UBCH5 family of E2 ubiquitin-conjugating enzymes through a carboxyl-terminal U-box (23). Parkin is another E3 ligase (24) that is targeted to substrate by Hsp70 (25). For some proteins, such as the GR, only CHIP promotes degradation, whereas for others, such as nNOS, CHIP and parkin are functionally redundant in promoting degradation (26). Overexpression of either CHIP or parkin increases ubiquitination of polyglutamine-expanded ataxin-3 and reduces its cellular toxicity in a manner that is promoted by Hsp70 (15, 25). Interest has focused on CHIP because it is found in aggregates of huntington, androgen receptor, ataxin-1, and ataxin-3 (15, 27–29), and CHIP overexpression suppresses aggregation and protein levels in cellular disease models (15, 27, 29). The notion that CHIP is a critical mediator of the neuronal response to misfolded proteins is buttressed by the observations that overexpression of CHIP in a *Drosophila* model of SCA1 (29) and a mouse model of SBMA (30) suppresses toxicity and that HD transgenic mice haploinsufficient for CHIP display an accelerated disease phenotype (27).

Most of what is known about the Hsp70 role in the degradation of polyglutamine-expanded proteins comes from Hsp70 overexpression experiments. To enhance a mechanistic understanding of Hsp70-dependent processes in general, it would be useful to have a small molecule inhibitor of Hsp70, much as geldanamycin has been so useful in probing Hsp90-dependent effects. To this end, the Gestwicki laboratory employed a high throughput chemical screen to identify compounds that inhibit Hsp70 ATPase activity. An inhibitor identified in the compound library was methylene blue, which was shown to interact with purified Hsp70 by NMR spectroscopy (31). Methylene blue reduced tau levels in both cellular and animal models of tauopathy (31), although it was not established that this effect was due to an effect of methylene blue on Hsp70. Methylene blue has been demonstrated to affect multiple systems, most notably cGMP signaling; thus, its action is not directed against Hsp70 as a single target.

Our goal here is to determine the usefulness of methylene blue as a research tool for probing Hsp70-dependent effects in three well established systems of increasing complexity, from the purified Hsp90/Hsp70-based chaperone machinery to a ubiquitinating system from reticulocyte lysate, to inhibition of polyQ AR degradation in cells. We first show that methylene blue inhibits the generation of steroid binding activity of the glucocorticoid receptor, an established physiological action of Hsp70 (1). Activation of GR steroid binding activity by purified chaperones requires Hsp70 (32), and we show that the methylene blue inhibition of activation is specific for the Hsp70 component of the Hsp90/Hsp70-based, multiprotein chaperone machinery. We then use methylene blue as a tool to probe the pathway regulating ubiquitination of neuronal nitric-oxide synthase. Using the classic system that was originally used to resolve the components of the ubiquitin-protein ligase pathway (33), we show that nNOS ubiquitination by the DE52-retained fraction of rabbit reticulocyte lysate is Hsp70-dependent. Methylene blue inhibits nNOS ubiquitination, and the block in ubiquitination is overcome by the addition of purified Hsp70. Additionally, nNOS ubiquitination is inhibited by anti-CHIP serum. This suggests that Hsp70-directed CHIP E3 ligase activity is responsible for nNOS ubiquitination in this system. Finally, we examine the effects of methylene blue in cells on the degradation of the polyQ AR with 112 glutamines (AR112Q) and a truncated amino-terminal fragment of the androgen receptor containing the expanded polyglutamine tract (trAR112Q). We show that inhibition of Hsp70 by methylene blue impairs AR112Q degradation and enhances ligand-dependent aggregation. In contrast to the effects on the full-length androgen receptor, we show enhanced degradation of an amino-terminal fragment of the expanded glutamine androgen receptor in the presence of methylene blue and that this is mediated by homeostatic induction of macroautophagy.

EXPERIMENTAL PROCEDURES

Materials—HeLa cells were purchased from the American Type Culture Collection. Phenol red-free Dulbecco's modified Eagle's medium was from Invitrogen, and charcoal-stripped calf serum was from Thermo Scientific Hyclone Products (Thermo Fisher Scientific, Waltham, MA). Untreated rabbit reticulocyte lysate was purchased from Green Hectares (Oregon, WI). [1,2,4,6,7-^{3}H]Dexamethasone (85 Ci/mmol) was from GE Healthcare, FuGENE 6 was from Roche Applied Science, and MG132, E64d, pepstatin A, anti-GST IgG and methylene blue (M9140) were from Sigma. ^{125}I-Conjugated goat-anti-mouse IgG was obtained from PerkinElmer Life Sciences. Horseradish peroxidase-tagged goat anti-rabbit IgG was from Millipore (Temecula, CA). The N27F3-4 anti-72/73-kDa Hsp70 monoclonal IgG (anti-Hsp70) and the AC88 monoclonal against Hsp90 were from StressGen Biotechnologies (Ann Arbor, MI). Affinity-purified IgG against nNOS was from BD Transduction Laboratories. The FiGR monoclonal IgG used to immunoadsorb the mouse GR was provided by Dr. Jack Bodwell (Dartmouth Medical School, Lebanon, NH). The BuGR2 monoclonal IgG used to immunoblot the mouse GR and rabbit anti-CHIP antibody were from Affinity Bioreagents (Golden, CO). The AR antibody (N-20) was from Santa Cruz Biotechnology (Santa Cruz, CA), GAPDH antibody was from Abcam (Cambridge, MA), LC3 antibody was from Novus Biologicals (Littleton, CO), and p62 (carboxyl-terminal) antibody was from American Research Products (Belmont, MA). GST-tagged ubiquitin was from Boston Biochem (Cambridge, MA). The cDNA for rat nNOS was provided by Dr. Solomon Snyder (The Johns Hopkins Medical School, Baltimore, MD). Plasmid encoding AR112Q was provided by Dr. Kenneth Fishbeck (National Institutes of Health), and plasmids encoding amino-terminal-truncated AR16Q and AR112Q were from Dr. Diane Merry (Thomas Jefferson University, Philadelphia, PA).

Expression and Purification of nNOS, Hsp90, Hsp70, Hsp40, Hop, and p23—Rat nNOS was expressed in Sf9 insect cells using a recombinant baculovirus and purified by 2′,5′-ADP-Sepharose and gel-filtration chromatography as described previously (34). Heme was added as an albumin conjugate during

the expression to convert all of the nNOS to the holo-nNOS dimer (34). Hsp90 and Hsp70 were purified from rabbit reticulocyte lysate by sequential chromatography on DE52, hydroxylapatite, and ATP-agarose as described previously (35). YDJ-1, the yeast ortholog of Hsp40, was expressed in bacteria and purified by sequential chromatography on DE52 and hydroxylapatite as described previously (36). Recombinant human Hop (Hsp organizing protein) and p23 were purified as described by Kanelakis and Pratt (37). For overexpression of Hsp70, the full-length human Hsp70 cDNA was amplified by PCR from clone pET23hsp70, kindly provided by Dr. David Toft (Mayo Clinic, Rochester, MN) using a 5′ primer encoding a polyhistidine tag followed by a hemagglutinin epitope. The 1.9-kb PCR fragment was digested with EcoRI and XhoI and cloned into pcDNA4/HisMax-C vector (Invitrogen). The entire coding region of Hsp70 was verified by sequencing.

GR·Hsp90 Heterocomplex Reconstitution—Mouse GR was expressed in Sf9 cells, and cytosol was prepared as described previously (38). Receptors were immunoadsorbed from aliquots of 50 μl (for measuring steroid binding) or 100 μl (for Western blotting) of Sf9 cell cytosol by rotation for 2 h at 4 °C with 14 μl of protein A-Sepharose precoupled to 8 μl of FiGR ascites suspended in 300 μl of TEG buffer (10 mM TES, pH 7.6, 50 mM NaCl, 4 mM EDTA, 10% glycerol). Immunoadsorbed GR was stripped of endogenously associated Hsp90 by incubating the immunopellet for an additional 2 h at 4 °C with 350 μl of 0.5 M NaCl in TEG buffer. The pellets were then washed once with 1 ml of TEG buffer followed by a second wash with 1 ml of Hepes buffer (10 mM Hepes, pH 7.4). For GR·Hsp90 heterocomplex reconstitution by reticulocyte lysate, immunopellets containing GR stripped of chaperones were incubated with 50 μl of lysate and 5 μl of an ATP-regenerating system (50 mM ATP, 250 mM creatine phosphate, 20 mM magnesium acetate, and 100 units/ml creatine phosphokinase). For heterocomplex reconstitution with purified proteins, immunopellets containing stripped GR were incubated with 15 μg of purified Hsp90, the indicated μg of purified Hsp70, 0.6 μg of purified Hop, 6 μg of purified p23, 0.125 μg of purified YDJ-1 adjusted to 55 μl with HKD buffer (10 mM Hepes, pH 7.4, 100 mM KCl, 5 mM dithiothreitol) containing 20 mM sodium molybdate, and 5 μl of the ATP-regenerating system. The assay mixtures were incubated for 20 min at 30 °C with suspension of the pellets by shaking the tubes every 2 min. At the end of the incubation, the pellets were washed twice with 1 ml of ice-cold TEGM buffer (TEG with 20 mM sodium molybdate) and assayed for steroid binding capacity and for GR-associated Hsp90.

Assay of Steroid Binding Capacity—Washed immune pellets to be assayed for steroid binding were incubated overnight at 4 °C in 50 μl of HEM buffer (10 mM Hepes, pH 7.4, 1 mM EDTA, 20 mM molybdate) plus 50 nM [^{3}H]dexamethasone. Samples were then washed 3 times with 1 ml of TEGM buffer and counted by liquid scintillation spectrometry. Steroid binding is expressed as counts/min of [^{3}H]dexamethasone bound/FiGR immunopellet prepared from 50 μl of Sf9 cell cytosol.

Ubiquitination of nNOS by DE52-retained Fraction of Reticulocyte Lysates—The DE52-retained fraction of rabbit reticulocyte lysate was prepared as described by Hershko *et al.* (33). Purified nNOS (0.6 μg) was incubated for 1 h at 37 °C with 4.5 μl of DE52-retained fraction of rabbit reticulocyte lysate (final concentration, 7 mg of protein/ml), 0.3 mg/ml bovine serum albumin, 8.3 μM GST-tagged ubiquitin, 1 mM dithiothreitol, 2 μl of the ATP-regenerating system, 1 μl of Complete Mini protease inhibitor mixture, 0.6 mM *N*-acetyl-Leu-Leu-Nle-CHO (Nle is norleucine), and 0.8 μM ubiquitin aldehyde (deubiquitination inhibitor), adjusted to a final volume of 20 μl with 50 mM Tris, pH 7.5. Methylene blue was added to yield the indicated final concentration, with all samples containing a final concentration of 0.1% ethanol vehicle. Incubations were terminated by boiling with an equal volume of SDS-sample buffer containing 8 M urea and 2 M thiourea.

Gel Electrophoresis and Western Blotting—GR immune pellets were resolved on 12% SDS-polyacrylamide gels and transferred to Immobilon-P membranes. The membranes were probed with 0.25 μg/ml BuGR2 for GR, 1 μg/ml AC88 for Hsp90, or 1 μg/ml anti-Hsp70. The immunoblots were then incubated a second time with ^{125}I-conjugated counter-antibody to visualize the immunoreactive bands. For experiments with nNOS, aliquots (25 μl) from the ubiquitination reactions boiled in SDS-sample buffer were resolved on 5% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with anti-nNOS (1:8000) followed by horseradish peroxidase-conjugated counter-antibody. Immunoreactive bands were visualized with the use of enhanced chemiluminescence reagent (Super Signal, Pierce) and X-Omat film (Eastman Kodak Co.). The monoubiquitinated nNOS bands were scanned, and the relative densities were determined with ImageJ software (rsb.info.nih.gov). Relative densities for three experiments are presented in *bar graphs* as percent of control ± S.E. Statistical probability is expressed as $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.0001$ (***).

For analysis of AR protein expression, HeLa cells were grown in 6-well dishes in phenol red-free Dulbecco's modified Eagle's medium supplemented with 10% charcoal/dextran-stripped fetal calf serum. Cells were transfected with FuGENE 6 transfection reagent using 3 μl of FuGENE 6 and 1 μg of DNA. Twenty-four hours post-transfection, cells were pooled and replated then treated as indicated starting 48 h post-transfection. After incubation, cells were washed with phosphate-buffered saline, harvested, and lysed by sonication in radioimmune precipitation assay buffer containing phosphatase and proteinase inhibitors. Protein samples were electrophoresed through 4–20% SDS-polyacrylamide gradient gels and transferred to Immobilon-P membranes using a semidry transfer apparatus. Immunoreactive proteins were detected by chemiluminescence, and relative densities of bands were quantified as described above.

Assay of Caspase Activity—Caspase activity was determined by measuring cleavage of the fluorescent substrate DEVD-aminofluoromethylcoumarin using the ApoTarget caspase-3/CPP 32 fluorometric protease assay kit (Invitrogen) 48 h post-transfection. Fluorescence intensity was measured using a Fluoroskan Ascent FL fluorometer (Thermo Electron Corp.).

RESULTS

Methylene Blue Inhibits Hsp70 Action on the GR—To bind steroid with high affinity, the GR must be assembled into a

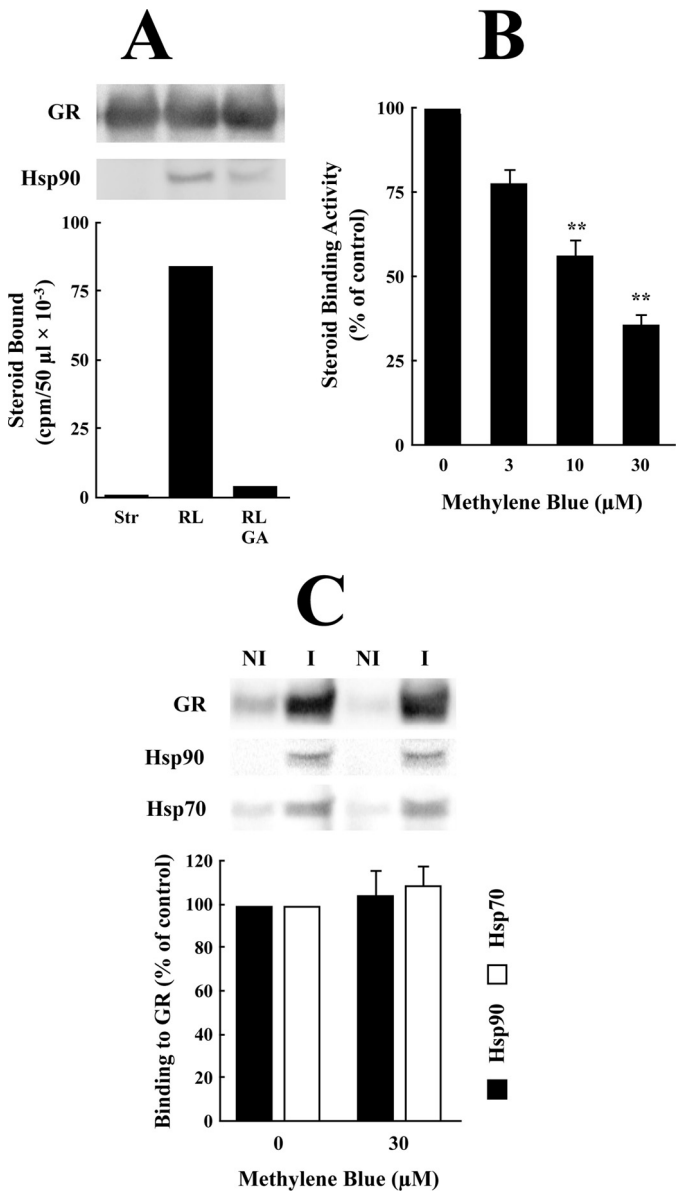

FIGURE 1. **Methylene blue inhibits generation of steroid binding activity but not GR·Hsp90 heterocomplex assembly by reticulocyte lysate.** *A*, the assay is shown. GR stripped of Hsp90 (*Str*) was incubated with reticulocyte lysate (*RL*) in the absence or presence of 10 μM geldanamycin (*GA*). Immunopellets were washed and Western-blotted for GR and Hsp90. Duplicate pellets were incubated with [^{3}H]dexamethasone to assay steroid binding activity. *B*, methylene blue inhibits generation of GR steroid binding activity. Stripped GRs were reactivated by reticulocyte lysate in the presence of the indicated concentrations of methylene blue. **, different from control at $p < 0.01$. *C*, methylene blue does not inhibit GR·Hsp90 heterocomplex assembly. Stripped nonimmune (*NI*) or immune (*I*) GR pellets were incubated with reticulocyte lysate in the absence or presence of 30 μM methylene blue. Immunopellets were washed and Western-blotted for GR, Hsp90, and Hsp70. A typical radiogram of a Western blot is shown *above the bar graph*. GR-specific Hsp90 (*solid bars*) and Hsp70 (*open bars*) were determined in six separate experiments and are presented in the graph relative to the zero (0) methylene blue control set at 100%.

heterocomplex with Hsp90 (1). These heterocomplexes are assembled by a multichaperone machinery present in lysates of all eukaryotic cells. Fig. 1*A* shows that incubation of an Hsp90-free GR immunopellet with rabbit reticulocyte lysate generates GR·Hsp90 heterocomplexes that bind steroid, and both formation of heterocomplexes and generation of steroid binding activity are reduced when the Hsp90 inhibitor geldanamycin is present. Methylene blue also inhibits the generation of steroid binding activity by reticulocyte lysate in a concentration-dependent manner (Fig. 1*B*). The formation of GR·Hsp90 heterocomplexes by reticulocyte lysate requires Hsp70 (1), and our presumption was that if methylene blue act as an Hsp70 inhibitor, it might inhibit Hsp70 binding to the GR. However, in the presence of 30 μM methylene blue, which inhibits generation of a majority of the steroid binding activity, the same amount of Hsp70 is bound to the GR as in control incubations without methylene blue (Fig. 1*C*). Surprisingly, the methylene blue-treated samples also contain the same amount of Hsp90 (Fig. 1*C*), distinguishing this effect from the action of the Hsp90 inhibitor geldanamycin.

To determine whether methylene blue inhibition of steroid binding activity is specific to an effect on Hsp70, we examined the effect of methylene blue on generation of steroid binding activity by a purified five-protein assembly system composed of Hsp90, Hsp70, Hop, Hsp40, and p23 (1). In this system both Hsp90 and Hsp70 are required for steroid binding (1, 32), and maximal generation of steroid binding activity in this system requires 15 μg of Hsp90 and 15 μg of Hsp70 in the incubation mixture. In the experiment of Fig. 2*A*, the stripped GR was incubated with the 5-protein system containing low amounts (0.5–5 μg) of Hsp70 in the presence or absence of 30 μM methylene blue. As the concentration of Hsp70 is increased, there is more generation of steroid binding activity both in the presence and absence of methylene blue (Fig. 2*A*). However, when the percent inhibition of reactivation of GR steroid binding is plotted as a function of the amount of Hsp70, we find that the extent of inhibition decreases as the amount of Hsp70 is increased (Fig. 2*B*), supporting the notion that Hsp70 function is inhibited by methylene blue. Consistent with this interpretation is the finding that methylene blue is a more potent inhibitor of steroid binding reactivation at lower Hsp70 concentrations (Fig. 2*C*). In contrast, when Hsp70 is present in a nonlimiting amount but Hsp90 is limiting, methylene blue has the same potency at each Hsp90 concentration (Fig. 2*D*).

These data suggest that it is the Hsp70 component of the multiprotein chaperone machinery that is inhibited by methylene blue. Methylene blue does not inhibit Hsp70 binding to the GR (Fig. 1*C*), but it may affect Hsp70 function once it has bound to the receptor. The first step in GR·Hsp90 heterocomplex assembly is the ATP-dependent priming of the GR to form a GR·Hsp70 complex that can interact with Hsp90 and Hop. This complex undergoes another ATP-dependent step to yield the high affinity steroid binding form of the receptor (38). Hsp90 is required to open the steroid binding cleft in the GR, and the amount of steroid binding activity generally reflects the amount of Hsp90 recovered in GR heterocomplexes. Notably, this is not what is seen in Fig. 1*C*.

Hop binds independently via an amino-terminal tetratricopeptide repeat domain to Hsp70 and via a central tetratricopeptide repeat domain to Hsp90 (39). This brings the two essential chaperones together into a more efficient machinery for heteroprotein complex assembly (32). Hsp90 can be present in GR heterocomplexes because it is bound via Hop

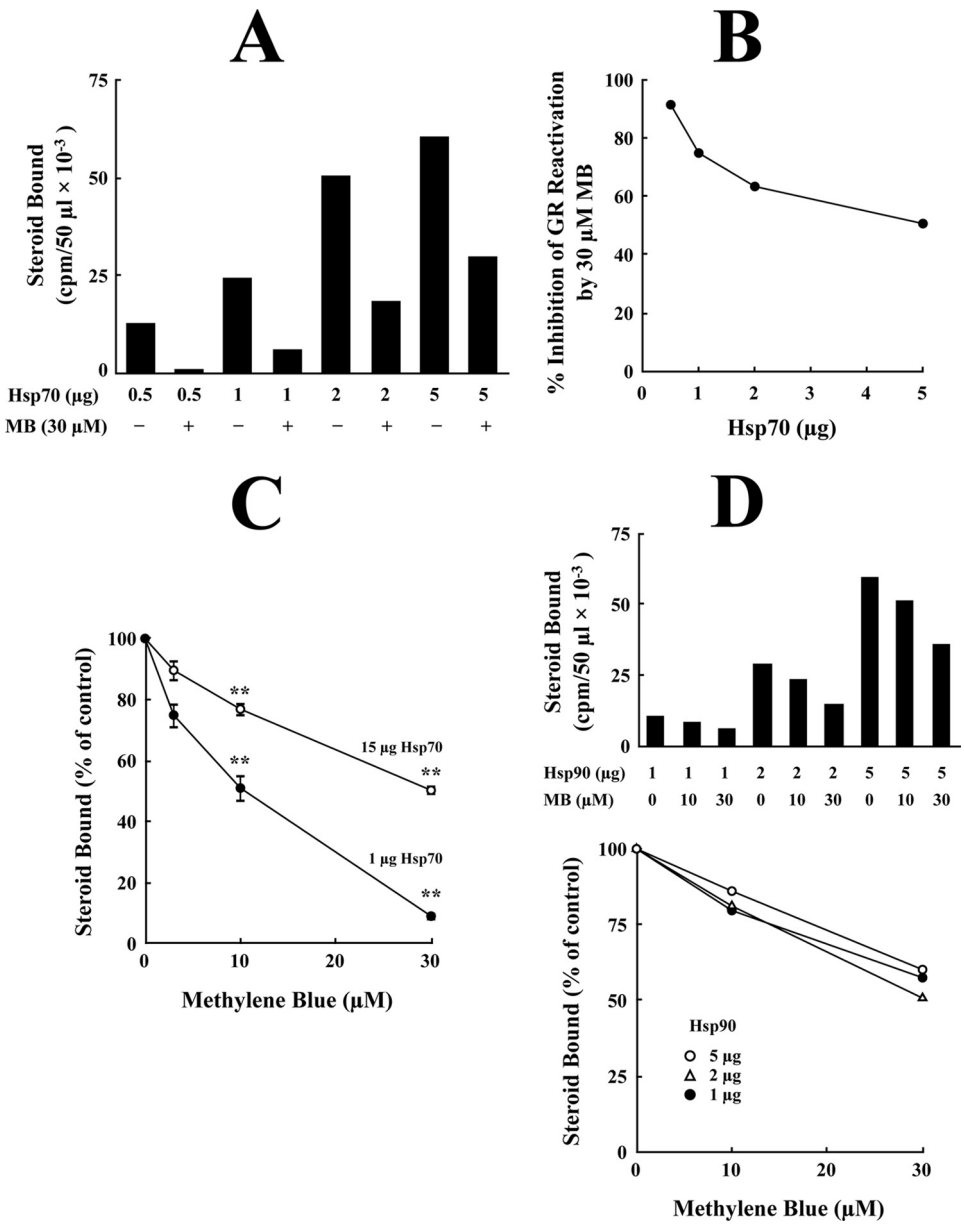

FIGURE 2. **Methylene blue inhibition of generation of steroid binding activity by the purified five-protein assembly system varies with the concentration of Hsp70.** *A*, stripped GR immune pellets were incubated with Hsp90, Hop, Hsp40, and p23 in the presence of the indicated concentrations of Hsp70 and in the presence or absence of 30 μM methylene blue (*MB*). Immune pellets were washed, and steroid binding activity was assayed. *B*, a plot of the data of *panel A* as % inhibition of reactivation of steroid binding activity by methylene blue at each concentration of Hsp70 is shown. *C*, concentration dependence of methylene blue inhibition of GR reactivation in the presence of 1 (●) or 15 (○) μg of Hsp70. **, different from control at $p < 0.01$. *D*, shown is concentration dependence of methylene blue in the presence of 15 μg Hsp70 and limiting amounts of Hsp90. The *upper panel* presents the cpm of steroid bound, and the *lower panel* presents binding as a % of the control without methylene blue.

to receptor-bound Hsp70. This does not require a direct interaction of Hsp90 with the Hsp70-primed receptor to yield steroid binding activity (32). This is illustrated with the Hsp90 inhibitor geldanamycin in Fig. 3*A*. When GR·Hsp90 heterocomplexes are assembled with the 5-protein system containing Hop, a substantial amount of Hsp90 is present in the GR immune pellet formed in the presence of geldanamycin (*lane 3*). However, when heterocomplexes are assembled without Hop, only a trace amount of Hsp90 is present in the geldanamycin-treated sample (*lane 5*). To test if this is the case with methylene blue, we incubated stripped GR with the five-protein system minus Hop in the presence and absence of the inhibitor. As shown in Figs. 3, *B* and *C*, both in the presence and absence of Hop, steroid binding is inhibited by methylene blue, but the amount of Hsp90 in GR immunopellets is similar to that in the controls. This suggests that methylene blue does not inhibit Hsp70 promotion of Hsp90 binding to the GR, but it inhibits priming of the receptor by Hsp70 that allows the GR to interact productively with Hsp90 to open the steroid binding cleft.

Use of Methylene Blue as a Tool to Determine Hsp70 Dependence of nNOS Ubiquitination—Because a good Hsp70 inhibitor has not been available, it has been difficult to establish whether or not ubiquitination events are Hsp70-dependent. It is known, for example, that the reaction of certain inactivators in the heme/substrate binding cleft of nNOS triggers its ubiquitination and degradation (40, 41) and that overexpression of either CHIP or parkin promotes nNOS degradation (26, 42). These data suggest that Hsp70 may be involved. Ubiquitination of purified nNOS by a purified ubiquitinating system using CHIP as the E3 ligase is promoted by purified Hsp70 (42, 43), but it is not known if nNOS ubiquitination by a physiological ubiquitinating system is Hsp70-dependent. Thus, we used this system to test the effectiveness of methylene blue in detecting the Hsp70 dependence of nNOS ubiquitination.

We have previously reported that nNOS is ubiquitinated in human embryonic kidney cells and in rat brain cytosol (40) and that the ubiquitination is mimicked by incubating purified nNOS with an extract of rabbit reticulocyte lysate, ubiquitin, and ATP (40, 43). The extract of reticulocyte lysate contains all material that is retained by a DE52 column, and this DE52-retained fraction is the same as lysate "fraction II" that has been extensively used to study protein ubiquitination (33). The DE52-retained fraction contains Hsp70 and its cochaperone Hsp40 as well as the ubiquitinating enzymes, with all of the components present in the same ratios as exist in reticulocyte lysate (40, 44). To

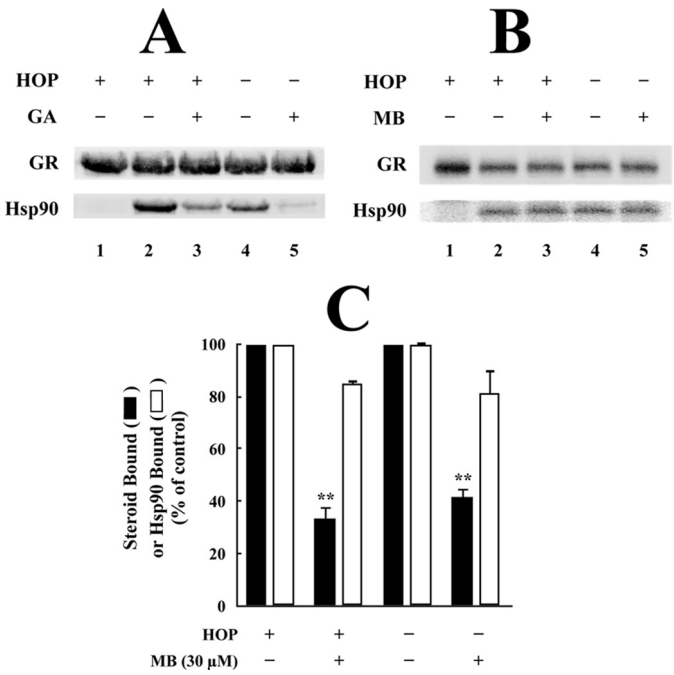

FIGURE 3. **Effect of methylene blue on GR·Hsp90 heterocomplex assembly in the presence and absence of Hop.** Stripped GR was incubated in the presence of the 5-protein system containing 15 μg Hsp70 (+*Hop*) or in the presence of the system minus Hop (−*Hop*) with or without 10 μM geldanamycin (*GA*) in *A* or 30 μM methylene blue (*MB*) in *B*. Immunopellets were washed and immunoblotted for GR and Hsp90. *Lane 1*, stripped GR; *lanes 2–5*, stripped GR incubated with chaperone mix for 20 min. *C*, the *bar graph* shows the results of four experiments where duplicate pellets were assayed for steroid binding activity, and the relative amount of GR-bound Hsp90 was determined by scanning immunoblots as in Fig. 1*C*. GR-bound Hsp90 in methylene blue samples is presented as a % of the untreated controls. **, different from control at $p < 0.01$.

determine whether ubiquitination by this system requires Hsp70, nNOS was incubated with the DE52-retained fraction in the presence of increasing concentrations of methylene blue. As shown in Fig. 4*A*, methylene blue inhibits nNOS ubiquitination. The concentration of Hsp70 in this ubiquitinating system is ~5% of Hsp70 in the reticulocyte lysate experiments of Fig. 1, and therefore, much lower concentrations of methylene blue are effective at inhibiting ubiquitination. Importantly, the inhibition produced by 1 μM methylene blue is largely overcome when purified Hsp70 is added to the incubation mix (Fig. 4*A*, *lane 7*). This indicates that methylene blue inhibits the Hsp70-dependent E3 ligase step in ubiquitination and not the E1 or E2 enzymes, which are not Hsp70-dependent. These data suggest that methylene blue may be a useful reagent to detect Hsp70-dependent effects, much as geldanamycin has been useful to probe for Hsp90-dependent effects.

Although overexpression of CHIP promotes nNOS degradation (42) and CHIP directs nNOS ubiquitination in a purified ubiquitination system (42, 43), it is not known if CHIP is the dominant E3 ligase for nNOS ubiquitination by a physiological ubiquitination system. To assess this, purified nNOS was incubated with the DE52-retained fraction of reticulocyte lysate in the presence of anti-CHIP antibody. As shown in Fig. 4*B*, nNOS ubiquitination is markedly reduced in the presence of anti-CHIP serum (*lane 4*) compared with nonimmune serum (*lane 3*). Taken together, the data of Fig. 4 suggest that nNOS ubiquitination by this model physiological ubiquitinating system is both Hsp70-dependent and CHIP-dependent, with the ubiquitinating activity being inhibited by methylene blue.

Effect of Methylene Blue on polyQ AR Degradation in Cells—Because transient overexpression of Hsp70, Hsp40, or CHIP reduces levels of polyQ AR in models of SBMA (16, 26, 30), it seems clear that the mutant AR can undergo Hsp70/CHIP-dependent proteasomal degradation. However, it is not known whether this is the major pathway or a minor pathway of polyQ AR degradation in the absence of overexpression of major proteins of the degradation pathway. To determine whether Hsp70 normally plays a role in polyQ AR degradation, HeLa cells expressing AR112Q were treated first with the AR agonist R1881 to activate the receptor and then with 10 μM methylene blue to inhibit Hsp70-dependent degradation. As shown in the immunoblot of total cellular AR112Q in Fig. 5*A*, methylene blue promotes accumulation of AR112Q both in the absence (*lane 3*) and presence (*lane 4*) of R1881. The accumulation of AR is particularly striking in the sample treated with both R1881 and methylene blue where there is also the accumulation of high molecular weight AR112Q oligomers seen with ligand-dependent aggregation. The quantitation of the methylene blue effect on the full-length AR112Q is shown in Fig. 5*B*. The fact that the proteasome inhibitor MG132 produces similar AR112Q accumulation (Fig. 5*C*) supports the model that methylene blue inhibits Hsp70-dependent degradation by the ubiquitin proteasome pathway.

Although methylene blue inhibited degradation of the full-length androgen receptor, it had opposite effects on amino-terminal fragments of the receptor containing a glutamine tract flanked by ~50 amino acids. These truncated fragments lack the receptor ligand binding domain and, therefore, are not clients of the Hsp90/Hsp70-based chaperone machinery. As shown in Fig. 6*A*, expression of both trAR16Q and trAR112Q was markedly decreased by methylene blue. This effect was dose-dependent (Fig. 6*B*) and abrogated glutamine-length dependent cytotoxicity (Fig. 6*D*). As similar fragments of mutant huntingtin are preferentially degraded by macroautophagy, we sought to determine whether the protective effect of methylene blue was associated with the induction of this alternative protein degradation pathway. We found that methylene blue increased levels of LC3-II, a marker of autophagosomes (Fig. 6*A*) and that the dose dependence of this induction coincided with diminished trAR112Q levels (Fig. 6*B*). We observed a similar induction of LC3-II in cells treated with either methylene blue or MG132 (Fig. 7*A*), indicating that inhibition of Hsp70-dependent ubiquitination is as potent an inducer of macroautophagy as blockade of the proteasome. The notion that methylene blue increased autophagic flux is supported by the super-induction of LC3-II in cells treated with both methylene blue and the lysosomal protease inhibitors E64d and pepstatin A (Fig. 7*B*) and by the observation that p62 did not accumulate in methylene blue-treated cells (Fig. 6*A*). Methylene blue also increased LC3-II in cells expressing full-

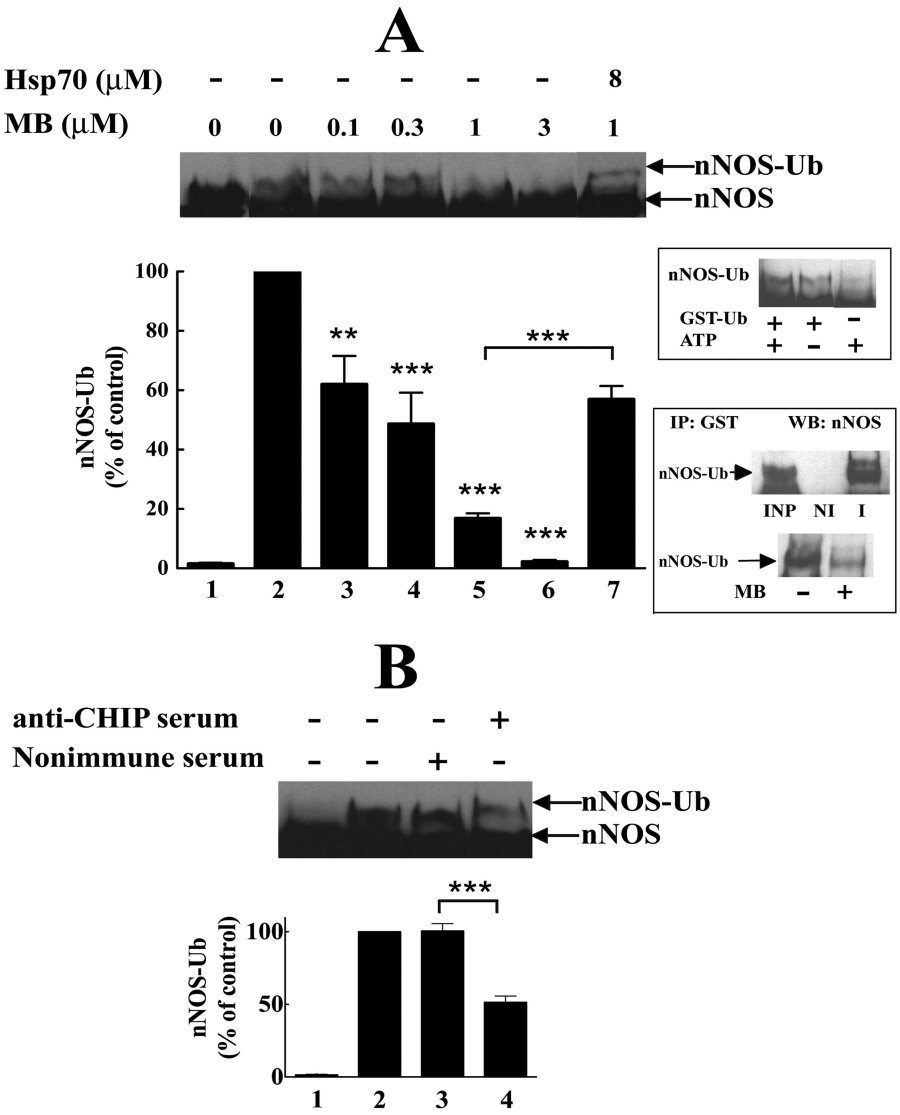

FIGURE 4. **nNOS ubiquitination by the DE52-retained fraction of reticulocyte lysate requires Hsp70 and CHIP.** *A*, methylene blue (*MB*) inhibits nNOS ubiquitination. Purified nNOS was incubated for 1 h at 37 °C with the DE52-retained fraction of reticulocyte lysate, ATP, GST-ubiquitin, and the indicated concentrations of methylene blue. In addition, 8 μM purified Hsp70 was added to a sample of the DE52-retained fraction containing 1 μM methylene blue. Samples were Western-blotted by probing with anti-nNOS. *Lane 1*, incubation time 0; *lanes 2–7*, incubation time 1 h. For the *bar graph*, the relative amount of monoubiquitinated nNOS (*nNOS-Ub*) in replicate experiments was determined by scanning and expressed as % of the 1-h control without methylene blue. The values are the mean ± S.E. ($n = 3$). *Asterisks over the columns* denote significantly different from control, and *asterisks over the line* denote that condition 7 is significantly different from condition 5. The *top inset* shows controls without GST-Ub and without added ATP (note: the stock, DE52-retained fraction contains 0.5 mM ATP.) The *bottom inset* (*top row*) shows an aliquot of input (*INP*) ubiquitinated nNOS of which 50 μl aliquots were immunoadsorbed with nonimmune (*NI*) or α-GST (*I*) IgG and immunoblotted (*WB*) with α-nNOS. The *bottom row* shows the effect of 3 μM methylene blue on samples immunoadsorbed with α-GST and immunoblotted with α-nNOS. *B*, CHIP is the major E3 ligase for nNOS ubiquitination. Purified nNOS was incubated with the DE52-retained fraction of reticulocyte lysate as above but in the presence of 1% nonimmune serum or 1% anti-CHIP serum. *Lane 1*, incubation time 0; *lanes 2–4*, incubation time 1 h.

length AR112Q (Fig. 6*C*). However, the full-length AR is not efficiently degraded by autophagy as ligand-dependent nuclear translocation moves the receptor to a compartment devoid of this degradation pathway. We conclude that inhibition of Hsp70 by methylene blue leads to the compensatory induction of macroautophagy, a pathway that preferentially degrades truncated androgen receptor fragments and thereby diminishes toxicity.

Finally, to confirm that cellular effects of methylene blue were mediated by the inhibition of Hsp70, we overexpressed Hsp70 along with full-length AR112Q. Overexpressed Hsp70 inhibited the accumulation of high molecular weight AR112Q oligomers and the induction of LC3-II that occurred following methylene blue treatment (Fig. 8). These data indicate that methylene blue acts through Hsp70 in cells to target these protein quality control pathways.

DISCUSSION

Several laboratories have been interested in developing small molecule inhibitors of Hsp70 for potential use in the treatment of cancers as well as neurodegenerative diseases characterized by the accumulation of aberrant proteins (44–49). Methylene blue was identified in a screen for compounds that inhibit Hsp70 ATPase activity (31). Unlike geldanamycin, which binds in the unique nucleotide binding pocket of Hsp90 and produces effects that are quite specific for inhibition of Hsp90 family proteins in eukaryotes (1), methylene blue has multiple cellular and molecular targets, including multiple neurotransmitter systems, ion channels, and enzymes (for review, see Ref.50). Although modulation of cGMP signaling is often considered its most significant effect, the redox properties of methylene blue are utilized in the treatment of methemoglobinemias and ifosfamide-induced encephalopathy and probably account for its use as an antimicrobial agent (50). Given its multiple molecular targets, methylene blue would seem *a priori* to be an imprecise research tool for probing Hsp70-dependent effects. Yet we indicate here that this readily available compound can be used in both subcellular and cellular systems for this purpose.

Using an established subcellular system where both Hsp70 and Hsp90 are required components, we show that the generation of steroid binding activity by the GR is inhibited by methylene blue. The effect occurs without inhibition of the binding of either Hsp70 or Hsp90 to the receptor (Fig. 1). Using the purified five-protein·Hsp90 heterocomplex as-

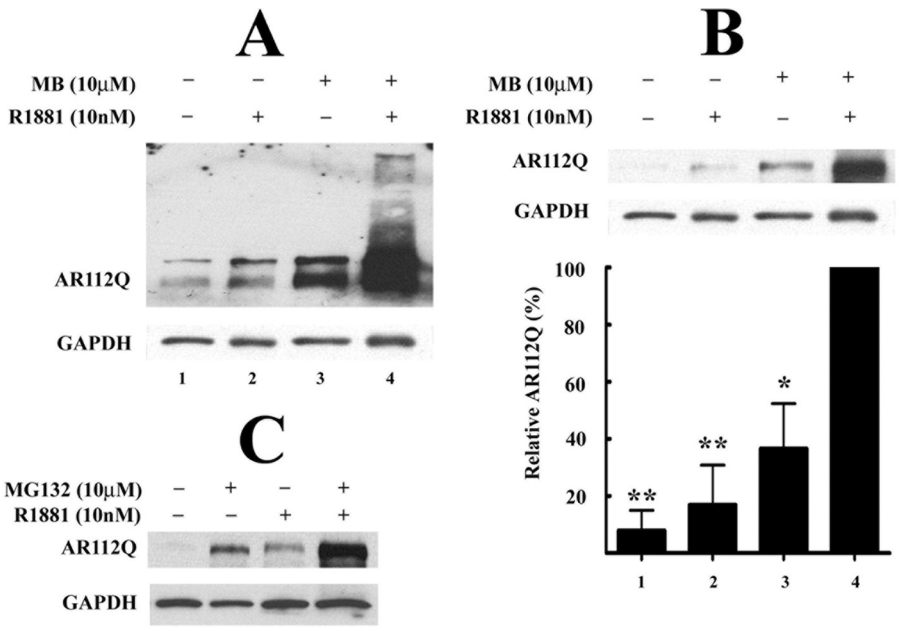

FIGURE 5. **Methylene blue inhibits AR112Q degradation in HeLa cells.** *A*, HeLa cells expressing AR112Q were incubated at 4 °C and treated sequentially with R1881for 30 min and then with 10 μM methylene blue (*MB*) for 30 min. Cells were then incubated at 37 °C for 8 h. Protein lysates were collected and analyzed by Western blot for AR expression. GAPDH controls for equal loading are shown. *B*, shown is a short exposure of the Western blot in *panel A* (*top*) and quantification of the AR signal (*bottom*) relative to the amount in cells treated with R1881 plus methylene blue. Data are the mean ± S.E. *C*, procedures are the same conditions as above except that 10 μM MG132 was substituted for methylene blue.

sembly system, we show that methylene blue inhibition of the activation of steroid binding activity depends upon the concentration of Hsp70 (Fig. 2). Although steroid binding activity is inhibited in the presence or absence of Hop, there is no depletion of GR-bound Hsp90 under either condition (Fig. 3). This suggests that the Hsp70 priming of the receptor, which is required for Hsp90-dependent opening of the steroid binding cleft, is inhibited by methylene blue. This priming step requires the ATPase activity of Hsp70 (51), and the ATPase activity is inhibited by methylene blue (31). Taken together, the GR experiments suggest that methylene blue inhibits an established physiological action of Hsp70 in a cell-free system.

Overexpression of CHIP has been shown to promote proteasomal degradation of a wide variety of normal and aberrant proteins. Although overexpression of CHIP promotes the proteasomal degradation of nNOS (42), there is clear redundancy in E3 ligase action on nNOS (26), and overexpression of one E3 ligase could favor a normally minor pathway of ubiquitination. Thus, it was not previously established that Hsp70-dependent CHIP activity is the principal physiologic pathway for nNOS ubiquitination. Methylene blue causes virtually complete inhibition of nNOS ubiquitination by the DE52-retained fraction of reticulocyte lysate (Fig. 4*A*). This finding suggests that all of the nNOS ubiquitination by the reticulocyte system may be Hsp70-dependent. Similarly, inhibition of ubiquitination by anti-CHIP antibody (Fig. 4*B*) suggests that CHIP is a major E3 ligase for nNOS in reticulocytes. Even though methylene blue affects a variety of biochemical process, including a well established inhibition of the NOS enzymes (52, 53), these data demonstrate that it can be used as a research tool to identify Hsp70-mediated processes in cell-free systems.

Using this tool, we explore the role of Hsp70 in controlling the proteostasis of the expanded glutamine androgen receptor in a cellular system. The Hsp90/Hsp70-based chaperone machinery binds to the carboxyl-terminal domain of the receptor and, like its action on the GR, regulates opening of the steroid binding cleft to permit ligand binding (1, 6). In this system Hsp90 functions to prevent androgen receptor unfolding. The extent to which this activity regulates stability of the expanded glutamine androgen receptor was previously demonstrated in *Hsf1*$^{-/-}$ cells that cannot mount a stress response (8). Treatment of these cells with geldanamycin or radicicol promotes androgen receptor degradation, demonstrating that Hsp90 normally functions to stabilize the receptor. Our data support a model in which ligand-dependent activation of the polyglutamine androgen receptor leads to Hsp70-dependent degradation through the ubiquitin proteasome pathway. Ubiquitination of the androgen receptor may be mediated by CHIP and other Hsp70-dependent E3 ligases that function redundantly (26). Here we show that methylene blue prevents degradation of the expanded glutamine androgen receptor and promotes the accumulation of aggregated species in an Hsp70-dependent manner.

The contrasting effects of methylene blue on degradation of trAR112Q are striking. We show that methylene blue promotes degradation of amino-terminal fragments of the receptor, thereby ameliorating glutamine-length dependent toxicity. These androgen receptor fragments include the glutamine tract flanked by ~50 amino acids (54) and, therefore, lack the ligand binding domain. In the absence of this domain, these proteins are not Hsp90 clients whose stability is regulated by the Hsp90/Hsp70-based chaperone machinery. Truncated fragments of the huntingtin protein are primarily degraded by macroautophagy (55, 56), and it is likely that these androgen receptor fragments are handled similarly. We show that methylene blue promotes induction and flux through the autophagic pathway. This homeostatic response likely reflects impairment of Hsp70-dependent degradation through the ubiquitin proteasome pathway. Others have observed similar induction of macroautophagy with genetic mutants that block degradation by the proteasome (57). These results suggest that degradation of trAR112Q is facilitated by methylene blue due to the compensatory activation of the pathway through which it is normally degraded.

Our findings demonstrate the effectiveness of methylene blue as a chemical tool to study Hsp70-dependent functions

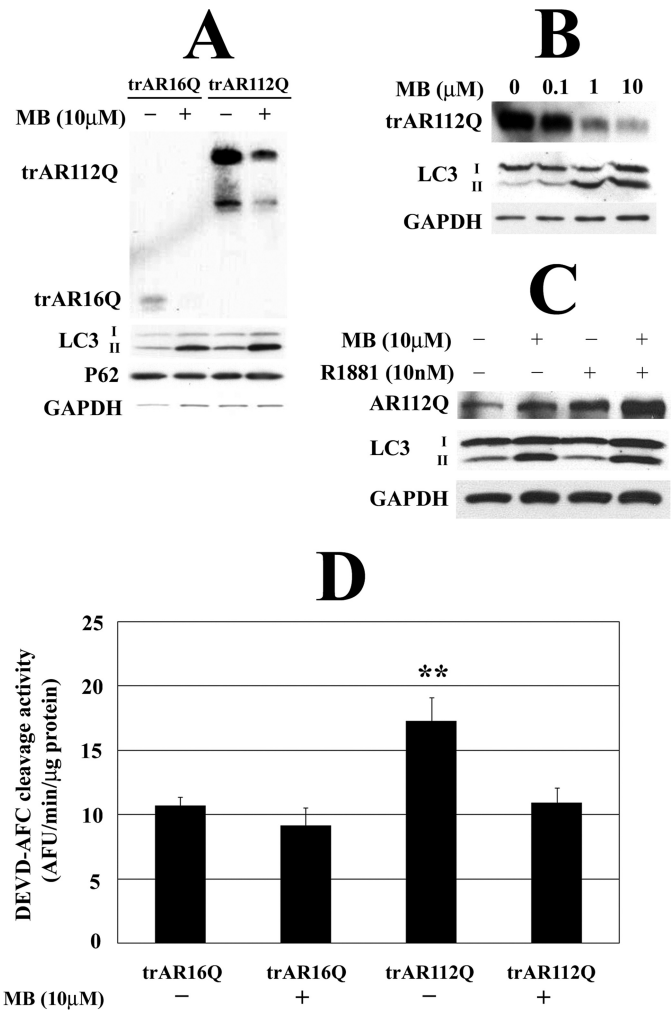

FIGURE 6. **Methylene blue enhances degradation of amino-terminal AR fragments and activates autophagy.** HeLa cells expressing trAR16Q or trAR112Q were treated with indicated concentrations of methylene blue (*MB*) or vehicle control for 24 h. *A*, protein lysates were collected and analyzed by Western blot for expression of AR, LC3, and p62. GAPDH controls for loading are shown. *B*, dose-dependent effects of methylene blue on trAR112Q and LC3 expression were determined by Western blot. *C*, shown is the effect of methylene blue on LC3-II levels in cells expressing full-length AR112Q. *D*, caspase activity was determined by measuring cleavage of the fluorescent substrate DEVD-aminofluoromethylcoumarin (*AFC*). Data are reported as the mean ± S.E. **, Different from all other samples at $p < 0.01$.

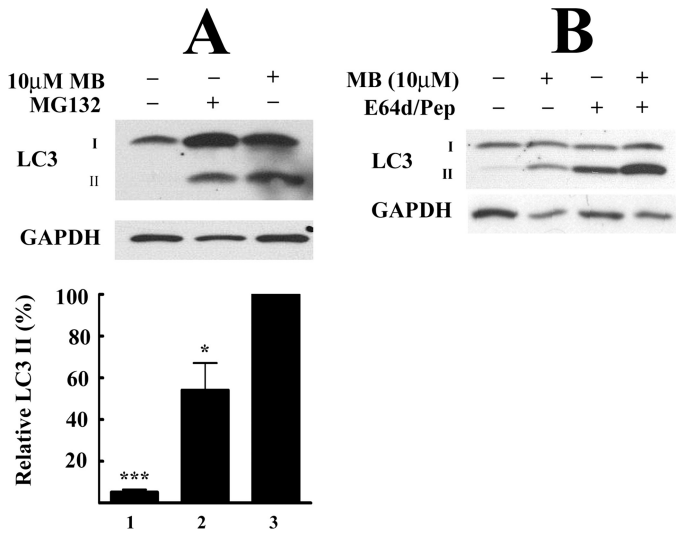

FIGURE 7. **Autophagic flux is increased by methylene blue.** *A*, HeLa cells were treated with 10 μM methylene blue (*MB*) or MG132 for 24 h. Protein lysates were collected and analyzed by Western blot for expression of LC3 and GAPDH (*top*). Quantification of the LC3-II/GAPDH signal relative to the amount in cells treated with methylene blue (*bottom*) is shown. Data are the mean ± S.E. Shown are differences from controls at $p < 0.05$ (*) or $p < 0.001$ (***). *B*, HeLa cells were treated as indicated with 10 μM methylene blue, 10 μg/ml E64d plus pepstatin A, or in combination. LC3 and GAPDH levels were determined by Western blot.

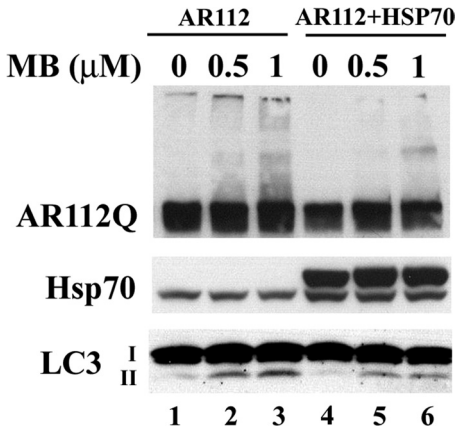

FIGURE 8. **Overexpression of Hsp70 diminishes the effects of methylene blue on levels of AR112Q and LC3-II.** HeLa cells expressing AR112Q alone (*lanes 1–3*) or with His-tagged Hsp70 (*lanes 4–6*) were incubated at 4 °C and treated sequentially with R1881for 30 min and then with 0.5 or 1 μM methylene blue for 30 min. Cells were then incubated at 37 °C for 8 h. Protein lysates were collected and analyzed by Western blot for AR, Hsp70, and LC3. His-tagged Hsp70 migrates slightly more slowly than the endogenous protein. Endogenous Hsp70 and LC3-I levels serve as loading controls.

in well established systems. Few other small molecule inhibitors of Hsp70 are available, and their potential use as chemical probes to define Hsp70-mediated effects in models of neurodegenerative diseases and cancer offers great promise. Small molecule inhibitors could be used to define the role of Hsp70 in regulating the function, trafficking, and turnover of proteins that are clients of the Hsp90/Hsp70-based chaperone machinery and may be used to probe the role of Hsp70 in protein quality control decisions. For example, 2-phenylethynesfulonamide, a recently described inhibitor of Hsp70, is reported to decrease CHIP-Hsp70 association and alter autophagy although in this case autophagic flux may be impaired (49). In contrast to 2-phenylethynesfulonamide, methylene blue treatment of cells did not decrease the amount of CHIP co-immunoadsorbed with Hsp70 (data not shown), suggesting that the two compounds inhibit Hsp70 by quite different mechanisms. Future studies will help define the experimental and therapeutic utility of this emerging class of small molecules.

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