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REVIEW

Role of Chaperones in Nuclear Translocation and Transactivation of Steroid Receptors

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Molecular chaperones assist proteins to reach their mature and functional conformation. It has become apparent in recent years that chaperones function as part of a multiprotein heterocomplex that is potentially involved not only in protein folding, but also in intracellular trafficking and in targeting proteins for degradation. In the case of steroid receptors, the activity of the chaperone heterocomplex, as well as the proteins comprising the heterocomplex, has an effect on the observed ligand-dependent transcriptional activity of the receptor. The direct interaction between chaperones and steroid receptors makes them potential therapeutic targets in a number of pathologic conditions. In the case of cancers with steroid receptor involvement, such as breast and prostate cancer, the inhibition of chaperone activity may inhibit tumor cell growth. Conversely, enhancement of chaperone activity may be beneficial in disorders of protein misfolding, as in the case of androgen receptor aggregates found in Spinal and Bulbar Muscular Atrophy.

Key Words: Steroid receptor; chaperone; heat-shock protein; trafficking; ligand affinity.

Introduction

The native states of proteins in vivo are reached with the assistance of molecular chaperones *(1,2)*. The primary eukaryotic chaperones are thought to include the heat-shock proteins Hsp90 and Hsp70, as well as Hop/p60, p23, BAG-1, Hsp40/DnaJ/HDJ-2, and the large immunophilins (Cyp40, FKBP51, and FKBP52) (reviewed in ref. *3*). Subsets of these proteins compose multiprotein complexes, referred to as the chaperone heterocomplex *(4)*, that assemble on target proteins. Although some molecular chaperones are strongly induced on heat shock or stress, most are constitutively expressed at high levels under normal physiologic conditions *(5,6)*. Hsp90, one of the most extensively characterized eukaryotic chaperones, is essential in yeast, *Drosophila*, and mice *(7,8)*. Hsp90 has been demonstrated to interact with a number of cellular signaling proteins *(9)* including calmodulin *(10)*, the c-Raf serine/threonine kinase *(11)*, pp60 v-src tyrosine kinase *(12,13)*, the heme-regulated eIF-2α protein kinase *(14)*, and casein kinase II *(15)*. However, much of what is known about the activities of Hsp90 and other chaperones comes from studies of their interaction with steroid hormone receptors *(16)*.

Steroid hormone receptors are transcription factors whose transcriptional activity is predominantly regulated by binding to cognate ligands. In the absence of hormone, the unliganded receptor (aporeceptor) exists in a dynamic chaperone heterocomplex. Chaperones interact with steroid receptors in a transitory but ordered manner to facilitate the folding of the receptor and stabilize it in a conformation receptive to ligand binding *(16)*. However, affinity of the aporeceptor to its ligand can be modified by the composition of the proteins in the heterocomplex *(3,17,18)*. Some members of the heterocomplex may also contribute to nuclear-cytoplasmic trafficking and receptor turnover. The chaperone heterocomplex, therefore, has the capacity to influence the observed transcriptional activity of nuclear steroid receptors. Other nuclear receptors, such as thyroid hormone receptor (TR), vitamin D receptor (VDR), the all-*trans*-retinoic receptor (RAR), and the 9-*cis*-retinoic acid receptor (RXR), do not efficiently associate with Hsp90 in vitro. Hsp90 and the chaperone heterocomplex may be important for these nuclear receptors to function in vivo *(19)*, but little is known about these potential interactions.

Assembly of Chaperone Heterocomplex with Steroid Receptors

Using in vitro reconstitution systems, it appears that the chaperone heterocomplex assembles with the steroid receptor in a stepwise process. Hsp70 and Hsp90 are among the initial constituents that bind to the newly translated receptor, but it is unclear if they do so as part of a preassembled

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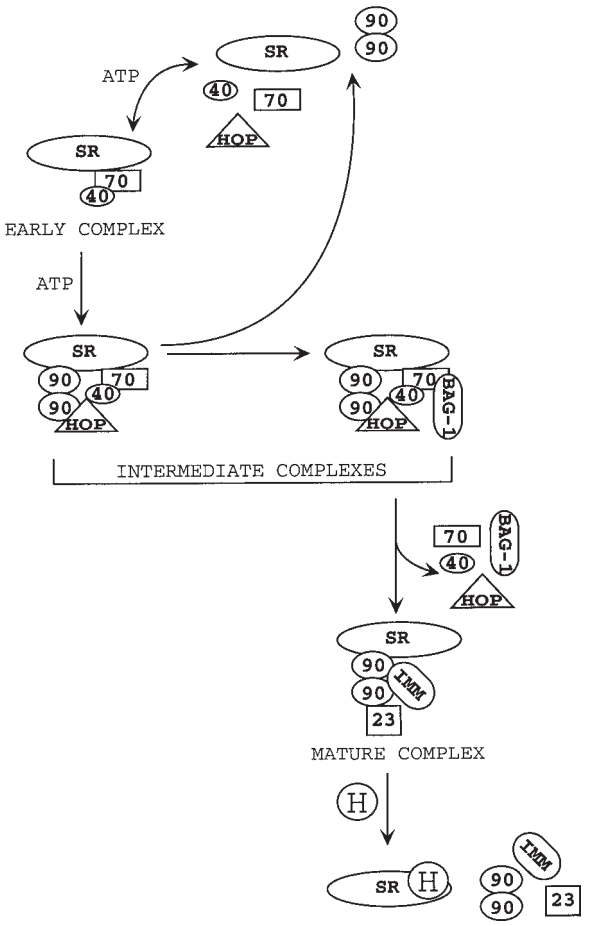

Fig. 1. Model of assembly of the chaperone heterocomplex with steroid receptors. For discussion, see text. SR, steroid receptor; 70, Hsp70; 90, Hsp90; 40, Hsp40; IMM, immunophilin; H, steroid hormone receptor ligand.

"foldosome" complex *(4)*, if they assemble on the receptor *(20)*, or if both events occur in vivo. When the time course of the assembly of the heterocomplex with the progesterone receptor (PR) was examined in reticulocyte lysate, Hsp70 and Hop showed early, transitory recovery peaks. By contrast, Hsp90, p23, and the immunophilins showed a more gradual accumulation on PR to a plateau *(3,21)*. The binding of Hsp70 to proteins is coupled to adenosine triphosphate (ATP) hydrolysis by Hsp70, which is enhanced by Hsp40 *(1)*. These observations together suggest that the initial phase of receptor-hetercomplex assembly involves the ATP-dependent association among the receptor, Hsp70, and Hsp40 (Fig. 1). Hop interacts with adenosine 5'-diphosphate (ADP)-bound forms of Hsp70 and a dimer of Hsp90 *(22)*. Therefore, in an intermediate phase of assembly, Hop interacts with Hsp70 and assists the recruitment of Hsp90 *(3,4,18)*. Finally, Hsp70 and Hop dissociate from the heterocomplex, probably with the assistance of BAG-1, which binds to the ATPase-binding site of Hsp70 and promotes the release of ADP, thus weakening the interaction between Hsp70 and the hydrophobic ligand-binding domain (LBD) of the receptor *(23,24)*. The release of Hop and Hsp70 allows p23 and the immunophilins to bind the heterocomplex and form the mature complex. As discussed below, once part of the mature complex, the steroid receptor shows maximal steroid-binding ability.

The mature PR and glucocorticoid receptor (GR) (and presumably other steroid receptors) heterocomplexes are not inherently stable and dissociate from the receptor in the absence of ligand *(4,21)*. In cell-free assembly systems, Hsp90-Hsp70-Hop binding to PR reaches half-maximal levels at 2.5 min. However, Hsp90 binding is lost from PR with a half-life of about 5 min *(21)*. This indicates that at least in the cell-free state, most of the receptor is in a relatively stable mature complex and available for hormone binding. The dynamic nature of heterocomplex formation is consistent with the known transient interaction of chaperones with other target proteins (reviewed in ref. *1*). The dynamic nature of the assembly/ dissassembly process may be important in considering the biological roles of the chaperone heterocomplex.

Chaperones and Steroid Receptor Ligand Binding

Because Hsp90 and other members of the heterocomplex were initially isolated in association with aporeceptors, it was suggested that steroid receptor function was repressed by the heterocomplex. However, genetic analyses in yeast demonstrated that yeast strains expressing a reduced level (<5%) of Hsp90 also had a reduced level of GR and estrogen receptor (ER) transcriptional activity, suggesting that Hsp90 may interact with aporeceptors to maintain an activatable conformation *(25)*. Hsp90 has been shown to interact with GR *(26)* and PR *(27)* through their ligand binding domains (LBDs). In the case of ER, Hsp90 interacts with both the LBD and the C-terminal portion of the DNA-binding domain *(28)*. The LBD of steroid receptors is thought to be capable of a high degree of flexibility based on X-ray crystallographic data indicating that ligand binding induces a dramatic conformational change *(29,30)*. Therefore, Hsp70 and Hsp90 may recognize and stabilize the flexible LBD, allowing more efficient ligand binding.

Experimental evidence suggests that several members of the chaperone heterocomplex influence hormone binding. Ligand binding of PR, GR, the androgen receptor (AR), and the mineralocorticoid receptor (MR) is significantly reduced in yeast strains harboring Hsp90 mutants *(31–33)*. In cell-free reconstitution experiments using rabbit reticulocyte lysate, both GR and PR require Hsp90 to maintain hormone ligand-binding capacity *(21,34)*. However, it is unclear whether all steroid receptors require continuous contact with Hsp90 to assume a high-affinity steroid-binding conformation. Whereas this is apparently the case for GR and PR, ER can be dissociated from the Hsp90 heterocomplex and retain a high-affinity hormone-binding conformation *(3,16)*. Interaction between the steroid receptor and Hsp90 alone, however, is probably insufficient for full

reconstitution of hormone binding. Using purified heterocomplex constituents, GR has been shown to minimally require Hsp90, Hsp70, p23, and Hop to generate the full hormone-binding activity observed in rabbit reticulocyte lysate *(35)*. Hop contributes to ligand-binding activity by enhancing the rate of Hsp90 and Hsp70 assembly *(18)*. The effect of p23 is to stabilize the receptor-Hsp90 interaction *(36)*, contributing to ligand binding and ultimately to enhanced transcriptional activity. Overexpression of some heterocomplex members in transfection experiments has been found to enhance the observed transcriptional activity of steroid receptors. Ectopic expression of p23 in MCF-7 human breast cancer cells increases ER-mediated transcriptional activation *(37)*. Similarly, a BAG-1 isoform, BAG-1L, enhances AR transcriptional activity in transient transfection assays in COS-7 cells *(38)*. One mechanism through which these effects may occur is to drive heterocomplex assembly toward the formation of mature complexes with the receptor. A greater population of the receptor molecules would then be stabilized in a conformation optimal for ligand binding.

Members of the chaperone heterocomplex may also negatively modulate receptor hormone binding. The immunophilin FKBP51 decreases the affinity of GR *(17)* and PR *(3)* for their cognate ligands. FKBP51 reduces the affinity of PR for progesterone by two- to three-fold *(3)*. GR binding to dexamethasone was reduced by 40–50% when FKBP51 was incorporated into the heterocomplex *(17)*. FKBP51 expression is induced by glucocorticoids in some GR-expressing cell lines *(39)*. Smith *(3)* has proposed that these observations may represent a mechanism through which naive cells can be conditioned for a decreased response to secondary hormone exposure. In this model, cortisol induces a robust response in naive cells including an increase in the level of FKBP51 expression. With subsequent cortisol exposure, FKBP51 is incorporated into the heterocomplex interacting with GR, which causes an attenuated response *(3)*. The mechanism through which FKBP51 decreases receptor ligand binding is currently not known.

Upon steroid binding, the heterocomplex dissociates from the receptor. The conformational change of the ligand-binding pocket induced by hormone binding is thought to destabilize the interaction between the receptor LBD and the heterocomplex *(16)*. In the case of PR, progesterone prevents Hsp70 and Hop from associating with the receptor *(21)*. In this system, Hsp90 is prevented from binding the holoreceptor although ligand binding does not accelerate the dissociation of Hsp90 from PR *(21)*. These observations raise the question as to whether both agonists and antagonists of steroid receptors induce the release of the heterocomplex. This has been addressed for the endogenous AR expressed in LNCaP cells derived from a human lymph node metastasis of the prostate. The LNCaP AR carries a point mutation (T877A) that allows it to be transcriptionally activated by both androgens and an antiandrogen hydroxyflutamide. However, the LNCaP AR is unable to be transcriptionally activated by the antiandrogen casodex (ICI 176334). Veldscholte et al. *(40)* observed that Hsp90 and Hsp70 dissociated from the LNCaP AR when bound to activating ligands (i.e., androgen and hydroxyflutamide) but remained associated with casodex-bound AR. These observations suggest that there may be a class of steroid receptor antagonists that are unable to release the receptor from the chaperone heterocomplex. However, this is probably not a universal mechanism of steroid receptor antagonists since the antiestrogen RU 58668 can induce the dissociation of Hsp90 from ER *(41)*.

Role of Chaperones in Protein Degradation

It has been suggested that if chaperones fail to promote appropriate protein folding they facilitate rapid degradation *(42)*. The mitochondrial Hsp70 and Hsp40 homologs cooperate with the protease Pim1 to degrade abnormal proteins *(42,43)*. Another study has indicated that Hsp70 is required for the degradation via the ubiquitin pathway for some but not all of its substrates. Immunodepletion of Hsp70 from reticulocyte lysate inhibits the degradation of actin, glyceraldehyde-3-phosphate dehydrogenase, and histone 2A but not bovine serum albumin or lysozyme, all known targets for ubiquitin tagging and degradation by the 26S proteasome *(44,45)*. On the other hand, mutations of p53 that stabilize the interaction between p53 and Hsp70 impair the degradation of the mutant p53 molecule *(46)*.

The ability of chaperones to influence the degradation of steroid receptors has not been extensively studied to date. Geldanamycin, an antibiotic that prevents the release of Hsp90 from steroid receptors and inhibits the binding of p23 to the heterocomplex *(47,48)*, causes the enhanced degradation of GR in HeLa cells *(49,50)*. The effect of geldanamycin on receptor degradation may not occur for all steroid receptors, however. Treatment with geldanamycin does not alter PR levels *(48)*, indicating that a potential role for Hsp90 or p23 in protein degradation may be specific to certain steroid receptors.

The antiestrogen ICI 182780 (ICI) prevents ER from localizing to the nucleus. This antiestrogen is also known to reduce the half-life of the ER protein *(51)*. Although the mechanism by which ICI increases the degradation rate of ER is not fully understood, it has been suggested that the ICI-bound ER is inhibited from dimerization, recognized as a misfolded protein by the chaperones, and targeted for degradation *(51)*.

The heterocomplex cochaperone BAG-1 has been shown to link Hsp70 to the proteasome *(52)*. However, as mentioned, a BAG-1 isoform enhances AR-mediated transcription *(38)* and contributes to the maturation of the chaperone heterocomplex *(23,24)*. If BAG-1 does contribute to steroid receptor degradation, it may do so only in the context of mutant or misfolded receptors. Alternatively, BAG-1-mediated association of Hsp70 with the proteasome may

not result in the degradation of all substrates. For example, BAG-1 may mediate the degradation of other components related to transcription by steroid receptors such as proteins removed through chromatin remodeling *(53)* or through targeting the proteasomal degradation of receptor corepressors *(54)*. The proteasomal component mSug1/p45/TRIP1 is a putative chaperone that interacts with ER, RXR, VDR, and TR in a ligand-dependent manner *(53,55)*. Unlike BAG-1, over-expression of mSug1 inhibits transcription by TR and RXR *(55)*, suggesting that mSug1 may play a role in down-regulating hormone-mediated transcription by some nuclear receptors by enhancing proteolytic degradation.

Chaperones and Nuclear Transport of Steroid Receptors

Prior to the cloning of steroid receptors, centrifugation experiments monitoring binding of radiolabeled ligands suggested that hormone-free receptors are located in the cytosol as a 9S heterocomplex and that ligand binding induces dissociation of the heterocomplex, receptor dimerization, and translocation to the nucleus, where it exists in a 4S form (reviewed in ref. *16*). However, in these experiments "cytosol" and "nuclear" fractions were not completely separated by the methods used *(56)*. Once antibodies to steroid receptors became available, immunohistochemical studies showed that the subcellular localization of steroid receptors in the absence of their cognate ligand differed among receptors.

In the absence of ligand, GR and MR are predominantly localized to the cytoplasm. Some studies have found that cytoplasmic GR colocalizes to tubulin-containing microtubules *(56,57)*. On exposure to hormone, these receptors translocate to the nucleus *(4,58)*. The subcellular localization of AR is less clear. AR is found to be predominantly nuclear in prostate tissue from cancer patients treated with androgen ablation therapy *(59)*. AR is also found to be nuclear in the tissues of castrated and intact rats *(60,61)*. However, ectopic expression of AR in certain AR-negative cell lines, such as COS-1, results in a cytoplasmic localization of AR in the absence of ligand and a nuclear localization of AR in response to androgen *(62)*. ER and PR, by contrast, are predominantly nuclear in both the absence and presence of hormone *(51,63–66)*. Experiments using heterokaryons have demonstrated that the unliganded ER and PR are not static in the nucleus but constantly shuttle between the nucleus and the cytoplasm *(51,63)*. The nuclear localization of apo-ER and -PR is due to the cooperative interaction of the proto-nuclear localization sequences (NLSs) that constitutively localize the receptors to the nucleus *(67)*. The NLS sequences of the ligand-free GR are prevented from cooperative interaction by the positioning of the LBD. On ligand binding, the conformation of the LBD is altered so that it no longer masks the NLS regions, and the receptor is able to translocate to the nucleus *(67,68)*.

Because hormone free nuclear receptors are normally present in both the nucleus and cytoplasm, it is possible that the heterocomplex may assemble in both cellular compartments, or may shuttle with the receptors between the nucleus and cytoplasm. Although biochemical and immunocytochemical analyses indicate that Hsp90 is predominantly a cytoplasmic protein, it is also found at a low level (~3%) in the nucleus *(16,69,70)*. In cotransfection experiments in COS-7 cells, a fraction of exogenous Hsp90 was found to localize to the nucleus with PR and ER in the absence of hormone *(41,70)*, suggesting that Hsp90 may be carried into the nucleus with its target proteins. A similar situation may occur for Hsp70. Hsp70 was initially characterized as a cytoplasmic protein that concentrated in the nucleus in response to heat shock or chemical stress and then relocalized to the cytoplasm during recovery. In cell fusion studies, Hsp70 was observed to cycle across the nuclear membrane in the absence of stress stimulus *(71)*. Hsp70 concentrates in the nucleus of cells overexpressing c-myc, suggesting that Hsp70 may also enter the nucleus in association with other target proteins, including apo-ER or -PR *(72)*. However, Hsp70 may not be *necessary* for translocation of all of its target proteins. Inhibition of Hsp70 binding in HeLa cells using an Hsp70 antibody prevents transport of the SV40 T antigen and nucleoplasmin into the nucleus but does not influence the dexamethasone-induced transport of GR *(73)*.

The immunophilin FKBP52 is predominantly a nuclear protein but approximately 20% of FKBP52 is cytoplasmic and colocalizes with microtubules *(74,75)*. Because FKBP52 coimmunoprecipitates with the intermediate and heavy chains of dynein, a protein family involved in vesicle movement along microtubules *(75)*, and because antibodies to FKBP52 inhibit dexamethasone-induced nuclear translocation of GR *(74)*, it has been suggested that FKBP52 may be involved in the trafficking of GR from the cytoplasm to the nucleus along the cytoskeleton *(4,76)*. On the basis of protein cross-linking studies, FKBP52 is not thought to contact hormone-free steroid receptors directly but instead interacts with Hsp90 *(77)*. Although an Hsp90-FKBP52 containing complex may be involved in the nuclear-cytoplasmic shuttling observed for the unliganded ER and PR, it is unlikely to be involved in the translocation of hormone-bound GR to the nucleus since GR dissociates from Hsp90 upon ligand binding. It remains possible that steroid receptors associate with an FKBP52-cytoskeleton transport complex through an as yet unidentified protein.

However, it is unclear to what extent GR or other steroid receptors require the cytoskeleton for nuclear-cytoplasmic movement. The VDR is associated with microtubules while in the cytoplasm, and microtubule-disrupting agents inhibit VDR transcriptional activity response to 1,25-dihydroxyvitamin D_3 and nuclear localization *(78,79)*. GR, and possibly other steroid receptors, may translocate from the cytoplasm to the nucleus by two independent pathways, one of

which involves the cytoskeleton. Dexamethasone-induced nuclear localization of GR is inhibited by the serine/threonine protein phosphatase inhibitor okadaic acid. When cells are treated with colcemid, a disruptor of cytoskeketal microtubules, okadaic acid is no longer able to prevent ligand-induced GR entry into the nucleus *(80)*. This suggests that under physiological conditions, a dephosphorylation event is involved in GR shuttling, and these processes involve the cytoskeleton. Geldanamycin also inhibits the movement of ligand-bound GR to the nucleus in a cytoskeleton-dependent manner *(81,82)*. However, because Hsp90 is not associated with hormone-bound receptors, the action of geldanamycin may be indirect and occur through another Hsp90 target.

Because disruption of microtubules, microfilaments, or intermediate filaments does not prevent GR from translocating to the nucleus in the presence of dexamethasone *(82)*, GR may also be able to localize to the nucleus by a separate mechanism, possibly involving diffusion. GR has two NLS domains, NL1 and NL2. NL1-mediated import operates through binding to the general nuclear import protein, importin α *(68,83,84)*. The mechanism of action of the NL2 domain, which is importin α independent *(68)*, is unknown, but it would be interesting to know if the cytoskeleton-associated and cytoskeleton-independent mechanisms of GR nuclear shuttling were separately controlled by separate NLS domains.

The assembly of the chaperone heterocomplex with unliganded steroid receptors may therefore occur in both the nucleus and cytoplasm. It is unclear, however, to what extent the chaperone proteins play a role in trafficking of the liganded or unliganded receptor. In the case of GR, and possibly AR, a cytoplasmic ligand-dependent interacting protein that was capable of enhancing nuclear import could potentially enhance receptor transcriptional activity. A possible example of this class of coregulators is tuberin, the product of the tuberous sclerosis gene 2. Tuberin has been identified as a coregulator of GR, VDR, and the peroxisome proliferator–activated receptor alpha (PPARα). In transfection experiments, tuberin enhances the ligand-dependent transactivation of PPARα and VDR by two- to threefold. However, it inhibits GR transcription in the presence of dexamethasone by approximately 50% *(85)*. Tuberin is a cytoplasmic protein and has recently been described as a chaperone *(86)*. However, it has not yet been determined whether tuberin exerts a coregulatory effect on nuclear receptors by altering transport or through other mechanisms.

It is also possible that proteins that regulate nuclear export of steroid receptors could influence the observed receptor transcriptional activity. To date, chaperone proteins have not been implicated in the nuclear export of receptors. However, the nuclear export factor Ran *(83)* is identical to ARA24, a coactivator of AR *(87)*. It has not yet been determined whether the ectopic expression of Ran/ARA24 enhances AR expression indirectly by making more importin molecules available in the cytoplasm, by enhancing the rate of AR mRNA export, or by exerting an activity independent of its nuclear export function *(88)*.

Conclusion

The interaction between steroid receptors and the chaperone heterocomplex is necessary for receptor transcriptional activity in response to hormone. Both genetic studies *(25)* and in vitro reconstitution experiments *(18,35)* have demonstrated that chaperones are necessary to establish the receptor in a conformation permissive for ligand binding. Alterations in the level of some of the chaperones of the heterocomplex have been shown to enhance (e.g., p23 and BAG-1) or repress (e.g., FKBP51) the observed ligand-mediated transcription of the steroid receptor *(17,37,38)*. Although not as well studied, chaperones may also alter the protein stability of steroid receptors and influence their subcellular localization. Because the activity of chaperones can alter the observed transcriptional activity of a steroid receptor, they can be considered steroid receptor coregulators. A coregulator, as used here, is a protein that interacts with the receptor directly or through an interacting complex to influence the transcriptional activity of the receptor. The coregulator may be localized to the nucleus or cytoplasm and does not necessarily interact with the basal transcription machinery. A number of steroid receptor coregulators have recently been identified as being predominantly localized to the cytoplasm *(85,89)*. The study of chaperones can provide an indication of the mechanisms through which some of these factors may influence the transcriptional activity of steroid receptors.

The direct and essential interaction of chaperones with steroid receptors makes them potential therapeutic targets for cancers with steroid receptor involvement, such as breast and prostate cancer. The benzoquinone ansamycin compounds, such as geldanamycin and herbimycin A, specifically bind to the nucleotide-binding pocket of Hsp90 *(47,90)* and interfere with the appropriate formation of the heterocomplex disrupting steroid receptor function *(49)*. Because Hsp90 also is required for the stability of a number of kinases associated with oncogenic transformation, compounds such as geldanamycin also have a general antitumor effect *(90)*. Preclinical pharmacologic evaluation of geldanamycin indicated that geldanamycin is cytotoxic to a wide range of tumor-derived cell lines but does not affect the viability of differentiated cells *(91)*. However, the presence of serious side effects with geldanamycin, especially hepatotoxicity, has limited its clinical development *(91)*.

While in tumors it may be desirable to inhibit the activity of certain chaperones, in other pathological conditions it may be advantageous to enhance chaperone activity. AR contains an N-terminal polyglutamine region of variable length, and expansion of the AR polyglutamine region causes the neuromuscular disorder Spinal Bulbar Muscular Atrophy (or

Kennedy disease) *(92)*. The polyglutamine expansion causes AR to accumulate in cellular aggregates due to protein misfolding and a failure of proteolytic processing. In vitro, AR aggregate formation is significantly reduced by the ectopic expression of Hsp40 or a combination of Hsp40 and Hsp70 *(93,94)*. Similarly, expansion of the polyglutamine tract of ataxin-1 results in ataxin aggregate formation and causes spinocerebellar ataxia type 1 (SCA1). Overexpression of an Hsp40 family member resulted in a 60% decrease in ataxin-1 aggregates in a tissue culture model of SCA1 *(95)*. These observations suggest that stimulation of chaperone activity could be of potential therapeutic benefit in at least some disorders caused by protein misfolding.

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