Hsp90 is a direct target of the anti-allergic drugs disodium cromoglycate and amlexanox

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Hsp90 (heat-shock protein 90) alone can act to prevent protein aggregation and promote refolding *in vitro*, but *in vivo* it operates as a part of a multichaperone complex, which includes Hsp70 and cohort proteins. Since the physiological function of Hsp90 is not yet fully understood, the development of specific antagonists might open new lines of investigation on the role of Hsp90. In an effort to discover Hsp90 antagonists, we screened many drugs and found that the anti-allergic drugs DSCG (disodium cromoglycate) and amlexanox target Hsp90. Both drugs were found to bind directly wild-type Hsp90 via the N- and C-terminal domains. Both drugs strongly suppressed the *in vitro* chaperone activity of native Hsp90 towards citrate synthase at 1.5–3.0 μM. Amlexanox suppressed C-terminal chaperone activity *in vitro*, but not N-terminal chaperone activity, and inhibited the association of cohort proteins, such as cyclophilin 40 and Hsp-organizing protein, to the C-terminal domain of Hsp90. These data suggest that amlexanox might disrupt the multichaperone complex, including Hsp70 and cohort proteins, both *in vitro* and *in vivo*. Although DSCG inhibited the *in vitro* chaperone activity of the N-terminal domain, the drug had no effect either on the C-terminal chaperone activity or on the association of the cohort proteins with the C-terminus of Hsp90. The physiological significance of these interactions *in vivo* remains to be investigated further, but undoubtedly must be taken into account when considering the pharmacology of anti-allergic drugs. DSCG and amlexanox may serve as useful tools for evaluating the physiological significance of Hsp90.

Key words: amlexanox, anti-allergic drug, chaperone, citrate synthase, cohort protein, disodium cromoglycate, Hsp90, rhodanese.

INTRODUCTION

Hsp90 (heat-shock protein 90) is an abundant molecular chaperone protein under physiological conditions [1,2]. At elevated temperatures, the concentration of Hsp90 increases severalfold, suggesting a protective function of this protein under heat stress [3,4]. Hsp90 alone can act to prevent protein aggregation and promote refolding *in vitro*, but *in vivo* it operates as a part of the multichaperone 'machinery' in the cytosol, which includes Hsp70, peptidyl–prolyl isomerases and other cohort proteins [5]. Several Hsp90-associated cohorts contain multiple copies of the TPR (tetratricopeptide repeat) motif, a degenerate consensus sequence that mediates protein–protein interactions in diverse cellular pathways [6]. The TPR domains of peptidyl–prolyl isomerases, such as FKBP52 (FK506 binding protein 52), CyP40 (cyclophilin 40) and PP5 (phosphoprotein phosphatase 5), mediate the binding of these proteins to Hsp90 [7–9]. Only a specific subset of the TPR proteins can bind to Hsp90. The Hsp70 cohort protein Hip (Hsp-interacting protein), which contains four TPR domains, could not bind directly to Hsp90 [10]. Unfolded polypeptide substrates associate first with the Hsp70 system and are then bound by Hsp90 via Hop (Hsp-organizing protein), which interacts with both Hsp70 and Hsp90 (which consists of Hip, Hop, Hsp90 and Hsp70, comprising an intermediate chaperone complex) [11,12]. Further remodelling results in the mature chaperone complex, consisting of Hsp90, p23 and FKBP52 or CyP40 [8]. In eukaryotes, cytoplasmic Hsp90 acts as a specific chaperone for many signal-transduction proteins, such as steroid hormone receptors [13–15] and protein kinases [16,17]. Because Hsp90 is essential for maintaining the activity of many signalling proteins, it has a key role in signal-transduction networks in the cell.

It has been reported previously that Hsp90 possesses two chaperone sites located in the N- and C-termini [18,19]. The highly conserved 25 kDa N-terminal domain contains the binding site for ATP, and also for geldanamycin and radicicol, which specifically target Hsp90 [20–22]. Geldanamycin and radicicol inhibit the ATPase activity of Hsp90 with nanomolar affinity, and inhibit the binding of the cohort protein p23 to Hsp90 [23,24]. Crystallography studies have shown that geldanamycin occupies the ATP-binding cleft within the N-terminal domain, which shares a similar tertiary structure with the GHKL (gyrase/Hsp90/histidine kinase/MutL) family proteins, such as bacterial topoisomerase, gyrase B and the mismatch-repair protein MutL [21]. The C-terminal domain also binds to partially folded substrate proteins in an ATP-independent manner. Thus Hsp90 contains at least two independent chaperone sites, which suppress protein aggregation with different specificities. A relatively high concentration (100 μM) of geldanamycin partially inhibits *in vitro* chaperone function of the N-terminal domain [19]. Novobiocin, a gyrase inhibitor, also compromises Hsp90 function similarly to that of geldanamycin [25]. However, whereas its binding site in the gyrase overlaps with the ATP-binding site, the binding site in Hsp90 is located in its C-terminus [25,26]. Deletion of amino acids 660–680 of Hsp90α abolishes both ATP and novobiocin binding, indicating this segment to be a part of a novel ATP-binding site [26]. Although the effect of novobiocin on the *in vitro* chaperone activity of Hsp90 is not clear at present, the drug at 1 mM concentration interferes with the association of p23 and Hsp70 with Hsp90 [25].

Abbreviations used: CS, citrate synthase; CyP40, cyclophilin 40; DSCG, disodium cromoglycate; FKBP52, FK506 binding protein 52; GST, glutathione S-transferase; Hsp90, heat-shock protein 90; Hip, Hsp-interacting protein; Hop, Hsp-organizing protein; PP5, phosphoprotein phosphatase 5; TPR, tetratricopeptide repeat; TPCK, tosylphenylalanylchloromethane.

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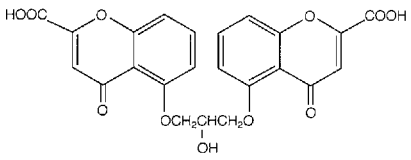
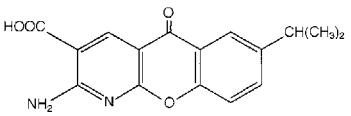


DSCG



Amlexanox

Figure 1 Chemical structures of DSCG and amlexanox

The important role of Hsp90 in intracellular signal-transduction processes, together with the specific inhibition of Hsp90 by low-molecular-mass compounds, make it a potent new drug target. We have focused our attention on discovering novel agents that are capable of inhibiting Hsp90 function. The availability of antagonists which interact solely with Hsp90 should permit understanding of the physiological role of this protein family in cellular responses. There are many reports on drug evaluation or clinical effects of anti-allergic drugs, but none has shown the precise molecular targets of the drugs. In the present study, we have used two structurally distinct anti-allergic drugs, DSCG (disodium cromoglycate) and amlexanox, as ligands for affinity chromatography and examined direct binding of these drugs to Hsp90. Both drugs selectively bound to Hsp90, and their binding sites were identified. Furthermore, DSCG and amlexanox suppressed the *in vitro* chaperone activities of Hsp90 on the thermal inactivation and aggregation of CS (citrate synthase), and the refolding of denaturated rhodanese. This paper describes in detail the binding of DSCG and amlexanox to Hsp90, and the effect of the drugs on the chaperone activity of Hsp90.

MATERIALS AND METHODS

Materials

DSCG {5,5′-[(2-hydroxy-1,3-propanediyl)bis(oxy)]bis (4-oxo-4H-1-benzopyran-2-carboxylic acid) disodium salt}, novobiocin and geldanamycin were purchased from Sigma. Amlexanox {2-amino-7-(1-methylethyl)-5-oxo-5H-[1]benzopyrano(2,3-b) pyridine-3-carboxylic acid} was from Takeda Pharmaceutical Co. Chemical structures of the two anti-allergic drugs are shown (Figure 1). EAH-Sepharose 4B, epoxy-activated Sepharose 6B and glutathione–Sepharose were purchased from Amersham. Mitochondrial CS was purchased from Roche Diagnostics. Rhodanese was purchased from Sigma.

Drug-affinity chromatography and identification of the anti-allergic drug-binding proteins

The coupling of DSCG with EAH-Sepharose 4B resin was carried out as described previously [27]. Briefly, the drug (150 mg) dissolved in 1 ml of *N*,*N*′-dimethylformamide was added to the EAH-Sepharose (7 ml; 5 g of wet mass), and *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodi-imide hydrochloride (EDC; 0.5 g) suspended in 10 ml of *N*,*N*′-dimethylformamide was added to the mixture. After adjusting the pH to 5.0, the mixture was incubated with gentle shaking for 48 h at 25 °C. The resin was washed with 50 % *N*,*N*′-dimethylformamide and then incubated with 0.2 M sodium acetate (8 ml) and acetic anhydride (4 ml) for 30 min at 0 °C, and, after addition of acetic anhydride (4 ml), was incubated further for 30 min at 25 °C. Amlexanox (150 mg) was coupled with epoxy-activated Sepharose 6B (5 g of wet mass), according to the manufacturer's instructions.

Bovine brain (5 g) was homogenized with 5 volumes of 20 mM Tris/HCl, pH 7.5, and centrifuged at 15 000 ***g*** for 20 min at 4 °C. The extract obtained from high-speed centrifugation was used as bovine brain extract. The extract was applied to the column (1.5 cm × 5 cm), equilibrated in the same buffer as that used for homogenization, and washed with 20 volumes of the same buffer. After washing the column, binding proteins were eluted with 5 mM ATP or 5 mM DSCG in 20 mM Tris/HCl, pH 7.5, followed by 1 M NaCl-containing buffer. The eluted proteins were analysed by SDS/PAGE and stained with Coomassie Brilliant Blue. The resultant anti-allergic drug-binding proteins were subjected to SDS/PAGE and transferred to PVDF membranes. After staining with Ponceau S, the protein bands were cut out and digested with lysyl-endopeptidase. Following overnight incubation at 37 °C, the proteolytic fragments were separated by HPLC (model LC10A; Shimadzu) with a C18 reversed-phase column (TSK gel ODS-80X; Tosoh) with a linear gradient of 0–80 % (v/v) acetonitrile in the presence of 0.1 % (v/v) trifluoroacetic acid at flow rate of 1 ml/min. The amino acid sequence of each proteolytic fragment was determined with an automated protein sequencer (model 476A; Applied Biosystems).

Isolation and identification of anti-allergic drug-binding fragments derived from Hsp90

Bovine brain Hsp90 was purified as described previously [28]. Purified Hsp90 (5 mg/ml) was digested mildly by TPCK (tosylphenylalanylchloromethane)-treated trypsin (0.5 μg/ml) for 30 min at 37 °C. The digests were applied to a DSCG– or an amlexanox–Sepharose column, eluted first with 20 mM Tris/HCl, pH 7.5, containing 5 mM ATP or DSCG, and then with buffer containing 1 M NaCl. Each eluate was electrophoresed on SDS/PAGE and blotted on to PVDF membranes. The amino acid sequence of the 35 kDa fragment was determined as described above. Eluates were also analysed by reversed-phase HPLC chromatography, and the amino acid sequence of the purified peptide was determined as described above.

Construction and purification of Hsp90 mutants

N- and C-terminally truncated mutants N90 and C90 (residues 1–236 and 526–732 respectively) were prepared by PCR using, as template, human Hsp90α cDNA (EMBL accession number X15183; kindly provided by Dr Kazunari Yokoyama, Department of Biological Systems, Gene Engineering Division RIKEN, Tsukuba Institute BioResource Center, Tsukuba, Japan). The oligonucleotides employed as PCR primers featured a *Bam*HI restriction enzyme site 5′ to the ATG start codon (sense primer), and different stop codons at an adjacent *Sma*I restriction enzyme site at the 3′ end of the cDNA (antisense primers). The PCR products were subcloned into pT7Blue T-vector (Novagen) and sequenced using an Applied Biosystems 377 DNA Sequencer. *Bam*HI/*Sma*I fragments were inserted into the corresponding sites of pGEX4T-2 (Amersham). Mutant ΔEEVD protein [where 'EEVD' is the C-terminal extension (Glu-Glu-Val-Asp) of Hsp90] was prepared by PCR with pGEX4T-2-C90 as the template, using

the specific primers 5′-GGATCCGCAGCAAAGAAACACCTG-GAGATA-3′ and 5′-CCCGGGTCACATGAGTGATGTCGTCAT-C-3′. Construction of mutant ΔEEVD was performed as described above. Mutants AAA (Glu^{651} and Asp^{653} changed to alanine), A-GAAA (Glu^{720}, Asp^{722}, Asp^{723} and Asp^{724} changed to alanine) and AAA-and-AGAAA (combination of AAA and AGAAA) were produced using the GeneEditor *in vitro* Site-Directed Mutagenesis System (Promega). Using pGEX4T-2-C90 as a template, the following mutagenic primers were employed: AAA, 5′-CAA-AAGGCAGCGGCTGCTAAGAACGACAAG-3′; AGAAA, 5′-CACCCCTTGCAGGAGCTGCCGCCACATCAC-3′; AAA-and-AGAAA, 5′-CAAAAGGCAGCGGCTGCTAAGAACGACAAG-3′ and 5′-CACCCCTTGCAGGAGCTGCCGCCACATCAC-3′. Mutant cDNAs were sequenced using an Applied Biosystems 377 DNA Sequencer. The expression vector was introduced into *Escherichia coli* BL21(DE3) cells. Bacteria were grown at 37 °C in LB (Luria–Bertani) medium supplemented with ampicillin (100 mg/l). The proteins were induced by adding 1 mM isopropyl β-D-thiogalactoside when the attenuance at 600 nm (D_{600}) $\approx$ 1. Cells were collected by centrifugation 5 h after induction, and the proteins were extracted. GST (glutathione S-transferase) fusion proteins were purified using glutathione–Sepharose chromatography, according to the manufacturer's instructions. In some cases, GST was removed from fusion proteins by thrombin digestion. Incubations at 37 °C for 3 h were performed with 50 units of thrombin/mg of protein in 20 mM Tris/HCl, pH 8.0. GST was removed by adsorption on to glutathione–Sepharose. As judged from SDS/PAGE, this method yielded the GST–N90 and GST–C90 fusion proteins, and the other mutants, with greater-than-95 % purity.

Cloning of CyP40 and Hop and recombinant protein production

Bovine CyP40 (GenBank® accession number BAA03159) and rat Hop (GenBank® accession number CAA75351) cDNAs were prepared by PCR with the bovine-brain cDNA library and rat-liver cDNA library (Clontech) as a template, and the following specific primers: for CyP40, 5′-GAATCCATGTCGCATCCA-TCCCCCCAGGC-3′ and 5′-CCCGGGTCAGGCAAACATTTT-TGCATAAGC-3′; and for Hop, 5′-GAATCCATGGAGCAGG-TGAATGAGCTAAAG-3′ and 3′-CTCGAGTCACCGAATTGC-GATGAGACCCAC-5′. The PCR products were subcloned into pT7blue T-vector (Novagen) and sequenced using an Applied Biosystems 377 DNA Sequencer. The *Bam*HI/*Sma*I fragment of CyP40 and the *Bam*HI/*Xho*I fragment of Hop were inserted into the corresponding sites of pGEX4T-2 (Amersham). Recombinant proteins were expressed and purified as described above.

Solid-phase anti-allergic drug-binding assay

Total protein (25 μg per assay) was incubated with DSCG– or amlexanox–Sepharose (50 μl) in 0.1 M NaCl and 20 mM Tris/HCl buffer, pH 7.5 (the binding buffer), with or without the prior addition of ATP, DSCG, amlexanox (suspended in 2 % polyvinylpyrrolidone), novobiocin or geldanamycin for 2 h at 4 °C with gentle rotation. The beads were then washed with the ice-cold binding buffer. Bound proteins were eluted by boiling in SDS sample buffer and then analysed by SDS/PAGE.

Protein binding studies

In direct binding studies between Hsp90 and recombinant GST–CyP40, GST–CyP40 pre-absorbed on glutathione–Sepharose was rotated for 3 h at 4 °C with 25 μg of the purified Hsp90 equilibrated in the same binding buffer (100 μl), as described above. After pelleting by microcentrifugation, the gels were subjected to replicate washes (4 × 1 ml), boiled in SDS sample buffer and examined for protein retention by SDS/PAGE. Binding studies of GST–C90 with CyP40 or Hop were also performed as described above.

In vitro chaperone assays

The effect of Hsp90 and anti-allergic drugs on the thermal aggregation of mitochondrial CS at 43 °C was monitored as described previously [29]. To monitor thermal aggregation, the concentration of CS used was 0.075 μM in 40 mM Hepes buffer, pH 7.4, in the presence or absence of Hsp90 (0.15 μM) and in the presence of Hsp90 and each of the two anti-allergic drugs, DSCG and amlexanox, at a concentration of 1.5 μM. Light scattering of CS was monitored over 90 min at an optical wavelength of 500 nm in an Amersham Ultrospec 3000 UV-Vis spectrophotometer equipped with a temperature control unit using semi-micro-cuvettes (1 ml) with a path-length of 10 mm. The ability of GST–N90 and GST–C90 proteins to inhibit rhodanese aggregation was assayed essentially as described previously [18]. GST–N90 and GST–C90 proteins were equilibrated for 5 min at 25 °C in 95 μl of 50 mM Hepes and 100 mM NaCl, pH 7.0. After adding 5 μl of 37.5 μM rhodanese in 6 M guanidinium chloride and 50 mM Hepes, pH 7.0, the rate of rhodanese aggregation was determined by measuring the increase in absorbance at 400 nm. Aggregation was essentially completed after 15 min.

Thermal inactivation of CS

CS (0.075 μM) was incubated in 40 mM Hepes, pH 7.5, at 43 °C in the presence of Hsp90 (0.3 μM) or BSA (2 mg/ml). During the incubation, aliquots were withdrawn at intervals of 2–10 min, and the enzyme activity was determined as described previously [29].

RESULTS

Identification of DSCG- and amlexanox-binding proteins from bovine brain

We have utilized two immobilized anti-allergic drugs to develop an affinity chromatography technique for analysing Hsp90. Extracts of bovine brain were applied to the DSCG- and amlexanox-coupled affinity columns. Proteins were eluted with 5 mM ATP or 5 mM DSCG, followed by 1 M NaCl-containing buffer. Because of its insolubility, elution with amlexanox was not examined. As shown in Figure 2, the proteins from the DSCG and amlexanox columns were pooled into four major groups of approx. size 90, 32, 17 and 10 kDa. It is noteworthy that the SDS/PAGE patterns of the proteins isolated by the two columns are essentially the same. Although DSCG and amlexanox each have a distinctive molecular structure (Figure 1), they have similar efficacy in terms of the inhibition of IgE-mediated degranulation of mast cells and basophils. Thus it can be presumed that these two anti-allergic drugs may interact with their target proteins and affect degranulation pharmacologically in a similar manner. To determine the partial amino acid sequence of each protein, lysyl-endopeptidase was used for digestion of the PVDF-blotted proteins, and then digested peptides were separated by reversed-phase HPLC followed by amino acid sequencing. A computer homology search of the NBRF (National Biomedical Research Foundation)

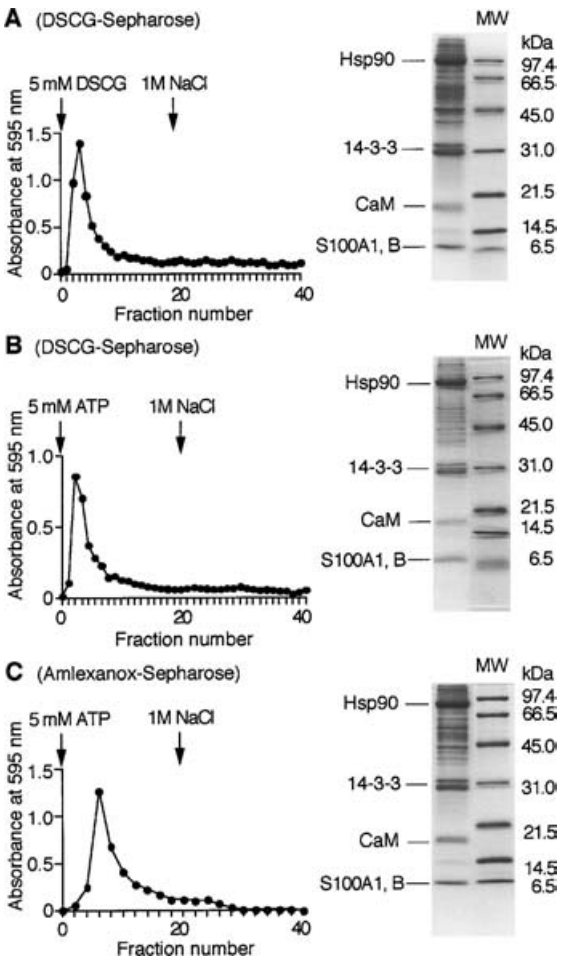


Figure 2 Identification of anti-allergic drug-binding proteins from bovine-brain extract

(**A**) Elution profile (left panel) and SDS/PAGE (right panel) of the proteins obtained on DSCG–Sepharose affinity chromatography. The elution was performed as described in the Materials and methods section. The SDS/polyacrylamide gel shows 5 mM DSCG eluate, as indicated in the elution profile. (**B**) Elution profile (left panel) and SDS/PAGE (right panel) of DSCG–Sepharose affinity chromatography. SDS/PAGE shows 5 mM ATP eluate, as indicated in the elution profile. (**C**) Elution profile (left panel) and SDS/PAGE (right panel) of amlexanox–Sepharose chromatography. SDS/PAGE shows 5 mM ATP eluate, as indicated in the elution profile.

protein sequencing data and SwissProt database revealed the identities of the 90-, 32-, 17- and 10-kDa-group proteins to be Hsp90, 14-3-3 protein, calmodulin (CaM) and a mixture of S100A1 and S100B respectively (results not shown).

Identification of the binding site of Hsp90 to DSCG and amlexanox

Many questions regarding the DSCG- and amlexanox-binding sites of Hsp90 remain unanswered. To investigate the anti-allergic drug-binding domains, we analysed the binding of the trypsin digests of Hsp90 to the drug-coupled Sepharose columns. Hsp90 was digested mildly with trypsin, and the digests were applied to the DSCG- and amlexanox-affinity columns. After washing, bound peptides were eluted with 5 mM ATP, followed by 1 M NaCl. The eluted peptides were separated on a reversed-phase column. Figure 3(A) shows peptide maps of the eluted peptides obtained from the DSCG and amlexanox columns. The elution profiles of DSCG- and amlexanox-bound peptides were quite similar. Amino acid sequence analyses were performed on the two major peptides from each column. Peptides 1 and 2 from each column (as labelled in Figure 3A) contained the same respective sequences, both of which were in the environment of residue 621, relative to the C-terminal end of Hsp90α (Figure 3C). Since large proteolytic fragments are not retained on the C18 reversed-phase column, the ATP eluates from the affinity column were subjected to PVDF blotting, followed by protein sequencing analysis. The ATP eluates of digested Hsp90 showed three major bands of approx. 35 kDa (Figure 3B). These SDS/PAGE patterns clearly indicated that the peptides isolated from the DSCG and amlexanox columns were essentially identical, and that the sequences were within the N-terminal end of Hsp90α (Figure 3C). These results identify the short C-terminal sequences (621–632 and 691–732) and the 35 kDa N-terminal fragment of Hsp90 as the specific binding sites for DSCG and amlexanox.

Analytical binding assays of purified Hsp90 and truncated mutants of Hsp90 (N90 and C90) to DSCG– and amlexanox–Sepharose

To exclude the possibility that the interactions between the two drugs and Hsp90 are indirect or non-specific, we performed an *in vitro* analytical binding assay using native Hsp90 and the truncated mutants of Hsp90, N90 (1–236) and C90 (629–732). Both native Hsp90 and its mutants strongly bound to DSCG– and amlexanox–Sepharose in the absence of ATP, and were eluted by SDS sample buffer (Figure 4A). Binding of Hsp90 and its mutants to DSCG–Sepharose was only partially competed by ATP. In contrast, binding of native Hsp90, N90 and C90 to amlexanox–Sepharose was more effectively competed by an excess of soluble ATP (5 mM). Thus these anti-allergic drugs directly and specifically targeted native Hsp90, and its two distinct domains (i.e. N90 and C90) were able to bind independently to the DSCG- and amlexanox-affinity matrices.

To characterize further the binding of Hsp90 to the drugs, we examined the ability of soluble DSCG, amlexanox (suspended in 2% polyvinylpyrrolidone), novobiocin and geldanamycin to block binding to solid-phase DSCG or amlexanox. The data in Figure 4(B) demonstrate that soluble DSCG is able to block the binding of Hsp90 to DSCG-coupled beads with an IC_{50} value of between 0.2 and 0.3 mM, whereas soluble amlexanox (10 mM) and novobiocin (10 mM) are able to only slightly compete with DSCG-coupled beads for the binding of Hsp90. In the presence of ATP (10 mM) or geldanamycin (100 μM), the binding of the protein was inhibited approx. 40% by the drugs. When the amlexanox-coupled beads were tested in a similar fashion, soluble amlexanox inhibited binding with an IC_{50} of between 0.2 and 0.3 mM, whereas the IC_{50} values for novobiocin and ATP were approx. 3 mM and 7 mM respectively (Figure 4C). DSCG at a concentration of 10 mM caused approx. 40% inhibition, and geldanamycin at 100 μM had no inhibitory effect (Figure 4C). Since the amount of DSCG or amlexanox bound to the resin is not known and may vary from batch to batch, these values only reflect relative IC_{50} values.

Effect of DSCG and amlexanox on the interaction of Hsp90 with CyP40 and Hop

Structurally related TPR-containing cohort proteins, such as CyP40, FKBP52 and Hop, mediate interactions with a common cellular target, Hsp90 [7–9]. Furthermore, the Hsp90 C-terminal extension (EEVD) has a major role in the interaction between Hsp90 and TPR-containing cohort proteins [9]. In order to determine the effect of DSCG and amlexanox on the interaction between the EEVD extension at the C-terminus of Hsp90 and its

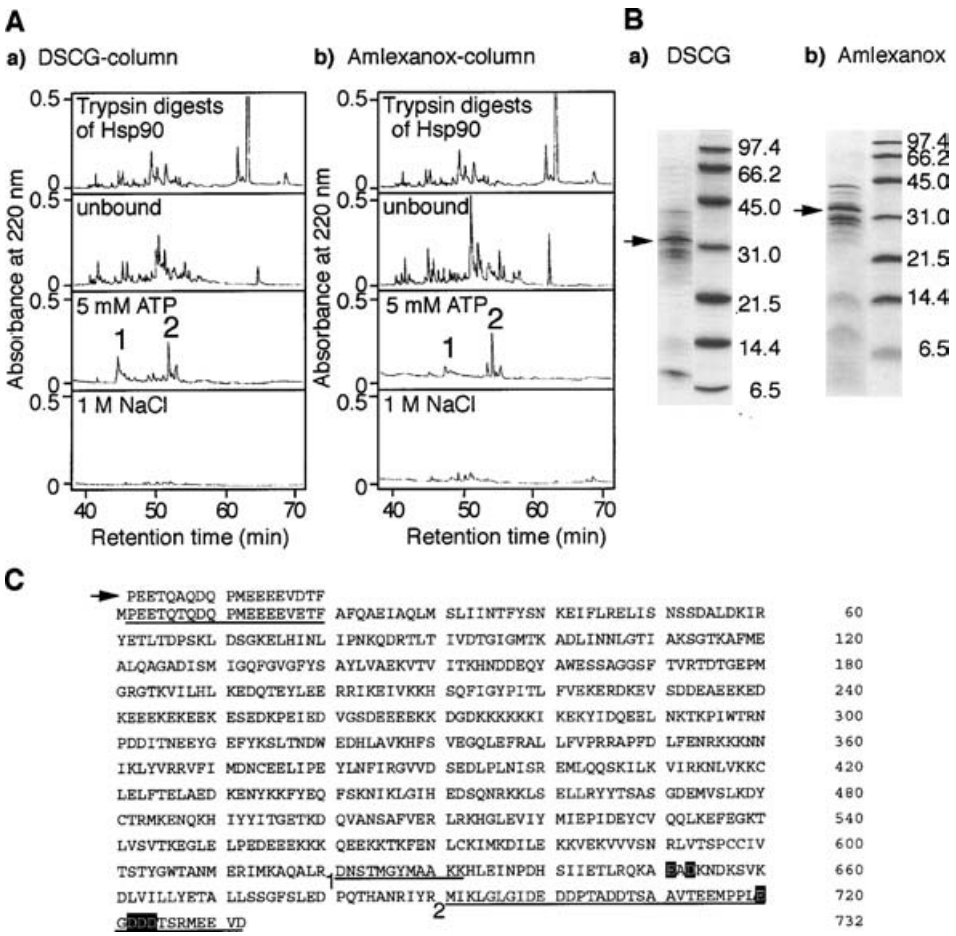


Figure 3 Determination of putative DSCG- and amlexanox-binding sites of Hsp90

Hsp90 digested with TPCK-treated trypsin was applied to a DSCG (**A**, **a**)- or an amlexanox (**A**, **b**)-derivatized Sepharose column. After washing, the peptides were eluted with 5 mM ATP, followed by 1 M NaCl. Input tryptic peptides, unbound peptides, ATP-eluted peptides and NaCl-eluted peptides were individually separated on a C18 reversed-phase column with HPLC. The sequences of ATP-eluted peptides, indicated by peaks 1 and 2 in (**A**, **a** and **b**), are underlined (1 and 2) in the amino acid sequence of human Hsp90α (**C**). ATP-eluted peptide mixtures from the DSCG (**B**, **a**) or amlexanox (**B**, **b**) column were then subjected to SDS/PAGE, followed by PVDF blotting. The 35 kDa fragments from both columns (indicated by arrows) were sequenced. The N-terminal amino acid sequence of the 35 kDa peptide is also shown in (**C**) (indicated by the arrow). The corresponding sequence is underlined. Acidic residues, shown in white lettering on a reversed-out background, were mutated to alanine, as described in the Materials and methods section.

cohort proteins, we analysed the binding of GST–Cyp40 to Hsp90 in the presence of these drugs (Figure 5A, part a). Hsp90 was incubated with the GST–CyP40-coupled glutathione–Sepharose beads with or without each anti-allergic drug. Hsp90 showed binding to GST–CyP40-coupled Sepharose in the absence of both drugs (Figure 5A, part a). In contrast, in the presence of graded dose of amlexanox (0.1–1 mM), the binding of the protein was inhibited by the drug (Figure 5A, part a). The binding of CyP40 to GST–C90-coupled Sepharose was also inhibited by amlexanox (Figure 5A, part b). The results of the binding experiments using Hop to GST-C90 (Figure 5A, part c) were similar to those for Hsp90 and GST–CyP40 (Figure 5A, part a) and CyP40 and GST–C90 (Figure 5A, part b). DSCG (1 mM), on the other hand, had no effect either on Hsp90 binding to the GST–CyP40-coupled Sepharose beads (Figure 5A, part a) or on CyP40 or Hop binding to GST–C90 (Figure 5A, parts b and c). These results suggest that amlexanox inhibits the binding of CyP40 and other cohort proteins to Hsp90 via an interaction with C-terminal EEVD residues of Hsp90.

A report by Ramsey et al. [30] indicates that a cluster of six acidic amino acids adjacent to the EEVD sequence, namely Glu^{651}, Asp^{653}, Glu^{720}, Asp^{722}, Asp^{723} and Asp^{724}, is involved in binding to FKBP52 or Hop. To analyse the anti-allergic drug-binding sites in detail, the interaction of the C-terminal point mutants with DSCG– and amlexanox–Sepharose was analysed. The amino acid sequences of each Hsp90 mutant are shown in Figure 3(C). Recombinant GST–C90, point mutants or GST alone (25 μg of each) were mixed with the drug-coupled Sepharose beads. After washing, bound proteins were eluted with SDS sample buffer. GST–C90 bound to both DSCG– and amlexanox–Sepharose beads (Figure 5B). In this comparative binding experiment, all the mutants as well as GST–C90 bound quantitatively to DSCG–Sepharose beads, and were eluted with SDS sample buffer (Figure 5B, upper panels). In contrast, ΔEEVD, AAA (Glu^{651} and Asp^{653} changed to alanine), AGAAA (Glu^{720}, Asp^{722}, Asp^{723} and Asp^{724} changed to alanine) and AAA-and-AGAAA (combination of AAA and AGAAA) mutants interacted weakly with amlexanox–Sepharose beads, and proteins detected in the unbound fractions were increased (Figure 5B, lower panels). These results show that acidic amino acid residues in the C-terminal extension of Hsp90 are necessary for the binding of this protein to amlexanox.

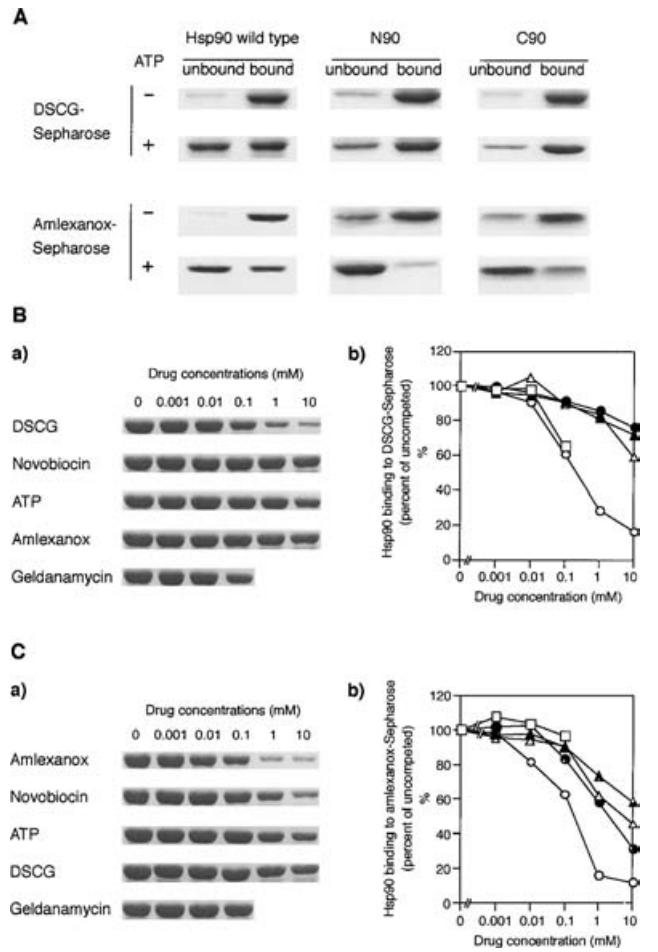


Figure 4 The specificity of Hsp90 binding to DSCG and amlexanox

(**A**) The binding of purified Hsp90α, recombinant N90 (residues 1–236) and C90 (residues 629–732) mutants to DSCG– and amlexanox–Sepharoses. Purified Hsp90 (25 μg), N90 (25 μg) or C90 (25 μg) was each incubated with DSCG–Sepharose (50 μl) or amlexanox–Sepharose (50 μl) in a total volume of 150 μl in the presence (+) or absence (–) of 5 mM ATP for 1 h at 4 °C. Bound and unbound proteins were subjected to SDS/PAGE. (**B** and **C**) The specificity of Hsp90 binding to DSCG–Sepharose (**B**) and amlexanox–Sepharose (**C**). (**B**, **a**) and (**B**, **b**) show the competitive effect of increasing concentrations of soluble DSCG (○), novobiocin (●), ATP (△), amlexanox (▲) or geldanamycin (□) on the binding of purified Hsp90α to DSCG beads. (**C**, **a**) and (**C**, **b**) show the competitive effect of increasing concentrations of soluble amlexanox (○), novobiocin (●), ATP (△), DSCG (▲) or geldanamycin (□) on the binding of purified Hsp90α. Variable amounts of soluble drugs were added to the Hsp90 solution before the addition of the drug-coupled beads. Material remaining bound to the beads after four washes in the washing buffer was analysed by SDS/PAGE (**B**, **a** and **C**, **a**). The amount of bound Hsp90 was quantified by using an image analyser (LAS-1000 plus; Fuji Film). Hsp90 binding to the drug-coupled beads was expressed as the percentage of that uncompeted (**B**, **b** and **C**, **b**).

Effect of DSCG and amlexanox on the *in vitro* chaperone activity of Hsp90 and its truncation mutants

One of the characteristic features of molecular chaperones is their ability to suppress the aggregation of proteins under stressed conditions. To analyse the functional properties of the two anti-allergic drugs, we studied their action in the protein folding reactions of Hsp90 *in vitro*. The thermal unfolding and aggregation of mitochondrial CS was used as a typical *in vitro* assay system. In a CS aggregation assay, Hsp90 apparently interacts transiently with early unfolded intermediates and, as a consequence, CS is effectively stabilized in the presence of Hsp90 [29]. In the present study, a 2-fold molar excess of Hsp90 suppressed the heat-induced (43 °C) aggregation of CS almost completely (Figures 6A and 6B). DSCG greatly (≈70%) suppressed the chaperone activity at a molar ratio of 1:10 of Hsp90:DSCG (Figure 6A). A 10-fold molar excess of amlexanox over Hsp90 also inhibited the chaperone activity almost completely (Figure 6B). The spontaneous aggregation of CS was not affected in the presence of DSCG or amlexanox (Figures 6A and 6B) and neither did the drugs cause any optical changes in Hsp90 in the light-scattering analysis (results not shown).

Analyses of the heat-induced loss of CS activity provide additional information. As shown in Figure 6(C), CS rapidly lost its activity on incubation in the absence of Hsp90 at 43 °C [29]. When CS and Hsp90 at a molar ratio of 1:4 were co-incubated for 30 min, the enzyme retained nearly 30% activity (Figure 6C). In control experiments at 37 °C, co-incubation with Hsp90 did not influence the specific activity of the enzyme (results not shown). These results suggest that Hsp90 recognizes and binds CS molecules that unfold during thermal stress, and protects the enzyme from loss of activity [29]. Both DSCG and amlexanox completely suppressed the stabilizing effect of Hsp90 at a molar ratio of 1:10 (Figure 6C).

It has been shown that Hsp90 contains two independent chaperone sites: one located at the N-terminus of the molecule, and the other at the C-terminus. The two chaperone sites differ in substrate specificity and ATP-dependence in the full-length protein, and appear to contribute independently to the chaperone activity [18,19]. Since both the N-terminal and C-terminal fragments of Hsp90 suppressed aggregation during refolding from chemically denatured substrate proteins, in the present study the chaperone activity of GST–N90 and GST–C90 was determined by measuring aggregation in an assay with rhodanese (denatured with 6 M guanidinium chloride), as described in the Materials and methods section. The aggregation was found to be suppressed significantly when diluting buffer contained increasing concentrations of GST–N90 (Figure 7A). Although the chaperone activity of GST–N90 was found to be less potent than that of GST–C90, more than 80% of the aggregation was suppressed by the protein (Figures 7A and 7B). DSCG inhibited the chaperone activity of GST–N90 (Figure 7C), but had little or no effect on the activity of GST–C90 (Figure 7D). In contrast, amlexanox had no effect on the chaperone activity of GST–N90 (Figure 7E), but significantly interfered with the ability of GST–C90 to inhibit rhodanese aggregation (Figure 7F).

DISCUSSION

Previous studies have made clear that Hsp90 interacts with a variety of proteins involved in fundamental cellular processes, such as hormone signalling, intracellular signal transduction and cell cycle control [2]. In this context, Hsp90 mediates the function of many key regulatory proteins, such as steroid receptors, mutated p53 and a number of tyrosine and serine/threonine protein kinases [15–17]. Many cohort proteins that play an important role in the folding of different subsets of proteins via the Hsp90 pathway have also been identified [6]. Hsp90 operates as part of a multichaperone complex which includes Hsp70, peptidyl–prolyl isomerases and other cohort proteins [2,13,31]. As reviewed by Young et al. [5], these multiple interactions involving Hsp70 and other molecular chaperones have made it more difficult to investigate Hsp90. Furthermore, geldanamycin has been very useful to examine the cellular function of Hsp90 and to identify previously unidentified substrate proteins that depend on the N-terminal chaperone activity of Hsp90 [30]. On the basis of earlier progress in biochemical, structural and pharmacological studies, Scheufler et al. [32] and Scheibel and Buchner [33] proposed an

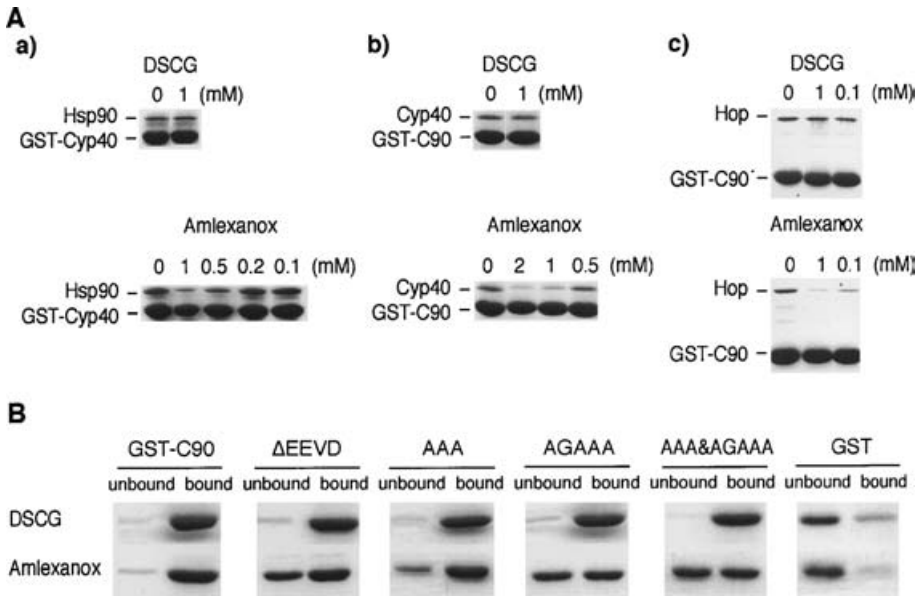


Figure 5 Effect of DSCG and amlexanox on the binding of Hsp90 and its C-terminal mutant to cohort proteins and binding to GST–C90 mutants

The experiments were performed as described in the Materials and methods section. (**A**) SDS/PAGE showing binding of cohort proteins to Hsp90 and its C-terminal mutant (GST–C90). (**A**, **a**) Binding of GST-CyP40 to Hsp90. (**A**, **b**) Binding of CyP40 to GST-C90. (**A**, **c**) Binding of Hop to GST-C90. (**B**) SDS/PAGE showing binding of GST-C90 mutants to the anti-allergic drug-coupled beads.

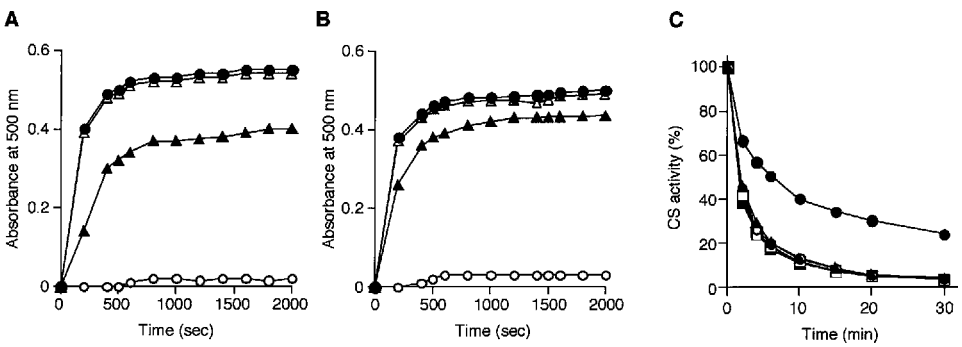


Figure 6 Effect of DSCG and amlexanox on the inhibition of thermal aggregation of CS by Hsp90

Concentrations of Hsp90 and drugs were 0.15 μM and 1.5 μM respectively. (**A**) Thermal aggregation of CS in the presence of Hsp90 (○), DSCG (△; obscured by other data in the Figure), Hsp90 and DSCG (▲) and in the absence of Hsp90 (●). (**B**) Thermal aggregation of CS in the presence of Hsp90 (○), amlexanox (△), Hsp90 and amlexanox (▲) and in the absence of Hsp90 (●). (**C**) The effect of DSCG and amlexanox on the protection of thermal inactivation of CS by Hsp90. Concentrations of Hsp90, BSA, DSCG and amlexanox were 0.3 μM, 2 mg/ml, 3 μM and 3 μM respectively. CS (0.075 μM) was incubated in 40 mM Hepes, pH 7.5, at 43 °C in the presence of BSA (△; obscured by other data in the Figure), Hsp90 (●), DSCG and BSA (□), amlexanox and BSA (■), Hsp90 and DSCG (▲) and Hsp90 and amlexanox (○). At the times indicated, aliquots were withdrawn, and the enzyme activity was determined as described in [29].

Hsp90–multichaperone complex as a potential drug target. Since the physiological function of Hsp90 is not yet fully understood, the development of the specific antagonists might open new lines of investigation on the role of Hsp90 *in vivo*.

As one approach to screen Hsp90 antagonists, in the present study we have attempted to use two structurally distinct anti-allergic drugs, DSCG and amlexanox, as molecular probes for the affinity chromatography of Hsp90. The results indicate that both drugs directly bind to Hsp90, and the protein is eluted by free DSCG or ATP (Figure 2). These data demonstrate the feasibility of using immobilized anti-allergic drugs for rapid purification of Hsp90.

In the present study, we have used a rapid affinity chromatography-based method to identify anti-allergic drug-binding regions within Hsp90. Using a trypsin digest of Hsp90, the N-terminal 35 kDa domain and short fragments (621–632 and 691–732) in the C-terminal domain were able to bind to the DSCG and amlexanox matrices, and were eluted with ATP (Figure 3). In addition, both recombinant N-terminal (1–236) and C-terminal (629–732) fragments, as well as native Hsp90, bound to the anti-allergic-drug matrices. ATP efficiently competed with the fragments for binding to immobilized amlexanox (Figure 4). Recently, Marcu et al. [25] identified the second ATP/novobiocin-binding site in the C-terminal domain, which was different from the well-established N-terminal one. The peptide 657–677 seems to be critical for ATP and novobiocin binding, because the corresponding deletion mutant was not retained by ATP–Sepharose and excess 657–677 peptide inhibited the binding of Hsp90 mutant to ATP–Sepharose and novobiocin–Sepharose [25]. More recently, Garnier et al. [34] proposed that the second C-terminal ATP-binding site is localized between amino acids 538–630, compatible with a Rossmann-fold motif known to bind ATP. Binding of purified Hsp90 to amlexanox–Sepharose was competed not only by excess ATP (IC_{50} 3 mM), but also

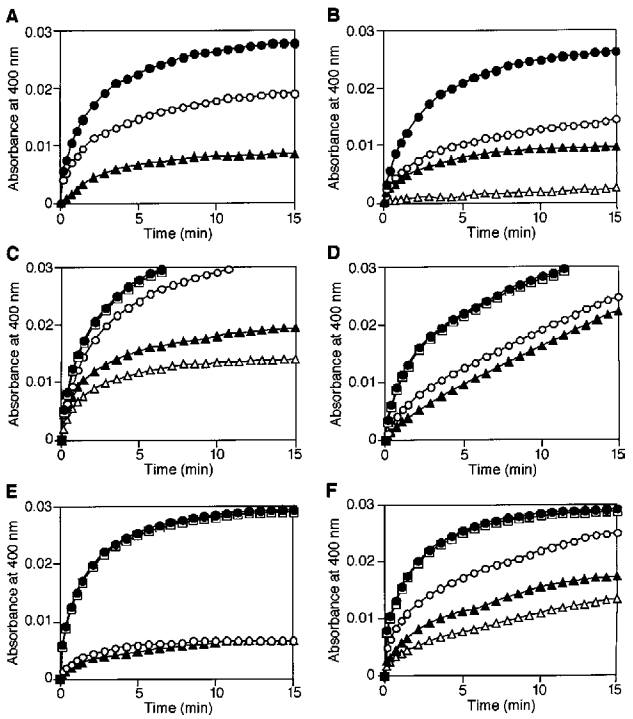


Figure 7 Effect of DSCG and amlexanox on the chaperone activity of N- and C-terminal mutants of Hsp90 during the time course of rhodanese refolding

The experiments were performed as described in the Materials and methods section. (**A**) Aggregation of rhodanese in the absence of GST–N90 (●) and the presence of GST–N90 at 3.5 μM (○) or 7.0 μM (▲). (**B**) Aggregation of rhodanese in the absence of GST–C90 (●) and the presence of GST-C90 at 0.7 μM (○), 1.7 μM (▲) or 3.5 μM (△). (**C**) Aggregation of rhodanese in the absence of GST–N90 (●), in the absence of GST–N90, but in the presence of 1 mM DSCG (□), in the presence of 7.0 μM GST–N90 (△), 7.0 μM GST–N90 + 0.4 mM DSCG (▲) and 7.0 μM GST–N90 + 1 mM DSCG (○). (**D**) Aggregation of rhodanese in the absence of GST–C90 (●), in the absence of GST–C90 but in the presence of 1 mM DSCG (□), in the presence of 1.7 μM GST–C90 (▲) and 1.7 μM GST–C90 + 1 mM DSCG (○). (**E**) Aggregation of rhodanese in the absence of GST–N90 (●), in the absence of GST–N90 but in the presence of 0.2 mM amlexanox (□), in the presence of 7.0 μM GST–N90 (○) and 7.0 μM GST–N90 + 0.2 mM amlexanox (▲). (**F**) Aggregation of rhodanese in the absence of GST–C90 (●), in the absence of GST–C90 but in the presence of 0.2 mM amlexanox (□), in the presence of 3.5 μM GST–C90 (△), 3.5 μM GST–C90 + 0.1 mM amlexanox (▲) and 3.5 μM GST–C90 + 0.2 mM amlexanox (○).

novobiocin (IC_{50} 7 mM) (Figure 3C). Our data also provide further support for the second ATP-binding site in the C-terminal domain. A high concentration (10 mM) of ATP, amlexanox and novobiocin only slightly competed for binding to immobilized DSCG. Thus these data indicate that amlexanox and DSCG have similar binding sites on Hsp90 (i.e. N90 and C90), but the ATP-sensitivity of the binding is different. The discrepancy between the ATP-sensitive elution from the DSCG–Sepharose using the trypsin digests of Hsp90 and the relative insensitivity using recombinant fragments (N90 and C90) remains to be explained.

In contrast with the N-terminus, the three-dimensional structure of the C-terminal domain of Hsp90 is still unknown. The C-terminal domain contains a second source of molecular-chaperone activity, which has different substrate specificity and ATP-dependency from the N-terminal chaperone activity [18]. In the present study, the thermal inactivation and aggregation of mitochondrial CS and the aggregation of chemically denatured rhodanese during refolding processes were chosen as typical *in vitro* chaperone assays. Hsp90 binds transiently to unfolding intermediates of the thermally unfolding CS. Upon release from Hsp90, the intermediates are able to refold rapidly to the native state [29]. Previous data have shown that the chaperone function of wild-type Hsp90 and its N-terminal domain is only partially inhibited by the high concentration (100 μM) of geldanamycin [19]. The effect of another Hsp90 antagonist, novobiocin, on the *in vitro* chaperone activity of Hsp90 remains to be investigated [25]. Results of the present study have shown that DSCG and amlexanox strongly suppress the chaperone activity of Hsp90 towards CS at 1.5–3.0 μM.

Although geldanamycin inhibits the N-terminal chaperone activity *in vitro*, it has no effect on the function of the C-terminus [19]. Therefore we examined the specificity of the drugs for individual chaperone sites in both the mutants as well as wild-type Hsp90. The present findings indicate that DSCG inhibits the N-terminal chaperone activity but not the C-terminal activity (Figures 7C and 7D). In contrast, amlexanox inhibits C-terminal chaperone activity (Figure 7F). These data indicate that DSCG disrupts the chaperone activity in a manner similar to that of geldanamycin, and that amlexanox belongs to a distinct class of the Hsp90 antagonists which suppress C-terminal function of Hsp90. The C-terminal domain contains the binding site for co-chaperone proteins [29,35]. The EEVD sequence at the extreme end of the C-terminus of Hsp90 is important for cohort proteins, such as PP5, FKBP52 and CyP40, and different cohort proteins compete for binding to the same site [9,36], whereas the p23 binding site is located in the N-terminal domain [31]. Characterization of the point mutations of the acidic residues in the C-terminal region of Hsp90 demonstrates that there appear to be different binding determinants for different cohort proteins: mutations at Glu^{729}, Glu^{730} and Asp^{732} at the C-terminus of Hsp90 interfere with binding of PP5, FKBP52 and Hop; mutations at Glu^{720}, Asp^{722}, Asp^{723} and Asp^{724} inhibit binding of FKBP52 and PP5, but not of Hop; mutations at Glu^{651} and Asp^{653} do not affect binding of FKBP52 or PP5, but inhibit Hop binding [30]. These observations strongly suggest the important role of the C-terminal extension of Hsp90 for its recognition of the cohort proteins. Results of the present study show that the EEVD sequence and a cluster of six amino acids in the C-terminal region of Hsp90 are essential for the binding of amlexanox to Hsp90 (Figure 5B). Further evidence of the physiological importance of amlexanox binding to Hsp90 is provided in the present study by observation that the drug interferes with the association of the protein with both Hop and CyP40 (Figure 5A). Since Hop and Cyp40 are components of the Hsp90-based multichaperone complex, the present data suggest that amlexanox may disrupt such a multichaperone complex *in vivo* and *in vitro*.

In the present work, we examined *in vitro* interaction of Hsp90 with two anti-allergic drugs, DSCG and amlexanox. These interesting pharmacological agents are used in the therapy of allergic diseases, including bronchial asthma. The results show that both anti-allergic drugs target Hsp90. Furthermore, amlexanox suppresses C-terminal chaperone activity *in vitro* and inhibits cohort protein association with the C-terminal domain of Hsp90. Although DSCG suppresses the chaperone function of the N-terminal domain, the drug has no effect on the function of the C-terminus of Hsp90. The physiological significance of these interactions *in vivo* remains to be investigated further, but undoubtedly must be taken into account when considering pharmacology of anti-allergic drugs. Anti-allergic drugs, such as DSCG and amlexanox, may serve as useful pharmacological tools for elucidating the multiple roles of Hsp90 in fundamental cellular processes.

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