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# Hsp90 Inhibition Accelerates Cell Lysis

## ANTI-Hsp90 RIBOZYME REVEALS A COMPLEX MECHANISM OF Hsp90 INHIBITORS INVOLVING BOTH SUPEROXIDE- AND Hsp90-DEPENDENT EVENTS*

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**The 90 kDa heat shock protein, Hsp90, is an abundant molecular chaperone participating in the cytoprotection of eukaryotic cells. Here we analyzed the involvement of Hsp90 in the maintenance of cellular integrity using partial cell lysis as a measure. Inhibition of Hsp90 by geldanamycin, radicicol, cisplatin, and novobiocin induced a significant acceleration of detergent- and hypotonic shock-induced cell lysis. The concentration and time dependence of cell lysis acceleration was in agreement with the Hsp90 inhibition characteristics of the N-terminal inhibitors, geldanamycin and radicicol. Glutathione and other reducing agents partially blocked geldanamycin-induced acceleration of cell lysis but were largely ineffective with other inhibitors. Indeed, geldanamycin treatment led to superoxide production and a change in membrane fluidity. When Hsp90 content was diminished using anti-Hsp90 hammerhead ribozymes, an accelerated cell lysis was also observed. Hsp90 inhibition-induced cell lysis was more pronounced in eukaryotic (yeast, mouse red blood, and human T-lymphoma) cells than in bacteria. Our results indicate that besides the geldanamycin-induced superoxide production, and a consequent increase in cell lysis, inhibition or lack of Hsp90 alone can also compromise cellular integrity. Moreover, cell lysis after hypoxia and complement attack was also enhanced by any type of Hsp90 inhibition used, which shows that the maintenance of cellular integrity by Hsp90 is important in physiologically relevant lytic conditions of tumor cells.**

The 90 kDa heat shock protein (Hsp90)[1] is a central part of a chaperone meshwork chaperoning a large number of substrate proteins (1–5). Besides being a partner of a large number of co-chaperones and substrates, Hsp90 binds to filamentous actin and tubulin (6–8) and the involvement of the cytoskeleton in the traffic of Hsp90 substrates has also been demonstrated (5, 9). Together with other chaperones, like Hsp27 and Hsp70, Hsp90 is involved in cytoprotection (10–12).

Cell lysis is one of the most commonly used methods to test cellular integrity. Moreover, lysis rate anomalies (13, 14) together with diffusional anomalies (15, 16) were used as important arguments for the organization of the cytoplasm. Since cellular integrity is preserved after a partial cell lysis to a large extent (13, 14), partial lysis provides a highly sensitized, but still somewhat organized cellular system, where the contribution of various components to both the cytoplasmic organization and cellular stability can be studied.

The original aim of the present study was to examine whether Hsp90 inhibition induces any change in the rate of cell lysis induced by mild detergent treatment or hypotonic shock. The rationale behind these experiments was to test, whether Hsp90, a cytoprotective chaperone, binding to "thousand-and-one" substrates and other proteins is involved in the maintenance of cellular integrity (17), and whether its inhibition renders cells more "lysis-prone." The first experiments were very promising: geldanamycin, a well-established Hsp90 inhibitor (18, 19) induced a significant increase in lysis rate of various cells. However, later experiments demonstrated that the extent of geldanamycin-induced enhancement of cell lysis was dependent on the experimental conditions, namely, if cells were shaken during the experiment or not. At this time the first results of geldanamycin-induced superoxide generation appeared (20, 21). These results turned our attention to examine the contribution of superoxide-related *versus* Hsp-related events to diminished cellular integrity after Hsp90 inhibition. Using various Hsp90 inhibitors (18, 19, 22–26) as well as anti-Hsp90 hammerhead ribozymes we demonstrated that besides a putative increase in membrane fragility by geldanamycin-induced superoxides, inhibition or lack of Hsp90 alone also results in a compromised cellular integrity. Moreover, cell lysis after hypoxia and complement attack was also enhanced by any type of Hsp90 inhibition used, which shows that the maintenance of cellular integrity by Hsp90 is important in physiologically relevant lytic conditions of tumor cells. Our results show

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[1] The abbreviations used are: Hsp90, 90 kDa heat shock protein; Brij-58: $C_{16}E_{20}$, polyethylene glycol hexadecyl ether; CDDP, cisplatin; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPI, diphenyleneiodonium chloride; GA, geldanamycin; GP, the inactive geldanamycin analogue, geldampicin; GSH, reduced glutathione; LDH, lactate dehydrogenase; NB, novobiocin; RA, radicicol; Tiron, 4,5-dihydroxy-1,3-benzene disulfonic acid disodium salt.

the first successful use of an anti-Hsp90 ribozyme in manipulating Hsp90 levels, and demonstrate a novel element of Hsp90-related cytoprotection: its role in the maintenance of cellular integrity.

## EXPERIMENTAL PROCEDURES

*Materials*—Cell culture media were from Invitrogen. The pcDNA3 vector for hammerhead ribozyme cloning and LipofectAMINE were obtained from Invitrogen, restriction enzymes and other molecular biology reagents were from Promega (Madison, WI). The anti-Hsp90 antibody, anti-Raf-1, anti-Lck rabbit polyclonal antibodies, goat anti-rabbit and anti-mouse IgG horseradish peroxidase conjugates were from Santa Cruz Biotechnology (Santa Cruz, CA). The Bradford reagent for protein concentration measurements was a Bio-Rad (Richmond CA) product. Geldanamycin and geldampicin (18) were kind gifts from Drs. Len Neckers and Robert J. Schultz (National Institutes of Health, Bethesda, MD). Radicicol (22) was a courtesy of Drs. Mitsunobu Hara and Hirofumi Nakano (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan). DPH (1,6-diphenyl-1,3,5-hexatriene) was from Molecular Probes (Eugene, OR). The enhanced chemiluminescence kit was from Amersham Biosciences. All other chemicals used were from Sigma Chemical Co.

*Culture of Jurkat Cells and Drug Treatments*—The human T-lymphocyte cell line (Jurkat, J32) was provided by M. Kamoun (Department of Pathology and Laboratory Medicine, Philadelphia, PA) and was cultured as described earlier (26). For all experiments $2 \times 10^5$ cells (at a cell density of $2 \times 10^5$ cells/ml) were used unless otherwise indicated. Drug treatments were always carried out in complete medium unless otherwise mentioned, for 2 h at 37 °C with 5% constant $CO_2$ supply. Cell viability was monitored by trypan blue exclusion all the time before starting the experiment. Concentrations of the N-terminal Hsp90 inhibitors, geldanamycin (18, 19) and radicicol (22), the ineffective geldanamycin analogue, geldampicin (18), the C-terminal inhibitor, cisplatin (22, 24) and novobiocin, which inhibits nucleotide binding to Hsp90 at both termini (24, 25) were optimized using the drugs at concentrations, where their Hsp90 inhibition is fully exerted, but no drug-induced cell toxicity is seen. Under "no-shaking" conditions cells were maintained at 25 °C in an Eppendorf incubator for 10 min after drug preincubation. Shaking of cells was performed for 10 min in an Eppendorf Thermomixer at a speed of 1000 rpm. Lysis conditions were standardized to have 20–25% lysis. This has been achieved using 0.005% Brij-58 for 10 min at room temperature. 100% cell lysis was obtained by sonicating cells for 30 s with a sonicator (Sonic 300, Artek Systems, Farmingdale, NY) on ice. After detergent treatment or sonication cells were centrifuged at room temperature for 10 min at 800 × *g* in an Eppendorf centrifuge (Model 5402). Supernatants were processed for protein determination, lactate dehydrogenase analysis, or immunoblot measurements.

*Protein Content*—Protein concentration of cell lysates was measured by the Bradford method (27) using bovine serum albumin as standard.

*Lactate Dehydrogenase Measurements*—The activity of lactate dehydrogenase (LDH) was measured using the direct spectrophotometric assay of Wroblewski and LaDue (28) in the presence of pyruvate and NADH. In 2 ml of a 50 mM Hepes buffer (pH 7.4) containing 30 μM pyruvate and 30 μM NADH 10 μl of Jurkat cell supernatant were added after the indicated treatments with drugs and detergents, and changes in optical density were measured at 340 nm for 5 min. Special care was taken to avoid the absorbtion of LDH to the cuvette wall during the activity measurement. The percentage of LDH release was calculated by dividing the activity of LDH in the supernatant by the LDH activity measured after complete cell lysis achieved by sonication. None of the detergents and drugs affected LDH activity, when added directly to the reaction mixture at the concentrations used in whole cell experiments.

*Superoxide Production*—Superoxide production in geldanamycin-treated Jurkat cells was assayed by the lucigenin-enhanced chemiluminescence method (29). $2 \times 10^6$ Jurkat cells ($2 \times 10^6$ cells/ml) after a treatment with various drugs were washed free of the used inhibitors, resuspended in 10 mM Hepes (pH 7.4), and added to the scintillation vial containing 5 μM lucigenin in the same Hepes buffer. Lucigenin chemiluminescence with and without cells was recorded as cpm (counts per minute) for about 10 min in 0.1-min intervals in a Beckman LS7800 liquid scintillation counter using a single photon mode. Chemiluminescence values were corrected to background chemiluminescence without added cells.

*Membrane Fluidity Measurements*—The procedure was adapted from Revathi *et al.* (30). Jurkat cells after a treatment with the drug indicated were incubated with 1 μM 1,6-diphenyl-1,3,5-hexatriene (DPH) for 15 min in the dark in triplicates. Cells were washed free of DPH, resuspended in 2 ml of phosphate-buffered saline, and the fluorescence was immediately measured using a steady-state spectrofluorimeter (M300-Edinburgh Instruments) in 1-cm path length quartz cuvettes with an excitation and emission wavelength of 357 and 430 nm, respectively. Fluorescence polarization was measured after adapting cells in dark with constant stirring.

*Construction of Anti-Hsp90 Hammerhead Ribozymes*—The design of anti-Hsp90 hammerhead ribozyme was adapted from the studies of Little and Lee (31). The ribozyme was designed to cleave in a highly conserved segment of the coding region of Hsp90 at its N or C terminus, respectively. Partially overlapping nucleotides for both Hsp90α and Hsp90β N-terminal and C-terminal regions were synthesized. These sense and antisense oligonucleotides were annealed at room temperature for 15 min in a 50 mM Tris-HCl (pH 7.6) buffer. Prior to cloning, these oligonucleotides were further extended with 5 units of Klenow polymerase in a standard PCR buffer (50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl, and 10 μM dNTP mix) for 60 min at 37 °C. The end-filled products were precipitated using ethanol, resuspended in sterile double distilled water, and double digested with *Hin*dIII and *Not*I. For cloning the cloning vector, pcDNA3 with an hCMV promoter and a polylinker containing the bovine growth hormone poly(A) sequence was selected. In the vector the upstream ATG (at nucleotide position 995) after the *Xba*I and *Apa*I sites was disrupted by introducing a stop codon followed by *Xho*I, *Nhe*I, *Not*I, and *Sac*II restriction sites. The vector was digested with *Hin*dIII and *Not*I restriction enzymes, the digested vector was dephosphorylated using calf intestinal phosphatase at 37 °C for 15 min and the enzyme was inactivated by phenol/chloroform extraction. The anti-Hsp90 hammerhead ribozyme oligonucleotides were then inserted as described in Sambrook *et al.* (32) at the *Hin*dIII and *Not*I sites within the multiple cloning site of pcDNA3. Ligation reactions were carried out in a total volume of a 10 μl buffer containing 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM dithiothreitol, 5% polyethylene glycol-8000, the double-digested insert and the vector in a molar ratio of 3:1 as well as 1 unit of T4 DNA ligase at 16 °C for 12 h. XLN-blue competent cells were transformed with the ligation mix, and plated on Luria-Bertani (LB) agar plates containing 60 μg/ml ampicillin. The colonies were selected from the plate, and were subjected to plasmid miniprep. The positive clones were selected by Southern blot analysis using the respective end-labeled, annealed antisense oligonucleotide.

*Transfection and Screening of Jurkat Cells*—Jurkat cells were transfected with either pcDNA3 (as a control), or the respective anti-Hsp90 hammerhead ribozyme using the polycationic reagent, LipofectAMINE. The entire transfection protocol was performed according to the manufacturer's instructions in a 96-well plate (NUNC™, Nalgene Nunc International, NY) in multiples. Control and ribozyme-transfected cells were screened for cell viability using trypan blue dye exclusion. Neomycin selection was not feasible, since the rate of cell death with ribozyme transfections was constantly increasing showing the importance of high Hsp90 levels for the survival of eukaryotic cells. Hsp90 content was checked by an immunoblot with anti-Hsp90 antibody. Cells on the second day of transfection, where cell death was not yet much prevalent were used for further experiments.

*SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis*—Cell lysates were mixed with Laemmli buffer (33) containing 100 μM dithiothreitol, boiled for 5 min, and the samples were subjected to 10% SDS-PAGE. Proteins were transferred from the gel to nitrocellulose membrane using a semidry protein gel transfer apparatus. Transfer of proteins was confirmed by Ponceau-S staining, and the blot was processed for Western blot analysis using a primary antibody followed by a horseradish peroxidase-conjugated secondary antibody. Labeled bands were visualized using an enhanced chemiluminescence kit.

*Bacterial Culture and Bacterial Protoplasts*—DH5-α (34) *Escherichia coli* strain (a gift from Dr. András Váradi, Institute of Enzymology, Hungarian Academy of Sciences, Budapest, Hungary) was grown in liquid LB. Bacteria from the mid-log phase of growth (at 37 °C) were collected and were subjected to mild lysozyme treatment to digest the cell wall. Protoplasts were collected by spinning cells at 2000 rpm for 2 min. Protoplasts were stored in 0.9% NaCl isotonic solution, and the osmotic lysis conditions were standardized as to get 20–25% of lysis. This has been achieved using 0.4% NaCl. Incubation with various concentrations of geldanamycin (from 0.001 to 5 μM) was performed at 25 °C for 60 min to see the effect of the drug on cell lysis. Distilled water was used to achieve 100% lysis of protoplasts.

*Yeast Cultures, Yeast Spheroplasts, and Protoplasts*—Wild-type strains of bakers' yeast, *Saccharomyces cerevisiae* (S-288) and fission yeast, *Schizosaccharomyces pombe* (L-972) were obtained from Department of Microbiology and Biotechnology, St. István University, Buda-

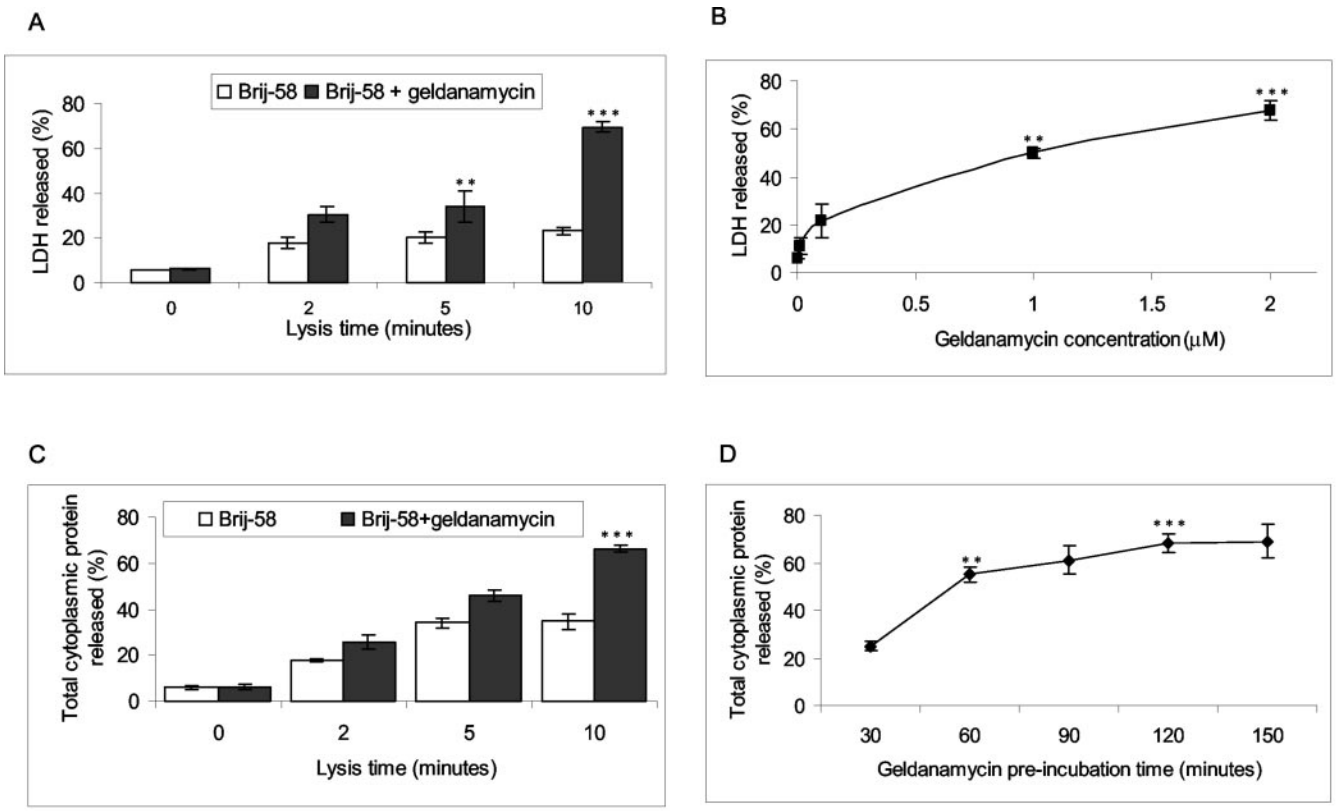


FIG. 1. **Geldanamycin increases detergent-induced lysis of Jurkat cells.** $2 \times 10^5$ cells ($2 \times 10^5$ cells/ml) were incubated in the absence (*open symbols*) or presence (*filled symbols*) of GA at final concentrations and times indicated. Cells were lysed with 0.005% Brij-58, and the activity of released LDH or amount of total proteins was measured as described under "Experimental Procedures." 100% lysis was achieved by sonication and results were normalized to this value. *Panel A*, effect of geldanamycin treatment on LDH release. Cells were incubated without or with 2 $\mu$M geldanamycin for 2 h and lysed with Brij-58 for the times indicated. *Panel B*, concentration dependence of geldanamycin-induced increase of LDH release. Cells were incubated without or with geldanamycin for 2 h at final concentrations indicated, and lysed with Brij-58 for 10 min. *Panel C*, effect of geldanamycin treatment on total protein release. Cells were incubated without or with 2 $\mu$M geldanamycin for 2 h and lysed with Brij-58 for the times indicated. *Panel D*, time dependence of geldanamycin-induced increase of total cytoplasmic protein release. Cells were incubated with 2 $\mu$M geldanamycin for times indicated and lysed with Brij-58 for 10 min.

pest, Hungary and were grown in liquid YEPD medium. Yeast cells from the mid-log phase of growth (at 30 °C) were used for making yeast spheroplasts and protoplasts. First, yeast cells were treated with 0.1 mg/ml of lyticase in case of *S. cerevisiae* (35) and 1 mg/ml lysing enzyme in case of *S. pombe* (36) at 30 °C for 30 or 45 min to make spheroplasts or protoplasts, respectively. Spheroplasts and protoplasts were collected in isotonic 1 M sorbitol, and were incubated with geldanamycin. Lysis conditions were standardized to have 20–25% lysis. This has been achieved using 0.65 M sorbitol. 100% cell lysis was achieved by treating cells with distilled water.

*Hypoxia-induced Cell Lysis Measurements*—The method to induce chemical hypoxia was adapted from Wang *et al.* (37). A stock solution of cobalt chloride was made in sterile double distilled water and various concentrations (25 $\mu$M to 2 mM) were used for a 2-h treatment to induce hypoxia in Jurkat cells. 0.2 mM $CoCl_2$ concentration for $2 \times 10^6$ cells/ml was chosen as the optimum to have 15 to 20% cell lysis, without any cellular damage (cell integrity was measured by trypan blue exclusion). The treatment was carried out both in presence and absence of 2 mM glutathione along with various Hsp90 inhibitors.

*Complement-induced Cell Lysis Measurements*—$2 \times 10^6$ Jurkat cells/ml were subjected to immune-mediated cell lysis using mouse and human serum in separate experiments. Serum from mouse showed very high cytolysis even at very low serial dilution (data not shown), hence, human serum was chosen for the experiments shown. The human serum concentration was optimized to have ~20% cell lysis (judged by both protein estimation and microscopic examination). Exponentially growing Jurkat cells were treated with various anti-Hsp90 drugs for 2 h both in presence and absence of 2 mM glutathione followed by addition of 5 $\mu$l of 1:5 diluted human serum in 0.5 ml RPMI 1640-complete medium (serum enhanced the complement-mediated cell lysis hence, all the lysis experiments were performed in complete medium). Cells were then further incubated for 10 min at 30 °C. Cells were subsequently washed with phosphate-buffered saline (pH 7.6) and centrifuged for 10 min at 2000 rpm and their supernatant was collected for measuring the cell lysis as the percent of total cytoplasmic protein released.

*Statistical Analysis*—Data are presented as means ± S.E. of minimum three independent experiments unless otherwise indicated, and analyzed with unpaired Student's $t$ test. $p < 0.05$ was accepted as indicating a statistically significant difference compared with controls. In figure legends: *, $p < 0.05$;**, $p < 0.01$; ***, $p < 0.001$.

## RESULTS

*Geldanamycin Induces an Accelerated Lysis of Jurkat Cells*—In order to test, whether Hsp90, a cytoprotective chaperone forming a complex with a lot of cytoplasmic proteins is involved in the maintenance of cellular integrity (17) Jurkat cells were subjected to mild detergent lysis with Brij-58. Lysis conditions were optimized to achieve an ~20% lysis of lactate dehydrogenase (LDH) in a 10-min detergent treatment (Fig. 1*A*, *open bars*). Preincubation of Jurkat cells with geldanamycin, a specific inhibitor of Hsp90 (18, 19) induced a significant increase in the extent of released LDH (Fig. 1*A*, *filled bars*). In agreement with previous data (18, 19, 26) geldanamycin treatment alone, without additional Brij-58 lysis did not induce a significant lysis of Jurkat cells (data not shown). Geldanamycin-induced lysis was not specific to Brij-58, but could be observed if primary cell lysis was induced by other detergents, like the plasma membrane specific digitonin or by hypotonic shock (data not shown). The effect was concentration dependent, showing a saturation above 2 $\mu$M geldanamycin (Fig. 1*B* and data not shown).

The geldanamycin-induced enhancement of the release of lactate dehydrogenase, a well-known cytoplasmic marker protein might be the consequence of a heretofore unknown inter-

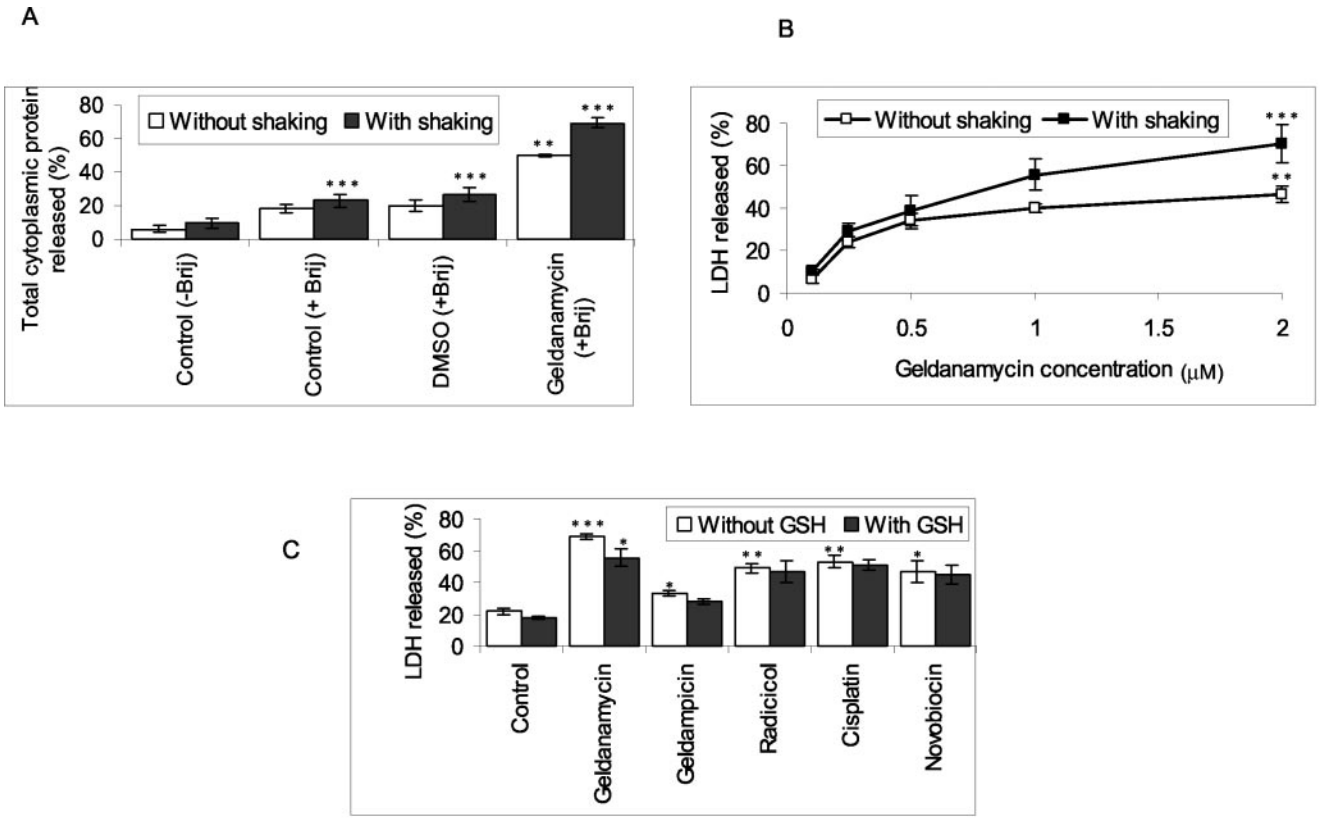


FIG. 2. **Geldanamycin- (but not other Hsp90 inhibitors) induced increase of Jurkat cell lysis is dependent on oxidative conditions.** $2 \times 10^5$ cells ($2 \times 10^5$ cells/ml) were incubated in the absence or presence of various Hsp90 inhibitors for 2 h at a final concentration of 2 $\mu$M unless otherwise indicated. Subsequently, 0.005% Brij-58 was added to the cells and one set was maintained without shaking (*open bars*), another with shaking (*closed bars*) for an additional 10 min, and the amount of released total proteins or the activity of lactate dehydrogenase (*LDH*) were measured as described under "Experimental Procedures." Two controls, one without Brij-58, another with Brij-58 alone were also included in the experiments. 100% lysis was achieved by sonication and results were normalized to this value. *Panel A*, effect of shaking to geldanamycin-induced additional release of total cytoplasmic proteins. Cells were incubated with geldanamycin for 2 h at a final concentration of 2 $\mu$M without (*open bars*) or with (*filled bars*) additional shaking and lysed with Brij-58 for 10 min, where indicated. *Panel B*, effect of shaking on the concentration dependence of geldanamycin-induced increase in LDH-release. After GA incubation at final concentrations indicated, cells were incubated with Brij-58 without (*open squares*) or with (*filled squares*) shaking. *Panel C*, effect of glutathione (*GSH*) on the increase of LDH release induced by various Hsp90 inhibitors. Cells were incubated without or with 2 $\mu$M GA, 2 $\mu$M GP, 2 $\mu$M RA, 2 $\mu$M CDDP, or 0.1 mM NB for 2 h in the absence (*open bars*) or presence (*filled bars*) of 2 mM reduced glutathione, and lysed with Brij-58 for 10 min.

action between LDH and Hsp90, where geldanamycin would dissociate LDH from Hsp90, and cause its accelerated release. However, the geldanamycin-induced enhancement of cell lysis was a general feature of all cytoplasmic proteins, including Hsp90 itself, Hsp90 substrates, like the Raf and Lck kinases and total cytoplasmic proteins (Fig. 1*C* and data not shown). The extent of maximal release was similar for LDH and total proteins showing that the inhibition of Hsp90 induced a fairly general destabilization of cellular structures. Geldanamycin-induced enhancement of cell lysis was not instant but required at least a 1-h preincubation with the drug to be effective (Fig. 1*D*). Our data are in good agreement with the concentration and time dependence of geldanamycin-induced changes in Hsp90 substrate proteins (18, 19, 26).

The geldanamycin-induced additional cell lysis was similar to the increase in cell lysis after disruption of cytoskeletal elements with colchicine or cytochalasin (data not shown). However, the effect of geldanamycin was not changed (data not shown) if lysis was performed in the presence of actin or tubulin stabilizing buffers (38, 39), which suggests that the geldanamycin-induced effect is not a direct consequence of cytoskeletal disorganization.

A highly similar pattern of geldanamycin-induced increase in Brij- or hypoosmotic shock-induced hemolysis was observed in mouse red blood cells. Time and concentration dependence of geldanamycin-induced additional hemolysis was very similar to the lysis rates obtained with Jurkat cells (see abstract published as Ref. 40, and data not shown) showing the generality of the effects observed.

*Geldanamycin-induced Additional Cell Lysis Is Partially Dependent on Oxidative Stress*—Interestingly, there was a marked difference in the extent of geldanamycin-induced additional lysis, if cells were shaken during the experiment or not. When geldanamycin treatment was combined with additional shaking, a larger increase in cell lysis was observed (Fig. 2*A*). Shaking the cells after geldanamycin treatment, but before detergent treatment might help to disrupt cellular structures otherwise preventing the faster release of LDH and cytoplasmic proteins in the presence of Brij-58. However, shaking-induced differences were larger at larger geldanamycin concentrations (Fig. 2*B*), which suggested that the two effects are not independent from each other. Reaching this point in our experiments the first results of geldanamycin-induced superoxide generation appeared (20, 21). What if shaking provided an additional oxygen inducing a larger amount of superoxides? The addition of reduced glutathione as an antioxidant to the incubation medium reduced the geldanamycin effect (Fig. 2*C*). On the contrary, the effect of other Hsp90 inhibitors, like the much more effective N-terminal inhibitor, radicicol (22), the C-terminal inhibitor, cisplatin (23, 24) and novobiocin, which inhibits nucleotide binding to Hsp90 at both termini (24, 25) was not changed by glutathione addition (Fig.

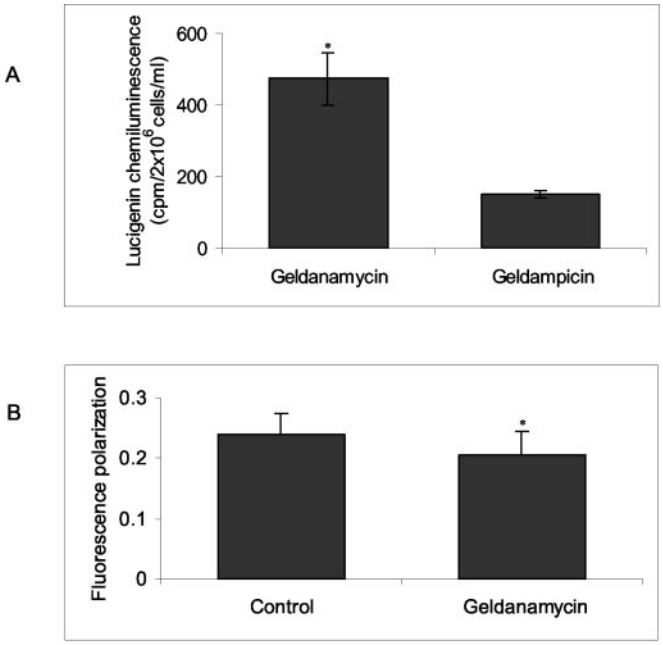


FIG. 3. **Effect of geldanamycin on superoxide production and membrane fluidity.** $2 \times 10^5$ cells ($2 \times 10^5$ cells/ml) were incubated in the absence or presence of GA or GP for 2 h at final concentrations of 2 $\mu$M, respectively. Superoxide production or fluorescence polarization of the membrane probe DPH were measured as described under "Experimental Procedures." *Panel A*, effect of geldanamycin and geldampicin on superoxide production. Cells were incubated without or with geldanamycin, and their superoxide production was measured. Data were normalized to background superoxide level of control cells. *Panel B*, effect of geldanamycin on membrane fluidity. Cells were incubated without or with geldanamycin and their membrane fluidity was measured.

2*C*). Geldampicin, an ineffective geldanamycin analogue (18) did not induce a large increase in cell lysis, and the effect of all other Hsp90 inhibitors was inbetween the control and geldanamycin-induced level being roughly equal with the lysis after the simultaneous addition of geldanamycin and reduced glutathione (Fig. 2*C*). These data raised the possibility that the geldanamycin-induced additional cell lysis was a result of both geldanamycin-induced superoxide production and Hsp90 inhibition.

*Geldanamycin Induces Superoxide Production in Jurkat Cells and an Increased Membrane Fluidity*—Observing an oxidative stress-related component of geldanamycin-induced additional cell lysis we wanted to obtain a direct evidence for geldanamycin-induced superoxide production in Jurkat cells. Indeed, geldanamycin, but not the inactive analogue, geldampicin (18) induced a significant increase in lucigenin chemiluminescence (Fig. 3*A*), which is a clear indication of geldanamycin-induced superoxide production in Jurkat cells.

To assess, if geldanamycin affects the status of Jurkat cell membranes we opted to measure membrane fluidity by measuring the fluorescence polarization of the commonly used probe, 1,6-diphenyl-1,3,5-hexatriene (DPH, Ref. 30). Geldanamycin induced a significant decrease in DPH fluorescence polarization (Fig. 3*B*). The change in fluorescence polarization (0.034) was higher than several physiologically significant changes reported in the literature (41–43). On the contrary to our results with geldanamycin, another N-terminal inhibitor of Hsp90, radicicol (22) did not induce a significant change in DPH fluorescence polarization (data not shown). Since detergent treatment causes difficulties in the interpretation of fluorescence polarization data, these experiments did not give a direct analysis of the membrane status after a combined treatment of Hsp90 inhibitors and Brij-58. However, our data suggest that geldanamycin-induced superoxide production may lead to an increased membrane fluidity, membrane disorganization in Jurkat cells, which may contribute to their increased sensitivity to physiological lysis conditions.

*Anti-Hsp90 Hammerhead Ribozymes Reveal a Truly Hsp90-dependent Component of Geldanamycin-induced Additional Lysis of Jurkat Cells*—Since none of the Hsp90-inhibitors have truly specific effects to Hsp90, (geldanamycin induces superoxide production independent of Hsp90, Refs. 20 and 21; radicicol is an inhibitor of citrate lyase, Ref. 44; cisplatin and novobiocin both have a wide spectra of effects at the concentration they inhibit Hsp90) we wanted to use a tool which really specifically inhibits Hsp90 to assess the ratio of superoxide-dependent and Hsp90-dependent components of geldanamycin effects. Utilizing the idea of Little and Lee (31) to diminish Grp94, we constructed two anti-Hsp90 hammerhead ribozymes specific to the N and C termini of Hsp90 mRNA (Fig. 4*A*). Both ribozymes cleave a sequence, which is the same in Hsp90-$\alpha$ and Hsp90-$\beta$ and conserved in a wide range of species (Fig. 4*B*). Transfection of Jurkat cells with the anti-Hsp90 hammerhead ribozymes resulted in an efficient reduction of Hsp90 levels, while keeping the pattern of total cellular proteins intact (Fig. 4*C*). According to our expectations, both the N-terminal and the C-terminal anti-Hsp90 ribozymes, either alone or in combination induced a significant acceleration of Brij-induced Jurkat cell lysis (Fig. 4*D*; the difference between $Me_2SO$- and GA-treated cells was statistically significant at a level of $p < 0.001$). Interestingly, the C-terminal ribozyme was somewhat less efficient than the N-terminal, which might be related to its smaller degree of homology (Fig. 4*A*). On the contrary, LipofectAMINE-treatment or vector transfection had only marginal effects (Fig. 4*D*). Ribozyme-induced additional lysis could be further increased by geldanamycin treatment. This increase was normalized by the addition of glutathione in all cases (Fig. 4*D*). Substitution of glutathione with the superoxide scavenger, Tiron (45) or the NO synthase, FADH and NADPH oxidase inhibitor, diphenyleneiodonium chloride (DPI; Refs. 21 and 46) gave smaller, but similar effects (data not shown). The effect of DPI was the smallest of all antioxidants studied, showing a rather small contribution of specific redox systems to Jurkat cell lysis. The ribozyme experiments gave a strong support to our conclusion that geldanamycin induces a destabilization of Jurkat cells by both superoxide- and truly Hsp90-dependent mechanisms. Hsp90 seems to be important in the maintenance of cellular integrity.

*Geldanamycin-induced Lysis Acceleration in Bacterial and Yeast Cells*—Observing a role for Hsp90 to maintain cellular integrity we wanted to study, whether cells with a less important contribution of Hsp90 to their viability and with a smaller degree of cellular organization than the human Jurkat cell line, namely bacterial or yeast cells are sensitive to geldanamycin-induced additional lysis or not. Lysis conditions were optimized to achieve a 20–30% lysis of total cytoplasmic proteins. On the contrary to our results with Jurkat cells, geldanamycin induced no significant additional lysis in *E. coli* (Fig. 5). In *S. cerevisiae* and *S. pombe* yeast cells the additional lysis after geldanamycin treatment was significant, but smaller than that of Jurkat cells (see Figs. 1*B* and 5*A*). As in the case of Jurkat cells, the lysis rate was strongly dependent on the extent of shaking in all cell types studied. No shaking induced no appreciable lysis, however, vigorous shaking induced a close-to-maximal cell lysis suggesting that bacterial and yeast protoplasts lacking a sophisticated cellular architecture are more sensitive to cell lysis than eukaryotic cells (data not shown and Fig. 5*B*). Since we could not be sure that geldanamycin inhibits the bacterial

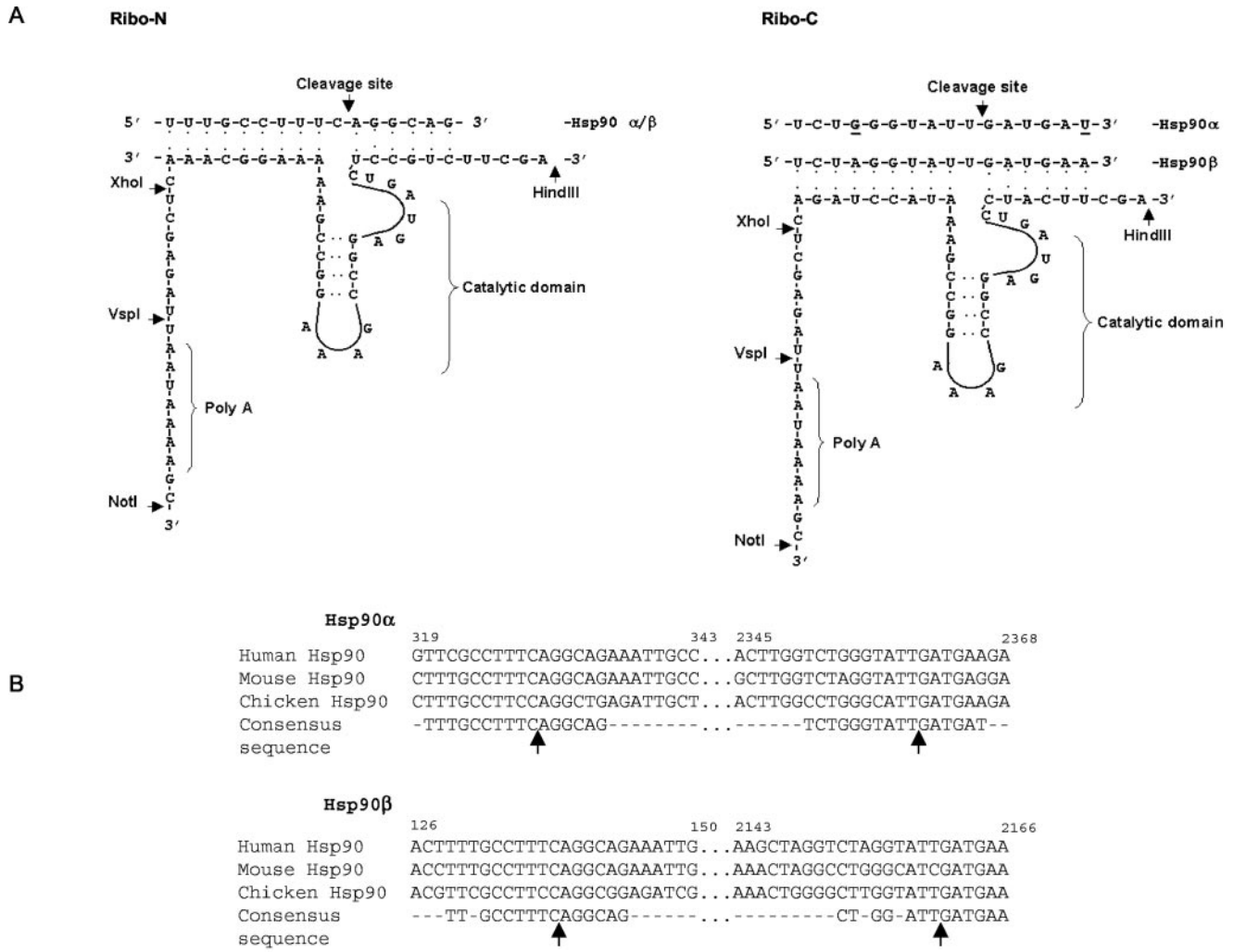


FIG. 4. **Effect of anti-Hsp90 hammerhead ribozymes on detergent-induced lysis of Jurkat cells.** *Panel A*, structure of anti-Hsp90 hammerhead ribozymes. The structure of the mRNA segments containing a conserved region of the N terminus (Hsp90-N) or the C terminus (Hsp90-C) of human Hsp90-$\alpha$ and Hsp90-$\beta$ mRNA as well as the respective hammerhead ribozymes is shown. The cleavage sites of various restriction enzymes, the mRNA cleavage site and the poly-A sequence are indicated. *Panel B*, conservation of ribozyme target sequences in Hsp90. Sequences of human, mouse and chicken Hsp90-$\alpha$ and Hsp90-$\beta$, as well as their consensus sequences in the vicinity of the ribozyme cleavage site (marked with an *arrow*) are shown for both the N- and C-terminal regions of anti-Hsp90 hammerhead ribozymes. Nucleotide numbers refers to human Hsp90. *Panel C*, Hsp90 content of ribozyme-transfected Jurkat cells. Anti-Hsp90 hammerhead ribozymes were constructed, and Jurkat cells were transfected as described under "Experimental Procedures." *a*, immunoblot analysis of control and ribozyme-transfected Jurkat cell lysates with polyclonal anti-Hsp90 antibody. *b*, Coomassie Blue-stained SDS-PAGE of control or ribozyme-transfected Jurkat cell lysates. Lanes from *left* to *right*, *Marker*, molecular weight standards; *+Ribo*, N+C ribozyme-transfected cells; and *Con*, control cells. *Panel D*, effect of anti-Hsp90 hammerhead ribozymes on detergent-induced lysis of Jurkat cells. $2 \times 10^5$ ($2 \times 10^5$ cells/ml) control, vector-, anti-N-terminal, anti-C-terminal, or both anti-N- and C-terminal Hsp90 hammerhead ribozyme-transfected cells were incubated in the absence or presence of GA and reduced GSH for 2 h at final concentrations of 2 $\mu$M or 2 mM, respectively. Cells were lysed with 0.005% Brij-58 for 10 min and the activity of released LDH was measured as described under "Experimental Procedures." 100% lysis was achieved by sonication, and results were normalized to this value.

Hsp90 homologue, HtpG similarly than eukaryotic Hsp90 we have heat preconditioned bacterial cells at 44 °C and measured their lysis rate. Elevation of molecular chaperones in bacterial cells did not induce any change in cell lysis rate (data not shown) indicating that in bacterial cells chaperones are not playing a prominent role in the maintenance of cellular integrity.

*Hsp90 Inhibition Enhances Hypoxia-induced Cell Lysis*—To address the physiological significance of our cell lysis experiments using mild detergent treatment, we wanted to study if Hsp90 inhibition induces an enhanced cell lysis in hypoxia, a usual phenomenon in tumors. Hypoxia was induced using cobalt chloride (37). Geldanamycin and radicicol both caused a significant increase of hypoxia-induced cell lysis in Jurkat cells. The effect was reduced in presence of glutathione (Fig. 6*A*). Other Hsp90 inhibitors, such as cisplatin and novobiocin showed ~50% less enhancement of hypoxia-induced Jurkat cell lysis, and, on the contrary to geldanamycin and radicicol, addition of glutathione had no significant effect on the extent of lysis (Fig. 6*A*). To see and compare the differences between Hsp90 inhibitor-induced cellular effects and those after ribozyme-targeted Hsp90 inhibition, ribozyme-transfected Jurkat cells were subjected to hypoxia and hypoxia-induced cell lysis was amplified to more than 2-fold in ribozyme-transfected cells (Fig. 6*B*). Vector-transfected cells did not show any significant change. Addition of geldanamycin to ribozyme-transfected cells showed no further change in hypoxia-induced cell lysis. Similarly, addition of glutathione did not reduce the combined effects of anti-Hsp90 ribozyme and geldanamycin (Fig. 6*B*).

*Hsp90 Inhibition Enhances Complement-mediated Cell Lysis*—To demonstrate the physiological significance of the enhanced cell lysis associated with Hsp90 inhibition further, we have chosen the complement-mediated immune lysis of the

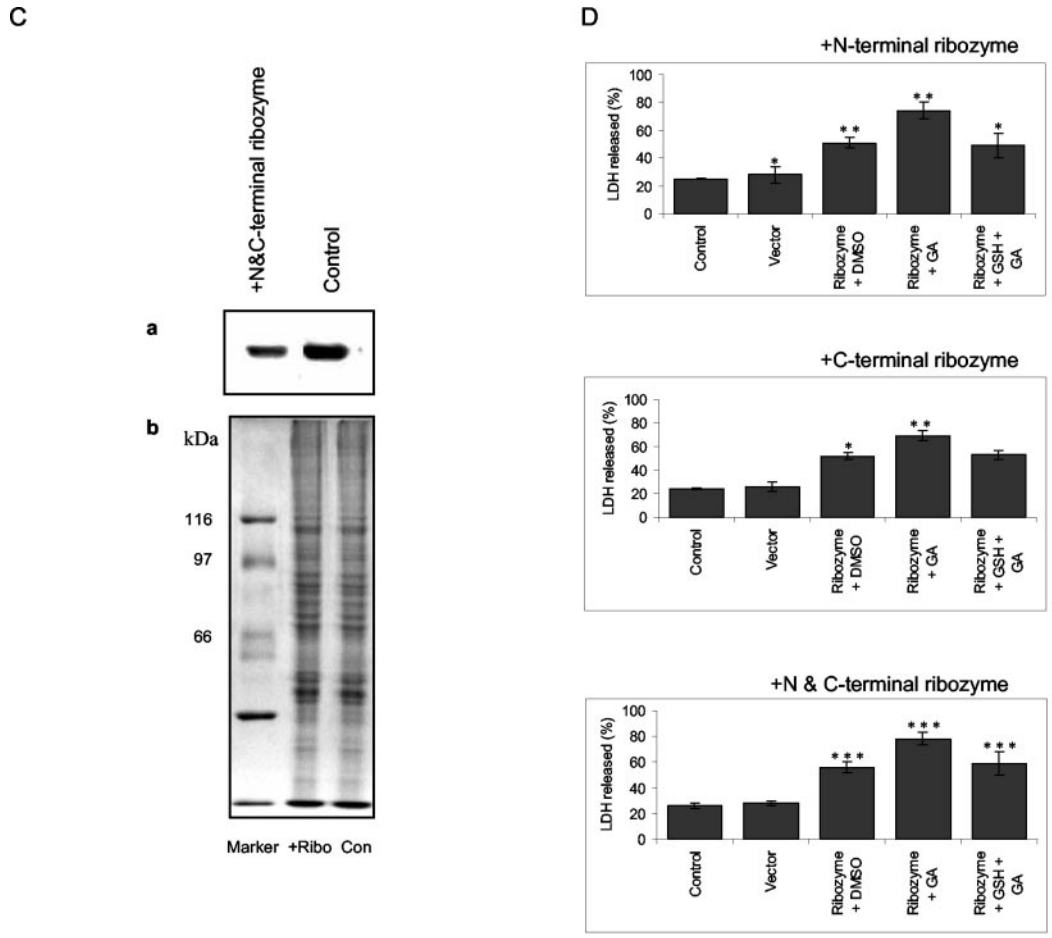


FIG. 4.—*continued*

malignant Jurkat cell line, as a model of immune attack on human tumor cells. Though all effective Hsp90 inhibitors showed some increase of cytolysis when Jurkat cells were preincubated with them before the addition of human serum, again geldanamycin and radicicol showed the only significant differences (Fig. 6*C*). Addition of glutathione induced a slight decrease in cell lysis (Fig. 6*C*). The Hsp90 antisense ribozyme induced a massive increase in complement-mediated cell lysis, which was only marginally affected with the addition of geldanamycin without or with glutathione suggesting that Hsp90 loss is the major cause of increased cytolysis after complement attack (Fig. 6*D*).

## DISCUSSION

One of the major findings of the present report is that the Hsp90 inhibitor, geldanamycin has a dual action in damaging cellular integrity: roughly half of its effects come from an accelerated superoxide production, but the other half of increased cellular fragility is a direct consequence of diminished Hsp90 function. Additionally, cell lysis after hypoxia and complement attack was also enhanced by any type of Hsp90 inhibition used, which shows that the maintenance of cellular integrity by Hsp90 is important in physiologically relevant lytic conditions of tumor cells.

Accelerated superoxide production is a recently observed feature of geldanamycin, the first Hsp90-independent function of this highly specific Hsp90 inhibitor (20, 21). Superoxide production has been first implicated in geldanamycin action as an alternative route of endothelial NO synthase function leading to an uncoupled superoxide production parallel with a decreased NO synthesis (47). The role of Hsp90 as a "switch" from superoxide to NO production was also demonstrated in neural NO synthase (48). However, later studies extended these findings and showed that geldanamycin is able to increase superoxide production independently of endothelial NO synthase activation both in *in vivo* and *in vitro* systems, possibly by its quinone group, which may participate in redox cycling (21). Similar data were reported on neural NO synthase (20). Importantly, the non-quinone Hsp90 inhibitor, radicicol had no direct superoxide producing effect (20). This is in agreement with our findings, that in contrast to the effects of geldanamycin, radicicol action cannot be attenuated by reduced glutathione (Fig. 2*C*). An earlier study proposed that radicicol might exert its antimalarial action *via* heme-dependent free radical generation but this assumption has not been tested directly (49). However, a recent study indicated that radicicol converts endothelial NO synthase from an NO producer to a superoxide generator independently of a direct superoxide production (50). Interestingly, the geldanamycin structural analogue, geldampicin was much less potent superoxide activator than the parent compound (Fig. 3*A*). Geldampicin contains a piperidine derivative directly attached to the quinoidal segment of the drug (18), which possibly hinders its participation in redox cycling by stabilization redox-independent oxygen binding to geldampicin.[2]

Geldanamycin-induced superoxides may induce lipid-peroxidation, which would lead to a significant membrane damage partially explaining the increased cell lysis observed in our study. However, our membrane fluidity data suggest that this

[2] Z. Riedl and J. Jakus, personal communication.

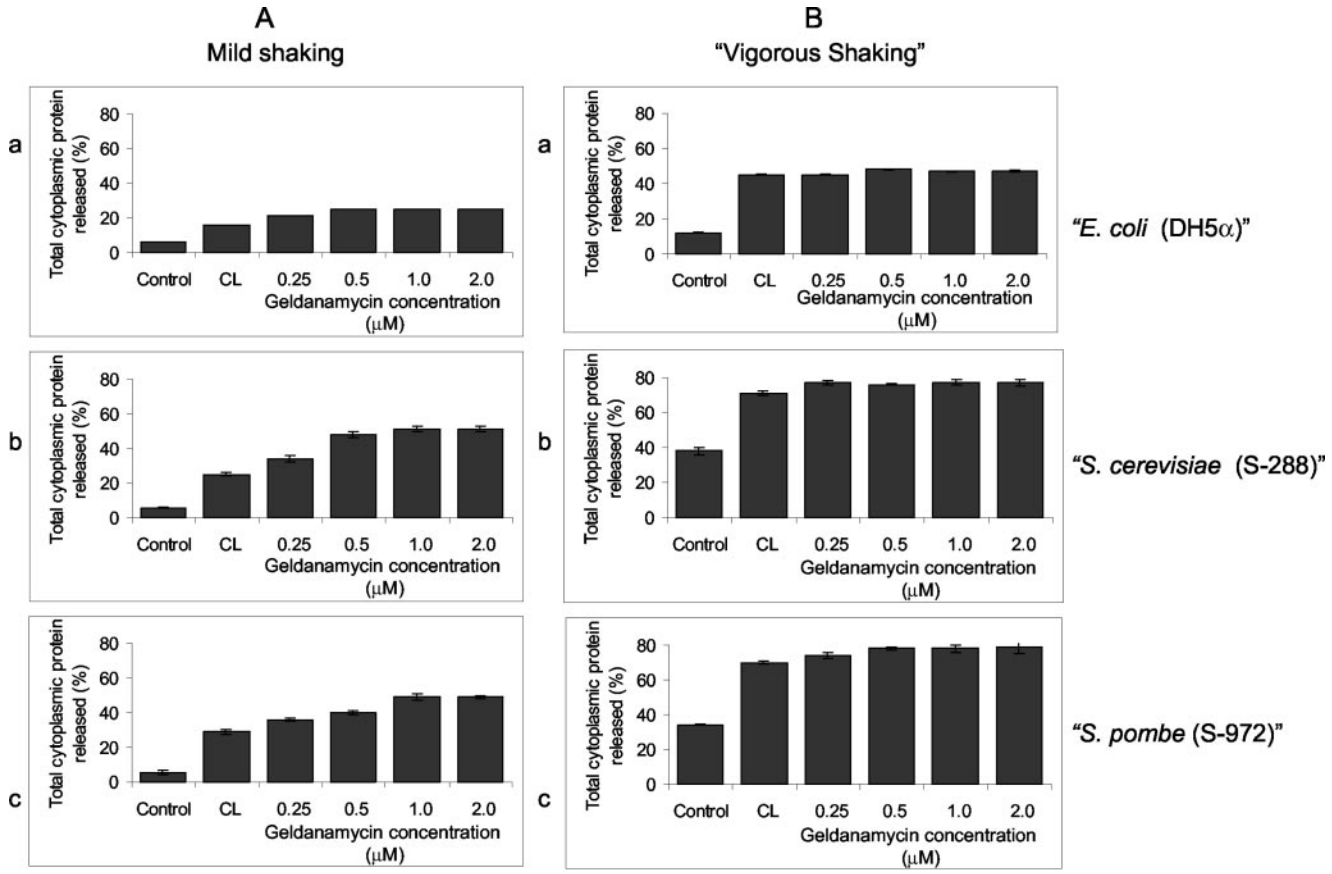


FIG. 5. **Effect of geldanamycin on the lysis of bacterial and yeast cells.** Bacterial protoplasts from *E. coli* cells (DH5α strain) were made from mid-log phase as described under "Experimental Procedures." Similarly, *S. cerevisiae* (S-288 strain) and *S. pombe* (S-972 strain) cells from 5-ml overnight cultures in YEPD medium were inoculated to a 100 ml culture in 1:100 dilution and cells were further incubated at 30 °C with constant shaking for 12 h. Yeast protoplasts were made as described under "Experimental Procedures." $2 \times 10^5$ ($2 \times 10^5$ cells/ml) protoplasts were treated with geldanamycin at final concentrations indicated for 2 h and were subjected to mild hypotonic lysis using 0.45 M NaCl or 0.65 M sorbitol in case of bacteria or yeast, respectively. Lysis was measured without shaking (data not shown), with mild shaking (2,000 rpm; *panel A*) and with vigorous shaking (10,000 rpm; *panel B*). Lysed protoplasts were centrifuged and the released total protein was measured as described under "Experimental Procedures." 100% lysis was achieved by distilled water treatment and results were normalized to this value. *Control* is the background cell lysis without any hypotonic shock, *CL* represents control lysis by hypotonic shock without any pretreatment. The data presented are the average of ten individual experiments. Geldanamycin-induced changes are statistically significant at a level of $p < 0.001$.

might not be the case. Lipid peroxidation induces a decrease in membrane fluidity (51, 52). In contrast, we have found an increased membrane fluidity after geldanamycin action (Fig. 3*B*). Increased membrane fluidity may be concomitant with an increased membrane fragility (53). Thus geldanamycin seems to contribute by a partially oxidation-dependent, but heretofore unknown mechanism to the observed increase in membrane sensitivity. It cannot be excluded, however, that Hsp90 inhibition alone, independently of any geldanamycin-induced oxidative changes also induces an increase in membrane disorganization.

As a possible example of oxidation-independent membrane action of geldanamycin, Suttitanamongkol *et al.* (54) published an interesting study, which showed that geldanamycin treatment alone induced a lysis of platelets by inducing extensive membrane blebbing and disrupting the plasma membrane structure. These data raise the possibility that the geldanamycin-enhanced lysis of Jurkat cells is due to a detergent effect of the drug. However, several of the following pieces of evidence refute this assumption. (i) In our studies geldanamycin alone did not induce a significant lysis of Jurkat cells (Ref. 26 and data not shown). (ii) Suttitanamongkol *et al.* (54) used geldanamycin at a high concentration (18 μM), which most probably produced aspecific effects. A recent study (55) showed that geldanamycin is accumulated in various cells. However, in our experiments detergent treatment was performed after the excess of geldanamycin has already been washed away, and the geldanamycin-induced sensitization for lysis also occurred in experiments (*i.e.* when applied together with hypoxia or complement-induced lysis) when detergent treatment was not performed. This excludes the possibility that geldanamycin was especially enriched in detergent-treated cells. (iii) The effect of geldanamycin was gradually developed over a long preincubation period extending for several hours in our experiments (see Fig. 1*C*). This slowly developing action is a typical feature of geldanamycin effects (18, 19, 26). On the contrary, detergent effects are prompt, requiring seconds to minutes to develop. (iv) The structural analogue of geldanamycin, geldampicin did not induce the acceleration of Jurkat cell lysis (Fig. 2*C*). Detergent effects are not so sensitive to minor changes in detergent structure. (v) The structurally unrelated Hsp90 inhibitors, radicicol, cisplatin or novobiocin gave a smaller, but significant increase in Jurkat cell lysis compared with that observed after geldanamycin treatment (Fig. 2*C*). (vi) Last but not least, we have analyzed Jurkat cell structure after geldanamycin treatment with electron microscopy and saw no signs of membrane blebbing or disintegration (data not shown).

Recent studies indicated a role of Hsp90 in maintaining the membrane raft structure. Geldanamycin treatment efficiently dissociated several Hsp90-related protein complexes in lipid rafts (56, 57). Since lipid rafts are usually isolated after a nonionic detergent extraction, the similar experimental condi-

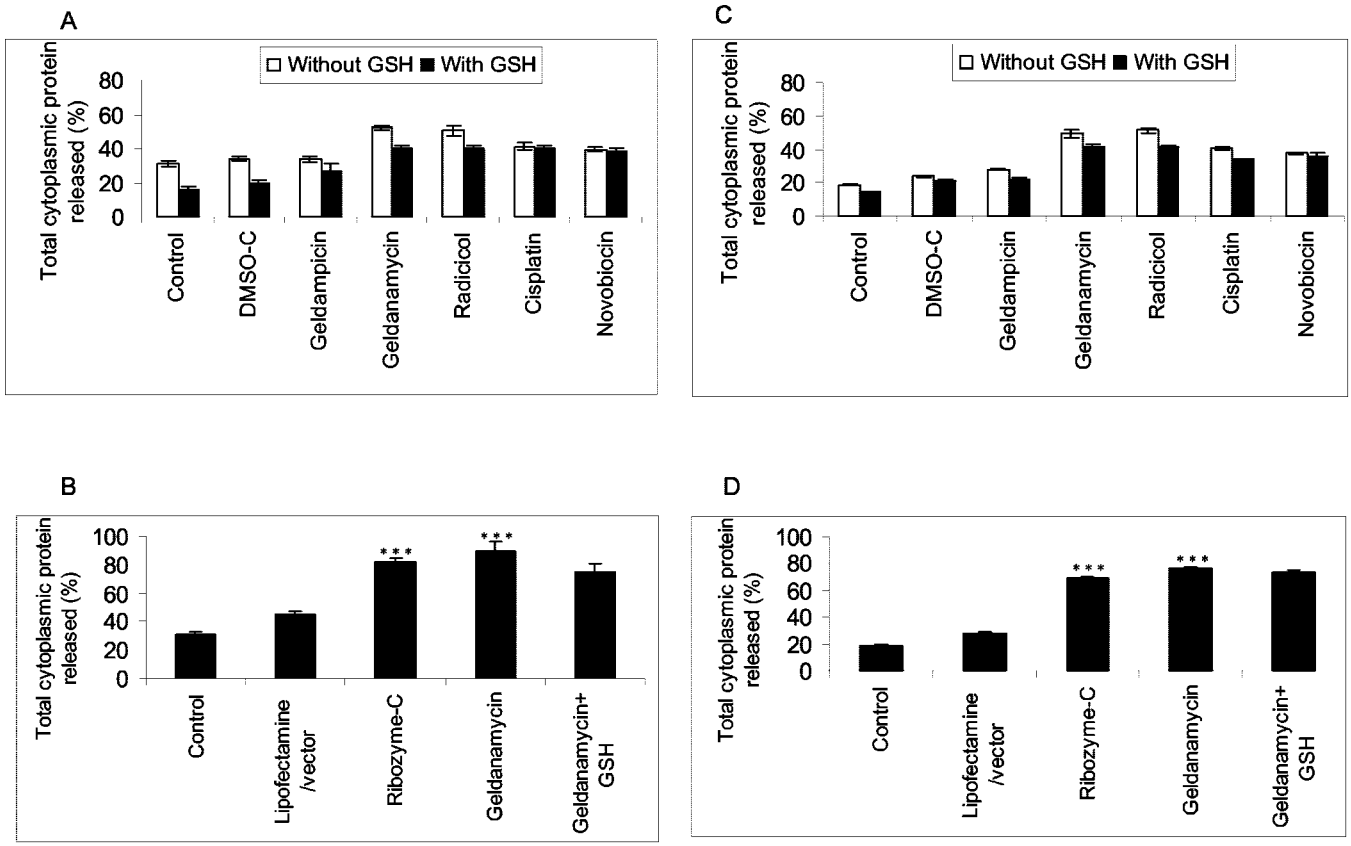


FIG. 6. **Effect of geldanamycin on hypoxia (*panels A* and *B*) and complement (*panels C* and *D*)-induced cytolysis.** *Panel A*, $2 \times 10^6$ Jurkat cells were preincubated with 0.2 mM $CoCl_2$ for 2 h together with geldanamycin both in the absence (*open bars*) and presence (*filled bars*) of glutathione and their lysis was measured as described under "Experimental Procedures." *Panel B*, effect of anti-Hsp90 hammerhead ribozymes on hypoxia-induced cell lysis. Jurkat cells were transfected with vector or anti-Hsp90 ribozyme, and cell lysis was measured after $CoCl_2$ incubation as described in *panel A* and under "Experimental Procedures." To distinguish the oxidation induced effects from Hsp90 effects antioxidant glutathione was added during geldanamycin treatment. *Panel C*, $2 \times 10^6$ cells were preincubated with various anti-Hsp90 inhibitors in the absence (*open bars*) or presence (*filled bars*) of glutathione. Cells were lysed with human serum for 10 min at 30 °C and cell lysis was measured by estimating the percent total cytoplasmic protein release as described under "Experimental Procedures." *Panel D*, effect of anti-Hsp90 hammerhead ribozymes on complement-induced lysis. Jurkat cells were transfected with vector or anti-Hsp90 ribozyme and complement-induced cell lysis was measured both in presence and absence of antioxidant glutathione together with geldanamycin as described in *panel C* and under "Experimental Procedures." Data represent mean ± S.D. of three independent experiments.

tions argue for a role of Hsp90 inhibition induced raft disorganization in the decrease of cellular integrity observed in our studies.

Our experiments with anti-Hsp90 hammerhead ribozymes demonstrated that a diminished Hsp90 function *alone* compromises cellular integrity (Fig. 4*D*). The role of Hsp90 can be a direct disorganization of cellular structures after the disruption of various Hsp90-related complexes or an indirect action of Hsp90 inhibition via the incorrect folding of an Hsp90 client protein important in the maintenance of cellular integrity. At present we cannot exclude any of these possibilities. Our data showing a prolonged action after Hsp90 inhibition (Fig. 1*C*) suggest the contribution of an Hsp90 client protein to the effects observed.

The observation, that Hsp90 inhibition does not affect the lysis of bacterial protoplasts, and has a smaller enhancement of yeast protoplast lysis than that of higher eukaryotic cells (Fig. 5) is in good agreement with the increasing role of this chaperone in the maintenance of cellular life from prokaryotes to yeast and higher eukaryotes (1–5) as well as with the development of more and more sophisticated cytoskeleton in these organisms. Hsp90 is well known to interact with filamentous actin and tubulin (6–8) and the involvement of the cytoskeleton in the traffic of Hsp90 substrates has been repeatedly demonstrated (5, 9). These findings raise the possibility that Hsp90 might contribute to an increased cellular integrity by the maintenance of cytoskeleton-related structural elements in eukaryotic cells.

Hsp90 inhibitors (geldanamycin analogues, radicicol, and purine scaffold inhibitors) were recently introduced to the clinical practice as anticancer agents (58, 59). Our findings may help to establish a novel element of the mechanism of action of these drugs by showing the role of Hsp90 inhibition to sensitize cells for various lytic events. To assess the decreased cell stability after the inhibition of Hsp90 function in experiments, which are more relevant to physiological conditions than mild detergent treatment, or hypotonic shock, we have examined the effect of Hsp90 inhibitors and the disruption of Hsp90 by anti-Hsp90 ribozyme on hypoxia-induced and complement-mediated cytolysis of Jurkat cells. Both conditions mimic quite well the lytic conditions usual for tumor cells. Our results demonstrated a clear enhancement of cell lysis under both conditions after any type of Hsp90 inhibition used. Our findings suggest the probability of a general contribution of Hsp90 to maintain cellular integrity. The role of heat shock proteins in natural cell reactivity is well demonstrated (60). Similarly, immune cell-mediated lysis is also associated with the production of superoxides (61). Our results show that both effects take place when Hsp90 inhibitors enhance cell lysis, but our anti-Hsp90 ribozyme experiments show that the inhibition of Hsp90 itself, seems to be predominant. Sodium arsenite was shown to sensitize Jurkat cells for immune-mediated cytolysis (62). How-

ever, in this case the complexity of the stress response both in tumor and bystander cells as well as the relative toxicity makes the treatment non-suitable for selective tumor therapy. On the contrary, selective depletion of Hsp90 seems to be an effective mode of cell sensitization to both hypoxia- and immune-mediated cell lysis, which adds a novel element to the mechanism of action of Hsp90 inhibitor drug candidates. This phenomenon may help the immune system to attack tumor cells. Similarly, a lysis sensitization may cause a shift from tumor cell apoptosis to necrosis, which gives a further help for the activation of the immune system (63).

In summary, our experiments demonstrated that a diminished Hsp90 function *alone* compromises cellular integrity. This effect is characteristic to eukaryotic organisms, and is extended by a putative increase in membrane fragility partially due to the increased superoxide production by geldanamycin, an Hsp90 inhibitor. Our results show the first successful use of an anti-Hsp90 ribozyme in manipulating Hsp90 levels, and demonstrate a novel element of Hsp90-related cytoprotection: its role in the maintenance of cellular integrity.

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