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Pharmacological induction of Hsp70 protects apoptosis-prone cells from doxorubicin: comparison with caspase-inhibitor- and cycle-arrest-mediated cytoprotection

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Abstract

Selective modulation of cell death is important for rational chemotherapy. By depleting Hsp90-client oncoproteins, geldanamycin (GA) and 17-allylamino-17-demethoxy-GA (17-AAG) (heat-shock protein-90-active drugs) render certain oncoprotein-addictive cancer cells sensitive to chemotherapy. Here we investigated effects of GA and 17-AAG in apoptosis-prone cells such as HL60 and U937. In these cells, doxorubicin (DOX) caused rapid apoptsis, whereas GA-induced heat-shock protein-70 (Hsp70) (a potent inhibitor of apoptosis) and G1 arrest without significant apoptosis. GA blocked caspase activation and apoptosis and delayed cell death caused by DOX. Inhibitors of translation and transcription and siRNA Hsp70 abrogated cytoprotective effects of GA. Also GA failed to protect HL60 cells from cytotoxicity of actinomycin D and flavopiridol (FL), inhibitors of transcription. We next compared cytoprotection by GA-induced Hsp70, caspase inhibitors (Z-VAD-fmk) and cell-cycle arrest. Whereas cell-cycle arrest protected HL60 cells from paclitaxel (PTX) but not from FL and DOX, Z-VAD-fmk prevented FL-induced apoptosis but was less effective against DOX and PTX. Thus, by inducing Hsp70, GA protected apoptosis-prone cells in unique and cell-type selective manner. Since GA does not protect apoptosis-reluctant cancer cells, we envision a therapeutic strategy to decrease side effects of chemotherapy without affecting its therapeutic efficacy.
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Abbreviations: AIF, apoptosis-inducing factor; 17-AAG, 17-allylamino-17-demethoxy-GA; DOX, doxorubicin; FL, flavopiridol; GA, geldanamycin; Hsp70, heat-shock protein-70; Hsp90, heat-shock protein-90; HSF1, heat-shock transcription factor1; PTX, paclitaxel; PMA, phorbol ester

Introduction

Chemotherapy causes apoptosis in certain cancer and normal cells, leading to both therapeutic and side effects. Broadly, all cells could be subdivided into apoptosis-prone and reluctant. As a canonical example, HL60 cells are apoptosis prone. Thus, HL60 cells undergo apoptosis in response to chemotherapeutic agents with diverse mechanisms of action: doxorubicin (DOX) and etoposide (topoisomease-II inhibitors), paclitaxel (PTX; microtubule active agent), flavopiridol (FL; an inhibitor of transcription) and proteasomal inhibitors.[1–6] In contrast, apoptosis-reluctant cells, including common cancer cell lines, undergo either slow (nonapoptotic) types of cell death or cycle arrest.[7] For example, K562 leukemia cells are apoptosis reluctant, because they express the Bcr-Abl anti-apoptotic kinase, which in turn induces heat-shock protein-70 (Hsp70).[8] Similarly, transfection of Hsp70 renders HL60 cells apoptosis reluctant.[8,9]

Hsp70 blocks several steps of the apoptotic cascade: upstream from mitochondria, release of cytochrome *C* and apoptosis-inducing factor (AIF), nuclear import of AIF, activation of procaspases-9 and -3, and even downstream of active caspase-3.[8,10–15] Hsp70 renders cells resistant to chemotherapy.[8] Similarly, heat shock protects colon cancer cells from TRAIL-induced apoptosis.[16] By inducing Hsps, heat shock can prevent apoptosis in cardiomyocytes.[17,18] Also, hyperthermia protected mice from tumor necrosis factor[19] and gamma radiation.[20] Yet, heat shock cannot be safely used in patients. Obviously, expression of ectopic Hsp70 is not suitable for clinical applications too. What is needed is pharmacological induction of Hsp70.

Geldanamycin (GA) and its analog 17-allylamino-17-demethoxy-GA (17-AAG) binds to heat-shock protein 90 (Hsp90).[21] Hsp90 is a major repressor of the heat-shock transcription factor 1 (HSF1).[22] By binding Hsp90, GA via HSF1 induces Hsp70, resembling heat shock response.[22,23] On the other hand, GA and 17-AAG are cytotoxic and can induce apoptosis in cancer cells. 17-AAG is undergoing clinical trials as an anticancer drug. Furthermore, numerous reports (including ours) have demonstrated that GA potentiated the cytotoxicity of some anticancer drugs.[5,24–28] Yet, in such studies, GA was used to deplete antiapoptotic kinases in apoptosis-reluctant cancer cells. The goal of this study is to investigate Hsp70-mediated effects in apoptosis-prone

HL60 cells and to determine conditions for cell-type selective cytoprotection.

Results

GA induces Hsp70 in parallel with cytoprotection

GA-induced Hsp70 in a dose-dependent manner (Figure 1a). In contrast, Hsp90 was unchanged. DOX neither induced Hsp70 nor prevented GA-induced Hsp70 (Figure 1a). Importantly that induction of Hsp70 correlated with the ability of GA to protect HL60 cells from DOX (Figure 1b). GA minimally inhibited cell survival, as measured by MTT assay (Figure 1b, open circles). In the absence of GA (*X*-axis: GA = 0), 500 ng/ml DOX completely inhibited cell survival. GA abrogated DOX-induced cytotoxicity in a dose-dependent manner, coincident with Hsp70 induction (Figure 1).

GA prevents caspase-9 activation and apoptosis

In HL60 cells, DOX activated the intrinsic pathway of apoptosis that is manifested by cleavage of caspase-9 (Figure 2a). This was followed by PARP cleavage (Figure 2a) and the appearance of apoptotic morphology (Figure 2b) and cell death, as evidenced by cessation of cell metabolism (Figure 2b). GA did not cause cleavage of caspase-9 and PARP in HL60 cells (Figure 2a). GA was preferentially cytostatic at this time point (Figure 3 and data not shown). As expected, GA-induced Hsp70 and blocked caspase-9 activation and PARP cleavage caused by DOX (Figure 2a) and prevented nuclear fragmentation (Figure 2b). This in turn resulted in cytoprotection of HL60 cells (Figure 2c). Thus, GA protected HL60 cells by blocking the intrinsic apoptotic pathway activated by DOX.

GA prevented apoptosis but not cell-cycle arrest

GA arrested HL60 cells in G1 phase of the cell cycle (Figure 3). Low concentrations of DOX (50 ng/ml) cause G2 arrest in HL60 cells without appearance of TUNEL-positive cells. Cytotoxic concentrations of DOX (500 ng/ml) caused apoptosis, as evidenced by TUNEL-positive cells, associated with accumulation of cells with S-phase DNA content (Figure 3). Importantly, GA completely blocked apoptosis caused by 500 ng/ml DOX (Figure 3: GA + DOX 500).

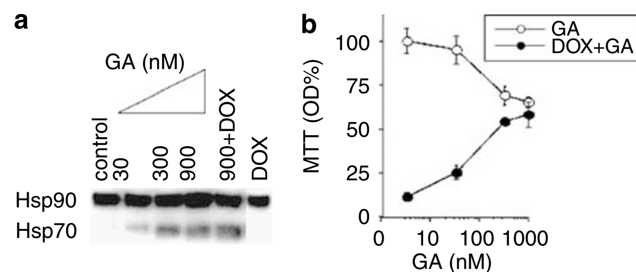

Figure 1 Effects of GA on Hsp70 and cytotoxicity of DOX. (**a**) HL60 cells were treated with indicated concentrations of GA and 300 ng/ml DOX. After 16 h, Hsp70 and Hsp90 were measured by immunoblot. (**b**) HL60 cells were treated (in triplicate) with indicated concentrations of GA: either GA alone (empty circles) or GA plus 500 ng/ml DOX (closed circles). At GA = 0, HL60 were treated with DOX alone or not treated (control). After 36 h, MTT assay was performed as described in Materials and Methods. Results are shown as percent of control (M ± m)

GA and 17-AAG have similar effects on Hsp70 and cell death

Next we investigated whether other Hsp90-active agents induce Hsp70 and protect HL60 cells from DOX-induced cytotoxicity. 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) is a less toxic analog of GA and is currently undergoing clinical trials. Like GA, 17-AAG induced Hsp70 in a dose-dependent manner (Figure 4a). Both agents abrogated cytotoxic effects of DOX, consistent with a dose-dependent induction of Hsp70. 17-AAG had two to three-fold lower potency compared with GA. Noteworthy, due to an excessive cytotoxicity, high concentrations (10 000 nM) of GA and 17-AAG could not sufficiently protect cells from DOX. Optimal protective concentrations were 500–2000 and 200–1000 nM

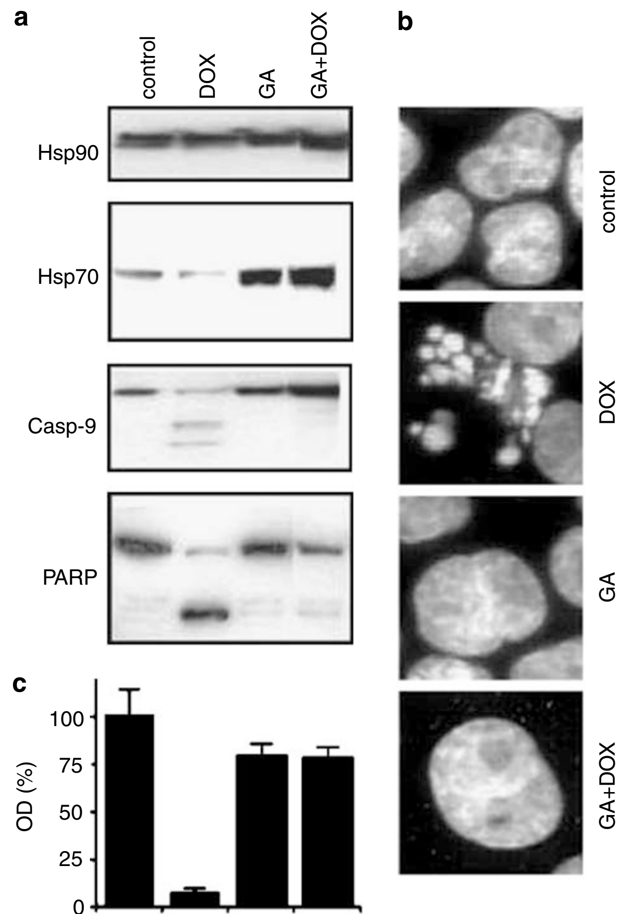

Figure 2 Effects of DOX and GA on apoptosis: (**a**). HL60 cells were treated with 500 ng/ml DOX, 90 nM GA and their combination as indicated. After 16 h, immunoblot for Hsp70, Hsp90 and caspase-9 was performed. After 20 h, immunoblot for PARP was performed. (**b**) After 20 h, DAPI staining for nuclear fragmentation was performed as described in Materials and Methods. (**c**) After 36 h, MTT assay was performed as described in Materials and Methods

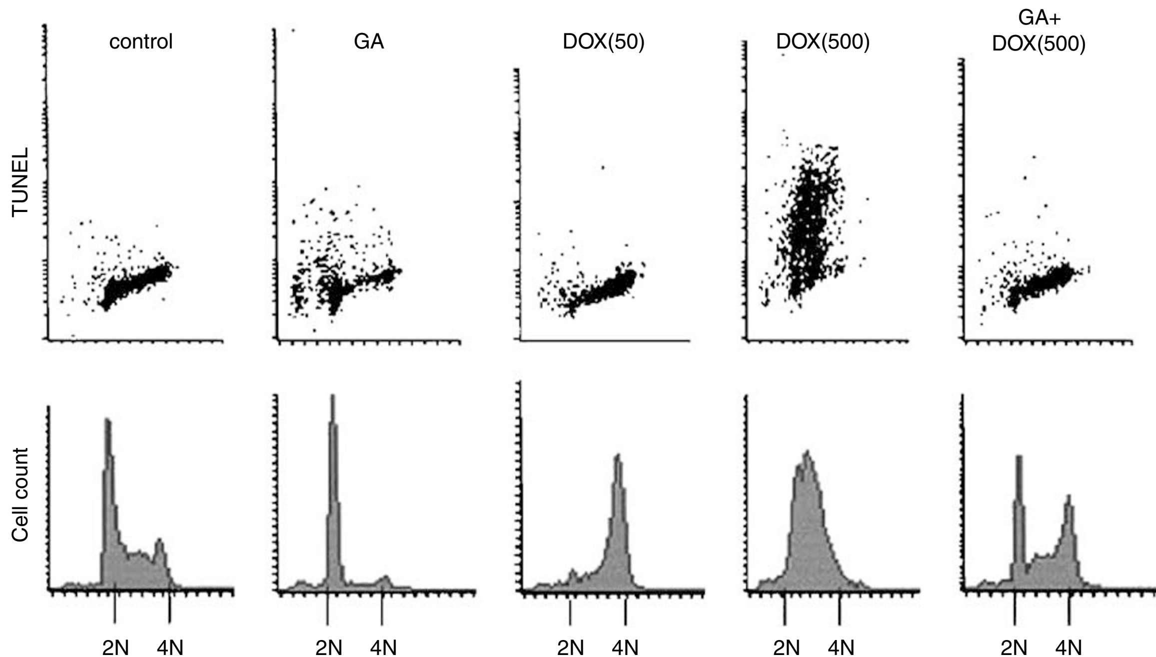

Figure 3 Effects of DOX and GA on TUNEL assay and cell-cycle distributions. HL60 cells were treated with 300 nM GA, 50 ng/ml DOX (50) or 500 ng/ml (500) either alone or in combinations. After 20 h, TUNEL assay and DNA contents were measured as described in Materials and Methods

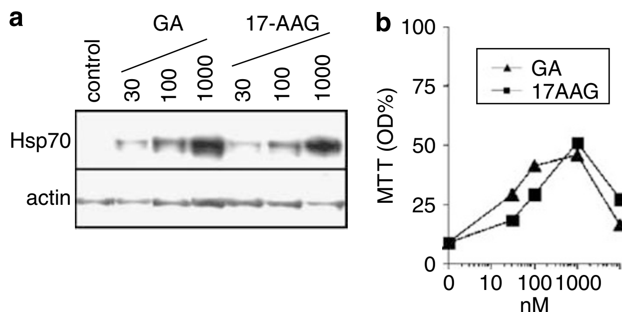

Figure 4 Comparison of GA and 17-AAG. (**a**) HL60 cells were treated with indicated concentrations of GA and 17-AAG. After 16 h, cells were lysed and immonoblot for Hsp70 and actin was performed as described in Materials and Methods. (**b**) HL60 cells were treated with 500 ng/ml DOX plus the indicated concentrations of GA and 17-AAG. (Note: at concentration zero – there is DOX alone). After 36 h, MTT assay was performed as described in Materials and Methods. Results are shown as percent of control (untreated HL60 cells)

for 17-AAG and GA, respectively (Figure 4b and data not shown).

Cytoprotection by GA requires transcription and translation

In the presence of CHX, an inhibitor of translation, GA did not protect cells from DOX (Figure 5). Similarly, GA did not protect cells from ActD and FL (Figure 5). Both ActD and FL are potent inhibitors of transcription and prevented GA-induced Hsp70 (data not shown). GA protected cells from cytotoxicity of topo-I and topo-II inhibitors such as camptothecin (CAM), etoposide and metaxantrone (Figure 5 and data not shown). However, by damaging and intercalating DNA, very high concentrations of DOX and other DNA-damaging drugs can

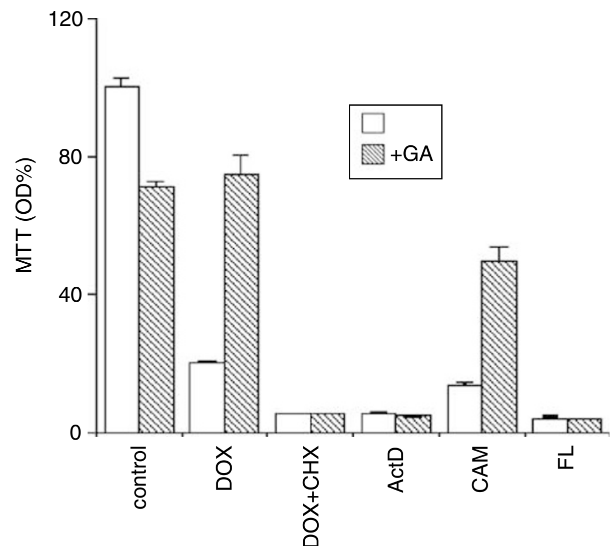

Figure 5 Effects of GA on the cytotoxicity of inhibitors of transcription and topoisomerase inhibitors. HL60 cells were incubated with indicated anticancer drugs (open bars) plus 300 nM GA (closed bars). DOX: 500 ng/ml doxorubicin; DOX + CHX (cycloheximide 10 μg/ml); ActD: 2 μg/ml actinomycin D; CAM: 500 nM camptothecin; FL: 500 nM flavopiridol. MTT assay was performed after 36 h

inhibit transcription. In agreement, GA did not protect HL60 cells from the cytotoxicity caused by DOX at concentrations above 2 μg/ml (Figure 6).

siRNA Hsp70 abrogated cytoprotection

Thus, cytoprotection by GA requires transcription and inhibition of global transcription prevents cytoprotective effects.

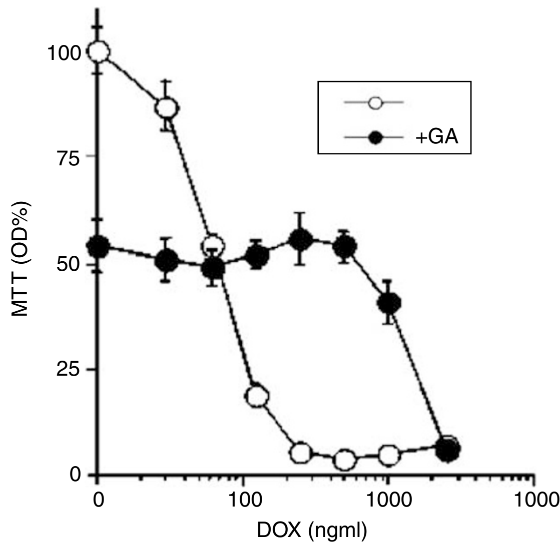

Figure 6 Cytoprotection from different concentrations of DOX. HL60 cells were incubated without (open circles) or with (closed circles) 300 nM GA and with the increasing concentrations of DOX. After 36 h, MTT assay was performed as described in Materials and Methods

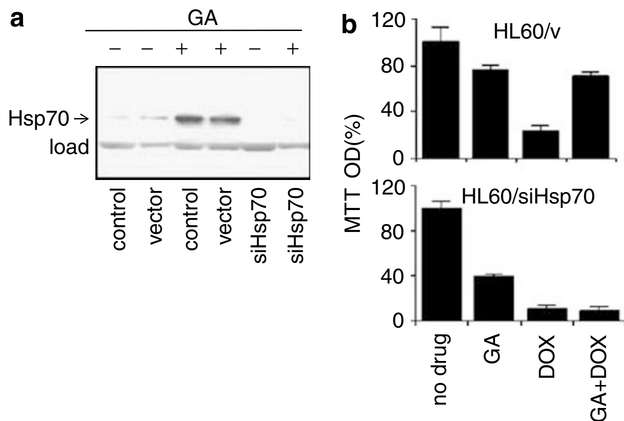

Figure 7 Effects of siRNA-Hsp70 on cytoprotective activity of GA. HL60 cells were transfected with vector (HL60/v) and with siRNA-Hsp70 (HL60/siRNA), as described in references 8 and 43. (**a**) Nontransfected HL60 cells (control), cells transfected with vector or siRNA-Hsp70 were treated with 500 nM GA (+) or left untreated (−). After 16 h, cells were lysed and immunoblot for Hsp70 was performed. (**b**) HL60/v and HL60/siHsp70 were treated with 500 nM GA, 500 ng/ml DOX, GA + DOX or left untreated (control). After 36 h, MTT assay was performed

Next, we investigated whether specific abrogation of Hsp70 precludes cytoprotection. As shown in Figure 7, siRNA Hsp70 prevented the induction of Hsp70. These cells become sensitive to cytotoxicity of GA (Figure 7b). Furthermore, this abrogated cytoprotective effects of GA.

Hsp70 protects cells from DOX, mimicking GA

Next, we investigated whether expression of Hsp70 substitutes for GA-mediated cytoprotection from DOX. We used both K562 cells with high endogenous Hsp70 and HL60/Hsp70 cells, transfected with Hsp70 (Figure 8, inset). K562 cells have high basal levels of Hsp70,[8] which cannot be further induced by GA. Unlike HL60, K562 and HL60/Hsp70 cells were resistant to DOX (Figure 8). GA protected HL60 cells from DOX but did not affect HL60/Hsp70 cells, which were already protected by Hsp70 (Figure 8). Therefore, GA-mediated cytoprotection could not be observed in cells that are resistant to DOX (apoptosis-reluctant cells). Furthermore, GA slightly sensitized K562 cells to DOX. This can be explained by depletion of the antiapoptotic Bcr-Abl caused by GA, as shown previously.[5,8] In HL60/Hsp70 cells, resistance to GA was due to inhibited apoptosis, as shown in Figure 9. Whereas DOX caused nuclear fragmentation in parental HL60 cells, either Hsp70 or GA completely blocked it (Figure 9).

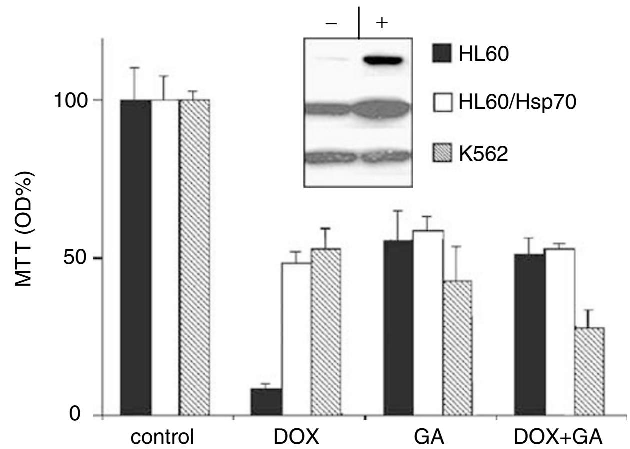

Figure 8 Effects of endogenous and ectopic Hsp70 on cell resistance to DOX and cytoprotective activity of GA. HL60 cells, HL60 cells were transfected with vector (HL60/Hsp70) and K562 cells were treated with 500 nM GA, 500 ng/ml DOX, GA + DOX or left untreated (control). After 36 h, MTT assay was performed. Inset: Basal levels of Hsp70 (−) and levels of Hsp70 following treatment with 500 nM GA for 16 h

Finally, like HL60, U937 leukemia cells are apoptosis prone and express low basal levels of Hsp70 (Figure 10a). In U937 cells, DOX causes rapid cell death with disintegration of apoptotic cells (Figure 10b). 17-AAG induced Hsp70 in U937 cells and prevented disintegration and death of DOX-treated U937 cells (Figure 10).

Comparison of three types of cytoprotection

Besides Hsp70, GA also induces cell-cycle arrest in HL60 cells. Cell-cycle arrest can protect from the cytotoxicity of cycle-dependent chemotherapy. For example, low concentrations of DOX (50 ng/ml), by arresting HL60 cells in G2, prevented apoptosis caused by PTX and docetaxel.[29] Here we compared cytoprotection caused by GA and low cytostatic concentrations of DOX (low DOX). Unlike GA, low DOX did not prevent cell death caused by high concentrations of DOX (Figure 11b). This indicates that apoptotic effects of DOX are not cell-cycle dependent. As expected, low DOX prevented cell death caused by PTX (Figure 11b). Low DOX did not prevent cell death caused by FL, suggesting that its cytotoxicity is not cell-cycle dependent. Whereas GA could not abrogate cytotoxicity caused by FL (FL inhibits transcription, preventing Hsp70 induction), pharmacological inhibitors of caspases such as Z-VAD-fmk were very effective

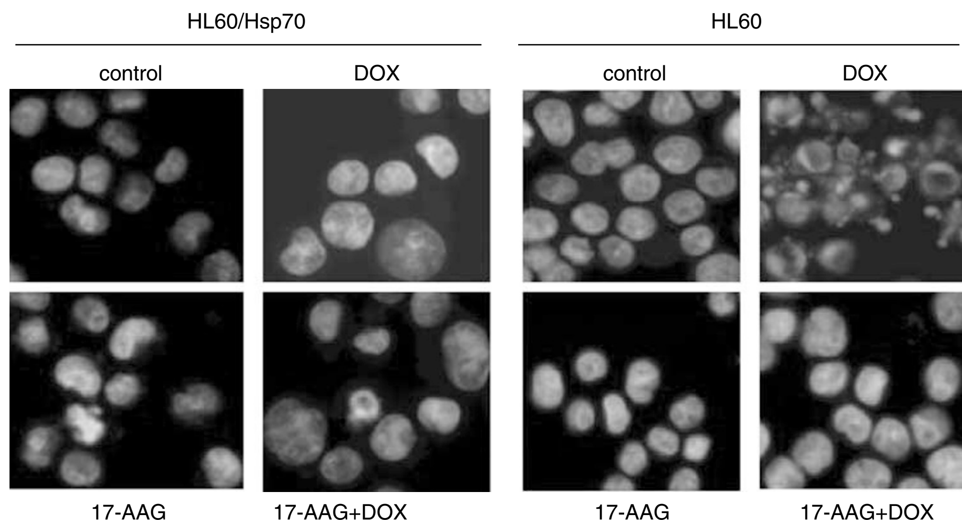

Figure 9 Effects of Hsp70 overexpression on apoptosis. HL60 cells transfected with Hsp70 (HL60/Hsp70) and HL60 cells were treated with 500 ng/ml DOX, 1000 nM 17-AAG and their combination as indicated. After 20 h, DAPI staining for nuclear fragmentation was performed as described in Materials and Methods

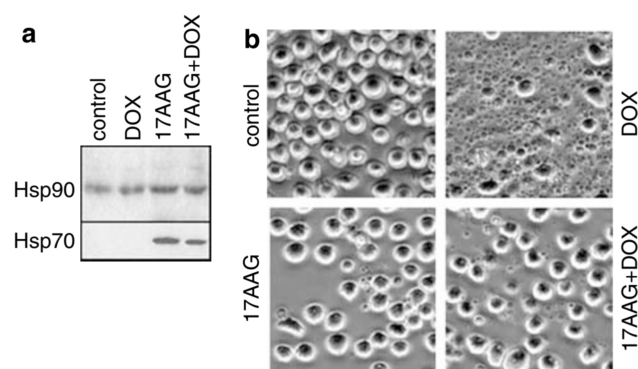

Figure 10 Cytoprotective effects of 17-AAG in U937 cells. U937 cells were treated with 500 ng/ml DOX, 1000 nM 17-AAG and their combination as indicated. (**a**) After 16 h, Hsp70 and Hsp90 were determined by immunoblot. (**b**) After 16 h, microphotograph of live culture was taken

(Figure 11c). Interestingly, Z-VAD-fmk poorly abrogated the cytotoxicity of DOX and PTX (Figure 11c). Finally, PMA (phorbol ester) induces macrophage-like differentiation of HL60 cells. These differentiated HL60 cells are resting and apoptosis reluctant.[30] Not surprisingly, pre-treatment with PMA prevented the cytotoxicity of both PTX and DOX (Figure 11). Pre-treatment with PMA was less effective against FL, which, by inhibiting transcription, can prevent differentiation.

Discussion

Here we showed that GA blocked the activation of the intrinsic apoptotic pathway and prolonged survival of HL60 cells treated with DOX and some other inhibitors of topoisomerases II and I (daunomycin, etoposide, camptothecin). GA did not protect HL60 cells from inhibitors of transcription ActD and FL. Like ActD, super-high concentrations of DOX (above 2 μM) inhibit transcription; and GA did not protect cells from super-high concentrations of DOX. Yet, such high concentrations of DOX are above therapeutic levels. Noteworthy, that GA did not protect HL60 cells from cisplatin and radiation (data not shown), which damage not only DNA but also proteins (and perhaps require intact Hsp90 functions for cytoprotection). In summary, GA protected against apoptosis-induced DNA-damaging topoisomerase inhibitors. The cytoprotective effect of GA correlated with Hsp70 induction. Both CXH and siRNA Hsp70 abrogated the cytoprotection. Conversely, transfection with Hsp70 rendered HL60 cells resistant to chemotherapy. Since DOX did not induce apoptosis in Hsp70-transfected cells, GA did not affect cytotoxicity of DOX in such cells. Taking together these data indicate that induction of Hsp70 is a main mechanism of cytoprotection by GA and 17-AAG.

In addition, cytoprotection can be achieved by cell-cycle arrest and synthetic caspase inhibitors. First, by causing G2 arrest, pre-treatment with low cytostatic (50 ng/ml) concentrations of DOX effectively protected cells from PTX but not FL and DOX. This further indicates that, unlike PTX, which kills cells strictly in mitosis, the cytotoxicity of both FL and DOX is not cell-phase dependent, at least in apoptosis-prone HL60 cells. Second, caspase inhibitors, such as Z-VAD-fmk, directly inhibit caspases and do not require transcription. Therefore, Z-VAD-fmk inhibited FL-induced apoptosis, whereas GA was absolutely ineffective. In contrast, GA demonstrated superior protection against DOX. In fact, Hsp70 can inhibit late stages of apoptosis, block events downstream from caspase-3.[11] Also, Hsp70 inhibits caspase-independent events and lysosomal-mediated cell death[31] and may be superior as an inhibitor of chemotherapy-induced cytotoxicity. Cytoprotection is clinically useful, only if it is selective, meaning that cancer cells are not protected. For example, cell-cycle-based cytoprotection exploits the differences in cell-cycle regulation between normal and cancer cells.[32] Similarly, caspase inhibitors can protect selectively parental cells but not Pgp-expressing multidrug-resistant cells, which pump Z-VAD-fmk and Z-DEVD-fmk out.[33] Cytoprotection by GA is selective: GA does not protect apoptosis-reluctant cancer and leukemia cells[5,27] including

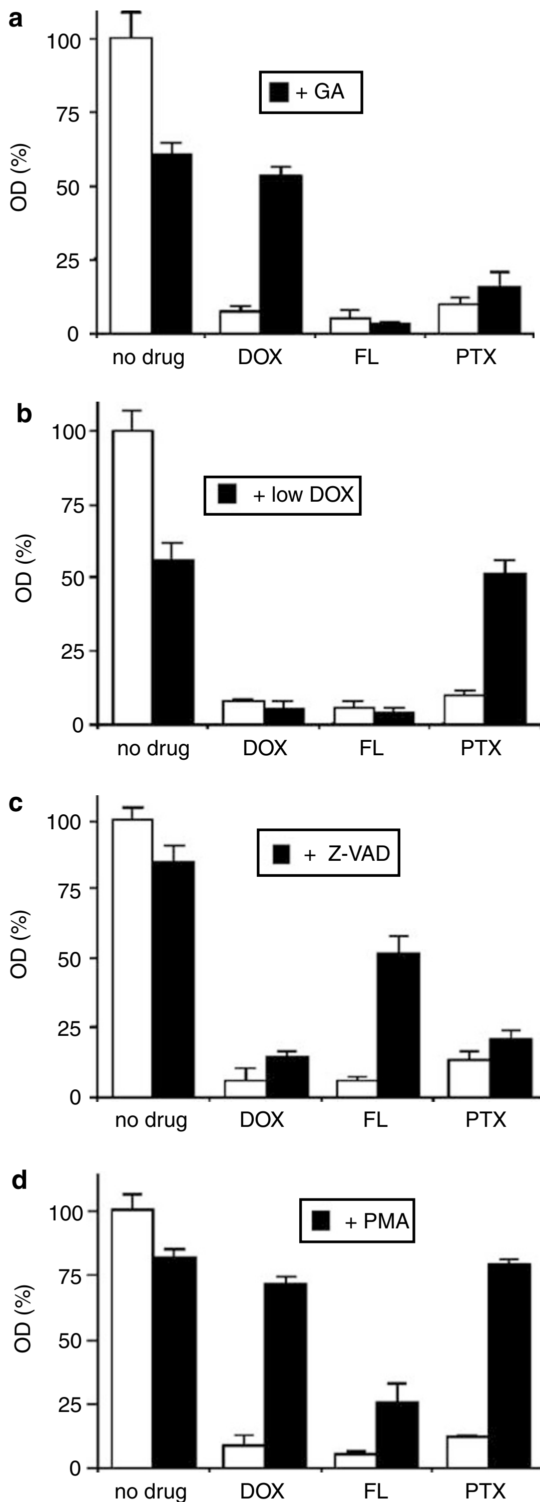

Figure 11 Comparison of cytoprotective activities of GA, low DOX, Z-VAD-fmk and PMA. First, HL60 cells were (**a**) treated with 300 nM GA, (**b**) pretreated for 12 h with 50 ng/ml DOX (low DOX), (**c**) treated with 20 μM Z-VAD-fmk or (**d**) pretreated for 24 h with 50 nM PMA (closed bars). Then, cells were treated with cytotoxic agents: either with 500 ng/ml DOX (DOX), or 500 nM flavopiridol (FL) or 60 nM paclitaxel (PTX). After 36 h, MTT assay was performed as described in Materials and Methods

Hsp70-expressing cells (Figures 8 and 9). Furthermore, effects of GA are both cell-type and drug-sequence dependent.[28] Effects of Hsp70 are also cell-type selective. For example, elevation of heat-shock protein did not attenuate radiation and etoposide-induced apoptosis in lung cancer cell lines.[34] Furthermore, GA can induce apoptosis in oncogene-dependent cancer cells. Numerous antiapoptotic kinases such as Raf-1, EGF-R, ErbB, Akt, Bcr-Abl, depend on Hsp90 for their stability and activity. By promoting degradation of these kinases, GA causes cytostatic and cytotoxic effects.[5,26,35,36] Also, by depleting these oncoproteins, GA sensitizes cancer cells to chemotherapy.[25,37] For example, GA sensitizes Bcr-Abl-transfected HL60 cells to standard chemotherapeutic agents, such as DOX (a topoisomerase-II inhibitor).[5] Our data can explain previously inexplicable observations in the literature. For example, it has been shown (as expected) that the kinase inhibitor herbimycin A sensitized Bcr-Abl-expressing cells to cytotoxicity of topoisomerase II inhibitor (etoposide). Unexpectedly, hebrimycin A protected HL60 and U937 cells from the cytotoxicity of etoposide.[2] This can be explained taking into account that hebrimycin A is an Hsp90-active agent which, like GA, induces Hsp70.[38]

17-AAG and other Hsp90-active agents are currently undergoing clinical trials to treat common cancers.[21,39] It is expected that these agents will be especially effective in drug combinations. In selected types of cancer (oncogene-addictive cancers with Bcr-Abl, EGF-R), a combination of 17-AAG and DOX are synergistic. On the other hand, 17-AAG and DOX may be selectively antagonistic in certain apoptosis-prone normal cells. Most normal cells such as fibroblasts and smooth muscle cells are resistant to chemotherapy. It's toxicity to apoptosis-prone hematopoietic, mucosal, cardiomyocites, colonic epithelial, hair follicules that causes side effects of DOX. There is no adequate model for such apoptosis-prone cells *in vitro*. Therefore, HL60 and U937 cell lines (albeit leukemic) might represent cell types that are protectable by GA. There are three prerequisites for GA-mediated cytoprotection. First, in HL60 cells, basal Hsp70 levels are low and GA induces Hsp70. Second, in HL60 cells, DOX causes caspase-dependent apoptosis that is Hsp70 inhibitable. Third, GA by itself does not cause significant apoptosis in HL60 cells.

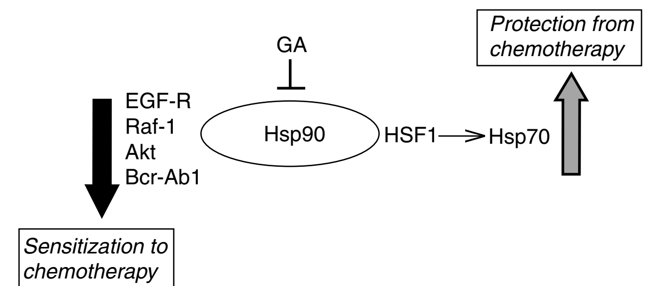

Figure 12 Dual effects of Hsp90-active agents on cell sensitivity to chemotherapy. By binding to Hsp90, GA causes depletion of antiapoptotic kinases (Raf-1, Bcr-Abl, Akt, EGF-R) and induces Hsp70. Therefore, they may simultaneously protect cells from and sensitize cells to chemotherapy. Sensitization is observed in apoptosis-reluctant cancer cells that are dependent on antiapoptotic hsp90 client kinases (Bcr-Abl, Akt). In contrast, in apoptosis-prone cells with low basal levels of Hsp70, GA antagonizes chemotherapy

Noteworthy, GA is considered for therapy of neurodegenerative diseases due to its Hsp70-dependent antiapoptotic effects in neuronal cells.[40] Similarly, we expect that Hsp70-inducing agents may decrease the toxicity of chemotherapy in apoptosis-prone normal (and cancer) cells. Simultaneously, GA may sensitize cancer cells that depend on antiapoptitcic kinase (Bcr-Abl, EGF-R, Akt) to DOX (Figure 12). Finally, DOX can induce nonapoptotic mitotic catastrophe and cell senescence in cancer cells.[41] When mitotic catastrophe and cell senescence is the goal of therapy, then prevention of apoptosis in normal cells may decrease side effects without decreasing therapeutic efficacy of chemotherapy.

Materials and Methods

Cell lines

HL60, U937, K562 human leukemia cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). HL60/Bcr-Abl and HL60/Hsp70 were described previously.[5,8,42,43] Reagents: Taxol was a Bristol-Myers product (Bristol-Myers, Princeton, NJ, USA). FL was obtained from the Development Therapeutics Program (NCI) and was prepared as 10 mM stock solution in DMSO. Cisplatin, camptotecin, FR901228, etoposide, GA and 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) were obtained from the Developmental Therapeutics Program, National Cancer Institute (Bethesda, MD, USA). DOX (Adriamycin), actinomycin D (ActD), cycloheximide (CHX), etoposide and 4,5 dimethyl-2-yl 2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Immunoblot analysis

Cells were lysed and soluble proteins harvested in TNES buffer and proteins resolved as previously described.[5,6] Immunoblot for PARP was performed using rabbit polyclonal anti-human PARP (Upstate Biotechnology, Lake Placid, NY, USA), for Bcr using monoclonal anti-human Bcr antibodies N 2 (Oncogene Science, Calbiochem, San Diego, CA, USA), mouse monoclonal antihuman tubulin and actin (Sigma, St. Louse, MO, USA), rabbit polyclonal anti-human Raf-1 (C12, Santa Cruz, CA, USA), monoclonal anti-human Hsp90 and Hsp70 (Upstate Biotechnology, NY, USA), mouse monoclonal anti-human caspase-9 (Pharmingen, San Diego, CA, USA) and caspase-3 (Transduction Lab., Lexington, KY, USA).

MTT assay

A total of 15 000 leukemia cells were plated in 0.1 ml in 96-well flat bottom plates and then exposed to tested agents (final volume 0.2 ml per well). At the indicated times, 20 μl of 5 mg/ml MTT solution in PBS were added to each well for 4 h. After removal of the medium, 170 μl of DMSO were added to each well to dissolve the formazan crystals. The absorbance at 540 nm was determined using a Biokinetics plate reader (Bio-Tek Instruments, Inc, Winooski, VT, USA). Triplicate wells were assayed for each condition and S.D. will be determined.

Flow cytometry

Cells were harvested, washed with PBS and resuspended in 75% ethanol in PBS and kept at 4°C for at least 30 min. Cells were resuspended and incubated for 30 min in propidium iodide staining solution containing 0.05 mg/ml propidium iodide (Sigma), 1 mM EDTA, 0.1% Triton-X-100 and 1 mg/ml RNAse A in PBS. The suspension was then analyzed on a Becton Dickinson FACScan. DNA content frequency histograms were measured using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). To calculate percentage of cells in respective phases of the cell cycle the DNA content frequency histograms were deconvoluted using the MultiCycle software (Phoenix Flow Systems, San Diego, CA, USA).

In situ DNA strand break labeling (TUNEL assay)

Cells were rinsed with PBS, fixed in 1% methanol-free formaldehyde for 15 min at room temperature and stored in 70% ethanol at −20°C for at least 1 h. The cells were then rinsed twice with PBS for 5 min. DNA strand break labeling was performed using the APO-BRDU kit provided by Phoenix Flow Systems (San Diego, CA, USA). After washing with PBS, cells were stained with propidium iodine (5 μg/ml PI) dissolved in PBS containing RNase A, for 20 min. Cellular fluorescence was measured using a FACScan flow cytometer (Becton-Dickinson).

Analysis of cell viability

Cells were incubated with Trypan blue and then blue (dead) cells and transparent (live) cells were counted in a hemocytometer.

Nuclear fragmentation assay

Cells were fixed with 90% ethanol with 10% glacial acid and stained with DAPI as described previously.[5] Nuclei were visualized under UV microscopy.

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