Schedule-Dependent Synergy between the Heat Shock Protein 90 Inhibitor 17-(Dimethylaminoethylamino)-17-Demethoxygeldanamycin and Doxorubicin Restores Apoptosis to p53-Mutant Lymphoma Cell Lines

Ana I. Robles,1 Mollie H. Wright,1 Bheru Gandhi,1 Steven S. Feis,1 Christin L. Hanigan,1 Adrian Wiestner,2 and Lyuba Varticovski1

Abstract

Purpose: Loss of p53 function impairs apoptosis induced by DNA-damaging agents used for cancer therapy. Here, we examined the effect of the heat shock protein 90 (HSP90) inhibitor 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (DMAG) on doxorubicin-induced apoptosis in lymphoma. We aimed to establish the optimal schedule for administration of both drugs in combination and the molecular basis for their interaction.

Experimental Design: Isogenic lymphoblastoid and nonisogenic lymphoma cell lines differing in p53 status were exposed to each drug or combination. Drug effects were examined using Annexin V, active caspase-3, cell cycle, and cytotoxicity assays. Synergy was evaluated by median effect/combination index. Protein expression and kinase inhibition provided insight into the molecular mechanisms of drug interaction.

Results: Presence of mutant p53 conferred increased survival to single agents. Nevertheless, DMAG showed synergistic toxicity with doxorubicin independently of p53 status. Synergy required exposure to doxorubicin before DMAG. DMAG-mediated down-regulation of CHK1, a known HSP90 client, forced doxorubicin-treated cells into premature mitosis followed by apoptosis. A CHK1 inhibitor, SB-218078, reproduced the effect of DMAG. Administration of DMAG before doxorubicin resulted in G1-S arrest and protection from apoptosis, leading to additive or antagonistic interactions that were exacerbated by p53 mutation.

Conclusions: Administration of DMAG to doxorubicin-primed cells induced premature mitosis and had a synergistic effect on apoptosis regardless of p53 status. These observations provide a rationale for prospective clinical trials and stress the need to consider schedule of exposure as a critical determinant of the overall response when DMAG is combined with chemotherapeutic agents for the treatment of patients with relapsed/refractory disease.

The DNA damage response pathway is a surveillance network that halts the cell cycle in the presence of damaged DNA to prevent genomic instability (1). This signal transduction network is composed of DNA damage sensors that recruit phosphatidylinositol 3-kinase–like proteins, including ataxia telangietasia mutated (ATM) and ATM and Rad3-related (ATR), which in turn phosphorylate and activate effector kinases, such as the serine-threonine kinases CHK1 and CHK2. A complex cascade of phosphorylation events further activates proteins involved in cell cycle control, DNA repair, and transcriptional regulation (1, 2). As a result, the cell cycle is delayed and DNA repair or apoptosis ensues. A key downstream target of DNA damage checkpoint activation is the p53 transcription factor. TP53 is a tumor suppressor gene that is functionally inactivated in more than half of human cancers through gene deletion or mutation (3, 4). The p53 protein is normally expressed at low levels, but on exogenous (radiation, chemotherapeutic agents) or endogenous (free radicals) DNA damage, it becomes stabilized and activated as a transcription factor. Depending on cellular context and the level of DNA damage, p53 activation leads to sustained cell cycle arrest, senescence, and/or apoptosis (5, 6). Cells that express wild-type (WT) p53 undergo G1-S and G2-M arrest after DNA damage. Cells that express mutant p53 or are p53-null bypass G1-S arrest and must rely on the G2-M checkpoint to undergo DNA repair (2). It has been proposed that the transient arrest in G2-M is essential for recovery from genotoxic stress and that its abrogation may...
result in sensitization of p53-deficient tumor cells to DNA-damaging agents and radiation (7, 8). In fact, studies of drug combinations using the protein kinase inhibitor UCN-01 (7-hydroxyauristosporine, NSC 638850) have shown that exposure to UCN-01 increases chemotheraphy-induced apoptosis in cells with disrupted p53 function through abrogation of the G2 checkpoint (9, 10). Although UCN-01 was originally identified as a protein kinase C inhibitor, it also blocks CHK1 activity, and this is thought to result in loss of G2 checkpoint control and increased cell death of p53-defective cells (8). Clinical trials are currently under way using UCN-01 alone and in combination with other agents (11, 12).

Heat shock protein 90 (HSP90) is a major molecular chaperone involved in the conformational folding of many cellular proteins, collectively referred to as “client proteins” (13). HSP90 client proteins include a wide variety of signal-transducing regulators of cell growth and differentiation. The binding of HSP90 to client proteins in a multiprotein chaperone complex is ATP dependent and serves a dual function. In the presence of ATP, HSP90 aids proper folding and stabilizes proteins, whereas, in its absence, unfolded client proteins are targeted for degradation through the proteasome pathway (14). HSP90 inhibitors, the naturally occurring ansamycin antibiotic geldanamycin (NSC 122750) and its analogues, 17-AAG (17-allylamino-17-demethoxygeldanamycin, NSC 330507) and DMAG (17-(dimethylaminoethylamino)-17-demethoxygeldanamycin, NSC 707545), block the ATP-binding pocket of HSP90 and inhibit the essential ATPase activity, leading to destabilization and eventual degradation of HSP90 client proteins. It is becoming apparent that inhibition of HSP90 might be a viable option for the treatment of cancer (15). Binding of HSP90 to 17-AAG is 10–100-fold higher in cancer cells than in normal cells. Moreover, cancer cells seem to be particularly dependent on growth-promoting and survival signal transduction pathways that are affected by the inhibition of HSP90. This is exemplified by the sensitization of BCR/ABL–positive leukemia cells through HSP90 inhibition (16, 17). Other potential tumor targets include those driven by so-called “oncogene addiction,” a term that describes tumor cells that are more sensitive to blockage of constitutively active growth-promoting pathways than normal cells. From a broader perspective, down-regulation of multiple signal-transducing pathways of cell growth and differentiation by HSP90 inhibitors may provide an advantage over single-target agents for treatment of tumors bearing multiple oncogenic mutations (18). 17-AAG and DMAG are effective, alone or in combination with other agents, in preclinical models of cancer (19–21) and are currently in phase I/II clinical trials (22–25). Because HSP90 inhibitors have the potential to affect many cellular pathways, the mechanism by which they exert antitumor activity is complex and not well understood.

Doxorubicin is a topoisomerase inhibitor that intercalates into DNA to induce DNA damage and oxidative stress, which result in cell cycle arrest and apoptosis (26). Treatment for patients with non-Hodgkin’s lymphoma is based on doxorubicin-containing combination chemotherapy, of which cyclophosphamide-Adriamycin-vincristine-prednisone (Oncovin) regimen (cyclophosphamide, doxorubicin, vincristine, and prednisone) is most frequently used (27). Mutations in TP53 in patients with non-Hodgkin’s lymphoma are associated with a more aggressive clinical course (28) and relapse (29).

It has been proposed that the best way to exploit the novel mechanism of action of HSP90 inhibitors is by combination with conventional cytotoxic drugs or other molecularly targeted agents (14). We found that the schedule of drug administration determines the efficacy of HSP90 inhibitors when combined with the DNA-damaging agent doxorubicin. Because clinical trials will likely include patients with relapsed/refractory disease, many of whom will bear mutations in TP53, we investigated how the lack of functional p53 affects the response. We found that clinically achievable concentrations of DMAG abrogated CHK1 expression and doxorubicin-induced G2/M arrest. Addition of DMAG to doxorubicin-primed cells had a synergistic effect on survival, regardless of p53 status, and sensitized p53-deficient cells to doxorubicin.

Materials and Methods

Cell lines. TK6, NH32, and WTK1 are EBV-immortalized human lymphoblastoid cell lines, with a population doubling time of 14 to 18 hours (30). TK6 and WTK1 were provided by Dr. Howard Liber (Colorado State University, Fort Collins, CO) and NH32 was provided by Dr. E. Chuang (National Cancer Institute). BJAB, SUDHL-4, OCI-Ly3, and OCI-Ly10 are non-Hodgkin’s lymphoma cell lines. OCI-Ly3 and OCI-Ly10 are ABC-subtype diffuse large B-cell lymphoma cell lines (WT TP53) and were provided by Dr. Louis Staudt (National Cancer Institute, Bethesda, MD). BJAB is a Burkitt-like lymphoma cell line that carries a His193Arg mutation in TP53 (31). It has a population doubling time of ~36 hours (data not shown). We found no record of TP53 status of the diffuse histiocytic lymphoma cell line SUDHL-4. All cell lines were maintained at 5 × 10^5 to 10 × 10^5 cells/mL in RPMI 1640, supplemented with 10% fetal bovine serum, glutamine, and 1% penicillin-streptomycin in a humidified 37°C incubator with 5% CO2.

Drugs. Doxorubicin was obtained from Sigma (St. Louis, MO) and dissolved in PBS as 10 mmol/L stock aliquots that were frozen and diluted in media immediately before use. The HSP90 inhibitor geldanamycin and its analogues, 17-AAG and DMAG, were obtained from Cancer Therapy Evaluation Program under Material Transfer Agreement with Kosan Biosciences. The indolocarbazole derivative SB-218078 (Calbiochem, San Diego, CA) acts as a potent inhibitor of CHK1 (32). HSP90 inhibitors and SB-218078 were dissolved in DMSO as 10 mmol/L stock aliquots that were frozen and diluted in media immediately before use.

Growth inhibition assay. Cells were seeded at 20,000 per well in 96-well plates. Serial dilutions of doxorubicin and/or HSP90 inhibitors in medium were added by sextuplicate. Dose–response curves to single drugs at 24 and 48 hours after exposure were generated to determine the range of concentrations to be used in combination. For drug interaction studies, drugs were sequentially added (with the second compound introduced with a 24-hour delay) in a final volume of 100 μL. Cytotoxicity was measured using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI), a colorimetric method for determining the number of viable cells based on bioreduction of a tetrazolium compound [3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)] by metabolically active cells. After 24 or 48 hours of exposure to a single drug or after a total of 48 hours in sequential addition experiments, 20 μL of MTS reagent were added to each well and the plates were incubated in a humidified 37°C incubator with 5% CO2 for 1 to 4 hours. Absorbance at 490 nm was recorded using a 96-well plate reader. For consistency across experiments and to ensure a linear response between cell number and absorbance, the background-corrected target absorbance value for untreated cells was kept at 0.9 to 1.0 in all plates. Data were averaged and normalized against the nontreated controls to generate dose-response curves.
Drug interaction analysis. The effects of drug combinations were evaluated using CalcuSyn (Biosoft, Ferguson, MO), a software based on the multiple drug-effect equation of Chou-Talalay (33). This method defines the expected additive effect of agents used in combination and then quantifies synergy or antagonism by determining how much the combination effect differs from the expected additive effect. Values of fraction affected (Fa) were calculated from cytotoxicity assays on 96-well plates using the following formula:

\[
Fa = 1 - \frac{\text{background} - \text{corrected Abs490 treated}}{\text{background} - \text{corrected Abs490 untreated}}
\]

These values represent the level of effect or survival loss achieved by each drug or drug combination. Such actual experimental data were entered into the CalcuSyn interface and used to calculate combination index (CI) values, a quantitative measure of the degree of drug interactions for a corresponding Fa. Serial CI values over an entire range of drug-effect levels (Fa) were then calculated. These data were used to generate Fa-CI plots, from which synergy or antagonism can be identified. Additivity, synergy, and antagonism are defined as CI = 1, CI < 1, and CI > 1, respectively.

Flow cytometry. Induction of apoptosis was evaluated through quantification of several variables by flow cytometry. Binding of phosphatidyl-serine residues to Annexin V-FITC is indicative of early changes in cell membrane composition (Oncogene Research Products, Cambridge, MA). Positive staining with phycocerythrin-conjugated anti–active caspase-3 antibody (measured by BD Biosciences, San Jose, CA) is indicative of caspase activation. Presence of cells with fragmented DNA is a later indicator of apoptosis. Annexin V and caspase-3 assays were done according to instructions from the manufacturers. For DNA analysis, cells were washed in ice-cold PBS and fixed in 70% ethanol. Fixed cells were centrifuged at 1,200 rpm for 5 minutes and stained by incubation in PBS containing 50 ng/µL propidium iodide (Sigma) and 50 µg/mL DNase-free RNase (Roche, Indianapolis, IN).

Histone H3 phosphorylation was evaluated using a phospho-specific antibody essentially as described (34). Briefly, ethanol-fixed cells were permeabilized in 0.25% Triton X-100/PBS on ice for 15 minutes, followed by blocking in 1% bovine serum albumin/PBS for 10 minutes at room temperature. Cells were exposed to phospho-histone-H3 (Ser10) antibody (Cell Signaling Technology, Danvers, MA) at 1:25 dilution or immunoglobulin G control in 1% bovine serum albumin/PBS for 30 minutes at room temperature. On washing, cells were exposed to FITC-conjugated goat anti-mouse antibody for 30 minutes in the dark. Finally, they were washed and counterstained by exposure to FITC-conjugated goat anti-mouse antibody for 30 minutes in the dark. Finally, they were washed and counterstained by

Immunoblotting. Cell pellets were washed with ice-cold PBS and lysed on ice in 50 mMol/L HEPES buffer containing 1% NP40, 150 mMol/L NaCl, 0.5 mMol/L EDTA (pH 8), 10% glycerol, 5 mMol/L sodium orthovanadate (pH 10), 10 mMol/L sodium fluoride, 2 mMol/L sodium pyrophosphate, and a protease inhibitor cocktail (Roche). After a 20-minute incubation, lysates were centrifuged at 14,000 rpm for 10 minutes, and the protein content of the supernatants was determined using a Bradford-based protein assay kit (Bio-Rad, Hercules, CA). Aliquots of total cell protein (40-50 µg/lane) were denatured by boiling in loading buffer and loaded onto 8% or 10% gradient SDS-polyacrylamide gels (Invitrogen, Carlsbad, CA), depending on the sizes of target proteins. Proteins were separated by electrophoresis at 125 V and then electrotransferred to nitrocellulose membranes at 30 V for 2 hours. Membranes were blocked at room temperature for 1 hour in TBS/0.05% Tween 20 (TBS-T) containing 5% nonfat dry milk (monoclonal antibodies) or 5% bovine serum albumin (polyclonal antibodies) and exposed to primary antibodies overnight at 4°C in blocking solution, with gentle rocking. The following commercial primary antibodies were used: p53 (Ab-6) and actin (Ab-1) from Oncogene Research Products; CHK2 from Stressgen Biotechnologies (Victoria, BC); survivin from Novus Biologicals (Littleton, CO); CHK1, phospho-specific CHK1(S317), phospho-specific CHK2(T68), phospho-specific p53(S15), HSP70, HSP90,cdc25C, phospho-specific cdc25C(S216), AKT, and cleaved poly(ADP)ribose polymerase from Cell Signaling Technology. Membranes were washed twice in TBS-T and incubated with species-specific horseradish peroxide–labeled secondary antibodies in TBS-T for 1 hour. Finally, the membranes were washed in TBS-T three times and the proteins were visualized by enhanced chemiluminescence (Amersham, Piscataway, NJ).

Statistical analysis. Survival and apoptosis data are expressed as means ± SD and means ± SE, respectively. Statistical analysis was done with two-tailed Student’s t test.

Results

Functional status of p53 modulates the response to doxorubicin and HSP90 inhibitors. We have previously shown that doxorubicin-induced apoptosis is dependent on functional p53 using p53-isogenic lymphoblastoid cell lines (26). TK6 cells express WT p53. NH32 is a subclone of TK6 with a TP53-targeted deletion and is p53-null (p53−/−). WTK1 was derived from the same donor but expresses mutant p53 due to a homozygous inactivating Met237Ile mutation in TP53 leading to protein accumulation and loss of transactivation (MT p53).

Doxorubicin exposure resulted in loss of cell viability in cells with WT p53 TK6 cells with IC50 ≈ 200 nmol/L, whereas IC50 > 2 µmol/L was observed for p53−/− NH32 or MT p53 WTK1 cells (Fig. 1A). A similar trend was observed in lymphoma cell lines (Fig. 1B), with IC50 ≈ 200 nmol/L for OCI-Ly3 and OCI-Ly10 (WT p53) and IC50 > 3 µmol/L for BIAB and SUDHL-4 (MT p53; Fig. 1B). A consistent reduction in apoptotic markers, including staining with Annexin V-FITC, and cleavage of poly(ADP)ribose polymerase, a surrogate marker for caspase-3 activation, were observed in p53−/− and MT p53 cells (Supplementary Fig. S1). Doxorubicin exposure also resulted in p53 protein phosphorylation and accumulation in WT p53 TK6 cells (Supplementary Fig. S1B). Analysis of p53 protein expression after exposure of SUDHL-4 cells to doxorubicin showed a high basal level of p53 that was not increased by DNA damage (Supplementary Fig. S1C), consistent with a p53-mutant phenotype.

The presence of mutant p53 led to enhanced survival in response to DMAG (Fig. 1C and D). Interestingly, lymphoblastoid cells were similarly sensitive to geldanamycin and DMAG, but 10-fold higher doses of 17-AAG were required to achieve similar toxicities (Supplementary Fig. S2). These results are in agreement with preclinical studies (21, 35) and indicate that DMAG may have greater bioavailability than 17-AAG. Based on this information, we did most of the experiments described below using DMAG.

Schedule-dependent synergy of DMAG and doxorubicin.

We evaluated the effect of each drug and their combination on the cell cycle. Low individual doses of both drugs were selected from dose-response curves (Fig. 1) aiming for >75% viability after 24-hour exposure in all cell lines. The same low doses of 30 nmol/L doxorubicin and 100 nmol/L DMAG were chosen for these experiments. Exposure to 100 nmol/L DMAG resulted in G1-S arrest within 24 hours in lymphoblastoid and lymphoma cell lines regardless of p53 status (Fig. 2A). These observations are consistent with previously reported loss of pRb...
phosphorylation and G1 arrest in breast cancer cells after treatment with 17-AAG (36).

A 24-hour exposure to 30 nmol/L doxorubicin led to 75% to 80% viability in WT p53 cell lines and >90% viability in p53/C0/C0 and MT p53 cell lines. This modest effect on viability was associated with major p53-dependent differences in cell cycle distribution. WT p53 TK6 and OCI-Ly3 cells accumulated transiently with 2N and 4N DNA content (corresponding to G1-S and G2-M arrest, respectively) within 24 hours. After 48 hours, 15% (TK6) and 30% (OCI-Ly3) cells presented with sub-G1 DNA content, indicative of DNA fragmentation and apoptosis. In contrast, p53/C0/C0 (NH32) and MT p53 (WTK1 and BJAB) cells showed no G1-S arrest, rapid arrest in G2-M, and a negligible increase in the fraction of cells with sub-G1 DNA content after 48 hours (Fig. 2A and Supplementary Fig. S3).

When 100 nmol/L DMAG was added to doxorubicin-exposed cells after a 24-hour delay, we observed loss of cells with G2-M DNA content and appearance of cells with fragmented sub-G1 DNA content, regardless of p53 status (Fig. 2A). In contrast, accumulation of cells in G1-S following exposure to DMAG was not altered by subsequent treatment with doxorubicin (Fig. 2B), indicating that sustained G1-S cell cycle arrest induced by DMAG was protective from apoptosis. These data suggest that the outcome of DMAG and doxorubicin combinations could be dependent on the schedule of exposure and that doxorubicin toxicity could be potentially enhanced through bypass of G2-M checkpoint control promoted by DMAG.

**DMAG enhances doxorubicin toxicity in a schedule-dependent manner.** Next, we examined whether addition of DMAG to doxorubicin-primed cells would result in a lower IC50 for doxorubicin in lymphoma cell lines. To this end, 100 nmol/L DMAG was applied to cells that had been exposed for 24 hours to serial dilutions of doxorubicin. Survival was assessed by MTS assay after a total of 48 hours. We found that sequential addition of DMAG to doxorubicin-treated BJAB cells resulted in a 5-fold reduction in IC50 for doxorubicin, from 200 nmol/L (48-hour dose-response curve) to 40 nmol/L (Fig. 3A).

To evaluate the role of schedule of exposure as a determinant of the overall response, we analyzed the interaction of doxorubicin and DMAG on cell survival using a standard criteria for synergy, the median effect/combination index method (33). Survival was measured by sextuplicate using standard MTS assay. CI values were calculated from experimental data points and a Fa-CI plot was constructed by simulating CI values over the entire range of Fa values from 5% to 95% using CalcuSyn software. Additivity, synergism, and antagonism are defined as CI = 1, CI < 1, and CI > 1, respectively. This software required a selection of a constant ratio for calculation of CI. Because we observed close to 80% viability in all cell lines on exposure to either 30 nmol/L
doxorubicin or 100 nmol/L DMAG, we selected a constant 1:3 molar ratio doxorubicin/DMAG for calculation of CI. Addition of DMAG to doxorubicin-treated cells (doxorubicin→DMAG) was synergistic over an entire range of drug-effect levels, with CI = 0.4 at Fa = 0.5 indicative of a highly synergistic interaction (Fig. 3B, open circles). Exposure to DMAG before doxorubicin (DMAG→doxorubicin) not only showed lack of synergy but also resulted in an antagonistic effect (Fig. 3B, closed circles) with CI > 2.0 at Fa = 0.5. The antagonistic effect of DMAG was more evident at lower drug concentrations (Fa < 0.5), most likely relevant for use in vivo (21). Simultaneous administration of DMAG and doxorubicin resulted in additive or antagonistic effects as well (data not shown).

To extend our observations, we exposed three other human lymphoma cell lines, SUDHL-4, OCI-Ly10, and OCI-Ly3, to single drugs or combinations of doxorubicin and DMAG. The drug molar ratio was maintained at 1:3 doxorubicin/DMAG, although one set of experiments was carried out at a 1:1 doxorubicin/DMAG molar ratio as well. We found that the sequential addition of DMAG to doxorubicin-treated cells was again highly synergistic (Table 1), with CI = 0.3 to 0.7 at Fa = 0.5. Schedule-dependent synergy was observed for both WT p53 (OCI-Ly10 and OCI-Ly3) and MT p53 (BJAB and SUDHL-4) lymphoma cell lines. Similar schedule-dependent synergy was observed when isogenic lymphoblastoid cell lines were used (Table 1). Reversal in the order of drug exposure resulted in nearly additive or antagonistic effects (CI = 0.8-5 at Fa = 0.5). The highest degree of antagonism was observed in MT p53 cell lines BJAB and SUDHL-4.

**HSP90 client protein expression in response to single drugs and drug combinations.** To begin to examine the molecular basis for schedule-dependent synergy and antagonism between doxorubicin and DMAG, we analyzed the expression of heat shock proteins and HSP90 clients after exposure of MT p53

---

**Fig. 2.** The effect of combination treatment with doxorubicin and DMAG on cell cycle progression depends on the schedule of administration. A. Lymphoblastoid and lymphoma cell lines were exposed to 30 nmol/L doxorubicin for 24 hours before addition of 100 nmol/L DMAG and harvested 24 hours later for cell cycle analysis by staining with propidium iodide. B. BJAB cells were exposed to 100 nmol/L DMAG followed by 30 nmol/L doxorubicin with a 24-hour delay and harvested for cell cycle analysis. Percentages of cells with sub-G1 (fragmented DNA), G0-G1 (2N), and G2-M (4N) DNA content are indicated.
BJAB cells to single agents and their combinations. Consistent with earlier studies (35), exposure to DMAG effectively inhibited HSP90, as evidenced by induction of HSP70, with a minor effect on expression of HSP90 itself (Fig. 3C). The extent of down-regulation of HSP90 client proteins was dependent on the length of exposure to DMAG. Specifically, loss of expression of AKT, cdc25C, and CHK1 was observed as early as 24 hours on initial exposure, whereas loss of expression of survivin was only observed after 48 hours of exposure, and expression of mutant p53 remained unchanged (Fig. 3C and D).

Signal transduction pathways activated in response to doxorubicin exposure involve ATM-dependent phosphorylation of the checkpoint kinases CHK1 and CHK2 (37). In the absence of functional p53, these kinases are critical for maintaining G2-M arrest. Consistently, we found that CHK1 and CHK2 were phosphorylated on Ser317 and Thr68, respectively, on exposure to doxorubicin (Fig. 3D). When DMAG was added after doxorubicin, the levels of total and phosphorylated CHK1 were substantially reduced, whereas only a small decrease was observed in CHK2 activation (Fig. 3D). These results indicate that synergy between DMAG and doxorubicin is associated with loss of G2-M checkpoint control through the combined effect of HSP90 inhibition on the stability of CHK1 and possibly other HSP90 client proteins that participate on cell cycle control after DNA damage.

**The apoptotic response to doxorubicin is enhanced by DMAG in a schedule-dependent manner.** Doxorubicin intercalates into DNA to induce DNA damage and oxidative stress that result in cell cycle arrest and apoptosis (26). Therefore, we examined whether the combination with DMAG resulted in a schedule-dependent increase in the apoptotic response of lymphoma cells to doxorubicin. Results from the quantification of three independent apoptotic markers are shown (Fig. 4). The earliest apoptotic marker studied was Annexin V (Fig. 4A). Exposure to 100 nmol/L DMAG alone resulted in 13% apoptosis at 24 hours and 27% at 48 hours. Exposure to 30 nmol/L doxorubicin alone resulted in 15% apoptosis at 24 hours and 36% at 48 hours. Addition of DMAG to doxorubicin-primed cells with a 24-hour delay (doxorubicin → DMAG) resulted in 64% apoptosis, which is higher than expected for an additive response, and consistent with the schedule-dependent synergy observed in survival assays. In contrast, exposure to DMAG for 24 hours before doxorubicin (DMAG → doxorubicin) resulted in 32% apoptosis, which is less than expected for an additive response. Similar results were obtained for caspase-3 activation (Fig. 4B). Exposure to 100 nmol/L DMAG alone resulted in 6% and 10% caspase-3 activation at 24 and 48 hours, respectively. Exposure to 30 nmol/L doxorubicin alone resulted in 15% and 36% caspase-3 activation at 24 and 48 hours, respectively. Addition of DMAG to doxorubicin-primed cells (doxorubicin → DMAG) resulted in 55% caspase-3 activation, which is higher than expected for an additive response, whereas the reverse schedule (DMAG → doxorubicin) resulted in 14% caspase-3 activation, which is the expected value for an additive interaction. The fraction of cells with sub-G1 DNA content is shown (Fig. 4C). DNA fragmentation is the latest marker of
apoptosis and lower overall values are anticipated. DNA fragmentation values are consistent with data obtained with Annexin V and caspase-3.

DMAG induces bypass of the G2-M checkpoint and premature mitosis. Altogether, our data indicate that addition of DMAG to doxorubicin-primed cells enhances doxorubicin toxicity by forcing cells into premature mitosis in the presence of unrepaired DNA damage. To test this hypothesis, we costained single-drug–treated and doxorubicin–DMAG–treated BJAB cells with an antibody specific for the phosphorylated form of histone H3 and the DNA marker propidium iodide. Histone H3 is phosphorylated exclusively during mitosis, and an antibody that specifically recognizes its phosphorylated form has been used to identify mitotic cells and distinguish them from G2 cells in a flow cytometric assay (34). In this assay, the fraction of cells that incorporates propidium iodide with a G2 DNA content and costains with anti–phospho-histone H3 antibody is considered to be at the onset of mitosis. Expression of phospho-histone H3 was <1% in control or single-drug–treated cells with G2 DNA content, but it increased to 18% when DMAG was added to doxorubicin-treated cells (Fig. 5A). This data is consistent with abrogation of the doxorubicin-induced G2-M checkpoint and premature mitotic entry.

Inhibition of CHK1 sensitizes lymphoma cells to doxorubicin. Depletion of the HSP90 client CHK1 by 17-AAG has previously been linked to increased sensitivity to DNA damage

<p>| Table 1. Schedule-dependent synergy of DMAG and doxorubicin |
|----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Cell line</th>
<th>p53 status</th>
<th>Ratio doxorubicin/DMAG</th>
<th>CI at IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>BJAB</td>
<td>Mutant</td>
<td>1:3</td>
<td>0.4 ± 0.1*</td>
</tr>
<tr>
<td>SUDHL-4</td>
<td>Mutant</td>
<td>1:3</td>
<td>0.4</td>
</tr>
<tr>
<td>OCIs</td>
<td>Mutant</td>
<td>1:1</td>
<td>0.5</td>
</tr>
<tr>
<td>OCI-Ly3</td>
<td>Wild-type</td>
<td>1:3</td>
<td>0.3 ± 0.1*</td>
</tr>
<tr>
<td>OCI-Ly10</td>
<td>Wild-type</td>
<td>1:3</td>
<td>0.7 ± 0.2*</td>
</tr>
<tr>
<td>TK6</td>
<td>Wild-type</td>
<td>1:3</td>
<td>0.3</td>
</tr>
<tr>
<td>NH32</td>
<td>Null</td>
<td>1:3</td>
<td>0.4</td>
</tr>
<tr>
<td>WTK1</td>
<td>Mutant</td>
<td>1:3</td>
<td>0.4</td>
</tr>
</tbody>
</table>

*Expressed as mean ± SD from three independent experiments.

Fig. 4. The apoptotic response to doxorubicin is modulated by DMAG in a schedule-dependent manner. BJAB cells were exposed to 30 nmol/L doxorubicin or 100 nmol/L DMAG alone for 24 or 48 hours or to combinations in which the second drug was added with a 24-hour delay. Cells were harvested at indicated times and the frequency of three independent apoptotic markers was analyzed in parallel by flow cytometry. Dot plots depict Annexin V–FITC binding (A) and activation of caspase-3 (B). C, histograms depict the presence of cells with sub-G1 DNA content as detected by labeling with propidium iodide. The percentage of cells that is positive for each marker is indicated in the respective graphs.
We hypothesized that depletion of active CHK1 on exposure to DMAG might mediate sensitization of lymphoma cells to doxorubicin. To test this hypothesis, we exposed BJAB cells to increasing concentrations of the CHK1 inhibitor SB-218078 (32), added 24 hours after doxorubicin. Exposure to SB-218078 alone in concentrations of up to 5 μmol/L produced negligible toxicity but significant G1-S arrest (Fig. 5B). Addition of increasing concentrations of SB-218078 to doxorubicin-treated cells caused a dose-dependent bypass of G2-M arrest and a concomitant dose-dependent increase in cells with fragmented DNA (Fig. 5B). Although expression and phosphorylation of CHK2 were unaffected by SB-218078, dose-dependent losses of CHK1 and phosphorylated CHK1 and of the CHK1 target cdc25C and its phosphorylated form were observed (Fig. 5C). Cleavage of the caspase-3 substrate poly(ADP)ribose polymerase was also consistent with apoptosis (Fig. 5C). These results indicate that inhibition of CHK1 results in sensitization of lymphoma cells to doxorubicin and support our hypothesis that CHK1 is a critical target of DMAG-mediated synergy.

Discussion

Our study addresses an important variable in clinical trial design that is frequently empirically defined: how drug schedule modulates the effect of DNA-damaging agents used in combination with molecularly targeted drugs. We found that synergy between doxorubicin and the HSP90 inhibitor DMAG was schedule dependent and required pretreatment with doxorubicin for 24 hours before the addition of DMAG. The combination of both drugs in this sequence enhanced the sensitivity of MT p53 cells to doxorubicin. Reversing the order of drug administration resulted in antagonistic effect in MT p53 cells and only additive toxicity in WT p53 cells. These observations are particularly important for the design of phase II clinical trials, which include patients with relapsed/refractory disease, frequently bearing MT p53.

It has been reported that HSP90 inhibitors sensitize cancer cells to conventional therapies, including Taxol (41), gemcitabine (38), cytarabine (40, 42), cisplatin (42), and radiation (19, 43), and enhance apoptosis induced by molecularly targeted agents, such as imatinib (44, 45), SN38 (39), and histone deacetylase inhibitors (46). It has been noted that cell type and drug sequence influence the effect of HSP90 inhibition on the activity of cytotoxic drugs. HSP90 inhibitors can be cytoprotective when administered together with or before chemotherapeutic agents, such as Taxol. It has been speculated that induction of G1 arrest after exposure to HSP90 inhibitors leads to the cytoprotective effect when cells are pretreated with HSP90 inhibitors. Addition of 17-AAG to Taxol-treated cells increased apoptosis, whereas exposure to 17-AAG before Taxol led to G1 arrest and protection from apoptosis in breast cancer cell lines (41). Similarly, we found that exposure to DMAG resulted in sustained G1 arrest that protected lymphoma cells from subsequent treatment with doxorubicin. The cytoprotective effect of HSP90 inhibitors may also be mediated by HSP70 (47), presumably through its binding to apoptotic factors, leading to hindrance of one or more steps in the apoptotic cascade (48). Induction of HSP70 has been observed in many cell types following inhibition of HSP90 and is believed to be a consequence of transcriptional activation of heat shock factor 1 (49). In our study, HSP70 was induced on
exposure to DMAG regardless of sequence of administration, and its expression was not associated with the overall response to drug combinations. We found in preliminary studies that simultaneous administration of doxorubicin and HSP90 inhibitors did not lead to synergistic toxicity (data not shown). Thus, we have no evidence that HSP70 exerts protection from apoptosis in lymphoma cells.

It seems that the key to optimizing synergistic interactions between DNA-damaging agents, such as doxorubicin, and HSP90 inhibitors is to understand their effect on the signal transduction pathways involved in cell cycle control. Many studies have addressed the role of p53 activation in response to radiation and chemotherapy (50–52). The signal transduction pathways activated by doxorubicin are well characterized (37) and evident in lymphoma cells used in this study. Doxorubicin induced G_1-S and G_2-M arrest followed by apoptosis in WT p53 cells, whereas MT p53 cells accumulated in G_2-M. Because HSP90 has numerous cellular clients (18), blockade of multiple signal transduction pathways could contribute to sensitization by HSP90 inhibitors. This complexity has hindered the understanding of molecular mechanisms of interaction. However, different client proteins exhibited different kinetics of response to HSP90 inhibition. Although it has been described as a HSP90 client (53), we did not observe loss of expression of MT p53 on exposure to DMAG. Survivin, a protein involved in inhibition of mitochondrial apoptosis and regulation of mitosis, has also been described as a HSP90 client (54), and its degradation following 17-AAG has been associated with sensitization of pediatric leukemia to imatinib (44). In our study, degradation of survivin required 48 hours of exposure to DMAG and did not contribute to synergy observed in the doxorubicin–DMAG combination. Furthermore, loss of expression of survivin observed in the DMAG–doxorubicin combination did not lead to increased apoptosis, indicating that survivin may not be a critical target for DMAG-mediated sensitization in lymphoma. In contrast, degradation of other HSP90 clients, including AKT, CHK1, and cdc25C, was evident within 24 hours of exposure to DMAG and correlated with increased toxicity drug combination. In this regard, CHK1, a key transducer of G_2-M-phase arrest in response to DNA damage (2), has previously been implicated in the sensitization to chemotherapeutic agents by HSP90 inhibition (38–40).

Abrogation of G_2- and S-phase arrest by DMAG has previously been associated with enhanced tumor cell radiosensitivity, although no molecular mechanism was proposed to support this observation (43). We found that accumulation and phosphorylation of CHK1 observed on doxorubicin-induced G_2-M arrest were substantially reduced when DMAG was administered 24 hours after doxorubicin. Furthermore, a specific CHK1 inhibitor added to doxorubicin–primed cells reproduced DMAG-mediated synergy on induction of apoptosis. We propose that the main effect of HSP90 inhibition in the context of DNA damage-induced cell cycle arrest in lymphoma cells is related to the abrogation of specific signal transduction pathways essential for recovery from genotoxic stress. Apoptosis induced by doxorubicin is enhanced more effectively by HSP90 inhibition in cells that lack functional p53 and must rely on G_2-M checkpoint for DNA repair.

Molecularly targeted agents are likely to be used in combination with standard chemotherapeutic regimens for patients with metastatic disease (14). Mutation and deletion of p53 are frequently associated with resistance to therapy, recurrence, and poor prognosis. It is timely to investigate how novel molecularly targeted agents interact with conventional therapies to sensitize tumors that lack functional p53. In our study, reduced sensitivity to doxorubicin- and DMAG-induced apoptosis was evident in MT p53 lymphoma cell lines. Similar dependency of HSP90 inhibitor–induced apoptosis on p53 status was reported in colon cancer cells exposed to geldanamycin or 17-AAG (55). Here, we showed that synergy from the combination doxorubicin and DMAG is determined by the schedule of exposure to each drug. Whereas schedule-dependent synergy is independent of p53 status, antagonism in cells pretreated with HSP90 inhibitors is more pronounced in cells without functional p53.

Altogether, our observations provide a rationale for further preclinical testing and stress the need to consider schedule of exposure as a critical determinant of the overall response when designing clinical trials that combine DMAG with DNA-damaging agents for patients with relapsed/refractory disease.

Acknowledgments

We thank Dr. Curtis C. Harris for his support and advice and Dr. Charles Vinson for critical revision of the manuscript.

References


Schedule-Dependent Synergy between the Heat Shock Protein 90 Inhibitor 17-(Dimethylaminoethylamino)-17-Demethoxygeldanamycin and Doxorubicin Restores Apoptosis to p53-Mutant Lymphoma Cell Lines

Ana I. Robles, Mollie H. Wright, Bheru Gandhi, et al.