Hsp90 Inhibition Depletes Chk1 and Sensitizes Tumor Cells to Replication Stress*

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DNA damage and replication stress activate the Chk1 signaling pathway, which blocks S phase progression, stabilizes stalled replication forks, and participates in G2 arrest. In this study, we show that Chk1 interacts with Hsp90, a molecular chaperone that participates in the folding, assembly, maturation, and stabilization of specific proteins known as clients. Consistent with Chk1 being an Hsp90 client, we also found that Chk1 but not Chk2 is destabilized in cells treated with the Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG). 17-AAG-mediated Chk1 loss blocked the ability of Chk1 to target Cdc25A for proteolytic destruction, demonstrating that the Chk1 signaling pathway was disrupted in the 17-AAG-treated cells. Finally, 17-AAG-mediated disruption of Chk1 activation dramatically sensitized various tumor cells to gemcitabine, an S phase-active chemotherapeutic agent. Collectively, our studies identify Chk1 as a novel Hsp90 client and suggest that pharmacologic inhibition of Hsp90 may sensitize tumor cells to chemotherapeutic agents by disrupting Chk1 function during replication stress.

Hsp90 is a ubiquitously expressed and abundant molecular chaperone that participates in the folding, assembly, maturation, and stabilization of specific proteins (known as clients). Hsp90 carries out these functions as an integral component of a multiprotein chaperone complex that contains additional chaperones and co-chaperones (reviewed in Refs. 1–3). Hsp90 regulates client function by cycling between two physiologically important states. In its ATP-bound state, Hsp90 interacts with co-chaperones Cdc37, p23, and an assortment of immunophilin-like proteins, forming a complex that stabilizes and protects client proteins from proteasomal degradation (1–3). In its ADP-bound form, Hsp90 recruits Hsp70 and p60/Hop, forming a complex that targets clients for proteasomal degradation.

Many new Hsp90 clients have been discovered recently with the aid of the Hsp90 inhibitors geldanamycin, radicicol, and their derivatives. These agents occupy the Hsp90 ATP-binding site, mimicking the ADP-bound state and targeting clients to the proteasome for degradation (4–7). Several of these clients, such as Akt, Her2/Neu, Bcr-Abl, and Raf-1, are important participants in signaling pathways that drive tumor cell proliferation and survival (8–11). In addition to their role in proliferation, the constitutive activation of these signaling proteins also enhances the survival of tumor cells following DNA damage. Thus, Hsp90-directed therapy has been viewed as a mechanism to simultaneously target numerous oncogenic signaling pathways and sensitize cells to chemotherapeutic agents (2, 12).

Although the oncogenic signaling pathways enhance survival following DNA damage, DNA damage also activates checkpoint signaling pathways that play pivotal roles in the survival of genotoxin-treated cells. The DNA damage-activated checkpoint pathways are evolutionarily conserved signaling pathways that regulate cell cycle progression, programmed cell death, and DNA repair (reviewed in Refs. 13 and 14). One checkpoint signaling pathway that has emerged as a key regulator of cellular responses is the Chk1 signaling pathway, which is activated by replication stress and various types of DNA damage. Following replication fork stalling, single-stranded regions of DNA accumulate that bind the single-stranded DNA-binding protein RPA (15–18). RPA binding is then followed by the chromatin recruitment of the phosphatidylinositol 3-kinase-related kinase ATR and its binding partner ATRIP (18). The stalled fork also recruits DNA polymerase α (16, 17), which then participates in the Rad17-dependent recruitment of the PCNA-like Rad9-Hus1-Rad1 (9-1-1) clamp complex to chromatin (17, 19). Once bound to chromatin, the 9-1-1 complex facilitates the ATR-mediated phosphorylation and activation of Chk1 (reviewed in Ref. 20).

Activated Chk1 performs several functions that promote cell survival. First, Chk1 increases the time available for DNA repair by arresting cells in G2. This arrest occurs when Chk1 phosphorylates Cdc25C and Cdc25A, two cell cycle phosphatases that activate the Cdk1-cyclin B complex (reviewed in Ref. 21). Second, activated Chk1 slows progression through S phase by blocking the firing of unirradiated origins of the replication (22–25). In this checkpoint, Chk1 phosphorylates Cdc25A, leading to Cdc25A degradation (24, 26, 27). Because Cdc25A is required for activation of Cdk2 complexes, which then control the firing of origins of replication, activation of this pathway blocks S phase progression (28–31). Third, Chk1 stabilizes stalled replication forks. Although the relevant substrate is not known, in the absence of Chk1 function, the stalled forks irreversibly collapse. Correspondingly, disruption of the Chk1 signaling pathway is associated with increased sensitivity to genotoxins. For example, cells lacking the upstream regulators Hus1 and Rad9 are highly sensitive to ultraviolet radiation and the replication inhibitor hydroxyurea (32, 33). Additionally, cells in which Chk1 has been deleted by gene targeting are sensitive to replication stress.

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the replication inhibitor aphidicolin and ionizing radiation (25). Consistent with this finding, UCN-01, a pharmacologic Chk1 inhibitor (34, 35), also sensitizes cells to genotoxins (36–41). Taken together, these observations have raised the possibility that inhibitors of the Chk1 signaling pathway may be useful clinical agents to sensitize tumor cells to genotoxic chemotherapy agents; however, no clinically viable Chk1 inhibitor has yet emerged.

Although a variety of previous studies have implicated Hsp90 function in signaling pathways regulating cell growth, survival, and apoptosis, the role of Hsp90 in checkpoint signaling has not been explored. Moreover, it remains unclear how Hsp90 inhibitors sensitize tumor cells to genotoxic chemotherapy agents. In the present study, we investigated whether Hsp90 regulates genotoxin-induced checkpoint signaling pathways. We report that Chk1 is an Hsp90 client that is lost from cells treated with 17-allylamino-17-demethoxygeldanamycin (17-AAG),\(^1\) a geldanamycin derivative that is used in clinical trials for the treatment of tumors. Collectively, these studies suggest a novel mechanism by which Hsp90 inhibitors sensitize cells to anti-tumor chemotherapeutic agents.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Cell Cycle Analysis, and Cell Transfection—**HeLa, OVCAR3, and ML-1 cells were grown in RPMI 1640 (BioWhittaker) supplemented with 10% fetal bovine serum. The cell cycle was analyzed by culturing trypanosized cells in 0.1% sodium citrate, 0.1% Triton X-100, 50 \(\mu\)g/ml propidium iodide, and 1 \(\mu\)g/ml RNase A for 30 min at room temperature. Cell cycle profiles were obtained by flow cytometry and analyzed with CellQuest software (Becton Dickinson). For HeLa cell transfections, the cells were trypsinized (0.5 \(\times\) \(10^6\) cells/transfection), reseeded in RPMI 1640 containing 10% fetal calf serum, and resuspended in 0.5 ml of the same medium. Plasmid DNA (40 \(\mu\)g/transfection) dissolved in RPMI 1640 was then added to the cells and incubated for 5 min. The cell-DNA mix was transferred to a 0.4-cm electroporation cuvette and electroporated with a 10-ms, 350-V pulse in a BTX T320 square wave electroporator. The cells were then replated and cultured for 20–24 h.

**Reagents and Antibodies—**17-AAG was obtained from R. Schultz (Developmental Therapeutics Program, National Cancer Institute) and from T. Mueller (Kosan Biosciences). Purified GST-Cdc25C (amino acids 200–256) was prepared as described (42). \(\gamma\)-\[^{32}\text{P}\]ATP (4500 Ci/\(\text{mmol}\)) was purchased from ICN Radiochemicals. Polyclonal anti-Chk1 and anti-Chk2 and monoclonal anti-Chk2 antibodies were provided by Junjie Chen (Mayo Foundation) (43). Monoclonal antibody to Hsp90 (H9010) has been previously described (44). HA-conjugated agarose (sc-7392AC) and polyclonal Chk1 (sc-7898) antibodies were purchased from Santa Cruz Biotechnology and used according to the manufacturer's instructions. Antibodies recognizing human Rad9, Rad1, Hus1, and Rad17 were raised as previously described (45, 46). Anti-phospho-Chk1 (Ser^345\)) was purchased from Cell Signaling Technology. Biotinylated anti-HA monoclonal antibody 3F10 was purchased from Roche Applied Science. Streptavidin and protein A-conjugated horseradish peroxidase was from Amersham Biosciences. The anti-Cdc25A antibody (Abk) was purchased from Neomarkers. HA-tagged expression vectors for Hus1, Chk1, and c-Raf have been described previously (35, 45, 47).

**Chk1 Kinase Assays—**HeLa cells (1 \(\times\) \(10^6\)) were transfected with 2 \(\mu\)g of pEFT-BO5-Chk1-HA\(^2\) and 38 \(\mu\)g of empty vector as described above. Following overnight incubation, the cells were pretreated with Me.SO or 17-AAG for 1 h prior to treatment with genecitabine. Following an additional 1-h incubation, the cells were lysed, and HA-tagged Chk1 was immunoprecipitated using HA-conjugated agarose following a previously described Chk1 assay procedure (48). The immunopurified Chk1 was then incubated with \(\gamma\)-\[^{32}\text{P}\]ATP and the Chk1 substrate GST-Cdc25C (encoding amino acids 200–256 of Cdc25C) at 30 \(^\circ\)C for 30 min. The reactions were terminated by adding 4 \(\times\) SDS-PAGE sample buffer. The kinase reactions were resolved on a 12.5% SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane.

\(^1\) The abbreviations used are: 17-AAG, 17-allylamino-17-demethoxygeldanamycin; GST, glutathione S-transferase; HA, hemagglutinin; MTS, 3-(4,5-dimethylythiazol-2-yl)-5-(3-carboxymethy phenol)-2-(4-sulphonyl)-2H-tetrazolium.

**RESULTS**

**The Hsp90 Inhibitor 17-AAG Selectively Destabilizes Chk1—**Many key signaling proteins in mammalian cells are Hsp90 clients. One way to identify potential Hsp90 clients is to examine whether a given protein is destabilized when cells are treated with an Hsp90 inhibitor. To examine whether any of the proteins in the Chk1 signaling pathway are potential Hsp90 clients, we treated HeLa cells with 1 \(\mu\)M 17-AAG, a concentration that maximally disrupts Hsp90 function (49, 50). The cellular levels of Rad9, Hus1, Rad1, Rad17, ATR, and Chk1 were then examined by immunoblotting at various time points after 17-AAG addition (Fig. 1A). The levels of Rad9, Hus1, Rad1, Rad17, and ATR were not affected by 17-AAG treatment. In contrast, Chk1 levels were reduced by an 8-h 17-AAG treatment and were maximally suppressed after 24 h of exposure (Fig. 1 and data not shown). Radicicol, a structurally unrelated Hsp90 inhibitor also depleted Chk1 (data not shown). We also

**Immunoprecipitations—**HeLa cells were plated in 10-cm dishes and grown to 90% confluence prior to cell lysis. Approximately 3 \(\times\) \(10^7\) cells were lysed for 10 min on ice in a buffer containing 10 mM HEPES, pH 7.4, 150 mM KCl, 10 mM MgCl\(_2\), 0.1% Nonidet P-40, 20 mM \(\beta\)-glycerophosphate, 1 mM sodium orthovanadate, 20 \(\mu\)g/ml aprotinin, 10 \(\mu\)g/ml pepstatin, 20 mM microcin-LR, and 20 \(\mu\)g/ml leupeptin. To identify the Chk1-Hsp90 interaction, the buffer described above was supplemented with 20 mM sodium molybdate. The lysates were centrifuged for 10 min at 4 \(^\circ\)C at 15,000 \(\times\) \(g\). The clarified lysates were then immunoprecipitated with the indicated antibodies. Washed immunoprecipitates were resolved on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were probed with antibodies as described above to detect associated proteins.

**Immunoblotting—**The cell lysates were prepared as described for immunoprecipitations. The cell lysates were mixed with an equal volume of 2\(\times\) SDS-PAGE sample buffer, boiled for 5 min, and resolved on 10% SDS-polyacrylamide gels. Anti-phospho-Chk1 (Ser^345\)) immunoblotting was performed according to the manufacturer's directions (Cell Signaling Technology). To detect HA-tagged proteins that co-immunoprecipitated with Hsp90, the immunoprecipitates were immunoblotted with biotinylated anti-HA antibody. Biotinylated anti-HA antibody was detected with streptavidin-conjugated horseradish peroxidase. All other immunoblots were performed as previously described (45, 46).

**Cell Viability—**The cells were treated with varying concentrations of genecitabine and 17-AAG for 24–48 h and stained with trypan blue. Viable (trypan blue-excluding) and nonviable (trypan blue-stained) cells were then counted using a hemocytometer. 3-(4,5-dimethylythiazol-2-yl)-5-(3-carboxymethylphenyl)-2-(4-sulphonyl)-2H-tetrazolium (MTS) assays were performed by plating 15,000 cells/well in 96-well plates, treating with drugs as described, and processing according to the manufacturer's directions (Promega).

**Radiolabeled proteins were visualized using an Amersham Biosciences storm 840 Phosphorimager.**

**FIG. 1. 17-AAG leads to the selective loss of Chk1.** A, HeLa cells (3 \(\times\) \(10^7\)) were treated with 1 \(\mu\)M 17-AAG for the indicated times. The cell lysates were prepared, and equal amounts of protein were immunoblotted for Atr, Hus1, Chk1, Chk2, and actin. Rad17, Rad9, and Rad1 were immunoprecipitated prior to immunoblotting. B, OVCAR3 cells were treated with 17-AAG for the indicated times, and the cell lysates were immunoblotted for Chk1 and Chk2. C, ML-1 cells were treated with 17-AAG for the indicated times, and the cell lysates were immunoblotted for Chk1 and Chk2.
examined whether Chk1 disappeared in OVCAR3 cells (Fig. 1B), an ovarian tumor line, and ML-1 cells (Fig. 1C), a myeloid leukemia cell line. In both cell lines, 17-AAG promoted Chk1 loss, demonstrating that the effect of 17-AAG on Chk1 levels is not limited to a single cell line.

To determine whether another checkpoint protein kinase was affected by Hsp90 inhibition, we also examined Chk2 levels in all three 17-AAG-treated cell lines (Fig. 1). Like Chk1, Chk2 is also activated by DNA damage but responds primarily to double-stranded DNA breaks in an ATM-dependent manner. Unlike Chk1, Chk2 levels did not decrease with 17-AAG treatment. Taken together, these results indicated that Chk1 is selectively lost when Hsp90 is inhibited.

Chk1 levels are low in cells in G1/G0. (51). The fact that geldanamycin and 17-AAG induce G1 and G2/M arrest (50, 52–54) raised the possibility that 17-AAG-induced Chk1 loss was merely the result of cell cycle redistribution caused by 17-AAG. To address this question, we devised a strategy to arrest cells in S phase, where Chk1 levels are high (51). HeLa cells were blocked in S phase by treating them with a low concentration of gemcitabine, a nucleoside analog that is incorporated by DNA polymerase into replicating DNA, blocking further chain elongation (55, 56). Gemcitabine-treated HeLa cells accumulated in S phase (Fig. 2A) and activated the Chk1 signaling pathway as evidenced by Chk1 phosphorylation on Ser\textsuperscript{345} (Fig. 2B), a site that is phosphorylated by ATR and is essential for Chk1 activation (57, 58). As previously reported, treatment with 17-AAG alone resulted in an accumulation of cells in G1 and G2/M (Fig. 2A). In contrast, when the cells were pretreated with gemcitabine, which slowed their progression through S phase, and then treated with 17-AAG, they remained in S phase. Under these conditions, Chk1 levels were also dramatically reduced (Fig. 2B, lane 4), and, correspondingly, Chk1 phosphorylation on Ser\textsuperscript{345} was not detected. This demonstration that Chk1 is lost in S phase-arrested cells indicates that Chk1 loss is not due to cell cycle arrest in G1.

**Fig. 2.** 17-AAG-induced Chk1 loss occurs in S phase-arrested cells. A, HeLa cells were treated with nothing (Control) or with 70 nm gemcitabine for 6 h. The cells were then treated with 1 \mu M 17-AAG for an additional 15 h. The cell populations were then split, and one portion was stained with propidium iodide and analyzed by flow cytometry. B, the remaining cells were lysed, and equal amounts of protein were electrophoresed and sequentially immunoblotted for phospho-Chk1 (Ser\textsuperscript{345}) and total Chk1. The asterisk denotes a nonspecific band detected by the antibody. Con, control; Gem, gemcitabine.

**FIG. 1.** Chk1 is an Hsp90 client. A, HeLa cell lysates were immunoprecipitated with the indicated antibodies and immunoblotted for Hsp90. PI, preimmune. B, HeLa cells were transiently transfected with HA-tagged Hus1 (10 \mu g), Chk1 (40 \mu g), or c-Raf (20 \mu g). Twenty-four h later, the cells were treated with vehicle or 1 \mu M 17-AAG for 1.5 h and lysed. The clarified cell lysates were immunoprecipitated (IP) with anti-Hsp90 monoclonal antibody H9010, fractionated by SDS-PAGE, and immunoblotted for the HA epitope using biotinylated anti-HA monoclonal antibody (top panel). A putative c-Raf proteolytic fragment is denoted by an asterisk. The blot was stripped and rebotted to detect immunoprecipitated Hsp90 (bottom panel). The cell lysates were immunoblotted with anti-HA to demonstrate expression of the fusion proteins (center panel).

**Chk1 Interacts with Hsp90**—Because many proteins that are destabilized by 17-AAG treatment are bona fide Hsp90 clients, the results in Figs. 1 and 2 raised the possibility that Chk1 may also be an Hsp90 client. An additional characteristic of Hsp90 clients is that they interact with Hsp90. To evaluate a potential Chk1-Hsp90 interaction, we immunoprecipitated endogenous Chk1 and the previously identified Hsp90 client Akt (59, 60) from HeLa cell lysates (Fig. 3A). No Hsp90 was found in the preimmune serum control, whereas both Akt and Chk1 co-immunoprecipitated with Hsp90. We also performed the reciprocal co-immunoprecipitation experiment. However, because Chk1 co-migrates with immunoglobulin heavy chain, we always detected the co-migrating immunoglobulin heavy chain when we immunoblotted for Chk1 (data not shown). To circumvent this problem, we transiently transfected HeLa cells with HA-tagged Chk1 and immunoblotted the immunoprecipitates with biotinylated anti-HA monoclonal antibody, which allowed detection of Chk1 in the Hsp90 immunoprecipitates without interference from the heavy chain (Fig. 3B). As has been reported previously for other Hsp90 clients (61–63), Hsp90 inhibition decreased the interaction between Hsp90 and Chk1. As controls, we also examined the interaction between Hsp90 and two additional HA-tagged proteins: Hus1, which is not an Hsp90 client, and c-Raf, a bona fide Hsp90 client. As expected, Hus1 did not co-precipitate with Hsp90, whereas c-Raf associated strongly with Hsp90. Like the Hsp90-Chk1 interaction, the Hsp90-c-Raf interaction was also disrupted by 17-AAG. These results, in conjunction with the results in Fig. 1, strongly suggest that Chk1 is indeed an Hsp90 client.

**The Chk1 Signaling Pathway Is Not Rapidly Inhibited in 17-AAG-treated Cells**—The results presented above demonstrated that Chk1 is an Hsp90 client and that Chk1 is slowly lost from 17-AAG-treated cells. However, Hsp90 inhibition occurs quickly following 17-AAG treatment, and some clients, such as the progesterone receptor, rapidly lose their ability to respond to stimuli when Hsp90 is inhibited (64). To determine whether Hsp90 inhibition rapidly affects the ability of Chk1 to respond to upstream signals and to be activated by genotoxic stress, we pretreated cells with 17-AAG for 1 h and asked whether Chk1 was still phosphorylated on Ser\textsuperscript{345} by its up-
stream activating kinase ATR. As shown in Fig. 4A, 17-AAG did not block Chk1 phosphorylation, indicating that even when Hsp90 is inhibited, this client still responds to upstream signals. Moreover, these results also indicate that short term Hsp90 inhibition does not block any of the upstream events required for Chk1 phosphorylation. To assess the effect of Hsp90 inhibition on Chk1 catalytic activity, we developed a Chk1 assay using transiently expressed HA-tagged Chk1 in HeLa cells. (We were unable to analyze endogenous Chk1 activity in any cell line we assayed (data not shown).) Treatment of HeLa cells with gemcitabine activated the catalytic activity of Chk1 (Fig. 4B), and this activation was not blocked by a 1-h pretreatment with 17-AAG. Taken together, these results suggest that Hsp90 is not required to continuously maintain Chk1 in a state that is able to receive and respond to upstream activating signals.

**17-AAG-mediated Chk1 Loss Blocks Cdc25A Loss**—Chk1-mediated Cdc25A phosphorylation leads to Cdc25A proteolytic destruction, thereby blocking cell cycle progression (24, 27, 65–67). To explore whether 17-AAG-induced Chk1 loss affects Cdc25A destruction, we treated HeLa cells with 17-AAG for 1 h and then with gemcitabine for an additional 4 h (Fig. 4C). During this period the level of Chk1 in HeLa cells is close to that found in untreated cells (Fig. 4A). Consistent with our Chk1 kinase assays (Fig. 4B), Chk1 was not lost in HeLa cells treated with 17-AAG for this short time period and did not block the gemcitabine-induced Cdc25A destruction, demonstrating that Chk1 remained functional. To address whether 17-AAG-induced Chk1 loss would affect Cdc25A levels, we treated HeLa cells with gemcitabine for 6 h to induce S phase arrest and activate Chk1. We then added 17-AAG, incubated for an additional 12 h to deplete Chk1, and assessed Cdc25A levels (Fig. 4D). Cdc25A was undetectable in the gemcitabine-treated cells, demonstrating that Cdc25A levels remain low even after 18 h of continuous exposure to gemcitabine. In contrast, the addition of 17-AAG during the last 12 h of this incubation restored the Cdc25A levels. Thus, these results show that 17-AAG leads to Chk1 loss and the corresponding loss of Chk1-dependent cellular responses to genotoxic stress.

**Disruption of Chk1 Activity Sensitizes Cells to Gemcitabine-induced Cell Death**—Chk1 plays an integral and essential role in activation of the S phase checkpoint (22–25), which stabilizes stalled DNA replication forks and blocks the firing of late acting origins of replication. Previous work has shown that ML-1 cells arrested in S phase with cytotoxic doses of gemcitabine are rapidly killed when the Chk1 inhibitor UCN-01 was added to the arrested cells (41). Because Chk1 is lost following 17-AAG treatment, we reasoned that 17-AAG might also sensitize tumor cells to the S phase arrest induced by gemcitabine. Therefore, we evaluated the potential of 17-AAG to sensitize gemcitabine-treated ML-1 cells. Because gemcitabine is only effective in S phase cells, and 17-AAG arrests cells in G1 and G2, we first arrested cells in S phase by treating for 24 h with gemcitabine. We then added 17-AAG and cultured the cells for an additional 24 h. Cell viability was assessed by trypan blue staining 24 and 48 h after 17-AAG addition. As controls, one set of samples was incubated with 20 nM gemcitabine alone, and one set was incubated for 17-AAG for the indicated time. The results show that 17-AAG leads to Chk1 loss and the corresponding loss of Chk1-dependent cellular responses to genotoxic stress.

**Fig. 4. 17-AAG does not rapidly affect the Chk1 activation pathway.** A, HeLa cells were pretreated as indicated with Me2SO (Control) or with 1 μM 17-AAG for 1 h and then treated with 500 nM gemcitabine for 1 h. The cell lysates were sequentially immunoblotted for phospho-Chk1 (Ser345) and total Chk1. B, HeLa cells transiently expressing HA-tagged Chk1 were pretreated with Me2SO (Control) or 1 μM 17-AAG for 30 min and then treated with 500 nM gemcitabine for an additional 1 h. HA-tagged Chk1 was immunopurified and Chk1 kinase activity was assessed using GST-Cdc25C as a substrate in an in vitro kinase assay. C, HeLa cells were pretreated as indicated with 1 μM 17-AAG for 1 h and then treated for an additional 4 h with 500 nM gemcitabine. The cell lysates were prepared, and a portion was sequentially immunoblotted for Chk1 and actin. D, Cdc25A was immunoprecipitated from the remaining cell lysates and immunoblotted. D, HeLa cells were pretreated with 500 nM gemcitabine for 6 h and then treated with 17-AAG for an additional 12 h. The cell lysates were prepared, and equal amounts of protein were analyzed as in C. Con, control; Gem, gemcitabine.

**Fig. 5. 17-AAG sensitizes cells to gemcitabine-induced cell death.** A, ML-1 cells were pretreated for 24 h with nothing (Control) or 20 nM gemcitabine. 1 μM 17-AAG was then added, and the cell viability was assayed by trypan blue staining 24 and 48 h after 17-AAG addition. As controls, one set of samples was incubated with 20 nM gemcitabine alone, and one set was incubated for 17-AAG for the indicated time. The values (± S.D.) are the average (n = 4) percentages of dead cells calculated by dividing the number of trypan blue positive cells by the total number of cells. B, HeLa and OVCAR3 were pretreated with 100 and 30 nM gemcitabine, respectively, for 24 h and then treated with 1 μM 17-AAG for an additional 24 h. The cell viability was assessed using the MTS assay. The values (± S.E.) are the averages of three independent experiments and are plotted as percentages of untreated cells. Con, control; Gem, gemcitabine.
killed few cells at the 24-h time point. In contrast, the combination of SAG and gemcitabine killed markedly more cells at this time point. At the 48-h time point, extensive cell death occurred when the drug combination was used, whereas SAG alone and gemcitabine were much less toxic as single agents. To extend these studies to other tumor cell types, we also analyzed the effects of these drugs on HeLa and OVCAR3 cells. Both cell lines were pretreated with minimally cytotoxic concentrations of gemcitabine for 24 h and then treated with Me$_3$SO or SAG for an additional 24 h, and the cell viability was quantitated with MTS assays. As was seen with the ML-1 cells, SAG and gemcitabine caused minimal decreases in cell viability in the OVCAR3 and HeLa cells (Fig. 5B). In contrast, treatment with both drugs lead to reduced viability. Taken together, these studies demonstrate that SAG dramatically sensitizes gemcitabine-arrested tumor cells.

**DISCUSSION**

In this report we demonstrate that 1) Chk1, a key component of the checkpoint activated by replication stress, is an Hsp90 client; 2) SAG inhibitors lead to the loss of Chk1 and a corresponding disruption of Chk1-mediated Cdc25A destruction; and 3) SAG sensitizes tumor cells arrested in S phase by gemcitabine. Collectively, these results shed new light on the regulation of the Chk1 signaling pathway and suggest a novel mechanism to sensitize tumor cells to gemcitabine.

Since the discovery that geldanamycin inhibits Hsp90 function, numerous Hsp90 clients have been identified, and our understanding of the role of Hsp90 in cellular physiology has expanded at a rapid pace. These studies have demonstrated that the tyrosine kinases Her2/Neu and Bcr-Abl and the serine/threonine kinases Raf-1 and Akt, all of which regulate cell cycle proliferation and survival, are Hsp90 clients (reviewed in Refs. 2 and 12). Because these signaling pathways are constitutively activated in many tumor cells and Hsp90 is overexpressed in tumor cells, Hsp90 inhibitors may be useful antineoplastic agents that can simultaneously disrupt multiple signaling pathways that drive tumor cell proliferation and survival. In addition, however, several studies have shown that geldanamycin and its derivatives also sensitize tumor cells to genotoxic chemotherapy agents (2). One potential explanation for the sensitization is that proliferation and survival signaling pathways, such as the Raf-1 and Akt pathways, are inactivated by Hsp90 inhibition, thereby increasing the sensitivity of cells to genotoxins. However, the Chk1 signaling pathway also plays a major role in the survival of genotoxin-treated cells. Because SAG treatment depletes Chk1, the present results strongly suggest that Hsp90 inhibition also sensitizes tumor cells to genotoxins by disrupting Chk1 function, thus defining another mechanism by which Hsp90 inhibitors may sensitize tumor cells to genotoxins.

Chk1 plays an essential role in maintaining cell viability in genotoxin-treated cells (22–25, 68). Following replication fork stalling and the accumulation of single-stranded DNA, the components of the Chk1 signaling pathway are recruited to DNA, where they mediate Chk1 activation (20). Once activated, Chk1 blocks the firing of unfired origins by targeting Cdc25A for degradation (24, 26, 27). This likely protects the genome by preventing additional DNA replication when damage is present. The Chk1 signaling pathway also stabilizes stalled replication forks (25, 68, 69). Although the relevant Chk1 substrate(s) is not known, studies have clearly shown that in the absence of Chk1 function, stalled forks collapse (25, 69). Even after the removal of the replication inhibitor, Chk1-deficient cells do not resume proliferation, and the cells do not enter mitosis (25). Consistent with this idea, treatment of SAG-phased-arristed cells with UCN-01 provoked rapid apoptosis of the S phase cells without cell cycle progression (40, 41). Collectively, these findings indicate that Chk1 plays a critical role in maintaining cell viability following replication stress, and they suggest that the disruption of Chk1 signaling may be an effective method to sensitize tumor cells to chemotherapy agents.

The Chk1 inhibitor UCN-01 has been viewed as a novel agent to sensitize tumors to genotoxins. However, several problems have emerged in clinical trials with UCN-01. The drug avidly binds a human serum protein, limiting bioavailability (70). Moreover, UCN-01 has an exceptionally long half-life in humans (25 days) and causes pronounced dose-limiting toxicities, such as hyperglycemia (70, 71), which may be due to the fact that the drug inhibits a wide spectrum of protein kinases (72). The studies presented here suggest that SAG, a drug that is undergoing early clinical testing in cancer patients (73), may represent a viable pharmacologic approach to disrupt Chk1 function in patients treated with S phase-active chemotherapeutics. In addition, SAG may have the added benefit of concurrently blocking additional signaling pathways that also contribute to chemoresistance. Taken together, the findings presented here suggest that sequencing of replication inhibitors and SAG may be critical to achieve maximal tumor responses. In particular, a clinical trial in which cancer patients are first treated with replication inhibitors to arrest tumor cells in S phase followed by SAG to deplete Chk1 (and other survival-promoting signaling pathways) may reveal a substantial clinical benefit to such drug combinations.

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