The Role of Checkpoint Kinase 1 in Sensitivity to Topoisomerase I Poisons*

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Agents that target topoisomerase I are widely utilized to treat human cancer. Previous studies have indicated that both the ataxia telangiectasia mutated (ATM)/checkpoint kinase (Chk) 2 and ATM- and Rad 3-related (ATR)/Chk1 checkpoint pathways are activated after treatment with these agents. The relative contributions of these two pathways to survival of cells after treatment with topoisomerase I poisons are currently unknown. To address this issue, we assessed the roles of ATR, Chk1, ATM, and Chk2 in cells treated with the topoisomerase I poisons camptothecin and 7-ethyl-10-hydroxycamptothecin (SN-38), the active metabolite of irinotecan. Colony forming assays demonstrated that down-regulation of ATR or Chk1 sensitized cells to SN-38 and camptothecin. In contrast, ATM and Chk2 had minimal effect of sensitivity to SN-38 or camptothecin. Additional experiments demonstrated that the Hsp90 inhibitor 17-allylamo-17-demethoxygeldanamycin, which down-regulates Chk1, also sensitized a variety of human carcinoma cell lines to SN-38. Collectively, these results show that the ATR/Chk1 pathway plays a predominant role in the response to topoisomerase I inhibitors in carcinoma cells and identify a potential approach for enhancing the efficacy of these drugs.
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the response of cells to camptothecin analogs. Shao et al. (38) reported that UCN-01, an inhibitor of Chk1 (39–41), diminishes the S-phase arrest observed after camptothecin. Subsequent studies detected 9-1-1 clamp loading (42, 43) and Chk1 activation (43, 44) after treatment of log-phase cells with camptothecin or camptothecin derivatives. Additional experiments demonstrated that interruption of the ATR/Chk1 pathway by expression of a dominant negative totothecin or camptothecin derivatives. Additional experiments suggested that Chk1 thrombosis (44) and targeted disruption of the Rad9 (43) or Hus1 genes (45) reduces the survival of mammalian cells exposed to the nanomolar concentrations of camptothecin derivatives that are encountered in the clinical setting (25, 46). Parallel studies in yeast have suggested that deletion of genes encoding 9-1-1 complex subunits, the 9-1-1 clamp loader, or the ATR homolog Mec1 also sensitizes cells to topoisomerase I poisoning (47).

The preceding studies have implicated the ATM/Chk2 and ATR/Chk1 pathways in the response to topoisomerase I poisons but have not identified their relative contributions. To address this issue, the present study utilized siRNA, gene-deleted cells, and pharmacological manipulations to reexamine the relative roles of the ATM/Chk2 and ATR/Chk1 pathways in protecting cells from camptothecin derivatives. Results of this study not only demonstrate a more prominent role for the ATR/Chk1 pathway but also identify a potential strategy for enhancing the cytotoxicity of topoisomerase I poisons.

EXPERIMENTAL PROCEDURES

Materials—17-AAG was obtained from the Developmental Therapeutics Program, National Cancer Institute (Bethesda, MD). SN-38 was a kind gift from L. P. McGovern (Pharmacia Upjohn, Kalamazoo, MI). Reagents were purchased from the following suppliers: Opti-MEM medium and Lipofectamine 2000, Invitrogen; and camptothecin, Sigma. Antibodies to the following antigens were purchased from the indicated manufacturers: Akt, PDK1, phospho-Ser345-Chk1, and phospho-Ser21/9 glycogen synthase kinase-3 (β) (Cell Signaling Technology (Beverly, MA); total glycogen synthase kinase-3β, Cell Signaling Technology (Beverly, MA); total Chk1, total Chk2, and Chk1, Santa Cruz Biotechnology (Santa Cruz, CA); and ATM, β-actin, and Chk1, Sigma. Murine antibodies against Chk2, Hsp90, and Topo-I (H11350) were kindly provided by B. Vogelstein (Johns Hopkins University, Baltimore, MD) and grown in medium A supplemented with 100 units/ml penicillin G and 100 μg/ml streptomycin (medium A). HCT 116 human colon cancer cells and their derivatives were grown in medium A supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin. HCT 116 (HCT 116) cells/plate in 60-mm (HeLa) or 35-mm dishes (T98G and H9262) were plated in 35-mm tissue culture dishes and incubated overnight. On day 2, after cells were washed twice with Opti-MEM medium, 2 ml of Opti-MEM were added to each plate. Four hundred nmol of control siRNA 1 (Dharmacon, Lafayette, CO), ATR siRNA (49), Chk1 siRNA (18), PDK1 siRNA (50), or ATM siRNA (51) were complexed with 100 nmol/ml Lipofectamine 2000 in 0.5 ml of Opti-MEM for 20 min. Following addition of the lipid-siRNA complexes to the cells, the cultures were incubated for 7–9 h before addition of 1 ml of Opti-MEM containing 30% fetal bovine serum. The transfections were repeated on day 3. On day 4, the cultures were trypsinized and replated in 100-mm tissue culture dishes containing medium B. On day 5, cells were harvested for immunoblotting or exposed to drugs as described below.

 Colony Assays—HeLa and U2OS cell clonogenic assays were performed on the siRNA-transfected cells 48 h after the second transfection. The cells were trypsinized, plated at 300 cells/plate in replicate 60-mm dishes containing medium A, allowed to adhere for 4 h, and treated with the indicated concentrations of SN-38 or camptothecin for 24 h. Following drug treatment, cells were washed with RPMI 1640 medium, cultured for 7 days in medium A, and stained with Coomasie Brilliant Blue. Colonies containing ≥50 cells were counted.

FIG. 1. Effect of SN-38 on phosphorylation of Chk1 and Chk2. A, dose-response curve. Log-phase HeLa cells were treated for 6 h with diluent (0.1% Me2SO, lane 1) or SN-38 at 12.5, 25, 50, 100, or 200 nM (lanes 2–6, respectively). At the end of the incubation, whole cell lysates were prepared, subjected to SDS-PAGE, transferred to nitrocellulose, and probed with reagents that recognize phospho-Ser345-Chk1, phospho-Thr68-Chk2, total Chk1, total Chk2, or, as a control, β-actin. B, time course. Log-phase HeLa cells were treated with 100 nM SN-38 for the indicated length of time. At the completion of the incubation, whole cell lysates were analyzed as indicated in A.

Colonial forming assays were performed in untransfected HeLa cells and the other cell lines with the following modifications: 1) transfection was omitted; 2) cells were plated at 250 (T98G), 300 (HeLa), or 500 (HCT 116) cells/plate in 60-mm (HeLa) or 35-mm dishes (T98G and HCT 116) in their respective media and allowed to adhere for 14–16 h; and 3) after the 24-h drug exposure, cells were cultured for 7–8 (HeLa, HCT 116) or 9–10 days (T98G) to allow colonies to form.

Immunoblotting—After treatment with drug or diluent as indicated in the figure legends, cells were washed three times with ice-cold RPMI 1640 medium containing 10 mM HEPES (pH 7.4 at 4 °C) and solubilized by addition of 6× guanidine hydrochloride containing 250 mM Tris-HCl (pH 8.5 at 20 °C), 10 mM EDTA, 1% (v/v) 2-mercaptoethanol, and 1 mM freshly added phenylmethylsulfonyl fluoride directly to the plates. After preparation for electrophoresis as described previously (52), aliquots containing 50 μg of protein (determined by the bicinchoninic acid method) (53) were separated on SDS-polyacrylamide gels containing 5–15% (w/v) acrylamide gradients, electrophoretically transferred to nitrocellulose, and probed with immunological reagents as described previously (54). Alternatively, cell lysates were prepared from siRNA-transfected cells and probed by immunoblotting as described previously (55).

Statistical Analysis—Clonogenic experiments in tissue culture cell lines were performed a minimum of three times. The method of Chou and Talalay (56) was employed as described previously (57–59) to determine whether the effects of the SN-38/17-AAG combination were synergistic.

RESULTS

A Predominant Role for the ATR/Chk1 Pathways in Camptothecin Sensitivity—Previous studies demonstrated that Chk1 and Chk2 are both activated after treatment with the topoisomerase I poisons topotecan (44) or camptothecin (43). Consistent with these results, treatment of HeLa cells with the camptothecin derivative SN-38, the active metabolite of the widely used chemotherapeutic agent irinotecan (25, 46), resulted in the concentration- and time-dependent phosphorylation of Chk1 on Ser345 and Chk2 on Thr68, sites that are required for activation of the kinases (Fig. 1). Chk1 phosphorylation was detected after treatment for 6 h with SN-38 at concentrations as low as 12.5 nM (Fig. 1A) and was present within 1 h after treatment with 100 nM SN-38 (Fig. 1B). Likewise, phosphorylation of Chk2 was evident after treatment with 12.5 nM SN-38 (Fig. 1A) for 6 h or after treatment with 100 nM SN-38 for 1 h (Fig. 1B).

To assess the potential importance of activating the ATR/Chk1 pathway, HeLa cells were transfected with an siRNA duplex oligonucleotide that down-regulates ATR (Fig. 2A, inset), treated for 24 h with increasing concentrations of SN-38, washed, and incubated for 7 days so that surviving cells could
form colonies. Results of this analysis (Fig. 2A) demonstrated that ATR down-regulation increased SN-38 sensitivity, with a 3-fold decrease in the IC_{50}. Likewise, siRNA-mediated down-regulation of Chk1 (Fig. 2B, inset) was accompanied by a 2-fold decrease in the SN-38 IC_{50} (Fig. 2B). Similar results were observed with the parent drug, camptothecin (Fig. 2C). These results are consistent with previous studies showing a comparable effect after disruption of ATR signaling by conditional expression of a dominant negative ATR construct (44) or by Rad9 deletion (43). siRNA to PDK1, a polypeptide that is discussed in greater detail below, diminished the levels of PDK1 as well as phosphorylation of the PDK1 substrate (Fig. 2D, inset) Akt on Thr^{308}, a site phosphorylated by PDK1 and required for Akt activation. However, PDK1 siRNA did not sensitize HeLa cells to SN-38 but instead slightly decreased the antiproliferative effects of this agent (Fig. 2D).

Several additional observations argue against the possibility that the sensitizing effects of siRNA targeting ATR or Chk1 are due to nonspecific toxicity. First, the cloning efficiencies of HeLa cells transfected with ATR siRNA and Chk1 siRNA were 87 ± 10% (mean ± S.D., n = 4) and 74 ± 19% (n = 9), respectively, of cells treated with control siRNA, ruling out the possibility that the siRNA was killing the bulk of the cells and allowing outgrowth of a nonrepresentative population. Second, whereas Chk1 siRNA sensitized cells to camptothecins (Fig. 2C) as well as antimetabolites such as cytarabine, it had no effect on sensitivity to ionizing radiation (Fig. 2E), consistent with predominant involvement of the ATM pathway in the response to DNA double-strand breaks.

To rule out the possibility that the results obtained were unique to HeLa cells, U2OS human osteosarcoma cells were transfected with ATR siRNA. Once again, the cells were sensitized to SN-38 (Fig. 2F). Because U2OS cells contain an intact p53 tumor suppressor protein (60), these results also rule out the possibility that ATR siRNA-induced sensitization to camptothecins occurs only in cells with an inactive p53 pathway.

In contrast to ATR or Chk1 down-regulation, down-regulation of components of the ATM/Chk2 pathway had a much smaller effect on SN-38 sensitivity. Transfection of HeLa cells with ATM siRNA depleted ATM (Fig. 3A, inset) but had minimal effect on SN-38 sensitivity (Fig. 3A). In view of this surprising result, similar experiments were performed using the parent drug, camptothecin. Once again little sensitization was observed (Fig. 3B). Importantly, the same siRNA transfectants were sensitized to ionizing radiation (Fig. 3C). To further examine the effect of interrupting the ATM/Chk2 pathway, SN-38 sensitivity was compared in parental HCT 116 colon cancer cells and their Chk2 \textsuperscript{-/-} derivative. Once again, little difference in SN-38 (Fig. 3D) or camptothecin (Fig. 3E) sensitivity was observed. When combined with the results in Fig. 2, these observations suggest that the ATR/Chk1 pathway plays a predominant role in protecting cells from topoisomerase I poisons.

Synergistic Effects of SN-38 and Hsp90 Inhibitors—In view of these results, we predicted that Chk1 depletion by other means would also sensitize cells to the antiproliferative effects of SN-38. Recent results demonstrated that Chk1 is a client of the Hsp90 chaperone complex and, correspondingly, showed that treatment with the Hsp90 inhibitor 17-AAG resulted in selective down-regulation of Chk1 without changes in other known components of the ATR/Chk1 and ATM/Chk2 pathways (55). Examination in HeLa cells demonstrated that Chk1 down-regulation is evident at 125 nM 17-AAG (Fig. 4A), a concentration that is readily sustained for >24 h in the clinical setting (61). Like Chk1, other Hsp90 client proteins such as PDK1 and Akt1 were depleted, whereas no change in topoisomerase I levels was observed (Fig. 4A).
FIG. 3. Effect of ATM siRNA or Chk2 gene deletion. A and B, 2 days after the second transfection with ATM siRNA, cells were exposed for 24 h to the indicated concentrations of SN-38 or camptothecin. At the completion of the drug treatment, cells were washed with drug-free medium and allowed to form colonies. Inset in A, whole cell lysates were prepared from additional transfected cells and analyzed for ATM levels by immunoblotting. C, 2 days after the second transfection with ATM siRNA, cells were subjected to the indicated dose of radiation from an α137Cs source and incubated in drug-free medium for 8 days to allow colonies to form. D and E, parental or Chk2−/− HCT 116 cells were treated for 24 h with the indicated concentration of SN-38 or camptothecin and then washed and incubated in drug-free medium until colonies formed. Inset in D, whole cell lysates from the same cultures of parental and Chk2−/− cells were subjected to SDS-PAGE and immunoblotting. Hsp90 served as a loading control. Error bars, ±S.D. from triplicate samples.

FIG. 4. Synergistic effect of SN-38 and the Hsp90 inhibitor 17-AAG in HeLa cells. A, log-phase HeLa cells were treated for 24 h with diluent (0.1% Me2SO, lane 1) or 17-AAG at 62.5, 125, 250, 500, and 1000 nM (lanes 2–6, respectively). At the end of the drug treatment, whole cell lysates were prepared as described under “Experimental Procedures.” Aliquots containing 50 μg of protein were subjected to SDS-PAGE and probed with reagents that recognize the indicated antigens. β-Actin served as a loading control. B and C, HeLa cells were treated for 24 h with the indicated concentrations of SN-38 alone (B), 17-AAG alone (C), or a 1:6 ratio of SN-38 to 17-AAG (B and C). The final Me2SO concentration was 0.2% in all cultures. At the completion of the incubation, cells were washed and incubated in drug-free medium until colonies formed. Error bars, ±S.D. from triplicate samples. D, combination index plot derived from the data in B and C. Data were analyzed by the method of Chou and Talalay (56), according to the assumption that effects of the two agents are mutually exclusive. A combination index of <1.0 indicates synergy. Results of this analysis are equivalent to isobologram analysis (62). Results calculated under the assumption that effects of the agents are mutually nonexclusive are also indicated.
To assess whether loss of Hsp90 clients was accompanied by enhanced SN-38 sensitivity, cells were treated for 24 h with increasing concentrations of SN-38, 17-AAG, or a combination of both agents. To facilitate subsequent analysis, the combination consisted of the two agents at a fixed ratio determined by their respective IC50 values, and the fixed ratio was applied at a number of concentrations that included one-half, five-eighths, three-fourths, seven-eighths, and 1.5 times the individual IC50 values. This approach has been widely applied in previous analyses of new drug combinations (56–59). Results of this analysis showed that the combination demonstrated greater antiproliferative effects than either agent alone (Fig. 4, B and C). Formal analysis by the method of Chou and Talalay (56) was performed under the assumption that effects of the two agents are mutually exclusive, an assumption that renders this approach equivalent to the isobologram method (62). This analysis (Fig. 4D) demonstrated that effects of the two agents were synergistic, as evidenced by a combination index of <1 over a wide range of concentrations (56). In repeated experiments, the combination index was 0.75 ± 0.09 (mean ± S.D., n = 3) at the IC50.

To rule out the possibility that this synergy was unique to the HeLa cell line, HCT116 colon cancer cells and T98G human glioblastoma cells were subjected to similar analyses. Immunoblotting demonstrated that 17-AAG induced down-regulation of Chk1 in these cell lines at even lower concentrations (Fig. 5, A and D), with no effect on topoisomerase I levels (data not shown). Once again, the antiproliferative effects of the SN-38/17-AAG combination were greater than those of either agent alone (Fig. 5, B and E; data not shown). Analysis by the method of Chou and Talalay (56) indicated synergy in both cell lines (Fig. 5, C and F), with combination indices of 0.63 ± 0.12 (n = 8) and 0.65 ± 0.10 (n = 3), respectively, at the IC90 values.

**FIG. 5.** Effects of the 17-AAG/SN-38 combination in HCT 116 colon cancer and T98G glioblastoma cells. A and D, HCT 116 (A) or T98G (D) cells were treated for 24 h with diluent (0.1% Me2SO, lane 1) or 17-AAG at 62.5, 125, 250, 500, or 1000 nM (lanes 2–6, respectively). After drug treatment, whole cell lysates were prepared. Aliquots containing 50 μg of protein were subjected to SDS-PAGE followed by immunoblotting with reagents that recognize the indicated antigens. β-Actin served as a loading control. B and E, HCT 116 (B) or T98G (E) cells were treated for 24 h with SN-38 alone or the SN-38/17-AAG combination at a fixed ratio of 1:8 (B) or 1:2 (E). At the end of the incubation, cells were washed and incubated in drug-free medium until colonies formed. Error bars, ± S.D. from triplicate aliquots. C and F, combination index plots. Data from the experiments shown in B and E, along with simultaneously derived results obtained after treatment with 17-AAG alone (see Fig. 4C), were analyzed by the method of Chou and Talalay (56). A combination index of <1.0 indicates synergy.

**DISCUSSION**

Results of the present study demonstrated that Chk1 down-regulation enhances sensitivity to the topoisomerase I poison SN-38, whereas down-regulation of ATM or deletion of Chk2 has a much smaller effect. Additional experiments showed that 17-AAG, which down-regulates Chk1, and SN-38 synergize in killing cells. These results have potentially important implications for the mechanism of action of topoisomerase I poisons and future clinical development of these agents.

Early studies indicated that fibroblasts and lymphoblastoid lines from ataxia telangiectasia patients are hypersensitive to camptothecin (34–37). These studies suggested an important role for the ATM/Chk2 pathway in response to topoisomerase I poisons and provided support for a model in which the cytotoxicity of topoisomerase I poisons results from the formation of DNA double-strand breaks as a consequence of a collision between advancing replication forks and drug-stabilized topoisomerase I-DNA covalent complexes. More recent studies found that disruption of the ATR/Chk1 pathway also sensitizes cells to topoisomerase I poisons (43, 44, 63). Because this pathway is robustly activated by agents that stall advancing replication forks (64), activation of this pathway by topoisomerase I poisons was initially somewhat surprising. The present results using siRNA constructs not only confirm that down-regulation of ATR or Chk1 sensitizes cells to topoisomerase I poisons, but they also suggest that the ATR/Chk1 pathway plays a more prominent role than the ATM/Chk2 pathway in the response to these agents.

These results appear to be at odds with earlier reports suggesting that ATM inactivation enhances camptothecin sensitivity. Several differences between these studies might contribute to the disparate conclusions. First, the results may be due to cell line differences. The earlier studies utilized fibroblast
and lymphoblastoid cell lines, whereas the present study employed epithelium-derived cancer cell lines. Second, the earlier studies compared cell lines from different individuals, raising the possibility that factors other than ATM status affected the results. In contrast, the present study used a single cell line treated with siRNA and an isogenic pair of cell lines differing in targeted deletion of Chk2 to reduce the number of uncontrolled variables. Third, the siRNA-mediated depletion of ATM was incomplete and, therefore, might have been less effective than the ATM inactivation seen in ataxia telangiectasia cells. It is important to point out, however, that this ATM down-regulation was sufficient to sensitize the cells to ionizing radiation (Fig. 3C). Moreover, deletion of Chk2 did not markedly alter SN-38 or camptothecin sensitivity (Fig. 3, D and E). Collectively, our results in two model systems suggest that down-regulation of the ATM/Chk2 pathway has a limited effect on sensitivity to topoisomerase I poisons.

Additional experiments in a wider range of model systems will be warranted to further confirm the conclusion that the ATR/Chk1 signaling pathway plays a predominant role in response to topoisomerase I poisons. Nonetheless, the present observations suggest the interesting possibility that stalling of advancing replication forks, rather than generation of double-strand breaks, is important in the antiproliferative effect of topoisomerase I poisons at the nanomolar drug concentrations that are achieved in the clinical setting (25, 46).

The present results also demonstrated that the antiproliferative effects of SN-38 are enhanced in a synergistic fashion by simultaneous administration of the Hsp90 inhibitor 17-AAG. These effects were observed in HeLa cells (Fig. 4D), which have a nonfunctional p53 pathway as a consequence of papilloma virus E6 protein expression (65), as well as HCT 116 cells, which have an intact p53 pathway (66) (Fig. 5C). These synergistic effects were observed at 17-AAG concentrations in the 50–300 nM range. Recent studies have demonstrated that concentrations of 17-AAG and its active metabolite 17-aminogeldanamycin exceed 300 nM for 16 and 24 h, respectively, after drug administration in the clinical setting (61). At these concentrations, Chk1 down-regulation is observed in a variety of cell lines (55) (Figs. 4A and 5, A and D). These observations provide a rationale for further preclinical and possible clinical studies of this combination.

Because 17-AAG induces the down-regulation of a variety of Hsp90 clients in addition to Chk1 (67, 68), it is possible that effects on other Hsp90 client proteins contribute to the observed synergy. Previous studies from other laboratories suggested that down-regulation of PDK1 (69) and Akt (70, 71) might contribute to the antiproliferative effects of 17-AAG alone or in drug combinations. In the present study, PDK1 siRNA blocked the activating phosphorylation of Akt (Fig. 2D, inset) but did not sensitize cells to SN-38 (Fig. 2D), most likely because inhibition of the phosphatidylinositol-3-kinase/PDK1/Akt pathway inhibits the S-phase progression required for the cytotoxic effects of topoisomerase I poisons. These observations suggest that Hsp90 clients contribute differentially to the sensitization observed in Figs. 4 and 5 and further highlight the potential importance of Chk1 down-regulation. Although the results shown in Fig. 2 strongly suggest that Chk1 depletion contributes to the effectiveness of the SN-38/17-AAG combination, additional studies are required to assess the potential role of other Hsp90 clients in this synergy.

In summary, the present results highlight the importance of the ATR/Chk1 pathway in survival of cancer cells after treatment with topoisomerase I poisons. In addition, these studies demonstrate that Chk1 down-regulation by siRNA or 17-AAG can sensitize cancer cells to camptothecin derivatives. These observations raise the possibility that inhibition of the ATR/Chk1 pathway might enhance the therapeutic effects of topoisomerase I poisons.

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