Drg1, a Novel Target for Modulating Sensitivity to CPT-11 in Colon Cancer Cells

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Abstract

Treatment of the human colon cancer cells Hct116 with SN-38 (an active metabolite of CPT-11) resulted in G2 cell cycle arrest without induction of apoptosis. However, subsequent treatment of SN-38-treated Hct116 cells with fluoropyridol induced apoptosis. One of the genes markedly up-regulated during cell cycle arrest by SN-38 and suppressed during apoptosis by SN-38 followed by fluoropyridol in Hct116 cells is Drg1. We found that Drg1 had profound effects on SN-38 sensitivity. Inhibition of endogenous Drg1 expression in Hct116 cells by stable expression of an antisense (AS) Drg1 cDNA increased the sensitivity of cells to undergo apoptosis by SN-38. Clonogenic and apoptosis assays with AS Drg1-expressing cells showed a 2-fold decrease in the IC50 and a 4-5-fold increase in induction of apoptosis with SN-38. Conversely, the forced expression of Drg1 in SW620 cells increased the resistance of these cells to SN-38-induced apoptosis by 2-5-fold. Moreover, when xenografted in mice, AS Drg1-expressing Hct116 cells were 3-fold more sensitive to CPT-11 as compared with vector transfected Hct116 cells. Similarly, tumors established from Drg1 overexpressing SW620 cells were more resistant to CPT-11 as compared with tumors established from vector-transfected SW620 cells in mice. Taken together, our data suggest that Drg1 is a novel gene that plays a direct role in resistance to CPT-11. Inhibition of Drg1 may provide a new means to increase the sensitivity of colon cancer cells to CPT-11.

Introduction

This year alone >130,000 new patients will be diagnosed with colon cancer in the United States. Many of these patients will require chemotherapy in either the adjuvant or metastatic setting. The topoi3 inhibitor CPT-11 has been approved as a part of first-line therapy in the treatment of patients with metastatic colon cancer. However, the response rate to CPT-11 alone is only 20%. When combined with 5-fluorouracil and leucovorin, the response rate improves to 40%, but the survival advantage over 5-fluorouracil/leucovorin alone is small (1, 2). The low response rate to CPT-11 alone implies an intrinsic resistance to CPT-11 (3, 4). The mechanisms of resistance to CPT-11 are unclear. CPT-11 belongs to a class of drugs called Camptothecins, which poison eukaryotic DNA topol (5). Point mutations in topol have been identified in camptothecan-resistant prostate and lung cancer cell lines (6, 7). However, topol levels have not been shown to correlate to CPT-11 resistance (8). The presence of intact G1 and G2 checkpoints has been shown to influence the sensitivity to CPT-11 (9). Other factors associated with CPT-11 sensitivity include loss of p53 (10) and the presence of the DNA mismatch repair gene hMLH1 (11).

Materials and Methods

Cell Culture and Drug Treatments for Cell Lines. The human colon cancer cell lines Hct116 and SW620 were purchased from American Type Culture Collection (Rockville, MD). The cell lines were maintained in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum, penicillin, and streptomycin at 37°C in 5% carbon dioxide. All of the cultures were tested as Mycoplasma free. The stock solutions of SN-38 (5 mM in DMSO; supplied by Dr. Patrick McGovern, Pharmacia and Upjohn, Kalamazoo, MI) and fluoropyridol (4.5 mM in water; graciously supplied by Dr. Edward Sausville, National Cancer Institute, Bethesda, MD) were stored at −20°C, and drugs were diluted in the medium before use. The xenografted cell lines were routinely maintained in medium supplemented with 200 μg of G418 (Life Technologies, Inc., Rockville, MD).

Apoptosis Assays. Apoptosis in drug treated cells was measured by QFM as described previously (13). In brief, the cells were cultured for 48–72 h (~60% confluent) and treated with indicated drugs. At the end of treatment, adherent cells were trypsinized, pooled with floating cells, washed with PBS, and fixed in 3% paraformaldehyde. Cells were stained with 4',6-diamidino-2-phenylindole (Sigma, St. Louis, MO) for 30 min at room temperature in the dark. The aliquots of cells were then prepared and duplicate samples of 400 cells each were counted and scored for the incidence of apoptotic chromatin condensation using an Olympus BH2-DM2U2UV Dichtemometor Mirror cube filter (Olympus, Lake Success, NY). Cells with condensed and fragmented chromatin were scored as apoptotic cells.

RDA of cDNA. Total RNA was extracted from cells treated with different schedules of 20 nM SN-38 and 150 nM fluoropyridol by cesium chloride method as described in Current Protocols in Molecular Biology (14). Polyadenylated cDNA was synthesized and RDA analysis was performed as described in Current Protocols in Molecular Biology (21). The use of RDA allows for the detection of differentially expressed genes. Expression of Drg1 was confirmed by RNA blot hybridization and SGH expression was visualized by the appropriate deprotection procedures.
mRNA was prepared from total RNA using Oligotex spin columns (Qiagen, Valencia, CA). To identify differentially expressed genes in Hct116 cells during SN-38 followed by no drug treatment (no apoptosis, cell cycle arrest) versus SN-38 followed by flavopiridol (apoptosis), we used a modified form of RDA of cDNA. The testor population, from which the target genes were sought, was comprised of cDNAs from Hct116 cells treated with SN-38 for 24 h followed by no drug for 24 h, whereas the driver consisted of cDNAs from Hct116 cells treated with SN-38 for 24 h followed by flavopiridol for 24 h. cDNA was made from polyadenylated RNA derived from each population, and the driver population was subtracted from the testor population using PCR-select kit (BD Biosciences, Palo Alto, CA). The cDNA fragments identified by this technique were subcloned and sequenced to determine their identities.

Northern blot analysis was carried out to confirm the differential expression. Total RNA (20 μg) was electrophoresed on a 1% agarose-phosphate buffer gel, blotted onto Hybond-N nylon membranes (Amersham Biosciences, Piscataway, NJ), and RNA was cross-linked by UV Stratallinker (Stratagene, La Jolla, CA). The membranes were hybridized with a 32P-labeled Drg1 cDNA probe in Expresshyb hybridization solution (BD Biosciences). The probe was labeled previously by random priming [32P]dCTP incorporation using a random-primer labeling kit (NEN Life Science, Boston, MA). The probe was purified by passing through a Sephadex Quick Spin column (Roche Molecular Biochemicals, Indianapolis, IN).

Plasmid Construction and Transfection. The Drg1 cDNA was obtained by screening a human prostate cDNA library using a 32P-labeled Drg1 cDNA fragment (identified in RDA screen) as a probe. The 1.2-kb coding region was amplified by PCR. The PCR product was purified, blunted by filling in with Klenow, and cloned into pCR2.1 vector (Invitrogen) by filling in with Klenow in sense and AS orientation. Hct116 cells were transfected with AS Drg1 and SW620 cells with sense Drg1 plasmid using FUGENE (Roche Molecular Biochemicals). As a negative control, cells were also transfected with vector alone. Exponentially growing Hct116 or SW620 cells (1 × 10⁶) were transfected with 7.5 μg of plasmid DNA on a 100-mm dish. The cells were selected in 600 μg/ml G418 for 2 weeks, and clones were isolated by cloning cylinders. Transfected clones were confirmed by Western blotting using rabbit polyclonal Drg1 antibody.

Western Blot Analysis. Cells were lysed with buffer containing 50 mM HEPES-KOH (pH 7.5), 150 mM NaCl, 1 mM of each EDTA, NaF and DTT, 2.5 mM EGTA, 0.1% Tween 20, 10% glycerol, 10 mM β-glycerophosphate, 0.1 mM Na,VO₄, 0.2 mM phenylmethylsulfonyl fluoride, and 10 μg/ml of each aprotinin and leupeptin. The cells were additionally disrupted by passing through a 21-gauge syringe 10 times, and lysates were clarified by centrifugation (10 min at 10,000 × g). Soluble protein (20 μg) was resolved by 10% SDS-PAGE and transferred onto Immobilon-P membranes (Millipore Corp., Bedford, MA). The equal loading of proteins was confirmed by amido black staining. The membranes were probed with either Drg1 rabbit polyclonal or tubulin mouse monoclonal (Calbiochem, San Diego, CA) antibodies. The membranes were treated with a secondary sheep antimouse-horseradish peroxidase or donkey antirabbit-horseradish peroxidase antibody for 1 h at room temperature. The fluorescent signal was detected by Super Signal West Pico Chemiluminescent (Pierce, Rockford, IL) according to the manufacturer’s protocol.

Colony Formation Assays. One-thousand cells were plated, in triplicates, in 100-mm plates and incubated for 24 h to allow cells to adhere. Cells were treated with various doses of SN-38 (1–20 nM) or DMSO (0.1%) for 24 h. At the end of treatment the medium-containing drug was replaced with drug-free medium and cells were allowed to grow for 10 days to form colonies. The resulting colonies were stained with 0.01% (w/v) crystal violet for 30 min and counted. Control plates contained approximately 300–400 colonies. The IC₅₀ is defined as the drug concentration that inhibits colony formation by 50%.

Xenograft Growth Assay. The general procedure used in the experiments has been described previously (15). Athymic-NCI-nu male mice between the age of 8 and 10 weeks were inoculated s.c. in both flanks with 5 million vector and Drg1 plasmid-transfected Hct116 or SW620 cells mixed with Matrigel (Becton Dickinson). Drug treatment was started on day 7 after the inoculation of cells with the maximum tolerated dose of CPT-11 (100 mg/kg). The average tumor volume at the day of treatment was 48–62 mm³. Total RNA from the control group was given vehicle (PBS) alone. The drug was administered i.p. twice a week, for a total of five injections. Ten mice were treated in each cohort. Tumors were measured every 3–4 days with calipers, and tumor volumes were calculated by the formula 4/3 × π × r² (r = larger diameter + smaller diameter/4). The percentage of tumor regression was calculated as percentage of ratio of difference between baseline and final tumor volume to the baseline volume. These studies were performed in accordance with the “Principles of Laboratory Animal Care” (NIH publication No. 85–23 released 1985).

Biostatistical Analysis. All of the in vitro and in vivo experiments were performed in duplicate and repeated at least three times unless otherwise indicated. The statistical significance of the experimental results was determined by the two-sided t test.

Results

Flavopiridol Augmented the Effect of SN-38 in Hct116 Cells. We have shown previously that treatment of Hct116 cells with SN-38 induced a G₂ cell cycle arrest (12). In clonogenic assays SN-38 induced a permanent cell cycle arrest and cells stay as viable single cells (12). We examined the combination of flavopiridol and SN-38 in the Hct116 cells, in a concurrent and sequential manner, for the induction of apoptosis. The cells were treated with 20 nM SN-38 and 150 nM flavopiridol in four separate schedules, i.e., individually for 24 h (SN₃₄ or F₃₄), concurrently for 24 h ((SN+F₃₄), and sequentially with SN-38 for 24 h followed by flavopiridol for 24 h (SN₃₄+F₃₄) or the same drugs given in reverse sequence (F₃₄→SN₃₄). Fig. 1A shows the percentage of cells that undergo apoptosis with each treatment condition. SN-38 as a single agent did not induce significant apoptosis in the Hct116 cells. The addition of flavopiridol to SN-38-treated cells (SN₃₄→F₃₄) induced apoptosis that was significantly greater than the other treatment conditions tested [SN₃₄→F₃₄ versus SN₃₄→ND₃₄, P < 0.005; SN₃₄→F₃₄ versus F₃₄→SN₃₄, P < 0.001; and SN₃₄→F₃₄ versus (SN+F₃₄), P < 0.05].

RDA of cDNA of SN-38 and SN-38/Flavopiridol-treated Hct116 Cells. We hypothesized that altered expression of one or more genes is reflective of (and potentially involved in) the processes of progression of tumor cells from cell cycle arrest (induced by SN-38 alone) to cell death (SN-38 followed by flavopiridol). Using RDA, we identified the Drg1 gene as being induced by SN₃₄→ND₃₄ (the point of maximum G₂ cell cycle arrest), and suppressed by SN₃₄→F₃₄ (the condition exhibiting greatest apoptosis). The induction of Drg1 was confirmed by Northern blot analysis using the 32P-labeled Drg1 cDNA fragment, identified in the RDA screen, as a probe. As shown in Fig. 1B, Drg1 mRNA levels were induced 5-fold in Hct116 cells treated with SN-38 followed by no drug (SN₃₄→ND₃₄), as compared with untreated cells (ND₃₄). The addition of flavopiridol to SN-38-treated cells (SN₃₄→F₃₄) suppressed the mRNA expression of Drg1 to basal levels (ND₃₄). The corresponding Western blot indicated a 2–3-fold increase in Drg1 protein expression after SN₃₄→ND₃₄ compared with untreated controls (Fig. 1C). The addition of flavopiridol to SN-38-treated cells (SN₃₄→F₃₄) resulted in a 2-fold decrease in Drg1 protein expression as compared with SN₃₄→ND₃₄ treated cells. Drg1 protein levels were also lower in the concurrent treatment [(SN+F₃₄), Fig. 1C], which showed greater potentiation of apoptosis, as compared with any of the conditions tested with SN-38 alone (SN₃₄ or SN₃₄→ND₃₄; Fig. 1A).

Decreased Drg1 Expression by Stable Expression of AS Drg1 in Hct116 Cells Exhibits Increased Sensitivity to SN-38-induced Apoptosis. To investigate the relationship between Drg1 expression and chemosensitivity, we generated Hct116 cells in which endogenous Drg1 expression was inhibited by stable expression of AS Drg1 cDNA. As shown in Fig. 2A, Drg1 expression was decreased 3-fold in AS clones 9 and 18 as compared with vector-transfected cells. To test the sensitivity of AS Drg1 clones to SN-38, AS18 and vector-transfected cells were exposed to various concentrations of SN-38 and examined for apoptosis by QFM. As shown in Fig. 2B, treatment with

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500 nM SN-38 for 24–48 h induced a 4–5-fold higher degree of apoptosis in the AS18 cells as compared with vector-transfected cells. Similar results were obtained with the AS9.

The differential sensitivity of AS Drg1 clones to SN-38 was also confirmed by colony formation assays. These results indicated that the IC50 was decreased from 3.6 nM for vector-transfected cells to 1.8 and 2 nM for AS9 and AS18 clones, respectively (Fig. 2C). To determine the correlation between the increased sensitivity to SN-38 and Drg1 suppression, Western blot analysis was performed on these cells.

Treatment of the vector-transfected cells with SN-38 for 24 h or SN-38 for 24 h followed by no drug for 24 h (SN24→ND24), at increasing concentrations from 20 nM to 1 µM, resulted in a 2–3-fold induction of Drg1. Treatment of the AS18 cells, under identical conditions, showed a consistent suppression of Drg1 expression when compared with the corresponding vector-treated controls (Fig. 2D).

Stable Expression of Drg1 in SW620 Cells Exhibit Decreased Sensitivity to SN-38-induced Apoptosis. The colon cancer cell line SW620 expresses very low levels of Drg1. To additionally establish the role of Drg1 in chemosensitivity, SW620 cells were engineered to stably express Drg1. As shown in Fig. 3A, clone 5 (SDrg5) showed a 10-fold increase in Drg1 protein expression as compared with vector-transfected SW620 cells. To test the sensitivity of Drg1 overexpressing clones to SN-38, we exposed clone SDrg5 and vector-transfected cells to various concentrations of SN-38 and examined the cells for
A representative experiment is presented; were repeated at least three times with similar results, and data from one of the replicates. The SDrg5 cells were treated with indicated concentrations of SN-38 and stained with 4',6-diamidino-2-phenylindole as described in Materials and Methods. The studies were repeated at least three times with similar results, and data from one of the representative experiments is presented; bars, ± SD.

**Fig. 3.** Effect of Drg1 overexpression in SW620. A, Western blot analysis of SW620 cells stably expressing Drg1. The SW620 cells were transfected with vector-expressing Drg1 cDNA or vector alone, and clones were isolated after selection in G418. The cell lysates were prepared from the isolated clones, and Western blot analysis with Drg1 antibody was performed as described in “Materials and Methods.” The equal loading was confirmed by amido black and tubulin expression. B, Drg1 expression in SW620 lowers the percentages of cells undergoing apoptosis by SN-38 treatment. SW620 vector and SDrg5 cells were treated with indicated concentrations of SN-38 and stained with 4',6-diamidino-2-phenylindole as described in “Materials and Methods.” The studies were repeated at least three times with similar results, and data from one of the representative experiment is presented; bars, ± SD.

**Fig. 4.** The effect of CPT-11 on growth of established Drg1-modulated Hct116 or SW620 cells xenografts in nude mice. Mice were treated with CPT-11 according to the protocol described in “Materials and Methods.” Tumor volumes were measured as described in “Materials and Methods,” and mean log volume [calculated as (final volume − baseline volume)/baseline volume] was plotted against time (days). The control and flavopiridol-treated animals were sacrificed before 6 weeks because tumor size reached the maximum allowable limits set by Memorial Sloan Kettering Cancer Center. A, growth curves of tumors established as xenografts from Hct116 vector-transfected or SDrg5 clone either untreated or treated with CPT-11. B, growth curves of tumors established as xenografts from SW620 vector-transfected or SDrg5 clone either untreated or treated with CPT-11.

**Discussion**

Intrinsic chemotherapy resistance is a major obstacle to the successful treatment of cancer. The ability to undergo cell cycle arrest and induce a repair mechanism describes an intrinsic resistance to many DNA-damaging agents (16, 17). This process protects the cell from the cytotoxic effects of the DNA-damaging agent. Indeed, increased sensitivity to CPT-11 and other DNA-damaging agents has been observed in cells that are unable to undergo a G2 cell cycle arrest after drug exposure (9, 10, 18). It has been shown previously that treatment of Hct116 cells with radiation, doxorubicin, or etoposide induces a state of permanent cell cycle arrest, creating a “lawn” of single cells that are unable to form colonies (19). Treatment of Hct116 cells with SN-38 also results in permanent cell cycle arrest (12). This state of cell cycle arrest induced by SN-38 could explain the relative resistance to this agent in vitro in the Hct116 cells. It may also explain the relatively low rates of response observed with this agent in xenografts and clinical trials.

There is recent appreciation that apoptosis, and not cell cycle arrest, results in higher tumor regressions and cures (19). Therefore, it is essential to create conditions that induce apoptosis in the SN-38–resisted cell population. We achieved this by treating the SN-38–resisted Hct116 cells with flavopiridol (12). This transition from cell cycle arrest to cell death presented a model system to identify new genes that were differentially expressed under these two treatment conditions (SN-38 alone versus SN-38 followed by flavopiridol). The identification of the Drg1 gene then lead us to create cell lines in...
which Drg1 was either suppressed or overexpressed. Our results indicate that inhibition of endogenous Drg1 expression in Hct116 cells by stable expression of AS Drg1 cDNA increased their sensitivity to SN-38. Conversely, the overexpression of Drg1 in SW620 cells increased the resistance of these cells to SN-38. Moreover, tumors established from AS Drg1-expressing Hct116 cells were more sensitive to CPT-11 and exhibited a greater reduction in tumor volume compared with tumors established from vector-alone-transfected Hct116 cells. Similarly, tumors established from Drg1 overexpressing SW620 cells were more resistant to CPT-11 as compared with tumors established from vector-alone-transfected SW620 cells. These results indicate that Drg1 is a bona fide target gene for resistance to CPT-11 in these colon cancer cells.

Drg1 is known to belong to a Drg family of four genes that share 57–65% amino acid identity (20). Drg1 is the most studied gene among this family. The Drg1 mRNA is 3 kb and is expressed in several normal tissues including prostate, ovary, intestine, placental membrane, and colon (21). Drg1 has been identified previously through differential screening techniques during stress response, hormone responses, cell growth, and differentiation (21–25). However, the functional significance of Drg1 in these processes is not known. Previous studies have shown that the constitutive overexpression of Drg1 in MCF7 and EJ tumor cells decreases their proliferation rates, and these cells form smaller colonies in soft agarose (22). We also observed a trend toward decreased growth rate in untreated tumors established from Drg1 overexpressing cells (SW620 SDr5g clone) and increased growth rate of untreated tumors established from AS Drg1 cells (AS18 and AS9 clones) compared with tumors established from vector-transfected cells. However, none of these differences were statistically significant (P < 0.5).

It has been shown that Drg1 mRNA expression is decreased in colon adenomas and adenocarcinomas as compared with normal colon mucosa (21). The enforced constitutive expression of Drg1 in the metastatic colon cancer cell line SW620 has been shown to induce morphological changes that are indicative of differentiation including up-regulation of the expression of several colonic epithelial cell differentiation markers (23). Drg1 overexpression in SW620 also reduced in vitro invasion through Matrigel and in vivo liver metastasis in nude mice (23). Northern blot studies of Drg1 expression in five matched pairs of colon cancer primary and liver metastases indicated a down-regulation in two and complete loss of Drg1 mRNA expression in three metastatic lesions (23). This would suggest that Drg1 serves as an antimitotic gene, and loss of Drg1 increases the metastatic potential of the colon cancer cells. We have examined the expression of Drg1 by immunohistochemistry in 18 matched pairs of primary colon cancers and liver metastases. Results from this study indicated no difference in the protein expression of Drg1 between the colon primary and the liver lesions (26). In fact, in a study of 131 patients with metastatic colon cancer, all of the liver metastases from these patients expressed Drg1, and there was no association between Drg1 protein expression by immunohistochemistry and the number of metastases present (26). This study would not support Drg1 as an antimitotic gene.

The clinical effect of CPT-11 on Drg1 expression in colonic metastases is unknown. However, a clinical trial of sequential CPT-11 followed by flavopiridol is now underway. Serial tumor biopsies are planned to examine changes in Drg1 expression under these experimental conditions. Thus far, the Phase I trial indicates that the combination is well tolerated, and levels of flavopiridol can be achieved in the plasma that are associated with Drg1 suppression in vitro (27). Thus, there are now clinical trials in which Drg1 modulation by CPT-11 and subsequent suppression by flavopiridol will be closely examined.

We have reported previously that treatment of Hct116 cells with SN-38 induces a G2 arrest with inhibition of cyclin B1-associated cdc2 kinase activity (12). Furthermore, subsequent treatment of these G2 arrested cells with flavopiridol resulted in continued inhibition of cyclin B1-associated cdc2 kinase and persistent arrest of the cells in G2. The G2 arrest induced by flavopiridol on the SN-38 treated cells is because of flavopiridol’s direct inhibition of cdc2 kinase (12). Flow cytometry analysis with MPM-2 and propidium iodide staining of SN-38-treated Drg1 AS clones showed no change in the percentage of cells arrested in G2 (data not shown). This is consistent with our flavopiridol studies and would indicate that abrogation of the G2 checkpoint is not the mechanism by which Drg1 suppression sensitizes these cells to SN-38.

Various possibilities can be envisioned to explain the role of Drg1 in differential sensitivity to CPT-11. In fact, the suppression of Drg1 also resulted in increased sensitization to docetaxel (data not shown). This would suggest that Drg1 plays an even greater role in chemotherapy sensitization. It is possible that Drg1 exerts its function by affecting the regulators of the apoptotic machinery, such as Bcl-2 family members, or by directly activating caspases, the executors of apoptosis. It is also possible that Drg1 affects the various signal transduction pathways such as PKB (protein kinase B), c-Jun-NH2-terminal kinase, or mitogen-activated protein kinase pathways that in turn regulate the induction of apoptosis on damaged cells. Studies to examine the mechanism for chemotherapeutic sensitization by Drg1 suppression are now underway. In summary, our results point to Drg1 as a novel gene that modulates CPT-11 sensitivity both in vitro and in vivo. Validating Drg1 as a molecular determinant for chemosensitivity to CPT-11 would establish Drg1 as a new target for improving the efficiency of a drug that has become a cornerstone of colon cancer therapy.

References
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