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Effect of Penclomedine (NSC-338720) on Telomere Fusions, Chromatin Blebbing, and Cell Viability with and without Telomerase Activity and Abrogated p53 Function

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ABSTRACT. Telomeres, or chromosome ends, are essential in maintaining chromosomal integrity. Telomeres consist of a short hexameric sequence, 3′-TTAGGG-5′, repeated in tandem arrays added to chromosomes by the ribonucleoprotein enzyme telomerase. In this study, we assessed whether penclomedine, a novel synthetic pyridine compound presently being evaluated in clinical trials for its anticancer activity, influences telomere fusions (chromosome end-to-end associations) and telomerase activity in cells in culture. We found that penclomedine reduced the mitotic index, induced chromosome end associations in all phases of the cell cycle, and rapidly induced chromatin blebbing in a concentration-dependent manner in both cervical carcinoma (HeLa) cells and in normal human fibroblasts (AG1522). However, the effectiveness of the drug was much more pronounced in HeLa cells. In addition, there was a drug-mediated, concentration-dependent decline in telomerase activity noted in the HeLa cells that correlated with a decrease in mitotic index and an increase in telomere fusions. Interestingly, when the mitotic index, chromatin blebbing, and telomere fusions were compared between the telomerase positive (HeLa) and negative (AG1522) cell types, penclomedine affected chromatin stability to a greater extent in those cells with detectable telomerase activity. In addition, telomerase positive colorectal carcinoma cells with abrogated p53 (RC-10.3 cells) were more sensitive to penclomedine than were telomerase positive cells with wild-type p53 (RKO cells). These studies suggest that penclomedine may have a therapeutic advantage in killing tumor cells that are positive for telomerase activity and defective in p53 function. *Copyright © 1997 Elsevier Science Inc.* BIOCHEM PHARMACOL 53;3:409–415, 1997.

KEY WORDS. penclomedine; tumor; chromosome end associations; blebbing; telomerase; p53

Chromosome end associations seen at metaphase involve telomeres, which are essential for the stability and complete replication of eukaryotic chromosomes [1, 2]. Telomeres are structures consisting of repeat sequences of DNA and telomere-associated proteins that cap human chromosomes [see Refs. 3–5 for reviews]. In human and most other mammals, the telomeric repeats are composed of repetitive tracts of hexameric sequence TTAGGG [6], which are added enzymatically to chromosomal DNA ends by the ribonucleoprotein complex, telomerase [7, 8]. In normal cells that do not have detectable telomerase activity, telomeres are not maintained and progressively shorten with each cell division, eventually resulting in inhibition of cell proliferation.

Telomerase activity has been detected in a variety of immortal human and mouse cell lines, tumor and germ cell lines, as well as some normal somatic cell types [9–19]. We recently observed that telomerase activity is highest in metastatic tumor cells [20], which may reflect a higher proliferation rate as recently described by Holt *et al.* [21]. In the present study, we investigated the ability of penclomedine, a drug shown previously to produce inhibition of DNA synthesis through an unknown mechanism(s), to affect telomerase activity as well as chromosomal structure. We examined the effect of penclomedine on the mitotic index, telomere fusions, and chromatin blebbing in cells with and without detectable telomerase activity.

Previous studies revealed that penclomedine produces chromosome aberrations in the mouse, although it does not

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¶ *Abbreviations:* PCC, premature chromosome condensation; PCR, polymerase chain reaciton; TRAP, telomere replication amplification protocol; TRF, terminal restriction fragment; and BrdU, bromodeoxyuridine.

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induce DNA strand breaks and interstrand cross-linking [22]. Penclomedine is currently in clinical trials based on its specific antitumor activity both in human and mouse breast tumor models [23, 24]. In the present study, we sought to determine if penclomedine induced chromatin changes at chromosome ends in the form of telomere fusions (chromosome end-to-end associations). Here we report the effects of penclomedine on telomere fusions, chromatin blebbing, telomere length, telomerase activity, and cell viability in normal fibroblasts and in HeLa and RKO cell lines. Our findings indicate that penclomedine induces a decrease in the mitotic index, an increase in both telomere fusions and chromatin blebbing, and an inhibition of telomerase activity.

MATERIALS AND METHODS

Materials

Penclomedine [(3,5-dichloro-2,4-dimethoxy-6-trichloromethyl)-pyridine] was obtained from the Pharmaceutical Resource Branch (Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute).

Cell Culture

Human cervical carcinoma cells (HeLa) were obtained from Dr. J. Bedford, University of Colorado. Low passage normal human fibroblasts (AG1522) were obtained from the Aging Cell repository (Camden, NJ). Both the HeLa and AG1522 cell lines were grown according to procedures described previously [20, 25]. Colorectal carcinoma cells (RKO) and their p53 abrogated derivative, RC-10.3, were obtained from Dr. M. Kastan, Johns Hopkins University, and grown according to the procedure described previously [20]. RC-10.3 cells are RKO cells that contain a pCMV-E6 expression construct [26]. Penclomedine was dissolved in DMSO and added to the cell culture. The final concentration in the medium was 0.2%. Appropriate concentrations of DMSO as drug vehicle were added to control cell cultures.

Cytotoxicity

RKO and RC-10.3 cells in exponential growth were treated with different concentrations of penclomedine for various periods of time. Cell viability was monitored by trypan blue exclusion, and cell counts were made by both hemacytometer and electronic counting (Coulter Electronics Inc., Hialeah, FL).

Chromosome Studies

MITOTIC CHROMOSOMES. Metaphase chromosomes were prepared by a procedure described earlier [27, 28]. Mitotic indices were counted (500 cells per sample) both from treated and control cells. Giemsa-stained chromosomes from 100 metaphases were analyzed for chromosome end associations, and 400 cells were analyzed for chromatin blebbing [29].

INTERPHASE CHROMOSOMES. Telomeric associations in interphase chromosomes from the G_1- and G_2-phases were examined in PCC¶ spreads following the protocol described earlier [29]. Discrimination between mitotic and interphase chromosomes was achieved through differential staining [25], rendering it easy to identify interphase chromosome end associations in G_1 as well as G_2 cells [29]. One hundred differentially Giemsa-stained PCC spreads from both G_1- and G_2-phase cells were examined microscopically for chromosome end associations.

Determination of S Phase Cells

Cells were treated with BrdU (Sigma Chemical Co., St. Louis, MO) at a concentration of 60 μg/mL. BrdU-labeled cells were detected by using biotin-labeled antiBrdU (Caltag Laboratories, Inc., San Francisco, CA) and horseradish peroxide reaction. After the fixation of slides, they were immersed in 0.07 N NaOH for 2 min and then washed thoroughly with PBS, pH 8.5. AntiBrdU was applied to slides, and preparations were incubated at 37°C for 30 min in a humidified chamber. The peroxidase staining procedure as described by Oncor (Gaithersburg, MD) was utilized to detect cells with BrdU label.

DNA Extraction

The procedure used for extraction of DNA was the same as that described previously [29]. In brief, cells were trypsinized and lysed, and proteins were digested in 10 mM Tris–HCl (pH 8.0), 100 mM NaCl, 25 mM EDTA, 0.5% SDS, and 0.1 mg/mL proteinase K at 50°C overnight. Following two extractions with phenol and one with chloroform, DNA was precipitated with ethanol and dissolved in TE buffer [10 mM Tris–HCl (pH 8.0), 1 mM EDTA].

Fluorescent In Situ *Hybridization (FISH)*

A biotinylated telomeric probe obtained from Oncor was used for *in situ* hybridization. The procedure has been described previously [29, 30]. The telomeric signals were examined in metaphases.

Determination of Telomere Length

For measuring TRF lengths, a measure of the relative size of telomeres, DNA was digested with restriction enzymes (*Rsa*I and *Hinf*I; GIBCO/BRL, Grand Island, NY) that do not cut TTAGGG sequences and processed as described earlier [29]. Prior to restriction digestion, a sample of each DNA was electrophoresed to verify its integrity. The digested DNA samples were loaded on 0.8% agarose gels, which were run in the presence of ethidium bromide at 2 V/cm in TAE buffer [0.04 M Tris-acetate (pH 8.3), 1 mM

EDTA]. The fractionated DNA was depurinated *in situ* by a 20-min incubation in 0.25 N HCl, subsequently denatured, and then nicked with 0.5 M NaOH, 1.5 M NaCl (two times, 20 min) and neutralized with 0.5 M Tris–HCl (pH 7.5), 3 M NaCl (two times, 20 min). The DNA was transferred to a nitrocellulose membrane in 20× SSC (3 M NaCl, 0.3 M sodium citrate, 66 mM Na_2HPO_4) overnight and baked at 80°C under vacuum for 2 hr. TTAGGG oligonucleotide probe was used for hybridization as previously described [29]. Briefly, the membrane was prehybridized for 1 hr and then incubated in 5× SSC, 0.5% SDS at 48°C with ^{32}P-end-labeled $(TTAGGG)_5$ for 12 hr and finally washed with 2× SSC at 48°C (10 min each) before exposure to Kodak XAR film for 1–2 days.

Telomerase Assays

To assay telomerase activity, the telomere repeat amplification protocol (TRAP) was performed with a modification of the procedure of Kim *et al.* [11]. Briefly, the assay was performed in two steps: (1) telomerase-mediated extension of an oligonucleotide primer (TS), which serves as a substrate for telomerase, and (2) PCR amplification of the resultant product with the oligonucleotide primer pairs. Cultured cells (10^5) were pelleted in 1.5 mL microcentrifuge tubes for 5 min at 6000 rpm using an Eppendorf centrifuge. Medium was removed carefully, and the cells were quickly stored at −80°C or immediately lysed. Cells were lysed with 200 μL of ice-cold lysis buffer for 3 min. The extract was assayed in 50 μL of reaction mixture containing 50 μM of each deoxynucleoside triphosphate, 344 nM TS primer (5′-AATCCGTCGAGCAGAGTT-3′), 0.5 μM T4gene32 protein (U.S. Biochemicals, Cleveland, OH), 2 μCi [α-^{32}P]TTP (3000 Ci/mmol) (Amersham, Arlington Heights, IL), and 2 U of Taq DNA polymerase (GIBCO/BRL) in a 0.5-mL tube that contained the CX primer (5′-CCCTTACCCTTACCCTTACCCTAA-3′) at the bottom sequestered by a wax barrier (Ampliwax; Perkin-Elmer, Foster City, CA). After a 30-min incubation at room temperature for telomerase-mediated extension of the TS primer, the reaction mixture was heated at 90° for 90 sec to inactivate telomerase. It was subjected to 31 PCR cycles at 94°C for 30 sec, 50°C for 30 sec, and 72°C for 45 sec. As a control, 5 μL of extract was incubated with 1 μg of RNase (5 Prime—3 Prime, Boulder, CO) for 20 min at 37°C prior to the telomerase assay. The PCR product was separated by electrophoresis on a 10% polyacrylamide gel. To obtain semiquantitative information about levels of telomerase activity, an internal telomerase assay standard [31] was included that could be amplified by the same two primers used in the telomerase activity assay. The sample without drug was considered to represent total (100%) telomerase activity; results were plotted as a percent of total telomerase activity versus that observed in cells after penclomedine treatment. DNA synthesis was determined as described previously [22].

RESULTS

To assess the influence of penclomedine on telomere fusion and telomerase activity, confluent HeLa cells and AG1522 fibroblasts were trypsinized and plated at low density. These two cell types were selected because: (1) both cell types have similar cell doubling times of about 21 hr, and (2) both have similar distribution of G_1, S and G_2/M phase cells. After 24 hr of subculturing, cells were exposed to various concentrations of penclomedine for different lengths of time. Since penclomedine was dissolved in DMSO, all of the treatment and control groups were exposed to similar final concentrations of DMSO.

Mitotic Index

To determine the influence of penclomedine on the mitotic index, cells were treated with concentrations of penclomedine ranging from 0 to 80 μg/mL for a period of 4 hr. Cells were then washed and further incubated for 4 hr in the presence of colcemid. As shown in Fig. 1, the mitotic index declined with an increase in drug concentration; the effect was more pronounced in HeLa cells than in AG1522 fibroblasts.

Telomere Fusions

To examine whether penclomedine could induce telomere fusions (chromosome end-to-end associations), cells were treated with various concentrations of drug for a period of 4 hr, washed with fresh medium, and then incubated in medium in the presence of colcemid for 2 hr prior to examination of chromosome end associations in metaphase spreads. As shown in Fig. 2, there is a concentration-dependent increase in the frequency of cells with chromosome end associations. Drug-induced chromosome end as-

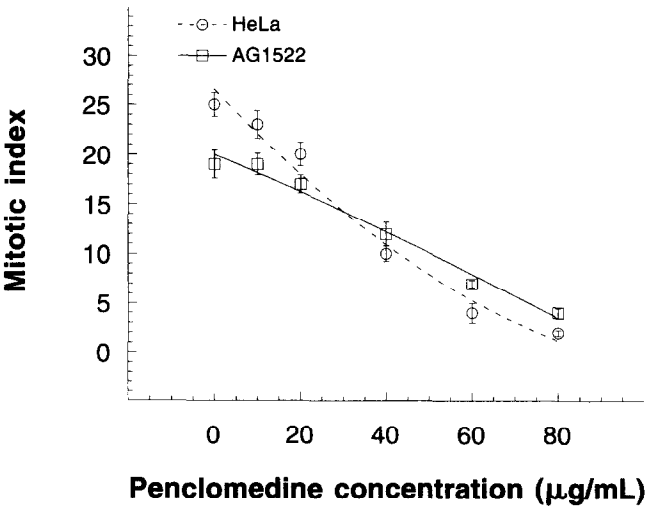

FIG. 1. Percent cells at mitosis (mitotic index) 4 hr after penclomedine treatment of HeLa and AG1522 cells. Data are presented as means ± SD. For each experiment, 500 cells were scored for mitotic index.

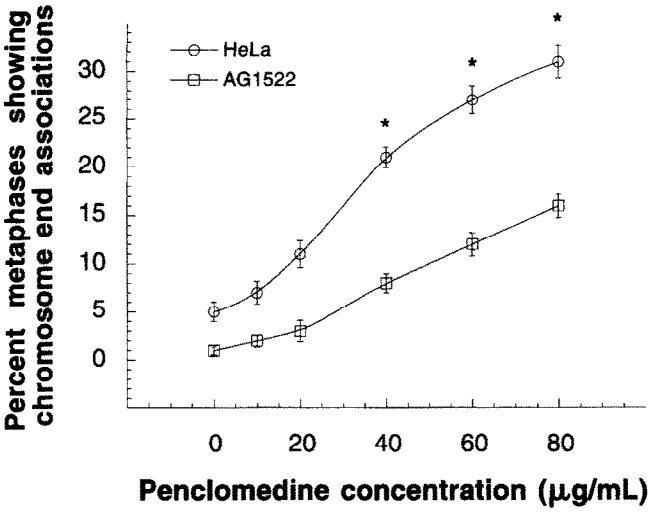

FIG. 2. Percentage of cells with chromosome end associations after treatment with different concentrations of penclomedine. Cells were treated for 4 hr with penclomedine, washed with fresh medium, and incubated with colcemid for 4 hr. Metaphases (100) were analyzed for chromosome end associations. Data are presented as means ± SD. Key: (*) $P \leq 0.05$.

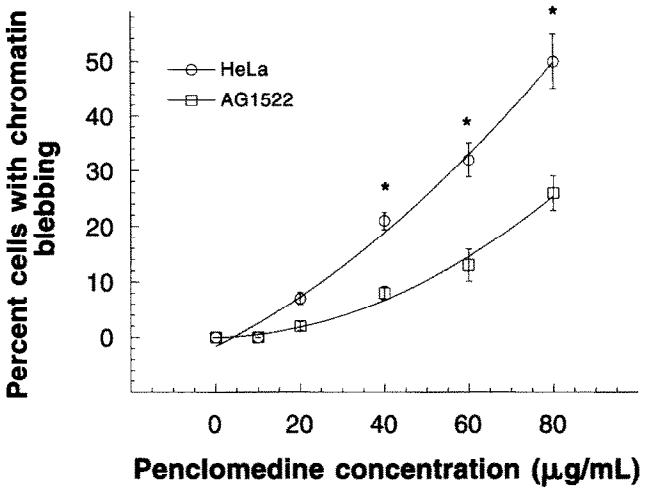

FIG. 3. Percentage of cells with chromatin blebbing after 12 hr of treatment with different concentrations of penclomedine. Cells were treated with drug for 12 hr, washed, fixed, and stained with Giemsa to determine the chomatin blebbing. Five hundred cells were examined for each time point. Data are presented as means ± SD. Key: (*) $P \leq 0.05$.

sociations were significantly higher ($P \leq 0.05$) in HeLa cells in comparison with AG1522 cells at drug concentrations of 40 μg/mL and above.

To determine whether penclomedine induced chromosome end associations in interphase cells, G_1- and G_2-phase cells were examined. After drug treatment (4 hr), cells were washed and immediately fused with the HeLa mitotic inducer cells. Chromosome end associations were observed in G_1- as well as G_2-phase cells (Table 1).

Since a dramatic effect on induction of telomere fusions was observed, we then determined if penclomedine induced global chromatin alterations (i.e. nuclear morphology and blebbing). To determine the influence of drug on chromatin blebbing, cells were exposed to penclomedine for 12 hr. As shown in Fig. 3, penclomedine induced chromatin blebbing in a concentration-dependent manner. Interestingly, the drug induced more chromatin blebbing in HeLa cells than in normal fibroblasts, and the differences were statistically significant ($P \leq 0.05$) at concentrations 40 μg/mL and above. To determine whether the chromatin blebbing was confined to S-phase, cells were exposed to BrdU for a period of 1 hr prior to treatment with penclomedine. The S-phase cells were then detected by using the antiBrdU antibody. Chromatin blebbing was seen in S-phase as well as non-S-phase cells.

The time-dependent formation of chromatin blebs after exposure of cells to a single concentration (80 μg/mL) of penclomedine was then examined. Drug-mediated blebbing was observed as early as 2 hr after addition of drug (data not shown). There was a higher incidence of chromatin blebbing in HeLa cells as compared with AG1522 fibroblasts at all time points beyond 2 hr. The cell type-dependent incidence of drug-mediated chromatin blebs was especially evident at 8 and 12 hr of drug exposure.

To examine whether penclomedine could produce delayed genomic instability, HeLa cells were exposed to penclomedine at 40 μg/mL for 12 hr. Cells surviving this treatment were grown for about 30 mean population doublings. They were then examined for chromosome end associations at metaphase. However, no difference was observed between control and drug-treated cell survivors.

TABLE 1. Percentage of HeLa cells with penclomedine-mediated chromosome end-to-end associations seen at different phases of the cell cycle

Drug concentrations (μg/mL)	G_1-phase	G_2-phase	Metaphase
0	7	4	5
40	23	25	21

Frequencies of cells with chromosomal end associations seen at different stages of the cell cycle in 100 cells at each concentration of drug. There were significant differences ($P \leq 0.001$) in chromosome end associations seen between drug-treated and untreated cells.

Detection of Telomeric Signals and Telomere Terminal Length Analysis

Since penclomedine produced telomeric fusions rapidly, we determined if this was due, at least in part, to loss of telomeric sequences. To address this question, we used two different approaches. First, we compared telomeric signals using a telomeric specific $(TTAGGG)_n$ biotinylated probe for FISH analysis. Hybridization of the telomeric probe showed no statistically significant differences in the telomeric signals between drug-treated and control cells (data not shown). Further, the telomeric signals were seen at the

chromosome end association sites. Since cytological observations do not provide an accurate measure of telomere length, this was determined by measuring TRF length in *RsaI* and *HinfI* digested DNA from cells exposed acutely to drug or from those cells that survived 30 mean population doublings post exposure to penclomedine. No effect on telomere length was observed either after acute exposure or in the drug survivors (data not shown).

Inhibition of Telomerase Activity by Penclomedine

To examine the influence of telomerase activity, cells were treated with different concentrations of penclomedine for a period of 4 hr. Cells were washed and examined for telomerase activity using the TRAP procedure (Fig. 4). As shown in Fig. 5, there is a concentration-dependent inhibition of DNA synthesis as well as telomerase activity. To determine whether inhibition of telomerase activity was independent of S-phase of the cell cycle, we examined telomerase activity in M-phase HeLa cells. Accumulation of mitotic cells was accomplished as described previously [25]. Mitotic cells were treated with drug for 4 hr, washed, and examined for telomerase activity. Telomerase activity was seen in mitotic control cells; however, there was a concentration-dependent decline in telomerase activity observed in drug-treated mitotic cells when compared with controls held in mitosis arrest for the same period of time but without being exposed to penclomedine (data not shown). Since trypan blue exclusion indicated a large amount of cell death at the higher concentrations of penclomedine, the decrease in telomerase activity is probably due to an indirect toxicity drug effect.

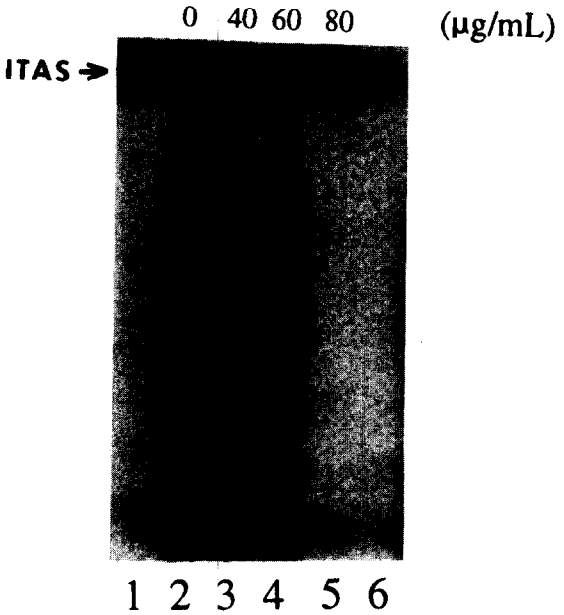

FIG. 4. Autoradiograph showing telomerase activity in HeLa cells after treatment with different concentrations of penclomedine. An equal number of cells (1000) was used for each TRAP assay. Note that there was a concentration-dependent inhibition of telomerase activity by penclomedine. ITAS stands for internal telomere amplification standard. Lanes 1 to 6 represent the products of telomerase assay after treatment of cells with the drug for 4 hr. Lane 1 represents lysis solution; lane 2 represents cells treated with 0 µg/mL of drug; lane 3 represents cells treated with 40 µg/mL of drug; lane 4 represents cells treated with 60 µg/mL of drug; lane 5 represents cells treated with 80 µg/mL of drug; and lane 6 represents lysis solution without ITAS.

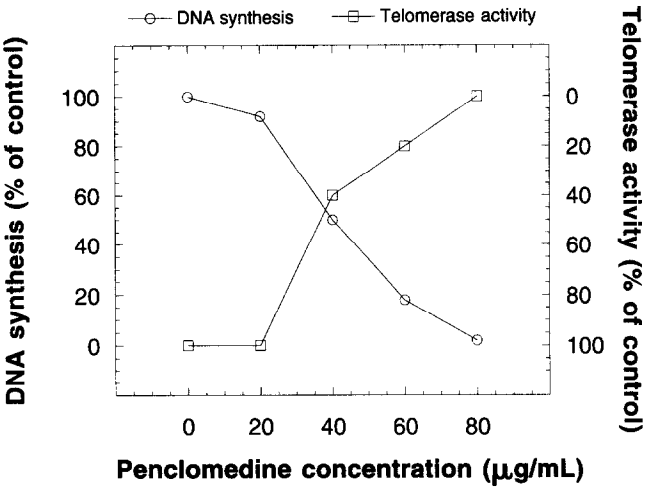

FIG. 5. Influence of inhibition of DNA synthesis and telomerase activity by penclomedine. HeLa cells were incubated with different concentrations of penclomedine for 4 hr. After treatment, cells were washed and split into two parts. One part was used for examining DNA synthesis and the other for telomerase activity. For the examination of DNA synthesis, cells were further incubated for 1 hr in the presence of [*methyl*-^{3}H]thymidine. The incorporation of thymidine was determined by liquid scintillation counting. Telomerase activity was measured by the TRAP procedure.

Influence of p53 *Abrogation on Penclomedine-Induced Cell Death*

Abrogation of p53 has been linked with gene amplification and drug resistance [32, 33]. To examine whether abrogation of p53 altered penclomedine-induced cell death and inhibition of telomerase activity, RKO human colorectal carcinoma cells with wild-type p53 and their p53 abrogated derivative (RC-10.3) cells were treated with penclomedine. Cell viability was determined by trypan blue exclusion and cell count. As shown in Fig. 6, there was a concentration- and time-dependent increase in cell kill in both cell types. However, statistically significant differences in cell viability by penclomedine were observed between the two cell types; RC-10.3 cells with abrogated p53 were more sensitive to penclomedine. Inhibition of telomerase activity by penclomedine in RKO and RC-10.3 was similar (data not shown).

DISCUSSION

Exposure of cells to penclomedine results in chromosome gaps, breaks, and exchanges in mouse leukemic P388 cells *in vivo* [22]. The data suggest a drug or drug-mediated interaction with DNA that is consistent with alkylating activity [22]. To further understand the mechanism(s) of pen-

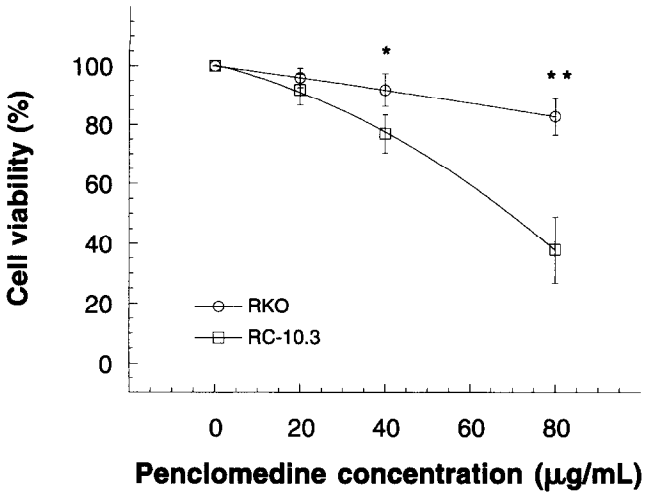

FIG. 6. Influence of p53 abrogation on penclomedine-induced cell kill. RKO cells with wild-type p53 and RC-10.3 cells with abrogated p53 were treated with different concentrations of penclomedine for 8 hr. A concentration-dependent decline in cell viability was found in both cell types. Note that an enhanced loss of cell viability was seen in RC-10.3 cells. Data are presented as means ± SD. Key: (*) $P \leq 0.05$, and (**) $P \leq 0.001$.

clomedine-mediated cell injury, we have assessed the influence of this agent on telomeres and telomerase activity. The present studies revealed that exposure of cells to penclomedine produces a reduction in mitotic index, an increase in telomere fusions, and an increase in chromatin blebbing. These drug-mediated changes were significantly greater in HeLa and RKO cells, which are telomerase positive, than in AG1522 fibroblasts, which do not contain detectable levels of telomerase activity. The studies suggest, but certainly do not prove, that cells containing telomerase activity may be more sensitive to penclomedine than cells in which telomerase activity may be either much lower or undetectable.

Telomerase has attracted interest as a possible target for cancer therapeutics. The apparent absence of measurable telomerase activity in many (but not all) normal cells, while almost all human primary tumors have telomerase activity, raises the possibility that telomerase inhibitors may have utility in the treatment of cancer. Recent experiments indicating that inhibition of telomerase in HeLa cells (via expression of antisense telomerase RNA) resulted in gradual telomere erosion and subsequent inhibition of cell proliferation [34] support this possibility. In addition, somatic cell hybrids made between normal telomerase negative cells and immortal cells expressing telomerase have greatly reduced telomerase activity, and cell growth eventually ceases, supporting the notion that there are normal gene products that can modulate telomerase activity [35]. In the present series of investigations, we have shown that penclomedine (or a metabolite) appears to have the ability to produce alterations of telomere stability. The drug produced an increase in telomere fusions (chromosome end-to-end associations) in a concentration-dependent manner, although no detectable effects were observed on telomeric signals through FISH analysis or on telomere length.

In this report, we found that cells expressing telomerase appeared more sensitive to the effects of penclomedine as measured by cytotoxicity. A similar observation regarding the relative cytotoxicity of penclomedine was also made in normal human bronchial epithelial cells versus their tumor-derived cell line (unpublished observations).

Another interesting finding revealed in the present studies was the greater sensitivity of cells with mutant p53 to penclomedine in contrast to those cells with normal p53 function. Most tumor cells have p53 mutations that result in unchecked cell proliferation. Abrogation of p53 function has been linked with genomic instability, gene amplification, and drug resistance [32, 33]. It is important to identify chemotherapeutic agents that have preferential toxicity to cells that possess both p53 mutations and significant telomerase activity.

Recently, there have been several reports of down-regulation of telomerase activity when cells are quiescent and after induction of terminal cell differentiation [21, 36, 37]. Since terminal differentiation induces global chromatin alterations, influencing the function of several genes, inhibition of telomerase activity could be the consequence of those chromatin changes. Additionally, the alkylating activity of penclomedine (or its metabolites) could greatly contribute to the chromatin damage and bleb formation noted in the present study. Such drug-mediated chromatin damage could, in turn, contribute to alteration of gene function. Clearly, the exact mechanism(s) of penclomedine in producing chromatin-mediated injury, inhibition of telomerase activity, and relative increased activity against p53 mutant cells needs to be defined more carefully. The present study demonstrates, however, that a single agent can produce pleotropic responses in malignant cells which may, in turn, lead to preferential tumor cell death.

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