Lethal and Cytokinetic Effects of Anguidine on a Human Colon Cancer Cell Line

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ABSTRACT

Anguidine is a fungal metabolite with antitumor activity in a murine colon cancer model. Because of disappointing results in clinical trials, we analyzed the lethal and cytokinetic effects of anguidine on cultured human colon cancer cells. The studies revealed a moderate reduction in survival only after prolonged drug exposure. Continuous incubation with anguidine for longer than 48 hr produced a moderate increase in the percentage of S-phase cells and a slight decrease in the proportion of cells in G1, by pulse cytophotometry. An immediate reduction in the cumulative labeling index for cells continuously exposed to trifluidine and anguidine and a rapid decrease in the cumulative mitotic index for cells continuously exposed to Colcemid and anguidine indicated a block at the G1, into S and G into mitosis transitions.

Tumoricidal activity of anguidine in a cultured human colon cancer line is poor and requires prolonged exposure. The kinetic data reflect an almost frozen state of the cell cycle.

INTRODUCTION

Anguidine (diacetoxyscirpenol, NSC 141537) is a drug that is presently undergoing clinical trials in cancer patients. It belongs to the 12,13-epoxytrichothecene group of sesquiterpenes. Compounds in this class are derived from the phytotoxic metabolites of Fusarium equiseti and several related plant parasitic fungi (7, 12, 17). Anguidine is the most abundant and easily accessible of these compounds.

In vitro studies have shown that certain of the 12,13-epoxytrichothecenes inhibit protein synthesis and, to a lesser extent, DNA production (24). No significant effect on RNA synthesis has been noted. Derivatives such as crotocin and trichodermin reversibly decrease protein synthesis by inhibiting chain elongation. Anguidine, however, irreversibly blocks protein synthesis by inhibiting protein chain initiation through degradation of polyribosomes (24, 26, 30, 35, 38).

Early preclinical studies demonstrated inhibition of tumor growth by anguidine in several continuous cell lines such as HEP2, BHk, L1210 (1, 21), and P388 (B. K. Bhuyan, unpublished data) and in certain experimental animals (23, 34). Toxicity to hematopoietic and other proliferating normal tissues was also noted (34). Initial screenings at the National Cancer Institute, Bethesda, Md., revealed >100% increase in the life span of P388 leukemia-bearing mice and approximately a 50% increase in the life span of animals with L1210 leukemia given i.p. anguidine. In both models the best results were obtained with chronic administration rather than intermittent courses (29).

Recently, Corbett et al. (10, 11) have found anguidine to possess activity in a transplantable, chemically induced mouse colon adenocarcinoma. Of 10 mice treated with anguidine as a single agent (5.6 mg/kg weekly for 4 doses beginning on Day 17 after transplantation), 9 had >50% regression in tumor weight and 4 were cured. When anguidine was utilized in simultaneous combination with 5-fluorouracil (30 mg/kg/dose), moderate potentiation was found, with 9 of 10 animals responding and with 7 cured (10, 11).

A Phase I trial has recently been conducted at the University of Texas System Cancer Cancer M. D. Anderson Hospital (28). The recommended dosage in this study, 4.0 to 5.0 mg/sq m/day × S was utilized in a Phase II study of patients with adenocarcinoma of the colon at our institution. Of 42 colon cancer patients treated, only 2 had partial responses (27). In view of the disparity between results in murine and human colon cancer, we have tested the efficacy of anguidine as a cytotoxic agent in a human colon cancer cell line.

Tissue culture studies provide an economical, fast, and efficient method to investigate a variety of biological responses in a controlled systematic fashion. Although no simple translation from in vitro to in vivo systems is always possible, many mammalian cells respond similarly in the 2 situations. The major assumption of in vitro studies is that the response of cultured cells will reflect that of in vivo cells once the drug has reached the neoplastic elements (13).

In this communication we report the lethal and cytokinetic effects of anguidine on cultured human colon cancer cells as a function of drug concentration and exposure time.

MATERIALS AND METHODS

Drug. Anguidine (NSC 141537), manufactured by Ben Venue Laboratories, Inc., Bedford, Ohio, was obtained from the Division of Cancer Treatment, National Cancer Institute, NIH, Bethesda, Md. 20014, and was supplied in 5-mg ampuls as a lyophilized powder with 10 mg of mannitol. Reconstitution was routinely accomplished with 0.9% NaCl solution, immediately prior to each experiment.

Cell Line. A human carcinomaembryonic antigen-producing adenocarcinoma cell line (LoVo), described in detail elsewhere (15), was utilized. Monolayers are maintained in Ham’s F-10 medium supplemented by 20% fetal calf serum,
glutamine, vitamins, and antibiotics. Cells seeded in 60-mm Petri dishes at a concentration of $5 \times 10^4$ cells/dish reach exponential growth in about 36 hr. Exponential growth with a doubling time of 36.3 hr, a LI of 31%, a generation time of 29.3 hr, $T_{G1}$ of 14.7 hr, $T_{S}$ of 10.7 hr, $T_{G2}$ of 4.8 hr, and a growth fraction of 90% lasts for about 4 to 5 days. Thereafter, cultures enter a stationary phase defined by no exponential growth in about 36 hr. Exponential growth with a growth fraction of 90% lasts for about 4 to 5 days.

Survival Studies. Cell survival studies were performed as a function of drug concentration and exposure time (13). Stock cultures of LoVo cells were harvested by using hyaluronidase (100 IU/ml) followed by trypsin (2.5% in Solution A), each for 5 min at 37°C. Routinely, $5 \times 10^4$ cells were placed into 60-mm Petri dishes containing 3 ml of medium and incubated at 37°C in a 5% CO$_2$-95% air atmosphere for about 48 hr. The medium was then decanted from the plates, and 5 ml of the medium containing increasing concentrations of anguidine were added. Following drug exposure, the cells were washed twice with Hanks' balanced salt solution and harvested as described previously. Cells from each dish were counted with an electronic particle counter, and an appropriate number of cells were dispersed into Petri dishes. Each concentration-time point was investigated in triplicate. Plating efficiency controls (cells that did not receive the drug) were run in two batches of 6 each for each group of concentrations tested at a single exposure duration. After 3 weeks of incubation, colonies (>50 cells) formed per number of cells plated. The mean plating efficiency of controls was 0.52 (range, 0.47 to 0.57) for all concentrations of anguidine for 1 hr. Cells were harvested, washed, and processed for colony formation. Comparisons with the survival of exponentially growing cells were made with the 1-sided Student t test.

Cytokinetic Studies. Cells in exponential growth were treated with varying concentrations of anguidine for increasing intervals. After treatment, cells were washed twice with Hanks' balanced salt solution and harvested with pepsin (0.5%) in HCl (pH 1.7) for 5 min at 37°C, obtaining a monodisperson of >97%. Cells were washed with 0.9% NaCl solution and fixed in 70% ethanol. DNA fluorochromation was performed with 5 ml of ethidium bromide (25 µg/ml in Tris buffer with 0.6% NaCl solution, pH 7.4) for 10 min followed by 5 ml of mithramycin (50 µg/ml containing 7.5 mM MgCl$_2$ and 12.5% ethanol) to yield a final concentration of 12.5 µg/ml for ethidium bromide and mithramycin (3, 40). RNase (0.1%) in 0.3 M NaCl was added (40) for 5 min at room temperature. The samples were measured in a Phye ICP-11 pulse cytophotometer (Phye Co., Göttingen, Germany) (19, 20). Routinely, 30,000 to 50,000 cells were measured, and a 128-channel histogram was generated. Histograms were evaluated by a modification of the model of Fried (18, 22). The coefficient of variation for the G$_{10}$ compartment was routinely <5% (median, 2.2%; range 0.6 to 5.3%). All experiments were performed twice with duplicate samples per each concentration and time point. Differences between controls and treated samples were analyzed by utilizing a 1-way analysis of variance with the Student-Newman-Keuls multiple range test.

To investigate the influence of anguidine on the transition of cells from the G$_{1}$ to S phase, we measured the cumulative LI after continuous incubation of exponentially growing cultures with anguidine and [³H]dThd (1 µCi/ml; specific activity, 3.0 Ci/mmol). At regular time intervals, cells were washed twice with Hanks' balanced salt solution and harvested with hyaluronidase and trypsin as described previously. Cytokinetic studies were performed and processed for autoradiography with Kodak NTB-2 emulsion. For a determination of the percentage of labeled nuclei, 500 cells were counted. Cells with 5 or more grains above the background count, overlying the nucleus, were considered labeled.

For definition of the effect of anguidine on the transition of cells from the G$_{2}$ phase to mitosis, cells in exponential growth were incubated continuously with increasing concentrations of anguidine and Colcemid (demecolcine; 0.05 µg/ml; CIBA Pharmaceutical Co., Summit, N. J.). Cultures treated with Colcemid alone and with anguidine alone served as controls. At regular intervals cells from duplicate dishes were harvested. Cytokinetic preparations were made and stained with Wright Giemsa stain. The MI was calculated for 1000 cells/duplicate dish.

RESULTS

Survival Studies. Significant cell kill was not observed after a 1-hr exposure to anguidine regardless of the concentration used (range, 1.0 to 1000 µg/ml). Chart 1, which shows cell survival as a function of drug dose for 5 exposure intervals, demonstrates that only after prolonged treatment was survival reduced, reaching 20% after 72 hr of incubation, with anguidine concentrations >5 µg/ml. No signifi-
For investigation of a perhaps more clinically relevant situation, the effect of anguidine on cells in the stationary phase of growth was tested. Cytotoxicity for the 1-hr treatment of stationary phase cells was minimal with >87% survival for cells treated with 1, 10, 100, and 500 µg of anguidine per ml. This demonstrated no significantly greater cell kill achieved upon stationary phase as opposed to exponentially growing cultures (p > 0.05).

**Cytokinetic Studies.** DNA distribution changes as a function of time for exponentially growing cells exposed continuously to increasing concentrations of anguidine are plotted in Chart 3. Control cells revealed 34.3% of cells in S-phase DNA content, 50.2% of cells in G_i, and 15.5% of cells in G_s + M. These values remained stable for the 5-day period of the experimental interval. Very little change in cell cycle compartments was noted in histograms during the first 24 hr of anguidine exposure. In treated cultures, however, there was a moderate increase in the S-phase compartment and a smaller concomitant reduction in G_i, which occurred beyond 24 hr of anguidine incubation. After 72 hr of exposure to anguidine (50 µg/ml), for example, 46.3% of cells were in S phase, 42.5% of cells were in G_i, and 11.2% of cells were in G_s + M. The increase in the S-phase compartment of treated versus untreated cells at 72 hr incubation was readily apparent upon inspection of DNA histograms of control cells (Chart 4A) and cultures treated with anguidine (50 µg/ml) (Chart 4B). DNA distributions for the 3 treatment concentrations (1, 10, and 50 µg/ml) at all points were not significantly different from one another (p > 0.1), showing no clear dose-response relationship for cultures treated with anguidine in the concentration range of 1 to 50 µg/ml.

For investigation of the effect of anguidine on the transition of LoVo cells from G_i into S phase, exponentially growing cultures were continuously treated with increasing concentrations of anguidine in the presence of [3H]dThd (Chart 5). The LI of control cultures increased at a rate of approximately 5%/hr over the first 10 hr of exposure, reaching a plateau of 86% after 24 hr. The presence of anguidine (1 µg/ml) reduced the rate of dThd uptake to about 1%/hr over the first 10 hr of exposure and the plateau to 37%. Anguidine (50 µg/ml) further reduced the initial 10-hr dThd uptake rate to <0.75%/hr, although the plateau was similar to that obtained for the 1 µg/ml concentration. An additional finding noted for both concentrations tested was a reduction of the initial 2-hr labeling values, from 39% for control cultures to 27 and 18% for cells treated with 1 and 50 µg/ml of anguidine, respectively.

For a determination of the effect of anguidine on G_i into mitosis transition, cultures were continuously treated with Colcemid and increasing anguidine concentrations (Chart 6). Cultures treated with Colcemid alone demonstrated a progressive linear accumulation of cells into metaphase at 1.6%/hr, with 81.4% mitoses at 48 hr. Exposure of cells to the combination of anguidine (1 µg/ml) and Colcemid was associated with a slight initial rise in the MI to 7% after 12 hr, followed by a progressive decline. The 50 µg/ml concentration showed no increase in the MI above the untreated value of 2.4%. A similar experiment without Col-

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**Chart 1.** Survival of a human adenocarcinoma cell line (LoVo) as a function of increasing anguidine concentration.

**Chart 2.** Survival of LoVo cells as a function of length of exposure.

**Chart 3.** Survival of LoVo cells as a function of length of exposure.
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Chart 3. Temporal changes in cell cycle compartment distributions of LoVo cells exposed to increasing concentrations of anguidine.

Chart 4. Representative DNA histograms for LoVo cells exposed for 72 hr to control medium (A) and anguidine (50 μg/ml) (B). A relative increase in the S-phase compartment for the treated cells is noted. The coefficient of variations of the G2+M peak for both histograms is <1%.

Chart 5. Effect of uninterrupted treatment of LoVo cells with increasing concentrations of anguidine on the cumulative [3H]dThd, [3H]dTdR, Li. SA = Specific Activity.

Chart 6. Effect of uninterrupted treatment of LoVo cells with increasing concentrations of anguidine in the presence of Colcemid on the cumulative MI.

cemid is detailed in Table 1. The MI of LoVo cells treated with 1 and 50 μg/ml after 24 hr was 0.3 and 0.001, respectively, whereas the MI of untreated cells was 1.8 to 2.4%. The difference between treated and untreated cells is significant at $p < 0.02$, by the 1-sided Student t test.

DISCUSSION

Early studies showed anguidine to be highly cytotoxic, with activity noted for concentrations <$1 \mu g/ml$, in several continuous tumor cell lines (1, 21). Anguidine was also found to be an effective agent in experimental animal tumors (10, 11, 23, 34). Recently, anguidine has received considerable interest due to its distinct mechanism of action and its antitumor efficacy in experimental colon...
carcinoma (10, 11). Because of discouraging Phase II trials in patients with adenocarcinoma of the large bowel, we tested the activity of anguidine on a cultured model of human colon cancer. It proved to be an ineffective lethal agent on cultured human colon cancer cells, even at doses of 100 \( \mu \text{g/ml} \). Moderate cytotoxicity in LoVo cells was noted only after prolonged drug exposure. *In vitro* cytotoxicity from prolonged drug exposure was not related to the synthesis of a more active, new metabolite and was not due to a greater efficacy of the drug on stationary phase cells.

Cytokinetic effects of anguidine were rapid and striking. The cumulative LI and MI experiments indicate a marked effect of the drug on cell cycle progression from \( G_1 \) into \( S \) and from \( G_2 \) into mitotic phase. These effects were noted within 2 to 3 hr of anguidine exposure, demonstrating that anguidine enters the cell within this time period. Since cell survival was not affected with \(<24\text{ hr of anguidine exposure, we conclude that early anguidine-induced cell cycle perturbation is reversible. Thus an effect of this drug exists that is different from the irreversible degradation of polyribosomes reported previously (24, 26, 35, 38).}

This contrary relationship between cytokinetic effects and lethal cell damage is consistent with previous investigations by Barlogie et al. (2) and Drewinko et al. (14) on cultured human lymphoma cells. In this system a lack of correlation between early interference with cell progression and cytotoxicity was noted for bleomycin (2) and the epipodophyllotoxin derivative VP 16-213 (14). The current study supports the concept that cell lethality and inhibition of progression through the division cycle can result from different cellular mechanisms.

Analysis of cytokinetic effects by combined DNA distribution analysis supplemented by radioisotope studies was necessary to discover the marked cycle perturbation effects. This is because the minimal change in DNA distribution seen over the first \( 24\text{ hr of anguidine exposure can occur in 2 different situations: (a) in the absence of perturbation of the division cycle; and (b) when cell cycle traverse through all phases is affected to a similar degree. The use of methods such as cumulative LI and cumulative MI revealed that, in fact, perturbation occurred at several areas in the cell cycle. The data are consistent with an almost frozen state of the cell cycle. Thus the perturbation could not be ascertained from the serial DNA histograms alone, because cell cycle stage distribution does not change upon complete arrest of cycle traverse. The additional observation of a moderate increase in S-phase cells at 24 hr, peaking at 72 hr, is consistent with the incomplete early suppression of DNA synthesis documented in the cumulative LI experiment.

The complex perturbation exhibited by anguidine is in agreement with the proposed mechanism of action of the drug, since protein inhibition has been shown to affect the cell cycle in several areas. Cycloheximide and puromycin, for example, both protein synthesis inhibitors, suppress DNA synthesis and a \( G_2 \) traverse (16, 37). In addition, experiments with \( \beta \)-fluorophenylalanine have revealed the presence of a protein specifically associated with mitosis, synthesized during \( G_2 \) (32). \( \beta \)-Fluorophenylalanine, which also inhibits protein synthesis, blocks cultured human amnion cells in mitosis and prolongs metaphase time (31).

Our additional finding of DNA synthesis inhibition is consistent with investigations of other nonrelated protein synthesis inhibitors that exhibit a similar effect (4, 6, 16, 25, 33). The rapidity of DNA synthesis inhibition, however, might also suggest a primary action of these drugs on DNA synthesis, as argued by Bennett et al. (6). These 2 mechanisms cannot be discerned with the techniques we have used.

Although anguidine is a poor cytotoxic agent in the LoVo line, it has been demonstrated to be very effective in producing a reversible frozen cell cycle in these human colon cancer cells. Therefore usage of this drug in simultaneous combination with cycle specific agents could be antagonistic. Anguidine might be utilized, however, at a given interval prior to an executor agent. This has been done in animals by using cycloheximide with \( 1\beta \)-d-arabinofuranosylcytosine or nitrogen mustard, showing protection by cycloheximide of the bone marrow and gastrointestinal cells from damage induced by these phase-specific and non-cycle-specific agents (5, 36).

If a differential rate of recovery between normal and malignant cells from anguidine-induced perturbation exists, appropriate drug scheduling could allow higher doses of an executor agent to be utilized. This concept has been demonstrated by Capizzi et al. (8, 9) who showed an apparent increase in the therapeutic index of methotrexate when it was preceded by L-asparaginase. This was postulated to be secondary to inhibition of protein-dependent DNA synthesis by L-asparaginase, preventing the phase-specific action of methotrexate.

*In vitro* cytotoxicity studies on a human colon cancer cell line are in agreement with our early clinical experience in human adenocarcinoma of the colon, showing low activity. Cytokinetic results reveal active cycle perturbation and provide clues for alternative usage of anguidine by taking advantage of its effects on the cell cycle. Usage of human *in vitro* models may therefore provide useful leads for clinical drug usage and aid in the interpretation of clinical results and in the understanding of complex drug mechanisms.

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