Growth Status Significantly Affects the Response of Human Lung Cancer Cells to Antitumor Polyamine-Analogue Exposure

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ABSTRACT

Human solid tumors frequently have a relatively small growth fraction, which interferes with the action of many chemotherapeutic agents that target actively cycling cells. Several polyamine analogues are currently being developed for clinical application against human solid tumors including N4,N11-bis(ethyl)norspermine. Therefore, an effort was made to examine the effects of growth rate on polyamine-analogue efficacy.

Low growth fraction (LGF) cell cultures of the human non-small cell lung cancer cell line NCI-H157 were generated to partially mimic solid tumors with low mitotic indices. Log-phase cells were compared with LGF cells with respect to cell survival and biochemical effects after exposure to polyamine analogues. The results demonstrate generally that LGF NCI-H157 cells were sensitive to analogue treatment. However, the dose necessary to elicit a response in LGF cells was an order of magnitude higher than the dose needed in log-phase cells. Additionally, the biochemical effects of analogues were similar between log phase and LGF cells with regard to a down-regulation of polyamine biosynthesis as measured by ornithine decarboxylase activity and an increase in polyamine catabolism as indicated by an increase in spermidine/spermine N4-acetyltransferase activity. However, biochemical effects were less dramatic in the LGF cells than those observed in the log-phase cells.

The overall results of these studies suggest that the growth status of solid tumors can significantly affect the response to antitumor polyamine analogues, and growth fraction must be considered in the continued development and use of the polyamine analogues.

INTRODUCTION

Polyamines are small cationic molecules that are required for cell growth and survival. Because they are necessary for cell survival, agents that interfere with polyamine metabolism such as the polyamine analogues have been designed and studied as antitumor drugs (1, 2). Transport of polyamine analogues into cells disrupts the normal function of the natural polyamines and can cause cell-growth arrest and, in some cases, cell death. Several such analogues are currently in clinical trials as antitumor agents.

Many current antitumor drugs target tumor cells by disrupting key processes during cell division. They are selective only in those tumor cells that have a higher mitotic index than normal cells (3). However, because of the microenvironment within a solid tumor, the fraction of actively dividing cells in many tumors is low. These tumors with a LGF3 are frequently resistant to drugs that target only dividing cells. The cells used in this study, NCI-H157 cells, are a non-small cell lung cancer cell line that is known to be resistant to most chemotherapeutic agents, but is sensitive to polyamine-analogue treatment during log-phase growth (4).

Previous studies have shown that several log-phase lung tumor cell lines, including NCI-H157, respond to polyamine analogues treatment by decreasing the activity of ODC, the major biosynthetic enzyme, and increasing the catabolic enzymes SSAT activity and polyamine oxidase (5–11). This leads to a depletion of intracellular polyamine concentration, and can result in apoptosis (12, 13). However, the mechanisms of action proposed for most polyamine analogues do not include direct interference with DNA synthesis, distinguishing them from classical antitumor agents. Whether tumor cells need to be actively cycling to be sensitive to the newer polyamine analogues is an important question that has not been rigorously addressed. Therefore, the object of the current study was to use slowly growing NCI-H157 cell cultures to model LGF tumor cell response to polyamine analogues and compare their response to the response of log-phase cells. The NCI-H157 cell cultures with a LGF responded to polyamine-analogue treatment by increasing SSAT activity and undergoing cell death, as do log-phase cells. However, the depletion of natural polyamines, the extent of SSAT induction, and the response of ODC differ with respect to the specific analogue used. Additionally, the degree to which these responses occurred was different from

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3 The abbreviations used are: LGF, low growth fraction; ODC, ornithine decarboxylase; SSAT, spermidine/spermine N4-acetyltransferase; BENSm, N4,N11-bis(ethyl)norspermine; CPENSm, N4-ethyl-N11-(cyclopropyl)methyl-4,8-diazaundecane; CHENSm, N4-ethyl-N11-(cycloheptylmethyl-4,8-diazaundecane; BESp, N4,N11-bis(ethyl)spermine; FACS, fluorescence-activated cell sorter.
log-phase cells and this difference has important clinical implications.

MATERIALS AND METHODS

Chemicals. BENSpm was synthesized as reported previously (14). Chemicals for HPLC analysis were purchased from Sigma Chemical Co. (St. Louis, MO). CPENSpm and CHENSpm were synthesized as reported previously (12). BESpm was provided by R. Bergeron (University of Florida, Gainesville, FL). Stock solutions of all analogues were prepared in 10 mM aliquots in double-distilled water (ddH₂O) and stored at −10°C.

Cell Culture. NCI-H157 cells (ATCC no. CRL-5802) were derived from a human squamous lung cancer. They were cultured in RPMI medium (Life Technologies, Inc.) with 10% enriched calf serum (Gemini Bioproducts, Woodland, CA). Stock solutions of all analogues were prepared in 10 mM aliquots in double-distilled water (ddH₂O) and stored at −20°C.

Growth Fraction Determination. To determine when cell cultures had a LGF, cells were seeded at high density and counted daily in 0.04% trypan blue (Sigma Chemical Co.) in PBS (Life Technologies, Inc.). In addition, a colorometric mitochondrial activity assay that measures the ability of cells to reduce a tetrazolium compound into formazan using electron coupling was performed daily (Promega Celltiter Assay). Cells were considered to be LGF when both cell count stabilized and mitochondrial activity declined from that of log-phase cells. An increase in G₁ and a decrease in S-phase cells were confirmed by FACS analysis. This calculation was performed independently for each culture vessel used, from 96-well plates to T175 flasks. In T175 flasks, this occurred when cells were seeded at 1 × 10⁸ cells/flask in 30 ml of complete medium. Cells grew without any medium changes for 5 days, at which time treatments took place. Medium was not changed during any treatments.

FACS Analysis. To confirm growth fraction, FACS analysis was performed. Cells were harvested after treatment by trypsin, washed with PBS, counted, and at least 2 × 10⁸ cells were resuspended and stained with propidium iodide as reported previously by Vindelov et al. (15).

Enzyme and Polyamine Analysis. SSAT and ODC activity were determined by using C¹⁴-labeled substrates and by scintillation counting the end products produced as reported previously (5, 16). Intracellular polyamine concentrations were determined using high-performance liquid chromatography by the methods of Kabra et al. (17). Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA).

Cell Survival. Because the Promega Celltiter Assay did not accurately measure cell survival in LGF cells, cell survival was determined 96 h after treatment with polyamine analogues by standard trypan blue analysis for LGF cells. Growth curves of log-phase cells were also performed with both trypan blue and the Celltiter assay. These assays were found to be equally sensitive for log-phase cells and gave identical results (data not shown). Because the Promega Celltiter Assay did accurately and precisely measure survival in log-phase cells, log-phase cell survival was measured using the Promega Celltiter Assay.

RESULTS

To assess tumor cell growth and to define a decrease in growth fraction, cultures were counted and harvested daily to determine mitochondrial activity. We found that when cell counts were stable for ~3 days, mitochondrial activity decreased rapidly (Fig. 1) and FACS analysis indicated an increase in the proportion of cells in G₁/G₀ phase of the cell cycle from 43 to 84%. The proportion of cells in S-phase decreased from 31 to 5%, whereas the remaining cells were in G₂ phase (Fig. 2). The cells in these cultures are defined as LGF because the number of cells in S and G₂ are low compared with log-phase cells. Although cultures cannot completely mimic LGF tumors in vivo, they can act as a useful model under these circumstances.
Both LGF tumor cells and log-phase cells were treated with polyamine analogues for 96 h, and cell survival was determined. Dose-response data (Fig. 3A) illustrate that CHENSpm was the most toxic in LGF cells, with only 4.0% survival at the 10 μM dose, and 0.33% survival at 50 μM. CPENSpm was next at 16 and 1.4%, then BENSpm at 25 and 1.2%. BESpm was only slightly growth inhibitory at these doses, with survival after treatment being 87 and 83% for the 10 μM and 50 μM doses, respectively. There were few surviving cells at doses of 100 μM or greater in any of the analogues used. Although the analogues were effective at killing LGF cells, the doses at which toxicity is seen is higher in LGF cells than for log-phase cells for all drugs tested (Fig. 3B). Doses of 10 μM and 50 μM analogue in LGF cells were compared after 24 h with doses of 1 μM and 5 μM analogue in log-phase cells for additional assays. The 24-h time point was used because there were sufficient surviving cells to examine biochemical changes.

In NCI-H157 cells the rate-limiting enzymes in the anaobic and catabolic pathways of polyamine metabolism appear to be ODC and SSAT, respectively, and the activity of these enzymes in log-phase cells is frequently affected by treatment with polyamine analogues. The biosynthetic enzyme ODC was analyzed after a 24-h treatment with 10 μM of each analogue in LGF cells or 1 μM in log-phase cells. Untreated LGF cells had a lower average ODC activity than did log-phase cells. The average control value for LGF cells was 239 pmol product/mg protein/h, whereas the average value for log-phase cells was 2334 pmol product/mg protein/h, as would be expected for actively dividing cells. After exposure of LGF cells to the symmetrically substituted polyamine analogues BESpm and BENSpm, ODC activity was decreased slightly as compared with control. However, ODC activity was increased after treatment with the unsymmetrically substituted polyamines CPENSpm and CHENSpm (Fig. 4A). ODC can function as a stress-response gene in the cell, and, consistent with this occurrence, ODC activity increases in log-phase cells at the 1 μM dose of analogue with the exception of CHENSpm (Fig. 4B). At the more toxic 50 μM dose in LGF cells, ODC activity was below detection limits in BESpm, BENSpm, and CPENSpm, although ODC activity in the most effective analogue tested, CHENSpm, was 2-fold greater than in control levels (Fig. 4A). Log-phase cells responded with low ODC activity levels after the 5 μM dose of all of the drugs tested (Fig. 4B).

SSAT activity was analyzed after a 24-h treatment with each analogue in LGF cells (Fig. 5A). SSAT was significantly induced >100-fold with 10 and 50 μM BESpm and BENSpm. CPENSpm induced SSAT as well, although not as high as BESpm and BENSpm at the 10 μM dose (44-fold), but reached levels similar to the other analogues at the 50 μM dose. CHENSpm only modestly induced SSAT, demonstrating a 7- and 16-fold induction in the 10 and 50 μM does, respectively. Although the results clearly indicate that several of the polyamine analogues produce highly significant induction of SSAT, it is critical to note that log-phase cells illicit an induction of SSAT ~5-fold greater than observed LGF cells. The only exception to this is in the case of CHENSpm, where the induction was similar to results observed in LGF cells (Fig. 5B).

The analogue-induced alterations of both ODC and SSAT

![Figure 3](image-url) Effect of analogue treatment on NCI-H157 cell survival. A, LGF cells; B, log-phase cells. Values are the average of at least three independent experiments; each performed in triplicate.

![Figure 4](image-url) Effect of analogue treatment on ODC activity. A, treatment of LGF cells was either 10 or 50 μM for 24 h. The average ODC activity for all untreated samples was 239 pmol/mg/h. B, treatment of log-phase cells was either 1 or 5 μM for 24 h. Average ODC activity for all untreated samples was 2334 pmol/mg/h. Values are the average of at least three independent experiments. Error bars indicate SE.

![Figure 5](image-url) Effect of analogue treatment on SSAT activity.
activity led to significant changes in intracellular polyamine and analogue concentrations. With exposure to a 50 μM dose of BESpm, BENSpm, or CPENSpm, the natural polyamine content was dramatically reduced in LGF cells (Table 1). However, after exposure to the same dose of CHENSpm, natural polyamine levels remained similar to control levels, whereas CHENSpm was readily accumulated. The total positive intracellular charge contributed by polyamine content (natural polyamines and polyamine analogue) remained similar or lower than control levels after treatment with BESpm, BENSpm, and CPENSpm. However, the total intracellular positive charge attributable to polyamines after CHENSpm treatment was higher than total polyamine levels in control cells. In log-phase cells, analogue was transported into cells efficiently, and although natural polyamine levels were low, the total charge attributable to polyamine content was higher than control levels. The total positive charge attributable to polyamine levels was even higher in CHENSpm-treated cells because natural polyamines were present at concentrations that were similar to controls in addition to the positive charge contributed by CHENSpm (Table 1).

**DISCUSSION**

The microenvironment within a tumor is often hypoxic and nutrient deprived. As a result, the cells in this environment generally have a LGF. Many standard chemotherapeutic agents target tumor cells by interfering with DNA metabolism and cell division; in particular, the topoisomerase-targeting drugs such as daunorubicin act in this way (3). Tumors with growth fractions that are relatively low are typically resistant to these drugs.

Previous studies with polyamine analogues have shown that they are effective cytotoxic agents in many solid tumor cell lines when treated during the log phase. The symmetrically substituted polyamines BESpm and BENSpm and the unsymmetrically substituted polyamines CPENSpm and CHENSpm have been shown to be cytotoxic to several non-small cell cancer cell lines and other human tumor types including melanoma, prostate, and breast (7, 8, 18–20). The current studies expand on the previous studies by comparing the cellular response of LGF cultures of the human lung tumor cell line NCI-H157 to log-phase cultures after treatment with these analogues.

In this study, LGF cultures were created by seeding NCI-H157 cells at high density in a nutrient-deprived environment. Growth-rate, mitochondrial-activity, and cell-cycle analyses indicated that these cultures have LGF without the use of any pharmacologic agents. Although no culture can yet mimic precisely the environment of a solid tumor, these studies provide insight as to the differences between the cellular responses of slow-growing cells when compared with log-phase cells that are commonly used to test new drugs for antitumor treatment. We have found that the polyamine analogues BESpm, BENSpm, CPENSpm, and CHENSpm are all effective at killing LGF NCI-H157 cells; however, compared with the log-phase cells, LGF cells require a log increase in concentration.

The activity of ODC, a rate-limiting enzyme in the polyamine biosynthetic pathway, was down-regulated by treatment with BESpm, BENSpm, or CPENSpm in LGF cells when treated with either a 10 or 50 μM dose. Although this is consistent with previous studies in log-phase cells (21), when the response of LGF cells was compared with that of log-phase cells in this study, there were some differences in ODC activity. Exposure of log-phase cells to low doses of analogue (1 μM) induced ODC activity. This paradoxical induction of ODC has been attributed to a stress response and has been documented under many conditions, including after exposure to free radicals and in response to tissue injury, and has been shown to be essential for wound healing under some conditions (22–24). However, when higher doses of analogue are used to treat log-phase cells, ODC activity is significantly down-regulated, consistent with previous observations (9, 25, 26). At the higher dose, when the down-regulation of ODC by LGF cells is compared with log-phase cells, log-phase cells respond less dramatically. It is possible that higher levels of polyamines are required for cycling cells, as indicated by the higher steady-state ODC activity level, and they may be less able to shut down the polyamine biosynthetic pathway.

In log-phase cells, exposure to some polyamine analogues will initiate apoptosis (12, 13, 27, 28). In some cases the induction of apoptosis is thought to be associated with SSAT activity (7, 8, 12). In LGF cells, however, doses of BESpm, which are highly effective at super-inducing SSAT and depleting cells of natural polyamines, are not toxic to NCI-H157 cells. CPENSpm is less effective than BESpm at super-inducing SSAT activity and decreasing ODC activity at the 10 μM dose, but more effective at causing cell death. These results suggest that the mechanism by which analogues are toxic to LGF cells may be different from the mechanism that has been proposed for log-phase cells. One explanation for the difference in response of LGF cells versus log-phase cells may be attributable to the specific molecular function of polyamines in cell growth.
Growth Rate-dependent Response to Polyamine Analogues

undetectable after a 50 μM/H9262 dose. BESpdm causes cytotoxicity by depleting cells of polyamines, nor does it cause cytotoxicity by depleting cells of polyamines, nor does it interact with intracellular regulators of polyamine metabolism in the same way as the other analogues examined. It is also important to note that CHENSpm is transported into cells, whereas biosynthesis of natural polyamines continues producing total polyamine concentrations significantly higher than those in untreated NCI-H157 cells. It is possible that CHENSpm causes a lethal disruption of this balance by increasing total polyamine concentration and changing the net charge of the cells to a level that does not allow normal cellular functions to take place. These results are consistent with the findings of Poulin et al. (33) who demonstrated that high polyamine concentrations could lead to lethal effects under hypoosmotic conditions.

The mechanism of action of CHENSpm has been postulated to be different from that of the other analogues because it does not deplete log-phase cells of polyamines before induction of cell death (12, 27). In addition, cells treated with CHENSpm have a G2/M cell-cycle block instead of the G1/S block after treatment with other analogues (12, 30, 31), and CHENSpm has been shown to alter microtubule polymerization, whereas the other analogues have not (32). The response of LGF NCI-H157 cells to CHENSpm in this study gives us further information on the mechanism by which CHENSpm kills tumor cells. In both this and previous studies, NCI-H157 cells did not induce SSAT after CHENSpm treatment to the extent that they did after BESpdm, BENSpdm, or CPENSpm treatment (12). In addition, ODC activity, which is decreased after a 10 μM treatment and undetectable after a 50 μM treatment of BESpdm, BENSpdm, or CPENSpm, is actually >2-fold that in control levels at both doses of CHENSpm. These differences from the other analogues are apparent when examining intracellular polyamine levels as well. Although LGF cells exposed to BESpdm, BENSpdm, or CPENSpm show a decrease in natural polyamine levels after treatment, they remain near control levels after CHENSpm treatment. These results demonstrate that CHENSpm does not cause cytotoxicity by depleting cells of polyamines, nor does it interact with intracellular regulators of polyamine metabolism in the same way as the other analogues examined. It is also important to note that CHENSpm is transported into cells, whereas biosynthesis of natural polyamines continues producing total polyamine concentrations significantly higher than those in untreated NCI-H157 cells. It is possible that CHENSpm causes a lethal disruption of this balance by increasing total polyamine concentration and changing the net charge of the cells to a level that does not allow normal cellular functions to take place. These results are consistent with the findings of Poulin et al. (33) who demonstrated that high polyamine concentrations could lead to lethal effects under hypoosmotic conditions.

Although both LGF and log-phase cells are sensitive to analogue treatment, these data show that the cellular response to treatment is very different between the two conditions. Because the microenvironment of a tumor is hypoxic and nutrient deprived, many tumors have a growth fraction that is relatively low. Although the current experiments are not done in a hypoxic environment, the studies examine cultures that are nutrient deprived, many tumors have a growth fraction that is relatively low. Although the current experiments are not done in a hypoxic environment, the studies examine cultures that are nutrient deprived and have a LGF. These data indicate that to treat LGF cells with polyamine analogue in vivo, a higher dose may be needed, negatively impacting the therapeutic index of some of the current generation of polyamine analogues. Therefore, additional work is needed to develop agents with greater activity in LGF cells to maximize the effect of polyamine analogues on solid tumors.

REFERENCES


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