Effects of a New Amsacrine Derivative, N-5-Dimethyl-9-(2-methoxy-4-methylsulfonylamino)phenylamino-4-acridinecarboxamide, on Cultured Mammalian Cells

Frank Traganos, Connie Bueti, Zbigniew Darzynkiewicz, and Myron R. Melamed

Investigative Cytology Laboratory, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

ABSTRACT

The effects of N-5-dimethyl-9-(2-methoxy-4-methylsulfonylamino)-phenylamino-4-acridinecarboxamide (CI-921; NSC 343499), a lipophilic and water-soluble derivative of amsacrine (NSC 249992), on cell viability, growth, clonogenicity, and progression through the cell cycle were investigated in suspension cultures of Friend erythroleukemic cells and in adherent cultures of Chinese hamster ovary cells. CI-921 was less toxic toward stationary than toward exponentially growing Chinese hamster ovary cells; colony formation was inhibited by 50% following a 1-h pulse of 190 nM CI-921, respectively. Cell viability was unaffected in Friend erythroleukemic cell cultures at concentrations up to 50 nM, although growth was inhibited by 50% following 24 h of continuous exposure to 9.5 nM or a 1-h pulse of 67.5 nM CI-921. Constant exposure of Friend erythroleukemic cells to 10 nM CI-921 slowed proliferation and resulted in prolongation of cell transit through late S and G2 phases. Higher drug concentrations (50 nM) caused a complete cessation of growth marked by greatly suppressed cell transit through S phase and an irreversible block in G2 phase, about 30 min prior to division. In such cases, unbalanced growth was observed with total RNA and protein content of drug-treated cells increasing by 74 and 34%, respectively. Pulse exposure of cells to CI-921 resulted in transient accumulations of drug in the cell, with concentration-progressive decline following the time of exposure. The highest drug concentration (50 nM) was made up fresh on the day of the experiment and stored at -70°C.

INTRODUCTION

CI-921 (NSC 343499) is a 4-(N-methylcarboxamide)-5-methyl derivative of the antineoplastic agent amsacrine (NSC 249992). While amsacrine, a 9-anilino-acridine derivative first synthesized by Cain and Atwell (1), has been shown to be clinically active against leukemia and lymphoma (2, 3), its activity in solid tumors other than breast was considered insufficient to warrant further clinical trials (4, 5). In an attempt to develop an agent with activity against solid tumors, Baguley et al. (6-9) have synthesized a large series of analogues of amsacrine, of which CI-921 appears to show the most promise (10).

Amsacrine binds to DNA by intercalation (11) and has been shown to induce single- and double-strand breaks in the DNA of treated cells (12), probably mediated through the action of DNA topoisomerase II (13). The agent inhibits the growth of a spectrum of experimental tumors in vivo (14), resembling the anthracycline antibiotic daunorubicin in action but without the attendant severe cardiotoxicity (10). However, many cationic drugs of the DNA-intercalating type are relatively inactive against solid tumors, notwithstanding high activity against leukemias, primarily because of inadequate drug distribution to distant sites (8). From a variety of structure-activity studies, it was determined that greater lipophilicity increased the activity of amsacrine analogues against s.c. and i.c. implanted tumors (8). The 4,5-disubstituted amsacrine derivative, CI-921, is more hydrophilic than amsacrine due to the 4-methylcarboxamide group (10).

Such structural changes appear to have been successful in increasing the utility of CI-921 against solid tumors. Thus, while CI-921 is approximately as potent as amsacrine against murine leukemia cell lines, it is significantly more potent than amsacrine against a host of murine and human lung, breast, and melanoma cell lines, and against Lewis lung carcinoma, B16 melanoma, colon 38, and the MX-1 xenograft in vivo (10). Others (15) have demonstrated equivalent or greater activity of CI-921 compared to amsacrine and Adriamycin against the LC-12 squamous cell, and the Nettlesheim and Madison 109 lung carcinoma in vivo. In fact, recent studies have shown CI-921 to be active against Adriamycin-resistant mammary adenocarcinoma 16/C and to act synergistically with cisplatin in at least one mouse tumor model (15). A further indication of favorable drug distribution properties is the presence of activity following p.o. administration. Generally, DNA-binding agents are not effective when given p.o. (8). Of several 4,5-disubstituted derivatives tested, CI-921 retained exceptional oral activity, seemingly due to its increased solubility and low base strength (8, 10).

Baguley et al. (10) have described the synthesis, DNA-binding properties, in vitro toxicity, and in vivo antitumor activity of CI-921. The purpose of the present study is to characterize the cytokinetic effect of CI-921 in vitro in greater detail, and to define the conditions by which the drug may be most effectively utilized, either alone or in combination with other antitumor agents.

MATERIALS AND METHODS

Drugs

CI-921 was a generous gift of Dr. Bruce C. Baguley of the Cancer Research Laboratory and Department of Pathology, University of Auckland Medical School, Auckland, New Zealand. The stock solution of the drug (0.5 mM) was made up fresh on the day of the experiment in 70% ethanol. All further dilutions were made in HBSS to 100 times the final dilution. The final dilution was accomplished by adding 0.1 ml of the appropriate drug concentration to 10-ml cultures of cells. The final concentration of ethanol in culture was negligible, never exceeding 0.07%. Vinblastine sulfate was obtained from Sigma Chemical Co. (St. Louis, MO) and kept frozen as a stock solution (50 μg/ml) prepared in HBSS.

Cells

FL and CHO cells are maintained routinely as exponentially growing cultures in RPMI 1640 supplemented with 10% fetal bovine serum and 2 mM L-glutamine (GIBCO). FL and CHO cells are maintained routinely as exponentially growing cultures in HBSS. Flank implants, derived from passages 30-40, were maintained as serially transplanted tumors. Three tumor lines were utilized, either alone or in combination with other antitumor agents.
prepared by allowing the cells to grow to confluence and replacing the medium with fresh medium on 2 subsequent days; experiments were always begun on the third day after the cultures reached confluence.

**Clonogenicity Studies**

Two ml of CHO cells from exponentially growing cultures were seeded in six-well plastic plates (Costar, Cambridge, MA) at a concentration of 200 cells/ml for a total of 400 cells/well in conditioned medium. Following 1 h to allow cells to attach, the drug was added to duplicate wells, incubated for 1 h, and the wells were washed three times with prewarmed HBSS followed by the addition of 2 ml of drug-free medium. The plates were incubated for 6 days, fixed, and stained as previously described (16). Colonies consisting of 50 or more cells were scored on an inverted microscope; the plating efficiency of exponential CHO cells was typically 80-90%.

Stationary cultures were prepared as described above in 2-ml plates, treated for 1 h at various drug concentrations, washed as above, and trypsinized. The trypsinized cells were diluted in drug-free conditioned medium to a concentration of 200 cells/ml. Duplicate sets of plates received 2 ml of cell suspension (i.e., 400 cells/well). As above, the plates were scored for colony formation 6 days later; typically, cloning efficiency for untreated stationary cultures ranged from 60 to 80%.

**Drug Effect on Growth of Suspension Cultures**

Suspension cultures of FL cells in asynchronous exponential growth were split and treated with varying concentrations of CI-921, either continuously or for 1 h, followed by washing in HBSS and resuspension in drug-free medium. All cultures were adjusted to an original cell count of 1-2 x 10^6 cells/ml such that control cell growth would remain exponential over the course of the experiment; FL cells double approximately every 11.2 h (17) and enter near-plateau growth at a cell concentration of 1 to 2 x 10^6 cells/ml (18).

Triplicate cultures were counted at the indicated times and viability was checked by trypan blue dye exclusion. The number of viable cells per ml was determined with each point representing the mean of three measurements from a typical experiment. For the purpose of determining the ID_{so}, the 24-hr time points for continuous and pulse exposure were plotted, each for two separate experiments.

**Cytokinetic Studies**

Exponential cultures of FL cells were established and drug was added at the appropriate concentration. Aliquots (0.2 ml) were removed after 4, 8, and 24 h and were analyzed by flow cytometry as described below. Both exponentially growing FL and CHO cells were exposed to a 1-h pulse of CI-921, washed twice with HBSS, and resuspended in drug-free medium. The cell cycle distribution of these cultures were also analyzed by flow cytometry. Finally, stationary CHO cell cultures were treated for 1 h with the drug, washed with HBSS, trypsinized, and replated at a concentration (2 x 10^6 cells/ml) which would permit the onset of exponential growth; generally a lag of 3-4 h is observed with cultures maintained at confluence for 3 days. It should be noted that CHO cells as opposed to normal fibroblast cultures are not completely blocked in G_{0}-G_{1}; upon reaching high cell density, especially when fresh serum is added daily; typically 65-70% of the cells reside in G_{0}-G_{1} under these conditions, with the remainder in mid- to late S and G_{2} phase (not shown).

**Stathmokinetic Experiment**

Details of this procedure are given elsewhere (19). Briefly, vinblastine sulfate was added to multiple large volume (500 ml) cultures of exponentially growing FL cells at a final concentration of 0.05 μg/ml. Simultaneously, CI-921, at a final concentration of 10 and 50 nM was added to two separate cultures, the control culture receiving an equivalent volume of HBSS. A 5-ml aliquot of cells was withdrawn immediately from the control culture, washed once with HBSS, resuspended in 1 ml of HBSS, and fixed as previously described (19). Ten-ml aliquots were removed from all cultures hourly up to 7 h and fixed. Following storage overnight at 0-4°C, each sample was pelleted, resuspended in 1.0 ml HBSS, and treated for 30 min at 37°C with approximately 1000 units of RNase (RASE; Worthington Biochemical Corp., Freehold, NJ).

**Staining for Flow Cytometry**

Simultaneous Staining of DNA and RNA in Situ. The staining techniques used in this study have been described in detail (20, 21). To obtain simultaneous measurement of cellular DNA and RNA content, the metachromatic properties of the fluorescent dye AO were utilized (22). An aliquot of cells (0.2 ml) was removed directly from culture to which was added 0.4 ml of a detergent solution containing 0.08 N HCl, 0.15 M NaCl, and 0.1% Triton X-100. After allowing 30 s at room temperature for the cells to become permeable, AO (chromatographically purified; Polysciences, Inc., Warrington, PA) at 6 μg/ml in a 0.2 M NaHPO_{4}-0.1 M citric acid buffer (pH 6.0) containing 1 mM EDTA was added. Multiparameter flow cytometry measurement of 5 x 10^5 cells/sample was carried out within the next 10 min.

The stoichiometry of the staining reaction for both DNA and RNA has been established (23-25). Approximately 20% of the red luminescence (RNA) signal for both FL and CHO cells is not specific for RNA under these staining conditions. The exact value of non-RNA specific red luminescence was established for each experiment by performing the appropriate RNase digestion and staining of detergent permeabilized cells.

Simultaneous Staining of DNA and Total Protein in Situ. Staining of DNA and total cellular protein was accomplished on ethanol-fixed cells, collected at hourly intervals after addition of drug, by a modification of the technique of Crissman and Steinkamp (26). Cells were rehydrated in HBSS from fixative and treated with 2000 units of RNase at 37°C for 30 min. The cells were pelleted and stained with a 0.05-μg/ml solution of fluorescein isothiocyanate in 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid buffer at pH 8.0; alkaline pH is required to dissociate ε-amino groups (27). After allowing 30 min for staining of protein at room temperature, an equivalent volume of a 40 μg/ml propidium iodide (Sigma) in 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid buffer was added to stain double-stranded DNA. The resultant red (DNA) propidium iodide fluorescence and green (protein) fluorescein isothiocyanate fluorescence for 5 x 10^5 individual cells were recorded by flow cytometry as described below.

Simultaneous Staining of Native and Denatured DNA in Situ. Aliquots of fixed, rehydrated, and RNase-treated samples from the stathmokinetic experiments were analyzed for the extent to which DNA in situ could be denatured by acid conditions (19). Following incubation with RNase, 0.2 ml of cell suspension containing 2-4 x 10^6 cells were admixed with 0.5 ml of 0.2 M HCl-KCl buffer at pH 1.3. The cells were stained 30 s later with AO by addition of 2.0 ml of a 4-μg/ml dye solution in 0.1 M citric acid-0.2 M NaHPO_{4} buffer, at pH 2.6. All solutions and staining were at room temperature. Samples were measured within 10 min of addition of dye.

The percentage of mitotic cells present in each culture following vinblastine addition was estimated using the selective acid DNA denaturation method based on the relative proportion of denatured (red luminescence) DNA to total (green plus red luminescence) DNA expressed as the ratio r_{o}, (19). In addition, the staining reaction allows for the analyses of the percentage of cells in the various interphase compartments (G_{1}, early, mid-, and late S phase and G_{2} phase) based on total fluorescence measurements, i.e., the sum of green and red luminescence reflecting the amounts of native and denatured DNA, respectively (19).

**Fluorescence Measurements**

The integrated values of green and red fluorescence pulses and the fluorescence pulse width of the DNA-associated fluorescence signal of individual cells were obtained by use of an FC-200 flow cytometer (Ortho Diagnostic Instruments, Westwood, MA) interfaced to a Nova 1220 minicomputer (Data General Corp., Southboro, MA).

Interactive computer analysis programs were used to obtain mean values and normalized histograms of fluorescence for populations and subpopulations and to provide computer-generated graphic representation of the data (28).
RESULTS

Cell Survival

Exponentially Growing CHO Cells. Following a 1-h pulse of CI-921, CHO cells were plated and colonies consisting of 50 or more cells were counted 6 days after plating. The dose which inhibited colony formation by 50% under these circumstances was 80 nM CI-921 (Fig. 1A).

Stationary Phase CHO Cells. When CHO cells were allowed to achieve confluence and remained confluent for an additional two days prior to a 1-hr exposure to varying concentrations of CI-921, the dose which inhibited colony formation by 50% increased to 190 nM.

Inhibition of Growth of Asynchronous FL Cells

Continuous versus Pulse Exposure. Suspension cultures of exponentially growing FL cells continuously exposed to varying concentrations of CI-921 were inhibited from growing by 50% at a dose of 9.5 nM (Fig. 1B). Investigating the increase in cell number as a function of duration of exposure, it was observed that continuous exposure to 50 nM CI-921 prevented almost any increase in cell number, such that by 4 h there was a plateau in cell number of cultures exposed to that concentration of drug (not shown). At 10 nM, cell growth slowed after 4 h but cell number continued to increase up to 24 h, albeit more slowly than in untreated cultures.

If exponentially growing FL cells were exposed to varying concentration of CI-921 for 1 h, washed free of drug, and allowed to grow for an additional 24 h in the absence of drug, the ID_{50} was increased to 67.5 nM (Fig. 1B). At the time the cell counts in Fig. 1B were performed (24 h), regardless of the length of drug exposure (i.e., continuous or pulse), greater than 90% of the remaining cells were viable based on their exclusion of trypan blue (not shown).

Cell Cycle Progression

The effects of CI-921 on the cell cycle distribution of both FL and CHO cells were analyzed by comparing the single-parameter DNA distributions obtained by flow cytometry of AO-stained cells. Drug concentrations were carefully selected such that little or no cell death occurred over the time course of the experiment, simplifying cell kinetic analysis. Viability was monitored both by trypan blue dye exclusion and by retention of RNA (cytoplasm) as detected by the present staining technique. As previously described (19), increased cell fragility or cell death results in loss of AO red luminescence (RNA content) due to the detergent present in the staining reaction; isolated nuclei, normally only observed in the presence of cytotoxic drug concentrations, are easily distinguished from intact, viable cells by this technique.

FL Cells in Exponential Growth

Continuous Exposure. The fraction of cells in each cell cycle compartment (i.e., G_{1}, S, or G_{2}/M) remains constant over a 24-h period when care is taken to ensure asynchronous, exponential growth (Fig. 2A). This is typically the case when the cell concentration is maintained between 1 and 6 \times 10^{6} cells/ml. Continuous exposure to 1.0 nM CI-921 had little or no effect on the cell cycle distribution (Fig. 2A) or, for that matter, cell growth (Fig. 1B). However, 10 nM CI-921 dramatically perturbed cell growth. Four h of exposure to 10 nM CI-921 caused an accumulation of cells in S and G_{2}/M and thus a concomitant...
DECREASE IN G1 CELLS (FIG. 2A). LONGER EXPOSURE LED FIRST TO A FURTHER INCREASE IN G2M AND DECREASE IN G1 CELLS AND EVENTUALLY TO THE APPEARANCE OF CELLS WITH GREATER THAN A G2M DNA CONTENT (I.E., CELLS AT HIGHER PLOIDY LEVELS). RAISING THE DRUG CONCENTRATION TO 50 NM CI-921 CAUSED A SIMILAR, ALTHOUGH MORE DRAMATIC REGULATION OF CELLS. THE COMPLETE EVACUATION OF G1 AND NEARLY ALL S PHASE CELLS WAS SEEN, WITH THE NUMBER OF CELLS CONTAINING A DNA CONTENT EQUAL TO OR GREATER THAN A G2M CELLS ACCOUNTING FOR OVER 90% OF THE POPULATION FOLLOWING 24 H OF CONTINUOUS DRUG EXPOSURE (FIG. 2A). EVIDENCE PRESENTED BELOW SUGGESTS THAT BY RAISING THE DRUG CONCENTRATION FROM 10 TO 50 NM THERE WAS AN ALMOST IMMEDIATE CESSATION OF CELLS CAPABLE OF DIVIDING (I.E., ENTERING G1 PHASE), WHEREAS AT THE LOWER DRUG CONCENTRATION, G1 CELLS CONTINUED TO APPEAR, ALTHOUGH MUCH MORE SLOWLY, THAN IN CONTROL CULTURES (FIG. 2A).

Pulse Exposure. The effect of a 1-h pulse exposure of CI-921 on the cell cycle distribution of asynchronous, exponentially growing FL cells were similar to those obtained with continuous drug exposure, but differed in some important respects (Fig. 2B). Thus, the early effect (4 h) of a pulse exposure to 50 or 100 nm (and to a lesser extent, 10 nm) CI-921 was, as with continuous exposure to the drug, consistent with a slowdown of cells through the cell cycle resulting in an accumulation in G2M and G0 phases (Fig. 2B). However, 8 h after a 1-h exposure to 50 or 100 nm CI-921 nearly all cells had accumulated in G2M phase. Following a 1-h pulse exposure to 10 nm CI-921 there was a relatively small proportion of cells accumulated in S or G2M phase at 4 h, and it seemed that they subsequently divided resulting in a modest accumulation of G1 cells by 8 h (Fig. 2B). By 24 h, all effects of the drug had disappeared in the culture treated with 10 nm CI-921 (Fig. 2B). A significant number of cells in the cultures exposed to a 1-h pulse of 50 or 100 nm CI-921 either divided (note the decrease in G2M phase cells) or proceeded to grow at a higher ploidy level (Fig. 2B).

At the drug concentrations tested, pulse exposure was less effective in maintaining the S phase block resulting in a greater accumulation of G0 phase cells by 8 h. However, a short pulse exposure to 100 nm CI-921 did not hold the G2 block as effectively as continuous exposure to lower concentrations of CI-921, so that by 24 h considerable numbers of cells divided to reenter G1 and S phases.

CHO Cells in Exponential Growth

Pulse Exposure. The effect of a 1-h exposure of asynchronously growing CHO cells to 25, 50, or 100 nm CI-921 paralleled that observed for FL cells except that the slowdown in transit through S phase was prolonged (see the 8-h time points in Table 1). Nonetheless, by 24 h following the pulse exposure, many cells had divided and reentered the cell cycle at the two lower drug concentrations, although a 1-h pulse of 100 nm CI-921 continued to block a large number of cells in G2 phase (Table 1). As seen with FL cells, a proportion of the CHO cells failed to divide and were observed to have increased DNA content (Table 1).

CHO Cells in Stationary Growth

Pulse Exposure. Stationary phase CHO cells are relatively resistant to the cell kinetic perturbations of CI-921 at the concentrations tested (see Fig. 1A). In order to assure that the stationary cultures would not be affected by serum depletion and remain highly viable, their medium was changed daily. As a result, some cells (approximately 35%) were observed in S and G2M phases even 3 days after growth to confluence (Table 1). However, these cells cycle slowly (not shown), and it is apparent that a 1-h pulse exposure does not result in their accumulation in S or G2; after cells are washed free of drug and plated at a low cell density. All of these cells reach G2M by 4 h and a new cohort (approximately 25%) enter early S phase. By 24 h, asynchronous growth had been reestablished in all cultures (Table 1).

RNA Content

The present staining technique allows for simultaneous analysis of cellular RNA content (Fig. 3). Total RNA content, after subtraction of nonspecific fluorescence as assessed by enzymatic treatment, represents predominantly ribosomal RNA. As always, following drug treatment or other perturbations, the RNA content measured is the result of the balance between RNA synthesis and catabolism and not a direct measure of the effect of the perturbation on RNA synthesis alone. Agents which block cell transit through the DNA synthesis cycle often induce "unbalanced growth," e.g., a situation in which RNA or protein content increases or decreases relative to normal for that phase of the cycle (18). While RNA content for a specific cell cycle compartment can be readily obtained by multiparameter flow cytometry, we have introduced the additional measure of the ratio of RNA content as a fraction of the total nucleic acid content, i.e., the $a_r$ ratio. This provides a quantitative measure of unbalanced growth that can be normalized and therefore compared, for instance, between cells blocked in G1 and G2 phase (18). An example of the correlated DNA versus RNA distribution of exponentially growing FL cells is displayed in Fig. 3, and compared with equivalent distributions following continuous exposure for 24 h to 10 and 50 nm CI-921 (refer to Fig. 2A for the cell cycle distributions). Since the predominant effect of drug treatment under these conditions was to accumulate cells in G2M, samples of drug-treated and control cultures were mixed in approximately equal proportions and their G2M RNA content and $a_r$ ratio compared (Fig. 3). Although significant numbers of cells had accumulated in G2M following continuous exposure to 10 nm CI-921, neither their RNA content nor $a_r$ ratio appeared to differ from that of untreated cells. However, both the RNA and $a_r$ plots of G2M cells show two distinct populations following 24 h of exposure to 50 nm CI-921 (Fig. 3). In fact, there was a nearly 75% increase in RNA content
following either a 24-h exposure to 50 nM CI-921 or 24 h following a 1-h pulse of 100 nM CI-921 (Table 2).

### Protein Content

Aliquots of the samples analyzed above for RNA content were also stained with the dye combination propidium iodide and fluorescein isothiocyanate, which provided correlated DNA versus total protein distributions (Fig. 4). The two parameter distributions and single parameter histograms for DNA and protein content of cultures exposed for 24 h to 0, 1, 10, and 50 nM CI-921 are presented in Fig. 4. In addition, $\alpha_p$, or the ratio of protein to DNA content, is also presented. The culture of equivalent size in early, mid-, and late S phase (Fig. 5). Utilizing the second parameter, $\alpha_p$, which corresponds to the sensitivity of cells to denaturation, the G1, S, and G2 + M phases can be identified separately. A subcompartment of G1 phase can also be identified, termed G1a, which was shown to have a higher $\alpha_p$ value than early S phase cells (Fig. 5).

Although little change was obvious in the raw data following 1 h of treatment, by 7 h it was clear that fewer cells had accumulated in mitosis and more cells remained in late S phase in the drug-treated cultures (Fig. 5); no cells enter G1 phase due to the action of the stathmokinetic agent.

G1-S Transition. Inasmuch as the action of vinblastine prevents cells from reentering G1 phase, the kinetics of emptying of the G1 compartment may be analyzed (Fig. 6A). During the initial 2.25 h, the percentage of G1 cells in both control and drug-treated cultures decreased by 60-65% (Fig. 6A). Thereafter, the G1 compartment emptied at a faster rate, displaying exponential kinetics. Only during the last h of the experiment did the kinetics of exit in the drug-treated cultures differ from the control. By that point, the difference only represented about

### Table 2. Effect of CI-921 on RNA and protein content

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* Cultures were exposed to drug during exponential growth or after having been maintained at confluence for 3 days.

* All samples were compared after either 24 h continuous drug exposure or after 24 h growth in fresh medium following a 1-h pulse of CI-921.

* Numbers represent the ratio of the means of the G2M populations of drug-treated to control cells. The ratio of RNA content in CI-921-treated cells compared to control cells was determined following subtraction of nonspecific red luminescence obtained as a result of enzymatic degradation of cellular RNA.
Fig. 4. Effect of CI-921 on the total protein content of FL cells. The two-parameter histograms at the top represent the DNA versus protein distributions of FL cells exposed for 24 h to 0, 1, 10, or 50 nM CI-921. Bottom, single-parameter histograms of DNA content and the protein and αi distributions of the G2M population. Cell staining was accomplished using the dye combination propidium iodide (for DNA) and fluorescein isothiocyanate (for total protein) as in “Materials and Methods.” The αi ratio was obtained by dividing the G2M protein fluorescence by the DNA fluorescence for each cell individually in the G2M population. Dotted line, mean of the control αi ratio.

1.5% (3.0 to 1.5%), i.e., not much greater than the error in the measurement.

G1A-G1B Transition. Previous studies (30) have suggested that the G1 phase could be subdivided into compartments based on the sensitivity of cells to acid denaturation. It appears that αi reflects, among other things, the degree of nuclear chromatin condensation (19, 31) which, in turn, can be correlated with the functional state of the cell. Thus, G1B cells are believed to be G1 cells prepared to enter S phase, whereas G1A cells appear to represent immediately postmitotic cells which must enter G1B before proceeding into S phase (Fig. 5). Thus, cells in G1B are part of the deterministic portion of the cell cycle, proceed through G1B at a constant rate, and are responsible for the beginning or convex portion of the G1 (i.e., G1A plus G1B) exit curve. G1A, on the other hand, represents a compartment from which cell exit is stochastic (32), resulting in the exponential character or second portion of the G1 exit curve (Fig. 6). When analyzed separately, the G1A exit curve was represented by a logarithmically declining slope from the onset of stathmokinesis. Note that approximately 42% of all G1 cells resided initially in the G1A compartment (Fig. 6). At the drug concentrations and the length of exposure tested in this experiment, no change could be observed in the transit of cells from G1A to G1B.

Transit through S Phase. In the absence of drug treatment, the percentage of cells should increase in each S phase window (due to the exponential nature of the culture), reach a maximum at a point where the effect of the stathmokinetic agents on cell entrance into G1 becomes apparent, and then decrease according to a specific function (Fig. 6B). The time at which the curve reaches a maximum should be later for cells in mid- and late S phase cells, while late S phase cells appeared to still be accumulating at the last time point (Fig. 6B). A separate peak in late S phase can be observed in the contour map of the 7-h sample exposed to 50 nM CI-921 (Fig. 5) and is consistent with the observations made above in Fig. 2A.

G2-M Transition. Mitotic cells begin to accumulate almost immediately upon addition of the stathmokinetic agent. Since the cells were growing exponentially, the slope of the mitotic accumulation curve should also be exponential. Utilizing the slope of this curve, the growth rate could be calculated to be 0.062 h⁻¹, corresponding to a doubling time of 11.22 h. The addition of CI-921 at either concentration (10 or 50 nM) immediately affected the accumulation of cells in mitosis (Fig. 6C). Within 30 min, the M accumulation curve plateaued for the culture that received 50 nM CI-921, indicating no new cells

Fig. 5. Contour plots of the acid denaturation profiles of FL cells at various stages in the stathmokinetic experiment. The contour map at the top represents the 0-h control distribution of FL cells partially denatured by acid and stained with AO as in “Materials and Methods.” The αi ratio represents the sensitivity of DNA in situ to denaturation as reflected by the ratio of single-stranded to total (single- and double-stranded) DNA as measured by red luminescence divided by total (red plus green) fluorescence for each cell. The specific cell cycle compartments analyzed are shown for the t = 0 (control) and represent M (mitotic) cells, G1 cells (G1A and G1B), early, mid-, and late S phase cells, and G2 cells. Contour maps are also presented for control (left), 10 nM (middle), and 50 nM (right) CI-921 samples taken at 1 or 7 h after the start of stathmokinesis. Bottom, contour maps for the 1:1 mixtures of 0-h control and the 7-h CI-921-treated samples.
were entering M from G2 phase after that time (Fig. 6C). At the lower drug concentration (10 nm), cells continued to exit G2, although very slowly (Fig. 6C).

S-G2 Transition. By calculating the increase in the number of cells in G2 + M it is possible to evaluate the drug effect on the movement of cells from S phase to G2 and to approximate the duration of G2, i.e., the distance along the time axis between the M and the G2 + M accumulation curves (which should be parallel in the unperturbed culture). The action of the drug on the S-G2 transition appeared to occur after the first h of treatment. Thus, by 2 h there was a slowdown in cell entrance into G2 (Fig. 6C). However, since the change in slope of the M accumulation curve occurred much earlier (and since G2 phase lasts about 2.5 h) the effect of the drugs on the S-G2 transition could not have been responsible for the change in rate of cells entering M. At the lower drug concentration (10 nm), following an early delay, cells continued to enter G2 at only a slightly slower rate than normal (Fig. 6C). Both an early (1–3 h) and late (5–7 h) change in slope of the G2-M accumulation curve at the higher drug concentration (50 nm), indicated that a substantial S phase accumulation occurred in cultures receiving 50 nm CI-921 (Fig. 6B and C).

Effect of CI-921 on Chromatin

In addition to allowing for the determination of the percentage of cells in the various cell cycle compartments as described above, the sensitivity of DNA in situ to acid denaturation, as represented by the $\alpha_r$ ratio, has been shown to vary with changes in chromatin condensation arising from histone modification and/or alteration in protein-DNA interactions following drug intercalation (19). CI-921 appears to bind to DNA in cells by intercalation. If its major effect is to stabilize the DNA, then the acid denaturability would be reduced (i.e., $\alpha_r$ would decrease). By comparing the contour maps of FL cells exposed for 7 h to vinblastine plus 0, 10, or 50 nm CI-921 in Fig. 5, it was observed that among the remaining, predominately G2 phase cells the $\alpha_r$ ratio shifted to lower values. Whereas for control cells the $G_2 \alpha_r$ ratio was approximately 0.45, the peak of the distribution of FL cells treated with 50 nm CI-921 fell below a value of 0.4 (Fig. 5). To demonstrate that this shift in $\alpha_r$ was not due to variations in staining or machine artifact, etc., the 7-h samples were mixed with 0-h untreated control FL cells. Note that the $G_2$ population in the mixture of control and 10 nm CI-921-treated cells and the S and G2 populations from the mixture of control and 50 nm CI-921-treated cells were still shifted to lower $\alpha_r$ values (Fig. 5). In fact, in the mixture of control and 50 nm CI-921-treated cells, two peaks can be observed in the $G_2$ region, representing the control $G_2$ cells with higher $\alpha_r$ values and the drug-treated $G_2$ cells whose means was shifted to lower $\alpha_r$ values (Fig. 5).

It should be noted that since the dye used for staining the DNA in situ is also an intercalating agent, one might expect dye-drug competition for binding sites which could affect cell stainability. Thus, a change in stainability may either be as a result of exclusion of dye binding by the presence of intercalated CI-921 molecules, or enhanced dye binding may be due to cooperativity (e.g., unwinding of the DNA helix by CI-921 which would facilitate AO binding nearby). This would then be expected to alter the total fluorescence distribution, i.e., the sum of AO fluorescence due to binding to double-stranded (green fluorescence) and single-stranded (red luminescence) DNA. The slight increase in total fluorescence observed in the $G_2$ populations of drug-treated cultures may be due to altered stainability, or alternatively may be indicative of a group of cells prepared to enter a higher ploidy as is known to occur with this drug (c.f. Fig. 2).

DISCUSSION

The search for an amsacrine derivative with activity against solid tumors led Baguley et al. (6, 7) to synthesize a large number of analogues. Previous structure-activity studies demonstrated that certain substitutions on the acridine chromophore of amsacrine increased drug lipophilicity and water solubility. Both these traits increase drug distribution properties in vivo, an important goal in treating solid tumors.

The present study was designed to detail the cytokinetic effects of CI-921 on both suspension cultures of leukemic cells
and on exponentially growing and stationary phase cultures of epithelial cells. Toward this end, drug concentrations were chosen to ensure that cytotoxic effects (i.e., cell loss) would not complicate the analysis of the point of action of CI-921. Alternatively, it should be pointed out that the cell kinetic effects observed at the lower, cytostatic concentrations (such as the G2 accumulation observed for most intercalating agents), may differ from the cell-killing effects of a drug (intercalators generally kill cells in S phase) at high concentrations (33). In the present instance, both continuous and short-term (pulse) exposure of exponentially growing FL cells and pulse exposure of exponentially growing and stationary phase CHO cells were studied.

Continuous drug exposure of exponentially growing FL cells, i.e., for a length of time in excess of the normal doubling time of 11.5 h, resulted in a biphasic growth inhibition curve (Fig. 1B). The ID$_{50}$ for continuous exposure was 9.5 nM CI-921, but increasing the drug concentration 5 times only resulted in an additional 50% decrease in growth. None of the concentrations tested resulted in immediate cell death, since greater than 90% of all cells excluded trypan blue. Under such circumstances, it must be assumed that the cell counts (based on trypan blue dye exclusion) represent the maximum surviving fraction but do not take into account more subtle drug interactions which may affect subsequent cell proliferation. A 1-h pulse exposure of FL cells to CI-921 resulted in a dose-dependent inhibition of growth, although the ID$_{50}$ was approximately 7 times higher than that following continuous exposure (67.5 nM).

Periodic examination of the cell cycle distribution of exponentially growing FL cells grown in the continuous presence of varying concentrations of CI-921 revealed that concentrations in excess of 10 nM slowed progression of cells through S phase, resulting in an eventual accumulation of cells in G2 (Fig. 2A). However, only continuous exposure to 50 nM CI-921 totally prevented cell division, although some cells which failed to divide appear to have proceeded to enter a higher ploidy level (Fig. 2A).

Pulse exposure of exponentially growing FL cells resulted in an early but transient accumulation of cells first in S (4 h) and then in G2 (8 h) phase (Fig. 2B). However, by 24 h, cells continued to cycle at both the original and at a higher ploidy level (Fig. 2B).

Exposure of exponentially growing CHO cells to a 1-h pulse of CI-921 provided similar results to that observed with FL cells (Table 1). Pulse exposure of confluent CHO cell cultures under the conditions described showed little change in cell cycle distribution as compared to untreated cultures even at a concentration of 100 nM CI-921 (Table 1). This was not surprising, inasmuch as the cloning efficiency of these cultures was only reduced by 50% at a drug concentration nearly twice that used in the kinetic study (i.e., 190 versus 100 nM) (Fig. 1B).

Under conditions of drug concentration and time of exposure sufficient to block cells in G2 for extended lengths of time (e.g., continuous exposure to 50 nM CI-921), unbalanced growth, defined by increased total RNA (Fig. 3) or protein (Fig. 4) content, was observed. The increases measured for G2 phase cells were substantial when monitored at 24 h (Table 2). Alternatively, while unbalanced growth was observed in cultures of exponentially growing CHO cells, no effect could be demonstrated for CHO cell cultures treated while at confluence (Table 2).

A more detailed kinetic analysis was performed by studying the effects of continuous drug exposure in a stathmokinetic experiment. Both the 10 and 50 nM concentrations of CI-921 were studied (Fig. 5). The results confirm the data obtained from the DNA histograms measured periodically over a longer exposure time.

Mathematical analysis of the untreated culture revealed that the doubling time for FL cells in this experiment was 11.3 h and the transit times through G1, S, G2, and M phases were, respectively, 3.4, 5.1, 2.2, and 0.6 h (17). Neither drug concentration affected the exit of cells from G1 phase (Fig. 6A). The lower drug concentration had little effect on early S phase cells but slowed progression through mid- and late S phases by approximately 1 and 2 h, respectively (Fig. 6B). At a concentration of 50 nM, CI-921 delayed cell transit through early S by about 1.5 h, through mid-S by 3 h, and resulted in continued accumulation of cells in late S phase over the time course of the experiment (Fig. 6B).

None of the FL cells exposed to 50 nM CI-921 divided following a 30-min exposure (Fig. 6C). Those cells exposed to 10 nM CI-921 continued to divide, albeit more slowly than control cells (Fig. 6C), accounting for the continued presence of G1 cells in the long-term exposure experiments (Fig. 2A). Both drug concentrations altered the rate of transition from S to G2 phase as expected, with the higher drug concentration manifesting a more dramatic slowdown at longer exposure times (Fig. 6C).

The cytokinetic effects described above for CI-921 were, in many respects, similar to those obtained with amsacrine on human colon carcinoma (LoVo) and human lymphoma (T2) cell lines (34). As with CI-921, several times higher concentrations of amsacrine were required to inhibit proliferation of stationary as compared to exponentially growing cells by an equivalent amount (34). In pulse exposure experiments, amsacrine caused an ever-increasing accumulation of cells in G2 phase. Paralleling the results obtained with CI-921 (Fig. 2), short exposure (1 h) to modest concentrations of amsacrine caused a transient G2 block, while increased concentration or length of exposure led to a more stable G2 block (34).

In addition to providing a means of quantitating the cell cycle distribution during stathmokinesis, AO staining of cells following partial acid denaturation often provides a sensitive measure of drug effects on chromatin structure (25). Occasionally potential antitumor agents modify the interaction between the anionic DNA molecule and its counterions in chromatin, the nuclear proteins (histones). These modifications may take the form of: (a) direct binding to available regions of the DNA; or (b) cross-linking DNA and nuclear proteins; or (c) modifying DNA-protein interactions indirectly, by modifying the proteins. In any case, the net result may be either stabilization or destabilization of DNA in situ as reflected by its sensitivity to acid denaturating (low pH) conditions (35). In the case of intercalating agents, the effect is often observed as a stabilization of DNA in situ to denaturation and thus a lowering of the a$_r$ ratio measured by the AO staining (35). This appeared to be the case with CI-921; interphase cells exposed to the drug for 7 h showed a marked shift to lower a$_r$ values (Fig. 5). It is unlikely that the observed effect was simply due to interference of dye binding by the drug (both dye and drug are intercalators). A decrease in the a$_r$ ratio with no decrease in total fluorescence would require that the decrease in AO binding to single-stranded DNA caused by CI-921 be exactly compensated by a drug-induced increase in AO binding to double-stranded DNA. While possible, such a combination of effects appears unlikely, especially since the shift in a$_r$ was highly reproducible and could be demonstrated by mixing control and drug-treated cells before staining (Fig. 5, bottom). Therefore, it seems more plausible to assume that
CI-921, as a result of intercalation, stabilizes the DNA to acid-denaturing conditions.

In conclusion, CI-921 appears to bind to DNA in intact cells probably by intercalation and, at cytostatic concentrations, slows the transit of cells through S phase leading to the eventual accumulation in $G_2$ in a state of unbalanced growth. Cell division is inhibited within 30 min of administration, although some proportion of the cells appear to proceed to a higher ploidy level. At subcytostatic drug concentrations, CI-921 causes a slowdown of cell transit through late S and $G_2$ phases, but unbalanced growth is not apparent. Cells with a significantly reduced cycling population (e.g., stationary phase CHO cells) are more resistant to the action of the drug, especially when given as a pulse. Nevertheless, while CI-921 is not likely to prove superior to amsacrine or Adriamycin in the treatment of leukemias (although some Adriamycin-resistant tumors appear to be sensitive to CI-921), the unique physiochemical characteristics of the drug may enhance its utility for the treatment of solid tumors. Certainly, the preliminary studies on the efficacy of CI-921 in treating solid tumors such as the Lewis lung carcinoma (10) is encouraging in this regard.

REFERENCES


Effects of a New Amsacrine Derivative, *N*-5-Dimethyl-9-(2-methoxy-4-methylsulfonylamino)phenylamino-4-acridinecarboxamide, on Cultured Mammalian Cells

Frank Traganos, Connie Bueti, Zbigniew Darzynkiewicz, et al.


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