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Cytotoxicity of 5-Aza-2′-deoxycytidine in a Mammalian Cell System

Sean Davidson, Penelope Crowther, John Radley and David Woodcock

After the addition of 5-aza-2′-deoxycytidine, a potent inhibitor of DNA methylation and S-phase-specific cytotoxic agent, metaphase chromosomes of Chinese hamster ovary (CHO) cells exhibited a highly decondensed and extended morphology (numerous "fragile sites") at the first mitotic division. However, when a lethal dose of this drug was added in early G_1 phase to cells synchronised by mitotic selection, the majority subsequently divided at the same time as an untreated control cell population with few division abnormalities and with few of the more usual types of chromosome aberrations such as gaps, breaks and exchanges. The drug-treated cells also entered and completed the second S-phase without significant delay and it was only at the second mitosis after addition of 5-azadeoxycytidine that cells showed delays in entering mitosis and significant increases in abnormal divisions concomitant with a modest increase in chromosome aberrations. If cells in a tumour behave similarly, the tumour mass would be expected to double before any reduction in tumour burden could be expected to occur.
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INTRODUCTION

IT IS IMPORTANT to elucidate the detailed biological effects of 5-aza-2′-deoxycytidine (5-azadC) on three grounds. Firstly, 5-azadC has been shown to be an active antileukaemic agent and has undergone phase I and II trials with different types of human tumours [1–3]. 5-azadC is highly cytotoxic to mammalian cells with maximal sensitivity in S-phase [4]. However, the actual mechanism of cytotoxicity has not been definitively demonstrated. Secondly, 5-azadC is a potent inhibitor of DNA methylation in mammalian cells with approximately 10 times the activity on a molar basis than the corresponding ribonucleotide, 5-azacytidine (5-azaC) [5, 6]. Both these agents have been used extensively in studies to elucidate the role of DNA methylation in mammalian cells. Both agents have been shown to reactivate inactive X-chromosomes [7] and induce the conversion of undifferentiated cell lines into myocytes, chondrocytes and adipocytes [9, 8]. Thirdly, 5-azadC (and 5-azaC) induces unusual morphologies in metaphase chromosomes which have variously been

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interpreted as undercondensation, segmental premature chromosome condensation, or as fragile sites [10–14].

In this study, we have chosen to use the cells of the Chinese hamster ovary (CHO) CHO-K1 cell line to undertake an integrated investigation of the biological and biochemical consequences of the addition of a single dose of 5-azadC. These cells have a relatively simple karyotype and can be very effectively synchronised by mitotic shake-off without the use of chemical blocking agents [15, 16]. We present data on on the effects of 5-azadC on DNA synthesis, DNA methylation, cell survival, time course of appearance of different types of chromosome aberrations, and cell cycle progression as shown by flow cytometric measurements of DNA content and time lapse video microcinematography.

Our data emphasise the complexity of the events which occur when this type of agent is used experimentally to perturb the level of DNA methylation in mammalian cells and also may be relevant to the most appropriate design of clinical trials for the assessment of this agent as an antitumour drug. In addition, these data also appear to be relevant to one class of fragile sites observed in mammalian chromosomes.

MATERIALS AND METHODS

Cell culture

CHO-K1 cells were grown as monolayer cultures in the α-modification of Eagle's minimal essential medium with 10% fetal calf serum (both from Flow Laboratories) in sealed flasks with 10% CO_2, 5% O_2 and 85% N_2. The bulk culture of CHO-K1 cells were used in all procedures except for the analysis of chromosome aberrations in colcemid-arrested metaphases in which a subclone (designated SCC30) with a more stable chromosome complement and an unusually low background of chromosome aberration was employed [17]. 5-azadC was obtained from Sigma.

Cell synchronisation

Cells were grown to 70–80% confluency in roller bottles (Corning) and synchronised by mitotic detachment without the use of colcemid [15, 16]. Mitotic cells were collected at 0.5 h intervals by vigorous shaking. The first sample collected was always discarded. Usually, between 0.5×10^6 and 2×10^6 cells were collected from 3–6 bottles. This was repeated until the requisite number of cell samples had been collected.

Flow cytofluorometric DNA content analysis

Cell samples were fixed with cold 70% ethanol. The fixed cells were stained with chromomycin A3 (Sigma) and analysed on a Becton–Dickinson Facstar Plus flow cytometer [15, 16].

Clonogenicity assays

CHO cells were seeded at 120 cells per plate containing 3 ml α-medium. 5-azadC was added at concentrations of 0, 0.1, 1 or 5 μmol/l with seven plates/point. Plates were stained with 0.01% crystal violet after 7 days and colonies counted. Colonies with more than 50 cells were scored as viable [16]. Percentage survival was calculated as the average number of colonies per plate compared with the numbers in the untreated control.

DNA synthesis rates and levels of methylation

CHO cells from two 150 cm^2 flasks were combined and used to seed four 75 cm^2 flasks so that the cell populations in each flask were totally equivalent. 2 h later, after the cells had attached to the flasks, 5azadC was added to three of these flasks at 0.1, 1.0, or 5 μmol/l while the fourth was a no drug control. After 30 min, [methyl-^{3}H]methionine and [^{14}C]thymidine (Amersham) were added together with 10 μmol/l bromodeoxyuridine (to density label newly synthesised strands), 1 μmol/l fluorodeoxyuridine (to maximise bromodeoxyuridine incorporation), and sodium formate (to prevent labelling of the C-1 pool by the methionine) as described previously [15, 18]. Cells were lysed 3 h later and DNA was extracted, purified and banded in alkaline $CsCl/Cs_2SO_4$ gradients. Under these conditions, the newly synthesised strands are density labelled with bromodeoxyuridine and band at a higher density than the preexistant (light) strands. Effectively all of the ^{3}H label incorporated into DNA is due to methylation of cytosines at the 5-position [18]. Most DNA methylation is into the newly synthesised strands which are also labelled with ^{14}C ("immediate methylation"). However, some methylation of DNA strands continues in the preexistant light strands for up to several hours after strand synthesis ("delayed methylation") [19].

Analysis of metaphase chromosome aberrations

CHO (clone SCC30) cells in logarithmic growth were incubated in 0.1, 1.0 or 5 μmol/l 5-azadC. Colcemid (1 μg/ml) was added to different flasks at 0, 6, 12, or 18 h and cells fixed 6 h later as described previously [16]. This provided 6-hourly windows of metaphases covering 0–24 h after addition of drug. Each metaphase was scored for the appearance of its chromosomes, and classified as being normal, or as having segmented/extended chromosomes, having gaps and breaks, exchanges, or for having a tetraploid chromosome complement.

Time-lapse microcinematography

Synchronised cells were allowed to attach to a 25 cm^2 flask for at least 3 h. Before filming, cells were rinsed in PBS-EDTA to remove detritus and fresh medium added. For films of 5-azadC treated cells, drug was present at 5 μmol/l in the fresh medium. The flask was placed on the stage of an inverted phase contrast microscope with an Ikegami ICD-42E CCD video camera attached to a Mitsubishi HS-480E time-lapse video recorder (which records time and date). Cells were monitored using a 10× or 20× objective. Recordings were made at 80 × slower than normal.

Scoring the time-lapse microcinematography

Criteria for estimation of mitosis duration were based on those of Hurwitz and Tolmach [20]. The beginning of mitosis was defined as the time when the shape of the cell became a constant sphere, and mitosis lasted until a membrane could first be seen between the doublet of daughter cells. A division was scored as normal when division time was within two standard deviations of the division times exhibited by control CHO cells [17 (S.D.8) min] and time-in-interphase was within one standard deviation of the mean for the control [14 (4) h]. Some abnormal divisions were successful (producing at least two progeny) but of anomalous duration; this category included some tripolar divisions. Cells which rounded and divided, but subsequently rejoined (usually within 3 h) were classified as abortive divisions. Death in division was scored when a cell rounded, but did not divide, and subsequently died (as observed by cessation of membrane movement or detachment from the surface) or where a division proceeded, perhaps appearing normal, but one or both daughter cells died immediately afterwards. Interphase deaths were usually not preceded by cell rounding, but often involved a darkening of the cell interior and subsequent detachment from the surface. Some of the cells moved slowly around the field and at times obscured other cells, while some cells moved out of the field. The low number of attempted third divisions in the control was partly due to the field

Table 1. Effect of 5-azadC on DNA methylation, DNA synthesis and cell survival

Concentration 5-azadC (μmol/l)	DNA synthesis	Methylation		Survival
		Immediate	Delayed	
Control	100.0	100.0	100.0	100.0 (6.3)
0.1	104.2	33.3	73.7	48.7 (4.3)
1.0	96.8	2.0	45.6	< 0.1
5.0	79.0	9.0	30.2	< 0.1

Percentage of DNA synthesis, DNA methylation (delayed and imediate [15, 19]) and survival of CHO cells in the presence of a series of concentrations of 5-azadC relative to control CHO cells (no 5-azadC). Mean (S.E.) survival.

becoming too crowded due to replication for individual cells to be traced successfully. In cases where it was not possible to identify unambiguously which cell was dividing, that division was not included in detailed analyses. Thus the number of attempted divisions does not quite double every round (even after allowing for the deaths of the first division).

RESULTS

DNA methylation in newly synthesised strands in CHO cells is almost completely inhibited by the addition of 5 μmol/l 5-azadC to the medium (Table 1). However, 5-azadC had relatively little effect on DNA synthesis even at the highest concentration tested. The amount of methylation in pre-existing strands ("delayed methylation") [18, 19] was much less strongly affected than methylation in strands synthesised while the drug was present ("immediate methylation"). This reflects the mechanism of inhibition of the mammalian DNA methyltransferase by this

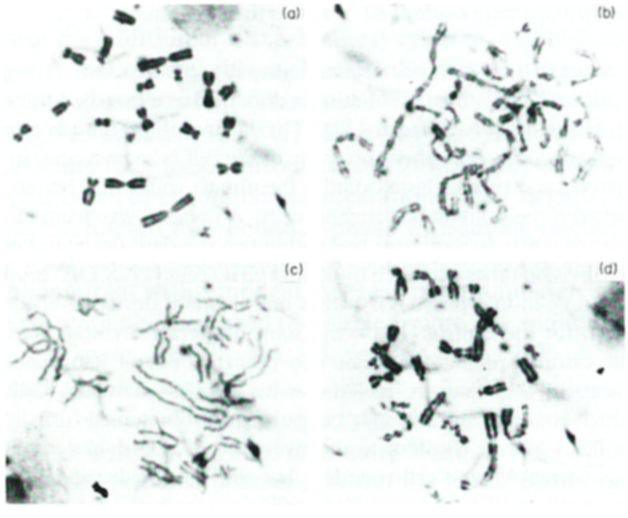

Fig. 1. Colcemid-arrested metaphase chromosomes of CHO (clone SCC30) cells for control cells (a) and 5-azadC-treated cells (b, c and d). (a) Normal morphology: chromosomes condensed, evenly stained and chromatids are closely associated; (b) segmented chromosomes: much longer than normal metaphase chromosomes which stain unevenly, indicating incomplete and variable condensation; (c) extended: chromosomes are much longer than normal and lack the close apposition between sister chromatids; and (d) a metaphase containing some chromosomes which are segmented and extended while others appear normal.

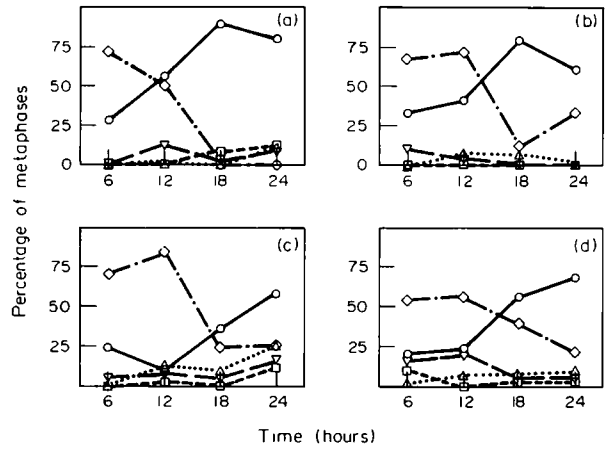

Fig. 2. Time-course of percentage of chromosome aberrations in CHO cells (clone SCC30) in (a) 0.1, (b) 0.5, (c) 1 and (d) 5 μmol/l 5-azadC. (○——○) Normal morphology; (◇--◇) segmented and/or extended; (△.....△) tetraploids; (▽_ _▽) chromosome and chromatid gaps plus breaks; (□_ _□) exchanges.

agent which involves covalent binding to 5-azadC incorporated into the DNA duplex [21, 22] and thus inhibition of DNA methylation primarily in regions of the chromatin which contain incorporated 5-azadC residues. The reduction in cell survival as measured by the clonogenicity of cells is quantitatively similar to the effect of 5-azadC on immediate methylation (Table 1).

In CHO-K1 cells, 5-azadC induces a variety of cytogenetically unusual metaphase chromosome forms, similar to those reported previously with other cell types [10–14]. The most frequent of the forms observed with CHO cells are illustrated in Fig. 1. The normal appearance of the chromosomes from colcemid-arrested metaphase spread is shown in Fig. 1a, with various aberrant morphologies observed after 5-azadC treatment in the remainder of the figure. The major aberrant form was for chromosomes to be unusually elongated (Fig. 1, b and c) with either segmentation (i.e. fragile sites [14]) (Fig. 1b) or without segmentation but with poor apposition of chromatid pairs (Fig. 1c). In another common variant (Fig. 1d), only a minority of the chromosomes in a metaphase show any sign of elongation or segmentation, with the remainder appearing normal.

The time course of the appearance of different abnormalities in the metaphase chromosomes was determined in an asynchronous population of cells (Fig. 2). A series of concentrations of 5-azadC (from 0.1 to 5 μmol/l) was added to cultures and portions of each population fixed every 6 h for 24 h (with colcemid being present for the last 6 h). After the first 6 h of 5-azadC treatment, the majority of metaphases contained segmented and/or highly extended chromosomes but with very few aberrations such as breaks, gaps, exchanges such as those induced by "conventional" cytotoxic agents. The frequencies of these highly elongated and apparently fragile chromosome forms decreased with time, particularly between 12 and 18 h, with metaphases with a normal appearance (and few breaks, gaps or exchanges) predominating after 18 h. This presumably reflects the spontaneous decay of 5-azadC in aqueous environments which, even without any metabolic effects of the cells, shows a $t_{\frac{1}{2}}$ of less than 16 h [23]. In general, conventional types of chromosome aberrations (breaks, gaps, exchanges) rise with time after 5-azadC treatment but are not present at high frequencies in metaphases at any time. The other major cytogenetic abnormality was the presence of cells with

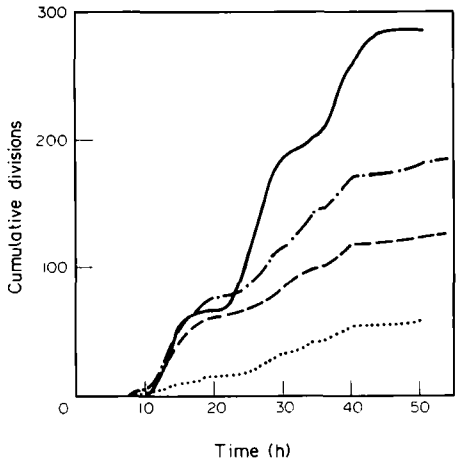

Fig. 3. Cumulative numbers of metaphases observed for control CHO cells and cells treated with 5 μmol/l 5-azadC compiled from three films of each category (representing initially 146 and 78 cells, respectively). The scale on the graph for the control cells (———) is 78/146 of that used for the drug-treated cells to normalise for different starting cell numbers. The number of unsuccessful divisions for control cells were negligible. 5-azadC-treated cells: (—·—·) attempted divisions; (_____) successful divisions; and (.....) unsuccessful divisions. (Attempted divisions is the sum of the unsuccessful and successful divisions.)

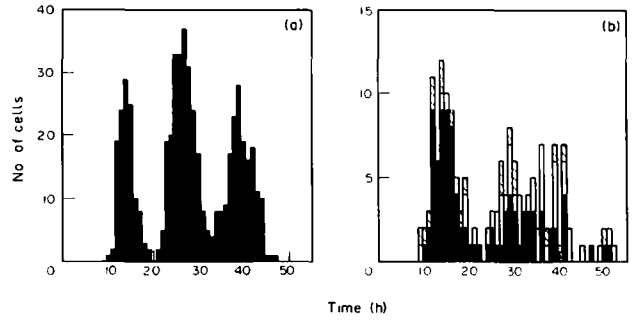

Fig. 4. (a) Number of cells undergoing mitosis during hourly intervals for control cells from three films (146 cells). (b) Number of mitoses observed during hourly intervals with drug treated cells from three films (78 cells). The filled sections of the bars represent normal divisions, diagonally hatched regions in bars indicate abortive divisions, and open sections of bars indicate deaths in division.

"tetraploid" chromosome complements. ("Tetraploid" is used loosely here since the original cells are not strictly diploid but contain a number of marker chromosomes.) Apart from the segmented/extended morphology, this was the predominant abnormal form shown by metaphases 6–12 h after 5-azadC addition at 5 μmol/l (Fig. 2d).

The normal cell cycle time for these cells was approximately 14 h [15, 16]. Hence, the predominantly normal metaphases observed 24 h after 5-azadC addition might represent cells which were entering a second mitotic division. However, cytotoxic agents characteristically cause delays in cell cycle progression. To assess the effect of 5-azadC on the various phases of the cell cycle, the CHO cells were synchronised by mitotic shake off. With this cell line, this can produce a cell population which retains close synchrony in cell division and DNA synthesis for at least two cell cycles without the use of any chemical synchronising agent [15, 16]. The time course of cell division in mitotically selected CHO cells was monitored by time-lapse video micrography and flow cytofluorometric DNA analysis.

Table 2. Analysis of time-lapse video data

Types of division	1st division		2nd division		3rd division	
	Control	5-azadC	Control	5-azadC	Control	5-azadC
Normal (%)	86	72	79	35	93	33
Protracted (%)	8	11	21	26	7	21
Abortive (%)	0	13	0	10	0	0
Death in division (%)	6	4	0	29	0	46
Attempted divisions	36	75	58	89	45	24
Interphase deaths	0	0	0	10	0	2

5-azadC (5 μmol/l) added in the G_1 phase of cycle 1. The values represent a compilation from three films for drug treated cells (78 cells) and one control film (36 cells).

The cumulative number of divisions in control and 5-azadC treated mitotically synchronised CHO cells as shown by time lapse video is presented in Fig. 3. Control cells exhibit two waves of division approximately 14 h apart. For cell populations in which 5 μmol/l 5-azadC had been added to the medium in G_1 phase 3 h after selection, the time of the first mitotic division was normal. Significant mitotic delay was only apparent at the subsequent division where only a minority of cells divided at the same time as the control population. When the types of mitotic division observed in control and 5-azadC treated populations are examined in more detail (Table 2 and Fig. 4), the principle mitotic abnormality seen in the first division cycle was abortive division: divisions in which the two daughter cells reunited into a single cell. Death in division in drug-treated cells was not significantly higher than in control cell populations. The control cells showed 6% death in division at the first division but not at subsequent divisions. This may reflect some form of mechanical damage to the cells resulting from the selection procedure. A few 5-azadC treated cells did not divide in over 60 h of filming although some movement of their surface indicated that they were still active. Abnormal divisions were frequently accompanied by blebbing of the cell membrane and the formation of "Bunches of Grapes" [24]. In some abortive divisions, a thin cytoplasmic bridge was visible between the two progeny which subsequently rejoined. At the second mitotic division after mitosis, drug-treated cells exhibited an increased frequency of a variety of mitotic abnormalities with the time of division being significantly extended beyond those observed in control cell populations (Fig. 4). The appearance of 5-azadC-treated cells became increasingly bizarre and diverse at later times of filming, with no discernible pattern of abnormality in morphology (not shown).

Flow cytofluorometry was used to analyse the rates of DNA synthesis and cell cycle progression in the second cycle after 5-azadC addition. Consistent with the time-lapse data, the majority of 5-azadC-treated synchronised CHO cells were in G_2/M phase 12 h after selection (9–11 h after addition of 5-azadC) (b). By 24 h after selection (c), the majority of 5-azadC-treated CHO cells were proceeding as a synchronous cohort about one third of the way through S-phase and had reached two thirds to three quarters of the way through S-phase by 27 h (d). By 30 h after selection (e), the majority of cells were in G_2/M with some G_1 cells present (probably representing cells which had just successfully divided). If there had been any delay in the G_1 of the second cell cycle after selection in 5-azadC-treated cells, the cells would not have entered

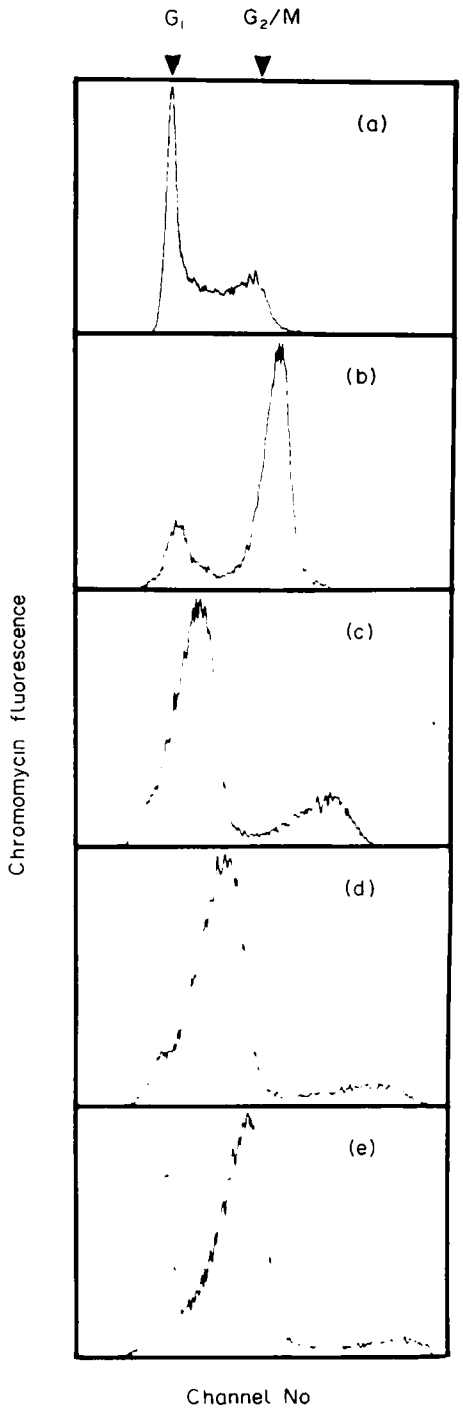

Fig. 5. Flow cytofluorometric analysis of syncronised CHO cells. The positions of G_1 and G_2/M populations are indicated at the top of the figure. (a) Asynchronous control cell population. (b)–(e): Cells incubated with 5 μmol/l 5-azadC. Cells were fixed (b) 12 h, (c) 24 h, (d) 27 h and (e) 30 h after mitotic selection.

the second S-phase until more than 20 h after selection (14 h total cell cycle time plus 6 h for G_1 [15, 16]). Delays in the second mitotic division after addition of the drugs to mitotically selected CHO cells might have been due to a block to initiation of DNA synthesis or to a slower rate of DNA synthesis in the second S-phase. However, in 5-azadC treated cells, the rate of progression through both the first and second S-phases was equivalent to the rate of progression in control, untreated populations (Fig. 5). Further, cells did not exhibit any significant delay in the G_1 phase following the first division (Fig. 5). Hence the 5-azadC treatment appears to have had a minimal effect in initiation on DNA synthesis in the second S-phase, despite the grossly abnormal morphology of the majority of chromosomes in metaphases of the first mitosis following drug addition.

The flow cytofluorometric DNA content analyses also showed the presence after the first division of a group of cells in the 5-azadC treated population which were hyperdiploid (approximately 20% of the total). These cells appear to represent the "tetraploid" subpopulation detected in the metaphase chromosome spreads (Fig. 2). Note that even this cell population was actively replicating its DNA and were progressing through S-phase in a reasonably synchronous manner at a rate comparable to that of the diploid population.

DISCUSSION

After a lethal dose of X-irradiation [20, 24] or of a DNA synthesis inhibitor such as cytarabine [16], some cells may undergo a number of divisions before death ensues. Hence the ability of cells treated with a toxic dose of 5-azadC to divide is not unusual in itself. What is unusual about the biological effects of 5-azadC compared to other agents which are cytotoxic to mammalian cells is the delayed nature of any effect on cell cycle progression. Agents which have proven useful as cytotoxic drugs in the treatment of human tumours usually have a fairly immediate effect on cell cycle progression in sensitive cell populations, whether the agent directly damages DNA (e.g. alkylating agents), inhibits topoisomerases (e.g. teniposide), affect microtubule assembly (e.g. vincristine and vinblastine which block cells at the first mitosis), or directly or indirectly inhibits DNA synthesis (e.g. cytarabine and methotrexate which inhibit progress through S-phase) [25]. Since 5-azadC has such a minimal effect in DNA synthesis, it is not surprising that it has quite different effects from DNA synthesis inhibitors. However, 5-azadC, once it is incorporated into DNA, is thought to inactivate the mammalian DNA methyltransferase through the formation of covalent adducts [21, 22]. Yet despite such DNA-protein adducts, progression through both the first and second phases after 5-azadC addition appears to occur at normal rates. Also, despite the grossly aberrant morphology of the majority of the first mitoses following 5-azadC addition with highly elongated and often segmented chromosomes, most mitoses occur at the normal time and with a minimal delay despite the undercondensed state of many chromosomes. The only exception to this appears to be the 10% to 20% of cells which, after 5 μmol/l 5-azadC, fail to undergo cytokinesis at the first mitosis and become tetraploid. Hence the cells have no significant problem segregating the pairs of sister chromatids despite their apparently "fragile" morphology. The extended and segmented sites on the chromosomes do not appear to be truly "fragile". If such decondensed regions commonly gave rise to DNA breakage (perhaps due to mechanical stresses of segregation or due to the action of endogenous nucleases), delays in progression through the subsequent S-phase would be expected. Moreover, the lack of significant number of acentric chromosome fragments inthe second division metaphases would argue against significant chromosome breakdown following the separation of these apparently "fragile" chromatids. Thus, the undercondensed regions of chromosomes at the first mitosis following 5-azadC addition most likely represent regions of the chromosomes in which the normal compaction processes have not proceeded to completion. Since 5-azadC is such an effective and specific inhibitor

of DNA methylation, this compaction is most likely due to undermethylation of regions of the genome. Note that this inhibition of DNA methylation during a single cell cycle can only produce hemimethylated DNA. Thus it appears that symmetrical methylation of certain regions of the genomic DNA is essential for the formation of fully compacted metaphase chromosomes. This effect appears fully reversible in that, after the 5-azadC has had time to decay, mitotic chromosomes at the second division have mostly regained their normal state of compaction.

As to the mechanism of cytotoxicity in these cells, we can formulate no definitive explanation from these data, and in particular no mechanism to explain the lack of a major effect on cell cycle progression until the second mitosis after addition of the 5-azadC. The closest correlation with cytotoxicity (loss of clonogenicity) is with the inhibition of DNA methylation in newly synthesised strands. Since 5-azaC and 5-azadC have been shown to induce changes in gene activity (presumably through their effects on DNA methylation [26]), it could be suggested that the most feasible mechanism for 5-azadC cytotoxicity is that this drug treatment results in changes in gene activity mediated by changes in DNA methylation patterns. The diversity of cell morphologies during the second drug treatment might be consistent with this hypothesis, although the appearance of cells lethally damaged with other types of agents can also be diverse (refs 16 and unpublished observations). However, if cells were ultimately dying due to loss of essential gene function, it seems unlikely that such an event would not also compromise the ability of cells to replicate their genome at a normal rate. Hence, loss of cell viability through loss of essential gene function seems somewhat unlikely as an overall mechanism.

It has also been shown that both 5-azadC and 5-azaC will induce differentiation in cells in culture [8, 9]. Apparently complex differentiated phenotypes such as striated muscle cells, chondrocytes which synthesise cartilage-specific proteins, and adipocytes have been observed in Swiss3T3 cells and C3H10T1/2 cells treated with these agents. Indeed, considering the inconsistencies of the available data with other more obvious mechanisms to explain the toxicity of 5-azadC, we would suggest that it is not unreasonable to propose that 5-azadC may cause the loss of clonogenicity of cells through the induction of terminal differentiation. Hence, in the experimental system employed here, the 5-azadC treated CHO-K1 cells may ultimately be dying through a parody of normal cellular senescence [27]. However, because these cells have undergone a variety of chromosome rearrangements during cellular transformation, such a differentiation process can have unpredictable consequences with a variety of aberrant cellular forms resulting. If this model is correct, this would imply that switching these cells onto the pathway to terminal differentiation requires one round of cell division and one or two rounds of DNA replication.

However, the most important implication of these data from a clinical perspective is that, assuming that the effect on CHO cells of 5-azadC is similar to its effect on tumour cell populations, cells of a tumour with a high proportion of cycling cells which have been terminally damaged by 5-azadC would be expected to double in number before significant reductions in growth rate or tumour mass could be expected. This is unlike other cytotoxic agents which have a more immediate effect on cell cycle progression [25]. This delay in any reduction of the rate of tumour proliferation might either render this agent unsuitable for use as a cytotoxic drug in cancer chemotherapy or mean that longer time intervals after treatment might be more appropriate for the assessment of efficacy of the treatment protocol with this drug.

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Interactions of Interferon-α_{2a} with 5′-Deoxy-5-fluorouridine in Colorectal Cancer Cells *in vitro*

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The biological activity of 5′-deoxy-5-fluorouridine (5′-dFUrd) depends upon intracellular enzymatic cleavage by pyrimidine phosphorylase to form 5-fluorouracil (5-FU). Interferon-α_{2a} (IFN-α) effect was analysed alone and combined with 5-FU or 5′-dFUrd, on proliferation inhibition of eight human colorectal cancer cell lines. The toxicity of 5-FU was enhanced by IFN-α in only one line (SW-480). In contrast, interactive enhancement of IFN-α was observed with 5′-dFUrd in five lines (WiDr, HT-29, 513, SW-480 and Co-115). In each of the lines showing potention by IFN/5′dFUrd but not by IFN/5-FU, cytoplasmic pyrimidine phosphorylase activity was increased after 5 days' incubation with IFN-α in a dose-dependent manner. Two lines (LISP-1 and SW-620) showed no potentiation of either 5-FU or 5′-dFUrd toxicity by IFN-α, and no change in pyrimidine phosphorylase activity. Potentiation of 5′-dFUrd effect by IFN-α may thus be explained by an enhancement of its conversion to 5-FU through stimulation of pyrimidine phosphorylase activity.
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INTRODUCTION

COLORECTAL CARCINOMAS are remarkably resistant to chemotherapy and, therefore, are mainly left to surgical treatment. Despite significant improvement in diagnosis and surgical techniques, decreased operative mortality and development of several chemotherapy and radiotherapy protocols, overall prognosis has not improved over the past 30 years. Large studies show survival advantage for adjuvant 5-fluorouracil (5-FU) and levamisole, and 5-FU, lomustine and radiotherapy [1, 2]. Clearly, new forms of effective systemic adjuvant therapy should be developed.

The anticancer activity of interferons (IFN) is presently beyond doubt for a limited number of cancers [3], but IFN therapy alone does not have much activity against colorectal cancer[4]. One of the most promising aspects of these cytokines is their ability to modulate the cytotoxic effects *in vitro* of chemotherapeutic drugs, as shown for vinblastine, cisplatin [5] or doxorubicin[6]. Synergistic effects of IFN with fluoropyrimidines *in vitro* have also been reported [7].

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Fluoropyrimidines are commonly used in clinical oncology, but their toxicity limits therapeutic application. The synthetic derivative 5′-deoxy-5-fluoridine (5′-dFUrd) [8] has raised marked interest due to higher therapeutic index as derived from *in vitro* and animal data and as compared to 5-FU or other related compounds [9]. It is also less potent in inducing leukopenia [9, 10] and is not as strongly immunosuppressive as other fluoropyrimidines [11, 12]. The biological activity of this prodrug is dependent upon its enzymatic cleavage to 5-FU by a pyrimidine phosphorylase [13–17], levels of which are markedly increased in human and animal tumours compared to normal tissues, with the exception of the intestinal tract [16, 18–20].

Recently, the combination of interferon α (IFN-α) plus 5-FU has been shown to give objective responses in 20 of 32 previously untreated patients with advanced colorectal carcinoma [21, 22]. This prompted us to analyse the *in vitro* effects of IFN-α on the cytotoxicity of 5-FU and of the prodrug 5′-dFUrd with human colon carcinoma cell lines.

MATERIALS AND METHODS

Cell lines

We used a panel of eight human colon cell lines, derived from primary tumours: WiDr (ATCC: CCL 218), HT-29 (ATCC: HTB 38), SW-480 (ATCC: CCL 228), SW-620 (ATCC: CCL 227), LISP-1 (obtained from Dr D. Lopez, Ludwig Institute for Cancer Research, Sao Paulo, Brazil), Co-115 [23], 513 and 411 (both established in our own laboratory). Cells were cultured