Europ. J. Cancer. Vol. 16, pp. 147–157
© Pergamon Press Ltd. 1980. Printed in Great Britain

0014-2964/80/0101-0147 $02.00/0

Lethal and Kinetic Effects of DDMP (2,4-diamino-5-(3′,4′-dichlorophenyl)-6-methylpyrimidine)

BRIDGET T. HILL with the technical assistance of R. D. H. WHELAN

Laboratory of Cellular Chemotherapy, Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, London WC2A 3PX, United Kingdom

Abstract—*The lethal and cytokinetic effects of DDMP have been investigated using a line of NIL 8 Syrian hamster cells by means of colony-formation, flow microfluorimetry, time-lapse cinematography and* ^{3}H*-thymidine labelling. Exponentially-growing NIL 8 cells exposed for* 1 hr *to increasing concentrations of DDMP showed initially a marked reduction in cell survival. The curve, however, plateaued at approximately* 40% *survival, even with drug concentrations in excess of* 100μg/ml. *'Quiescent' or 'plateau-phase' cultures were less sensitive (approximately 2-fold) to the lethal effects of DDMP. Exposure of synchronised cultures to DDMP for* 1 hr *killed cells in the S phase most effectively and had least effect on cells in the* G_2 *and M phases of the cycle. Cell cycle traverse was delayed markedly in S, and slightly in* G_2 *and M at sublethal drug concentrations, but was unaffected in* G_1*. These kinetic perturbations increased with increasing exposure time and with increasing drug concentrations, until drug levels were reached which induced no further cell kill since the plateau in the survival curve had been reached.*

These data suggest that, depending on the tumour cell kinetics, a prolonged exposure to a high drug concentration would be most effective in producing the greatest cytotoxic effect with DDMP alone.

INTRODUCTION

DDMP HAS been shown to be the most effective antitumour agent, amongst a series of 2,4-diamino-pyrimidines, on experimental tumours *in vitro* [1, 2] and *in vivo* [3–5]. The first clinical study [6] with DDMP had shown antitumour activity in children with acute leukaemia, but because of very severe myelosuppressive effects the drug was rejected as unsuitable for further trials. In retrospect, this toxicity may be explained by the inadequate treatment schedule (daily doses until toxicity), since it is now known that DDMP has a very long half-life in human serum, approaching 200 hr [7]. However, the observations that DDMP was particularly effective in killing cells resistant to methotrexate (MTX) [8], prompted a further evaluation of this compound. Experimental studies, using L5178Y lymphoblasts, confirmed that DDMP is effective in killing MTX-resistant cells and indicated: (1) that high concentrations for long exposure times should be used, and (2) that the concept of 'selective folinic acid protection' should be exploited by giving DDMP plus folinic acid (CF) simultaneously. In this way the drug-sensitive cells could be selectively protected from the lethal effects of DDMP but no such protection was afforded cells relatively drug-resistant [9–11]. These studies, which emphasized that the protection exerted by simultaneous CF was *not* identical when CF was used in a subsequent 'rescue', suggested that DDMP might be given more effectively and more safely in the clinic than in the past. Initial clinical trials have demonstrated that the combination of DDMP plus simultaneous folinic acid represents a useful addition to available anti-cancer drug treatment [12–17].

The antitumour activity of these diamino-pyrimidines has been attributed to their ability to function as 'anti-folate' compounds and inhibit the enzyme dihydrofolate reductase [8, 18]. However, while some recent studies have confirmed this observation in normal human cells [19], other studies with drug-resistant

Accepted 12 July 1979.

tumour cells have shown that they are much less effective inhibitors of this enzyme than is MTX [1]. In addition, a recent study showed that using a range of diamino-pyrimidine analogues which all inhibited dihydrofolate reductase, the various analogues produced a different pattern of response against certain solid tumours [20]. It is possible therefore that their cytotoxic effects may be associated with some other loci or mechanism of action.

In this report, the effects of DDMP at the cellular level have been examined in a Syrian golden hamster cell line (NIL 8 cells). Lethal cell damage was assessed by determining the effects of DDMP on the colony-forming ability of exposed cells. Drug sensitivity was more related to duration of exposure than to drug concentration and logarithmically-growing cultures were shown to be more sensitive than plateau-phase cells to the cell killing effects of DDMP. The survival of synchronised cells was much less when drug treatment was during G_1 and S compared with treating G_2 and M populations. The effects on cell cycle progression were monitored by flow cytofluorimetry and by following the entry of synchronised cells into DNA synthesis and mitosis. Cell cycle traverse was delayed predominantly in S being unaffected in G_1. There was evidence of a minor hold-up in G_2 and M at low drug concentrations.

MATERIALS AND METHODS

Material

DDMP was kindly provided for these studies as a gift from Dr. A. H. Griffith, the Wellcome Research Laboratories, Burroughs Wellcome Co., Beckenham, Kent, U.K. The drug was dissolved in a minimum quantity of ethanolic-HCl (4% w/v) and the diluted at least 100-fold in serum-free tissue culture medium prior to use, as described previously [21]. For cell synchronisation, thymidine was purchased from Sigma London Chemical Co., Kingston-on-Thames, Surrey, U.K., whilst hydroxyurea was donated by E. R. Squibb and Sons Ltd., Liverpool, U.K. The mithramycin used for flow microfluorimetric (FMF) analyses was the gift of Pfizer Co. Ltd., Kent, U.K., as Mithracin. All radioisotopes were purchased from the Radiochemical Centre, Amersham, Bucks, U.K. Other chemicals were obtained from Sigma Chemical Co., London or Fisons Scientific Apparatus, Loughborough, Leicestershire, U.K.

Cell cultures

NIL 8 cells isolated from Syrian golden hamster cell lines [22] were grown in Eagle's medium plus 10% calf serum at 37°C in a humidified 10% CO_2–90% air atmosphere. Cells for experiment were inoculated into Petri dishes (30mm dia.; Nunclon, Stafford, U.K.) at 5×10^4 cells/dish, except for FMF analyses where 90mm diameter dishes were used containing an initial inoculum of 1.5×10^5 cells/dish. Logarithmically-growing cultures were obtained 12–24 hr after subculture. Plateau-phase (or quiescent) cultures were obtained with cells grown to confluency (5 days after plating without medium changing) in which the cell count/dish had not increased over the previous 36 hr period. Cell counts were obtained using a Coulter Counter, model ZBI.

Cell survival was assessed by determining the ability of cells to form colonies. After drug treatments the cell monolayers were washed with phosphate-buffered saline (PBS) and trypsinized (0.025% trypsin as Tryptar from Armour Pharmaceuticals Co. Ltd., Eastbourne, U.K., for 5 min at 37°C) and then numbers of single cells were plated into Petri dishes and incubated at 37°C for 7–10 days, without any additional manipulation, to allow colony formation. Colonies were stained with Leishman's stain and then counted. A cell was considered to have retained reproductive capacity (viability) if it gave rise to a colony of 50 or more cells, as defined by Barranco *et al.* [23]. Under these conditions the plating efficiency was usually between 75–85%. Treated cultures were expressed as a percentage of the control (non-treated or solvent-treated) cultures, which were given the value of 100%. The mean and S.D. of the colony counts of 4 replicate cultures, with each experiment repeated 2 or 3 times, were computed to attain the survival curves which were produced by plotting on a semi-logarithmic scale, the percentage surviving fraction against time or drug concentration.

Synchronisation techniques

(1) Using low serum and hydroxyurea—the procedure used was a modification of that previously described [24]. NIL 8 cells were set up at a density of approximately 10^5 cells/dish (30mm dia) in Eagle's medium containing 10% calf serum (complete medium). Eight hr later the medium was changed to Eagle's medium containing 0.25% calf serum (i.e., low serum). After a further 64 hr the medium

was changed to Eagle's containing 10% calf serum plus 2.5 mM hydroxyurea. Twenty hours later the cells were washed with serum-free Eagle's medium and then complete medium, including 10% serum, was added to stimulate the cells to move through the cycle.

(2) Using low serum and a double-thymidine blockade—the procedure used was a modification of that described by Hittleman and Rao [25]. NIL 8 cells were set up as described above in (1) and left in medium containing low serum for 60 hr prior to changing the medium to Eagle's containing 10% calf serum plus 7.5mM thymidine. Nine hours later the thymidine was removed and fresh complete medium added to the cells for 5 hr. After this time the second thymidine block was introduced by adding back 7.5mM thymidine to the medium. Ten hours later the cells were washed with serum-free medium and were then ready for stimulation by fresh complete medium containing 10% serum.

The extent of synchrony achieved by these two procedures was monitored by total cell counts and pulse labelling cells with 1 μCi/ml of ^{3}H-thymidine (spec. act. 56 Ci/mmole) at various time intervals after the addition of fresh medium. In addition, entry into mitosis was monitored by time lapse cinematography and the percentage of labelled cells determined by autoradiography (see below). The results of typical experiments are shown in Fig. 1. Under these conditions at least 90% of the cells were radio-isotopically labelled within 3.5 hr of release from the blockade.

Autoradiography

Simultaneously with the release from the blockade 1 μCi/ml ^{3}H-thymidine was added to the cultures. Individual cultures were removed at subsequent half-hourly intervals, washed twice with PBS, fixed with methanol and extracted with 5% ice-cold TCA for 2 hr.

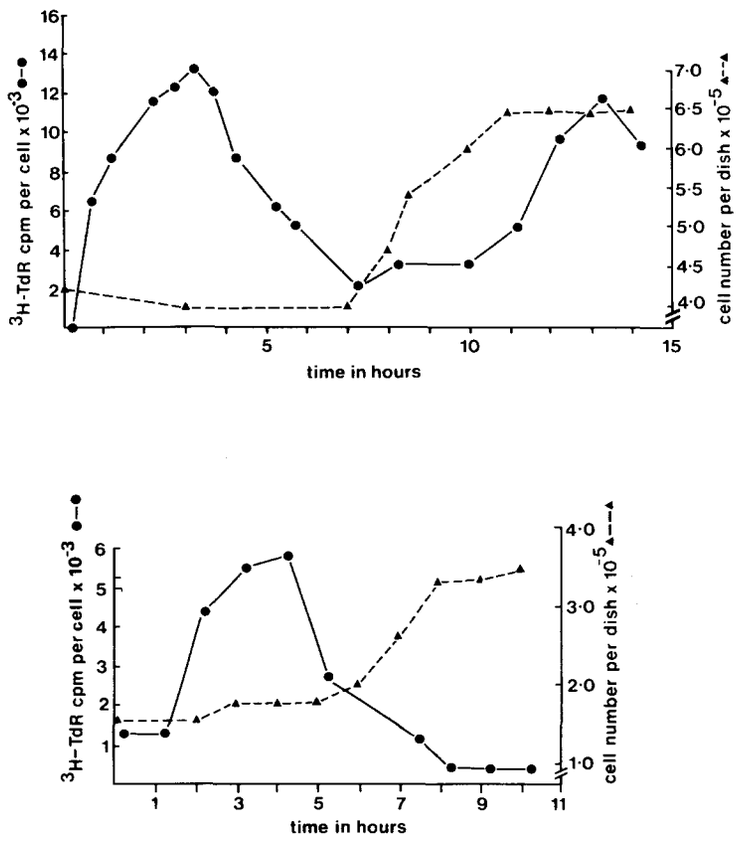

Fig. 1. ^{3}H-*thymidine incorporation after a* 30 min *pulse label and entry into mitosis judged by time-lapse cinematography of NIL 8 Syrian hamster cells after synchronisation using two different techniques: Top—sequential use of low-serum and then hydroxyurea; Bottom—sequential use of low-serum and a double-thymidine blockade.*

Cells were released from the blockade by the addition of fresh medium containing 10% *serum. The zero time points represent samples washed rapidly with fresh medium only.*

The fixed cells were washed twice with distilled water and air-dried. The cells were prepared for autoradiography using Ilford K5 dipping emulsion, exposed for 3 days and developed. The per cent labelled nuclei were determined on a total of 300 cells on duplicate dishes.

Monitoring effects on cell cycle progression

Synchronised cells were treated with DDMP for periods of 1 hr at varying times after stimulation by the addition of fresh complete medium. The effects of drug treatment, compared with control cultures, were determined by estimating: (1) DNA synthesis—the extent of incorporation of ^{3}H-thymidine into DNA was established by pulse-labelling cultures with 1 μCi/ml for 30 min at 37°C. The cells were then washed twice in ice-cold PBS and precipitated with 10% trichloroacetic acid (TCA) at 4°C for at least 1 hr. The acid-insoluble radioactivity was determined by filtering the samples through Millipore® filters Type HA, 0.45 μM). The filters were washed twice with 10ml of 5% ice-cold TCA, air-dried and their radioactivity measured in a toluene-based fluid using a Packard Tricarb scintillation counter, model No. 3855 with a 30% counting efficiency for tritium. (2) Entry into mitosis—the times at which cells were rounded up and underwent mitosis were estimated by time-lapse cinematography. This procedure was carried out using cells in Petri dishes and a Wild inverted microscope by Dr. P. Riddle. At least 100 cells were monitored in each analysis.

Flow microfluorimetry (FMF)

Drug effects on cell cycle progression of asynchronous cultures were analysed by serial measurements of the relative DNA content of the individual cells. Nuclei were prepared by the procedure of Marzluff *et al.* [26] with slight modifications [27]. The nuclei were ethanol-fixed and stained with mithramycin [28, 29] and measured in a Fluorescence Activated Cell Sorter (FACS-1, Becton Dickinson, Mountain Way, California). A full description of this machine is given elsewhere [30]. Briefly, this instrument measures both size and fluorescence of cells as they pass one by one through the focussed beam of a high-power argon ion laser. In this study, we were concerned only with cell fluorescence and for subsequent numerical analyses a graphic plot of the fluorescence histogram was made. Standard conditions of laser power, detector sensitivity and amplifier gain were used. These were: laser power 100mW at 457nm, fluorescence channel gain 8×1 and fluorescence channel photomultiplier tube high voltage 700V. Filters used were Ditric Optics 520nm 'cut-on' interference filters, together with a 520 series D coloured glass filter in the fluorescence channel. Routinely, 30,000 nuclei were measured from a sample concentration of approximately $2–10 \times 10^6$ nuclei/ml. DNA histogram evaluation was performed using the procedure recommended by Barfod [31].

Studies on the incorporation of radioactively-labelled precursors

Logarithmically-growing cells in medium containing 10% serum were treated with DDMP (3.125 μg/ml). At 1, 8 and 24 hr after treatment, the drug-containing medium was removed from the cell monolayers which were then washed with Hank's balanced salt solution at room temperature prior to incubation at 37°C in fresh Hank's solution with glucose containing the radio-isotope at a final concentration of 2 μCi/ml for 30 min. Additional cold precursors were added to a final concentration of 0.5 μM, except for deoxyuridine where a concentration of 0.1 μM was used. Triplicate samples of both drug treatment and control (non-treated) cells were analysed at each time point for radioactivity in acid-insoluble material. The radioactivity was estimated after collecting the material on Millipore® filters and processing as described above.

RESULTS

Lethal effects

Asynchronous cells. Figure 2 shows the lethal effects of a 1 hr exposure of both logarithmically-growing and plateau-phase NIL 8 cells to varying concentrations of DDMP. The survival curves are characterised by a logarithmic reduction in colony formation followed by a plateau, with no further significant increase in cell kill with increasing drug dosage (i.e., above 20 μg/ml). DDMP was more effective in reducing the colony-forming ability of cells in logarithmic growth than those in stationary-phase, with D_0 values differing by a factor of 2.5, being 7 and 17.5 μg/ml respectively, and the optimal cell kill achieved being 40% in stationary phase cells as opposed to 60% for logarithmically-growing cells. Drug concentrations less than 10 μg/ml of DDMP were without effect on the viability of stationary-phase cultures.

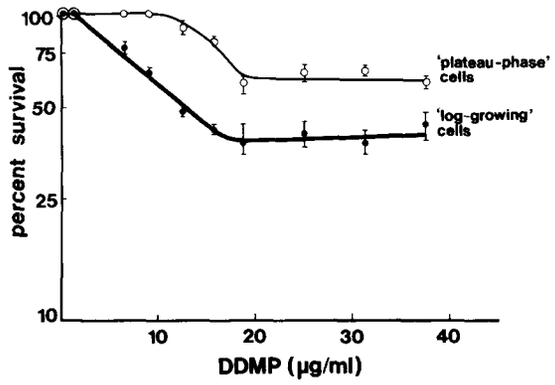

Fig. 2. The lethal effects of a 1 hr *exposure of logarithmically-growing and 'plateau phase' NIL* 8 *cells to varying concentrations of DDMP. The D_0 values (the dose required to reduce survival by* 25% *on the exponential parts of the survival curves) differ by a factor of* 2.5.

Figure 3 illustrates the time-dependent killing effects of DDMP on logarithmically-growing cells. There is increasing kill with increasing time over a 12 hr period. It is possible to achieve a 2-log cell kill with this concentration of drug (6.25 μg/ml) if cells are exposed for approximately 2 cell cycle times, namely 24 hr (results not shown).

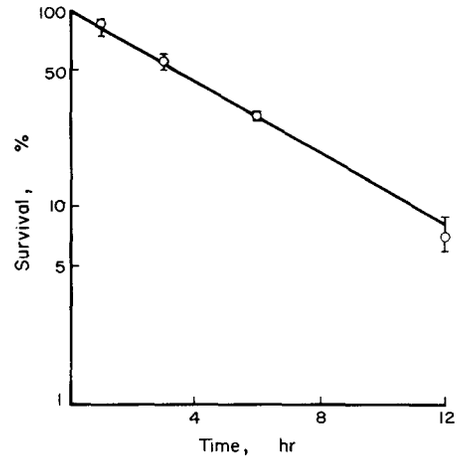

Fig. 3. The time-dependent killing effects of DDMP (6.25 μg/ml) *on logarithmically-growing NIL* 8 *cells.*

Synchronized cells. To analyse the effect of the cell age of NIL 8 cells to treatment with DDMP, synchronised cells were exposed to DDMP for 1 hr at all stages of their cell cycle. The dose of drug selected was 12.5 μg/ml which reduced the survival of asynchronous cells in logarithmic growth by approximately 50% (see Fig. 2). Cells treated with DDMP, under these conditins, showed fluctuations in survival as a function of position in the cell cycle, with an approximately 2.5-fold difference between the most (S phase) and the least (G_2 phase) sensitive cells (see Fig. 4). This cell cycle stage dependency was apparent irrespective of whether the cells were synchronised by hydroxurea (Fig. 4, left) or a double-thymidine block (Fig. 4, right).

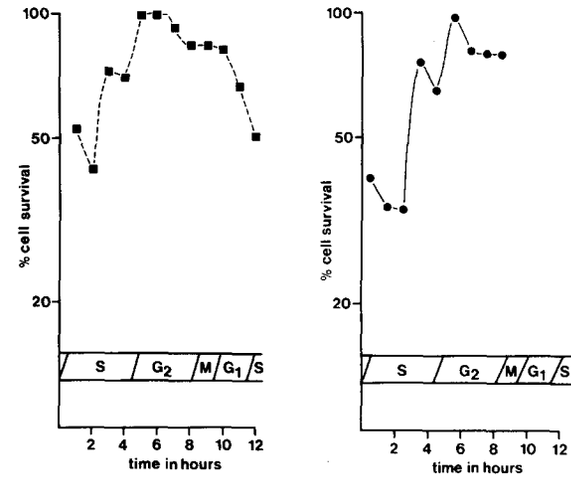

Fig. 4. The survival of synchronised NIL 8 *cells treated for* 1 hr *with DDMP* (12. 5 μg/ml) *at various stages of the cell cycle. Left—cells synchronised using low-serum followed by hydroxyurea. Right—cells synchronized using low-serum and a double-thymidine blockade.*

Effects on cell progression

Synchronous cells. Cells synchronised by treatment with hydroxyurea were exposed for 1 hr to a concentration of DDMP which allowed at least 90% cell survival as judged by colony-forming ability. This dose of 1.25 μg/ml was selected in an attempt to differentiate between the lethal or cell-killing effects of the drug and its ability to delay cell cycle progression. The effects of DDMP on the DNA synthetic phase were determined by treating the cells for 1 hr's duration during the 3 hr immediately following the release from the blockade and monitoring by pulse-labelling with ^{3}H-thymidine at 1 hr (Fig. 5, left) or 20 min intervals (Fig. 5, right). The fact that peak levels of ^{3}H-thymidine incorporation were similar in all control and drug-treated samples indicates that little or no cell kill was occurring under these conditions. The entry into DNA synthesis was not affected in cells treated with DDMP immediately following release from the blockade since ^{3}H-thymidine incorporation in both control and drug-treated cells increased at the same rate and reached a peak at approximately 4 hr. In contrast, treatment in mid-S-phase (i.e., 1–2 and 2–3

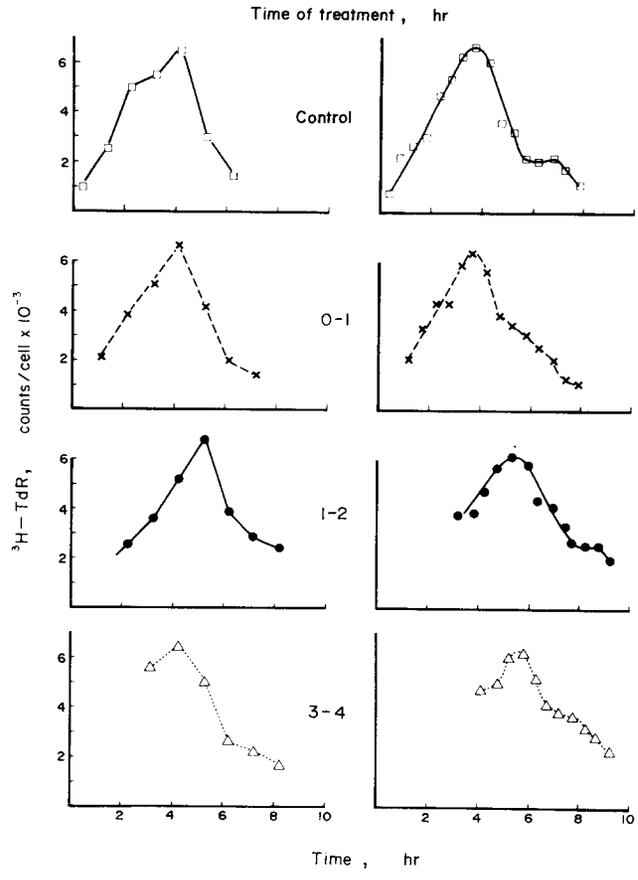

Fig. 5. The effects of a 1 hr *exposure to a low-dose of DDMP* (1.25 μg/ml) *on the progression of synchronised NIL 8 cells into DNA synthesis after release from the blockade induced by low-serum and hydroxyurea. Entry into S was monitored by pulse-labelling with* ^{3}H-*thymidine as described in the Materials and Methods section. The results from two such experiments are included to illustrate their reproducibility.*

hr after release from the blockade) delayed the further entry of the cells into DNA synthesis and extended the time spent in S, with the exit from this phase being delayed by approximately 1 hr.

The effects of DDMP treatment during early, mid and late S and in G_2 on the entry of synchronised cells into mitoses was examined using time-lapse cinematography. The results, listed in Table 1, indicate that the entry into M of cells treated immediately on removal of the blockade, or in late S occurred at a similar rate to control cells since between $7\frac{1}{2}$–9 hr approximately 75% of all the cells went through mitoses. These data suggest no delay in progression through the G_2 period. However, time-lapse cinematography showed that cells treated in the early and mid-S phase had a delayed entry into mitosis of approximately 1 hr which may be accounted for by the delayed exit of such cells from the S phase, recorded above. In addition there appeared a slight delay of cells entering M when they were treated with DDMP in mid-G_2.

Asynchronous cells. In an attempt to confirm these initial studies using synchronised cells, which indicated cell kill predominantly in the S-phase coupled with progression delay in this phase, asynchronous cultures were treated with DDMP at different concentrations for various exposure times and examined by flow microfluorimetry. Figure 6 shows the effects of exposure of cells to increasing DDMP concentrations for a fixed 24 hr period and the results are calculated in Table 2 and compared with survival figures. These data demonstrate that at a dose which allows 98% survival (i.e., 0.31 ng/ml) there is an indication of a build-up of cells in the S phase (Fig. 6B). This effect is exaggerated as the concentration is increased to 31 ng/ml (62% survival) (Fig. 6D), and this is still maintained at the higher doses of 310 ng/ml and

Table 1. Time of entry of synchronized cells into mitosis after treatment for periods of 1 hr *with* 1.25 μg/ml *DDMP as judged by time-lapse cinematography*

Period of drug treatment after release from blockade (hr)		Time of entry of >75% of the population into mitosis after release from blockade (hr)*
0–1		7.5–9.0
1–2		8.5–10.5
	'S phase'	
2–3		8.5–10.5
3–4		7.5–9.5
4–5		7.5–9.5
	'G_2 phase'	
5–6		8.0–10.0

*At least 100 cells were monitored in each analysis and the experiment was repeated at least twice.

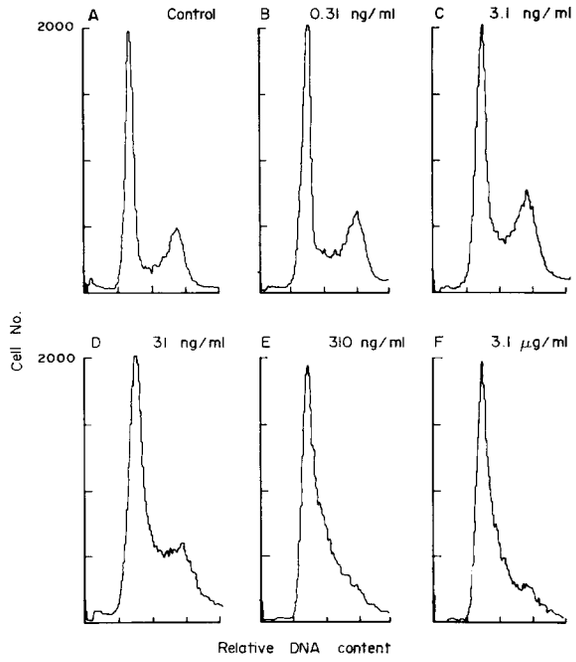

Fig. 6. The effects on the DNA distribution histograms of asynchronous NIL 8 cultures following a 24 hr exposure to increasing concentrations of DDMP.

3.1 μg/ml when cell kill is significant with survival being reduced to 38% and 30% respectively. This build-up of cells in the S phase suggests there is no delay in progression through G_1 induced by DDMP. At the low drug concentrations there is an indication of a slight build-up of cells in G_2 and M. However, at the higher lethal concentrations, there is a marked reduction in the G_2 and M populations which could result from a killing of cells in S phase and/or from a delayed progression through the S phase. At these lethal concentrations there is no evidence of accumulation in G_2 or M or delayed progression through these phases, and this is emphasized by the continued presence of a significant percentage of the population with a 2n DNA content characteristic of G_1 cells. Figure 7 and Table 3 show the results obtained following exposure of NIL 8 cells to a fixed DDMP concentration of 310ng/ml from FMF analyses and colony-forming assays for varying times. These data confirm the studies reported above but establish the temporal pattern of the alterations in the cell popu-

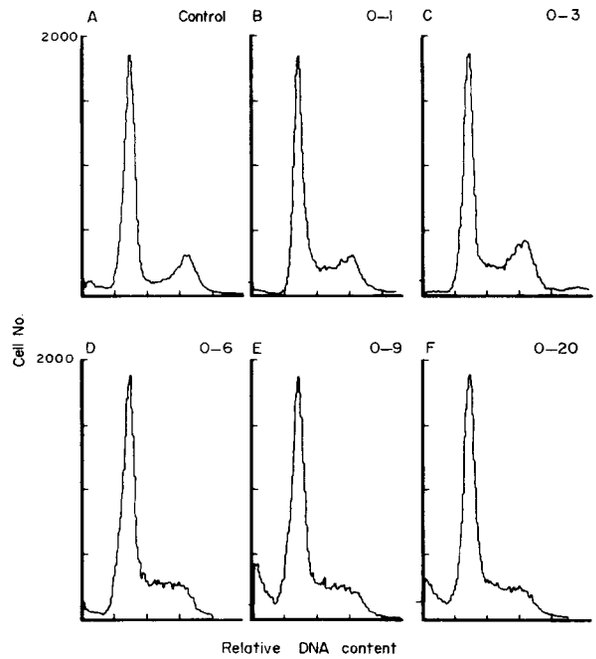

Fig. 7. The effects on the DNA distribution histograms of asynchronous NIL 8 cultures following exposure to a fixed DDMP concentration of 310 ng/ml *for varying times* (hr).

Table 2. A comparison of the cell cycle distribution analyses by FMF and cell survival following exposure of asynchronous NIL 8 cells for 24 hr *to increasing concentrations of DDMP*

Drug concetration (ng/ml)	Survival* (%)	Cell cycle distribution by FMF†		
		G_1	S	G_2+M
zero	100	59	19	22
0.31	98	49	26	25
3.1	82	46	26	28
31.0	62	46	36	18
310.0	38	60	28	12
3100.0	3	62	29	9

*Survival was assessed by determining colony-forming ability as described in the Materials and Methods section.

†These figures were calculated from the data in Fig. 7 by the method of Barfod [31] making allowance for the deviation of curves E and F from the normal distribution.

Table 3. A comparison of the cell cycle distribution analyses by FMF and cell survival following exposure of asynchronous NIL 8 cells to a fixed DDMP concentration of 3100 ng/ml *for varying time periods*

Time of drug exposure (hr)	Survival* (%)	Cell cycle distribution by FMF†		
		G_1	S	G_2+M
zero	100	66	16	18
0–1	86	62	21	17
0–3	56	53	24	23
0–6	29	52	32	16
0–9	7	60	29	11
0–20	3	57	29	13

*At least 100 cells were monitored in each analysis and the experiment was repeated at least twice.

†These figures were calculated from the data in Fig. 8 by the method of Barfod [31] making allowance for the deviations of curves E and F from the normal distribution.

lation. The build-up in the S phase occurs rapidly, it is apparent by 1 hr after treatment (Fig. 7B) and continues to increase over the next 6 hr and remains for 9–24 hr (see Fig. 7F and 6F). There is a slight build-up of cells in G_2 and M during the first 3 hr of treatment but by 6 hr the reduction in G_2 commences (see Fig. 7D), suggesting cell kill in the S phase, as confirmed by a reduction in colony-forming ability to 29% of control and remains low for 9–24 hr (Figs. 7E and F, and 6F).

To complement these studies the effects of DDMP treatment on the incorporation of tritiated precursors into DNA, RNA and protein was estimated. The results, using the same drug concentration as the experiments described in Fig. 3, are given in Fig. 8 and clearly demonstrate that under these conditions DDMP very markedly inhibits both the incorporation of ^{3}H-thymidine and ^{3}H-deoxyuridine into DNA. The effect of ^{3}H-deoxyuridine incorportion is seen most rapidly with reduction to approximately 40% of control occurring within 1 hr of drug treatment. DDMP also caused a marked reduction in the incorporation of ^{3}H-uridine into RNA which was apparent within 1 hr of treatment but was further reduced by 8 and 24 hr, finally reaching a value of approximately 30% of control values. The effects of DDMP on the incorporation of ^{3}H-leucine into TCA-insoluble material were much less marked, no inhibition was seen until 24 hr after treatment when a reduction of approximately 45% could be measured. These results confirm the very marked effects of DDMP on DNA synthesis, causing its inhibition which leads to cell kill, but also suggest an interference with overall RNA synthesis.

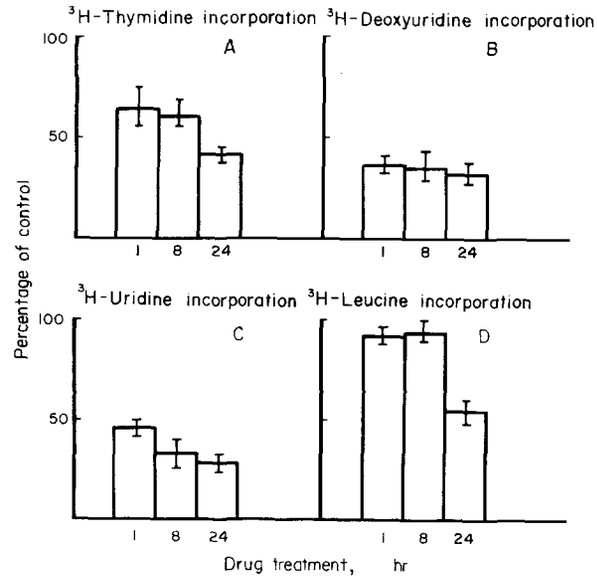

Fig. 8. The influence of DDMP treatment (6.25 μg/ml) *for various time periods on the incorporation of tritiated precursors into DNA, RNA and protein by NIL 8 cells. The incubation conditions are described in the Materials and Methods section. The mean values* ± S.E. *from triplicate samples from four experiments are provided as a per cent of control. Control values were as follows, expressed as* counts/min/10^6cells: ^{3}H-*thymidine:* 5500; ^{3}H-*deoxyuridine:* 1600; ^{3}H-*uridine:* 18,000; ^{3}H-*leucine:* 15,000.

DISCUSSION

These studies have shown that DDMP has cytotoxic activity against NIL 8 cells in culture, confirming the results of earlier *in vitro* studies [1, 2]. DDMP has more activity against logarithmically-growing as opposed to

stationary-phase cultures and its effectiveness increases with time of exposure. These observations are to be expected since subsequently, using synchronised cell populations, the S-phase was established as the major lethal site of action of DDMP.

The fact that when cells are exposed to DDMP for a fixed time period the survival curve eventually plateaus irrespective of increasing drug concentrations, suggests that DDMP is a class II agent according to the kinetic classification of antitumour agents, originally proposed by W. R. Bruce and his colleagues [32] and subsequently extended [33–35]. The cytotoxicity of class II agents is known to be more related to the duration of exposure than to dose, as shown for DDMP in this study. In this respect the long half-life of DDMP in man [7] represents a valuable characteristic of the drug for use in the clinic.

S phase cells were maximally susceptible to lethal cell damage, whilst cells in the G_2 and M phases were least affected. This result was obtained irrespective of whether cells were synchronised by pretreatment with hydroxyurea or a double-thymidine blockade. Therefore the method of synchrony used did not appear to influence the cells' subsequent response to drug treatment. This observation would be in agreement with other reported data. For example, comparable drug responses were seen using Chinese hamster cells arrested in G_1 either by hydroxurea [36] or by growth in isoleucine-deficient medium [37]. However, since both the methods of synchrony used here arrest cells at a similar point in the cycle, namely the G_1/S boundary or early S phase [25, 38], we have not been able to establish in this study whether the stage at which cells are synchronised influences their subsequent response to drug treatment. In this respect, although our initial attempts to collect cells by selective mitotic detachment for study were unsuccessful, we intend to pursue this approach further.

Interference with cell cycle traverse by DDMP was shown to occur predominantly in the S phase. Progression through G_2 and mitosis was not altered by lethal concentration of DDMP but a slight delay was observed with sub-lethal drug concentrations. Similar results were obtained using both synchronous and asynchronous cell populations, which would suggest that the procedures used for synchronisation did not influence the cells' subsequent response to drug treatment.

The effects of DDMP were seen very rapidly, i.e., within 1 hr after treatment. This observation is consistent with the earlier finding that the entry of DDMP into L5178Y cells occurred very rapidly with early association of the drug occurring within 5 min and a steady-state intracellular concentration being reached after 30 min incubation at 37°C [9]. In addition DDMP has been shown to exert an effect on the deoxyribonucleoside triphosphate concentrations in human cells within 15 min of drug addition [19].

This knowledge of the cellular pharmacokinetics of DDMP may prove useful when attempting to establish the mechanism of action of DDMP and also in deciding on suitable combinations of this agent with other anticancer drugs.

Acknowledgement—The help of D. Capellaro and D. Delia with the flow microfluorimetric analyses and that of Dr. P. Riddle with the time-lapse cinematography is gratefully acknowledged. The helpful comments of Drs. L. A. Price and J. H. Goldie and Professor K. Hellmann were much appreciated during the preparation of this manuscript. The artwork of Mrs. J. Minton, reproduced by the photographic department at the ICRF, and the secretarial assistance of Eileen Simmons and Cilla Conway provided valued support for this presentation.

REFERENCES

1. Bridget T. Hill, J. H. Goldie and L. A. Price, Studies concerned with overcoming resistance to methotrexate: a comparison of the effects of methotrexate and 2,4-diamino-5-(3′-4′-dichlotophenyl-)-6-methyl pyrimidine (BW 51097) on the colony-forming ability of L5178Y cells. *Brit. J. Cancer* **28,** 263 (1973).
2. Bridget T. Hill, L. A. Price and J. H. Goldie, Attempts to overcome methotrexate resistance: the value of drug-resistant cell lines. In *Chemotherapy. Proc. IXth International Congress of Chemotherapy.* (Edited by K. Hellmann and T. A. Connors) Vol. 7, p. 309. Plenum Press, New York (1976).
3. J. H. Burchenal, S.K. Goethchius, C. C. Stock and G. H. Hitchings, Diamino dichlorophenyl pyrimidines in mouse leukemia. *Cancer Res.* **12,** 251 (1952).

4. D. A. Clarke, S. M. Buckley, S. S. Sternberg, C. C. Stock, C. P. Rhoads and G. H. Hitchings, Effect of 2,4-diamino pyrimidines on mouse sarcoma 180. *Cancer Res.* **12,** 255 (1952).
5. K. Sugiura, Effects of various compounds on the Ehrlich ascites carcinoma. *Cancer Res.* **13,** 431 (1953).
6. L. M. Murphy, R. R. Ellison, D. A. Karnofsky and J. H. Burchenal, Clinical effects of dichloro- and monochlorophenyl analogues of diaminopyrimidines: antagonists of folic acid. *J. clin. Invest.* **33,** 1388 (1954).
7. D. R. Stickney, W. S. Simmons, R. L. DeAngelis, R. W. Rundles and C. A. Nichol, Pharmacokinetics of pyrimethamine (PRM) and 2,4-diamino-5-(3′-4′-dichlorophenyl)-6-methyl pyrimidine (DMP) relevant to meningeal leukaemia. *Proc. Amer. Ass. Cancer Res.* **14,** 52 (1973).
8. C. A. Nichol, Studies on dihydrofolate reductase related to the drug sensitivity of microbial and neoplastic cells. *Advanc. Enzyme Regul.* **6,** 305 (1968).
9. Bridget T. Hill, L. A. Price and J. H. Goldie, Methotrexate resistance and uptake of DDMP by L5178Y cells. Selective protection with folinic acid. *Europ. J. Cancer* **11,** 545 (1975).
10. J. H. Goldie, S. I. Harrison, L. A. Price and Bridget T. Hill, Impaired responsiveness to folinic acid protection in methotrexate-resistant L5178Y cells. *Europ. J. Cancer* **11,** 627 (1975).
11. Bridget T. Hill, L. A. Price, S. I. Harrison and J. H. Goldie, The difference between 'selective folinic acid protection' and 'folinic acid rescue' in L5178Y cells in culture. *Europ. J. Cancer* **13,** 861 (1977).
12. L. A. Price, J. H. Goldie and Bridget T. Hill, Methodichlorophen as an antitumour drug: preliminary report. *Brit. med. J.* **2,** 20 (1975).
13. L. A. Price and Bridget T. Hill, Clinical use of DDMP in cancer chemotherapy. In *Chemotherapy, Proc. IXth International Congress of Chemotherapy.* (Edited by J. D. Williams) Vol. 2, p. 48. Plenum Press, New York (1976).
14. L. A. Price and Bridget T. Hill, Clinical use of DDMP (2,4-diamino-5-(3′-4′-dichlorophenyl-6-methyl pyrimidine). *Proc. Amer. Ass. Cancer Res.* **17,** 59 (1976).
15. L. A. Price, Bridget T. Hill and J. H. Goldie, DDMP and selective folinic acid protein in the treatment of malignant disese: a further report. *Clin. Oncol.* **3,** 281 (1977).
16. P. Alberto, R. Peytremann, R. Medenica and M. Beretta-Piccoli, Clinical experience with a combination of DDMP, a diamino pyrimidine, and folinic acid (CF). *Proc. Amer. Ass. Cancer Res.* **19,** 346 (1978).
17. R. J. DeJager, J. J. Rodzynek, J. Klastersky and Y. Kenis, Phase I study of DDMP (2,4-diamino-5-(3′-4′-dichlorophenyl-6-methyl pyrimidine) and folinic acid (CF) administration. *Proc. Amer. Ass. Cancer Res.* **19,** 403 (1978).
18. G. H. Hitchings, G. B. Elion, H. van der Werff and E. A. Falco, Pyrimidine derivatives as antagonists of pteroylglutamic acid. *J. biol. Chem.* **174,** 765 (1948).
19. K. Ganeshaguru and A. V. Hoffbrand, Effects of DDMP and DDEP on the deoxyribonucleoside triphosphate concentrations in human cells. *Biochem. Pharmacol.* **26,** 543 (1977).
20. S. F. Zakrezewski, C. Dave and F. Rosen, Comparison of the antitumour activity and toxicity of 2,4-diamino-5-(1-adamantyl)-6-methyl pyrimidine and 2,4-diamino-5-(1(adamantyl)-6-ethylpyrimidine. *J. nat. Cancer Inst.* **60,** 1029 (1978).
21. Bridget T. Hill, The effectiveness of clinically useful antitumour agents as inhibitors of RNA polymerases. *Cancer Biochem. Biophys.* **2,** 43 (1977).
22. D. R. Critchley, K. A. Chandrabose, J. M. Grahem and I. Macpherson, Glycolipids of NIL hamster cells as a function of cell density and cell cycle. In *Control of Proliferation in Animal Cells.* (Edited by B. Clarkson and R. Baserga) p. 481. Cold Spring Harbor (1974).
23. S. C. Barranco, J. K. Novak and R. M. Humphrey, Response of mammalian cells following treatment with bleomycin and 1,3-bis(2-chloroethyl)-1-nitrosourea during plateau phase. *Cancer Res.* **33,** 691 (1973).
24. Bridget T. Hill, R. D. H. Whelan, V. J. Hemmings, and L. M. Franks, A method of synchronisation of normal and malignat tumour cells in culture. *Cell Biol. Internat. Rep.* **1,** 379 (1977).

25. W. N. HITTELMAN and P. N. RAO, The nature of adriamycin-induced cytotoxicity in Chinese hamster cells as revealed by premature chromosome condensation. *Cancer Res.* **35,** 3027 (1975).
26. W. F. MARZLUFF, C. E. MURPHY and R. C. C. HUANG, Transcription of ribonucleic acid in isolated mouse myeloma nuclei. *Biochemistry* **12,** 3340 (1973).
27. BRIDGET T. HILL, R. D. H. WHELAN and S. A. WHATLEY, Evidence that transcription changes in ageing cultures are terminal events occuring after the expression of a reduced replicative potential. *Mech. Age. Devel.* **8,** 85 (1978).
28. H. A. CRISSMAN and R. A. TOBEY, Cell cycle analysis in 20 minutes. *Science* **184,** 1297 (1974).
29. A. KRISHAN, Rapid flow cytofluorimetric analysis of mammalian cell cycle by propidium iodide staining. *J. Cell. Biol.* **66,** 188 (1975).
30. L. A. HERZENBERG, R. C. SWEET and L. HERZENBERG, Fluorescence-activated cell sorting. *Sci. Amer.* **234,** 108 (1976).
31. N. M. BARFOD, Flow microfluorimetric estimation of G_1 and G_2 chalone inhibition of the JB-1 tumour cell cycle *in vitro*. *Exp. Cell Res.* **110,** 225 (1977).
32. W. R. BRUCE, B. E. MEEKER and F. A. VALERIOTE, Comparison of the sensitivity of normal haematopoietic and transplanted lymphoma colony-forming cells to chemotherapeutic agents administered *in vivo*. *J. nat. Cancer Inst.* **37,** 233 (1966).
33. L. M. VAN PUTTEN and P. LELIEVELD, Factors determining cell killing by chemotherapeutic agents *in vivo*. II. Melphalan, chlorambucil and nitrogen mustard. *Europ. J. Cancer* **7,** 11 (1971).
34. BRIDGET T. HILL and R. BASERGA, The cell and its significance for cancer treatment. *Cancer Treat. Rev.* **2,** 159 (1975).
35. J. C. MARSH, The effects of cancer chemotherapeutic agents on normal haematopoetic precursor cells: a review. *Cancer Res.* **36,** 1853 (1976).
36. F. MAURO and H. MADOC-JONES, Age responses of cultured mammalian cells to cytotoxic drugs. *Cancer Res.* **30,** 1397 (1970).
37. R. A. TOBEY, Effects of cytosine arabinoside, daunomycin, mithramycin, azacytidine, adriamycin and camptothecin on mammalian cell cycle traverse. *Cancer Res.* **32,** 2720 (1972).
38. W. K. SINCLAIR, Hydroxyurea: differential lethal effects on cultured mammalian cells during the cell cycle. *Science* **150,** 1729 (1965).