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STUDIES WITH A 2,4-DIAMINO-5-(3′,4′-DICHLOROPHENYL)-6-METHYLPYRIMIDINE (DDMP)-RESISTANT L1210 LEUKEMIA CELL LINE WITHOUT CROSS-RESISTANCE TO METHOTREXATE

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Abstract—An L1210 leukemia cell line resistant to 2,4-diamino-5-(3′,4′-dichlorophenyl)-6-methylpyrimidine (DDMP) (L1210/DDMP) was developed *in vivo* by treatment of tumor-bearing mice. Resistance to DDMP was confirmed by subsequent *in vivo* survival experiments and by *in vitro* dose–response curves. The L1210/DDMP line demonstrated little cross-resistance to another folate analog, methotrexate (MTX). This was confirmed both *in vivo*, with survival experiments, and *in vitro*, using dose–response curves. A statistical analysis of the *in vivo* data confirmed DDMP resistance with lack of MTX cross-resistance. Dihydrofolate reductase (DHFR) activity in the L1210/DDMP/R_5 line was no greater than in the parent cell line (L1210/S), and the K_m of DHFR for dihydrofolate was the same in the L1210/DDMP/R_5 and L1210/S lines. The K_i for DHFR of the L1210/DDMP/R_5 cell line versus the L1210/S cell line was increased 3.0-fold for MTX and 3.5-fold for DDMP. Total accumulation of [^{14}C]DDMP was identical in the two cell lines. The explanation for the lack of MTX cross-resistance in the L1210/DDMP/R_5 line is unknown.

DDMP [2,4-diamino-5-(3′,4′-dichlorophenyl)-6-methylpyrimidine] is a substituted pyrimidine derivative active against experimental animal tumors and human cancer [1–3]. Its mechanism of action is thought to be related to its antifolate effect and there is strong evidence that the compound acts in a manner similar to the folate analogs [4]. Reduced folate in the form of N^5-formyl tetrahydrofolate will modify the toxic and therapeutic effects of DDMP in experimental animals [5]. It is a very strong inhibitor of dihydrofolate reductase (DHFR) with an inhibition constant (K_i) of 10^{-10} M for DHFR purified from L1210 cells [6]. Experimental tumors resistant to methotrexate (MTX) on the basis of impaired cellular transport may, however, be sensitive to DDMP [7]. A methotrexate-resistant L1210 cell line with elevated DHFR has demonstrated cross-resistance to DDMP [6].

During the course of investigations designed to evaluate the potential clinical role of antifolate combinations, a DDMP-resistant L1210 cell line (L1210/DDMP) was produced which, when tested *in vivo* and *in vitro*, was not cross-resistant to MTX. This paper describes initial studies with this cell line.

MATERIALS AND METHODS

L1210 murine leukemia cells and male BDF_1 (C57 black × DBA_2) mice were obtained from the Arthur D. Little Co. (Cambridge, MA). DDMP and DDMP-[2-^{14}C] (13.8 mCi/mmole) were provided as a gift from Dr. C. A. Nichol, Burroughs–Wellcome Co. (Research Triangle Park, NC). For *in vivo* use, DDMP was prepared as a suspension in 10% Emulphor, 10% absolute alcohol and 80% normal saline. For *in vitro* use, DDMP and [^{14}C]DDMP were dissolved in lactate and the pH was adjusted with NaOH. MTX was supplied as a lyophilized powder (Drug Development Branch, NCI, Bethesda, MD) and was reconstituted with sterile water just before use. Dihydrofolic acid (FH_2) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were obtained from the Sigma Chemical Co. (St. Louis, MO). Eagle's minimal essential tissue culture medium was obtained in powder form from Microbiologic Associates (Bethesda, MD) and ammonium sulfate was purchased from the Fisher Scientific Co. (Fair Lawn, NJ). Biofluor scintillation counting fluid was obtained from the New England Nuclear Corp. (Boston, MA). All routine chemicals were analytical grade.

In vivo *experiments*

The L1210/DDMP cell line was produced *in vivo* by subcutaneous (s.c.) injection of DDMP (40 mg · kg^{-1}) 1 day after intraperitoneal (i.p.) implantation of 10^6 L1210/S tumor cells during serial passages. Sensitivity of the cells to DDMP or MTX was assessed *in vivo* by determining the effect of a

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single injection of DDMP ($40\ mg \cdot kg^{-1}$, s.c.) or MTX ($3.0\ mg \cdot kg^{-1}$, i.p.) daily for 4 days, on the median survival time (MST) of tumor-bearing mice. Results are expressed as per cent increase in life span (% ILS) of a group of treated mice vs a group of untreated mice bearing the same tumor, and calculated according to the formula:

$$\%\ ILS = \left(\frac{MST\text{-treated group}}{MST\text{-untreated group}} - 1\right) \times 100\%.$$

For *in vivo* assessment, ten mice per group were used in each experiment.

The difference in % ILS between mice bearing DDMP-treated cells and mice bearing cells never previously exposed to DDMP (L1210/S) was analyzed statistically by the methods of Cox [8] and Peto *et al.* [9] to confirm the presence of resistance.

In vitro *experiments*

Drug sensitivity studies. Once tumor growth was evident (by the appearance of ascites) following *in vivo* treatment with DDMP, tumor cells were harvested, washed, and maintained in asynchronous logarithmic growth in medium supplemented with 10% fetal bovine serum, 1×10^{-5} M 2-mercaptoethanol, streptomycin sulfate (100 μg/ml), sodium penicillin G (100 units/ml), Hepes* buffer (20 mM) and L-glutamine (0.03%). Dose–response curves to DDMP and MTX were obtained after 48 hr of continuous exposure of cells to different concentrations of drug. The number of remaining cells was enumerated in a Coulter Counter model F. Cell sensitivity to drug was expressed as the concentration of drug which inhibited cell growth by 50 per cent (ID_{50}) according to the method described by Jackson *et al.* [10]. Drug resistance was expressed as the ratio of ID_{50} values for cells exposed to DDMP *in vivo* (L1210/DDMP) vs previously unexposed cells (L1210/S) passaged *in vivo* through six transplant generations.

Enzyme studies. DHFR was partially purified from a sonicated extract of L1210 cells by ammonium sulfate fractionation according to the method of Perkins *et al.* [11]. Cells were suspended in 50 mM Tris buffer, pH 7.5, and sonicated for 30 sec at setting No. 3 using a Branson model 185 cell disruptor. The sonicate was centrifuged at 27,000 *g* for 20 min at 4° in a Beckman J21B centrifuge. The supernatant fraction was adjusted to pH 5.1 with 0.1 N HCl, stirred for 15 min at 4°, and then respun at 27,000 *g* for 30 min at 4°. The resulting supernatant fraction was adjusted to pH 6.0 with 0.1 N KOH and then 34.6 g of ammonium sulfate was added to every 100 ml. This mixture was stirred for 20 min at 4°, and then spun at 27,000 *g* for 30 min. The precipitate was discarded, and to every 100 ml (original volume) of supernatant fraction was added 26.4 g ammonium sulfate. This was stirred for 1 hr at 4° and centrifuged as before. The supernatant fraction was discarded and the precipitate, containing the enzyme, was dissolved in 0.15 M potassium phosphate buffer, pH 6.8. The protein content was determined by the method of Lowry *et al.* [12], using bovine serum albumin (BSA) as standard.

DHFR activity was measured spectrophotometrically at 23° using a Beckman model 25 double-beam instrument. The reaction mixture contained 25 μl FH_2 (2.3 mM in 10% $NaHCO_3$ and 0.25 M 2-mercaptoethanol), 25 μl NADPH (2.4 mM aqueous), 35–160 μg enzyme and 0.15 M potassium phosphate buffer, pH 6.8, in a total volume of 1 ml. The reaction was initiated by addition of FH_2, and monitored spectrophotometrically at 340 nm.

For K_i determinations DHFR activity was measured as described except that 25 μl of appropriately diluted inhibitor (DDMP or MTX) was included in the reaction mixture to achieve the final desired inhibitor concentrations. The volume of buffer was accordingly adjusted to a final volume of 1 ml. For DDMP, the final concentration in the reaction mixture ranged from 5.6×10^{-7} M to 1.4×10^{-8} M to produce a range of inhibition from 10 to 90 per cent. MTX concentrations ranged from 8.25×10^{-9} M to 2.75×10^{-10} M and produced inhibition ranging from 8 to 85 per cent. Seventeen experimental points were used to determine the K_i of DDMP for DHFR from L1210/S and twenty points to determine the K_i of MTX for the same enzyme. For DHFR from L1210/DDMP/R_5 twelve experimental points were used to determine the K_i of DDMP and twenty points for the K_i of MTX. The apparent K_i ($K_{i,app}$) was determined from the DHFR inhibition curves by use of a modified version of the Fortran program of Henderson [13] for tight-binding zone B inhibitors.† The rationale for such an analysis is presented by Henderson [13, 14] and Jackson *et al.* [15], and is based on considerations raised by Straus and Goldstein [16]. Once the $K_{i,app}$ was computed, the true K_i was calculated according to the formula:

$$K_i = \frac{K_{i,\ app}}{1 + \dfrac{\text{substrate concentration}}{K_m}}. \qquad [15]$$

The molar concentration of the substrate, FH_2, was checked spectrophotometrically at 283 nm using a molar extinction coefficient of 19,000 at pH 8.3.

The K_m of FH_2 for DHFR of both control and DDMP-resistant cells was determined spectrophotometrically at 340 nm by addition of a limiting quantity of substrate to the reaction mixture and following the reaction to completion. K_m was calculated using an integrated form of the Michaelis–Menten equation derived from serial measurements of substrate concentrations over time [17].

[^{14}C]*DDMP accumulation studies.* Total [^{14}C]DDMP accumulation in L1210/S and L1210/DDMP/R_5 cell lines was compared using a filtration technique described by Sedwick *et al.* [18]. Cells (10^6/ml) were suspended in 5 ml of Eagle's medium, supplemented with 10% dialyzed fetal bovine serum, and incubated at 37°. After 5 min, [^{14}C]DDMP was added to a final specific activity of 0.7 μCi/ml (final concentration DDMP, 50 μM) and 10 min later a 0.5-ml aliquot of cell suspension was removed and placed in 5 ml of cold Tris–saline (50 mM Tris) buffer, pH 7.5. The cells were then poured onto Whatman GF/C glass fibre discs and

* Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

† Performed by Dr. L. I. Hart.

washed twice with 5 ml of cold buffer, taking care not to allow drying between washes. After the last wash the filters were dried and placed in glass scintillation vials and the cells were solubilized with 0.5 ml of 2 N NaOH and left sitting overnight at 50°. Subsequently, 0.5 ml of 3 N HCl was added, to minimize quench, and then 18 ml Biofluor was added and the samples were counted in a Beckman LS-230 scintillation counter with a counting efficiency for ^{14}C of 42 per cent. Results are expressed as disintegrations per minute (dpm) per 10^6 cells. The 10-minute accumulation was measured since a plateau of drug uptake occurs at this time [18].

RESULTS

In vivo *experiments*

Table 1 shows the effect of DDMP and MTX on the median survival time (MST) of mice bearing L1210/S tumor cells. When used as single agents, DDMP and MTX produce an ILS of 79 and 59 per cent respectively (passage 0). The effect of DDMP on the survival of mice bearing tumor cells exposed to the drug during serial passages is also shown (passages 1–8). The ILS produced by DDMP fell to 25 per cent after one passage, to 14 per cent after three passages, and to 0 per cent by the fifth passage, at which time DDMP resistance was considered complete. Even when the dose of DDMP was raised from 40 to 60 mg · kg^{-1}, there was only a small increase in survival (14 per cent) of mice bearing cells that had been exposed through eight passages to the lower drug dose. The degree of *in vivo* cross-resistance of the L1210/DDMP cells to MTX is also shown in Table 1. After exposure of cells to DDMP through three passages, when ILS had fallen from 79 to 14 per cent, the corresponding change in ILS to MTX exposure *in vivo* was minor (59–43 per cent). At the fifth passage, when *in vivo* DDMP resistance was complete (% ILS = 0), MTX continued to produce an ILS of 40 per cent. This cytotoxic effect of MTX on DDMP-resistant cells continued through eight passages, during which cells were continually exposed to DDMP. Cells exposed to a higher dosage of DDMP (60 mg · kg^{-1} s.c. × 1) through two further transplant generations also continued to demonstrate the absence of MTX cross-resistance.

A statistical analysis for the *in vivo* data confirmed that the above determinations were true indicators of DDMP resistance without MTX cross-resistance (see Appendix A).

In vitro *experiments*

Drug sensitivity. At passages 5 and 7, when *in vivo* DDMP resistance was complete (% ILS = 0), the ratio of the ID_{50} for L1210/DDMP/R_5 to that for L1210/S cells was 4.8 ± 0.5 (N = 7). The ID_{50} ratio of these cell lines for MTX was 1.00 ± 0.07 (N = 6), thus confirming the absence of cross-resistance to MTX using this *in vitro* test. Figure 1 illustrates representative dose–response curves of MTX and DDMP for the cell lines L1210/S and L1210/DDMP/R_7, indicating the shift of the DDMP dose–response with little change in the dose–response to MTX.

Enzyme studies. Table 2 shows studies involving DHFR from L1210/S and L1210/DDMP/R_5 cells. The results include DHFR activity, K_m for FH_2, and K_i for DDMP and MTX. DHFR activity, expressed as μmoles · min^{-1} · mg^{-1} protein, was similar in the L1210/S and L1210/DDMP/R_5 cell lines (0.179 and 0.160, respectively).

Enzyme activity was also measured from an L1210 line made resistant to both DDMP and MTX in combination (L1210/DDMP-MTX). This line was completely resistant to both drugs *in vivo*, and the ratio of the *in vitro* ID_{50} of L1210/DDMP-MTX to L1210/S was 5.8 for DDMP and 6.8 for MTX (each value represents the mean of three separate experiments). DHFR activity of this cell line was 0.396 μmole · min^{-1} · mg^{-1} protein, 2.5 times greater than for L1210/S and L1210/DDMP/R_5.

The K_m values of FH_2 for the enzyme from L1210/DDMP/R_5 and L1210/S cell lines were not significantly different (3.82×10^{-7} M and 4.03×10^{-7} M, respectively), and are similar to values previously reported from DHFR derived from other L1210 cells [15]. The K_i of MTX for DHFR from the L1210/S line was 2.4×10^{-12} M and for the L1210/DDMP/R_5 line, 7.1×10^{-12} M. The K_i of DDMP for DHFR from L1210/S and L1210/DDMP/R_5 lines was 2.5×10^{-10} M and 8.7×10^{-10} M, respectively.

[^{14}C]*DDMP accumulation studies.* DDMP is a

Table 1. Effect of DDMP and MTX on survival of L1210/S and the cross-resistance of L1210/DDMP-resistant cells to MTX *in vivo**

Passage	*In vivo* resistance to DDMP (% ILS ± S.E.)	*In vivo* cross-resistance of L1210/DDMP cells to MTX (% ILS ± S.E.)
0 (L1210/S)	79 ± 8 (3)†	59 ± 3 (6)
1 (DDMP/R_1)	25 (1)	
3 (DDMP/R_3)	14, 14 (2)	43, 43 (2)
5 (DDMP/R_5)	0 (2)	37, 43 (2)
8 (DDMP/R_8)	0 (1)	43 (1)
8‡ (DDMP/R_8)	14 (1)	57 (1)

* DDMP, 40 mg · kg^{-1}, s.c., day 1; MTX 3.0 mg · kg^{-1}, i.p., days 1–4.

† The numbers in parentheses indicate replicate experiments.

‡ Cells treated for eight transplant generations with DDMP 40 mg · kg^{-1}, s.c., day 1, and two further transplant generations with 60 mg · kg^{-1}, s.c., day 1.

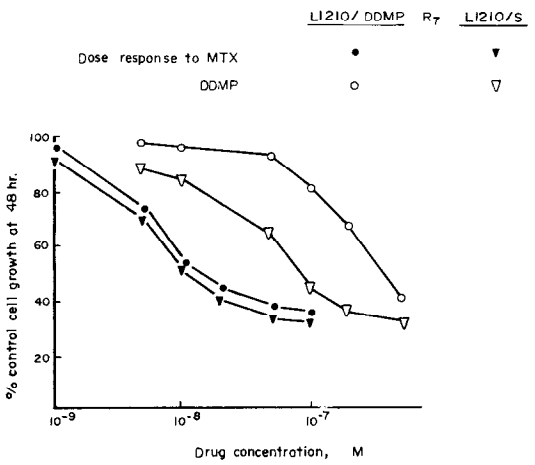

Fig. 1. *In vitro* dose–response curves of control cells and DDMP-resistant cells to MTX and DDMP.

Table 2. Results of studies with DHFR from control cells and resistant cells

Cell line	DHFR activity*	K_m FH_2	K_i DDMP ± 2 S.D.	K_i MTX ± 2 S.D.
L1210/S	0.179	3.82×10^{-7} M (N = 2)	$2.5 \pm 0.2 \times 10^{-10}$ M	$2.4 \pm 0.9 \times 10^{-12}$ M
L1210/DDMP/R_5	0.160	4.03×10^{-7} M (N = 2)	$8.7 \pm 1.4 \times 10^{-10}$ M	$7.1 \pm 0.6 \times 10^{-12}$ M
L1210/DDMP-MTX	0.396			

* DHFR activity (μmoles · min^{-1} · mg^{-1} protein).

lipophilic compound and the kinetics of drug transport are, therefore, difficult to measure. In order to obtain information regarding possible differences in DDMP transport between the parent and DDMP-resistant cell lines, total cellular drug accumulation was measured at a time when a plateau of drug uptake was expected [18]. The studies were repeated twice with <5.0 per cent variation. The mean accumulation of [^{14}C]DDMP at 10 min was 3888 dpm/10^6 cells for the parent line (L1210/S) and 3767 dpm/10^6 cells for L1210/DDMP/R_5.

DISCUSSION

Mechanisms of resistance to MTX have been well defined in experimental tumor systems. These mechanisms include impairment of cell membrane transport, increased levels of DHFR and altered affinity of the drug for DHFR [19, 20].

One approach to circumventing these mechanisms of resistance has been to use 'small molecule' antifolates (i.e. DDMP) that can more easily penetrate the cell membrane by passive diffusion. The pyrimidine derivatives DDMP and pyrimethamine are the most effective of these compounds in experimental tumor systems. The cytotoxic effects of DDMP *in vitro* and *in vivo*, in both mouse and man, can be prevented or reversed by the use of reduced folates [5, 21, 22]. *In vitro*, the inhibitory effect of DDMP on cell growth can be partially prevented by purines and thymidine [6]. Thus, the mechanism of cytotoxicity of this compound is thought to be related to its antifolate effect. It was, therefore, surprising to find a cell resistant to DDMP which did not display MTX cross-resistance.

The studies described in this paper were designed to examine aspects of the drug–enzyme interaction which might account for the absence of MTX resistance in this DDMP-resistant cell line. The activity of DHFR in the resistant line was not increased over control levels. This was not unexpected since, in a previous study, it was shown that an increased level of DHFR associated with acquired MTX resistance was accompanied by increased resistance to DDMP [6]. Moreover, increased DHFR activity produced by *in vivo* exposure of cells to MTX and DDMP in combination (L1210/DDMP-MTX) was associated with resistance to both agents (Table 2). There was no difference in the apparent K_m values of FH_2 for DHFR isolated from either the L1210/S or L1210/DDMP/R_5 lines.

Since the membrane transport routes of MTX and DDMP are felt to be different, the observed absence of MTX cross-resistance might be explained by a decreased transport for DDMP which does not affect MTX transport. This possibility was examined by measuring the accumulation of [^{14}C]DDMP in the two cell lines. At 10 min, when a plateau of drug

uptake was expected [18], the total accumulation of label in the two cell lines was almost identical. Due to the lipophilic nature of DDMP, cell transport kinetics are difficult to measure and differences in cellular transport K_m or V_{max} between the two cell lines cannot be ruled out on the basis of this data. It is possible that non-specific binding of [^{14}C]DDMP to the cell membrane might, in fact, mask differences in DDMP accumulation between the two cell lines.

When affinity of MTX or DDMP for DHFR was examined, both DDMP and MTX had a higher affinity for the enzyme from the L1210/S vs the L1210/DDMP/R_5 cell line. In both cell lines, MTX had a 100-fold greater affinity for DHFR than did DDMP. The K_i values of MTX and DDMP for DHFR from the L1210/S cell line are similar to those reported previously for other L1210 cells [6, 15]. The ratios of the K_i values of MTX and DDMP for the dihydrofolate reductases from L1210/DDMP/R_5 vs L1210/S cells are 3.0 and 3.5, respectively, suggesting a possible alteration in affinity of both drugs for the enzyme. Whether this small difference in K_i values is sufficient to explain the differential resistance might be tested by mathematical modeling [23].

Although it is not possible to determine the mechanism of DDMP resistance from these studies, the fact that resistance to one class of antifols can be produced without producing cross-resistance to another class of antifols is a potentially important finding. It suggests that there may be a clinical role for the use of these compounds in combination. Furthermore, clinical resistance to one antifol may not necessarily preclude the use of other classes of antifolate compounds. To use antifolates such as MTX and DDMP to their maximum advantage, the mechanisms of antifolate resistance in human tumors must be elucidated.

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REFERENCES

1. D. A. Clarke, S. M. Buckley, S. S. Steinberg, C. C. Stock, C. P. Rhoads and G. H. Hitchings, *Cancer Res.* **12**, 255 (1952).
2. J. H. Burchenal, S. K. Goetchius, C. C. Stock and G. H. Hitchings, *Cancer Res.* **12**, 251 (1952).
3. L. A. Price, J. H. Goldie and B. T. Hill, *Br. med. J.* **2**, 20 (1975).
4. K. Ganeshaguru and A. V. Hoffbrand, *Biochem. Pharmac.* **26**, 543 (1977).
5. F. M. Sirotnak, D. M. Dorick and D. M. Moccio, *Cancer Treat. Rep.* **60**, 547 (1976).
6. G. P. Browman, A. H. Calvert, G. A. Taylor, L. I. Hart and K. R. Harrap, *Eur. J. Cancer*, in press.
7. C. A. Nichol, *Adv. Enzyme Regulat.* **6**, 305 (1967).
8. D. R. Cox, *Jl. statist. Soc. Series* **B34**, 187 (1972).
9. R. Peto, M. C. Pike, P. Armitage, N. E. Breslow, D. R. Cox, S. V. Howard, N. Mantel, K. McPherson, J. Peto and P. G. Smith, *Br. J. Cancer* **35**, 1 (1977).
10. R. C. Jackson, G. A. Taylor and K. R. Harrap, *Neoplasma* **22**, 259 (1975).
11. J. P. Perkins, B. L. Hillcoat and J. R. Bertino, *J. biol. Chem.* **242**, 4771 (1967).
12. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
13. P. J. F. Henderson, *Biochem. J.* **135**, 101 (1973).
14. P. J. Henderson, *Biochem. J.* **127**, 321 (1972).
15. R. C. Jackson, L. I. Hart and K. R. Harrap, *Cancer Res.* **36**, 1991 (1976).
16. O. H. Straus and A. Goldstein, *J. gen. Physiol.* **26**, 559 (1943).
17. M. L. Sweat, L. T. Samuels and R. Lumry, *J. biol. Chem.* **185**, 75 (1950).
18. W. D. Sedwick, M. J. Fyfe, O. E. Brown, T. A. Frazer, M. Kutler and J. Laszlo, *Molec. Pharmac.* **16**, 607 (1979).
19. J. R. Bertino and R. T. Skeel, *Biochem. Pharmac.* **23**, (suppl. 2) 101 (1974).
20. D. Niethammer and R. C. Jackson, *Eur. J. Cancer* **11**, 845 (1975).
21. M. Murphy, R. R. Ellison, D. A. Karnofsky and J. H. Burchenal, *J. clin. Invest.* **33**, 1388 (1954).
22. P. Alberto, R. Peytremann, R. Medenica and M. Beretta-Piccoli, *Proc. Am. Soc. clin. Oncol.* **19**, 346 (1978).
23. R. C. Jackson and K. R. Harrap, *Archs Biochem. Biophys.* **154**, 192 (1973).

APPENDIX A

Five successive DDMP-treated tumor cell generations (DDMP/R_1, DDMP/R_3, DDMP/R_5, DDMP/R_7 and *DDMP/R_8) were tested *in vivo* for their resistance to DDMP and MTX as already described. Each tumor generation was implanted into three groups of animals.

Group 1 (control) received no therapy while group 2 and group 3 animals received MTX and DDMP, respectively. The survival time of each animal was noted. For each of the cell generations the survival curves of the treated and control groups were compared using the Log rank test procedure [9]. It was observed that MTX-treated animals consistently survived longer than control animals. The difference was highly statistically significant across all cell generations ($P < 0.001$). On the other hand, the DDMP-treated animals survived significantly longer than control animals only for cell generations DDMP/R_1 and DDMP/R_3. For all other cell generations there was no significant difference between the two treatments.

A formal test of the specific hypothesis that cells developed resistance to DDMP was next performed, using a regression model [8]. This model can establish the relative contributions of several prognostic variables which simultaneously affect survival time. The following five prognostic variables were used in the model:

$V_1 = 1$ if MTX was the treatment; = 0 otherwise.
$V_2 = 1$ if DDMP was the treatment; = 0 otherwise.
$V_3 = 1$ if the cell generation was DDMP/R_1, or DDMP/R_3; = 0 otherwise.
$V_4 = V_1 V_3$
$V_5 = V_2 V_3$ } These are 'interaction terms' in the model.

The model revealed that only V_1 and V_5 were statistically significant prognostic variables affecting survival ($P = 0.00001$ and 0.004, respectively). This implies that: (1) MTX-treated animals have an improved prognosis regardless of the cell generation, and (2) although neither the cell generation by itself nor DDMP by itself is a significant prognostic variable, the interaction between the two is significant. Thus, DDMP-treated animals have an improved prognosis in the early cell generations (DDMP/R_1 and DDMP/R_3) but not in the later cell generations.

* See Table 1.