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A Comparative Assessment of the in vitro Effects of Drugs on Cells by Means of Colony Assays or Flow Microfluorimetry

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Summary. *The effects of a range of anticancer drugs on murine neuroblastoma cells, used as a readily reproducible model system, have been compared by means of colony-forming assays and analyses by flow microfluorimetry (FMF). FMF provides the most rapid means of assessing the kinetic effects of drugs on cells. However, interpretation of these data is not clear-cut, since drug effects are highly dose-dependent and no distinction can be made easily between progression arrest and cell kill. Thus whilst FMF allows some qualitative assessment of the perturbing effects of cytotoxic drugs quantitative evaluation of cytotoxicity is still dependent on data from the more time-consuming cloning assays. However, when cells are treated with certain drugs, e.g., methotrexate, vincristine, or VM26, for only 1 h, negligible kill occurred as measured by colony formation. Therefore it appears necessary to prolong in vitro exposure time when testing these drugs or evaluating cytotoxicity of potential antitumour agents in vitro.*

Introduction

Interest has recently been renewed in drug sensitivity testing in vitro with the development of clonogenic assays for human tumours [10, 21]. These procedures are, however, very time-consuming and require a delay of about 2 weeks before results are available. In addition, when human tumours with low plating efficiencies, often $< 0.01\%$ [8, 15], are assayed a large sample volume is required. In the search for a faster and technically more satisfactory test procedure than in vitro colony formation, we examined the use of flow microfluorimetric (FMF) analyses. This procedure provides results within a few hours and allows a study of the kinetic effects of drugs on cells [5, 12, 13]. We have used a reproducible model system of murine neuroblastoma cells, which easily dissociate from monolayers into single-cell suspensions, allowing precise determinations of cell number and FMF analyses, and readily form colonies in agar.

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Materials and Methods

Chemicals. The drugs used, their solvents, and their sources are listed in Table 1.

Cell Cultures. Murine neuroblastoma cells (clone MNB/P_L) were maintained in RPMI 1640 (Gibco Bio-Cult, Renfrewshire, Scotland) plus 10% fetal calf serum (Flow Laboratories, Irvine, Scotland) at 37° C in a humidified atmosphere of 10% CO_2 in air. Cells were counted on the Coulter Counter Model ZBI and their doubling times was 20 h. Only data from mycoplasma-free cultures, as judged by testing for growth on nutrient agar and by direct microscopy, are presented.

Agar Colony-Forming Assays. From initial dose-response curves the appropriate drug concentrations were selected, the log cell kill calculated, and the number of cells plated adjusted so that approximately 100 colonies per dish were obtained. Cells in logarithmic growth were exposed to the drugs for a fixed time. The medium was removed, after which the cells were washed with serum-free medium, trypsinized (0.25 trypsin as Tryptar from Armour Pharmaceuticals, Eastbourne, England), and resuspended in fresh medium plus 1% serum at 37° C. The appropriate number of cells was plated in 5-cm dishes (Sterilin, Teddington, GB) in cloning medium consisting of 0.4% Difco Bacto-agar (Difco Labs., Detroit, USA) in medium containing 10% serum. The agar was allowed to solidify on ice prior to incubation at 37° C, and 14 days later the colonies were counted. A cell was considered to have retained reproductive capacity if it formed a colony of at least 100 cells. Under these conditions, the plating efficiency was 70%–90%. Colony formation by treated cultures was expressed as a percentage of the controls (non-treated or solvent-treated), which were given the value of 100%, and survival curves were constructed.

FMF. Effects of drugs on logarithmically growing cultures were analysed by measurements of the relative DNA content of the

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Table 1. The drugs used and their sources

Drugs	Source
Actinomycin D	Merck, Sharp & Dohme, Hoddesdon, GB
Adriamycin	Gift from Farmitalia Carlo Erba, Barnet, GB
Bleomycin	Gift from Lundbeck, Luton, Beds., GB
cis-Platinum	Boehringer Corporation (London), GB
Dibromodulcitol	Developmental Therapeutics Program (Chemotherapy), NCI, Bethesda, USA
DDMP[a]	Gift from Wellcome Research Laboratories, Beckenham, GB
Hydroxyurea	Gift from E.R. Squibb & Sons, Wirral, GB
ICRF 159 (Razoxin)	Gift from Dr. A. H. Creighton, Imperial Cancer Research Fund, London, GB
Melphalan	Wellcome Foundation, London, GB
Methotrexate	Gift from Lederle Laboratories, Gosport, GB
Mithramycin	Gift from Pfizer, Kent, GB
Peptichemio	Gift from Instituto Sieroterapico Milanese, S. Belfanti Milan, Italy
Vincristine and vindesine	Gifts from Eli Lilly, Basingstoke, GB
VM26 and VP-16-213	Sandoz AG, Basel, Switzerland

All the drugs were dissolved in RPMI 1640 medium except for dibromodulcitol, which was solubilized in dimethyl sulfoxide, and melphalan and DDMP, which were solubilized in 4.5% (w/v) ethanolic HCl, and then diluted at least 100-fold in medium before use
[a] DDMP, 2,4-diamino-5-(3′,4′-dichlorophenyl)-6-methyl pyrimidine

individual cells. Cell monolayers were exposed to the drug for 24 h, washed three times with phosphate-buffered saline, trypsinized (0.25% trypsin), then ethanol-fixed and stained with mithramycin (20 mg per ml in 20% ethanol and 300 m*M* $MgCl_2$) [11, 18]. Measurements were made in the fluorescence-activated cell sorter (FACS-1, Becton Dickenson, California, USA) according to the modifications described earlier [16]. Routinely 30,000 cells were measured for fluorescence from a sample of 2×10^6 cells per ml. Graphic plots of the fluorescence histograms were interpreted as recommended by Barfod [3].

Results

Colony-forming Assay Data

Previous reports referring to this technique generally have presented data collected after drug exposure times of either 1 or 24 h. We have compared the results obtained for both periods in this model system.

In *24-h drug exposures* logarithmically growing cells were treated with a range of concentrations of various antitumour drugs and their colony-forming ability was assessed. The survival curves are shown in

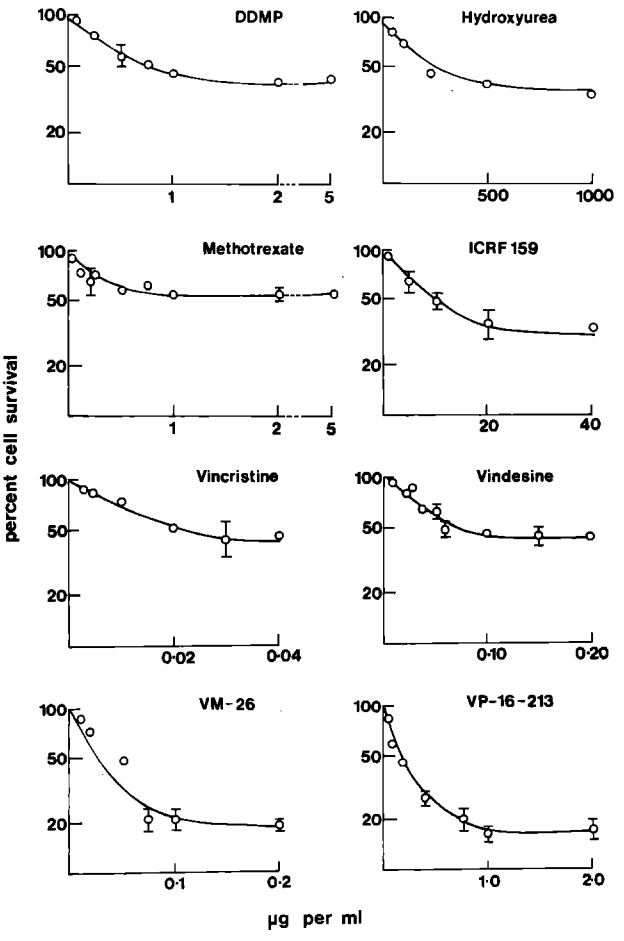

Fig. 1. The effects of a 24-h exposure to increasing drug concentrations on the colony-forming ability of murine neuroblastoma cultures. Each *point* represents the mean of four estimations with experiments repeated at least twice. Only SE in excess of 5% are included as *error bars*

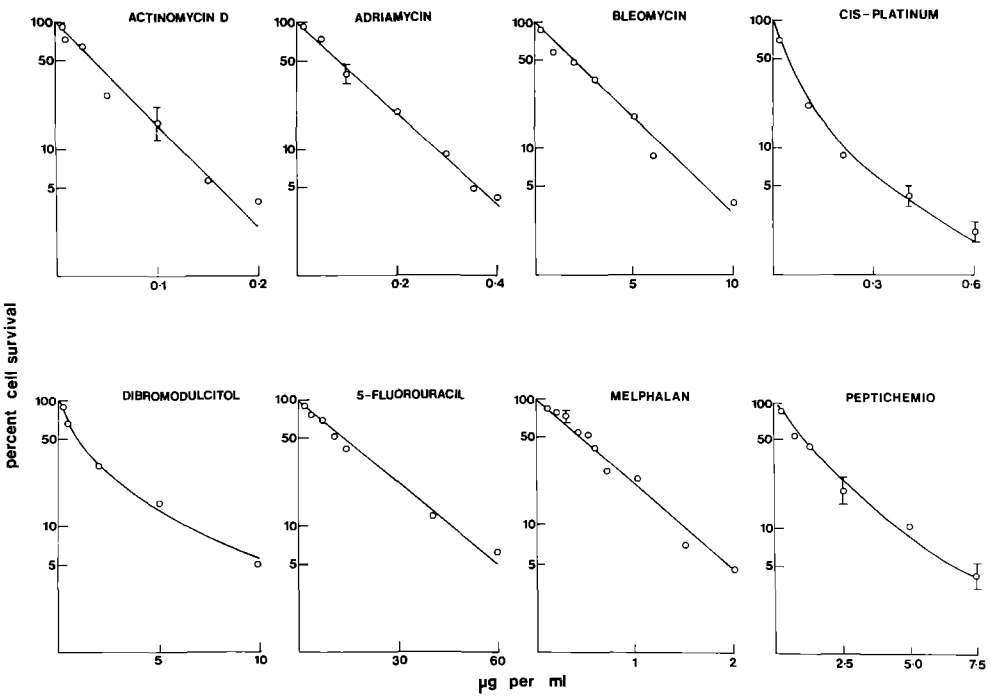

Fig. 2. The effects of a 24-h exposure to increasing drug concentrations on the colony-forming ability of murine neuroblastoma cultures. Each *point* represents the mean of four estimations with experiments repeated at least twice. Only SE in excess of 5% are included as *error bars*

Figs. 1 and 2 and are of two distinct types, namely exponential-plateau and exponential. Thus the drugs tested can be divided into two groups based on the shape of the survival curve obtained. Figure 1 shows the results with drugs where the survival curves plateau before a log cell kill is achieved, irrespective of increasing drug concentration. Depending on the drug used, this plateau region may occur at as high a level as 50%, e.g., with methotrexate, or at as low a level of 20%, e.g., with VM-26. Figure 2 illustrates data obtained where increasing drug concentrations result in increasing cell kill. In these cases drug concentrations could be selected which caused a cell kill in excess of 1 log.

In *1-h drug exposures,* logarithmically growing cultures were exposed to a few of the drugs studied above, but increased concentrations were used. The results obtained are summarised in Fig. 3, illustrating, as expected, that cell kill was consistently less after this shorter exposure. Again two types of survival curves were obtained, and the drugs selected fell into the same categories as those noted after 24 h of treatment. However, cell kill obtained with the drugs which resulted in plateauing of the survival curves namely methotrexate, vincristine, and VM-26, caused negligible kill when the exposure was limited to this 1-h period, irrespective of increasing drug concentrations.

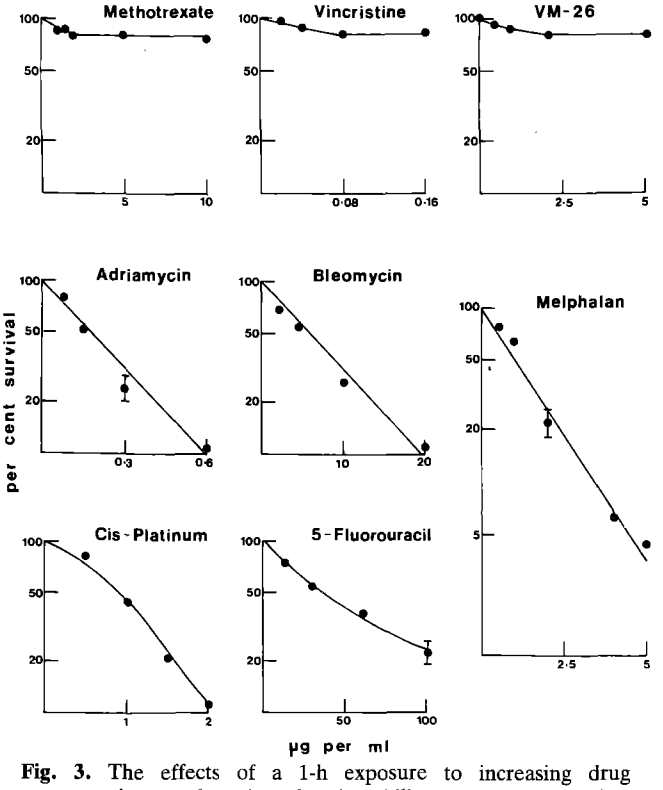

Fig. 3. The effects of a 1-h exposure to increasing drug concentrations on the colony-forming ability of murine neuroblastoma cells. Each *point* represents the mean of four estimations with experiments repeated at least twice. Only SE in excess of 5% are included as *error bars*

FMF Data

Results are presented only for 24-h drug exposures, since perturbations in the control pattern could not readily be achieved with 1-h drug exposures unless drug concentrations greatly in excess of those achievable in the plasma clinically [1] were employed. Two particular drug concentrations were selected from the data in Figs. 1 and 2, the first termed 'low dose', which allowed at least 80% cell survival and might provide information on the effects of drugs on cycle progression, and a second, higher concentration resulting in approximately 40% survival, which might establish the DNA pattern resulting from lethal concentrations of drugs without producing too much cell debris which could interfere with analyses.

The results are shown in Fig. 4. For the lower concentrations (left-hand panels) arrest/accumulation is indicated and noticeable (i) at the G_1/S boundary, by a broadening of the initial peak, for DDMP, 5-fluorouracil, methotrexate and, most dramatically, hydroxyurea; (ii) at the G_2/M phases with melphalan, peptichemio, adriamycin, dibromodulcitol, bleomycin, ICRF-159, vincristine and vindesine, and only slightly with *cis*-platinum; and (iii) additionally in the S phase with dibromodulcitol, peptichemio, and melphalan.

In attempts to analyse the lethal effects of drugs on cells, the results obtained at the higher drug concentrations (the right-hand panels in Fig. 4) need to be considered. Treatment with DDMP, 5-fluorouracil, methotrexate, or hydroxyurea results in a loss of the G_2/M populations. For actinomycin D the overall histogram shows little change from the control and hence could not be used to predict cell kill. Melphalan, peptichemio, and *cis*-platinum all result in a pronounced accumulation of cells in the G_2 and/or M phases where a block and/or cell kill has probably occurred. A substantial G_1 population, however, still remains. This contrasts with vincris-

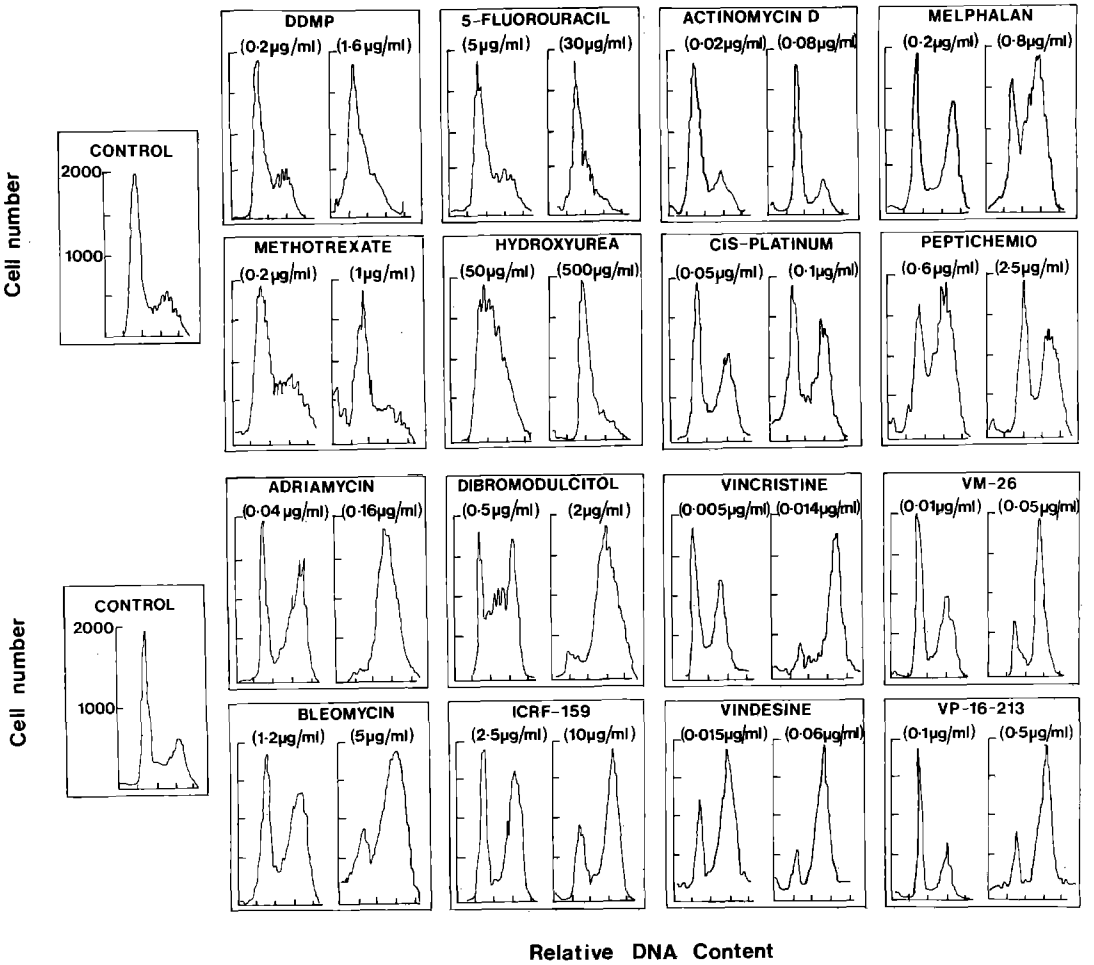

Fig. 4. The effects, monitored by FMF, on murine neuroblastoma cells of a 24-h exposure to 'low' or 'high' drug concentrations selected from the survival curves in Figs. 1 and 2, as described in the text, with the lower concentration (left-hand side) selected to illustrated effects on cell cycle progression, whilst the higher concentrations (see right-hand side) illustrate lethal effects

tine, vindesine, VM-26, VP-16-213, and ICRF-159, where arrest is almost totally in the G_2/M phases and there is a loss of both G_1 and S phase cells. The effects of adriamycin, bleomycin, and dibromodulcitol are characterised by a broad accumulation of most cells shifted away from G_1 into S and the G_2/M phases.

Discussion

In our search for a test procedure which would allow accurate evaluation of in vitro drug sensitivities, we compared data obtained from colony-forming assays with those from the simpler and more rapid FMF analyses. We assessed these methods in terms of (i) ease of performance, (ii) rapidity with which results can be obtained, (iii) ease of interpretation of results, (iv) reproducibility of data, and (v) ability to accurately predict the loss of a cell's reproductive capacity.

FMF analyses provide the most rapid means of assessing the kinetic effects of drugs on cells. The overall results obtained for the 16 drugs are similar to those given in previous reports on a few of these drugs in other cell systems. For example, G_2 accumulation was reported in human lymphoma cells with bleomycin, adriamycin, VP-16-213, and melphalan, with additional S and G_1 phase perturbations with the latter three drugs following prolonged drug exposure [4]. A comparison of VM-26 and VP-16-213 showed similar kinetic responses to both drugs [19]. Methotrexate, 5-fluorouracil, and hydroxyurea caused G_1/S arrest in L1210 cells [6] and a similar pattern of response was noted with DDMP in L5178Y cells [16]. The ability of vincristine to cause mitotic accumulation is well documented [9], and the newer derivative vindesine has been shown to have very similar lethal and kinetic effects on cells [17]. However, the interpretation of FMF data is not clear-cut. Drug effects are highly dose-dependent and no clear distinction can be made easily between progression arrest, which may or may not be reversible, and cell kill. It has been pointed out that there is no correlation between the degree of G_2 block and the magnitude of cell kill with VP-16-213, and an independent mechanism of killing and blocking has been suggested for bleomycin, since recovery from arrest did not predict for unimpaired reproductive capacity [13]. Hence the unreliability of using the cytokinetic changes to predict lethality needs to be stressed; but it is perhaps not surprising, since this technique allows no distinction to be made between cells which retain their proliferative capacity, those which remain capable of dividing a limited number of times, and dead or dying cells. Analyses can indeed be complicated by the presence of a variable population of dead or dying cells, especially with respect to debris. Therefore often only drug concentrations which reduce the survival of the population not greatly in excess of 50% can be tested accurately.

In contrast, colony-forming assays provide reliable and reproducible estimations of cells reproductive ability following drug treatment. The results obtained here with this model system were of interest since the patterns of cell survival after 24-h exposure to the various drugs tested confirm and extend the division of drugs as class II and class III agents according to the Kinetic Classification of Antitumour Drugs [7]. This simple in vitro model system may therefore be more convenient than the original spleen colony assay procedure for ascribing new agents to their particular class. These clonogenic assays, are, however, time-consuming to carry out and also fail to yield rapid results, since approximately 14 days are required for colony growth before assessment can be made.

The other question considered in this study was the choice of incubation time for drug exposure. Many groups conducting studies have used a 1-h incubation, considering it representative of exposure times to drugs in vivo when they are delivered as bolus treatment providing effective plasma levels for only 30 min to a few hours [1, 20]. This short exposure time also obviates problems of possible chemical instability or light sensitivity. However, it is the tumour drug levels that are important, and whilst unfortunately pharmacological data available in humans is sparse it is not necessarily valid to equate peak plasma and tumour drug levels. In addition, it is now common clinical practice to administer some drugs, e.g., methotrexate, 5-fluorouracil, and bleomycin, as infusions in an attempt to prolong drug exposure times. Our data suggest that for certain drugs, e.g., methotrexate, vincristine, and VM-26, a 1-h exposure time may be unsuitable for evaluating sensitivity, since negligible cell kill occurs within this period. This observation was also made for cytosine arabinoside, 5-fluorouracil, methotrexate, and hydroxyurea, in human lymphoma cells [14]. This point may be particularly relevant to the evaluation of potential anticancer drugs by in vitro testing, as recently suggested [2, 21, 22].

In conclusion, whilst FMF may allow some qualitative assessment of perturbing effects of drugs on progression through the cycle, quantitative assessment of cytotoxicity is still dependent on data from colony-forming assays. The duration of drug exposure for in vitro sensitivity testing should in some

cases be extended beyond 1 h, particularly if new compounds are being evaluated.

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