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Assessment of Antimetabolite Cytotoxicity: a Comparison of Clonogenic Assays and Tritiated Deoxyuridine Incorporation

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Abstract—*A comparison has been made between the cloning capacity and changes in tritiated deoxyuridine (6-[^{3}H]-UdR) incorporation of L1210 (murine leukaemia) and PMC-22 (human melanoma) cells treated with methotrexate (MTX), 5-fluorouracil (5-FU) and cytosine arabinoside (ARA-C). The labelling–cloning relationship was poor, with brief drug exposure times, but improved progressively after drug treatment of 1 cell-cycle time's duration. Labelling changes resulting from short-term exposure to drug (several hours) provided poor predictions of the cytotoxicity resulting from longer drug exposure.*

INTRODUCTION

THE MEASUREMENT of radiolabelled nucleoside incorporation into nucleic acids has been widely used in experimental animal and human tumours as an indirect measure of the rate of nucleic acid synthesis and to study the effects of drug treatment [1–4]. In animals the correlation between *in vitro* labelling following *in vivo* drug treatment and *in vivo* tumour response has generally been good [1, 2, 5]. However, studies of human tumours comparing *in vitro* label incorporation following *in vitro* drug treatment with clinical tumour response have reported both good [4, 6, 7] and poor correlations [3, 8, 9]. A common feature of studies reporting poor correlations was the use of antimetabolite drugs.

A possible explanation for the poor *in vitro* prediction of *in vivo* antimetabolite drug effect may be deduced from studies using cultured cell lines and tumour-bearing animals. Hryniuk [10] compared cloning capacity and [^{3}H]-UdR incorporation in L5178Y cells treated with MTX. He reported a linear correlation when the cells had been exposed to MTX for 7 hr, but with shorter drug exposure the correlation was poorer. Tattersall and Harrap [11] reported a good cloning—[^{3}H]-UdR incorporation in L5178Y cells treated with MTX and 5-FU only after drug exposure for 18–20 hr. Both these studies suggest that the time of cell exposure to MTX is important if changes in label incorporation are to reliably reflect cytotoxicity. In tumour-bearing animals Chabner and Young [2] reported that *in vivo* [^{3}H]-UdR incorporation into L1210 cells following MTX treatment did not parallel the ascites cell count until 20 hr after MTX administration. This treatment time factor has been overlooked in most label incorporation (*in vitro*)/tumour response (*in vivo*) comparisons using antimetabolite drugs, and this possibly accounts for the poor correlations reported.

We have systematically investigated the use of [6-^{3}H]-UdR incorporation as an index of antimetabolite drug cytotoxicity (assessed by clonogenic capacity). In a preliminary study the incorporation of other tritiated nucleosides (tritiated thymidine, deoxycytidine and uridine) was compared to clonogenic capacity in MTX-, 5-FU- and ARA-C-treated cells. The cell survival–labelling correlations with those nucleosides were inferior to those obtained with [6-^{3}H]-UdR, and these data have not been included in this report.

MATERIALS AND METHODS

The 3 cell lines used were L1210, L1210 (MTX-R)(dihydrofolate reductase amplification mutant-

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MTX resistant, courtesy of Dr. J. Bertino, Yale University School of Medicine, New Haven, CT, U.S.A.) and PMC-22 (human melanoma, courtesy of Dr. R. Whitehead, The Cancer Institute, Melbourne, Australia). The cells were grown as suspension cultures in RMPI-1640 medium containing 10% undialysed foetal calf serum (Flow Laboratories, New Haven, CT, U.S.A.). The L1210 (MTX-R) line was maintained in MTX 10^{-4}M. Twenty-four hours prior to drug addition the cells were suspended in fresh medium at concentrations of 5×10^4/ml for L1210 and 1×10^5/ml for L1210 (MTX-R) and PMC-22. Final drug concentrations in studies with L1210 and PMC-22 cells were: MTX (Lederle, Sydney, Australia): 5, 20 and 100 nM; 5-FU (Sigma Chemical Co., St. Louis, MO, U.S.A.): 1, 10 and 100 μM; ARA-C (Calbiochem., San Diego, CA, U.S.A.): 10, 100 and 1000 nM. In L1210 (MTX-R) MTX 100 and 1000 μM were studied.

All metabolic and cloning assays were performed at times related to the cell cycle of unperturbed logarithmically growing cells, viz. 0.25, 1 and 2 cell cycles after drug addition. Cell-cycle times were 12 hr for L1210 and 24 hr for L1210 (MTX-R) and PMC-22.

The soft agar cloning technique used has been previously described by Bradley and Metcalf [12]. Human erythrocytes were used as the feeder cells. Control and drug-treated cells were plated at a concentration of 100 cells per plate, while drug-treated cells were also cultured at 1000 cells per plate. Incubation times for L1210, L1210 (MTX-R) and PMC-22 cells were 10, 14 and 21 days respectively. Cell aggregates were scored as colonies if the diameter was greater than 0.1 mm. Because drug-treated cultures contained significantly fewer cells at the 1 and 2 cell-cycle points than the control cultures, it was necessary to include in the expression of cell survival a factor indicating the degree of cell loss in the flask prior to cloning. Such an expression is embodied in the colony-forming units (CFU)/ml equation:

$$\frac{\text{CFU/ml}}{(\%\text{ control})} = \frac{+\text{ colonies formed/100 treated cells}}{\text{colonies formed/100 control cells}} \times \frac{\text{cell conc. in treated suspension culture at the time of cloning}}{\text{cell conc. in control suspension culture at the time of cloning}} \times 100.$$

The mean cloning efficiencies of untreated L1210, L1210 (MTX-R) and PMC-22 cells were 100, 50 and 60% respectively.

Studies were conducted with [6-^{3}H]-UdR (Radiochemical Centre, Amersham, U.K.). Specific activity varied between 15 and 30 Ci/nmol. At the appropriate assay times duplicate samples of 2.5×10^6 cells were incubated with 2.5 μl of [6-^{3}H]-UdR (2.5 μCi, $2\text{–}8 \times 10^{-8}$M, depending on the label's specific activity) at 37°C for 20 min. The reaction was stopped by placing the tubes on ice for 10 min, then the cells were washed with Hank's balanced salt solution. The label was extracted in DNA and RNA fractions by treatment with 0.2 M perchloric acid (15 min at 4°C), 0.3 M sodium hydroxide (60 min at 37°C) and 0.5 M perchloric acid (30 min at 70°C).

Label incorporation was expressed as specific activity (dpm/μg DNA or RNA). DNA was assayed by a modification of the Burton diphenylamine reaction [13] and RNA was measured by 260 nm u.v. absorbance.

RESULTS

The survival curves for the 3 drug-treated cell lines are shown in Figs 1–3. Figs 1(A), 1(B) and 1(C) show the results of MTX-treated L1210, L1210 (MTX-R) and PMC-22 cells respectively, and a comparison of the L1210 and L1210 (MTX-R) curves shows the latter cell line to be approximately 10,000 times less sensitive to MTX. Because of the limited number of MTX concentrations it is impossible to reliably characterise the shape of the dose–response curve, but the L1210 and PMC-22 results suggest an exponential–plateau pattern. Cell survival was

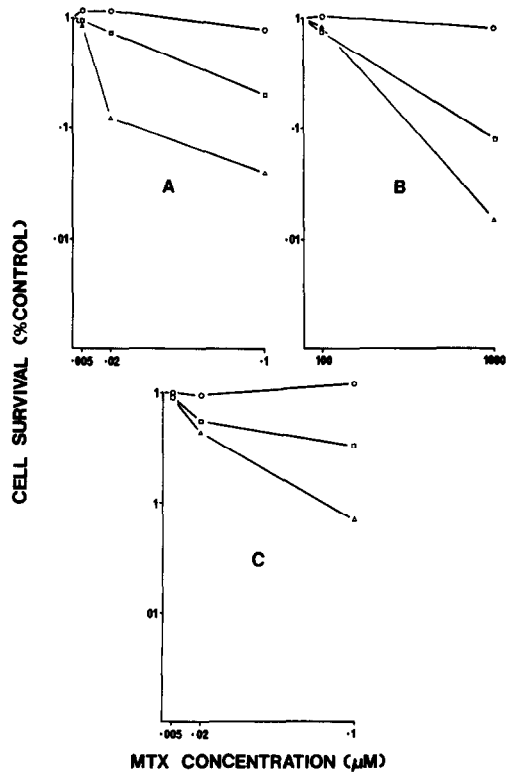

Fig. 1. Cell survival (% control) following MTX treatment. A, L1210; B, L1210 (MTX-R); C, PMC-22. ○——○, *Drug treatment for 0.25 cell-cycle times;* □——□, *1 cycle time;* △——△, *2 cycle times. Results are the means of 2 separate experiments.*

virtually unaffected after MTX exposure for 0.25 cell-cycle times. The survival results for 5-FU-treated L1210 and PMC-22 cells are presented in Figs 2(A) and 2(B) respectively. Both cell lines showed at least 3 logs cell kill at the 10^{-4}M/2 cell-cycle point, and the shape of the PMC-22 survival after treatment for 1 cell-cycle time suggests exponential cell kill in this cell line. Again, the 0.25 cell-cycle time survival was a poor reflection of longer exposure to 5-FU. Figs 3(A) and 3(B) show the survival of ARA-C-treated L1210 and PMC-22 cells respectively. The PMC-22 cells were 10-fold more sensitive to ARA-C than L1210 cells. As with MTX and 5-FU, ARA-C cytotoxicity was time- and dose-dependent, the dose-dependency being of an exponential–plateau type.

The results of [6-^{3}H]-UdR incorporation, measured as DNA specific activity, are contained in Table 1. Results are the means of duplicate samples from 2 separate experiments. In contrast to the cloning experiments, where results were time-related, the inhibition of [6-^{3}H]-UdR incorporation occurred rapidly and usually remained stable from the 0.25 cell-cycle time point onwards. The inhibition of [6-^{3}H]-UdR incorporation was related to drug concentration.

The comparison of label concentration and cloning results was performed by linear regression analysis. Exponential logarithmic and power curve-fitting analyses were also performed, but significance was only seen sporadically. The label incorporation–cloning correlations were

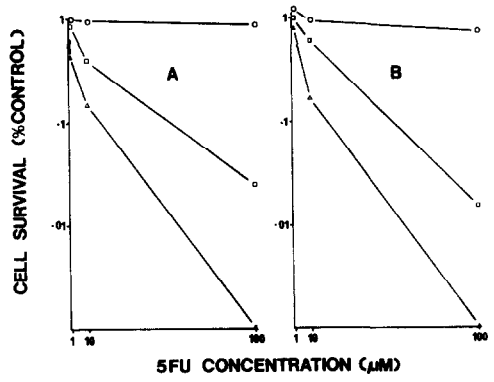

Fig. 2. Cell survival (% control) following 5-FU treatment. A, L1210; B, PMC-22. O——O, *Drug treatment for 0.25 cell-cycle times;* □——□, *1 cycle time;* △——△, *2 cycle times. Results are the means of 2 separate experiments.*

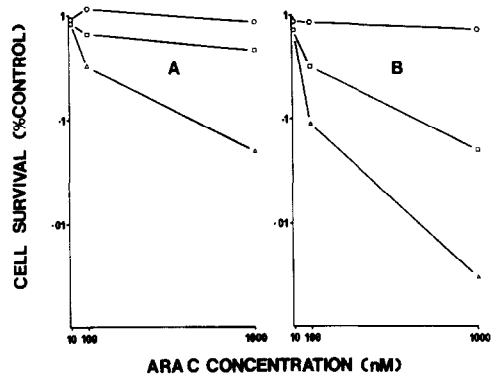

Fig. 3. Cell survival (% control) following ARA-C treatment. A, L1210; B, PMC-22. O——O, *drug treatment for 0.25 cell-cycle times;* □——□, *1 cycle time;* △——△, *2 cycle times. Results are the means of 2 separate experiments.*

Table 1. Drug-induced changes in [6-^{3}H]-UdR *incorporation*

Treatment time (cell cycles)	MTX concentration			5-FU concentration			ARA-C concentration		
				L1210					
	5nM	20nM	100nM	1μM	10μM	100μM	10nM	100nM	1000nM
0.25	101(2)	78(8)	15(1)	89(1)	16(2)	3(1)	68(0.5)	12(1)	2(0.5)
1	102(2)	43(9)	12(1)	77(1)	12(0.5)	1(0.5)	71(2)	22(2)	2(0.5)
2	90(1)	9(1)	1(0.5)	50(1)	8(0.5)	1(0.5)	77(2)	28(2)	0.5(0.25)
				L1210 (MTX-R)					
	100μM	1000μM							
0.25	73(5)	13(2)							
1	98(2)	6(2)							
2	66(11)	7(1)							
				PMC-22					
	5nM	20nM	100nM	1μM	10μM	100μM	10nM	100nM	1000nM
0.25	92(4)	74(6)	11(2)	101(3)	72(2)	17(1)	48(2)	14(2)	4(0.25)
1	99(2)	32(8)	1(0.5)	100(1)	82(13)	17(2)	72(2)	19(2)	10(2)
2	82(5)	15(3)	2(0.5)	87(5)	54(0.5)	24(2)	54(0.5)	10(1)	10(1)

Cells growing in the presence of drug were 'flash' labelled (20 min) at 0.25, 1 and 2 cell-cycle times after addition of drug. Incorporation was measured as DNA specific activity. Results are means of duplicate samples from 2 experiments. Results are expressed as percent of control. Figures in parentheses represent standard errors of the means.

stratified by time of drug exposure and drug used. In addition to the 0.25, 1 and 2 cell-cycle times analysed, a composite analysis of all time–dose points was performed in some cases because of a limitation inherent in the interpretation of regression analyses. Regression analysis measures variance in terms of the range of the data points' scatter. When this range is small (e.g. at the 0.25 cell-cycle time point) the variance of the data points appears relatively large. When there was a progressive improvement in the 0.25, 1 and 2 cell-cycle correlation coefficients a composite regression analysis was performed to determine whether the 'range-artefact' was a significant factor. If so, the composite correlation coefficient would be similar to the 2 cell-cycle coefficient.

The [6-^{3}H]-UdR incorporation–cloning capacity correlation coefficients are presented in histogram form in Fig. 4 and the statistical significance of the coefficient is shown in the histogram. Each correlation coefficient represents L1210 and PMC-22 data and, for MTX coefficients, L1210 (MTX-R) data. All correlation coefficients were positive.

The [6-^{3}H]-UdR-cloning correlations were significant with all 3 drugs at 1 and 2 cell-cycle times. The 2 cell-cycle time correlations were consistently the best and the lower composite coefficients suggested that the variance at the shorter drug exposure times was real. This was borne out by the [6-^{3}H]-UdR incorporation–survival scatter plots shown in Fig. 5. Figure 5(A) contains all time–dose points from MTX-treated cells. The diagonal line represents a one-to-one linear relationship and does not necessarily represent the line of best fit. This scatter plot confirms the large contribution to variance made by the 0.25 cell-cycle data points, and if these are disregarded the 1 and 2 cell-cycle data points approximate a one-to-one relationship. The 5-FU data (Fig. 5B) were similar but there was significant [6-^{3}H]-UdR incorporation, even when cloning capacity was severely reduced. In Fig. 5(C) it can be seen that the variance of the composite ARA-C data was largely attributable to the 0.25 cell-cycle points and, to a lesser degree, the 1 cell-cycle points. The 2 cell-cycle points approximate the drawn line.

The label incorporation–cloning correlations showed a consistent improvement with increasing drug treatment time. The increased variance of the 0.25 cell-cycle time was usually due to label incorporation changing faster than cloning capacity. This prompts the question: what is the relationship between labelling changes following short drug exposure and cloning changes following longer drug exposure? Table 2 contains correlation coefficients derived from a comparison of 0.25 and 2 cell-cycle [6-^{3}H]-UdR incorporations and 2 cell-cycle cloning results. None of the 0.25/2 cell-cycle correlations were significant, implying that short-term [6-^{3}H]-UdR perturbations do not predict cell kill resulting from more prolonged drug exposure.

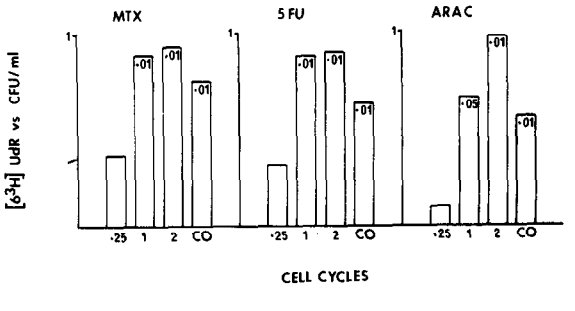

Fig. 4. Correlations between [6-^{3}H]-UdR incorporation and cell survival. The correlation coefficient (r) is represented on the ordinate and the significance of the coefficient is shown in the histogram. 0.25, 1 and 2: drug exposure times (cell cycles). CO: composite (all time–dose) correlation coefficient.

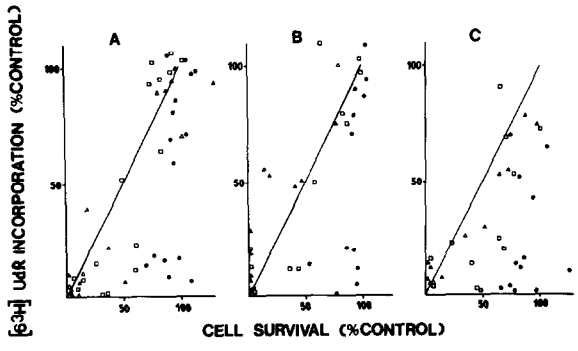

Fig. 5. Scatter plots of [6-^{3}H]-UdR incorporation vs cell survival (both as % control). A, MTX treatment; B, 5-FU; C, ARA-C. ●——●, drug treatment for 0.25 cell-cycle times; □——□, 1 cycle time; △——△, 2 cycle times.

Table 2. Short-term labelling changes vs long-term cloning changes

	0.25 cell-cycle [6-^{3}H]-UdR vs 2 cell-cycle cloning r*	2 cell-cycle [6-^{3}H]-UdR vs 2 cell-cycle cloning r
MTX	0.33	0.92
5 FU	0.42	0.89
ARA-C	0.17	0.97

Linear regression analysis was performed on 0.25 and 2 cell-cycle time [6-^{3}H]-UdR incorporation compared to 2 cell-cycle time cloning capacity. The correlation coefficients incorporate L1210 and PMC-22 results and for MTX coefficients only, L1210 (MTX-R) results. Results from all drug concentrations are included.
*Correlation coefficient.

DISCUSSION

These results confirm that [6-^{3}H]-UdR incorporation studies can usefully predict antimetabolite drug cytotoxicity. The correlation between [6-^{3}H]-UdR incorporation and cell survival after MTX and 5-FU treatment was anticipated because the cytotoxicity of these drugs is closely linked to the inhibition of dUMP methylation to TMP. In addition, the expanded dUMP pool resulting from this inhibition reduces the specific activity of labelled dUMP. However, the finding that [6-^{3}H]-UdR incorporation with 5-FU treatment persisted at appreciable levels in the face of a severe loss of cloning capacity (Fig. 5B) suggested that the inhibition of thymidylate synthetase was not the sole mechanism of 5-FU toxicity in these cell lines. The inhibition of [6-^{3}H]-UdR incorporation caused by ARA-C can be explained in terms of DNA polymerase inhibition or expansion of the dTTP pool or both. dTTP levels in cells treated with micromolar ARA-C for 2 cell cycles were 349 and 546% of control for L1210 and PMC-22 cells respectively. However, the 0.25 cell-cycle time dTTP levels were not elevated and yet [6-^{3}H]-UdR incorporation was suppressed to 2–5% of control. Under these circumstances the inhibition of DNA polymerase seems the more likely explanation.

The observation by Hryniuk [10] and Tattersall and Harrap [11] that the time of exposure to antimetabolite drug prior to [^{3}H]-UdR incorporation was relevant to the assessment of cytotoxicity has been confirmed and extended in this study. The analysis of [6-^{3}H]-UdR incorporation–cloning correlations showed the variance of the 0.25 cell-cycle time results to be markedly greater than the 1 and 2 cell-cycle time results, although most labelling changes reached their nadir by the end of 1 cell cycle and the 2 cell-cycle time correlations were generally better. This phenomenon resulted from increased cell death between 1 and 2 cell cycles and suggests that some cells may cycle a second time or that cytotoxicity correlates with the duration of cell-cycle inhibition. The variance of 0.25 cell-cycle time results was usually due to the rapid changes in label incorporation compared to slower impairment of cell survival. A similar disparity between cell survival and [^{3}H]-UdR incorporation rate was seen by Hryniuk [10] in L5178Y cells treated with MTX concentrations up to 10 μM. However, the correlation between L5178Y survival and [^{3}H]-UdR incorporation improved when [^{3}H]-UdR incorporation was 'corrected' for simultaneous changes in [^{3}H]-TdR incorporation (calculated [^{3}H]-UdR rate). When this correction factor was applied to the [6-^{3}H]-UdR results in this study, the cell survival–labelling correlation did not improve; rather, it deteriorated.

Many previous *in vitro/in vivo* chemosensitivity studies with antimetabolite drugs have used short-term (1/2–1 hr) drug exposures [3, 8, 9]. In most instances this arbitrary exposure time bore little relation to the time of clinical drug exposure and this may explain why the *in vitro/in vivo* correlations were often poor. The results from this study show that antimetabolite drug cytotoxicity is drug exposure-time-dependent and the labelling changes reflecting this are similarly time-dependent. The results comparing 0.25 cell-cycle labelling changes with 2 cell-cycle cloning capacities indicate that short-term labelling changes were poorly predictive *in vitro* cytotoxicity assays (clonogenic or metabolic), and drugs which have a phase-specific action should use a drug exposure time of at least 1 and preferably 2 cell cycles of the tumour cell line in question. This argument may not apply universally to all antimetabolite drugs, particularly if the cytoxicity is mediated by inhibition of RNA synthesis.

The application of this principle to *in vitro* chemosensitivity testing of fresh human tumours may pose problems because some tumours have prolonged cycle times. Cell-cycle times in a wide range of solid and haematological human tumours have been reported to range from 14 hr (bronchial cancer) to 260 hr (bronchial cancer) [14–16], but the modal cell-cycle time appears to be 40–50 hr. Although human tumours can be grown in tissue culture for 72 hr with apparent preservation of DNA and RNA synthesis [17], it is almost inevitable that some perturbation of nucleic acid metabolism would result from the conditions of culture. The severity and time-dependence of this derangement would need to be investigated before the principles derived from this study were applied to studies with fresh human tumours.

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