ASSAYS OF DRUG SENSITIVITY FOR CELLS FROM HUMAN TUMOURS: IN VITRO AND IN VIVO TESTS ON A XENOGRAFTED TUMOUR

A. E. BATEMAN, M. J. PECKHAM AND G. G. STEEL

From the Radiotherapy Research Department, Institute of Cancer Research,
Belmont, Sutton, Surrey

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Summary.—A human tumour which grows as a xenograft in immune-suppressed mice and forms colonies in vitro has been used to test the correlation between 2 methods of exposure of human tumour cells to chemotherapeutic agents. In vivo exposure to drugs was achieved by injection of tumour-bearing mice with each of 8 cytotoxic agents. For the in vitro exposure, cell suspensions were incubated for 1 h with the same series of drugs. The survival of tumour clonogenic cells was assayed in vitro after either treatment or dose-response curves were obtained. The 8 drugs were ranked according to their in vivo effect at doses equitoxic to mice, and according to their in vitro effect at concentrations designed to approximate to levels of drugs in human plasma. The ranks for in vivo and in vitro exposure correlated well.

It is common clinical experience that tumours of similar histology and stage show wide differences in response to cytotoxic drugs. This may be reflected clinically as differences in tumour-volume regression or, less commonly, as a difference between curative and non-curative therapy. Whilst factors such as drug distribution and metabolism, metastatic site and tumour volume may in part explain this variability, evidence from the experimental therapy of human tumour xenografts (Houghton et al., 1977; Nowak et al., 1978) supports the view that clinical differences in response reflect differences in intrinsic cellular chemosensitivity.

Since current methods of clinical drug evaluation are protracted and relatively imprecise, a predictive test of chemosensitivity would be advantageous for selecting active drugs from a range of currently used agents, and for testing new compounds. Previous attempts at in vitro tests for chemosensitivity of tumours have not met with great success (Mitchell et al., 1972; Berry et al., 1975) but methods which measure depression of colony-forming ability of the tumour cells appear more promising (Salmon et al., 1978). It is essential that the results of in vitro sensitivity tests satisfactorily reflect the tumour-cell kill that can be achieved in vivo. The present study is an attempt to validate an in vitro chemosensitivity test using a xenografted human tumour for which in vivo responses to drugs can be measured accurately in mice.

MATERIALS AND METHODS

The tumour used in this study was a poorly differentiated carcinoma of human pancreas (HX32, Courtenay & Mills, 1978) transplanted and passaged in the leg muscle of CBA/lac mice immune-suppressed by the method of Steel et al. (1978). In brief, mice were thymectomized at 4 weeks of age, and injected 2 weeks later with 200 mg/kg arabinosyl cytosine (Ara-C) i.p. 2 days before 900 rad whole-body 60Co irradiation.

In vivo chemotherapy.—Tumours were treated by i.p. injection of the host mouse with graded doses of chemotherapeutic drugs. Melphalan, adriamycin, cis-platinum (II) diammine dichloride (cis-Pt(II)) and
methotrexate were supplied by the Division of Cancer Treatment of the U.S. National Institutes of Health. Chlorambucil and hexamethylmelamine (HMM) were gifts from Professor Ross of the Institute of Cancer Research, and vinblastine sulphate (Velbe, Lilly and Co.) and thio-TEPA (Lederle Ltd) were also used. All drugs except chlorambucil and HMM were injected in saline. Melphalan was initially dissolved in 0·1M HCl and methotrexate in 2% NaHCO₃. Chlorambucil was dissolved in 2% HCl:98% ethanol and diluted with 4·5 volumes propane-1,2-diol and 4·5 volumes saline, and powdered HMM was homogenized in dimethyl-sulphoxide before addition of 9 volumes 5% Tween 80 in saline and re-homogenization.

Mice were treated when the diameter of the tumour-bearing leg was ~ 8 mm. They were killed 20 h after drug injection and the tumour was chopped, incubated in 2 mg/ml collagenase (Type 1, Sigma) in Ham’s medium for 30 min at 37°C followed by incubation in 0·05% Bactotryptsin in calcium- and magnesium-free saline for 5 min. The resulting cell suspension was poured through a sterile polyester mesh of pore size 25 μm and mixed with calf serum (10% of total volume). The refractile tumour cells were counted on a haemacytometer. Appropriate cell dilutions were made and cells were plated in 0·3% agar medium containing rat erythrocytes and 20% Special Bobby Calf Serum (SBCS, Gibco-Biocult) in Ham’s F12 medium as described by Courtenay and Mills (1978). One-ml agar cultures, containing either 300 control cells or up to 3 × 10⁴ treated cells were gassed with a 5% O₂, 5% CO₂, 90% N₂ mixture and fed after 1 and 2 weeks with 1·5 ml fresh medium. Cell colonies were scored after 3 weeks. The plating efficiency (PE) of the untreated tumour cells was ~ 30%. The ratio of PE of treated cells to PE of control cells was used to calculate the fraction of clonogenic cells surviving treatment. Control PE was determined in each experiment by plating cells from each of at least 2 untreated mice; 2 or 3 mice were given each test treatment, and the surviving fraction of tumour clonogenic cells was determined for each individual mouse.

In vitro chemotherapy.—Cell suspensions were prepared as above and aliquots of 10⁶ cells in 1 ml Ham’s medium plus 20% SBCS with various drug concentrations were set up without delay. These cultures were gassed with 5% O₂, 5% CO₂ and 90% N₂ before incubation at 37°C for 1 h followed by 2 washes in phosphate-buffered saline at 5°C and centrifugation at 600 g. Cells were resuspended in 1 ml Ham’s medium plus SBCS, aliquots were counted on a haemacytometer, and cells diluted and plated as above. In all assays heavily irradiated cells (given 10,000 rad) were added to give a total cell concentration of 10⁴/ml, to act as “feeder cells”.

All liquids that had come into contact with the human tumour material were autoclaved before disposal; all plastics and glassware were either incinerated or immersed in hypochlorite solution before re-use.

Assessment of the in vitro cytotoxic activity of the 8 agents was made at drug concentrations selected on the basis of available information on human pharmacology. Human plasma concentrations at different times after conventional therapeutic doses of drug were obtained from the literature and replotted on a linear scale. The integral over the first hour after administration of the drug, and the integral of the whole plasma clearance curve were measured graphically. For HMM the 1h peak value was the integral between 1·5 and 2·5 h after oral administration of the drug, as the peak plasma level occurred at 2 h (Bryan et al., 1968). For adriamycin the sum of unchanged adriamycin and adriamycinol levels was used because, among the many metabolites, only adriamycinol is known to be cytotoxic (Benjamin et al., 1977). The drugs used vary in their stability in in vitro systems. Melphalan, chlorambucil and thio-TEPA are the least stable and may have undergone some hydrolysis during the 1h incubation. The decision to use a 1h incubation for all the drugs was arbitrary. We recognize that the valid assessment of some drugs may require a longer or shorter time, and this will be the subject of subsequent research.

RESULTS

The clonogenic cell assay was used to measure cell survival after the HX32 tumours were exposed by injecting host mice with each cytotoxic agent. Fig. 1 shows the sensitivity of cells in this tumour, measured 20 h after a single
injection of each drug (tumours were left in situ for 20 h to allow completion of drug metabolism and to allow for any in vivo repair of drug damage). Drug dose has been expressed as a fraction of the LD₁₀, which is defined as the single i.p. dose of each agent which kills 10% of mice within 30 days of injection. The LD₁₀ data were obtained from the literature and from tests in CBA/lac mice exposed 2–3 weeks after 900 rad immunosuppressive treatment. The assumption has been made that LD₁₀ doses, which are by definition equitoxic to mice, are in proportion to the maximum tolerated doses in man. There is a basis for this assumption in reports comparing drug doses in small-animal lethality studies with maximum tolerated doses in human beings (Freireich et al., 1966; Mellett, 1974; Goldsmith et al., 1975).

Table I summarizes the in vivo data. Column 2 gives the LD₁₀ values used and Column 3 gives the surviving fraction of tumour cells at that dose. This fraction was read for each drug at the intersection of the dose–response curve with the vertical dotted line drawn at 1.0 on the abscissa. Fig. 1 shows the standard error of these values, as calculated from the regression analysis used to draw the curves, and the *t* test was used for differences between pairs of drugs. The resulting probabilities are shown in Table I and drugs are ranked accordingly. Column 4 gives the rank of each agent in order of decreasing cytotoxicity as used in the mouse.

**In vitro cell survival**

Figs. 2, 3 and 4 show the survival of clonogenic tumour cells exposed in vitro for 1 h to the 8 cytotoxic drugs. Data points shown are from at least 2 experiments for each drug. The concave form of the curves has been noted in other studies in vitro (e.g. Barranco et al., 1978). The in vivo and in vitro dose–response curves for HMM in Figs. 1 and 3 are qualitatively different. The in vitro data show a plateau-type response, with little reduction in
Figs. 2, 3 and 4.—Sensitivity of HX32 tumour cells to drug exposure in vitro. Data points from at least 2 experiments are shown. ♦ indicates survivals at the human plasma concentration over the peak hour (Level A from Table II). ◇ indicates survival at human plasma concentration over the total time measured (Level B from Table II). (Symbols as in Fig. 1.)

Surviving fraction between 1 and 10 μg/ml, whereas the in vivo data are fitted by an exponential curve for doses above $0.5 \times LD_{10}$. Rutty & Connors (1977) obtained low cytotoxicity of HMM in vitro in the absence of liver microsomes, and concluded that this drug owes its cytotoxicity.
to activation in vitro. Our method of in vitro exposure would, therefore, underestimate the possible effect of HMM in patients at concentrations above 1 μg/ml. We have therefore excluded HMM from the in vitro/in vivo comparison. A sample of the postulated active metabolite of HMM (pentamethyl monomethylyl melamine) was kindly given to us by C. J. Ruttty. This compound showed no plateau of response (Fig. 5). These results therefore support the conclusion that activation of HMM is needed for maximum cytotoxic effect.

In this preliminary study we have assessed the in vitro sensitivity of cells from HX32 tumour at concentrations derived by 2 alternative methods from human plasma clearance curves:

(a) On the hypothesis that the initial peak levels of drug determine the response, the average concentration was measured over the first hour after administration (or the average between 1.5 and 2.5 h after oral HMM) and these values are quoted as Level A in Table II. The solid arrows in Figs. 2–4 show the fraction of cells which survived incubation with Level A for 1 h.

(b) On the hypothesis that drug concentration × time is the effective parameter of drug exposure, the integral of the whole curve was measured. We then estimated the effect of exposing cells for 1 h at the concentration (Level B) that would give the same integral dose (concentration × time). The open arrows in Figs. 2–4 indicate the fraction of cells which survive 1 h incubation in vitro at Level B. The surviving fractions at the positions of these arrows were used to rank drugs for in vitro activity, as shown in Table III. For HMM, Levels A and B are both much higher than the concentration at which the plateau level of cell kill was reached in vitro and this drug therefore was omitted from the in vitro ranking.

Comparison of in vivo and in vitro assessments of chemosensitivity

As shown in Table III, there is good correlation between in vitro ranking based on plasma concentrations and in vivo ranking. The ranking at 1 h plasma levels correlates more closely to ranking at mouse LD10 doses than does the ranking at the doses equivalent to the total plasma clearance curve (Spearman rank correla-

![Graph of drug concentration vs. surviving fraction of clonogenic cells.](image)

**Graph**

**Fig. 5.**—A comparison of the in vitro action of HMM with that of its postulated active metabolite, pentamethyl monomethylyl melamine. Data from 3 experiments.

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**Table II.**—Integrals on drug concentration in human plasma (μg/ml/h)

| Drug        | Dose per patient (mg) | A* | B†  | Reference                  |
|-------------|-----------------------|----|-----|---------------------------|
| Melphalan   | 30                    | 0.77 | 1.28 (3) | Tattersall et al., 1978    |
| HMM         | 12/kg                 | 14  | 114 (24) | Bryan et al., 1968        |
| *cis*-Pt (II) | 20/m²               | 1.92 | 20.2 (20) | Malerbi (pers. comm.)     |
| thio-TEPA   | 0.3/kg                | 0.19 | 0.35 (4) | Mellett et al., 1962      |
| Chlorambucil | 10                   | 0.18 | 0.51 (6) | D. Newell (pers. comm.)   |
| Methotrexate | 200                  | 1.07 | 3.18 (20) | Calvert et al., 1978      |
| Vinblastine | 12–16                 | 0.136| 0.32 (4) | Owellen et al., 1977      |
| Adriamycin  | 60/m²                 | 0.3  | 1.65 (48) | Benjamin et al., 1977     |

* Integral over the period 0–1 h after administration (1.5–2.5 h for HMM).
† Integral of the whole plasma clearance curve up to the time (in h) shown in brackets.
Table III.—In vitro cytotoxicity of 8 drugs against HX32 cells

| Drug        | Surviving fraction at level A* (solid arrows) | Rank (A) | Surviving fraction at level B* (open arrows) | Rank (B) | In vivo rank† |
|-------------|-----------------------------------------------|----------|-----------------------------------------------|----------|---------------|
| Melphanan   | 0·17                                          | 1        | 0·041                                         | 2        | 1             |
| cis-Pt(II)  | 0·29                                          | 2        | 0·012                                         | 1        | 2             |
| Thio-TEPA   | 0·35                                          | 3        | 0·29                                          | 4        | 3·5           |
| Chlorambucil| 0·42                                          | 4        | 0·16                                          | 3        | 3·5           |
| Adriamycin  | 0·88                                          | 5        | 0·54                                          | 5        | 7             |
| Methotrexate| 0·93                                          | 6        | 0·88                                          | 6        | 5·5           |
| Vinblastine | 1·0                                           | 7        | 1·0                                           | 7        | 5·5           |

* From Table II.
† From Table I, omitting HMM.

The good correlation that we have found between in vitro and in vivo effects of drugs lends support to our original hypothesis. We had proposed that predictions of the relative efficacy of drugs against tumour cells in patients might be made:

(a) by assessing cytotoxicity against human tumour cells grown in immune-suppressed mice at doses of drug equitoxic to mice, and
(b) by assessing cytotoxicity to human tumour cells in vitro at drug concentrations found in patients.

Although at the present time there are unavoidable uncertainties in the translation of drug levels from in vivo to in vitro, and from man to mouse, this study has shown that plausible assumptions lead to a good correlation between responses seen in vitro and in the mouse. However, it was found to be impossible to assess HMM by an in vitro method because of its requirements for in vivo activation, and adriamycin, a drug with a wide spectrum of clinical activity, failed to show in vivo cell kill to the extent that would have been predicted by our in vitro studies. Other investigators have found adriamycin to be ineffective against mouse tumours, and this has been attributed to poor drug access (Sutherland et al., 1979).

The validity of the rankings obtained in this study depends on the errors involved in assessing cell survival at a given drug dose. For HX32 cells the in vivo dose-response curves for the 8 agents are exponential in most cases, and values for surviving fraction (SF) at the LD10 and its standard error can be calculated from the regression analysis. This enables the significance of differences between cytotoxicity of different drugs at the LD10 to be determined (assuming an accurate LD10 estimate). The pooling of data from several mouse strains and several laboratories should give a good estimate of LD10.

The in vitro dose-response curves are less well defined than the in vivo curves, but they are generally not exponential and have been drawn by eye. Thus the errors of SF for a given dose are not known unless experimental observations have by chance been made at that desired concentration. However, Table III shows that our assessment of drugs in vitro produces similar drug rankings, whether the effect is measured at the peak human plasma concentration or at the drug level equiva-
lent to the integral of the whole plasma clearance curve. Furthermore, both in vitro rankings correlate well with drug ranks assessed in vivo.

Salmon et al. (1978), using an alternative approach for defining in vitro sensitivity of cells from tumour biopsies, compared in vitro results with the response of patients. Their distinction between “resistance” and “sensitivity” in vitro was somewhat arbitrary, being based on the integral of a cell-survival vs drug-concentration curve with upper limits of 0-1 μg/ml for melphalan and bleomycin, and 0-2 μg/ml for the other drugs used. Available pharmacological data indicate that melphalan, for example, gives a peak plasma level in patients of ~1 μg/ml (Tattersall et al., 1978), which is higher than the maximum concentration used in vitro by Salmon and his colleagues (1978). If the 8 drugs of the present study had been assessed in vitro at a concentration of 1 μg/ml, chlorambucil would have been ranked as the most effective drug followed by (2) melphalan, (3) thio-TEPA, (4) cis-Pt(II), (5) adriamycin, (6) methotrexate and (7) vinblastine. This correlates poorly with the in vivo ranking (P > 0.05 for a Spearman rank correlation of 0.67). Thus the use of arbitrary drug concentrations in vitro precludes any effective ranking of drugs.

For the 8 drugs in this study, we conclude that HMM and adriamycin cannot be used in vitro to mimic in vivo response. For the other 6 drugs, 1h in vitro exposures can be used to predict the in vivo effect of a single injection, if in vitro concentrations approximating to drug levels in patients’ plasma are used.

Theoretically, the measurement of drug cytotoxicity at human plasma concentrations in vitro and at doses equitoxic to mice in vivo might both be expected to correlate with cytotoxicity in patients at drug doses equitoxic to man. The use of both these methods must be validated by studies on biopsy material from many human cancers and correlation of laboratory results with patients’ responses.

However, we have demonstrated, using one human tumour xenograft, that the 2 assays correlate well with one another, and we therefore feel encouraged in our attempts to use the in vitro test for alkylating agents and cis-Pt(II) to compare the response of tumour biopsy material with patient response to chemotherapy. A study of this type is in progress, using ovarian carcinoma cells.

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