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Validation of Clinical Predictive Value of *In Vitro* Colorimetric Chemosensitivity Assay in Head and Neck Cancer

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For chemosensitivity testing, a rapid *in vitro* colorimetric method (MTT assay) was used. Eleven head and neck cancer cell lines were investigated to distinguish five known active agents from five compounds inactive in phase II studies. Evaluation of the reliability of the assay for assessing drug sensitivity in this tumor cell population was done by correlating the *in vitro* results with reported *in vivo* response data.

Methotrexate and cisplatin (clinically active) and vindesine and doxorubicin (less active clinically) were recognized *in vitro* as active and correlated well with clinical experience. Bleomycin (clinically active) was ineffective against some cell lines. The *in vitro* findings for the clinically inactive drugs (deoxyazacytidine, lomustine, and carmustine) also corresponded. Amsacrine and etoposide, contrary to clinical experience, showed activity *in vitro*. Further comparison of MTT assay results with clinical data is warranted and essential before its use in large-scale drug screening studies.

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INTRODUCTION

In squamous cell carcinoma of the head and neck the clinical course becomes extremely severe when the tumour is no longer amenable to surgery or radiotherapy. Several standard anti-tumour agents, alone or in combination, achieve encouraging remission rates. However, response duration and survival from the onset of chemotherapy are short. Therapeutic results with investigational drugs have generally been disappointing. It would be a great advantage to have a preclinical *in vitro* test to screen new agents. The method should be widely applicable, rapid, simple and inexpensive. In addition, *in vitro* cytotoxic activity must correlate with the *in vivo* antitumour effect.

With these principles in mind, a study was designed to assess the *in vitro* chemosensitivity of a disease-oriented panel of eleven tumour cell lines to ten chemotherapeutic agents with an assay that has generated interest for drug screening. Squamous cell carcinoma of the head and neck was selected because of the existence of several clinically active and inactive agents. We used a colorimetric drug sensitivity assay [1] based on the biological reduction of a tetrazolium salt to a blue formazan product as indicator of viability. Several reports on this colorimetric MTT assay have compared it with other "standard" assays [2–4]. Our approach differed since we compared our assay results with clinical data. The objective was to investigate whether our panel of cell lines would correctly identify the known active agents and differentiate them from the compounds that were inactive in phase II studies of head and neck tumours.

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MATERIALS AND METHODS

Drugs

All chemotherapeutic agents except lomustine and deoxyazacytidine were purchased as formulated commercial products. Lomustine was provided by Rhone Poulenc (Brussels) as a dry powder. Deoxyazacytidine was supplied by the EORTC New Drug Development Office (Amsterdam). The drugs were solubilized in culture medium; lomustine was dissolved in a mixture of 2 parts ethanol to 8 parts 20% propylene glycol, followed by dilution in serum-free medium. The drugs were then added in 10 μl aliquots to the culture medium. Under no condition were the cells exposed to solvent concentrations in excess of 1%. Control cultures with solvent alone as well as controls of medium alone with and without the various drugs were included.

Drug concentrations

Five clinically active and five inactive drugs (Table 1) were tested at concentrations of 10, 1, 0.1 and 0.01 μg/ml. Drug exposure was continuous.

Human cell lines

Eleven human head and neck tumour cell lines were tested (Table 2). LICR cell lines were obtained from Dr M. Mareel (Ghent) and were established by Easty *et al.* [15]. The other cell lines were bought at the American Type Culture Collection (ATTC, Rockville). Cell lines were maintained as monolayers in Falcon plastic culture vessels. Dulbecco's modified Eagle's medium (Flow) was used for the LICR cell lines and the supplier's recommended medium for the ATCC cell lines. The culture media also contained penicillin (1%), streptomycin (5000 U/ml), L-glutamine (200 mMol/l) (Gibco) and 10% fetal calf serum (Gibco). Cells were harvested during exponential growth.

Table 1. Clinical response rates of single agents in head and neck carcinoma

Drug [ref]	Evaluable cases	Response rate (%)
Active		
Methotrexate (5)	361	35
Cisplatin (5)	255	24
Bleomycin (5)	298	18
Doxorubicin (5–7)	185	18
Vindesine (8)	119	13
Inactive		
Amsacrine (9, 10)	43	7
Lomustine (6, 11)	84	7
Carmustine (12)	18	5
Deoxyazacytidine (13)	27	0
Etoposide (14)	34	0

Drug exposure

Tumour cells were suspended in their respective culture media at a final concentration of 5×10^4/ml, except for FaDu and LICR(LON)HN-2 which were suspended at 2.5×10^4 and 10^5/ml. 100 µl of cell suspension were plated in a 96-well flat-bottom microtitre plate and treated with 10 µl of each drug at the specified final concentration. The plates were placed in a humidified 5% CO_2 atmosphere at 37°C for 4, 5 or 6 days. The optimal incubation times and cell seeding concentrations were determined from growth curves. Each experiment consisted of eight replicate wells and was done at least in triplicate with untreated controls.

Colorimetric assay and evaluation

The colorimetric assay was based on a method developed by Mosmann *et al.* [1]. It has been evaluated in our laboratory and others [2, 16, 17] and is described elsewhere in detail [18]. After subtracting the background absorbance, the relative percentage of metabolizing cells was calculated as the ratio of the absorbance after drug exposure to the absorbance of the controls.

Table 2. Human cell lines derived from squamous carcinomas of the head and neck

Cell line	Origin	Previous treatment†
LICR(LON)HN-1	Tongue	CT, RT; VCR, BLM, MTX, 5FU
LICR(LON)HN-2	Larynx	RT
LICR(LON)HN-3	Tongue	CT, RT; VCR, BLM, MTX, 5FU
LICR(LON)HN-4	Larynx	CT, RT; VCR, BLM, MTX, 5FU
LICR(LON)HN-5	Tongue	CT, RT; VCR, BLM, MTX, 5FU
LICR(LON)HN-6	Tongue	CT, RT; VCR, BLM, MTX, 5FU
SCC-4	Tongue	CT, RT; MTX
SCC-9	Tongue	—
SCC-25	Tongue	—
FaDu	Hypopharynx	—
RPMI 2650	Nasal septum*	RT

*Pleural effusion.

CT = chemotherapy; RT = radiotherapy; VCR = vincristine, BLM = bleomycin, MTX = methotrexate, and 5FU = 5-fluorouracil.

Data analysis

The mean result of all experiments with one cell line at one concentration was calculated from measurements on at least 48 different wells. One hundred and ten dose–response curves were plotted for all drugs. We used the area under semilogarithmic *in vitro* concentration–percent survival curves (AUC) to measure efficacy [19]. A cell line was considered to respond if the AUC, based on results obtained with the four drug concentrations, was reduced to 50% of control. The concentration that inhibited 50% growth (IC_{50}) was derived from the dose–response curves of those drugs that showed some effectiveness. A statistical test of the AUCs was done with analysis of variance. Results with cell lines originating from chemotherapy-treated patients were also compared with those from untreated patients. The clinical effectiveness of the tested drugs, used for comparison with *in vitro* results, was taken from published data [5–14]. In a final evaluation, the *in vitro* results and the clinical data were arranged in 2×2 tables and the significance of the association was tested [20].

RESULTS

In preliminary experiments, we found that for each cell line a given number of viable cells per well reduced a constant amount of MTT to its formazan reaction product. Differences between the cell lines in their capacity to metabolize MTT were observed. Since each cell line had its own control, this did not interfere with the calculated percent survival curves. The optimal incubation times and cell seeding concentrations were determined from growth curves in preliminary experiments, thus avoiding the plateau region for growth and selecting for the largest optical difference between day 1 and the end of the experiment. This approach led to variation in drug exposure but allowed the best experimental conditions for each cell line so that exponential growth and adequate absorbance readings in control wells were ensured.

A two-factorial analysis of variance of the AUC values showed no difference between cell lines originating from chemotherapy-treated patients and those from non-treated patients ($P = 0.19$); therefore, the cell lines were analyzed as one group. Methotrexate followed vindesine as the most effective drug, reducing the AUC by 50% in nine compared with 10 cell lines. Cisplatin and amsacrine had the same efficacy inhibiting cell lines. Bleomycin, etoposide, and doxorubicin reduced the AUC by 50% in 3, 3, and 4 cases. All cell lines were resistant to lomustine, carmustine and deoxyazacytidine. Significant differences were observed between the drugs. Figure 1 shows the effect of the drugs as measured by percent AUC reduction. A one-factorial analysis of variance of the AUC data for all drugs, pooled for each cell line, did not indicate any cell line to be more drug resistant or sensitive than another.

The IC_{50}s derived from the dose–response curves of the 7 drugs producing about 50% or more reduction in AUC are shown in Table 3. Table 4 shows that the mean drug concentration giving 50% growth inhibition was, except for doxorubicin, at least ten times smaller than the peak plasma concentrations that would be expected for an active drug. This mean drug concentration was calculated after deleting values greater than 1 µg/ml, since such results probably indicate cell line resistance.

Table 5 shows that the sensitivity of the test was 100% with a specificity of 60%. The positive predictive value was 71% and the negative predictive value was 100%.

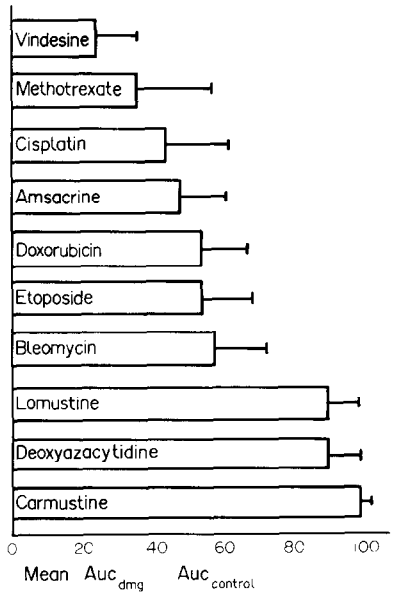

Fig. 1. Drug efficiency. Mean (S.D.) percentage of AUC relative to controls for eleven cell lines. AUCs based on dose–response curves obtained with four concentrations (10–0.01 μg/ml).

DISCUSSION

Several attempts have been made to develop an *in vitro* chemosensitivity test on human tumour biopsy specimens [27]. The clonogenic assay [28] has been the most extensively studied and clinically applied. However, application of this soft-agar cloning assay to fresh head and neck tumours has several limitations [29,30]. To eliminate some of the problems associated with fresh biopsy material, we chose to work with established cell lines. Furthermore, using the colorimetric method, we were able to circumvent some of the limitations of the clonogenic assay and to assess tumour cell survival in a semi-quantitative, rapid, and sensitive method. The colorimetric and clonogenic assays give similar results with other cell lines [2, 4]. However, the criteria for defining *in vitro* drug sensitivity or resistance have not been standardized in these or other assays.

We tested eleven head and neck tumour cell lines with ten drugs. Some of the cell lines were derived from tumours of patients previously treated with chemotherapy and/or radiotherapy. Contrary to expectations, Inoue *et al.* [31] and Von Hoff *et al.* [32] demonstrated in a different assay that specimens from such patients are not more resistant *in vitro*. We also found no difference in the response between cell lines derived from pretreated and untreated patients. Furthermore, we did not identify a cell line that was statistically more resistant or sensitive than another.

As has been done in clonogenic assays, we worked with a range of drug concentrations, used continuous drug exposure, and required at least 50% reduction of AUC to indicate *in vitro* drug efficacy. We also calculated the IC_{50} to compare the effect of the different drugs on each cell line and to analyze the IC_{50} to peak plasma concentration ratio. To define drug activity *in vitro*, we followed widely used criteria that require the testing of ten to twenty specimens with at least 10% of these being sensitive *in vitro*. All these cut-off points were selected arbitrarily.

We compared our *in vitro* results with published clinical data. A comparison with "standard assays" that are also insufficient predictors does not produce a useful validation. An ideal, non-biased validation of a chemosensitivity test with each of the drugs tested *in vitro* also evaluated prospectively as a single agent in the same patient cannot be done. However, the nearer this ideal is reached, the more relevant the results will be.

*Table 4. Mean IC_{50} and ratio of IC_{50} to peak plasma concentration**

Drug	Mean IC_{50} (μg/ml)	PPC (μg/ml)	Drug dosage [ref] (mg/m²)		Ratio
Methotrexate	0.05	0.5–5	25–100	21	0.1–0.01
Cisplatin	0.30	0.5–5	100	22	0.06
Bleomycin	0.65	1–10	15	23	0.65–0.065
Etoposide	0.55	30	290	24	0.018
Amsacrine	0.40	6.7	160	25	0.059
Doxorubicin	0.55	1	50	26	0.55
Vindesine	0.045	3.4	3	24	0.013

*Mean IC_{50} was calculated from Table 3 after deletion of values greater than 1 μg/ml. IC_{50}s larger than 1 μg/ml were considered to indicate cell line resistance.

Table 5. Association between in vitro *activity and clinical effectiveness for ten drugs*

	Clinic: Active	Inactive	
In vitro test			
+	5	2	7
−	0	3	3
	5	5	10

Table 3. Mean IC_{50} (μ/ml) based on dose–response curves

		Cell lines LICR1	LICR3	LICR4	LICR5	LICR6	SCC4	LICR2	SCC9	SCC25	FaDu	RMPI
Methotrexate	MTX	0.062	0.0075*	0.047	0.056	0.064	0.067	0.052	>10	>10	0.00065*	0.07
Cisplatin	DDP	6.0	0.015	0.65	0.35	0.062	0.45	0.85	0.017	0.2	0.041	0.5
Bleomycin	BLM	>10	0.62	0.085	0.9	0.6	0.59	>10	0.95	0.75	4.0	0.84
Etoposide	VP16	0.065	0.37	0.76	0.55	0.55	0.95	6.6	0.76	4.9	0.52	1.5
Amsacrine	AMSA	0.09	0.37	0.095	0.5	0.05	0.8	0.9	0.7	3.5	0.39	0.25
Doxorubicin	DXR	0.85	0.21	0.66	0.71	0.65	0.8	6.0	0.35	0.85	0.067	0.45
Vindesine	VDS	0.093	0.0083	0.037	0.059	0.0056	0.037	0.087	0.037	0.036	0.027	0.047

*Values are extrapolated.

The clinical response rates in Table 1 were taken from pooled data of trials with various criteria for patient entry with variable drug doses and schedules. The response rates should, therefore, be regarded as approximations. These studies were done with single agents and most patients had received previous cytotoxic therapy.

In our *in vitro* study, methotrexate and cisplatin (clinically active) were recognized as active. However, bleomycin (clinically active), ineffective against some cell lines, was designated as having intermediate activity. Clinically inactive drugs (deoxyazacytidine, lomustine and carmustine), correlated well, as did vindesine and doxorubicin, which were recognized as active *in vitro*. *In vitro*/clinical correlations for amsacrine and etoposide were inaccurate. Vindesine was the most potent of all drugs tested; however, the concentration of vinca alkaloids required to produce cytotoxicity in cell culture is very low. This observation might explain the high sensitivity of the cell lines since drug concentrations were high.

In a non-clonogenic technique, differences in chemo-sensitivity between cell lines and relative resistance to drugs have been attributed to the presence of a differing number of non-dividing cells [2]. When this cell population is large, due to cell cycle delay or inhibition of proliferation, the result of the assay becomes influenced by the non-dividing cells since no significant kill will be achieved. This situation, however, might mimic what happens in the patient. However, testing all cell lines with incubation for 1 h (results not shown) did not show this factor to be influential. Only drug exposure time seems crucial. The role of the non-dividing cells as well as drug dose, exposure time, and the establishment of non-arbitrary cut-off points needs further investigation.

The high number of true positives and the superior yield of positive *in vitro* tests are a favourable aspect of the MTT assay. The high predictive value of the positive test (i.e. the probability that a drug with a positive test will achieve clinical response) is of interest clinically. Attempts to evaluate predictive tests by using, almost exclusively, drugs known to be active in the clinic can only offer limited information, as do comparisons between different test systems. Our testing of ten drugs with known active and inactive clinical effects on eleven cell lines is still too small to draw a final conclusion. Notwithstanding the problems in assessing such tests and of collecting clinical data with which to make correlations, we believe that the patient and his disease is the starting point for cancer treatment research and not another assay. Despite these difficulties our data are encouraging and this method could produce a reliable drug screen in the future.

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Disposition, Pharmacokinetics, and Metabolism of ^{14}C-Fotemustine in Cancer Patients

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The pharmacokinetics and metabolism of intravenously infused ^{14}C-fotemustine (about 100 mg/m^2) were examined in 2 cancer patients. Plasma levels of radioactivity increased to a maximum of 4.1 and 5.5 μg equivalents per g when the infusion stopped then declined triexponentially with mean half-lives of about ½, 10 and 80 h for the initial, mid and terminal phases, respectively. Plasma levels of intact drug were lower, with maximum levels of 1.1 and 2.8 μg/ml, and declined monophasically with a half-life of about 24 min. Plasma clearance was high (1426 and 764 ml/min) with the volume of distribution based on areas of 47.7 and 26.4 l. Most of the radioactivity was eliminated in urine (50.1 and 61.3%) over 7 days with smaller amounts in the faeces (6.8 and 0.3%) and only minimal quantities (under 0.1%) as expired carbon dioxide. Metabolites of fotemustine were identified as chloroethanol and N-nitroso-1-imidazolone-ethyl-diethylphosphonate in plasma and as 1-hydantoin-ethyl-diethylphosphonate and acetic acid in urine.
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INTRODUCTION

THE NOVEL nitrosourea, fotemustine (diethyl-1-[3-(2-chloroethyl)-3-nitrosoureido] ethyl phosphonate), has significantly increased efficacy and reduced side-effects compared with similar drugs for the treatment of carcinomas such as malignant melanoma [1]. Induction treatment consists of a 1 h constant-rate intravenous infusion at a dose of up to 100 mg/m^2 over 3 consecutive weeks, followed by a 5 week rest, after which maintenance therapy is given every 3 weeks in responding patients. We have investigated the pharmacokinetics and metabolism of fotemustine in cancer patients.

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PATIENTS AND METHODS

Drugs and reference compounds

^{14}C-fotemustine was synthesised at the Commissariat à l'Energie Atomique to a specific activity of 1961 MBq/mmol. The radiochemical purity was established by high-performance liquid chromatography (HPLC) with radiochemical detection as 96.4 and 88.9% for patients 1 and 2, respectively. Non-radiolabelled fotemustine, N-nitroso-1-imidazolidone-ethyl-diethylphosphonate (NIEDP) and 1-imidazolidone-ethyl-diethylphosphonate (IEDP) were supplied by Laboratoires Servier, France, and chloroethanol was from Aldrich.

Patients

Patient 1. (F/65, Caucasian, 52.4 kg, 1.53 m^2). Ovarian carcinoma previously treated with five monthly courses of cyclophosphamide and doxorubicin as well as three subsequent treatments with melphalan. The most recent treatment of cinnarizine was discontinued 1 week before study.