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In Vitro Chemical Eradication of Small Cell Lung Cancer: Application in Autologous Bone Marrow Transplantation

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Abstract—*Autologous bone marrow transplantation raises the question of the possible reinjection of tumour cells together with marrow. This paper investigates the ability of chemical compounds other than cyclophosphamide derivatives to eradicate small cell lung cancer (SCLC) in bone marrow. The responsiveness of SCLC lines to* cis-*platinum, doxorubicin, VP*16, *Celiptium® (an ellipticine derivative), Ditercalinium®, a new drug belonging to the pyridocarbazole series, and Asta Z® were measured in an agar–agar clonogenic assay.* Cis-*platinum and Ditercalinium® exhibited a high tumouricidal effect. The low cloning efficiencies of SCLC lines in an agar–agar clonogenic assay did not allow tumour cells to be detected among bone marrow cells with sufficient sensitivity. Therefore* cis-*platinum and Ditercalinium® were tested on bone marrow–tumour cell mixtures cultured in a liquid medium allowing the detection of one tumour cell per* 10^4 *bone marrow cells. As* cis-*platinum exhibited a low myelotoxicity, it is proposed for purging bone marrows of patients with SCLC.*

INTRODUCTION

It has been proposed to treat solid tumours such as small cell lung carcinoma (SCLC) and neuroblastoma by chemicals at high dose followed by autologous bone marrow transplantation [1–4]. Unfortunately, these diseases frequently display bone marrow involvement, leading to a possible reinjection of tumour cells into the patients. To overcome this problem, many 'purging' procedures of the marrow have been proposed including immunological [5, 6], physical [7] or chemical treatments. An active metabolite of cyclophosphamide, Asta Z®, is commonly used for that purpose [8], although myelotoxicity, with a delay in marrow recovery, has been reported [9]. Two types of cancer cells can be isolated *in vitro* from SCLC, the 'classic' and the 'variant' types, differing not only in their morphology and their neuroendocrine properties but also in their growth properties [10]. The 'variant' cells might be considered as the most aggressive cancer cells and exhibit a high tumourigenicity in nude mice as compared to the cells of the 'classic' type.

The aim of this work was to propose a chemical, other than Asta Z®, able to purge SCLC from marrow. We chose a 'variant' cell line, NCI-N417, and a 'classic' one, NCI-H449, as models for culture. We first studied the efficiency of Asta Z®, *cis*-platinum, doxorubicin, VP16, Celiptium® (an ellipticine derivative) [11] and Ditercalinium® [12] (a new bis-intercalator) on these two SCLC lines. We then measured the granulocyte–macrophage progenitor cells (GM-CFU) content of normal human bone marrows treated with the most tumouricidal chemicals, i.e. Ditercalinium® and *cis*-platinum. Lastly, we established the efficiency of *cis*-platinum when NCI-N417 was mixed with normal human bone marrow.

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Abbreviations used: SCLC, small cell lung carcinoma; RPMI-10% HIFCS, Roswell Park Memorial Institute Medium 1640 supplemented with 10% heat inactivated foetal calf serum; IC_{50}, drug dose which inhibits the formation of 50% of colonies; HIFCS, heat inactivated foetal calf serum; GM-CFU, granulocyte–macrophage colony forming units.

MATERIALS AND METHODS

Drug supply

The following drugs were purchased: doxorubicin (mol. wt = 600), VP16 (mol. wt = 588.5; Sandoz Co., Rueil Malmaison, France), *cis*-platinum (mol. wt = 300; Bellon Co., Neuilly/Seine, France),

mafosfamide cyclohexylamine 7557 or Asta Z® (mol. wt = 500; Asta Werke Co., Bielefeld, F.R.G.), 9 hydroxy-6-methyl ellipticinium or Celiptium® (mol. wt = 363; Sanofi Co., Marne La Coquette, France) [11] and dimer of ethyl-piperidino-2-methoxy-10-pyrido[4-3 C]carbozolium 7H, chloride or Ditercalinium®, NSC 335153 (mol. wt = 1007; Bellon Co., Neuilly/Seine, France) [12]. Drugs were dissolved before use in sterile distilled water, except for *cis*-platinum for which sterile saline was used.

Human tumour cells

SCLC cell lines, NCI-H449 ('classic small cells') and NCI-N417 ('variant small cells') have been well documented [10, 13]. These cell lines were kindly provided by D. Carney (Mater Misericordiae Hospital, Dublin, Ireland). They were grown as floating tumour cell aggregates. All cell lines were maintained in 4 ml of RPMI 1640 with 10% heat inactivated foetal calf serum (HIFCS) in a 25 cm^2 Nunclon flask culture and were transferred periodically at a 1:3 ratio after passing aggregates through a fine Pasteur pipette. Cultures were made at 37°C in a humidified atmosphere containing 7.5% CO_2. In preliminary experiments, the defined RPMI-HITES medium as proposed by Carney *et al.* [14] was used; this medium consists of RPMI containing 5 μg/ml bovine insulin, 100 μg/ml human transferrin, 10^{-8} M hydrocortisone, 10^{-8} M 17-β-oestradiol, 3×10^{-8} M sodium selenite. All products were purchased from Sigma Co. (St Louis, Missouri, U.S.A.).

Normal human bone marrow cells

Mononuclear bone marrow cells were collected by bone aspiration from healthy patients under anaesthesia after informed consent. The cells were fractionated on Ficoll-Hypaque, washed in RPMI containing 50 IU heparin/ml and counted in Trypan blue using an haemocytometer.

Drug sensitivity of tumour cells

In order to study the chemosensitivity of pure SCLC lines, clonogenic agar–agar assays were performed. A monocellular suspension was achieved by trypsinization of the SCLC lines aggregates. To investigate the effect of trypsin on the cytotoxicity of the drug, the same amount of cellular aggregates was submitted to pipette disaggregation and then treated as described. Cell viability was estimated with Trypan blue. Incubation was for 1 h at 37°C in RPMI with and without drug at a cellular concentration of 10^5 cells/ml. Drug concentrations varied from 0.001 to 100 μM. After washing in RPMI and resuspension, cells were plated in a double agar layer (Noble, Difco Co., Detroit, U.S.A.) as described by Carney *et al.* [15]. 10^5 cells were plated in each 35 mm Petri dish in triplicate for each assay point. One day after plating, Petri dishes were inspected for clumping. After 14 days of culture in 7.5% CO_2 at 37°C, scoring of colonies of up to 50 cells was performed using a Magiscan II image analyser (Joyce Loeble Co.) [16]. Results were expressed as mean ± S.D. Cloning efficiencies were scored by the following formula:

$$\frac{\text{number of colonies}}{\text{number of plated cells}} \times 100.$$

Drug sensitivity of bone marrow progenitors cells

Granulocyte–macrophage colony forming unit (GM-CFU) content was measured in a methylcellulose assay as previously described [17]. Human placenta conditioned medium was used as colony stimulating factor. Total marrow cells (10^7/ml) were treated by various concentrations of drug in RPMI for 1 h at 37°C. After washing and appropriate dilution, 10^4 and 2×10^4 total marrow cells were plated in duplicate in 35 mm Petri dishes. After incubation for 10 days at 37°C in a humidified incubator with 7.5% CO_2, the number of colonies (more than 50 cells) was counted and survival fractions of GM-CFU were estimated. Experiments were carried out using several bone marrow samples from different donors (n) and results were expressed as mean ± S.D. of the mean.

Treatment of bone marrow–NCI-N417 line mixtures by drugs

Aggregates of NCI-N417 line ranging from 10 to 50 cells were obtained by syringe passage and mixed with bone marrow of healthy donors. To determine the exact percentage of SCLC in the bone marrow mixture, aliquots of cell aggregates were trypsinized and counted. Tumour cells in the mixture corresponded to 10%, 1% and 0.01% of the bone marrow cells always maintained at a final concentration of 10^7 cells/ml. Each tumour cell–bone marrow mixture was cultured in triplicate. After 1 h of drug treatment at 37°C, cells were washed and cultured in RPMI-10% HIFCS for 1 month with a weekly refeeding. Viable cells were then counted using Trypan blue.

RESULTS

1. *Effects of drugs on SCLC lines*

The responsiveness of NCI-N417 and NCI-H449 cell lines to drugs was measured in an agar–agar clonogenic assay. Their cloning efficiencies were 2.3% ± 0.3 and 1.7% ± 0.2, respectively. Tumour cells were plated after 1 h of drug exposure. Figure 1 shows the curves of cytotoxicity obtained with *cis*-platinum, doxorubicin, VP16, Celiptium®, Ditercalinium® and Asta Z®. Drug doses which inhibit the formation of 50% of colonies (IC_{50}) were

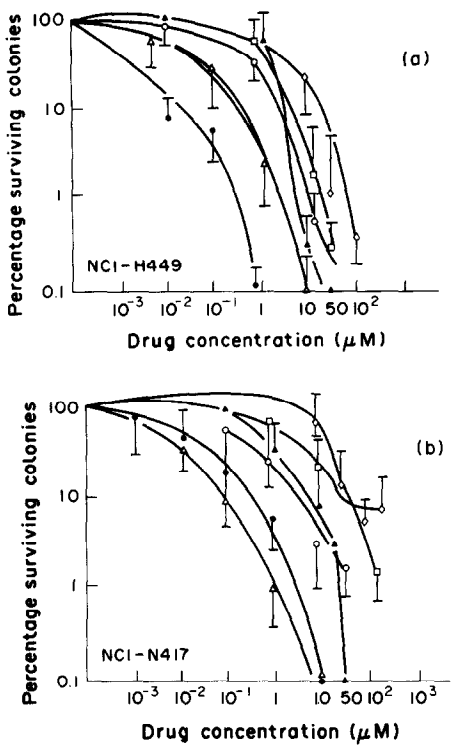

Fig. 1. Responses of NCI-H449 (A) and NCI-N417 (B) to various drugs in an agar–agar clonogenic assay. Cells in exponential growth phase were incubated with various drug concentrations for 1 h and further subcultured for 14 days in agar. —△—, *doxorubicin*; —●—, *Ditercalinium®*, —○—, *Celiptium®*; —▲—, cis-*platinum*; —◇—, *Asta Z®*; —□—, *VP16*. 100 μM *cyclophosphamide produced 92% colony survival in NCI-N417.*

estimated from these curves and presented in Table 1. At identical drug concentrations, cell lines displayed different responses according to the drug. NCI-N417, the variant cell line, is less sensitive to VP16, Asta Z® and Ditercalinium® than NCI-H449, the classic one. Unexpectedly, the variant line is more sensitive to doxorubicin and Celiptium® than the classic line while *cis*-platinum shows a similar efficiency on both lines. Ditercalinium® and doxorubicin are the most active. Asta Z® is much less active and produces a biphasic curve of cytotoxicity with the NCI-N417 cell line: a decrease of surviving colonies was followed by a plateau, suggesting the presence of a resistant cell subpopulation. As expected, cyclophosphamide was not toxic to NCI-N417, since this compound was not metabolized in its active form. Whatever the studied cell line, in the early period of culture and for low drug concentrations, Ditercalinium® led to the development of two types of colonies: normal sized and small sized colonies which will not develop later as reported for L1210 cells [18].

As described in Materials and Methods, the tumour cell aggregates were treated with trypsin to obtain a monocellular suspension. However this treatment, even applied in our mild conditions, might produce cells more sensitive to chemicals by modifying the structure of the membrane. Moreover, enzymatic treatment does not mimic the purging conditions used routinely. Therefore, as a control experiment, the same clonogenic assays were performed on the NCI-N417 line treated by *cis*-platinum after mechanical dispersion (successive passages in a Pasteur pipette). The IC_{50} measured from experimental curves is 0.85 μM which is not very different (0.65 μM) from that obtained with enzymatically dispersed tumour cells. This slight variation of IC_{50} might be accounted for by the trypsin treatment.

2. *Effects of drugs on normal human bone marrow*

Figure 2 represents the percentage of surviving GM-CFU as a function of concentration of either *cis*-platinum (A) or Ditercalinium® (B). In both cell lines, 50 μM *cis*-platinum or 10 μM Ditercalinium® treatment led to no tumour colony survival. Corresponding GM-CFU survivals were 9% ± and 0% respectively. Thus, *cis*-platinum is less cytotoxic than Ditercalinium®.

3. *Effects of drugs on NCI-N417 cells in suspension in human bone marrow*

To measure drug efficiency on tumour cells mixed with human bone marrow, it was first necessary to estimate the sensitivity of tumour cell detection. Culture in the RPMI-10% HIFCS was preferred

Table 1. Drug concentrations (μM) which inhibit the formation of 50% of colonies (IC_{50}) growing in agar from two SCLC lines

Drug	NCI-H449 'classic type'	NCI-N417 'variant type'
Doxorubicin	4×10^{-2}	5×10^{-3}
Ditercalinium®	1.2×10^{-3}	10^{-2}
Celiptium®	1	0.25
Cis-platinum	1	0.6
Asta Z®	5	20
VP16	3	18

Exponentially growing cells were treated by the drug for 1 h at 37°C, then plated in a clonogenic agar–agar assay [15]. Colonies were counted after 14 days of culture. IC_{50}s were obtained graphically from Fig. 1A and B.

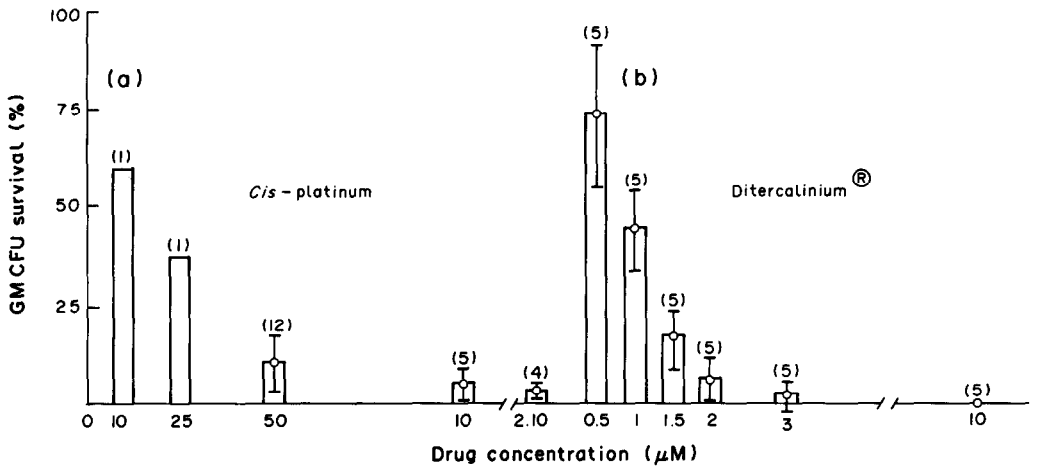

Fig. 2. In vitro effects of cis-*platinum (A) and Ditercalinium® (B) on normal human bone marrow myeloid progenitors. percentage of surviving GM-CFU colonies as a function of drug concentration. The number in parentheses represents number of different bone marrow samples analysed for each drug concentration, and bars indicate the standard deviation of the mean.*

to the clonogenic assay in agar as cloning efficiency was too low to detect a tumour cell involvement of less than 0.1%. Mixtures of normal bone marrow and of the two SCLC lines were therefore cultured at various ratios and the viability of tumour cells was determined. We could detect one tumour cell per 10^4 bone marrow cells [19]. As expected, in the absence of colony stimulating factor, bone marrow cells alone did not grow in RPMI-10% HIFCS.

Cellular suspensions of NCI-N417 line with normal human bone marrow were treated for 1 h with various concentrations of Ditercalinium® or *cis*-platinum. After washing, cells were cultured for 1 month in RPMI-10% HIFCS and viable cells counted using Trypan blue. Cultures were carried out for 1 month in order to prevent any possible delay in cell growth following the drug treatment. Results are presented in Table 2. No viable cells could be detected after this period of culture when suspensions were treated by either 25 μM *cis*-platinum or 5 μM Ditercalinium®. The effect of the bone marrow cell concentration on the responsiveness of tumour cells to the drugs was studied. Therefore, mixtures with a ratio of NCI-N417 cells in bone marrow of 1% and 0.01% were treated with 50 μM *cis*-platinum or 10 μM Ditercalinium®, considered as 'safe' bone marrow purging concentrations. Similarly, no viable cells could be detected. By contrast an untreated suspension of 0.01% of NCI-N417 cells exhibited aggregates of tumour cells with characteristic properties of the cell line (morphology and cloning efficiencies).

Table 2. Percentage of viable tumour cells as a function of drug concentration in a bone marrow cells suspension containing 10% *of NCI-N417 line*

Drug concentration (μM)	*Cis*-platinum	Ditercalinium®
0.5	ND	15 ± 6
1	63 ± 7	8 ± 3
5	39 ± 12	0
10	3 ± 4	0
25	0	ND
50	0	ND
100	0	ND

Mixtures of bone marrow cells and SCLC were treated for 1 h at 37°C at the indicated drug concentrations and the cells were cultured for 1 month. Results are expressed as a percentage of viable tumour cells as compared to an untreated mixture.
ND: not determined.

Considering the sensitivity of the liquid culture and the absence of viability for tumour cells 30 days after drug treatment, the data indicate that 50 μM *cis*-platinum or 10 μM Ditercalinium® induce a cell-kill of 4 logarithms (i.e. from a ratio of 10% to a ratio of 0.01%). It must be noted that 50 μM *cis*-platinum and 10 μM Ditercalinium® produce 10% and 0% GM-CFU survival, respectively (Fig. 2).

DISCUSSION

Autologous bone marrow transplantation permits the patients to rapidly recover their haematopoietic functions after high dose chemotherapy. However, the high frequency of bone marrow involvement in SCLC patients puts the physician in a situation where the advantage of a quick recovery of haematopoietic tissue is hampered by the possible reinjection of occult tumour cells into the patient. Current specialist practice is therefore to treat *ex vivo* the cytologically negative marrow before reinjection into the patient. Laboratories dealing with this problem aim to detect with a high sensitivity the tumour cells present in the patient's bone marrow and to eradicate tumour cells while preserving as much haematopoietic tissue as possible.

Cytological, immunological, physical and cell culture techniques could be applied to detect bone marrow involvement. Cytology is routinely the quickest technique, allowing harvesting and storage

of the marrow. This method further allows one tumour cell per 100 marrow cells to be detected. Morphological characteristics of aggregates are the only criteria that can be used to assess bone marrow involvement. Immunocytochemistry using monoclonal antibodies directed towards SCLC antigens [20] has also been proposed, although the sensitivity of such a technique is not well established. It has been recently shown that discontinuous gradient sedimentation increases the sensitivity of SCLC detection in bone marrow specimens [21]. Cultures in the RPMI-10% HIFCS liquid medium permits a detection of one tumour cell per 10^4 bone marrow cells. Similar results were obtained with RPMI-HITES, a serum-free medium previously used to favour the growth of SCLC from patient biopsies [14]. These data suggest that hydrocortisone, insulin, oestradiol, and sodium selenite do not afford an additional advantage for the growth of the two SCC lines in bone marrow.

Taking into account the low sensitivity of detection of tumour cells using cytology, *in vitro* chemical treatment of marrow is used to purge undetectable and possibly metastatic cells. Alkylating agents were first described [8] and are currently used. A derivative of these substances, mafosfamide cyclohexylamine or Asta Z®, is an active metabolite of cyclophosphamide Haematologic reconstitution in patients after purging of bone marrow has been studied. This compound produces a complete disappearance of GM-CFU bone marrow progenitors but the treated marrow is able to repopulate bones, indicating that marrow stem cells are functional [9, 22]. Nevertheless, with such an *in vitro* treatment of the marrow, aplasia recovery in patients occurs with a lag period ranging from 5 to 10 days during which infectious complications can occur. Therefore an *in vitro* treatment exhibiting a weaker toxicity towards bone marrow is required.

As shown in Table 1, NCI-N417, 'variant' cells, are more resistant to Ditercalinium®, Asta Z® and VP16 than NCI-H449, 'classic' cells. On the contrary, NCI-N417 cells are more sensitive to doxorubicin and Celiptium® than NCI-H449 cells while *cis*-platinum exhibits a similar efficiency on both lines. The variant type of SCLC has been previously shown to exhibit amplification of the c-*myc* proto-oncogene and to be related to the aggressiveness of the cancer [23]. Our data do not permit us to consider the variant type as the SCLC phenotype exhibiting chemoresistance. It must be pointed out that NCI-N417 and NCI-H449 cell lines were derived from patients with no prior therapy [13, 24]. Other studies on SCLC lines must be done, taking into account the previous treatment of the patient from whose tumour the line has been originated.

Doxorubicin, *cis*-platinum and Ditercalinium® appear to be the most active compounds (Fig. 1). It is well known that doxorubicin induces myelotoxicity. We therefore mainly studied *cis*-platinum and Ditercalinium®, potential candidates for obtaining a good therapeutic index in bone marrow purging. *Cis*-platinum was found as the less myelotoxic compound in a GM-CFU assay. Our data are in agreement with those of Ogawa *et al.* [25] who showed that, in the same experimental conditions, 50 μM *cis*-platinum led to a 50% GM-CFU survival of human bone marrow.

These experimental results lead us to propose the use of *cis*-platinum as a bone marrow purging agent in patients with SCLC and treated by chemicals at high dose followed by autologous bone marrow transplantation.

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