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Comparison of the Cytotoxicity of Amsacrine and its Analogue CI-921 against Cultured Human and Mouse Bone Marrow Tumour Cells

LAI-MING CHING, GRAEME J. FINLAY, WAYNE R. JOSEPH and BRUCE C. BAGULEY
Cancer Research Laboratory, University of Auckland Medical School, Auckland, New Zealand

Abstract—*Human and mouse bone marrow cells were cultured for* 1 h *in the presence of either the antileukaemia drug amsacrine or its* 4-*methyl*,5-[N-*methyl*]*carboxamide disubstituted analogue CI-*921, *before being plated in methylcellulose medium to determine the survival of granulocyte–macrophage colony forming units (CFU-GM). The drug concentration required for* 50% *reduction in survival was approx.* 0.4 μM *for both drugs and was similar for both human and mouse cells. A comparison of the two drugs was then made, at an added drug concentration of* 0.5 μM, *using cultured mouse L*1210 *and P*388 *leukaemia, Lewis lung carcinoma cell lines LLAK and LLTC, human Jurkat leukaemia, human histiocytic lymphoma U*937 *and human colon carcinoma SW*620. *The sensitivity of the mouse lines for amsacrine was in the order L*1210 > *P*388 > *LLAK* >*LLTC, similar to the* in vivo *sensitivity. The selectivity of CI-*921 *for L*1210 *versus bone marrow, and for LLAK versus L*1210 *or P*388, *was greater than that of amsacrine, again in keeping with its* in vivo *properties. The sensitivity of the human Jurkat and U*937 *lines for amsacrine was intermediate between that of L*1210 *and P*388, *while SW*620 *was resistant. The selectivity of CI-*921 *for Jurkat and U*937 *versus bone marrow was greater than that of amsacrine, suggesting that CI-*921 *could have additional advantages over amsacrine in the treatment of some tumours.*

INTRODUCTION

AMSACRINE, a 9-aminoacridine derivative first synthesized by Cain and Atwell [1], is now used in combination with agents such as cytosine arabinoside in the treatment of acute leukaemia [2]. Selected predominantly on the basis of testing with murine leukaemias, its narrow clinical spectrum of action raises the question of whether the use of another preclinical screening method employing a solid tumour would select a different compound in this series. The disubstituted derivative CI-921 (9-[[2-methoxy-4[(methylsulfonyl)-amino]phenyl]amino)-*N*,5-dimethyl-4-acridine carboxamide) was selected with an experimental solid tumour system, is superior to amsacrine against murine leukaemias and is curative against established murine Lewis lung tumours which are resistant to treatment by amsacrine [3, 4]. CI-921 has now undergone Phase II clinical trials [5, 6] and although a response has been obtained in non small cell lung cancer, it is not yet possible to decide whether CI-921 is superior clinically to amsacrine.

A question of great relevance to the clinical potential of CI-921 is that of why CI-921 is superior to amsacrine against a number of mouse tumours. Both amsacrine and CI-921 bind by intercalation to double-stranded DNA [3] and are thought to act by inducing the formation of covalent links between DNA and the enzyme topoisomerase II [7]. P/AMSA, a subline of P388 murine leukaemia cells with an altered topoisomerase II enzyme [8], is cross-resistant to both amsacrine and CI-921 [9]. On the other hand, P/ACTD, a multidrug-resistant P388 subline which is resistant to vincristine, doxorubicin and etoposide is sensitive to both amsacrine and CI-921 [9]. Continuous drug exposure growth inhibition assays have shown that CI-921 is approx. 4-fold more potent than amsacrine against a number of human and murine tumour cell lines [10–12], provided ascorbate was included in the

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Address for correspondence: Dr L.-M. Ching, Cancer Research Laboratory, University of Auckland Medical School, Private Bag, Auckland, New Zealand.

culture medium to prevent differential aerial oxidation of CI-921 [12]. Taken together, these results suggest that the two drugs differ in potency but not in mechanism of action.

Since *in vitro* studies have been carried out with tumour lines only, it has yet to be established whether the greater potency of CI-921 against tumour cells extends also to normal cells. In this report we have measured the relative toxicity of amsacrine and CI-921 against murine and human myeloid progenitor cells from the bone marrow. Bone marrow haemopoietic stem cells are of great interest since myelosuppression is the dose-limiting toxicity for both amsacrine and CI-921 [5]. Although it is not the most pluripotent of the bone marrow stem cells [13], the granulocyte–macrophage colony-forming-unit (CFU-GM) provides a convenient starting point for the investigation of comparative drug effects on normal host cells.

MATERIALS AND METHODS

Materials

Amsacrine and CI-921 (as the isethionate salts) were kindly provided by the Parke-Davis Division of the Warner-Lambert Company. Conditioned medium (CM) was prepared as previously described [14, 15] and provided a convenient source of bone marrow colony-stimulating factor as it promoted colony growth from both murine and human bone marrow precursors [16, 17]. Human peripheral blood was obtained from the Auckland Blood Tranfusion Service and leucocytes were enriched by Ficoll density fractionation (Ficoll-paque, Pharmacia, density 1.076 g/ml). The resulting human peripheral blood leucocytes (10^6/ml) were incubated in culture medium containing 1% phytohaemagglutinin (PHA; Wellcome Laboratories, U.K.) and 10% FCS, for 7 days at 37°C in a humidified atmosphere of 5% CO_2 in air. CM was sterilized by filtration through 0.45 μm filters and stored at 4°C.

Growth of bone marrow colonies

Mouse bone marrow was obtained by removal of femurs from (C57BL/6 × DBA/2)F_1 mice which were bred in the laboratory animal facilities under constant temperature and humidity with sterile bedding and food. Both epiphyses were removed and the interior shafts were rinsed with culture medium (α-MEM; Gibco). Human bone marrow, originating from accident victims during the course of routine post-mortems, was kindly provided by Professor F.J. Cairns, Department of Pathology, University of Auckland Medical School.

Bone marrow cells were incubated with or without drug at 37°C for 1 h in 10 ml culture medium supplemented with 10% foetal calf serum (FCS; Gibco NZ Ltd). Cells were then washed and contaminating erythrocytes lysed by osmotic shock. The remaining leukocytes were counted using an electronic cell counter (Coulter Electronics). The bone marrow cells were then plated for colony growth in culture dishes (35 mm, Nunc) in a 1 ml suspension of methylcellulose (0.8% w/v; Sigma) in culture medium with 10% FCS, 50 μM 2-mercaptoethanol and 30% CM. Dishes were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Colonies of greater than 200 cells were counted using a dissection microscope after 5 days for murine colonies and 12 days for human colonies. Generally, 7.5×10^4 mouse or 10^5 human bone marrow cells produced approx. 150 colonies per dish in the untreated control cultures. Electron microscopic studies confirmed that the cells in the colonies were immature granulocytes and monocytes (results not shown).

Clonogenic assays of tumour lines

The SW620 human colon carcinoma and U937 histiocytic lymphoma lines were obtained from the American Type Culture Collection, Jurkat human leukaemia from Professor James D. Watson, Department of Immunobiology, University of Auckland Medical School, LLAK and LLTC murine lung carcinoma as previously described [18], the L1210 mouse leukaemia from Arthur D. Little Inc., U.S.A., and the P388 mouse leukaemia line from National Cancer Institute, U.S.A. All tumour lines were maintained *in vitro* in the laboratory by serial passages in culture medium supplemented with 10% FCS and antibiotics (penicillin 100 units/ml, streptomycin sulphate 100 μg/ml). L1210 and P388 cells were grown in the presence of 50 μM 2-mercaptoethanol. Cultures in exponential phase of growth containing approximately $1–2 \times 10^5$ cells/ml were used for experiments.

Haematopoietic cells were incubated with or without drug in 10 ml culture medium with 10% FCS at 37°C for 1 h. Cells were then centrifuged, washed, counted and plated in 35 mm culture dishes in 1 ml of methyl cellulose (0.8% w/v) in culture medium with 10% FCS, 50 μM 2-mercaptoethanol. The number of colonies were counted after incubation at 37°C in 5% CO_2 in air after 5 days for murine tumour lines and 10 days for human tumour lines. Percentage survival was plotted against logarithmic drug concentration and fitted by a best fit function. The D_{50} was defined as the drug concentration required to reduce the number of surviving clonogenic cells in a culture by 50% with respect to control cells.

Clonogenicity assays with SW620 cells were performed as follows. Cells in 100 mm dishes (3.3×10^5/ml) were trypsinized, collected by centrifugation, and resuspended in growth medium at 10^5 cells/ml in polystyrene tubes (5 ml per tube). Cells were exposed to drugs for 1 h at 37°C, then centri-

fuged, washed and plated in 60 mm dishes (5 ml per dish). Colonies of at least 50 cells were counted after 14 days.

RESULTS

Sensitivity of mouse and human bone marrow cells to amsacrine and CI-921

Bone marrow cells were exposed to amsacrine and CI-921 at concentrations approximating those encountered *in vivo*. The cells were then plated for growth of CFU-GM colonies in methylcellulose cultures. Data from several experiments have been combined in Fig. 1, where each point represents a single determination. A least squares equation (linear or cubic, depending on which had the best correlation coefficient) was fitted to the data of Fig. 1, giving the following D_{50} concentrations for amsacrine and CI-921: mouse bone marrow, 0.36 and 0.35 μM, respectively; human bone marrow, 0.31 and 0.38 μM, respectively. Thus, both mouse and human bone marrow CFU-GM show similar sensitivities toward each drug.

Sensitivity of L1210 leukaemia cells to amsacrine, CI-921 and daunorubicin

To compare the chemosensitivity of a representative mouse tumour cell line with that of CFU-GM cells, we carried out clonogenic survival assays with the murine L1210 leukaemia line which is known to be highly sensitive to these compounds [19]. Daunorubicin, a widely used clinical antileukaemia agent [20], was used for comparison. The survival curves for L1210 and murine bone marrow CFU-GM are compared in Fig. 2. The D_{50} value for mouse bone marrow CFU-GM with daunorubicin was found to be 0.32 μM, similar to that found for amsacrine and CI-921. The fitted regression lines show the D_{50} values against L1210 to be 0.053, 0.021 and 0.010 μM for amsacrine, CI-921 and daunorubicin, respectively.

Sensitivity of mouse and human tumour cell lines to amsacrine and CI-921

A dose of drug sufficient to kill approx. 60% of either mouse or human bone marrow cells, was chosen to compare the cytotoxicity of a single concentration of amsacrine and CI-921 towards other tumour cell lines. Mouse L1210 and P388 cells, as well as two different Lewis lung carcinoma lines, were compared to mouse bone marrow, while human Jurkat leukaemia cells, U-937 histiocytic lymphoma cells and SW620 colon adenocarcinoma cells were compared with human bone marrow. The order of cell line sensitivity to amsacrine (Table 1) was: L1210 > U-937 > Jurkat > P388 > LLAK > LLTC > SW620, with the leukaemia and lymphoma lines more sensitive than the carcinoma lines. The order for CI-921 was similar, with the

Table 1. Survival of clonogenic cells following exposure to amsacrine (0.5 μM) *or CI-921* (0.5 μM) *for* 1 h

Cell source	Percentage survival	
	Amsacrine	CI-921
Mouse bone marrow	42	40
Human bone marrow	39	40
Mouse leukaemia L1210	1.1	0.5
Mouse leukaemia P388	8	2.4
Human leukaemia Jurkat	6.6	1.3
Human lymphoma U937	3.6	1.3
Murine lung carcinoma LLAK	12	1.5
Murine lung carcinoma LLTC	60	30
Human colon carcinoma SW620	65	55

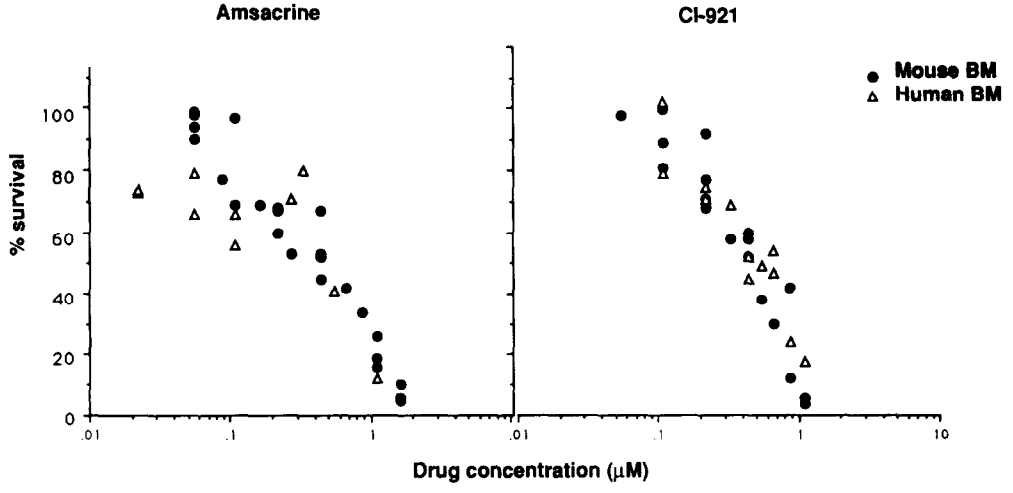

Fig. 1. Survival of murine and human bone marrow CFU-GM colonies after treatment with amsacrine or CI-921. Bone marrow cells were incubated for 1 h *with amsacrine or CI-921 then plated for growth of CFU-GM colonies.* (●) *Murine bone marrow CFU-GM at day* 5; (△) *human bone marrow CFU-GM at day* 12.

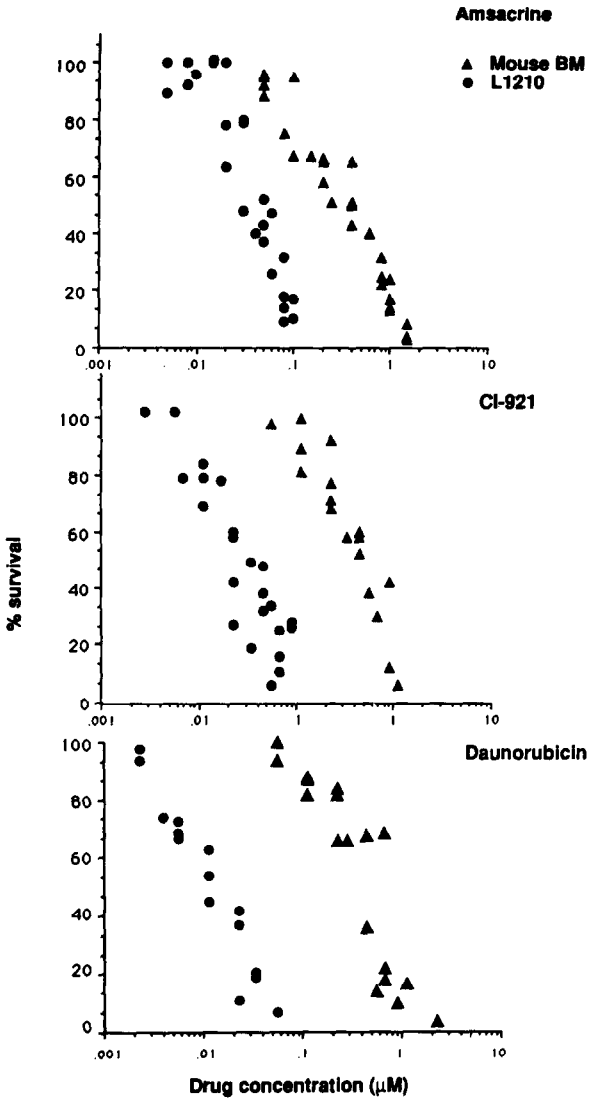

Fig. 2. Comparison of the sensitivity of L1210 murine leukaemia cells with that of murine bone marrow CFU-GM to amsacrine, CI-921 and daunorubicin. Cells were exposed to drug for 1 h then plated for colony growth. (●) L1210 colonies on day 5; (▲) murine bone marrow CFU-GM colonies on day 5.

exception of the Lewis lung carcinoma LLAK line which appeared particularly more sensitive to CI-921 than did the other carcinoma lines. The results in Table 1 also show that while the two drugs were equitoxic towards bone marrow, CI-921 was more potent than amsacrine against all the tumour lines tested.

DISCUSSION

We have compared the effects of amsacrine and CI-921 on bone marrow and tumour cells from both human and rodent sources. The results in Fig. 1 show that CI-921 and amsacrine have similar cytotoxic potencies toward normal bone marrow cells from humans and mice. The D_{50} following exposure to amsacrine or CI-921 for 1 h is approx. 0.35 μM for both human and mouse CFU-GM. This may be compared with the value of approx. 1.2 μM reported for human CFU-GM with amsacrine using an exposure time of 30 min [21].

The results in Fig. 2 show that all three drugs tested are selectively toxic to L1210 leukaemia cells. Beckman *et al.* [22] used ratios of D_{50} values for drugs against tumour cell lines and the corresponding CFU-GM cells as a measure of the therapeutic ratio. They showed for U937 cells that the therapeutic ratio was 1.35 for doxorubicin and 1.98 for 3′-deamino-3′-(3-cyano-4-morpholinyl) doxorubicin. The corresponding therapeutic ratios obtained here for U937 cells are 3.3 for amsacrine and 4.2 for CI-921 (data not shown). The therapeutic ratios for L1210 cells, determined from the data in Fig. 2, are 9 for amsacrine, 16 for CI-921 and 32 for daunorubicin. Thus, by this criterion CI-921 is more selective for L1210 cells than amsacrine but less selective than daunorubicin.

Because of the similarity of the CFU-GM survival curves for amsacrine and CI-921 we have compared tumour lines at a single drug concentration of 0.5 μM (Table 1), which was chosen to be in the range of *in vivo* exposure. Studies on the pharmacokinetics of amsacrine and CI-921 in mice indicate that average drug exposures, expressed as the area under the curve of a graph of free drug concentration versus time, after administration of the maximum tolerated dose are approx. 0.42 and 0.20 μmol.h/l, respectively [23]. In the *in vitro* experiments reported in Table 1, drug exposure was 0.5 μmol.h/l for total drug. Free drug fractions have not been determined under culture conditions but preliminary data suggest values of approx. 0.5 and 0.1, respectively, giving free drug exposures of 0.25 and 0.25 and 0.05 μmol.h/l, respectively.

Some degree of validation of the use of clonogenic assays as a measure of *in vivo* selectivity can be obtained by examining the results with mouse CFU-GM and tumour lines. The results in Table 1 show that the L1210 lymphoblastic leukaemia line is the most sensitive to amsacrine, followed by P388, LLAK and LLTC. This parallels the *in vivo* activity of amsacrine, where L1210 and P388 are highly sensitive [4], Lewis lung is marginally responsive [3] and LLTC cells are highly resistant [24]. The therapeutic ratios of CI-921 are higher than those for amsacrine for all cell lines tested, confirming the greater *in vivo* activity of CI-921 against all of these cell lines [3, 4, 24].

As the *in vitro* murine results appear to be predictive of *in vivo* responses, it is pertinent to ask what predictions can be made from the results with human cells. The Jurkat leukaemia and U-937 lymphoma lines are very sensitive to both drugs and, like all the other lines tested, are more sensitive to CI-921 than amsacrine (Table 1). On the other hand, the SW620 colon tumour, one of the more chemosensitive of the available colon tumour lines

[11], is highly resistant to both drugs. The *in vitro* clonogenic assay might profitably be used to investigate the sensitivity of other human tumour material to CI-921 in a search for sensitive tumours. This is particularly pertinent for lung tumour lines, in case the Lewis lung carcinoma models some types of human lung cancer.

It is not known whether the CFU-GM assay used here predicts for the chemosensitivity of the pluripotent stem cells which determine haematological toxicity and thus the limiting dose for both amsacrine and CI-921 in the clinic [5]. Although *in vivo* methods are available [25], the multipotential haemopoietic stem cell is not easy to clone *in vitro*. The CFU-GM is an early progeny of the multipotential stem cell [26], is the committed progenitor cell of the granulocyte–macrophage cell class, and can be easily cloned *in vitro*. As granulocytes represent 60% of the population of mature blood cells, CFU-GM have a major role in blood cell regeneration, and the CFU-GM assay may represent a highly relevant *in vitro* model for measuring bone marrow toxicity.

In conclusion, the studies show that CI-921 has greater *in vitro* selectivity than amsacrine against murine and human tumour cell lines, particularly leukaemia lines. Although the selectivity is only 2-fold better when measured in terms of ratios of D_{50} values for normal and tumour cells, the difference between 12% survival and 1.5% survival for cultured Lewis lung cells is probably enough to explain the difference in *in vivo* activity of the two drugs towards this tumour. CI-921 may also have a pharmacokinetic advantage in solid tumours [23]. If clonogenic data do predict *in vivo* responses, the results raise the possibility that CI-921 has superior activity to amsacrine in leukaemia and perhaps other human tumours.

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