Eur J Cancer Clin Oncol, Vol. 19, No. 11, pp. 1607–1613, 1983.
Printed in Great Britain.

0277-5379/83$3.00+0.00

Divergent Activity of Derivatives of Amsacrine (*m*-AMSA) towards Lewis Lung Carcinoma and P388 Leukaemia in Mice*

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Abstract—*A series of acridine monosubstituted derivatives of the antitumour agent amsacrine [4'-(9-acridinylamino)methanesulphon-m-anisidide] has been tested for activity against intraperitoneally inoculated P388 leukaemia and intravenously inoculated Lewis lung carcinoma growing in DBA/2J × C57BL/6J mice, and treated using a q4d × 3 intraperitoneal injection schedule. Whereas all derivatives tested exhibited moderate to high activity towards the leukaemia, activity against the lung tumour varied from inactive to curative. Amsacrine itself displayed low but statistically significant activity. Cyclophosphamide and 2-β-D-ribofuranosyl-thiazole-4-carboxamide (tiazofurin) were highly active. 5-Fluorouracil was active but doxorubicin, daunorubicin, ametantrone and mitoxantrone showed no significant activity. Since the Lewis lung carcinoma is responsive to a high proportion of agents active against solid tumours in the clinic, it is concluded that some derivatives of amsacrine could be considerably more active than amsacrine itself against human solid tumours.*

INTRODUCTION

AMSACRINE (*m*-AMSA; NSC 249992) was first reported to have antitumour activity by Cain and Atwell [1] and since this time has been the focus of an intensive analogue development programme in this laboratory [2]. Clinical trials, initiated in 1978, indicate amsacrine to have highly promising activity in the treatment of acute leukaemia, particularly in combination with other agents [3, 4]. It shows significant activity in malignant lymphoma and breast cancer, but little or no activity towards other solid tumours [5–7]. One of the aims of the current research programme in this laboratory is to develop analogues of amsacrine with a broader spectrum of clinical antitumour activity.

The most appropriate tumours for assessing amsacrine analogues would be those that are relatively resistant to common antitumour agents, and which respond best to those clinical agents which are useful in the treatment of human solid tumours. Following previous experience in this laboratory and because of the data available for other agents, we decided to use one of the mouse tumours in the screening panel of the National Cancer Institute (N.C.I.) U.S.A. The results of testing of a large range of clinical and experimental cytotoxic agents against a variety of tumours used at the N.C.I. has been reviewed recently bv Goldin and co-workers [8]. A summary of this data, in which 87 compounds were tested against 10 different tumours, is shown in Table 1. Goldin *et al.* [8] have delineated a subgroup of 12 clinical agents with activity against breast, colon and/or lung cancer. These are listed as 'subgroup I' agents in Table 1. (Etoposide is also a member of this group, but N.C.I testing data was not provided.) The remaining 19 agents, listed as 'Subgroup II' in Table 1, are active against haematological, childhood and gynaecological malignancies.

To be effective in predicting activity in human breast, lung or colon tumours, an experimental tumour system should show a good differential response between subgroup I and II agents.

Accepted 11 May 1983.
*This work was supported by the Cancer Society of New Zealand, Inc., by its Auckland Division and by the Medical Research Council of New Zealand.
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Table 1. *Response of different experimental mouse tumours to antitumour drugs in clinical use or in development*

	Clinical use category		Development category
	Subgroup I	Subgroup II	
L1210 leukaemia	12/12 100%	16/19 84%	40/56 71%
P388 leukaemia	11/12 92%	19/19 100%	46/51 90%
B16 melanoma	11/12 92%	16/19 84%	40/55 73%
Lewis lung	11/12 92%	7/18 39%	11/53 21%
Colon 26	10/12 83%	15/19 79%	36/51 71%
Colon 38	10/12 83%	14/19 74%	27/55 49%
CD8F1 mammary	11/12 92%	15/19 79%	37/56 66%
Xenograft s.c. MX-1	7/11 64%	5/15 33%	8/54 15%
s.c. LX-1	6/11 55%	3/16 19%	4/55 7%
s.c. CX-1	0/11 0%	0/15 0%	1/52 0.5%
s.r.c. MX-1	5/8 63%	7/12 58%	14/45 31%
s.r.c. LX-1	3/9 33%	2/9 22%	3/46 7%
s.r.c. CX-1	3/6 50%	1/9 11%	10/41 24%

Data are taken from a review by Goldin *et al.* [8]. Fractions indicate the number of active drugs out of the total number tested, with the percentage also shown. The 'clinical use' categories comprise the 31 compounds listed in Tables 4 and 8 of that review. Subgroup I comprises the 12 agents identified in that list as having clinical activity in breast, lung and/or colon cancer, namely CCNU, cyclophosphamide, doxorubicin, 5-fluorouracil, hexamethylmelamine, melphalan, methotrexate, methyl CCNU, mitomycin C, nitrogen mustard, *cis*-platinum and procarbazine. Subgroup II comprises the 19 remaining agents in that list, namely actinomycin D, amsacrine, asparaginase, BCNU, bleomycin, chlorambucil, chlorozotocin, cytosine arabinoside, dacarbazine, daunorubicin, D-O-norleucine, 6-mercaptopurine, methylglyoxalbisguanylhydrazone, neocarzinostatin, PALA, teniposide (VM 26), 6-thioguanine, vinblastine and vincristine. The 'development' category comprises 56 compounds in development, which are listed in Tables 10 and 11 of the review by Goldin *et al.* [8]. The data listed for xenografts comprises the subcutaneously implanted (s.c.) and subrenal capsule-implanted (s.r.c.) mammary, lung and colon xenografts.

Examination of Table 1 indicates that only the Lewis lung carcinoma, the subcutaneous lung xenograft and the subrenal capule-implanted colon xenograft provide more than 2-fold discrimination. It is interesting that the Lewis lung tumour and the human xenograft tumours are also the most stringent when applied to compounds in the 'development category' of Table 1.

This present study has utilised the Lewis lung carcinoma to evaluate the antitumour activity of a number of monosubstituted derivatives of amsacrine, selected as a representative subset of an earlier series [9]. All were highly active towards L1210 leukaemia and both *in vitro* and *in vivo* data have been presented previously [9, 10]. Results are compared with data for P388 leukaemia obtained in concurrent experiments using the same administration schedule (q4d × 3), route of drug injection (i.p.) and vehicle (30% v/v aqueous ethanol).

MATERIALS AND METHODS

Agents

Amsacrine analogues were all synthesised in this laboratory as previously described [9, and references therein]. Amsacrine analogues were dissolved in 30% v/v ethanol in water, and 0.1 ml was injected intraperitoneally for each dose. This solvent proved to be a suitable vehicle for dissolution of these agents, many being insufficiently soluble in water. Control animals received 30% alcohol alone, which was well tolerated with no obvious acute reaction. Subsequent life spans did not differ from animals receiving no vehicle. Doxorubicin (Farmitalia), daunorubicin (May and Baker), 5-fluorouracil (Hoffman-Laroche) and cyclophosphamide (Bristol-Myers) were obtained from the Auckland Hospital Pharmacy and dissolved in sterile water for injection. Mitoxantrone, ametantrone and tiazofurin were kindly provided bv the Warner-Lambert Company and were dissolved in sterile water for injection.

Mice

DBA/2J and C57BL/6J breeding pairs of mice were obtained through the generosity of Jackson Laboratories, Bar Harbor, ME, U.S.A. and used to initiate inbred colonies. Breeding mice were housed under constant temperature and humidity and with sterile bedding, water and food. BDF1

hybrid (DBA/2J × C57BL/6J) mice of either sex and of weights between 18 and 22 g were used for experiments. Individual groups of mice were matched for weight, and drug dosage was adjusted for pretreatment body weight.

Tumours

The P388 and Lewis lung cells used were kindly provided by the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, U.S.A. The cells were obtained as frozen stocks in 1977 and, after passage in carrier mice of the appropriate strain, were stored in liquid nitrogen. The P388 leukaemia was re-initiated from frozen stocks every 12 months and the Lewis lung tumour was re-initiated every 4 months.

P388 cells were passaged in DBA/2J female mice at weekly intervals by intraperitoneal injection of 10^6 cells. Cells were washed from the peritoneum after 7 days using sterile PBS (NaCl 8g/l, KCl 0.2 g/l, KH_2PO_4 0.2 g/l, Na_2HPO_4 1.15 g/l, $CaCl_2$ 0.1 g/l, $MgCl_2$ 0.1 g/l) and counted with an electronic particle counter. BDF1 hybrid mice were injected intraperitoneally with 10^6 nucleated cells for experiments.

Lewis lung tumours were grown subcutaneously and passaged in C57BL/6J mice every 14 days. The solid tumour was carefully dissected under aseptic conditions and disaggregated by passage through a 100-micron nylon monofilament mesh and a 26G needle. The resulting suspension contained mainly single cells (approximately 99%) with a few small clumps. Large nucleated cells were counted in 1% acetic acid using a haemocytometer. For drug testing cells (10^6 unless otherwise stated) were injected intravenously in PBS (0.2 ml) into tail veins of BDF1 mice of either sex.

Life extension assays

A typical experiment included 20 control mice (injected with vehicle alone) and 30 groups each of six mice which were treated with drug. At least three dose levels (at 1.5-fold increments) were used to encompass the optimal dose (defined as that giving the highest increase in lifespan without causing deaths from drug toxicity). Toxic deaths were those which occurred two standard deviations before the mean lifespan of control animals.

Mouse deaths were recorded daily. Time of death was averaged for groups of six mice and related to the mean day of death of the corresponding control group. Long-term survivors for P388 and Lewis lung tumours were recorded 50 and 60 days respectively after tumour inoculation. Life spans were calculated from the average of all animals dying up to these times, thus excluding long-term survivors, but including toxic deaths.

RESULTS

Characteristics of the tumours

The P388 and Lewis lung tumours were grown in accordance with the protocols suggested by the N.C.I., U.S.A. (ref. [10] and instruction 271E, protocol 3LL 39 for Lewis lung and 3PS31 for P388 leukaemia, provided by the N.C.I.). The characteristics of the tumours also conformed to these guidelines. The range of mean survival times in groups of control animals in P388 experiments was 10.0–12.8 days, with a mean of 11.3 days and a mean coefficient of variation of 7.0%. The mean survival times in groups of control mice in Lewis lung experiments varied from 15 to 18 days, with a mean of 16.7 days and a mean coefficient of variation of 15%. No long-term survivors were found among control animals.

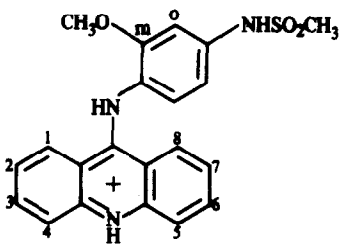
Animals were examined at various times after intravenous inoculation of Lewis lung cells. Multiple tumour foci were found in the lung but no tumours were found in the liver. Occasionally (incidence = 3%), in the course of experiments mice developed a solid tumour at the base of the tail. These mice were culled and excluded from analyses. In an experiment where animals were killed 9 days after tumour inoculation, an average of 23 tumours was observed on the surface of the lungs in each mouse. In animals which received subcutaneous implants of 10^6 tumour cells, lung metastases were found after termination at day 14.

In order to estimate the number of clonogenic cells in the tumour inoculum, animals were inoculated intravenously with different numbers of Lewis lung tumour cells. For an inoculation of 10^5 cells, one long-term survivor was found out of 12 animals, with the mean survival for the remainder of 22.4 days. For 10^4 cells, 9 out of 12 survived and the mean survival for the remainder was 29.7 days. Assuming Poisson statistics, this result provides an estimate of 27 clonogenic cells per inoculum of 10^6 cells, which is consistent with the number of tumour foci observed in the lungs.

Response of tumours to amsacrine analogues

Both P388 and Lewis lung tumours were initially treated using a q4d × 3 schedule beginning on day 1 after tumour inoculation. Results are shown in Table 2. All analogues were active towards P388 leukaemia, and all have previously been shown to be active against L1210 leukaemia using a qd × 5 schedule [9]. Over 10 assays using Lewis lung cells, amsacrine at the optimal dose (13.3 mg/kg on 5 occasions and 8.9 mg/kg on 5 occasions) provided an increase in

Table 2. Response of Lewis lung carcinoma and P388 leukaemia to amsacrine analogues



Compound	P388 leukaemia: drug days 1, 5, 9 Optimal dose (mg/kg)	P388 leukaemia: drug days 1, 5, 9 ILS max* (%)	Lewis lung: drug days 1, 5, 9 Optimal dose (mg/kg)	Lewis lung: drug days 1, 5, 9 ILS max (%)	Lewis lung: drug days 5, 9, 13 Optimal dose (mg/kg) Exp. 1	Exp. 2	Lewis lung: drug days 5, 9, 13 ILS max (%) Exp. 1	Exp. 2
Amsacrine analogues								
Parent	13.3†	78 ± 18‡	13.3†	38 ± 16‡	13.3†		42 ± 15‡	
3-CH_3	10.0	120(1)§	13.3	66	13.3	13.3	42	66
3-OCH_3	8.9	196(4)	—	—	13.3	8.9	52	15
3-F	45	224(1)	—	—	45	65	145	137(1)
3-Cl	20	232	—	—	30	30	140(4)	149(3)
3-Br	20	133(2)	20	247(5)	20	20	146(4)	145
3-I	30	137	—	—	30	45	97	86
3-NH_2	8.0	116	—	—	5.9	5.9	14	25
3-$NHCH_3$	3.0	117	2.7	11	1.8	2.7	5	14
3-$NHCOCH_3$	13.3	105	—	—	13.3	20	6	21
3-$NHCOOCH_3$	3.9	89	3.9	2	3.9	3.9	0	2
3-$N_3(CH_3)_2$	8.9	119	8.9	7	8.9	—	28	—
3-NO_2	5.9	73	5.9	0	5.9	5.9	21	2
3-$CONHCH_3$	30	115(1)	—	—	30	45	0	8
4-CH_3	20	172(3)	20	(6)	20	20	92	152
4-OCH_3	30	139(5)	—	—	30	45	143	147(2)
4-$CONH_2$	30	113	—	—	65	100	54	67
4-$CONHCH_3$	30	127	—	—	30	45	112	126(4)
4-$CONHCH_2CONH_2$	100	125(1)	—	—	65	100	11	8
Other agents								
Cyclophosphamide	225‖	216(5)	90‖	200(4)	225¶	—	185(5)	—
Tiazofurin (NSC 286193)	225	28	—	—	330	—	148(3)	—
5-Fluorouracil	65	104	—	—	65	—	59	—
Doxorubicin	3.9	79(1)	5.9	9	2.6	—	25	—
Daunorubicin	2.7	60	1.8	6	3.9	—	12	—
Mitoxantrone (NSC 301 739)	3-3	79(1)	—	—	3.9	—	27	—
Ametantrone (NSC 287 513)	27	62	—	—	30	—	17	—

*ILS max is the maximal percentage increase in lifespan, as defined in the text. For Lewis lung, an ILS max of less than 40% is considered non-significant in a single test [8].

†Optimal dose was 8.9 mg/kg in some experiments.

‡Mean ± standard deviation over several experiments.

§Figures in parentheses represent number of long-term survivors out of six mice.

‖Single dose, day 1.

¶Single dose, day 5.

lifespan of 38%. However, two agents, the 3-bromo and 4-methyl derivatives, provided cures of 5/6 and 6/6 animals respectively (Table 2). In order to provide a more discriminating assay whereby the most active compounds could be better ranked, the first day of the schedule for the Lewis lung tumour was changed to day 5 after tumour injection. This procedure had the advantage of providing a higher initial tumour cell population as a target for therapy. From the data in the previous section, it was likely that less than 100 clonogenic tumour cells were present at day 1, whereas, assuming a doubling time for these cells of 0.7 days[11], there would be in excess of 3000 cells on day 5.

Data using this day 5, 9, 13 schedule are also shown in Table 2. Duplicate experiments are shown separately for the Lewis lung tumour results, so that the degree of reproducibility of the tests is apparent. Over 14 assays, amsacrine provided a life extension of 42%, similar to that provided by the day 1, 5, 9 schedule. Results for the

two schedules are also similar for those compounds with low activity against this tumour. The main differences are for the two highly active compounds, and it is notable that although the activity using the day 5, 9, 13 schedule is reduced, several analogues provided a proportion of 60-day survivors.

Although scored according to the N.C.I. protocol at 60 days, some mice were kept for longer times. A small proportion (3 have been detected so far) of these died between 60 and 120 days after treatment, but the cause of death was not established. Examination of lungs of mice killed at 100 days showed no evidence of lung tumours.

Response to some other antitumour agents was also measured. Cyclophosphamide was highly effective when given as a single dose on either day 1 or day 5. Tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide), which has recently been found to be extremely active against the Lewis lung carcinoma by Robins *et al.* [12], was highly effective over a large range of doses. 5-Fluorouracil was also active. Contrary to the results of Goldin *et al.* [8], doxorubicin was not active in our system, at least with the dosage schedule and (i.p.) route of administration used here. Other workers have reported lack of activity with doxorubicin [13].

Daunorubicin, and two new anthracenedione derivatives, mitoxantrone and ametantrone, were also ineffective.

DISCUSSION

Amsacrine, an antitumour agent with little clinical activity against solid tumours, appears to have borderline but statistically significant activity towards the Lewis lung carcinoma in mice. The value of 25% life extension found by Goldin *et al.* [8] is within the range of 38 ± 16% found in our studies. Examination of a number of analogues of amsacrine, all of which are active against P388 leukaemia, reveals a striking divergence of activity against the lung tumour. All derivatives tested with amino or substituted amino substituents at the acridine 3-position are completely inactive. On the other hand, some compounds, such as the 3-halogen derivatives, the 4-methyl and the 4(*N*-methyl)carboxamide, are comparable with cyclophosphamide in their therapeutic activity and are considerably more dose-potent. The structure–activity relationships in the amsacrine series are thus quite different in these two different tumours growing in the same host and treated with the same schedule (q4d × 3).

Differences in the activity of amsacrine derivatives against these two different tumours could result from one or more of the following causes:

(*a*) *Differences in pharmacokinetics.* Although the route and schedule of drug administration is identical in the experiments in Table 2, the sites of tumour growth are different. For the P388 leukaemia, tumour and drug are administered to the same anatomical site. Lack of drug distribution to remote sites could therefore be responsible for the apparent lack of activity against the Lewis lung tumour.

(*b*) *Differences in tumour architecture.* Whereas P388 cells grow as an ascites, Lewis lung cells grow as solid tumours with possible barriers to drug penetration. Lack of drug penetration of the tumour could be responsible for lack of activity.

(*c*) *Differences in cell cycle kinetics.* Amsacrine demonstrates distinct cell cycle phase specificity [14] and selectivity for cycling vs non-cycling cells [15]. Analogues for which these parameters vary may have altered selectivity for cells in solid tumours.

(*d*) *Differences in cellular uptake or accumulation.* The intracellular free drug concentration of cationic drugs such as daunorubicin is regulated, probably by an energy-dependent outward transport mechanism [16]. This mechanism is presumably involved in cellular resistance to a variety of drugs, including amsacrine [17]. Differential transport effects with different amsacrine derivatives could be responsible for the variation of sensitivity of Lewis lung tumour cells.

(*e*) *Differences in target.* Amsacrine and its derivatives bind to double-stranded DNA [18] and cytotoxic activity is correlated with DNA binding [19]. Amsacrine induces the production of single- and double-stranded DNA breaks, as well as protein–DNA cross-links [20]. Various studies with derivatives of 9-anilinoacridine point to the great importance of the anilino substituents of the molecule for biological activity [21] and the suggestion has been made [18] that a ternary complex between amsacrine, DNA and protein could mediate the cytotoxicity of these compounds. If this is the case, it is possible that Lewis lung tumour cells and P388 leukaemia cells differ in their content of proteins able to produce such complexes.

Experiments in this laboratory are currently being devised to explore these possibilities. A comparison of drug effects on P388 cells growing either intraperitoneally or subcutaneously along the lines used in previous investigations for L1210 leukaemia cells [22] is in progress to assess the contributions of (a) and/or (b) above. A comparison of drug effects on cultured P388 or

Lewis lung tumour cells is also being made to assess the contributions of (d) and/or (e).

In conclusion, six monosubstituted derivatives of amsacrine have been identified as having very high activity against the Lewis lung tumour. They are the 3-fluoro, 3-chloro, 3-bromo, 4-methyl, 4-methoxy and 4-(*N*-methyl)carboxamide derivatives. The syntheses of all of these compounds, and their high activity against L1210 leukaemia in mice, have been reported previously [9, 23, 24]. The activity of the 4-methyl derivative (NSC 150440) against intraperitoneally, subcutaneously and intracerebrally inoculated L1210 leukaemia, as well as against first generation transplants of the C3H/HeJ spontaneous mammary tumour, has been previously reported [1]. The activity of disubstituted derivatives of amsacrine is currently being evaluated, with the aim of identifying the most active compound in this series. This will be submitted for clinical trial in the hope that the activity shown against the Lewis lung carcinoma will be reflected by a broadened clinical antitumour spectrum.

Acknowledgements—The authors would like to thank Drs W. A. Denny, G. J. Atwell and G. W. Rewcastle for resynthesis of some of the compounds used, Cherry Grimwade and Julie Harris for expert help in the animal testing, Dr S. B. Calveley for providing data on the histology of this tumour and Mrs S. A. Hill for preparing the manuscript.

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