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Effects of CI-921, an Analogue of Amsacrine, on Advanced Lewis Lung Tumours in Mice: Relevance to Clinical Trials*

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Abstract—*The 4-methyl-5-N-methylcarboxamide derivative of amsacrine (CI-921; NSC 343 499), which is now undergoing clinical trial, has been used to treat advanced Lewis lung tumours in mice. The response of the tumours was monitored by physical measurement, histological examination and flow cytometric analysis. The latter technique demonstrated that a single dose of CI-921 induced an efficient blockade of the tumour cell cycle in G2 phase. Histological assessment of lung nodules indicated a progressive increase in the cross-sectional area of both tumour cells and lung nodules after drug treatment. Under conditions providing a growth delay equivalent to a 6 log_{10} cell kill, and demonstrable histological destruction of tumour cells, the sizes of subcutaneous tumours did not decrease below the pre-treatment volumes, suggesting, at least for subcutaneous tumours, that changes in tumour size do not provide a good criterion for appraisal of drug effects. Schedule dependence studies on advanced lung tumours were carried out in an effort to provide information of specific relevance to clinical trials. A significant correlation of total dose with response was found, suggesting that clinical schedules allowing the total administered dose to be maximal should be used.*

INTRODUCTION

AMSACRINE, a derivative of 9-anilinoacridine synthesized by Cain and Atwell [1], is now widely used in the treatment of acute leukemia [2]. In the development of a second generation analogue of amsacrine with a broader spectrum of activity, two approaches have been used. Firstly the Lewis lung carcinoma [3] was utilized for *in vivo* screening of analogues since it was resistant to treatment by amsacrine [4]. Secondly, tissue culture assays with human cell lines were used to detect analogues with high activity towards cells cultured from solid tumours [5]. The combination of these two approaches led to the identification of a highly active analogue [6] of amsacrine, CI-921 (NSC 343 499; *N*,5-dimethyl-9-[-2-methoxy-4-methyl-sulphonyl aminophenylamino]-4-acridinecarboxamide; structure shown in Fig. 1), which is currently undergoing Phase I clinical trial [7].

Ideally, the results of preclinical studies should serve not only to identify active drugs but should also provide guidelines for clinical studies. Most preclinical schedule dependence studies are carried out on mice with transplantable leukaemias at an early stage of growth, whereas Phase I clinical studies are generally conducted with advanced stage carcinomas. A previous schedule dependence study of CI-921 utilized a mouse carcinoma as well as a leukaemia [8] but the effect of the drug on advanced disease was not investigated. We have attempted here to provide data which are more relevant to a clinical trial by treating advanced Lewis lung tumours which represent a significant tumour burden to the host.

As well as life extension and tumour growth delay assays, we have employed both histological and flow cytometric investigation of treated tumours in order to characterize the response of tumours to treatment by this agent. The results may have some relevance to the criteria for assessment of drug response in the clinical trials of this new agent.

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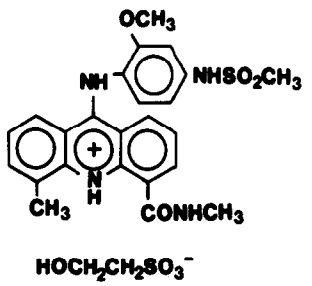

Fig. 1. Chemical structure of CI-921 (NSC 343 499).

METHODS AND MATERIALS

Establishment of solid tumours in mice

Lewis lung tumours were passaged every 14 days in carrier C57BL/6J mice by s.c. (subcutaneous) injection of 10^6 cells. Solid tumours were mechanically disaggregated by mincing with crossed scalpels and passage of the resulting suspension in PBS (NaCl 8 g/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) through a 100-μm nylon monofilament mesh and a 26 gauge needle, following which cells were counted in a haemocytometer. The resulting cell suspension was used to inoculate B6D2F1 hybrid mice either i.v. (intravenously) or s.c. with 10^6 cells. Further details have been published previously [4].

Flow cytometry

S.c. tumours were removed from mice, weighed and minced with crossed scalpels. A representative portion (300 mg) of the mince was placed in a glass centrifuge tube containing a small magnetic spin bar and weighed. A solution (1 mg/ml) of pronase (Calbiochem) in alpha-modified MEM containing 5% foetal calf serum was added to the minced tumour at a ratio of 1 ml per 60 mg tumour, the tube tightly capped and incubated with stirring at 37°C for 40 min. The cell suspension was then pipetted off the stir bar, centrifuged (900 ***g***, 5 min) and the pellet resuspended in medium plus serum (10 ml). The cells were washed by two more cycles of centrifugation and finally resuspended in growth medium and counted. The yield of cells was approximately 2×10^8 cells per gram. A portion of cells (10^6) was diluted by the addition of PBS (1 ml) containing 0.4% Triton X (Shell) and 10 μl of an ethanolic solution of diamidinophenylindole (0.2 mg/ml; Sigma) according to the procedure of Taylor [8]. Analysis was carried out using an Ortho Instruments ICP22A cytometer with a Model 2103 multichannel analyser. Proportions of cells in different phases of the cell cycle were estimated by drawing symmetrical curves over the G1- and G2-phase peaks, and measuring the corresponding areas.

RESULTS

Relationship between cytotoxic effect and tumour burden

The relationship between the therapeutic effect of intermittent schedule (q4d × 3) of CI-921 treatment and the tumour burden is illustrated for the Lewis lung carcinoma in Fig. 2, with data being pooled from a number of experiments. When treatment was initiated 1 or 5 days i.v. tumour inoculation (at which time only microscopic tumours were present) a high proportion of long-term survivors

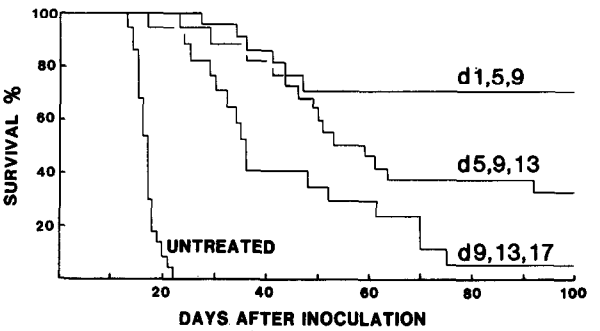

Fig. 2. Survival of mice inoculated i.v. on day 0 with 10^6 Lewis lung tumour cells and treated at various times later with CI-921 (20 mg/kg). Administration schedules (i.p.) for CI-921 were days 1, 5, 9, days 5, 9, 13 or days 9, 13, 17. Results are pooled from a number of experiments with 18–50 mice in each group.

(40–70%) was evident. However, with treatment commenced after 9 days, at which time plaques 1–2 mm in diameter were visible on the surface of the lungs, only one mouse survived for more than 200 days. Mice dying beween 50 and 200 days were checked at death and were found to have died from advanced lung tumours. Nevertheless, the median life span was more than twice that of the untreated mice. The median survival of 35 days corresponded to a tumour growth delay of 13 days, similar to that obtained when mice bearing advanced s.c. Lewis lung tumours are treated with CI-921 [6].

Schedule dependence of treatment of advanced lung tumours

Most preclinical schedule dependence studies employ single dose, daily and intermittent schedules. CI-921 was administered beginning at day 9 after tumour inoculation in a variety of such schedules, and the results are summarized in Table 1. Daily schedules of varying duration, and three intermittent doses separated by various intervals were compared. Two more complex schedules were designed to correspond in some aspects to Phase I clinical schedules (see Discussion).

Although the differences in response to different schedules in individual experiments were small, correlations between dose and life extensions could be tested statistically. For the Table 1 data, there were significant linear correlations between life extension and the total dose administered both for optimal doses at each schedule ($r = 0.85$;

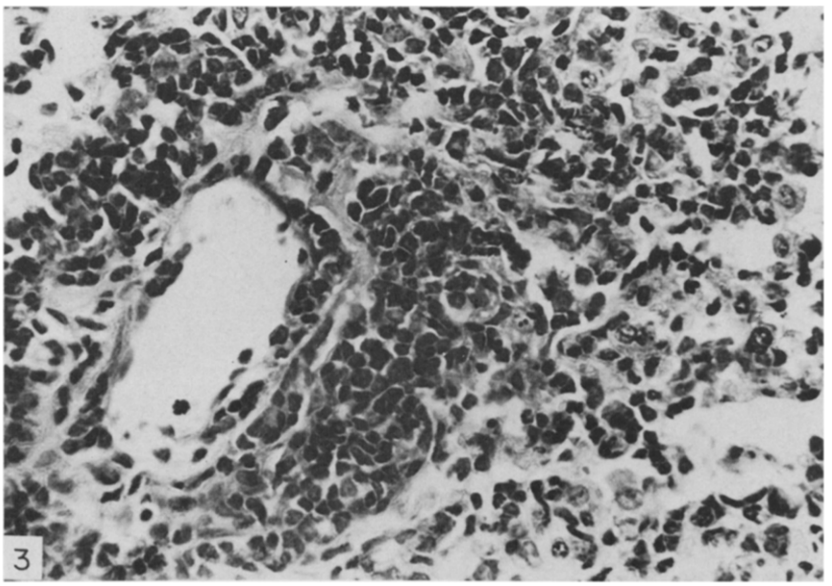

Fig. 3. *Histological appearance of untreated Lewis lung tumour growing as lung nodules* 9 *days after i.v. inoculation of* 10^6 *cells* (× 100)

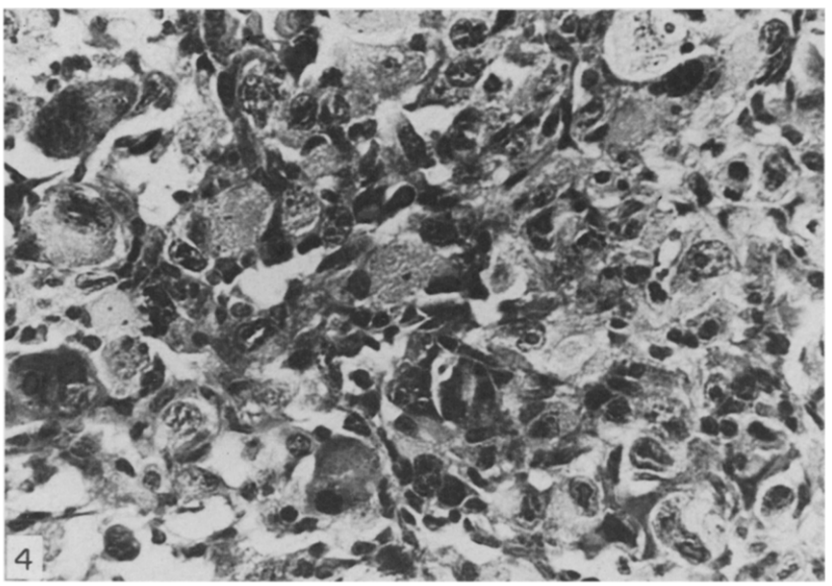

Fig. 4. *Histological appearance of treated tumour* 12 *days after inoculation and after i.p. treatment with CI-921* (20 mg/kg) *on day* 9 (× 100).

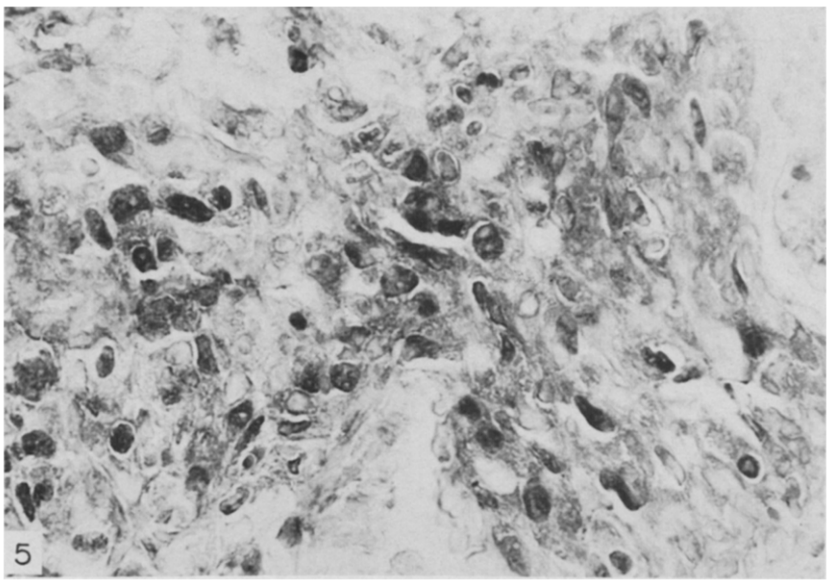

Fig. 5. *Histological appearance of treated tumour* 15 *days after inoculation, and after i.p. treatment with CI*-921 (20 mg/kg) *on days* 9 *and* 12 (× 100).

Table 1. Schedule dependence of activity of CI-921 against advanced Lewis lung tumours

Schedule	Individual dose (mg/kg)	Total dose (mg/kg)	ILS (%)
(a) Daily schedule, starting day 9 after tumour inoculation			
Day 9	45	45	79
Day 9	30	30	56
Days 9–13	5.9	30	58
Days 9–13	3.9	20	45
Days 9–17	5.9	53	82
Days 9–17	3.9	35	37
Days 9–23	3.9	59	89
(b) Three dose schedule, starting day 9			
Every 12 h	13.3	40	74
Every 12 h	8.9	27	58
Every 24 h	13.3	40	55
Every 2 days	20	60	86
Every 2 days	13.3	40	84
Every 3 days	20	60	112
Every 3 days	13.3	40	85
Every 4 days	30	90	110
Every 4 days	20	60	119
Every 4 days	13.3	40	55
(c) 'Clinical schedules', starting day 9, finishing day 29			
Every 10 days × 3	45	135	108
Every 10 days × 3	30	90	92
Every 4 days × 6	20	120	153
Every 4 days × 6	13.3	80	92
3 × 12 h every 10 days	13.3	120	123
3 × 12 h every 10 days	8.9	80	68

$P < 0.001$) and for all doses ($r = 0.79$; $P < 0.001$). High activity was obtained when the drug was administered every 4 days over a 20 day period. Administration of drug in a 3 × 12 h schedule, repeated every 10 days over the same period, was also effective.

Histological examination of lung tumours

Histological studies were carried out on tumours of mice treated with CI-921 (20 mg/kg) on a day 9, 12, 15 schedule. Prior to treatment, numerous tumour nodules were found scattered throughout the lung parenchyma. Some preference was found for growth around blood vessels, around bronchioli and in protrusions from the pleural surface (corresponding to surface plaques). The tumour cells were pleomorphic, small with a high nuclear to cytoplasmic ratio, densely stained, undifferentiated and showed no signs of necrosis (Fig. 3).

Three days after the first treatment, tumour cell colonies (nodules) were found to have increased in average aggregate cross-sectional area, and were composed of markedly swollen, pale staining cells including some multinucleate cells (Fig. 4). Three days after the second treatment there was a conspicuous reduction in the number of colonies. The tumours consisted predominantly of large foamy cells and giant cells together with fibroblasts and fibrous connective tissue. It was difficult to identify any tumour cells resembling those observed prior to treatment (Fig. 5). Lungs taken from animals 5 days after the third treatment showed no visible signs of surface plaques. Histological sections also showed mainly normal lung tissue with small and infrequent collections of indistinct dark cells, with occasional giant cells. Rarely, fibrous connective tissue pleural plaques were found.

Flow cytometric analysis of subcutaneous tumours

Flow cytometric studies of the response of the Lewis lung tumour to CI-921 could provide useful information on the response of advanced solid tumours to the drug. Such studies were not feasible in treated lungs because of the high proportion of host cells. Lewis lung tumours were therefore grown s.c.; previous studies have indicated a growth delay of approx. 11 days following treatment of advanced s.c. tumours with CI-921, with an absence of any significant decrease in tumour dimensions following treatment [6].

The effects of CI-921 were examined histologically (using the same schedule as for the lung tumours) to determine whether the drug response was similar to that obtained with lung tumour nodules. S.c. tumours before treatment (8 days after inoculation, 0.5–1.0 cm in diameter) were found to be highly cellular with a minimum of stroma but with numerous blood vessels. There was marked invasion of surrounding muscle tissue and extensive areas of clearly demarcated necrosis was observed. Three days after the first of three treatments (q3d × 3) with CI-921 (20 mg/kg), swollen cells and multinucleate giant cells were evident, as were conspicuous intercellular spaces. Cellular changes were uniform throughout the tumour, extending from blood vessels to areas of necrosis. Tumours taken 5 days after the third treatment showed an almost uniform change towards swollen, pleomorphic, sometimes multinucleate forms, with a loss of intensity of nuclear staining and an increase in eosinophilic staining of cytoplasm. Many cells exhibited pyknotic, fragmented or absent nuclei. Results were therefore similar to those histological changes noted after treatment of lung tumours.

Flow cytometric analysis of DNA content of cells from advanced s.c. tumours were then carried out. In untreated tumours, a large proportion of the cells were found to be in the S and G2 phases of the cell cycle (Fig. 6A). G1-phase tumour cells had a DNA content close to tetraploid and could thus be distinguished from host cells in the tumour. Approximately 45% of cells in the tumour were in S phase, consistent with previously published values of 45% for the labelling index of this tumour [9].

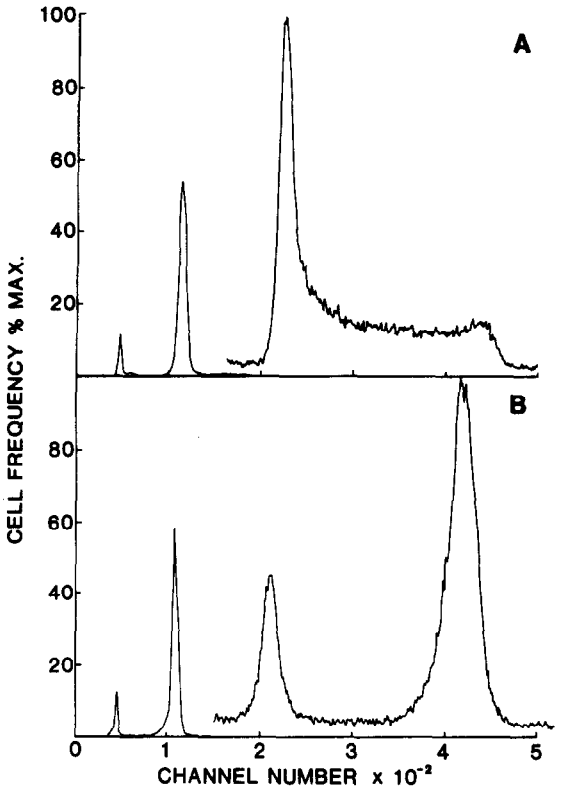

Fig. 6. Flow cytofluorometric profiles of DNA content of cells isolated by pronase digestion from s.c. Lewis lung tumours (0.5–1.0 g). *Tumours were removed from mice either before (A) or* 24 h *following (B) a single i.p. injection of CI-921* (30 mg/kg). *The profile peaks are from left to right: marker pigeon red blood cells (channel* 48), *host diploid cells (channel* 110, *on a different vertical scale), tetraploid (approximately) tumour cells (channel* 210) *and G2-phase tumour cells (channel* 420). *The host cells comprise approx.* 50% *of the total isolated cell population.*

The effect of a single dose of CI-921 (30 mg/kg) was then determined. The proportion of cells in G2 phase increased significantly by 8 h after treatment, and reached a maximum after 24 h (Fig. 6B). The rate of accumulation of cells in G2 phase after drug exposure was consistent with rapid cycling of a high proportion of the tumour cell population. A reduced degree of G2 arrest was obtained if the lower doses of CI-921 were utilized. If tumours were analysed 48 and 72 h after treatment, progressively lower yield of cells was obtained, although G2 arrest was still evident.

DISCUSSION

There are a number of difficulties in designing administration schedules for clinical trial based on experiments with mice. The cell division times, and the tumour doubling times, in tumours of patients (particularly with advanced disease) and in transplantable mouse tumours are considerably different. The rates of distribution, metabolism and elimination of drug in mice and humans are likely to be quite disparate. Furthermore, the recovery time for mouse host tissues, particularly bone marrow, is generally considerably shorter in mice than in humans. In spite of these problems, it appears to be worthwhile to examine experimental models in order to develop treatment principles which may be of relevance to clinical trials. In the present study we have employed mice with advanced carcinomas in order to determine both the schedule dependence to treatment and the response of tumour cells.

The dependence of antitumour effect, as measured by increases in lifespan, on the time of initiation of treatment is quite substantial (Fig. 2). This confirms the results of other studies [10, 11] showing that, as the tumour mass increases, the outcome of chemotherapy is negatively affected. The causes of this reduced chemotherapic efficiency can be attributed to the formation of pharmacologic sanctuaries, alteration of cell kinetics and development of drug resistance in some tumour cells.

The dependence of antitumour effect on treatment schedule (Table 1) is quite small, and in most cases the differences in response between two different schedules is not statistically significant. However, there is a highly significant linear correlation between response and total administered dose, which confirms a previous study with CI-921 using earlier stage experimental tumours [12]. The results suggest that the best clinical treatment schedule will be that which allows the maximal total dose at equivalent patient toxicity, and confirms clinical studies with other drugs [13].

Schedules can be adequately compared only when treatment is administered over the same time-frame. Three schedules have been compared (each the average of three separate experiments) where the time-frame is 30 days. The interval of 10 days in the mouse was considered to be approximately equivalent to the interval of 3–4 weeks used in humans between successive treatments, since host recovery is faster in the mouse. Similarly, the interval of 4 days in the mouse was considered to be similar to the interval of 1 week in humans. Because of the shorter doubling times of mouse cell lines than human cell lines, the 12 h interval in mice was considered to be equivalent to 1 day in humans. Thus the three (30 day time-frame) schedules in Table 1 were designed to reflect single dose treatment every 3 weeks, single dose treatment weekly, and three daily doses repeated every 3 weeks, which are currently being used in Phase I clinical trials of CI-921. In mice, the 4-day repeated schedule was best, significantly ($P < 0.05$) better than the single dose schedule, while the 3 × 12 h repeated schedule was intermediate. Assuming an 18 h doubling time for the *in vivo* tumour [6, 9] and no reversible growth inhibition, the best schedule provided a total cell kill of 11.7 $\log_{10}$ units, or almost 2 $\log_{10}$ units per dose. If the results of Table 1 can be extrapolated to the clinical situation, it could be concluded that a weekly administration schedule would allow the

highest total dose to be administered, and would provide the best chance of a response.

The response of treated tumours was measured both histologically and flow cytometrically. The earliest response was an accumulation of cells in G2 phase, and histological studies indicated that this was accompanied by an increase in the average diameter of the lung nodules, consistent with a physical enlargement of individual tumour cells. Although such lesions appeared to be virtually devoid of viable cells, they did not disappear until more than 1 week after initial treatment with CI-921. In the case of s.c. tumours, which were considerably larger at the time of treatment, even though a high degree of cell killing was evident, the physical size of the tumours did not decrease with time. The time required for dead cellular material to be removed from the tumour occupied approximately the same time as that required for tumour regrowth. If such a situation occurred in human tumours, a large antitumour effect in terms of cell kill would not be categorized as a partial remission because of the failure of the tumour dimensions to decrease. In this respect, CI-921 differs from cyclophosphamide [6] and tiazofurin [14 and unpublished results], which cause a similar growth delay but also significantly reduce the tumour dimensions. Different clinical criteria for response may have to be used for CI-921 in comparison with those used previously for other antitumour agents.

Flow cytometric analysis of s.c. Lewis lung tumours indicate that a single therapeutic dose of CI-921 is capable of producing a dose-dependent arrest of cycling tumour cells in G2 phase of the cell cycle. Examination of the kinetics of entry into G2 phase can therefore provide information about the rate of progression of cells through the cell cycle. In the case of the Lewis lung tumour, it is apparent that most of the tumour cells are in active progression. In a more slowly growing variant of the Lewis lung tumour, a similar kinetic experiment demonstrates the existence of a fraction of quiescent tumour cells [15]. The use of CI-921 in conjunction with DNA staining and flow cytometry [6, 15–17] promises to be a useful technique for stathmokinetic analysis of cell populations, complementing existing techniques with agents which arrest cells in mitosis.

CI-921 is capable of inducing significant regressions of advanced Lewis lung tumours, whereas the parental compound amsacrine is inactive. *In vitro* studies have shown that the relative toxicities of amsacrine and CI-921 are similar towards Lewis lung cells and P388 leukaemia cells [18] as well as towards human carcinoma cells and leukaemia cells [6]. *In vivo* studies suggest that CI-921 may have better distributive capacity, allowing effective killing of rapidly cycling tumour cells [18]. The clinical advantage of CI-921 may therefore be in its ability to distribute well within tumours. The best schedules, based on experimental tumours, will require multiple cycles of intermittent doses.

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