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# Selectivity of *N*-[2-(Dimethylamino)ethyl]acridine-4-carboxamide Towards Lewis Lung Carcinoma and Human Tumour Cell Lines *In Vitro**

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**Abstract**—N-[2-(*Dimethylamino*)*ethyl*]*acridine-4-carboxamide* (*AC*; *NSC* 601316) *is a chemically novel antitumour agent which is thought to interact with DNA topoisomerase II and which has DNA binding properties which are distinct from other acridine derivatives such as amsacrine and its disubstituted analogue CI-921. AC is one of the most active agents, experimental or clinical, against the Lewis lung carcinoma in mice. AC is the first acridine derivative in our hands to show higher activity against cultured Lewis lung cells than against leukaemia lines. AC is more active against two human leukaemia cell lines (U-937 and Jurkat) than against a melanoma line (MM-96) and is inactive against the HT-29 human colon line. With all cell lines tested, cytotoxicity was higher at AC concentrations of* 3–6 μM *than at* 15–20 μM. *AC at a concentration of* 20 μM *inhibited the cytotoxicity of amsacrine and CI-921, but not that of another topoisomerase-directed drug doxorubicin. A Lewis lung line which had been cultured for a long period was less sensitive than a line freshly isolated from mice, but sensitivity of the cultured line recovered after it was multiply passaged* in vivo. *Long-term cultures may therefore be less appropriate than short-term cultures for predicting effectiveness of AC* in vivo.

## INTRODUCTION

*N*-[2-(Dimethylamino)ethyl]acridine-4-carboxamide (AC; NSC 601316; see Fig. 1 for structure) was recently synthesized in this laboratory and found to have curative activity against the Lewis lung carcinoma in mice [1]. It was synthesized in a continuation of a programme which produced two other acridine derivatives, amsacrine [2] which is in clinical use for the treatment of acute leukaemia [3], and its 4-methyl,5-(*N*-methyl)carboxamide disubstituted derivative CI-921 [4] which is presently undergoing clinical trial [5].

Amsacrine has only marginal activity against Lewis lung carcinoma, whereas CI-921 has high activity [4] and AC is curative [1]. Thus, if the trials of CI-921 demonstrate significant activity against human tumours, AC will be an obvious candidate for further clinical studies. We have therefore commenced a comparison of the relative efficiency of killing of mouse and human tumour cells by AC.

AC inhibits the strand-passing activity of mammalian topoisomerase II and stimulates the formation of double-stranded DNA breaks and protein–DNA cross-links in cultured L1210 cells [6]. These results, together with the observed cross-resistance to a P388 leukaemia line with altered topoisomerase II activity, implicate topoisomerase II as the target of cytotoxic action of AC as well as of amsacrine and CI-921 [6]. However, AC differs in a number of respects from amsacrine, and it is not known which differences are responsible for its vey high activity against Lewis lung carcinoma. It is more lipophilic and the ionization of the acridine nitrogen is almost completely suppressed [1]. It binds to double-stranded DNA by intercalation, and kinetic studies using stopped flow techniques [7] have shown that the rate of dissociation of compounds of this type from the DNA intercalation site is much slower than that of amsacrine or CI-921.

In the present study, we address the question of cell line selectivity using clonogenic techniques. An established line (LLTC) derived from Lewis lung

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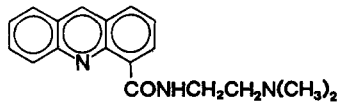


*Fig. 1. Structure of AC.*

carcinoma [8], as well as a newly developed line (LLAK) [9], have been compared with four human tumour lines (U-937 and Jurkat leukaemias; MM-96 melanoma; HT-29 colon) in their sensitivity to AC.

## MATERIALS AND METHODS

*Materials*

Acridine derivatives were synthesized in the laboratory or were made available from stocks at the Parke-Davis Division of the Warner-Lambert Company, Michigan, U.S.A. Doxorubicin was obtained from Farmitalia.

*Cell lines*

LLTC cells were adapted for *in vitro* growth from the Lewis lung tumour by Wilkoff and co-workers at the Southern Research Institute [8]. They were obtained from Dr R.C. Jackson (Warner–Lambert Company, Ann Arbor, Michigan, U.S.A.) in 1981. LLAK cells were established independently in culture from the original Lewis lung tumour as described [9] and used in experiments only at low passage number (less than 12).

MM-96 human melanoma cells (provided by Dr R. Whitehead, Ludwig Institute for Cancer Research, Melbourne) and HT-29 human colon carcinoma cells (provided by Dr J. Fogh, Sloan-Kettering Institute, New York), were maintained as described, except that antibiotics were used [10]. Human leukaemic cell lines Jurkat (provided by Professor J. Watson, University of Auckland) and U-937 (obtained from the American Type Culture Collection) were cultured as described [11].

*Development of LL23 line*

The LL23 cultured line was derived from the LLTC line by growing the latter as tumours in mice by serial transplantation as follows. LLTC cells were inoculated s.c. into B6D2F1 mice in which they grew progressively to reach a size of 1 $cm^3$ in about 3 weeks. Tumours were excised, disaggregated mechanically by passage through a nylon screen (100 μm mesh size) [9], and cells injected s.c. into a second group of host animals. Tumours were propagated as above over 23 successive transplantations covering a period of about 12 months. After 23 passages a tumour was excised, minced with crossed scalpels and disaggregated by pronase digestion [9]. Tumour mince (60 mg tumour/mg pronase/ml growth medium) was incubated with stirring for 40 min at 37°C, the cells recovered by centrifugation, and washed once to give a recovery of $7 \times 10^7$ cells/g tumour. Cultures were initiated at $10^4$ cells/ml under an atmosphere containing either 20% or 5% oxygen. The cells grew readily and at the same rate under both conditions, and all subsequent passages (less than 12) of this new cultured subline, designated LL23, were performed as for the parent LLTC cells.

*Clonogenicity assays*

LL sublines, MM-96, and HT-29 cells were plated at $10^5$ cells/ml in 100 mm dishes (15 ml growth medium per dish). Unless otherwise stated, cells were trypsinized 18 h later, collected by centrifugation and resuspended to a concentration of $10^5$ cells/ml in αMEM supplemented with FBS (10%) and antibiotics in polystyrene tubes (5 ml/tube). Cells were incubated at 37°C for 1 h in the presence of a range of concentrations of cytotoxic agents. Control (drug-free) tubes contained solvent at a concentration equivalent to the maximum concentration of solvent present in the drug-containing tubes. After incubation the cells were collected by centrifugation, washed twice, counted, and plated in 60 mm dishes. Cultures were fixed and stained after 10 (Lewis lung sublines), 12 (MM-96), or 14 (HT-29) days using methylene blue (5 g/l) in 50% aqueous ethanol. Colonies comprising over 50 cells were counted.

An experiment was performed to assess the cytotoxicity of acridine carboxamide towards cells from a large s.c. LLTC tumour, both immediately after tumour extirpation and disaggregation, and after 1 day in culture. The tumour was disaggregated by mincing and pronase digestion (cell recovery $1.6 \times 10^8$ cells/g) and cells exposed to acridine carboxamide as described above. Maximal colony-forming efficiency required that irradiated LLTC cells (35 Gy, $^{60}Co$ source) were added to give a total concentration of $10^5$ cells/dish with cultures incubated in the presence of 5% oxygen [12].

Jurkat and U-937 were cultured at $10^5$ cells/ml and after 1 day exposed to AC for 1 h as described for the other cells. Cells were cultured in 35 mm dishes containing an underlayer of 0.3% agarose and a plating layer of 0.2% agarose (both 1 ml) in humidified boxes for 12 days. Colonies were counted using an Olympus stereo microscope at 40-fold magnification.

## RESULTS

In vivo *tumour growth inhibition by AC*

AC was curative in 90% of mice with i.v. inoculated Lewis lung cells when treated i.p. with drug on days 5, 9 and 13 (100 mg/kg/dose) after tumour inoculation (Fig. 2; cf. Ref. [1]). The dose–response curve was steep and administration of a dose of 65 mg/kg × 3 induced 60% life extension with 27%

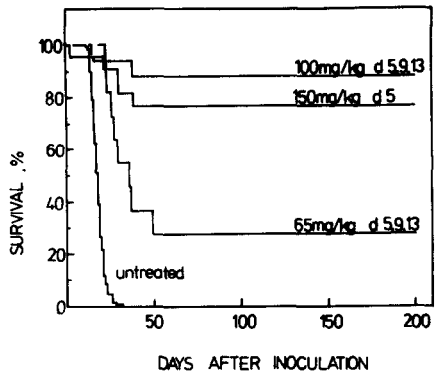


*Fig. 2.* In vivo *activity of AC against Lewis lung carcinoma. Tumour cells were injected i.v.and drug injected i.p. at the indicated time. Controls comprised approx. 20 mice* [4].

long term survivors. A single dose schedule was also effective (Fig. 2). It was not possible to test LLTC in this way because no lung colonies were formed following i.v. inoculation of cells. An experiment with s.c. LLTC tumours indicated that AC was inactive, extending the observation that this tumour is resistant to a variety of antitumour agents active against Lewis lung. LL23 cells were found to cause the growth of lung tumours following i.v. inoculation, although the number of lung nodules was more variable between mice and the average lifetime of untreated mice (20.7 days) was longer than that for Lewis lung (typically 16.7 days). AC was found to be active against this tumour, inducing a mean increase in survival time of 116%. However, no animals were cured by AC (100 mg/kg/dose on days 5, 9, 13), and a dose of 65 mg/kg × 3 was not significantly active.

AC was also tested against L1210 and P388 leukaemia lines using established protocols [2, 4] and showed much lower activity. Using a dose of 65 mg/kg administered on days 1, 5 and 9 after tumour inoculation, survival times were increased by 27 and 60%, respectively.

In vitro *cytotoxicity of AC towards Lewis lung sublines*

The cytotoxicity of AC towards three cultured sublines derived from the Lewis lung carcinoma was investigated. Exponentially growing cells were exposed to AC for 1 h, then washed free of drug and assayed for their ability to form colonies *in vitro*. The results indicate that the lines differ in their sensitivity to AC (Fig. 3) both in $D_{10}$ values and in the maximum achievable cell kill. The sensitivity of LLTC cells was less than for LLAK and LL23 (Table 1). Although LL23 appeared to be more sensitive than LLAK at 3 μM, this was not observed in other experiments (data not shown). High AC concentrations (15 μM) reproducibly caused less killing than at low concentrations (Fig. 3).

A comparison of AC responsiveness of the LLTC

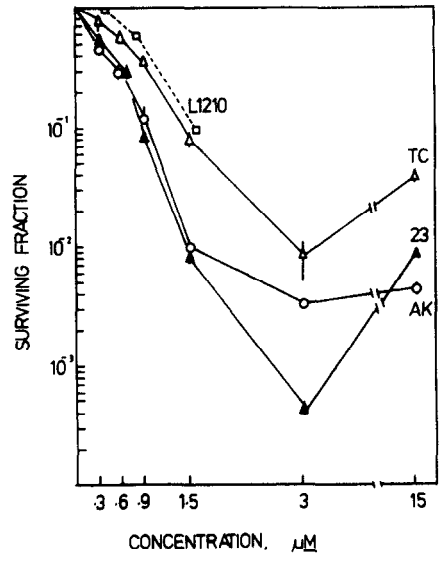


*Fig. 3. Cytotoxicity of AC towards LL sublines LLTC* (△), *LL23* (▲), *and LLAK* (○). *Cells initiated in culture 1 day previously at* $10^5$ *cells/*ml *were exposed to AC for* 1 h *at the concentrations shown. Bars in this and subsequent figures represent standard errors for duplicate cultures where they exceed the size of the symbol, unless stated otherwise. Data for L1210 cells* [6] *are included for comparison* (□).

line and its derivative LL23 line was made on 4 successive days to investigate the role of cytokinetic factors in drug sensitivity. After the first day in culture, cells were actively proliferating, and 2 and 3 logs of cell kill were observed for LLTC and LL23 cells respectively (Fig. 4). Again, higher AC concentrations caused less killing.

*Cytotoxicity of AC towards LLTC cells freshly explanted from a tumour*

An experiment was performed to investigate the sensitivity of LLTC cells prepared freshly from a s.c. tumour. Cells were recovered from a tumour (1.0 g) by pronase digestion and exposed to AC either immediately or following culture for 24 h, under which conditions a large proportion of cells commenced active proliferation as shown by flow cytometry (data not shown). The cells prepared directly from the tumour were totally refractory to AC at all concentrations tested but after culture, some 95% cells were killed at 4 μM AC (Fig. 5). Again, high concentrations of AC (20 μM) caused markedly less toxicity.

*Inhibition of other drug cytotoxicity by high AC concentrations*

The ability of AC at high concentrations to inhibit its own cytotoxicity (Figs. 3 and 4) prompted an investigation of the effect of high concentrations of AC on the cytotoxicity of other intercalating agents. LLTC cells were incubated for 20 min in drug-free growth medium or in medium containing AC at 20 μM. After 20 min, amsacrine, CI-921, or doxorubicin at several concentrations were added

*Table 1. Cytotoxicity of AC towards different cell lines*

| Cell line | Activity *in vivo* | *In vitro* cell kill (clonogenic assay) | |
|---|---|---|---|
| | | Max. | $D_{10}$ |
| Lewis lung lines* | | | |
| LLAK | Curative | >99.9% | 1.1 |
| LLTC | Inactive | 99% | 1.8 |
| LL23 | Active | 99.9% | 0.9 |
| Human tumour lines | | | |
| HT-29 (colon) | — | 40% | — |
| MM-96 (melanoma) | — | 80% | — |
| U-937 (lymphoma) | — | 90% | 2.0† |
| Jurkat (leukaemia) | — | >94% | 1.0 |

*LLAK was derived directly from the *in vivo* line; LLTC was a long term cultured line and LL23 is derived from LLTC after multiple *in vivo* passage. Data averaged from several experiments.

†Results of a second experiment, not shown in Fig. 7.

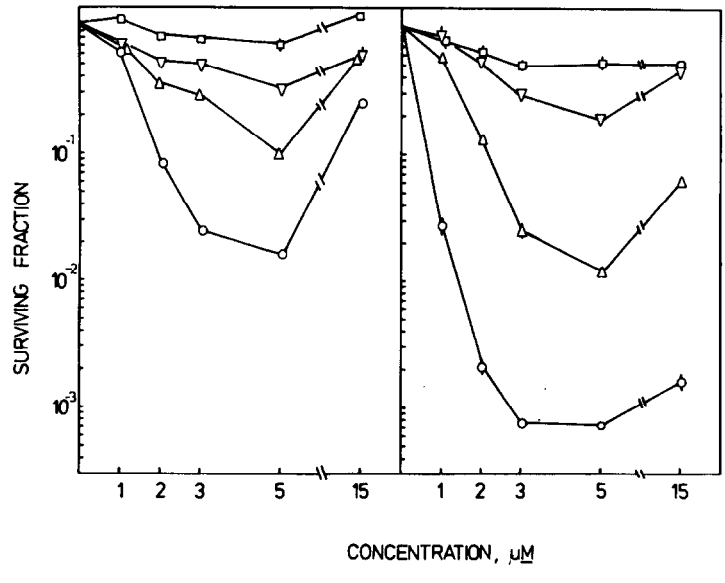


*Fig. 4. Cytotoxicity of AC towards LLTC and LL23 cells in cultures of varying proliferative activity. Cultures of LLTC (left panel) and LL23 (right panel) cells were initiated at $10^5$ cells/ml. Cells were trypsinized and assayed for sensitivity to AC after 1 (○), 2 (△), 3 (▽), and 4 (□) days.*

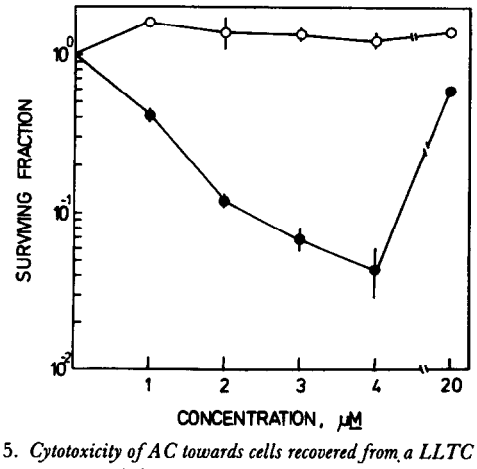


*Fig. 5. Cytotoxicity of AC towards cells recovered from a LLTC tumour. Cells were prepared from a LLTC tumour and exposed to AC either immediately (○) or 1 day after initiating cultures at $10^5$ cells/ml (●).*

to the tubes, and the incubation continued for another hour. Cells were then washed free of drug and clonogenicity determined as before. The results (Fig. 6) demonstrate that amsacrine alone at 8 μM, and CI-921 alone at 1.5 μM produced 99% cell kill. However, when incubated with cells in the presence of AC (20 μM), neither agent was able to inflict any increment in cell kill in excess of that due to AC alone (about 50% loss of clonogenic potential). A contrasting result is observed with a non-acridine drug, doxorubicin, for which similar survival curves were generated in the absence or presence of AC (20 μM).

In vitro *cytotoxicity of AC towards human cell lines*

Clonogenicity assays were carried out on human

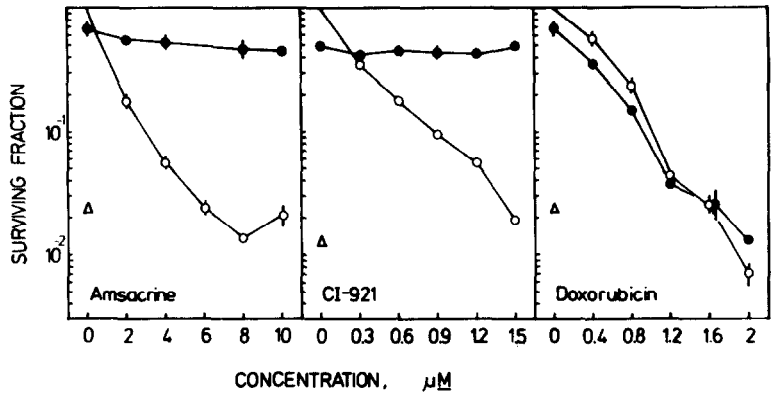


*Fig. 6. Effect of high concentrations of AC on cytotoxicity of other intercalating antineoplastic agents. Cells, prepared 1 day after initiating cultures at $10^5$ cells/ml, were incubated for 20 min in the absence of AC (○) or the presence of AC at 20 μM (●). After 20 min amsacrine, CI-921, and doxorubicin were added to the tubes at the concentrations shown on the abscissa, and the incubations continued for 1 h. Survival of clonogenic cells was then determined [9]. The triangle represents survival of cells exposed to AC (5 μM) alone for the 80 min duration of the assay.*

cancer cells. The histiocytic lymphoma U-937 showed a 90% loss of clonogenicity at 2.5–5 μM AC above which the cytotoxic effect declined to a minimum at 15 μM (Fig. 7). Similar results were obtained with the human Jurkat leukaemia line (Table 1). A human melanoma MM-96 and colon carcinoma HT-29 cell line also showed a biphasic response to AC, with maximum cell killing at 6–8 μM and a diminished effect at higher concentrations. Concentrations in excess of 30 μM also resulted in loss of viability (data not shown).

## DISCUSSION

AC has very high *in vivo* activity against Lewis lung carcinoma (Fig. 2), moderate activity against P388 and poor activity against L1210. This behaviour contrasts sharply with compounds in the amsacrine series, where activity measured with either

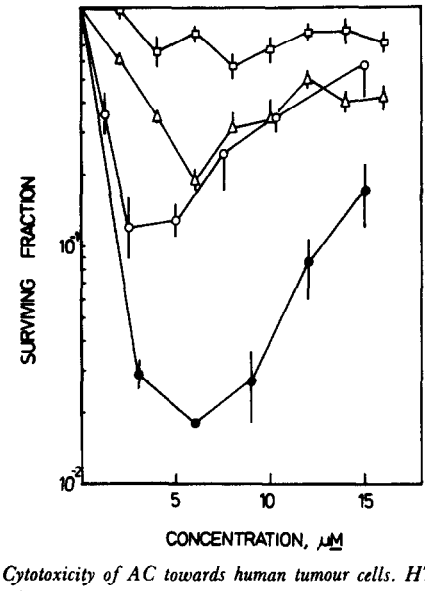


*Fig. 7. Cytotoxicity of AC towards human tumour cells. HT-29 (□), MM96 (△) and U-937 (○). LLTC (●) is included for comparison. Cells were exposed to AC for 1 h at the concentrations shown on the abscissa. Symbols represent means ± standard errors of three replicates.*

L1210 or P388 life extension assays is never lower than that found with Lewis lung [4]. Some explanation for this selectivity can be deduced from the present *in vitro* results. The mean $D_{10}$ concentrations (10% survival) of AC, amsacrine and CI-921, measured over 2–6 experiments, are 1.1, 0.9 and 0.37 μM for LLAK cells (Table 1 and unpublished results) and 1.6, 0.14 and 0.06 μM for L1210 cells [6]. Thus AC shows 1.5-fold selectivity for LLAK cells while the other two acridine derivatives show 6-fold selectivity for L1210 cells.

Cytokinetic cells also influence the *in vivo* cytotoxicity of AC. The results of Fig. 4 show a dependence of cytotoxicity on cellular cytokinetics, since cells in the late logarithmic phase of cell growth are considerably less sensitive to AC. Such cytokinetic resistance has been observed with amsacrine and CI-921 although the effects are not so marked [12]. As measured by clonogenic survival assays, LLTC cells removed directly from a subcutaneous mouse tumour were highly resistant to AC (Fig. 5) but became sensitive after culture for 24 h. Flow cytometric analysis of these cells indicated that the S-phase cell population was low in the original tumour but rose significantly in culture. In view of the established relationship between cell growth status, topoisomerase II activity and cytotoxicity which has been established for amsacrine [13–15], it is likely that AC acts most effectively against cells with high topoisomerase II levels.

The reduction of cytotoxicity in culture observed at AC concentrations around 10 μM (Figs. 3 and 4) suggests that AC might inhibit its own cellular toxicity. DNA intercalating cytotoxic agents are generally thought to act by arresting the enzyme topoisomerase II in a state where it is covalently cross-linked to DNA [16]. AC induces the formation of protein–DNA cross-links at low (1–2 μM) but not at high concentrations (20–50 μM) [6], a phenomenon also observed for 9-hydroxyellipticine

[17]. The most likely interpretation of this result is that high concentrations of AC prevent the formation of the covalent complex of topoisomerase with DNA.

As shown in Fig. 6, AC at a concentration of 20 μM not only inhibits its own action but also inhibits the cytotoxic action of two other topoisomerase-directed agents, amsacrine and CI-921. In this respect it resembles the DNA intercalator ethidium, which suppresses the cytotoxic effect of the topoisomerase II-directed drug etoposide [18]. The mechanism of this inhibition, and also the reason why the action of another drug in this class, doxorubicin (Fig. 6) is not affected, is not yet known.

One of the aims of this study was to determine whether human cell lines responded in a similar manner to that of the mouse lines tested. This was demonstrated in the results of Fig. 7 and Table 1. The most sensitive lines were the U-937 and Jurkat lines, which resemble the mouse L1210 line [6] in sensitivity. MM-96 melanoma cells were less sensitive, while HT-29 cells were almost completely resistant. A reproducible reversal of the cytotoxic effect of AC at concentrations of 10–20 μM, similar to that seen with mouse cell lines, was observed with human tumour cells (Fig. 7). None of the human cell lines was as sensitive, in terms of the maximum killing achieved, as either the LLAK or LL23 mouse lines.

In summary, the results show that a Lewis lung line (LLTC) which has been adapted over many passages to *in vitro* growth is less sensitive to AC than a freshly isolated line, and also that *in vivo* passage of LLTC for a number of passages restored its sensitivity. Two possible conclusions can be made from these results. The first is that serial long-term culture of cells from an *in vivo* tumour can significantly change both cytokinetic properties [12] and sensitivity to topoisomerase II-directed drugs. If this is the case, it is possible that the use of long-term cultures of human tumours such as the ones used in this study could seriously underestimate the cytotoxicity of drugs such as AC. Work is underway in this laboratory to measure drug effects on human tumour samples which have had a much shorter culture history.

The other possible conclusion is that prolonged *in vivo* passage of cells, for example Lewis lung cells, may both alter cytokinetics and increase sensitivity to drugs such as AC. Since the drug sensitivity of the original carcinogen-induced tumour is not known, we have no way of testing this hypothesis. It is clear that prolonged *in vivo* passage does not alter all tumours in this way since the Colon 38 tumour in our hands has had a long history of *in vivo* passage yet preserves cytokinetics similar to those of LLTC and a phenotype which is resistant to AC (unpublished results). In our view it is reasonable to suggest that certain human tumours may contain populations of rapidly dividing cells with the kinetic characteristics of LLAK cells, i.e. the apparent absence of a distinct G0 phase [12]. Further work will be required to detemine whether these populations exist and whether they are common in human neoplastic disease.

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