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Relationship of Cell Cycle Parameters to *in vitro* and *in vivo* Chemosensitivity for a Series of Lewis Lung Carcinoma Lines

Karen M. Holdaway, Graeme J. Finlay and Bruce C. Baguley

The sensitivity of three Lewis lung carcinoma sublines, which grow in culture and *in vivo*, and vary in *in vivo* drug sensitivity, have been compared using topoisomerase II poisons amsacrine, amsacrine analogue CI-921, doxorubicin and etoposide. D_{10} (drug concentration for 10% clonogenic survival) values were determined *in vitro* for low and high density cultures, and *ex vivo* for cells from subcutaneous tumours. The cytokinetic parameters of these populations were obtained by flow cytometric analysis of bromodeoxyuridine-labelled cells. Regression analysis showed that logarithmic D_{10} values were significantly correlated ($r > 0.95$) with G_1- and S-phase proportions and highly correlated ($r = 0.99$) with calculated G_1 transit times. The slopes of the regression lines were similar for all topoisomerase II poisons tested and it is suggested that this slope reflects the disappearance of topoisomerase II during G_1 phase.
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INTRODUCTION

ONE OF the goals of present day cancer chemotherapy is to determine, from a tumour biopsy, the spectrum of drug sensitivity of the patient's cancer cells. Three principal methods have been developed with this intent: drug sensitivity testing of cultured primary cancer cells in culture, inhibition of growth of tumour cell xenografts in immune deficient mice, and the analysis of biochemical, cytological or molecular characteristics of tumour cells which may predispose it towards responding to a given drug. One particular example of the third method which has found widespread clinical use is the technique of flow cytometric analysis of cellular DNA following labelling with 5-bromodeoxyuridine (BUdR) [1] and for some tumours there is an inverse correlation between the proportion of S-phase cells in the tumour and the response to chemotherapy with a variety of agents [2,3].

The use of model systems in which the cytokinetic properties of tumour cells can be related to chemosensitivity both *in vivo* and *in vitro* can provide valuable information relevant to the interpretation of clinical studies. Few studies have been carried out using variants of the same tumour cell line with varying cytokinetics and chemosensitivity. In this report we describe such a system using the murine Lewis lung adenocarcinoma, a tumour which initially arose spontaneously in $C_{57}B1$ mice, and which has a number of features which make it a good model for clinical carcinomas. It grows easily both *in vitro* and *in vivo* and is aneuploid, heterogeneous, metastatic and resistant to many, but not all, clinical antitumour agents. It has been used extensively as one of the main tumour models in the *in vivo* drug

Correspondence to B.C. Baguley.
The authors are at the Cancer Research Laboratory, University of Auckland School of Medicine, Auckland, New Zealand.
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screening programme of the National Cancer Institute[4]. Zacharski [5] has concluded that although Lewis lung has the cytological appearance of a large cell cancer, its rapid rate of growth, propensity to cause lethal metastases, as well as its susceptibility to combination chemotherapy, radiation and anticoagulant treatment, make it a good model for human small cell lung cancer (SCLC).

In the course of testing a cell culture line originally derived from Lewis lung [6] and designated LLTC, we found that it displayed reduced *in vivo* sensitivity to the cytotoxic agents cyclophosphamide, 5-fluorouracil(5-FU), tiazofurin, the amsacrine derivative CI-921 {9-[(2-methoxy-4-methylsulphonylamino)-phenylamino]-N,5-dimethyl-4-acridinecarboxamide} [7,8]. The cell line was also less sensitive to a variety of cytotoxic agents than was a line freshly derived from the original tumour and designated LLAK [7]. We developed a third line (designated LL23) by 23 consecutive *in vivo* passages of LLTC and found that this had sensitivity intermediate between those of LLAK and LLTC for amsacrine and some of its derivatives [7].

In this study, we show that the cytokinetics of these lines, determined both *in vivo* and *in vitro* using the BUdR methodology, are related directly to drug sensitivity. We have used the antitumour drugs amsacrine, amsacrine analogue CI-921, doxorubicin and etoposide, which are all thought to act by poisoning the cellular enzyme of DNA metabolism, topoisomerase II. The results have been interpreted in terms of a model in which the G_1 transit time in the cell cycle is a major determinant of drug sensitivity.

MATERIALS AND METHODS

Drugs

Amsacrine and CI-921 were prepared in the laboratory or were provided by Parke-Davis (Ann Arbor). Cyclophosphamide and etoposide (Bristol-Myers), and doxorubicin (Farmitalia) were obtained from Auckland Hospital, and BUdR was obtained from Sigma.

Tumour cell lines

The parental tumour, designated LL, was obtained in 1977 from the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda. The LLAK cell line was derived from LL in this laboratory [7]. The LLTC line, originally developed from LL at the Southern Research Institute, Birmingham, Alabama, was obtained in 1981 from Dr R.C. Jackson (Parke-Davis). The LL23 line was derived from LLTC [7].

In vivo *experiments*

LL cells were passaged *in vivo* in $C_{57}B1/6J$ or $B6D2F_1$ mice by subcutaneous injection of a suspension (10^6 cells) in 0.2 ml PBS (NaCl 8 g/l, KCl 0.2 g/l, Na_2HPO_4 1.15 g/l, KH_2PO_4 0.2 g/l, $MgCl_26H_2O$ 0.1 g/l, pH 7.4). Passaging was carried out by removing the tumour (approximately 7 mm diameter) from the carrier, mincing with a scalpel and disaggregating in PBS using a stomacher for 60 s (Lab-Blender 80). LLTC and LL23 cells were cultured in 25 cm^2 plastic flasks. Growth medium (GM) consisted of α-modified minimal essential medium (MEM) (Gibco) supplemented with fetal bovine serum (FBS; 10% v/v; Smith-Biolab, Auckland) and antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml). In order to propagate cells for injection into mice, cultures were established at 10^4 cells/ml in 100 mm dishes containing GM (15 ml). Cells were grown to at least 1.5×10^7 per dish, removed from the plastic using trypsin (0.1%, Difco) in citrate saline (trisodium citrate dihydrate 4.4 g/l, KCl 10 g/l, pH 7.3), collected by centrifugation, and 10^6 cells (0.1 ml) injected subcutaneously. Growth delay (GD) experiments were carried out as previously described [8] with treatment initiated when the mean tumour diameter was between 5 and 8 mm. Cyclophosphamide was administered as a single dose (220 mg/kg) and CI-921 was administered in three doses (each 20 mg/kg) with a 4-day interval between each dose. The time taken for the tumour to reach 4 times the initial volume was calculated for treated and untreated tumours and the GD defined as the difference between these two values.

Cytotoxicity assays

Survival curves for 1 h exposures of LLTC, LL23 and LLAK to cytotoxic drugs used previously published methods [7,10]. Survival of low and high density cells in culture was quantitated by initiating cultures at 10^5 cells per ml in 100 mm dishes containing 15 ml GM. After 1 or 4 days, cultures were trypsinised, the cells collected by centrifugation, and exposed to cytotoxic drugs in plastic tubes (10^5 cells per ml) at 37°C for 1 h, after which cells were collected by centrifugation, washed twice, resuspended in GM and counted in a Coulter counter. Various dilutions of cells were plated in 60 mm dishes (5 ml GM per dish) and cultured for 9–10 days, after which colonies were stained with methylene blue (Ajax Chemicals, Auburn, NSW), those containing over 50 cells counted, and the surviving clonogenic fraction calculated.

To obtain *ex vivo* tumour cells, mice with 5–10 mm diameter subcutaneous tumours were killed by cervical dislocation and the tumours excised and minced using crossed scalpels. The mince was placed in a glass vessel containing a small spin bar and incubated with stirring in GM containing pronase (Calbiochem) at 37°C for 40 min. The pronase was prepared in GM at 2 mg/ml, filter-sterilised (0.2 μm pore size) and used at a ratio of 1 mg pronase per 60 mg tumour tissue. At the conclusion of the digestion, large aggregates were allowed to settle, most of the cell suspension supernatant was removed, the cells were recovered by centrifugation, and washed once. Large, refractile cells were counted using a haemocytometer. Recoveries were $1–2 \times 10^8$ cells/g tumour. Cells were then exposed to cytotoxic drugs for 1 h. To estimate the surviving clonogenic fraction, various dilutions of cells were plated in 60 mm dishes to which irradiated LLTC cells (35 Gy) were added to maintain a constant number of 10^5 cells/dish. The cultures were incubated at 37°C in a humidified atmosphere of 5% oxygen, 5% carbon dioxide in nitrogen [10] for 10 days and colonies enumerated as described above.

The responses of cells recovered from LLTC tumours and cultured for 1 or 4 days were investigated by initiating cultures of freshly prepared cells at 10^5 cells/ml in an atmosphere of reduced oxygen concentration, as used above. Cells were collected by trypsinisation and centrifugation, and assayed for response to cytotoxic drugs as described above.

Flow cytometry

Cultured cells were labelled for 60 min with BUdR (10 μmol/l), while mice with subcutaneous tumours (approximately 7 mm diameter) were injected intraperitoneally with BUdR in PBS (100 mg/kg) and killed after 60 min. Solid tumours were minced with scalpels and the fragments converted to a single cell suspension in PBS (10 ml) using a stomacher. Single cell suspensions were passed through a 26 gauge needle. Suspensions

of cells from either *in vitro* or *in vivo* experiments were fixed in 70% aqueous ethanol at −20°C for at least 16 h.

Flow cytometry was performed using a combination of published methods [11,12]. 10^6 cells were centrifuged (200 *g*, 7 min), washed in PBS containing 2% FCS (10 ml) and resuspended in a minimal amount of PBS/FCS. The suspension was mixed with a solution of pepsin (0.2 mg/ml, Sigma) in 2N NCl (1 ml) incubated with occasional mixing at 37° for 30 min. The suspension was diluted to 10 ml with PBS/FCS, mixed and centrifuged (200 *g*, 7 min). The pellet was resuspended in a minimal amount of PBS/FCS and 0.1 M $NaBH_4$ (1 ml) was added with mixing. After 2 min, PBS/FCS was added to 10 ml and the suspension was centrifuged (200 *g*; 7 min) and washed twice with PBS/FCS (10 ml) and once with PBS/0.5% Tween 20 (2 ml). Cells were then resuspended in PBS/0.5% Tween 20 (50 μl) and stained with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-BUdR antibody (Beckton Dickinson; 10 μmol/l) for 1 h at room temperature. PBS (0.5 ml) was added and the cells centrifuged for 7 min and washed once with PBS (0.5 ml). The cells were then resuspended in PBS (0.45 ml) and 8 μl of propidium iodide (PI; Calbiochem; 1 mg/ml) and 50 μl of ribonuclease (Sigma; 10 mg/ml) was added. After standing at room temperature for 15 min the cells were centrifuged and some of the supernatant (300 μl) was removed. After resuspension the cells were analysed in a Becton Dickinson FACS IV cell sorter. Cells were excited at 488 nm with a 150 mW argon laser and the fluorescent light passed through 585/42 and 625/35 nm dichroic mirrors. Green and red fluorescence were measured with 530(15) and 625(17) nm [mean (S.D.)] band pass filters and analysed using Consort 40 software on a Digital Equipment Microvax computer. Chick erythrocytes were used to align the instrument and PI-stained mouse thymocytes were used for final adjustments. Cell cycle proportions were obtained from the contour plots. To estimate the G_1 phase transit times (assuming a constant $S/G_2/M$ transit), it was assumed that the minimum doubling time was 11 h [7]. Since the $S/G_2/M$ transit time of the most rapidly growing population (LLAK cells in exponential phase) was 8.7 h (79% of 11 h), the G_1 transit time of the other populations was calculated as [8.7 × (G_1 proportion)/($S/G_2/M$ proportion)].

RESULTS

In vivo *drug sensitivity of the Lewis lung lines*

The growth of the parent LL, as well as of LLTC and LL23 tumours, growing subcutaneously in BDF1 hybrid mice, was compared following treatment with CI-921, the most active of the four topoisomerase poisons against this tumour (Ref. 13 and unpublished results). Cyclophosphamide was also tested as a highly active reference drug. The initial doubling times of the three untreated tumours were 2.5 (LL), 2.0 (LL23) and 3.6 days (LLTC), as determined by tumour volume measurement. LLAK and LL23 were sensitive to CI-921 and cyclophosphamide, while LLTC was resistant (Table 1).

In vitro *drug sensitivity of the LL lines*

Cultures were grown to either low or high culture density and incubated for 60 min with the topoisomerase II poisons amsacrine, CI-921, doxorubicin and etoposide. The survival of cells was then measured in a colony-forming assay, and a representative result is shown in Fig 1. D_{10} values (concentrations for 10% survival) were calculated by averaging the results of a large number of experiments including that shown in Fig. 1 and those from published data [10]. These are summarised in Table 1.

Cells were also recovered from LL, LL23 and LLTc tumours and exposed to CI-921 for 1 h before determination of D_{10} values (Table 2). The sensitivity of LL and LLTC cells was also compared when taken either immediately from the mouse or

Table 1. Effects of CI-921 and cyclophosphamide on the growth of subcutaneous LL tumours

Tumour subline	Drug	Optimal dose (mg/kg)	Growth delay (days)
LL	CI-921	20	13.0
	Cyclophosphamide	220	11.3
LLTC	CI-921	20	0.6
	Cyclophosphamide	220	1.2
LL23	CI-921	20	8.5
	Cyclophosphamide	220	10.5

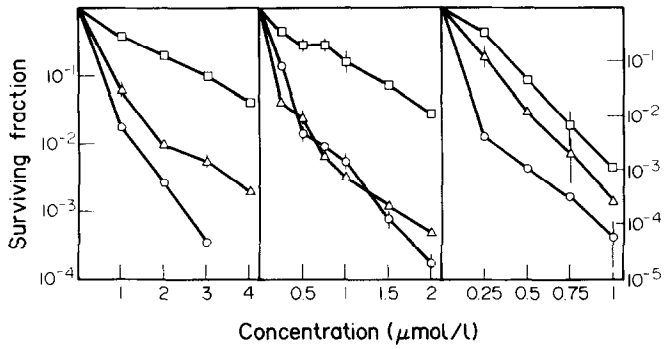

Fig. 1. Comparison of sensitivity of LLAK, LLTC and LL23 cell lines to intercalating drugs. LLAK (○), LL23 (△) and LLTC (□) cells were grown to low density and exposed to amsacrine (left panel), CI-921 (centre) and doxorubicin (right panel) for 1 h at 37°. Survival, as determined by colony counting, is plotted against drug concentration. Symbols represent arithmetic means (S.E.) of duplicate cultures.

Table 2. D_{10} values for various drugs using the three cell lines tested under conditions tested (low density growth, high density growth and cells recovered from subcutaneous tumours)

Subline	Condition	D_{10}(μmol/l) CI-921	Doxorubicin	Amsacrine	Etoposide
LLAK	Low density	0.2	0.093	0.52	
	High density	0.3			
	Ex vivo (LL)	1.2			
LLTC	Low density	0.70	0.41	2.5	7.7
	High density	4.6	2.35	10.2	45
	Ex vivo	7.2	2.75	15	
LL23	Low density	0.25	0.24	0.78	
	High density	1.95			
	Ex vivo	3.43			

Values are the means of various determinations including those in Figs 1 and 2, and from [10].

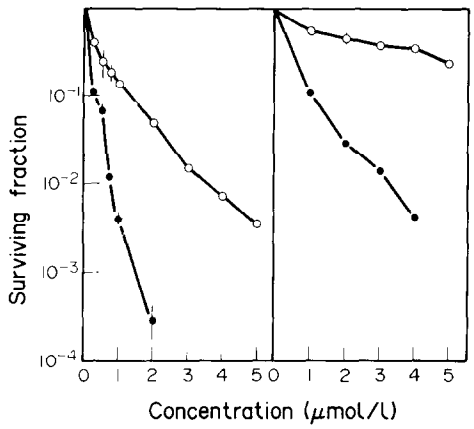

Fig. 2. Sensitivity of *ex vivo* LLAK and LLTC cells to CI-921 before or after culture. LLAK (left panel) and LLTC (right panel) cells were recovered from subcutaneous tumours and exposed to CI-921 either immediately (○) or after overnight culture at 10^5 cells/ml (●). Symbols represent arithmetic means (S.E.) of duplicate cultures.

after 1 day in culture. The results indicated that cells freshly isolated from LL tumours were substantially more sensitive than those from LLTC tumours. Less than 80% of LLTC cells were killed at 5 μmol/l CI-921, whereas killing of LL cells exceeded 99%. Moreover, after 1 day in tissue culture, cells of both lines had become much more sensitive to CI-921, but cytotoxicity towards LL still greatly exceeded that towards LLTC (Fig. 2).

Cytokinetics of Lewis lung cell lines

Cultures of LLAK, LLTC and LL23 were grown either to low or high culture density and labelled for 60 min with BUdR. Cells were also recovered from LL, LLTC and LL23 tumours 60 min after *in vivo* injection with BUdR. Following fixation and acid denaturation of the DNA, cells were labelled with anti-BUdR antibody, counterstained with propidium iodide and analysed (Fig. 3). LL and LLAK cells were found to be close to tetraploid in DNA content, as found by others [14], whereas LLTC and LL23 cells were 1.65 × diploid. A host cell population, as well as a tumour cell population, was evident for each

Table 3. Cell cycle parameters for the three cell lines tested (1–3 determinations) under conditions tested (low density growth, high density growth and cells recovered from subcutaneous tumours)

Type	Condition	Percentage of each phase		
		G_1	S	G_2/M
LLAK	Low density	21(1)*	62(7)	17(7)
	High density	30(1)	60(2)	10(2)
	Ex vivo (LL)	45(5)	40(10)	11(7)
LLTC	Low density	37(7)	58(2)	16(3)
	High density	53(9)	32(4)	15(3)
	Ex vivo	55(4)	25(4)	20(0)
LL23	Low density	29(1)	59(0)	12(1)
	High density	45(4)	34(1)	21(4)
	Ex vivo	50(5)	25(5)	25(3)

*Mean (S.E.).

of the *ex vivo* samples. The G_1 phase, S phase and G_2/M phase proportions, calculated from a number of experiments, are shown in Table 3.

Sensitivity to the drug CI-921, as defined by logarithmic D_{10} values, was related to the cell cycle parameters of the three Lewis lung lines growing under three different conditions (low density, high density and *ex vivo*). A high degree of linear correlation was obtained with G_1 phase proportion ($r = 0.98$) and with S phase proportion ($r = -0.96$). A very high degree of correlation was obtained when drug sensitivity was plotted against G_1 transit time (Fig. 4). G_1 phase transit times were calculated using two assumptions, firstly that the transit time for S/G_2/M phases was constant [15] and secondly, that the minimal cell cycle time for these cells was 11 h [7]. The correlation coefficients (using averaged data) for amsacrine, CI-921 and doxorubicin were all 0.99, and the slopes of the regression lines were very similar (Fig. 4).

DISCUSSION

The Lewis lung lines have been used as a model to relate *in vivo* drug sensitivity to *in vitro* chemosensitivity assays and cytokinetic profiles. At least in some cases, the latter two

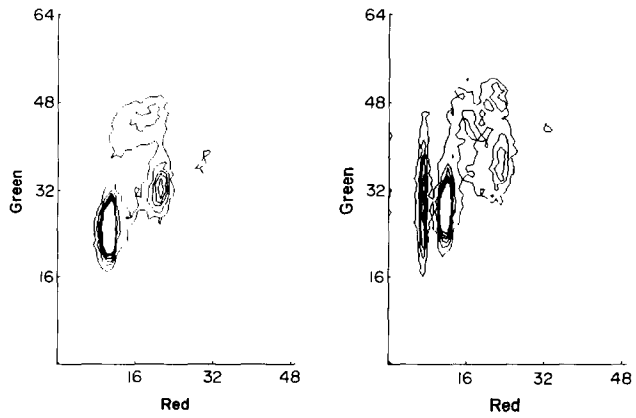

Fig. 3. Typical contour plots for cell suspensions double-labelled with propidium (red fluorescence) and anti-BUdR-DNA antibody (green fluorescence). Left hand panel: *in vitro* labelled LLTC. The left-most population comprises G_1 phase cells while the upper population comprises S phase cells. Right hand panel: *in vivo* labelled LLTC. The left-most population comprises host (diploid) cells present in the tumour cell suspension.

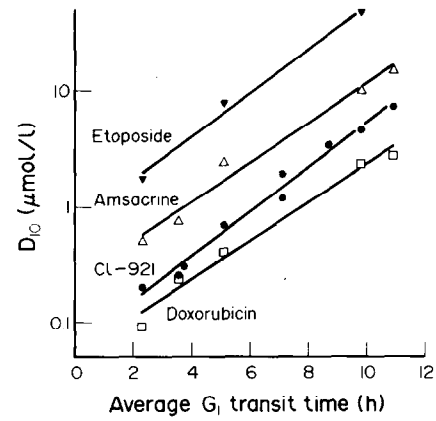

Fig. 4. Relationship between D_{10} values (taken from Table 2) and G_1 transit times (calculated from Table 3 using the assumptions described in the text) for etoposide (▼), amsacrine (△), CI-921 (●) and doxorubicin (□).

measurements can be made in human tumours, and it is the goal of many groups including ours [19], to utilise these measurements in the selection of appropriate chemotherapy for individual patients. The correlation of *in vivo* drug sensitivity with drug sensitivity and cytokinetics, as described here, provides a useful background to current attempts to predict clinical drug response from an analysis of tumour biopsy material.

The substantial differences in the *in vivo* drug sensitivity of LL, LL23 and LLTC tumours, shown in Table 1, might be related either to intrinsic differences in individual cells, or to divergences in tumour architecture, drug penetration or host–tumour interactions. We consider that host interactions are unlikely to make a major contribution to drug sensitivity for the following reasons. Firstly, the ability of all three lines to form lung colonies in recipient mice following intravenous injection of tumour cells, which varies with some tumour variants [16], has been found to be similar for all three cell lines (approximately 50 lung colonies per million injected cells). Secondly, no consistent differences in the expression of the relative abundance of the major H-2D or H-2K histocompatibility antigens, which can lead to altered tumorigenicity in the host [16], have been observed for the three lines using flow cytometric analysis (unpublished results).

One of the most notable features of the results is the close relationship beween G_1 and S phase proportions and sensitivity to topoisomerase II poisons. This is visualised particularly well when the G_1 phase transit time, calculated from G_1 phase proportion using the assumption that the G_1 phase is the major source of cell cycle time variability [15], is plotted against chemosensitivity in a clonogenic assay (Fig 4). Similar regressions are obtained for CI-921 (where a complete data set for all three lines was available), amsacrine, etoposide and doxorubicin, with the slopes of the regressions for all four cytotoxic drugs being very similar. This result supports the contention that all of these drugs act on a similar target, i.e. DNA topoisomerase II.

The sensitivity of cells to topoisomerase II poisons is related, to a first approximation, to the amount of active enzyme [17]. Furthermore, it is known that topoisomerase IIα activity decreases exponentially following the completion of the cell cycle [20]. Therefore, an attractive interpretation of the data in Fig. 4 is that relative drug sensitivity (D_{10}), which is determined by the survival of the most resistant 10% of the cell population, is related to the activity of topoisomerase IIα in this population, i.e. the longer the G_1 transit time, the lower the residual topoisomerase IIα and the greater the resistance. The calculated half-life of topoiosomerase activity, from Fig. 4, is 1.6 h, comparable with the half-life (for topoisomerase II protein content) of 1.0 h in a chicken hepatoma line [20].

The results (Tables 2 and 3) clearly show that for all three cell lines there is a difference in cytokinetic parameters, accompanied in each case by a change in chemosensitivity, between cells analysed *ex vivo* from mouse tumours and cells growing *in vitro* at low density (under conditions normally used for chemosensitivity testing). This emphasises the need to take regard of both intrinsic drug sensitivity and cytokinetics in the analysis of tumour samples from patients. It is of interest that in the LL tumour, a relatively high S-phase content is maintained even at high cell density. L1210 and P388 leukaemias, which are characterised by a high content of S-phase cells, even at high density *in vivo* (unpublished results), are, like LLAK, sensitive to a variety of agents. It is possible that such behaviour is associated with loss of a true G_0 phase [18] and, thus, with minimal degradation of topoisomerase IIα. If this is the case, then the identification of human tumours with short G_1 phase transit times should form an important part of the decision on treatment strategy for this class of chemotherapeutic agent.

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