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Formation of Cytosine Arabinoside-5′-triphosphate in Different Cultured Lymphoblastic Leukaemic Cells With Reference to Their Drug Sensitivity

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The accumulation of intracellular cytosine arabinoside-5′-triphosphate (Ara-CTP) is determined in five lymphoblastic cell lines: Molt 4, H9 and three newly established cell lines from paediatric patients, KFB-1, KFB-2, KFT-1. The cell lines KFB-1 and KFB-2 are B-lymphoblastic (B-ALL), the others are T-lymphoblastic leukaemic cells (T-ALL). The Ara-CTP levels were compared with the sensitivity of the cells to Ara-C. The cells were incubated at different concentrations (100 nM–100 μM) of Ara-C for 4 h or incubated for variable times (30 min–11 h) at 0.1, 1 and 10 μM Ara-C to form Ara-CTP. The Ara-CTP-concentrations were measured by high pressure liquid chromatography (HPLC). To determine the sensitivity of the cells to Ara-C, the MTT colorimetric-assay was used. The studies indicate that different B- and T-lymphoblastic leukaemia cell lines accumulate Ara-CTP to a markedly different extent. Ara-CTP plateau levels and sensitivity of the cells to Ara-C correlated well in four of the five cells lines studied.

Key words: Ara-CTP, high pressure liquid chromatography, acute lymphoblastic leukaemia, MTT assay
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INTRODUCTION

CYTOSINE ARABINOSIDE (Ara-C) is one of the most effective drugs in the treatment of acute lymphoblastic and myelogenous leukaemia (ALL, AML) [1]. The activity of this drug is dependent on the phosphorylation of the prodrug Ara-C to the active metabolite Ara-CTP [2, 3]. Ara-CTP acts as a DNA polymerase inhibitor by competing with the binding of deoxycytidine-triphosphate (dCTP), and is also incorporated into the DNA [4, 5]. Single strand breakage is induced.

Some investigations indicate a relationship between accumulation and retention of Ara-CTP and clinical parameters, like remission, and response to Ara-C [6–9]. Prolonged Ara-CTP retention is found to indicate higher duration of remission [9, 10]. Based on *in vitro* animal and patient studies, several mechanisms of resistance have been proposed: lower formation of Ara-CTP because of decrease or absence of deoxycytidine kinase (dCK), increase of cellular dCTP level, decreased incorporation of Ara-CTP into the DNA, an active repair mechanism of Ara-CTP/DNA, decreased membrane transport, increase in cytidine deaminase activity and shortened half-life for Ara-CTP [1].

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We investigated the intracellular accumulation of Ara-CTP in B- and T-lymphoblastic cell lines after incubation at different concentrations and different durations. To investigate the correlation between accumulation of Ara-CTP and sensitivity of the cells to Ara-C, we compared the Ara-CTP plateau levels with the IC_{50} of Ara-C.

MATERIALS AND METHODS

Materials

Ara-CTP, tartaric acid and other nucleotides were obtained from Sigma; ACN from Zinsser, KH_2PO_4 and H_3PO_4 from Baker and tetrabutylammoniumphosphate from Waters.

Cell lines

Five different lymphoblastic cell lines were used: commercially available Molt 4 and H9 and three newly established cell lines from our own paediatric patients, KFB-1, KFB-2 and KFT-1. These were kept in exponential growth at 37°C and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), penicillin (1%), streptomycin (1%) and 1 mM L-glutamine. The cells were routinely tested for mycoplasma by the Hoechst 33258 staining method [11].

The phenotyping of the newly established cell lines was based on morphology and immunoenzymatical results [12].

Cell preparation for Ara-CTP determination

Cells in the logarithmic growth phase (4×10^5–1×10^6 cells/ml) were incubated for 4 h with different concentrations (100 nM–100 μM) of Ara-C or incubated for variable times (30 min–11 h) with 0.1, 1 and 10 μM Ara-C. Viability was

tested by Trypan blue exclusion. All steps of the extraction procedure were performed on ice. First cells were washed twice with cold phosphate-buffered saline (PBS). After the supernatant was discarded, the cell pellet was resuspended in 300 µl PBS containing 100 mM tartaric acid to enhance recovery [13] and gently mixed with a vortex. After 100 µl were removed for cell counting, cells were extracted with 100 µl buffer. The buffer for the extraction was the mobile phase of the high pressure liquid chromatography (HPLC) procedure, which was adjusted to pH 2.35 with H_3PO_4. Following vigorous mixing with a vortex, and centrifugation at 1000 *g* for 10 min at 40°C, the supernatant was collected. Ara-CTP was stable after storage of the sample for 9 months at −20°C at a pH of 2.6–2.7. This pH was adjusted by adding 100 µl of the buffer (pH 2.35) to the cell pellet. We took the mobile phase for lysis of the cells to enhance chromatographic conditions without background peaks. The total number of cells was counted with an electronic haematocytometer.

Chromatographic conditions

The Ara-CTP concentration was determined by HPLC [13, 14]. Aliquots (25–200 µl) of the nucleotide extracts were injected into a Milton Roy liquid chromatograph equipped with the automatical Waters injector Wisp 712. The nucleotides were eluted from a reverse phase ODS2 C18-column (5 µ, 4,6 mm × 25 cm) and detected with a UV detector, using a wavelength of 280 nm. The detection limit of Ara-CTP was 5 pmol. The mobile phase was a solution of 0.1 M KH_2PO_4, 5 mM tetrabutylammoniumphosphate and 0.5% acetonitrile, which was adjusted to pH 2.6–2.7 with H_3PO_4. During the initial 16 min, a flow rate of 0.8 ml/min was used and increased linearly to 1.8 ml/min by 18 min and maintained until the end of separation, after 25 min. The retention times for the nucleotide triphosphates were: CTP 12 min, Ara-CTP 14 min, dCTP 16 min, ATP 21 min. The amounts of Ara-CTP and the other nucleotide triphosphates were measured by integrating the area under the peak compared to known concentrations of external standards. The AUC was calculated by the trapezoid method. The concentration of Ara-CTP, CTP and ATP in cells was calculated by dividing the amount of the nucleotide triphosphates in each sample analysed by the number of cells in the extract.

Stability of Ara-CTP to extraction of the cells

To stabilise Ara-CTP in samples, tartaric acid was added and the sample adjusted to a pH of 2.6–2.7 (as described previously). It is of great interest what happens to the amount of Ara-CTP during washing and pipetting until stability is guaranteed. Duplicate samples of 10 ml cell suspension with a density of 1×10^6 cells/ml were extracted at 0.5, 1, 1.5, 2 and 3 h after the end of incubation. The cells were stored on ice until lysis. The amount of Ara-CTP was determined as described previously.

Sensitivity of the cells to Ara-C

The sensitivity of the cells to Ara-C was measured by the MTT assay and expressed as the IC_{50} value, the concentration of Ara-C that inhibits cell growth by 50% in comparison to the control without Ara-C [15–18].

Eighty microlitres of the cell suspension, at a density of $2.5–5 \times 10^5$ cells/ml, was incubated on 96-well plates with different concentrations of 20 µl Ara-C in RPMI medium for various lengths of time, ranging from 2 to 5 days. The linearity of optical density was tested. The cells are maintained in RPMI medium supplemented with 10% FCS, penicillin (1%), streptomycin (1%) and 1 mM L-glutamine. Because of evaporation in the outer wells, these were filled with RPMI only. Six wells containing medium only were used for blanking the reader. Another six wells, containing cells and medium, were used to determine the control cell survival. After the end of incubation with Ara-C, 10 µl of the MTT solution (5 mg/ml in PBS) were added to each well. After shaking, the plates were incubated for 4 h. To optimise the MTT incubation time, various durations of exposure to MTT were tested, ranging from 1 to 8 h. Finally, 150 µl 0.04 M HCl in isopropanol was added. After vigorous mixing, the results were measured on a multiscan Elisa plate spectrophotometer using a test wavelength of 540 nm and a reference wavelength of 630 nm.

RESULTS

Ara-CTP accumulation

Figure 1 shows the relationship between the intracellular Ara-CTP accumulation after 4 h and the concentration of Ara-C in the medium. The accumulation of Ara-CTP in the T-lymphoblastic cell lines KFT-1, H9 and Molt 4 plateaus at about 69, 205 and 1033 pmol/10^6 cells. The plateau levels of the B-lymphoblastic cell lines KFB-1 and KFB-2 are 88 and 692 pmol/10^6 cells. The highest formation of Ara-CTP was more than 10 times higher than the lowest. These plateaus are reached at a concentration of 10 µM Ara-C. At 100 µM Ara-C, the Ara-CTP accumulation decreases by approximately 20% compared to the Ara-CTP-plateau level.

Figure 2 presents time dependence of Ara-CTP formation of the cell lines Molt 4, KFB-2, KFB-1 and H9 when incubated with 0.1, 1 and 10 µM Ara-C. The plateau levels were 108, 444 and 1126 pmol/10^6 cells for Molt 4; 45, 245 and 656 pmol/10^6 cells for KFB-2; 11, 46 and 86 pmol/10^6 cells for KFB-1; and 46, 60 and 209 pmol/10^6 cells for the cell line H9.

The plateau levels were reached within 3–4 hours. After an incubation of 11 h (not shown), the Ara-CTP-concentrations did not differ.

Stability of Ara-CTP to extraction of the cells

Our results on the stability of the Ara-CTP concentration from the end of incubation to extraction of the cells is shown in Figure 3. The Ara-CTP concentration remained constant in the

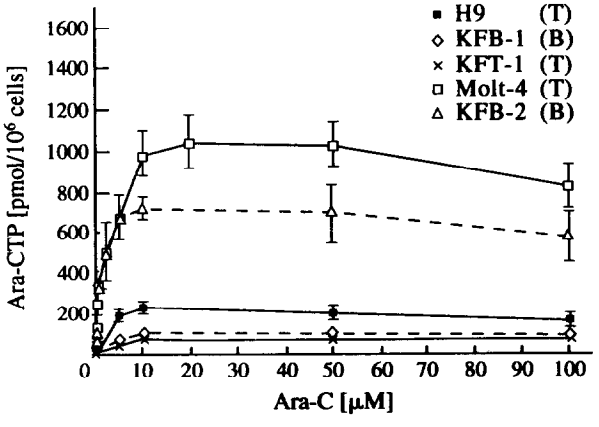

Figure 1. Accumulation of intracellular Ara-CTP in B- and T-lymphoblastic cell lines incubated with different concentrations of Ara-C for 4 h. Each value is the mean of three experiments run in duplicate. Bars = S.E.

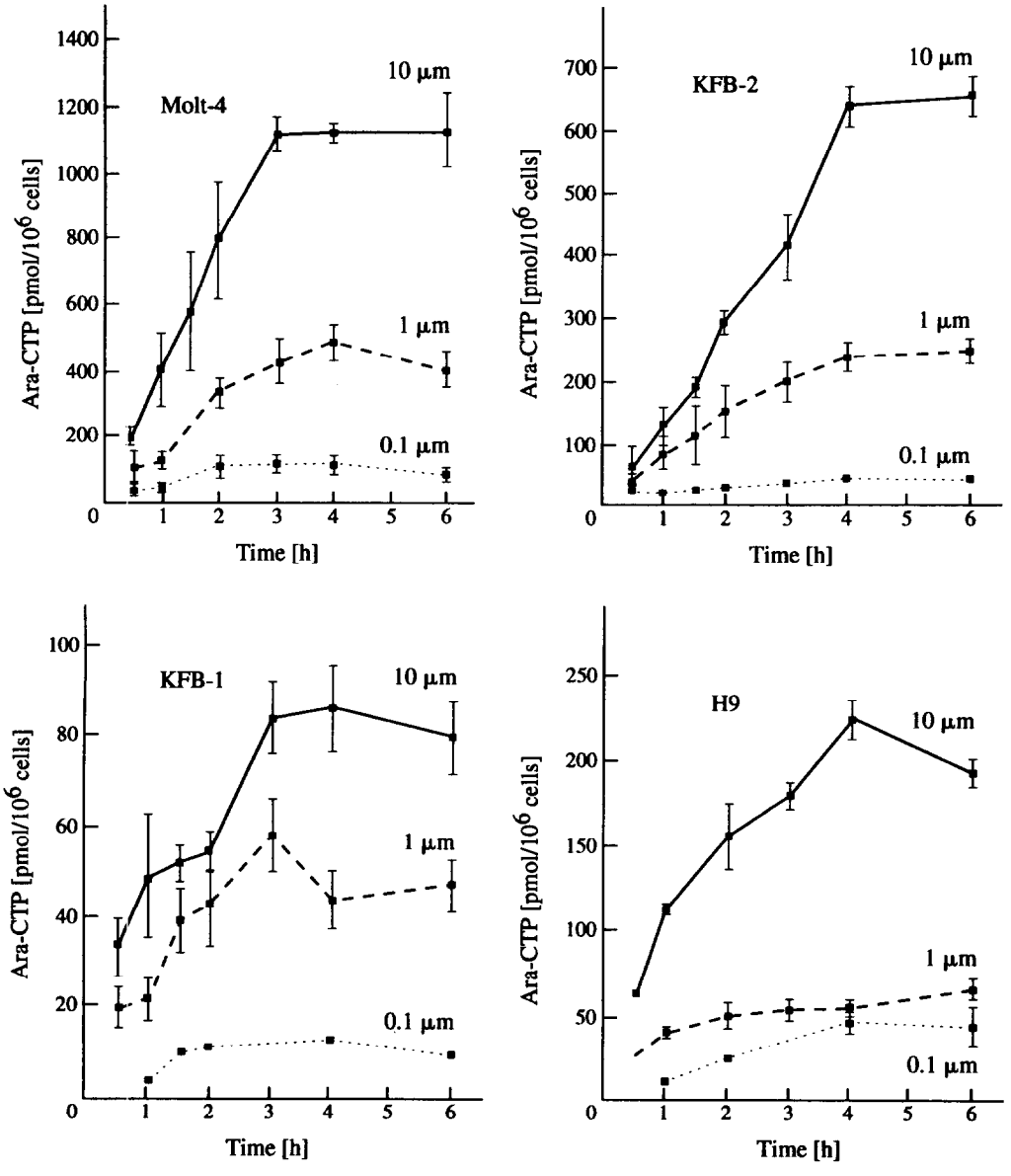

Figure 2. Relationship between accumulation of Ara-CTP and incubation time in the cell lines Molt 4, KFB-2, H9 and KFB-1. Ten millilitres of the cell suspension (4×10^5–1×10^6 cells/ml) were incubated for up to 6 h with 0.1, 1 and 10 µM extracellular Ara-C. Ara-CTP was quantitated as described in Materials and Methods.

Molt 4 and KFB-2 cell lines, which form high amounts of Ara-CTP. In the cell lines H9 and KFB-1, which show a low formation of Ara-CTP, the Ara-CTP concentration fell to 50 or 40% during 3 h compared to its former value at 30 min.

Sensitivity of the cells to Ara-C

The MTT assay was optimised by altering cell number, drug exposure time and MTT exposure as described in the Materials and Methods. The optical density was linearly related to the cell number in a range from 1×10^3 to 4×10^5 cells/well in all five cell lines. The growth rate for the cell lines Molt 4, KFB-1, KFB-2 and H9 was approximately 23–24 h, and for the cell line KFT-1, it was 29 h. In all cell lines, the shape of the dose–response curve was unchanged for 2-, 3- and 4-day assay duration. The 4-day assay was used because the cytotoxic effect of the drugs could be expected to be more marked with a longer duration. All cell lines were in log growth phase up to a duration of 4.5 days. To determine the influence of MTT duration, various incubation times (1–8 h) were used. A plateau of the absorbance value was reached between 3 and 4 h, so a standard MTT incubation time of 4 h was selected.

Table 1 shows the IC_{50} values of our studied cell lines, determined with the MTT assay. The greatest sensitivity (Molt 4) to Ara-C was 63 times higher than the lowest (KFT-1). The T-lymphoblastic cell lines also showed, in addition to a high sensitivity (Molt 4), very low sensitivity (KFT-1, H9) to Ara-C. Both B-lymphoblastic cell lines (KFB-1, KFB-2) were intermediate in their sensitivity.

The correlation between Ara-CTP accumulation and IC_{50} value is shown in Figure 4. Ara-CTP plateau level and sensitivity of the cells to Ara-C correlate well in the cell lines Molt 4, KFB-2, KFT-1 and H9, but not in the cell line KFB-1.

Nucleotide triphosphate levels

Table 1 shows ATP and CTP levels in the five lymphoblastic cell lines. ATP and CTP levels were similar in the cell lines Molt 4,

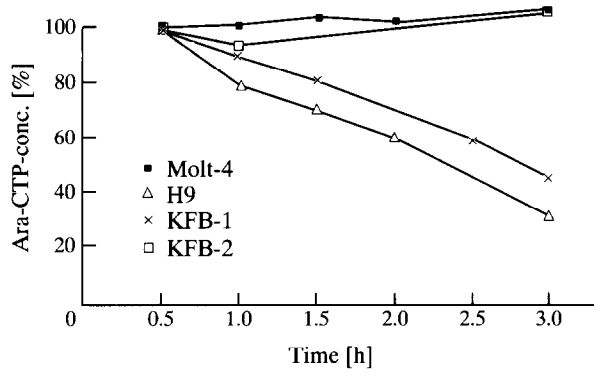

Figure 3. Stability of the Ara-CTP concentration from the end of incubation to extraction of the cells. Ten millilitres of cell suspension in a density of 1 × 10^6 cells/ml were incubated for 4 h with 10 μM Ara-C. After washing the cells, the sample is stored on ice water for variable times until the cells are extracted. Ara-CTP was analysed by high pressure liquid chromatography.

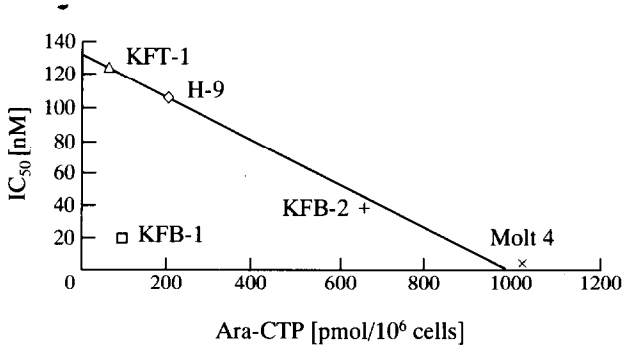

Figure 4. Correlation of Ara-CTP plateau level and IC_{50} of Ara-C. The sensitivity of the cells to Ara-C, measured by the MTT assay, was compared with the Ara-CTP plateau levels after an incubation of 4 h with 10 μM Ara-C. The correlation coefficient r^2 = 0.9865.

KFT-1, H9 and KFB-2. The nucleotide triphosphate levels in the cell line KFB-1 were significantly lower in comparison to the other cell lines. The CTP levels in KFB-1 were 5–7-fold less and the ATP levels were 27–37-fold less.

Quality control

To test the reproducibility of HPLC measurement, a 25-μl injection of 1 μl/ml Ara-CTP standard was evaluated. The intraassay variation coefficient was < 3% (n = 15), the day-to-day variation was < 5% (n = 13).

To test reproducibility of the HPLC method in cell extracts, cells were incubated at 10 μM Ara-C for 4 h (n = 30). Cell counting, pipetting and washing of the cells contribute to the imprecision of the overall Ara-CTP determination which was determined to be < 10% for all five cell lines. The precision was < 15% for an incubation with 1 μM Ara-C for 4 h (n = 15) and < 20% for an incubation with 10 μM Ara-C for 2 h (n = 15) in the cell line Molt 4.

DISCUSSION

Our studies indicate that high or low Ara-CTP-formation occurred in T and B-lymphoblastic leukaemic cell lines (Figure 1). Other investigators have described higher accumulation of Ara-CTP in T-lymphoblastic leukaemic cells compared with leukaemic cells of other phenotypes [19], but in these studies, cells were incubated for 30 or 90 min. We have demonstrated that plateau levels of Ara-CTP formation are reached within 3–4 h (Figure 2) as previously described [20]. In our study, the level at 30 min did not correlate with the ultimate plateau level. Moreover, the standard errors of the Ara-CTP concentration are much higher during the ascending part of the time–concentration curve than during the plateau. For investigations of Ara-CTP accumulation in different cell lines, the incubation period should be extended to 4 h or more.

In our study, the Ara-CTP concentrations of all four cell lines investigated remained constant up to 11 h. This is in accordance with results of Abe and colleagues [20], who used 4 and 8 h incubations, but differ from the observations of Gandhi and Plunkett [21], who noticed that Ara-CTP accumulation continued for 3 h, reached equilibrium and then decreased from 3 to 5 h.

Figure 1 shows that the Ara-CTP formation was saturated at exogenous concentrations of Ara-C in excess of 10 μM. These results agree with previous observations [22–25]. At 100 μM Ara-C, the Ara-CTP accumulation decreased by approximately 20% compared to the Ara-CTP plateau level, a phenomenon also observed by Gandhi and Plunkett in the myelogenous cell line K562 [21].

In our study, the highest formation of Ara-CTP was more than 10 times higher than the lowest. The same wide variation in the saturating level of Ara-CTP accumulation in patients' cells has been described by Jamieson and colleagues [26]. There are

Table 1. IC_{50} values and nucleotide triphosphate levels in different lymphoblastic leukaemic cell lines

Cell line	IC_{50} (nM)*	Ara-CTP (pmol/10^6 cells)	CTP (pmol/10^6 cells)	ATP (pmol/10^6 cells)
Molt 4	2.0 ± 0.2	1033	578.2	2820
KFT-1	126 ± 10	69	378.4	3816
H9	107 ± 11	205	535.7	2798
KFB-1	15.4 ± 1.1	88	78.2	103
KFB-2	31.2 ± 2.2	692	511.0	2917

To determine the nucleotide triphosphate levels, a cell suspension (4 × 10^5–1 × 10^6 cells/ml) was incubated for 4 h with 10 μM extracellular Ara-C. The concentration of Ara-CTP, CTP and ATP in the cells was calculated by dividing the amount of the nucleotide triphosphates in each sample analysed by the number of cells in the extract. The sensitivity of the cells to Ara-C was measured by the MTT assay as described in the Materials and Methods. *Each value is the mean of four to six experiments run in triplicate.

several mechanisms which influence Ara-CTP accumulation. First, the accumulation of Ara-CTP by exponentially growing cells is known to be influenced by the cellular dCTP concentrations [27, 28]. It has been reported that normal intracellular dCTP concentration causes severe feedback inhibition of the deoxycytidinekinase [29], which catalyses the first step of Ara-CTP formation. Second the Ara-CTP level not only reflects the rate of synthesis, but also the rate of degradation. Increased activity of dephosphorylation is correlated to lower Ara-CTP levels [20], and cell lines resistant to Ara-C have an increased cytidine deaminase activity [30]. Third, some investigations have shown that dTTP, dUTP and UTP can partly reverse the inhibitory action of dCTP on Ara-C phosphorylation in cell extracts. Fourth, accumulation of Ara-CTP by S-phase-enriched cells is 50% greater than in G1-phase-enriched cells [28]. Finally, cellular nucleotides are subject to degradation by non-specific phosphatases and their concentrations may decrease during preparation of cell extracts (see below).

The wide variation in the saturating level of Ara-CTP accumulation cannot be explained by one of these mechanisms, but could be a summary of several specific and non-specific mechanisms. The question remains unanswered.

Figure 3 shows the correlation between the stability and the concentration of Ara-CTP in the cells. The stability was poor in cells with low Ara-CTP concentrations. The decrease of Ara-CTP concentration follows a first order kinetic curve [28]. Dephosphorylation is important in Ara-CTP determination [31]. Our experience indicates that, during washing and lysis of the cells, Ara-CTP can be degraded, a phenomenon that is somewhat dependent on the initial Ara-CTP concentration. Therefore, the time period between incubation and lysis should be taken into account and should not exceed 30 min.

The intracellular Ara-CTP plateau level and sensitivity of the cells to Ara-C correlated well in four of the five cell lines studied. The cell line with the greatest amount of Ara-CTP formation (Molt 4) was most sensitive to Ara-C, while the cell line with the lowest Ara-CTP accumulation (KFT-1) was most resistant. Ara-CTP formation and sensitivity of the cells to Ara-C also correlated in the cell lines KFB-2 and H9. There was no relationship between these two parameters in the cell line KFB-1, which showed low Ara-CTP formation, but high sensitivity to Ara-C in the MTT assay. This suggests that, in this cell line, even low Ara-CTP concentration induces cell death. This could be due to a decreased level of dCTP in the cell. Ara-CTP acts as a competitive antagonist of dCTP. Lower Ara-CTP concentrations would be sufficient to inhibit the DNA polymerase in cells with a low dCTP level [27]. Kessel and colleagues [3] and Chou and associates [32] reported that similar levels of Ara-CTP may be expected to inhibit the growth of different cell lines equally, as long as there is no difference at the DNA polymerase level.

Incorporation of Ara-CTP into DNA is another important mechanism, with single strand breakage induced. If incorporation of Ara-CTP into DNA is increased, lower Ara-CTP concentrations would be able to induce single strand breakage and stop replication.

Different growth rates of the cell lines could be another reason for independence between Ara-CTP plateau level and sensitivity of the cells to Ara-C. In our studies, however, all cell lines show a very similar growth rate.

Presumably, in KFB-1, non-specific phosphatases are responsible for the lack of correlation. The low Ara-CTP level (Table 1), which parallels the level of CTP, supports this interpretation. All nucleotide triphosphates might have been degraded similarly. Thus, the Ara-CTP level measured could have been much higher in the intact cell. However, degradation of Ara-CTP in KFB-1 was not markedly different from cell line H9, 30 min to 3 h after lysis (Figure 2). It may be possible that fast acting phosphatases occurred in the first 30 min after lysis. Information on the distribution of activities of non-specific phosphatases is almost completely lacking in the literature. ATP and CTP levels provide a good marker to test the quality of cell extraction procedure and the stability of nucleotide triphosphates.

Many important questions, regarding the development of resistance and sensitivity to Ara-C, remain unanswered. Determination of the Ara-CTP plateau level is not sufficient to find a correlation between Ara-CTP saturation and sensitivity to Ara-C in all cell lines. Ara-CTP retention is also a very important mechanism. Further studies should investigate whether there is a better correlation between sensitivity of the cells to Ara-C and Ara-CTP retention, respectively. It would be of interest to investigate the level of dCTP, the dCK activity and the level of DNA polymerase [33] and in particular determination of Ara-CTP retention and dCK activity in comparison to ATP and CTP level could give more information on the effect of specific and non-specific enzyme activity on Ara-CTP levels.

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Primary Sequence Determination and Molecular Modelling of the Variable Region of an AntiMUC1 Mucin Monoclonal Antibody

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Polymerase chain reaction (PCR) products representative of the DNA sequence coding for the variable heavy (V_H) and the variable light (V_L) chains of an antiMUC1 mucin monoclonal antibody, C595, have been produced. These products were cloned, sequenced, and the primary amino acid sequences of the V_H and V_L regions deduced. The hypervariable complementarity determining regions (CDRs) and framework regions in the heavy and light chains were located, and homologies with canonical forms for the CDR loops L_1, L_2, L_3, H_1 and H_2 were identified by database searching. The structure for the H_3 loop was calculated directly. Computational molecular modelling was accomplished using the fully automated AbM package (Oxford Molecular, Oxford, U.K.). Energy minimisation was performed using the program InsightII (Biosym, San Diego, California, U.S.A.). The investigation provides a basis for the molecular analysis of the antigen binding site of the C595 antibody with the aim to identify key residues and interactions involved in the immune recognition of the C595 antibody defined epitope, which is expressed in the majority of breast and ovarian carcinomas.
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