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Evaluation of methotrexate sensitivity in human leukemia cell lines by an adenosine triphosphate bioluminescence assay

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To verify a recently developed *in vitro* tumor chemosensitivity assay (TCA) based on adenosine triphosphate (ATP) measurement for detection of methotrexate (MTX) sensitivity or resistance in human leukemias and solid tumors in which the antifol is indicated, we investigated the ability of the assay to discriminate between sensitivity and resistance to this antifolate in human leukemia cell lines sensitive (parental CCRF-CEM/S) and resistant (CCRF-CEM/E, CCRF-CEM/T and CCRF-CEM/P sublines) to MTX by virtue of known biochemical mechanisms. Correlation experiments with a standard cell growth inhibition assay and a radiometric method for measurement of thymidylate (dTMP) synthesis ([5-^{3}H]-2′-deoxyuridine tritium release assay) were performed. No significant differences were observed in the IC_{50} values for the four cell lines tested as determined by cell growth evaluation (cell number counts) and the ATP-TCA after a 72 h MTX exposure. After short-term (4 h) high-dose MTX exposure, no significant correlation between ATP-TCA and the classic [5-^{3}H]-2′-deoxyuridine tritium release assay was observed in both CCRF-CEM/S and CCRF-CEM/P cells. CCRF-CEM/T and CCRF-CEM/E displayed, instead, complete resistance with both methods. When using conditions proposed for clinical application (long-term exposure, i.e. 144 h) the ATP-TCA permitted the identification of cell lines highly resistant to MTX (CCRF-CEM/T and CCRF-CEM/E), while intermediate MTX resistance due to altered polyglutamylation was not detectable. Detection of this kind of resistance was obtained, as expected, using a short-term exposure (4 h) to MTX followed by a long-term efflux (72 h) in drug-free medium. On the basis of these results, ATP-TCA appears to be a suitable method for the evaluation of cytotoxicity induced by MTX.

Key words: ATP, bioluminescence, human leukemia, methotrexate, tumor chemosensitivity assay.

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Introduction

Methotrexate (MTX) is a folate antagonist inhibitor of dihydrofolate reductase (DHFR), successfully used as a single agent or more frequently as part of combination chemotherapy in the treatment of various solid tumors and lymphoid malignancies.[1]

As with other antineoplastic agents, the occurrence of natural or acquired resistance is considered to be the most important factor limiting the therapeutic effectiveness of MTX in the treatment of cancer patients.

In *in vitro* and *in vivo* tumor model systems resistance to MTX may occur by: (i) increased levels of DHFR, often as a result of gene amplification, (ii) alteration of DHFR with consequent reduction in enzyme affinity for MTX, (iii) decreased transport into the cell and (iv) reduced MTX polyglutamate formation.[1,2] Polyglutamylation represents a critical determinant of the cytotoxic action of the antifol. In contrast to the parent drug, polyglutamylated MTX species are in fact retained intracellularly for more prolonged periods of time and bind to DHFR as tightly as MTX. In addition, they inhibit several other folate-dependent enzymes with greater affinity than MTX.[3]

Consistent information is now available on the relative contributions to drug resistance clinically encountered in leukemia.[4–15] The frequency of occurrence of the different types of resistance is still largely unknown, however, especially in solid tumors.[16–18] Recently new sensitive molecular techniques to measure these events have been reported.[19,20] However, the need still exists to develop accurate methods to screen *in vitro* MTX cytotoxicity of tumor explants.

A number of tumor chemosensitivity assays (TCAs) have been developed for measuring *in vitro* drug sensitivity of established tumor cell lines or of human

tumor explants.[21-25] Those based on the inhibition of cell colony formation[26,27] and radioactive nucleic acid precursor incorporation[28] have been characterized by good sensitivity and accuracy.[21-23,25] However, technical problems such as feasibility, risks and costs associated with their use prevent their widespread application for clinical purposes, and have prompted investigators to develop new alternative TCAs for prediction of patient response to chemotherapy.

A TCA based on the measurement of adenosine triphosphate (ATP) by bioluminescence (ATP-TCA) has been recently proposed to evaluate drug sensitivity rapidly from fresh tumor samples.[29-32] ATP may represent an important biochemically based parameter of drug-induced cytotoxicity since its intracellular concentration correlates with the biomass of living cells[33] and it deteriorates immediately after cell death.[34] The ATP bioluminescence assay is extremely sensitive and allows extensive measurement of drug sensitivity in very limited numbers of cells.[35]

We have investigated the ability of this assay to discriminate between sensitivity and resistance to MTX in human leukemia cell lines (CCRF-CEM) sensitive or resistant to this antifol by virtue of known biochemical mechanisms[36-39] in comparison with standard methods of *in vitro* drug cytotoxicity measurement. In these cell lines we have previously reported a good correlation between results obtained with the ATP-TCA and a soft-agar colony forming assay.[40] The correlations between the ATP-TCA, a cell growth inhibition assay[41] and a radiometric method for measurement of thymidylate (dTMP) synthesis[28] following MTX treatment are reported herein using drug concentrations, exposure times, other assay conditions (i.e. tissue culture media) and parameters for interpretation of results useful for optimum discrimination of MTX sensitivity/resistance by the ATP-TCA.

Materials and methods

Chemicals

MTX sodium salt was obtained from Cyanamid Italia (Catania, Italy). Media, sera and antibiotics for tissue culture were purchased from Gibco (Grand Island, NY). Noble agar was purchased from Difco (Detroit, MI). Plastic ware was purchased from Costar (Milano, Italy). [5-^{3}H]-2′-Deoxyuridine was purchased from Moravek Biochemicals (Brea, CA). Reagents for the bioluminescent ATP-TCA were obtained from DCS Innovative Diagnostik Systeme (Hamburg, Germany).

Cell lines

A human T lymphoblast parental cell line sensitive to MTX (CCRF-CEM/S) and three derived sublines resistant to MTX by virtue of various biochemical mechanisms were used in this investigation. CCRF-CEM/E cells were resistant to MTX by virtue of increased DHFR activity;[38] CCRF-CEM/T cells showed a marked decrease in MTX transport.[37] Resistance to MTX occurred in the CCRF-CEM/P line by an impaired ability to form MTX polyglutamates.[36,39]

Cells were grown as suspension culture in RPMI 1640 medium supplemented with 10% horse serum (HS) and antibiotics at 37°C in a 5% CO_2 atmosphere and subcultured twice weekly. For other culture conditions and characteristics of these cell lines, see elsewhere.[36,39] All experiments were conducted on exponentially growing cells.

ATP-TCA

The ATP-TCA was performed on CCRF-CEM cell lines according to the manufacturer's instructions using a proprietary serum-free medium [complete assay medium (CAM)] as previously described.[30] The drug exposures were modified according to experimental purposes. Cells (1×10^3/well) were incubated for 144, 72 or 4 h with MTX at six serial dilutions [from 200 down to 6.25% of the standard test drug concentration (TDC)] at 37°C in a humidified 95% air/5% CO_2 atmosphere. The standard (100%) TDC for MTX was 2.8 μg/ml. This equaled the peak plasma concentration achieved by i.v. administration of 25 mg/m^2.[42] A fresh 800% TDC drug solution in CAM was used to prepare 200 to 6.25% TDC dilutions. The measurement of drug sensitivity was performed using 96-well microplates by adding 0.1 ml of cell suspension to 0.1 ml of CAM [no inhibition control cultures (MO)] or to 0.1 ml of maximum ATP inhibitor [maximum inhibition control cultures (MI)] or to 0.1 ml of appropriate MTX dilution. ATP cell content was determined at the end of a 144 or 72 h exposure, or after a 72 h incubation in drug-free medium following a 4 h drug exposure. At these times cellular ATP was extracted and then measured in a luminometer (MicroLumat 952; Berthold, Wilbad, Germany) using 0.05 ml of cellular extract injected with 0.1 ml of luciferin-luciferase reagent (DCS Innovative Diagnostik Systeme).

A count integration time of 10 s with a 4 s delay was used.

Tumor cell growth inhibition (TGI) values for each TDC were calculated according to the following equation:

$$TGI = 1.0 - \frac{(TDC) - MI)}{(MO) - (MI)} \times 100$$

where MO = mean counts for no inhibition control cultures, MI = mean counts for maximum inhibition control cultures and TDC = mean counts for replicate test drug cultures.

Area under the curve (AUC) values of the percentage of TGI versus the percentage of TDC were calculated by the trapezoidal rule. IC_{50} values were determined by plotting the percentage of tumor growth as a function of drug concentrations.

The inhibitory effects of MTX on the growth of parent and MTX-resistant cell sublines were also determined under conditions typical of other cytotoxicity methods (see below).

In case of comparative experiments with the cell growth inhibition assay, CCRF-CEM cell lines (1×10^3 cells/well) were plated onto a microplate in culture medium and exposed for 72 h to MTX concentrations ranging from 1 nM to 3 μM. Media used were either RPMI 1640 plus 10% HS or CAM.

In comparative experiments with the [5-^{3}H]-2′-deoxyuridine tritium release assay, cells were handled similarly, but drug exposure time was 4 h.

Cell growth inhibition assay

MTX effects on cell growth were evaluated as previously described.[41] In brief, the cells were prepared at a density of 5×10^4 cells/ml and distributed in duplicate 5 ml portions into tissue culture tubes, to which 0.05 ml of MTX solutions were added to reach final concentrations ranging from 1 nM to 3 μM. The cells were incubated at 37°C. After a 72 h exposure the cells were counted using a Coulter counter Model B (Coulter Electronics). The IC_{50} was determined by plotting the percentage of total cell division as a function of drug concentrations.[38]

[5-^{3}H]-2′-deoxyuridine tritium release assay

For evaluation of MTX effects on dTMP biosynthesis, the radiometric method of Rodenhuis *et al.*[28] was used. In this assay, the inhibition of dTMP biosynthesis, caused by antifolates, is estimated by measuring tritium release as ^3H_2O into the aqueous medium, when dTMP synthase replaces the 5-^{3}H group by a methyl group. The latter is formed following cell exposure to [5-^{3}H]-2′-deoxyuridine.

dTMP synthesis was measured after a 4 h MTX treatment (at concentrations of 1 and 3 μM for CCRF-CEM/S and CCRF-CEM/P, and 1 and 10 μM MTX for CCRF-CEM/T and CCRF-CEM/E) and after a 4 h efflux (i.e. 4 h after the end of drug exposure following washing and resuspension in drug-free medium).

Results

Comparison between the ATP-TCA and the cell growth inhibition assay

No significant differences were observed in the degree of cell growth inhibition obtained by the ATP-TCA and cell growth inhibition assay after 72 h exposure over a range of concentrations from 1 nM to 3 μM (dose-response curves not shown). IC_{50} values for the four cell lines tested are reported in Table 1.

CCRF-CEM/E and CCRF-CEM/T displayed comparable MTX-resistance ratios (i.e. IC_{50} MTX-resistant cells:IC_{50} MTX-sensitive parental cells), varying, respectively, from 54 to 62 and from 240 to 287, with the two methods.

CCRF-CEM/P cells, which exhibit an increased ability to survive short-term (4–24 h) high-dose treatment with the antifol, were, as expected,[39] about as sensitive as the parental line to long-term (72 h) low-dose treatment with both methods (resistance ratio 1.2).

Comparison between the ATP-TCA and the [5-^{3}H]-2′-deoxyuridine tritium release assay

After short-term (4 h) high-dose (1 and 3 μM) MTX exposure, dTMP synthesis observed in both CCRF-

Table 1. Inhibitory effects of 72 h exposure to MTX on the growth of CCRF-CEM parental and MTX-resistant cell lines

Cell line	IC_{50} M[a] (cross-resistance)[b]			
	Cell growth inhibition assay		ATP-TCA	
CCRF-CEM/S	1.0×10^{-8}		1.5×10^{-8}	
CCRF-CEM/P	1.2×10^{-8}	(1.2)	1.8×10^{-8}	(1.2)
CCRF-CEM/E	5.4×10^{-7}	(54)	9.3×10^{-7}	(62)
CCRF-CEM/T	2.4×10^{-6}	(240)	4.3×10^{-6}	(287)

[a] Concentration of MTX required to inhibit cell growth by 50% compared with untreated control cells.
[b] Ratio of IC_{50} CCRF-CEM MTX-resistant: IC_{50} parental CCRF-CEM cells.

CEM/S and CCRF-CEM/P cells was markedly reduced in a dose-dependent fashion as compared to control cultures. The percentage of inhibition of dTMP synthesis was 81.7 and 88.1 for CCRF-CEM/S cells and 71.5 and 76.7 for CCRF-CEM/P, at 1 and 3 μM MTX, respectively. In the same experimental conditions ATP cell content was also reduced, but to a much lower extent (maximum percent of inhibition at 3 μM MTX equal to 36.4 and 19 for CCRF-CEM/S and CCRF-CEM/P cells, respectively) (Figure 1A and B).

As expected, after a 4 h efflux period, the inhibitory effects of MTX on CCRF-CEM/S cells were maintained at levels similar to those observed at the end of the 4 h exposure, using both methods (Figure 1C). A rapid recovery of dTMP synthesis (above the 100% control value) was instead observed in CCRF-CEM/P cells. A concomitant increase in ATP cell content was also observed (Figure 1D).

CCRF-CEM/T and CCRF-CEM/E showed complete resistance after a 4 h exposure to MTX (1 and 10 μM) with both methods (dTMP synthesis and ATP content values always 90% or more of untreated controls). These data were also confirmed after a 4 h efflux period (data not shown).

Effects of culture medium on MTX sensitivity in the ATP-TCA

The inhibitory effects of MTX on the growth of CCRF-CEM lines were evaluated with the ATP-TCA in the presence of either RPMI 1640 medium supplemented with 10% HS or CAM.

Cell growth of all untreated cell lines was uniformly and markedly reduced in the presence of CAM as compared to RPMI 1640 (about 2.8-fold; data not shown). No significant differences were, however, observed in the degree of MTX cell growth inhibition obtained with the two media (Figure 2A and B). IC_{50} values calculated from dose-response curves obtained

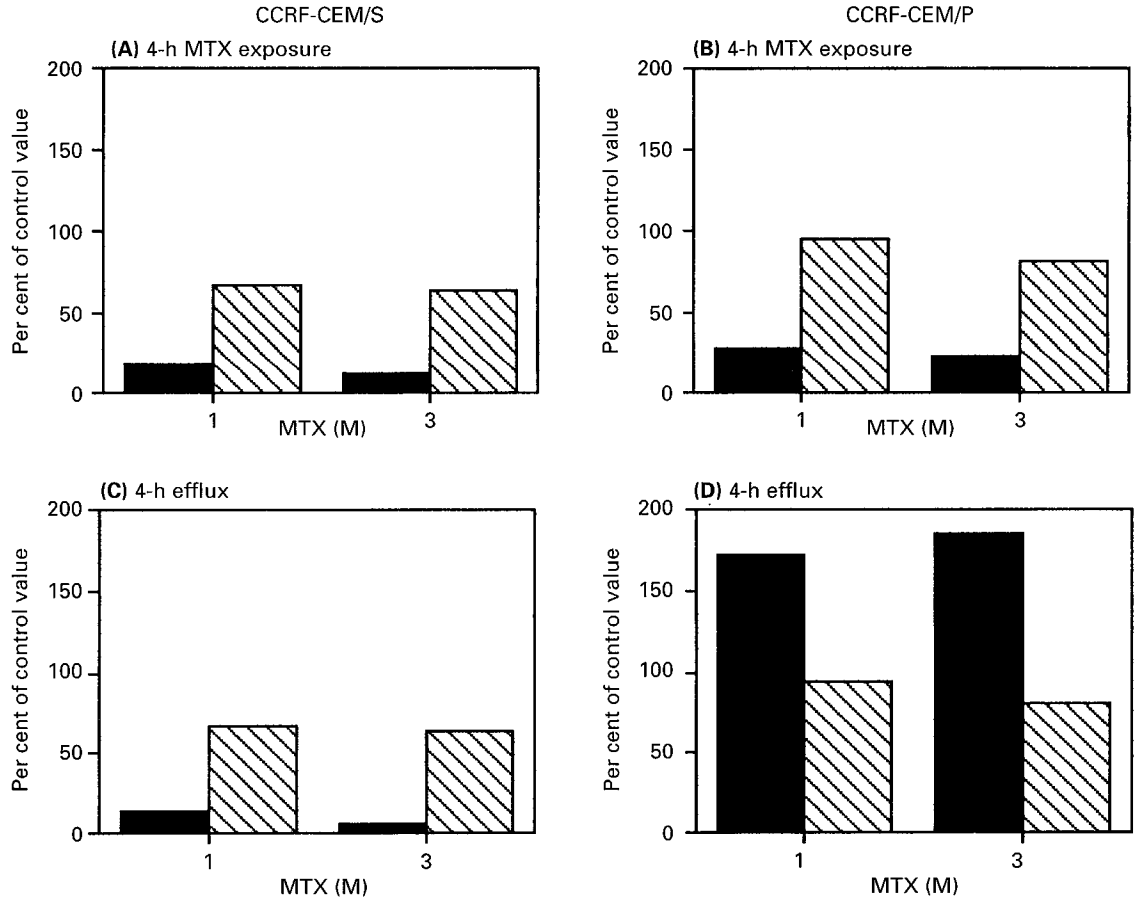

Figure 1. Cytotoxic effects of MTX on CCRF-CEM/S and CCRF-CEM/P cells after a 4 h drug exposure (A and B, respectively) and after a 4 h efflux (C and D, respectively) evaluated by the [5-^{3}H]-2′-deoxyuridine tritium release assay (filled bars) and the ATP-TCA (striped bars). Results are from a single representative experiment.

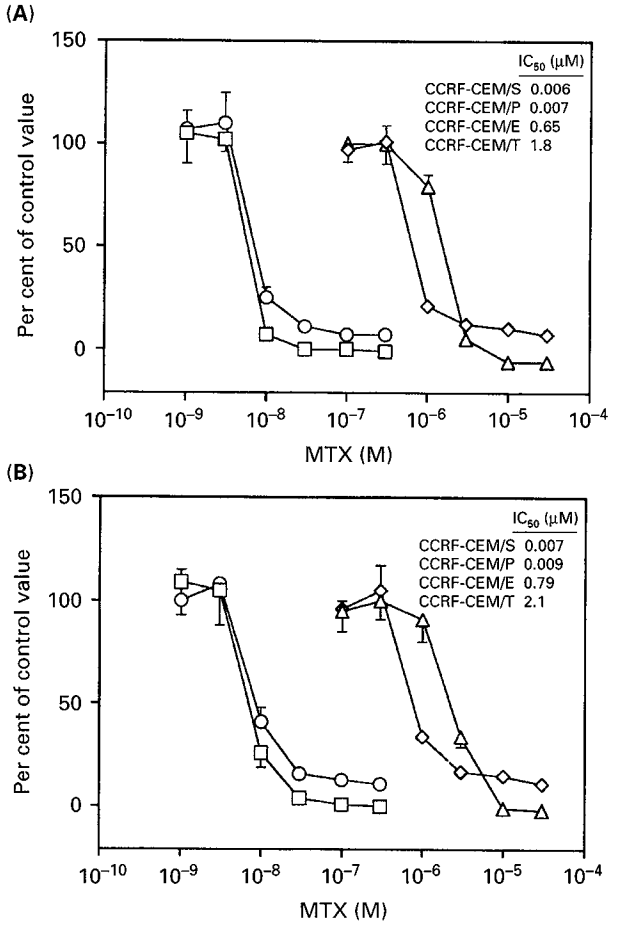

Figure 2. Inhibitory effects of MTX (72 h exposure) on growth of CCRF-CEM parental (□, CCRF-CEM/S) and MTX-resistant cell lines (○, CCRF-CEM/P; ◇, CCRF-CEM/E; and △, CCRF-CEM/T) in the presence of RPMI 1640 + 10% HS (A) or CAM (B) medium evaluated by the ATP-TCA. The results represent mean ± SD of three experiments in duplicate.

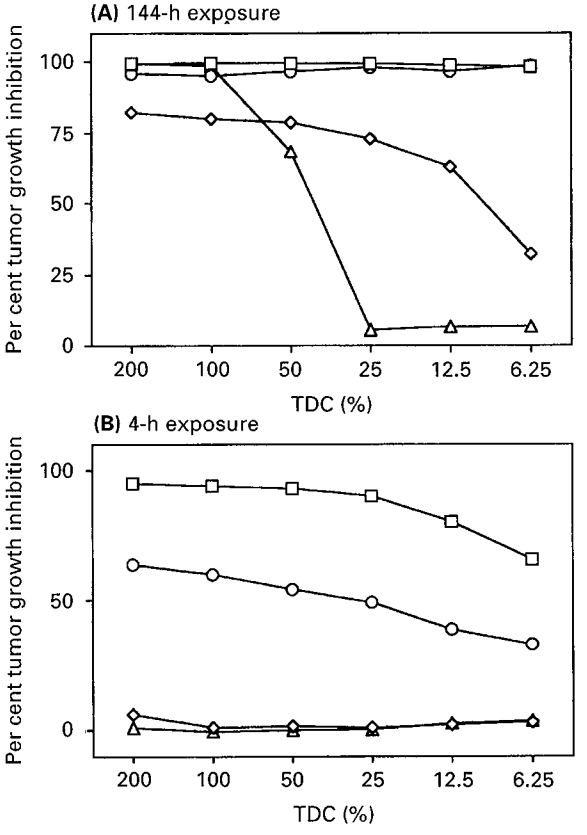

Figure 3. Inhibitory effects of a 144 h (A) and a 4 h (B) exposure to MTX (200 to 6.25% TDC) on the growth of CCRF-CEM parental (□, CCRF-CEM/S) and MTX-resistant cells (○, CCRF-CEM/P; ◇, CCRF-CEM/E; and △, CCRF-CEM/T) in the presence of CAM evaluated by the ATP-TCA. The 4 h exposure was followed by a 72 h efflux. Results are from a single representative experiment.

using CAM were comparable to those obtained with RPMI 1640 plus 10% HS.

Effects of drug concentrations and exposure times on MTX sensitivity in the ATP-TCA

We exposed CCRF-CEM cells to MTX concentrations varying from 200 to 6.25% of the MTX TDC (i.e. 2.8 μg/ml) based on clinical pharmacokinetics of MTX as proposed by Andreotti *et al.*,[30] for either long or short times (144 and 4 h, respectively) in CAM medium.

MTX growth inhibitory effects are reported as percent TGI (as described in Materials and methods) in Figure 3(A). CCRF-CEM/S and CCRF-CEM/P showed marked sensitivity to MTX with 90% or greater inhibition at all concentrations tested. CCRF-CEM/E and CCRF-CEM/T showed a dose-dependent inhibition of their growth. No inhibitory effects on CCRF-CEM/T cells were noted at low MTX concentrations (6.25-25% TDC), reaching almost complete inhibition at 100-200% TDC. CCRF-CEM/E displayed intermediate resistance with a maximum of 82.3% inhibition at 200% TDC and a minimum of 32.2% at 6.25% TDC.

The results were also plotted on a semilogarithmic scale as percent of control value, as a function of MTX concentrations (Figure 4, filled squares). The dose-response curves obtained using RPMI 1640 plus 10% HS (already reported in Figures 2(A) and 3(A)) are also reported (Figure 4, open circles) for comparison (72 h exposure to MTX). It is apparent that these results are in agreement with the classic 72 h experiments aimed

Table 2 Inhibitory effects of a 144 h exposure to MTX and of a 4 h exposure to MTX followed by 72 h efflux on the growth of CCRF-CEM parental and MTX-resistant cell lines expressed as area under curve (AUC) values

Cell line	AUC[a] (S/R ratio)[b]		
	144 h MTX exposure		4 h MTX exposure +72 h efflux
	200–6.25% TDC[c]	50–6.25% TDC[c]	200–6.25% TDC[c]
CCRF-CEM/S	19231	4332	17929
CCRF-CEM/P	18602 (1.0)	4257 (1.0)	11052 (1.6)
CCRF-CEM/E	15131 (1.3)	3047 (1.4)	273 (66.7)
CCRF-CEM/T	15107 (1.3)	1046 (4.1)	53 (338)

AUC sensitive cells/AUC resistant cells ratio (S/R ratio) is also reported.
[a] AUC calculated by the trapezoidal rule.
[b] Ratio of AUC of parental CCRF-CEM cells:AUC of CCRF-CEM MTX-resistant cells.
[c] Range of TDC used for calculation of AUC.

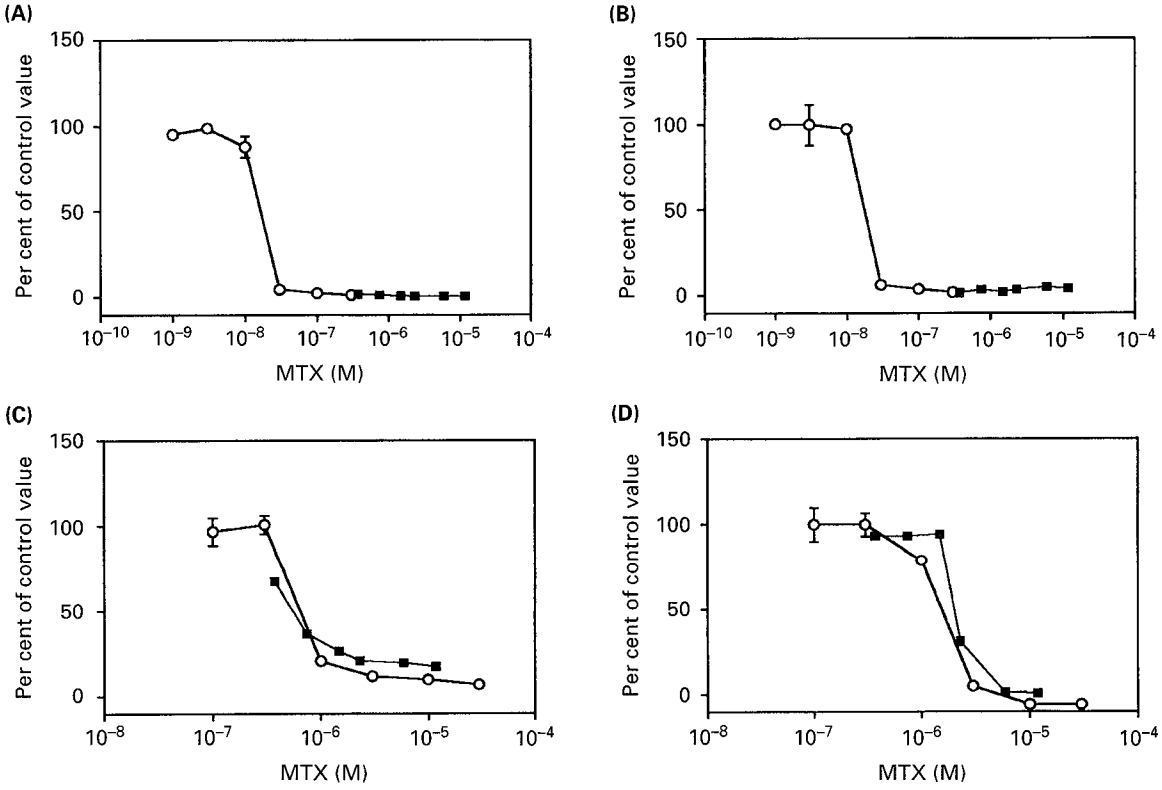

Figure 4. Inhibitory effects after 144 h (filled squares) exposure to MTX (200–6.25% TDC) on the growth of CCRF-CEM parental cells (CCRF-CEM/S) and MTX-resistant cells (CCRF-CEM/P, CCRF-CEM/E and CCRF-CEM/T) (A, B, C and D, respectively) plotted on a logarithmic scale (expressed as mol/l concentration) and compared to results obtained with a MTX concentration range aimed at identifying IC_{50} (72 h exposure; open circles).

at establishing the IC_{50} values (Table 1), even if application of the drug concentration range based on TDC did not permit calculation of the IC_{50} value of cell lines very sensitive to long-term MTX exposures such as CCRF-CEM/S and CCRF-CEM/P (Figure 4A and B). The dose-response curves for CCRF-CEM/E and CCRF-CEM/T were superimposable on those generated previously (Figure 4C and D), allowing determination of an IC_{50}.

The degree of sensitivity/resistance to MTX of the four cell lines was also calculated according to Andreotti *et al.*,[30] based on the AUC values of the entire concentration range tested (200–6.25% TDC) (Table 2, first column).

Based on the AUC values, a pattern of relative sensitivity was shown for CCRF-CEM/S and CCRF-CEM/P cells (19231 and 18602, respectively), while relative resistance was shown by CCRF-CEM/E and CCRF-CEM/T (15 131 and 15 107, respectively). However, differences in the AUC of sensitive cells/AUC of resistant cells ratio (S/R ratio) did not vary markedly among the four cell lines. The maximum S/R ratio was in fact 1.3 for the most resistant phenotype (CCRF-CEM/T).

Evaluation of sensitivity/resistance to MTX was also determined by calculating the AUC in the lower drug concentration range (50–6.25% TDC).

In this case the maximum S/R ratios were slightly higher for the two resistant cell lines CCRF-CEM/E and CCRF-CEM/T (1.4 and 4.1, respectively) (Table 2, second column).

When cells were exposed to the same drug concentration range (200–6.25% TDC) for a short time (4 h) followed by a 72 h efflux, the growth inhibitory effects of MTX, as measured by the ATP-TCA, were markedly reduced in all three resistant cell lines compared to controls (Figure 3B). In particular, CCRF-CEM/E and CCRF-CEM/T displayed complete resistance at all concentrations tested (200–6.25% TDC). Also CCRF-CEM/P showed, as expected under the assay conditions, intermediate resistance with a maximum TGI of 63.5 at 200% TDC.

Consequently, evaluation of sensitivity/resistance to MTX based on S/R ratios of AUC values distinguished this parameter more precisely in the four cell lines (Table 2, third column). S/R ratios of AUC values were in this case very similar to S/R ratios based on IC_{50} values (Table 1).

Discussion

The possibility for optimizing anticancer chemotherapy is today provided studying the chemosensitivity or resistance of tumor explants from cancer patients using innovative, highly reliable, biochemical, molecular or immunohistochemical methodologies, capable of detecting the expression of gene products responsible for the occurrence of these phenomena,[19,20] and by use of *in vitro* chemosensitivity tests.[21–23,25]

In the future, data obtained from these methods may contribute to the prediction of clinical response to specific antineoplastic agents, helping guide the choice of optimum pharmacological treatment for individual patients, so that active drugs are chosen and inactive drugs are excluded which only contribute to host toxicity.

The attempts to develop a laboratory method to evaluate *in vitro* tumor chemosensitivity and resistance valid for prediction of clinical response to therapy have been numerous. Results have not always been reliable, and techniques are often complex and costly.[21–23,25]

As well as technical problems specific for the various methodologies, clinical application of these methods has been limited by the lack of prospective studies demonstrating the real clinical predictability of TCA results,[43] even if a recent meta-analysis of more than 4000 patients suggests that it is useful to introduce this type of technology into clinical oncological practice.[22]

A TCA based on the measurement of ATP using the bioluminescence reaction of the luciferin-luciferase system has been developed. This technique is able to evaluate accurately and rapidly the *in vitro* sensitivity or resistance of small human tumor explants cultured in an appropriate selective medium (CAM) not containing serum.[30]

The application of this test to ovarian carcinoma is highly accurate in predicting cisplatin resistance,[29] both in pretreated and non-pretreated patients, as well as sensitivity to the combination of carboplatin and cyclophosphamide in chemotherapy-naive patients,[44] and, finally, the superiority of individualized over standard chemotherapy in a significant number of heavily pretreated patients.[45]

MTX is a widely used folate antagonist, potent inhibitor of DHFR, for which the clinically relevant mechanisms of resistance are now well known.[1,4–18]

New antifolate inhibitors of DHFR or of other folate-dependent enzymes (e.g. thymidylate synthetase and purine transformylases) which have been evaluated preclinically and clinically have also been recently described.[1,46–48] These drugs have proved useful as selective inhibitors of cells resistant to MTX due to reduced polyglutamylation or alterations at the DHFR level, in relation to their innovative pharmacological characteristics.

Therefore, the necessity to try out new methods able to correctly determine the *in vitro* cytotoxicity of these drugs is being addressed.

Several authors have previously described direct *in vitro* testing of MTX sensitivity in patient-derived solid tumors[49,50] and leukemia.[28,51] Natural or acquired resistance to MTX in fresh tumor specimens could be a possible explanation for the lack of activity of this antifol both in the human tumor stem cell assay[49,50] and in a metabolic assay (i.e. the MTT dye reduction assay),[51] but lack of sensitivity to this agent in these assays might also be artifactual.[50,51] A significant role in determining artificial resistance to MTX may be played by protective agents such as thymidine,

hypoxanthine and reduced folates in native sera.[52–54] The use of dialyzed sera or serum-free media has been proposed to provide an adequate solution for testing patient-derived tumor cells.

To verify whether the ATP-TCA represents a valid alternative to previously described methods for measuring MTX sensitivity or resistance in the clinical setting, we investigated the ability of this assay to distinguish these patterns in human leukemia cell lines (CCRF-CEM) sensitive or resistant to this antifol by virtue of known biochemical mechanisms.[36–39] We had previously reported a good correlation between the ATP-TCA and a soft-agar colony forming assay regarding their ability to discriminate between a MTX-sensitive parental CCRF-CEM line and MTX-resistance sublines.[40]

In this study we performed correlation experiments between the ATP-TCA, a cell growth inhibition assay[41] and a radiometric method for measurement of dTMP synthesis.[28] There was excellent agreement between the results obtained by long-term exposure (72 h) to MTX using the ATP-TCA and the standard cell growth inhibition assay[41] when the two methods were applied to CCRF-CEM MTX-sensitive and -resistant cell lines (Table 1), as found previously.[51,52]

When measuring cell damage by the ATP-TCA and the radiometric assay for measurement of dTMP synthesis[2] following short-term (4 h) exposure to relatively high concentrations (1 and 3 μM) of MTX some discrepancies were observed between results obtained in CCRF-CEM/S and CCRF-CEM/P cell lines (Figure 1). This finding may be due to the different biochemical end-points used in the two methods for quantitating cell damage. Changes in ATP content will necessarily reflect variations in cell viability which is affected only later (after 72–96 h) by MTX. Variations in dTMP synthesis instead occur rapidly in cells undergoing DNA synthesis at the time of drug exposure.[28] Since incorporation of precursors of DNA synthesis is a relevant phenomenon in these cell lines, in which 40–50% of the population is in the S phase of the cell cycle (unpublished data), a more marked and rapid inhibition of dTMP synthesis following cytotoxic drug treatment can be observed with the radiometric method than that of ATP as measured by the ATP-TCA.

An apparent agreement between the two methods has been observed at MTX concentrations of 1 and 10 μM for CCRF-CEM/T and CCRF-CEM/E cell lines which show a high level of resistance to the drug (data not shown).

While data obtained with the [5-^{3}H]-2′-deoxyuridine tritium release assay confirm previous measurements,[28,39] the results obtained with the ATP-TCA displaying absence of variations in the ATP cell content might be attributed to the lack of short-term sensitivity of this test.

In order to evaluate the role of the serum-free medium CAM on MTX cytotoxicity *in vitro* we compared the inhibitory growth effects of MTX on CCRF-CEM cell lines using RPMI 1640 plus 10% HS and CAM with the ATP-TCA (Figure 2). CAM is a proprietary medium specifically developed for minimizing growth of non-neoplastic cells in primary cultures of tumor explants during *in vitro* chemosensitivity testing.[30] In spite of the observed reduction in CCRF-CEM cell growth rate induced by CAM compared to RPMI 1640 medium containing serum, good agreement was observed in the degree of cytotoxicity induced by MTX in the presence of the two different media in all the cell lines tested (Figure 2).

When using conditions proposed by Andreotti *et al.*[30] for clinical application [CAM medium and long-term, i.e. 144 h, exposure to MTX concentrations related to drug levels achievable in the plasma following administration of conventional doses (25–100 mg/m^2) of the antifol in patients[3] (200–6.25% of the TDC)], results allowed identification of cell lines highly resistant to MTX by virtue of impaired transport (CCRF-CEM/T) or elevated DHFR levels (CCRF-CEM/E) (Figure 3A). Under these experimental conditions MTX resistance due to altered polyglutamylation (CCRF-CEM/P) was, as expected, not detectable despite analysis of results by different parameters (AUC 200–6.25% TDC or 50–6.25% TDC and IC_{50}) (Table 2).

The CCRF-CEM/P cell line in fact exhibits resistance to MTX if short-term high-dose exposures are used[39] due to defective polyglutamylation which favors MTX efflux and inhibits its activation to more cytotoxic metabolites.[1,3] However, under conditions of continuous low-dose exposure such as those applied experimentally, its resistance is completely overcome, displaying a growth inhibition curve similar to that shown by sensitive parent cells. The continuous presence of MTX concentrations even slightly higher than those of the target enzyme (DHFR) causes cytotoxic damage independent of the polyglutamylation process since non-conjugated MTX is an inhibitor of such enzyme as potent as its polyglutamate derivatives.[3] The difference in MTX sensitivity between CCRF-CEM/S and CCRF-CEM/P cells was instead observed after short-term high-dose exposure to MTX (i.e. 4 h exposure to the antifol followed by a 72 h efflux period in drug-free medium; Figure 3B).

These data document that short-term exposure to MTX and possibly to other anticancer drugs for which cytotoxicity is not only dose-, but also time-

dependent might also be of clinical relevance and warrant future evaluation of shorter exposure times (i.e. 1-4 h) than those routinely used in the ATP-TCA, as explored before in the human tumor clonogenic assay.[55] The optimum interpretation of results remains a critical issue to be addressed in proper use of the ATP-TCA.

The use of a limited range of MTX concentrations (200-6.25% TDC) in the ATP-TCA produced tumor growth inhibition patterns similar to those obtained using a wider concentration range (Figure 4); however, it prevented the determination of the IC_{50} value for some of the very sensitive cells (CCRF-CEM/S) investigated.

The results of a 144 h exposure to MTX expressed on the basis of the AUC calculation procedure (Table 2) can quantitate correctly the degree of sensitivity in the different CCRF-CEM cell lines studied, but these values do not correlate well with those obtained with the classic methods for determining sensitivity/resistance (IC_{50} values). This implies a considerable difference between IC_{50} ratios and AUC values for resistant versus sensitive parental cells. Following a 4 h exposure to MTX differences both in absolute values of AUC and IC_{50} for resistant versus sensitive parental cells and in their ratios demonstrated a more accurate discrimination between sensitivity levels as we observed using the classic cell growth inhibition assay. The discrepancies observed in the ratios between sensitivity/resistance of the four cell lines tested, as calculated by the AUC method and IC_{50} determination, pointed out, especially in the use of a 144 h exposure, the need for more exact refinement of the algorithms to evaluate the results.

Conclusions

On the basis of these results, the ATP-TCA appears to be a suitable method for the evaluation of cytotoxicity induced by MTX. The value of this assay for testing clinical sensitivity/resistance to MTX and other antifolates in tumor explants from patients with solid tumors and leukemias remains to be demonstrated. To optimize assay conditions for clinical application, additional studies on the role of drug concentrations titrated on peak plasma concentrations achievable with high-dose (1 g/m^2) MTX infusion regimens, different exposure times and parameters for interpretation of results are warranted. Results may be better interpreted by using threshold values measured in cell lines with a known mechanism of resistance when assaying tumor samples from patients, as demonstrated in this investigation with MTX.

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