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Methotrexate chemosensitivity by ATP luminescence in human leukemia cell lines and in breast cancer primary cultures: comparison of the TCA-100 assay with a clonogenic assay

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Chemosensitivity assays are widely used to predict the ability of tumor cell lines to respond to potential or existing cytotoxic drugs. In this study we have compared the cell cloning assay first described by Salmon and Hamburger with a recently developed assay which measures viable cell number by ATP luminescence. Methotrexate (MTX) was chosen as the test agent, since cell lines with varying degrees of sensitivity to this agent were readily available. The results shown good correlation between the two assays, both of which are able to discriminate between the various cell lines used. MTX inhibition of primary breast carcinomas and cell lines shows a steep dose–response curve with a threshold concentration above which increasing dose does not increase sensitivity. In solid tumors, the plateau is usually reached at a level well below 100% inhibition. The ATP luminescence assay allows discrimination of MTX sensitivity between breast carcinomas and has considerable technical advantages over the cloning assay.

Key words: ATP, chemiluminescence, chemotherapy, luciferase, methotrexate.

Introduction

Measurement of the sensitivity of human tumor cell lines and tumor explants to cytotoxic drugs is important for the evaluation of new anti-cancer agents. Such methods may also allow prediction of patient responses to single agent and combination chemotherapy.[1,2] However, technical problems associated with some chemosensitivity assays such as the soft agar clonogenic assay[3,4] have in the past limited their clinical use.[1,5] The recent development of ATP luminescence assays[6–11] may change this due to their ability to measure chemosensitivity rapidly from small tumor samples.

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In this study we have compared the ability of an ATP luminescence assay (TCA-100 assay)[8,9] and a widely used clonogenic assay[12] to determine the sensitivity to methotrexate (MTX) of various human leukemic T lymphoblast CCRF-CEM cell lines with known mechanisms of resistance to the antifol. We have also applied the TCA-100 assay to screen for *in vitro* sensitivity or resistance to MTX and the commonly used CMF combination (cyclophosphamide + methotrexate + 5-fluorouracil) in human breast cancer specimens.

MTX is a widely used antimetabolite for the treatment of various solid tumors, including breast cancer, and lymphoid malignancies.[13] As a folate antagonist inhibitor of dihydrofolate reductase (DHFR), its primary mode of action is to interfere with various folate-dependent metabolic steps involved in the biosynthesis of DNA and its cytotoxicity varies with the proliferative status of the cell population.[13] It is at present used mainly in combination to avoid the emergence of drug resistance, as well as to modulate or increase selectively the effects of other drugs. In the treatment of lymphoid malignancies MTX is mainly combined with 6-mercaptopurine in maintenance treatment of childhood acute lymphocytic leukemia and at moderate/high doses with leucovorin rescue in various combinations for non-Hodgkin's lymphomas.[13] MTX shows experimental and clinical synergy with 5-fluorouracil (5-FU) if used sequentially (i.e. MTX followed by 5-FU) and the combination of cyclophosphamide, MTX and 5-FU (CMF) is successfully used both in the adjuvant therapy of operable primary breast cancer and in the treatment of matastatic disease.[14] MTX resistance may occur as the result of decreased cellular uptake, amplification of the DHFR gene, structural alteration of DHFR leading

to reduced binding of MTX to the enzyme or reduced intracellular retention and activation of MTX by polyglutamate formation.[13]

Among the cytotoxicity assays available to study mechanisms of MTX resistance in cell lines,[15–18] the clonogenic assay represents one of the established reference methods. It is a cell proliferation assay, measuring the number of clone-forming cells present in the population tested following exposure of the cells to cytotoxic drugs. The main disadvantage of the clonogenic assay is poor practicability due to the extensive time required for execution of the test.

The ATP luminescence assay measures viable cell number by the extremely sensitive reaction of ATP with firefly luciferase in the presence of luciferin.[6,19,20] When used as a chemosensitivity assay, the assay is performed in a microplate to which drugs are added at varying dilutions with 1×10^6 cells/plate.[11] We were keen to test the ability of this new assay alongside a clonogenic assay and to examine its ability to discriminate between MTX-sensitive and -resistant leukemia cell lines. The TCA-100 ATP luminescence assay is currently undergoing clinical validation. We present data showing its ability to measure MTX chemosensitivity in breast tumors and to assess the contribution made by MTX to the efficacy of the CMF regimen in a series of tumors in which all three agents and the combination were tested simultaneously.

Materials and methods

Chemicals

For studies in Italy, MTX sodium salt was obtained from Cyanamid Italia (Catania, Italy) and was dissolved in water. Media, sera and antibiotics for tissue culture were purchased from Gibco (Grand Island, NY). Plasticware was purchased from Nunc (Roskilde, Denmark). For studies in Scotland, MTX was obtained from Lederle Laboratories (Gosport, UK) and 5-fluorouracil (5-FU) from David Bull Laboratories (Warwick, UK). Since cyclophosphamide is inactive *in vitro*, 4-hydroperoxycyclophosphamide (4-HC) obtained from Nova (Baltimore, MD) was used instead. All other necessary reagents were obtained from BATLE LE (Fort Lauderdale, FL).

Cell lines

A human T lymphoblast leukemic parental cell line sensitive to MTX (CCRF-CEM S) and three derived sublines resistant to MTX by virtue of various biochemical mechanisms have been used in this study. In particular, CCRF-CEM/E cells are resistant due to the presence of increased DHFR levels as described by Mini *et al.*;[15] CCRF-CEM/P and CCRF-CEM/T cells show resistance because of impaired polyglutamylation and transport of the antifol, respectively.[16,18] Cells were grown as suspension culture in RPMI 1640 medium supplemented with 10% horse serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a 5% CO_2 atmosphere, and subcultured twice weekly.

CCRF-CEM/E and CCRF-CEM/T were grown in the continuous presence of 0.2 and 1 μM MTX, respectively. CCRF-CEM/P, which were selected for MTX resistance by repeated short-term (24 h) exposure to a higher concentration (3 μM) of the drug and retained sensitivity to low-dose long-term exposure to the antifol, were cultured in its absence, showing a stable resistant phenotype. Under these conditions, the doubling time of exponentially growing sensitive and resistant cells varied between 21 and 28 h.

Clonogenic assay

Exponentially growing cells were prepared at a density of 5×10^4 cells/ml in RPMI 1640 medium supplemented with 10% horse serum and distributed in duplicate 5 ml portions into tissue culture tubes, to which 0.05 ml of drug solutions at concentrations varying from 1 nM to 30 μM were added on the basis of the expected IC_{50} values. The cells were incubated at 37°C and after 72 h cell viability was measured by the clonogenic assay described by Chu and Fischer.[12] Briefly, the number of cells cloned varied from 100 to 5000/plastic tube, depending upon the expected cell kill. Maximum cell inoculum (5000) allowed quantitation of cell kill in the *in vitro* clonogenic assay ≤ 3 logs. The average cloning efficiency for control cells was 40, 56, 26 and 25 for CCRF-CEM S, CCRF-CEM E, CCRF-CEM T and CCRF-CEM P, respectively. These values were normalized to 100%. Each condition was cloned in quadruplicate.

Tumors

Human breast adenocarcinomas were obtained from the surgical service at Ninewells Hospital as previously published.[10,11] Briefly, tumor biopsies which were not required for diagnostic purposes

were minced and incubated with a mixture of enzymes. [Tumor dissociation enzyme reagent (TDER); BATLE] overnight at 37°C. Following dissociation, the cells were washed and if viability was less than 50%, non-viable debris was removed by Ficoll-Hypaque sedimentation (Lymphoprep; Nycomed, Birmingham, UK). A total of 33 primary breast tumors were assayed simultaneously with MTX, 5-FU and 4-HC as single agents, and the CMF combination. All were classified histologically as infiltrating ductal mammary adenocarcinomas. Of the 33 tumors, 31 were primary and two secondary: a lymph node metastasis and a recurrence following lumpectomy.

TCA-100 assay

ATP luminescent assays (TCA-100) were performed according to the manufacturer's instructions and previously published methods.[8–10]

CCRF-CEM cell lines (1000 or 2000 cells/well) were first plated onto a microplate with RPMI 1640 medium implemented with 10% horse serum and exposed to MTX concentrations varying from 1 nM to 30 μM on the basis of the expected IC_{50} values. After 72 h incubation at 37°C ATP was extracted by the addition of 50 μl tumor cell extraction reagent (TCER; BATLE) to each well and the sample processed as described below.

Dissociated tumour cells were suspended in a serum-free culture medium (LMA, BATLE) at 20 000 cells/well. Serial dilutions of MTX and other drugs were made in polypropylene microplates (Costar, High Wycombe, UK) using concentrations approximating to the peak plasma concentrations achieved *in vivo*. Control wells without drugs and with a maximum inhibitor of ATP (MI, BATLE) were also present in the plate. Following addition of cells to all wells, the microplates were incubated in 5% CO_2 at 37°C for 6–7 days.

ATP was extracted in the wells by addition of 50 μl TCER to each well. Following vigorous pipetting to ensure extraction of ATP from all of the cells, 50 μl from each well was then transferred to a 3.5 ml plastic vial (Sarstedt, Numbrecht, Germany) and ATP measured by the injection of 55 μl luciferin and luciferase (BATLE) in an LB953 luminometer (Berthold, Wildbad, Germany). The percentage ATP was compared with control wells without drug to give a percentage survival:

$$\frac{\text{luminescence count (test)} - \text{luminescence count (MI)}}{\text{luminescence count (no drug)} - \text{luminescence count (MI)}} \times 100$$

The percentage inhibition was calculated as 100−% surviving cells. For comparison between TCA-100 results, the area under each curve (concentration × inhibition) was calculated by the trapezoidal rule.

Results

MTX-sensitive/resistance leukemia cell lines

The results from the various CCRF-CEM cell lines with different sensitivities to MTX assayed by the TCA-100 and the clonogenic assay are shown in Figure 1. These sublines have clearly shown different sensitivies to MTX with no significant difference between the results obtained from the two assays. IC_{50} values varied from 5.9×10^{-9} to 2.0×10^{-6} M, respectively, from parental sensitive line (CCRF-CEM/S) to the most resistant subline (CCRF-CEM/T) (Table 1). Dose–response curves showed a similar sigmoidal shape with both assay methods. When two different densities of cells (1000 or 2000/well) were used in the TCA-100 assay, no differences were observed in the degree of growth inhibition induced by MTX at each drug concentration. Consequently no significant variation was observed in the IC_{50} values (Table 2).

Some tumors and cell lines showed a constant degree of inhibition across the range of concentra-

Table 1. IC_{50} values for the experiments shown in Figure 1, showing the similarity of the results from both assay methods for the different cell lines [both methods are able to discriminate between parent (CEM/S), sensitive (CEM/P) and resistant (CEM/E, CEM/T) cell lines]

Method	CEM/S IC_{50}	CEM/P IC_{50}	CEM/E IC_{50}	CEM/T IC_{50}
Cloning assay	1.2×10^{-8}	1.8×10^{-8}	6.0×10^{-7}	3.4×10^{-6}
TCA-100	1.5×10^{-8}	1.8×10^{-8}	9.3×10^{-7}	4.3×10^{-6}

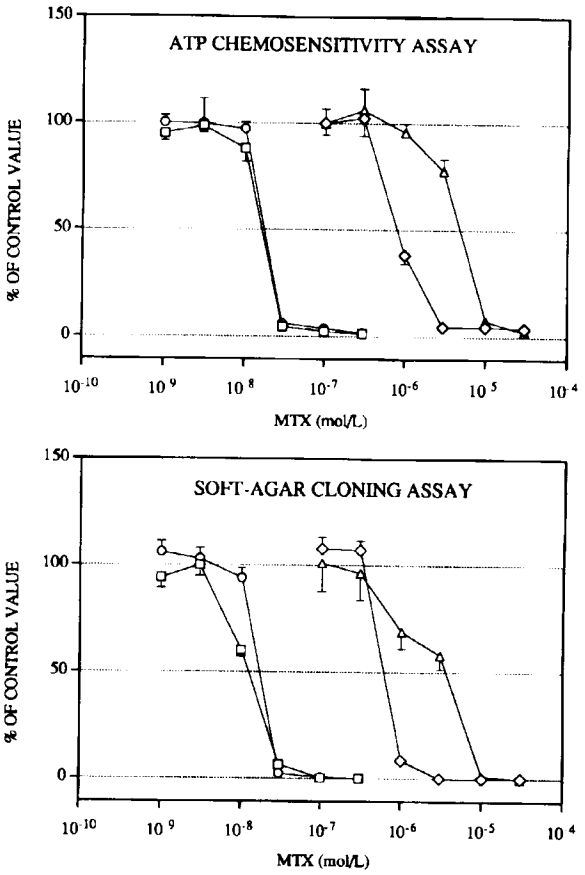

Figure 1. Growth inhibitory effects of MTX (72 h exposure) expressed as a percentage of control without drug, evaluated by TCA-100 method (upper panel) and soft-agar colony forming assay (lower panel) on CCRF/CEM parent (CEM/S, □), sensitive (CEM/P, ○) and resistant (CEM/E, ◇ CEM/T, △) cell lines. Each point represents the mean ± SD of six experiments.

tions usually tested for MTX (87.5–5600 nM). However, if further dilutions are performed below the minimum normally used (87.5 nM), a typical sigmoid dose–response curve is then seen,[21] suggesting that a maximum response less than 100% may occur with MTX.

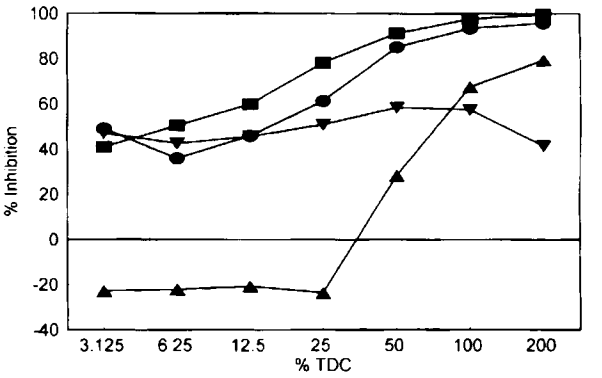

Figure 2. A typical TCA result showing the inhibition achieved at each test drug concentration (TDC) for MTX (▼), 5-FU (●), 4-HC (▲) and the combination of all three drugs (CMF) (■). In this case there is sensitivity to CMF which is largely accounted for by 5-FU alone. There is resistance to 4-HC and some sensitivity to MTX which may be reponsible for the enhanced sensitivity to the combination. The 100% TDC for MTX = 6.2 μM, for 5-FU = 173 μM and for 4-HC = 1.0 μM.

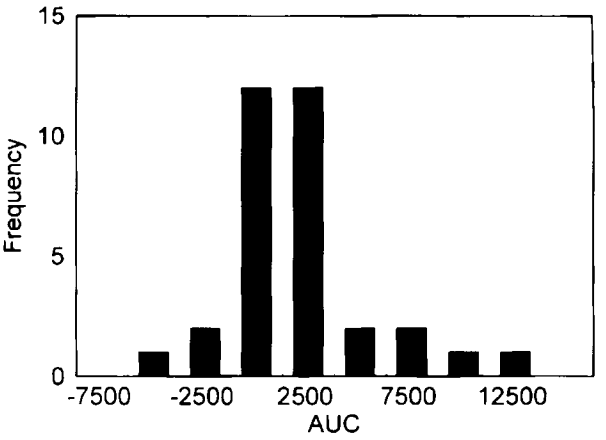

Figure 3. The area under the curve (concentration × inhibition) for 33 breast tumors assayed with MTX showing considerable heterogeneity of sensitivity. Most tumors showed mild sensitivity. The negative values represent complete resistance at all concentrations tested. While some of these negative results are due to different cell growth within the plate, this phenomenon is more common with antimetabolites and may represent an *in vitro* growth-promoting effect of MTX on some tumors.[11]

Table 2. IC_{50} values for TCA-100 assay results with the different cell lines seeded at either 1000 or 2000 cells/well showing that the assay is insensitive to variation in cell number

Cells/well	CEM/S IC_{50}	CEM/P IC_{50}	CEM/E IC_{50}	CEM/T IC_{50}
1000	1.8×10^{-8}	1.8×10^{-8}	9.9×10^{-7}	5.5×10^{-6}
2000	1.9×10^{-8}	1.9×10^{-8}	9.2×10^{-7}	5.9×10^{-6}

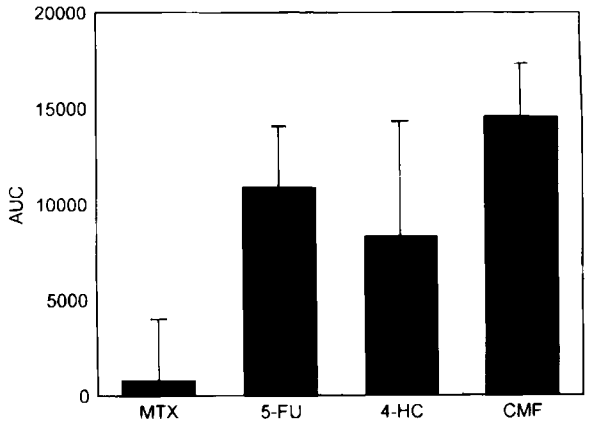

Figure 4. Comparison of AUC data for MTX, 5-FU, 4-HC and the CMF combination for 33 breast tumors. In general, breast tumors show resistance in the assay to MTX alone, but good sensitivity to the CMF combination. There is considerable variation in 5-FU and 4-HC sensitivities, as well as MTX between tumors.

Breast cancer primary cultures

Since it is able to use much smaller numbers of cells than most previous chemosensitivity assays, the TCA-100 assay is able to obtain results from surgical biopsies, needle biopsies or effusions. A typical assay result for MTX and the other drugs included in the CMF combination is shown in Figure 2. The MTX response is constant across the range of concentrations tested. This tumor shows resistance to 4-HC, but has considerable sensitivity to 5-FU, although 100% inhibition is not achieved until all three drugs are combined (CMF). Similar assays were performed for a series of 33 breast carcinomas, allowing the relative contribution of MTX to the CMF regimen to be assessed in a series of 33 breast carcinomas. While most tumors showed quite weak sensitivity to MTX (Figure 3), their response to the CMF regimen was good (Figure 4), reflecting additive effects between the agents making up this regimen. There was also considerable heterogeneity of MTX sensitivity between tumors (Figure 3), as previously described.[11]

Discussion

Clonogenic assays measure the size of the proliferating cell compartment in tumor cell populations, but in solid tumors only a small percentage of the cells are in the cell cycle. Consequently, clonogenic assays tend to be unreliable with solid tumor samples due to their low evaluability rate.[1,5] Such assays are now used mainly for research with cell lines. Clonogenic assays are also time-consuming to perform in comparison with ATP luminescence methods which measure total viable cell number, irrespective of their stage in the cell cycle. The TCA-100 assay is therefore better suited to the measurement of chemosensitivity in tumor cell preparations since cells have only to survive—not divide—in short-term culture to be measured. While normal cell survival can produce false results, in practice this is effectively limited by the use of serum-free medium and suspension culture.[7–9] Abnormally proliferating, benign cells offer a different problem and it is important to obtain a tissue diagnosis by histopathology or cytology with all specimens tested for chemosensitivity.[9] Rules for interpretation of assay results offer a further safeguard against false results.[9]

Cell lines

The growth inhibition curves obtained with both methods show marked sensitivity to MTX for CCRF-CEM/S and CCRF-CEM/P cells, while CCRF-CEM/E and CCRF/CEM/T cell are resistant to the antifol, in accordance with previously published data.[16,18] In both assays, cells were exposed continuously to MTX for 72 h before assessment. The CCRF-CEM/P cells exhibit resistance to MTX if short exposures are used[18] due to defective polyglutamylation, but under conditions of continuous exposure, their resistance is completely overcome with an IC_{50} similar to that shown by sensitive parent cells. Thus neither assay is suitable for evaluation of drug resistance in cases of altered polyglutamylation, which favors MTX efflux and inhibits its activation to more cytotoxic metabolites. The continuous presence of MTX concentrations higher than those of the target enzyme DHFR causes cytotoxic damage independent of the polyglutamylation process since non-conjugated MTX represents an inhibitor of such enzyme as potent as its polyglutamate derivatives. Short-term exposure to antimetabolite and other anticancer drugs for which cytotoxicity is not only dose-, but also time-dependent might also be of clinical relevance. The luminescence assay could be used with short exposure times to explore this further.

In this study, both assays produced very similar results with the CCRF-CEM cell sublines which were developed for the study of MTX resistance. While IC_{50} results are useful for comparison of such assays

with cell lines, 50% tumor cell kill is not the issue in real tumors: to be useful 90–100% kill is required, while in combinations lesser degrees of kill may be useful even at low concentrations. To take account of this, the area under the curve (AUC) representing concentration × inhibition at each dose can be used to allow comparison of results.[9]

Breast tumours

The breast tumors tested show resistance or low sensitivity to MTX as a single agent. Several other authors have previously described resistance to MTX of patient-derived solid tumors,[22,23] but the drug still appears to be useful in combination. The degree of chemosensitivity to MTX varied widely between tumors, a phenomenon which has also been observed with other single agents or combinations in breast cancer.[10,11] Preliminary data suggests that the TCA-100 assay has a predictive accuracy of 85% in breast cancer when the results are correlated with clinical outcome (unpublished data). Since single agent therapy with MTX would not be ethical, we are unable to correlate the MTX results with clinical outcome: studies in xenograft models would be desirable.

Conclusion

It is clear from this study that both the clonogenic and the TCA-100 assays can be used to determine chemosensitivity responses to MTX with cell lines. However, the TCA-100 assay has some advantages in that it can also be applied to tumor tissue and it requires less technical expertise than the clonogenic assay.

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