ELSEVIER

International Journal of Pharmaceutics 111 (1994) 25–30

international journal of pharmaceutics

In vitro assay of the effects of anticancer drugs on leukemic T-cells by flow microcalorimetry

Cristina P. da Silva [a,c], Catarina R. de Oliveira [b,c], Maria da Conceição P. de Lima [a,c,*]

[a] *Department of Biochemistry, Faculty of Sciences and Technology,* [b] *Laboratory of Biochemistry, Faculty of Medicine,* [c] *Center for Neurosciences, University of Coimbra, 3049 Coimbra Codex, Portugal*

Received 3 January 1994; accepted 6 April 1994

Abstract

The effects of the anticancer drugs methotrexate, vincristine, adriamycin and daunorubicin on leukemic T-cells were studied by flow microcalorimetry and conventional cell proliferation assays. Alterations in the metabolic heat power of cell suspensions due to drug treatment were measured, as a function of time and dose of exposure, and correlated with cell growth inhibition data. While no clear correlation was found for the compound vincristine, a good correlation between calorimetric and cytotoxicity data was obtained for the other drugs tested, suggesting a potential use of flow microcalorimetry for in vitro predictive tests of the effects of anticancer drugs.

Key words: Adriamycin; Anticancer drug; Cytotoxic assay; Daunorubicin; Flow microcalorimetry; Leukemic T-cell; Methotrexate; Vincristine

1. Introduction

Treatment of acute lymphoblastic leukemia is commonly based on the use of growth inhibiting compounds such as methotrexate (MTX), vincristine (VCR) and the anthracyclines adriamycin (ADR) and daunorubicin (DNR) (Bonadonna et al., 1988; Santoro and Gasparini, 1988). However, there is increasing evidence that the sensitivity of tumour cells to an antineoplastic treatment varies widely (Calman et al., 1980; Beck, 1987; Bradley et al., 1988). Therefore, in vitro evaluation of the effects of anticancer drugs on tumour cell populations, prior to the onset of a chemotherapeutic treatment, is of significant interest.

At present, the most commonly used methods for studying cytotoxic effects on cells in vitro are based on clonogenic assays (Adamson, 1991). These methods, however, have the disadvantage of being relatively slow. Microcalorimetry is a very simple and sensitive analytical technique for monitoring the metabolic activity of growing cells. Since it is a non-specific method which takes into account the total cell metabolism (anabolism and catabolism), any change in the status of a cell

Abbreviations: ADR, adriamycin; DNR, daunorubicin; MTX, methotrexate; VCR, vincristine.

* Corresponding author. Tel: 351-39-20190; Fax: 351-39-26798.

SSDI 0378-5173(94)00120-T

population induced by different agents can be observed (Crabtree and Nicholson, 1988; Schön and Wadsö, 1988a; Wadsö, 1988). If the breakdown of biomass is balanced by anabolism, cells will give rise to a constant heat production rate. On the other hand, if cells are maintained in a rich medium and growth occurs, the increased amount of biomass will be observed in a microcalorimeter as an increase in thermal power with time. In contrast, when cell proliferation is inhibited by the addition of a drug, a decrease in heat production rate will occur (Schön and Wadsö, 1988b; Wadsö, 1988; Brandão et al., 1992). Therefore, microcalorimetric methods may have some potential for in vitro predictive tests of the effect of anticancer drugs.

In the present work, we have studied the effects of four anticancer drugs (MTX, ADR, DNR and VCR) on the metabolism of human leukemic lymphoblasts (CCRF-CEM), using flow microcalorimetry. These results were correlated with data obtained from conventional cell proliferation assays.

2. Materials and methods

2.1. Materials

RPMI 1640 medium, methotrexate (sodium salt), adriamycin (hydrochloride), daunorubicin (hydrochloride) and vincristine (sulphate salt) were purchased from Sigma Chemical Co. (St. Louis, MO). Foetal calf serum was obtained from Gibco Laboratories (Grand Island, NY). All other chemicals were of analytical grade.

2.2. Cells and cell culture

The human T-lymphoblastoid cell line CCRF-CEM was obtained from the cell culture facility at the University of California, San Francisco, and was maintained in suspension in RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum, 20 mM Hepes (pH 7.4), 1 mM pyruvic acid, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were grown at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

2.3. Cell proliferation studies

For studying the growth inhibitory effects of the anticancer drugs, cells were incubated with the indicated concentrations of the drugs for 24 h, at an initial cell density of 4×10^5 cells/ml. An appropriate volume of drug vehicle was added to untreated cells. After this period of incubation, cellular growth was evaluated by cell counting in a Coulter counter or in a hemocytometer and cell viability was assayed by trypan blue exclusion. For each experimental condition tested, samples were prepared in duplicate and the second set of culture flasks was used in the calorimetric experiments.

2.4. Flow microcalorimetry

The calorimetric experiments were performed at 37°C in an LKB 2277 model microcalorimeter (Bioactivity Monitor), operating in the flow-through mode and with a sensitivity of 30 μW, full scale. The cell suspensions were kept under continuous magnetic stirring in the reaction vessel outside the calorimeter and were pumped into the measuring cell with an LKB 2232 microperspex-S peristaltic pump at a flow rate of 40 ml/h. Electrical calibrations of the instrument were always performed prior to the experiments.

Immediately before the microcalorimetric assays, drug-treated or untreated cells were harvested by centrifugation ($200 \times g$, 8 min) and resuspended in 5 ml of fresh culture medium. A baseline was first established by pumping 12 ml of culture medium through the calorimeter, after which the 5 ml suspension of cells was added. The initial cell density in the calorimetric experiments was in the range $0.2–2 \times 10^6$ cells/ml, depending on the preincubation conditions used. The microcalorimetric assays of cell metabolism were carried out in a closed circuit and the heat production rate was monitored for different periods of time (from 30 min up to a maximum of 21 h). Alternatively, untreated cells were pumped into the calorimeter and aliquots of stock solutions of the various compounds were added directly to the reaction vessel, giving the desired final drug concentrations. Cell number and viabil-

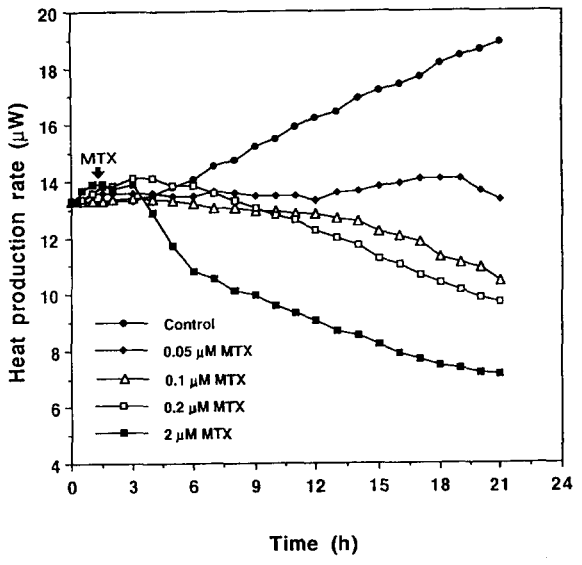

Fig. 1. Dose- and time-dependent effects of MTX on the metabolic heat production rate of CEM cells. Cells were harvested by centrifugation (200× *g*, 8 min), resuspended in 5 ml of fresh RPMI 1640 medium and kept at 37°C under constant magnetic stiring in a reaction vessel containing culture medium. Cells were pumped through the calorimeter at a flow rate of 40 ml/h, using a closed circuit. At the time point shown by the arrow, MTX was added at the final concentrations indicated. The metabolic heat power was recorded for 21 h (sensitivity 30 μW, full scale).

ity were also measured at the end of the calorimetric experiments by the procedures described above.

3. Results and discussion

In the present work, the metabolic activity of CEM leukemic T-lymphoblasts and the effects of several anticancer drugs upon cell metabolism were studied using flow microcalorimetry. As an example, Fig. 1 shows typical calorimetric curves obtained for CEM cells in the absence and in the presence of different concentrations of MTX. As demonstrated in Fig. 1, the thermal power recorded for a cell suspension in the absence of drug increased almost linearly with time, this effect reflecting the contribution of cell growth to the total heat production rate. On the other hand, following addition of the anticancer drug MTX, the thermal power remained either constant (for low drug concentrations) or decreased with time in a concentration-dependent manner. In the range of MTX concentrations investigated, such decreases in thermal power were clearly detected 5 h after addition of the drug to the reaction vessel. In contrast to MTX, direct addition of the compounds ADR, DNR or VCR to the cell suspensions produced only marginal effects on the total cell metabolism, within the time span used for the calorimetric experiments (data not shown). For studying the effects of these anticancer drugs upon CEM cell metabolism a more convenient procedure was to incubate the cells with the compounds for one generation time (approx. 24 h), prior to use in the calorimetric assays. For the compound MTX, similar results were obtained with both procedures.

Using such an experimental approach, a calori-

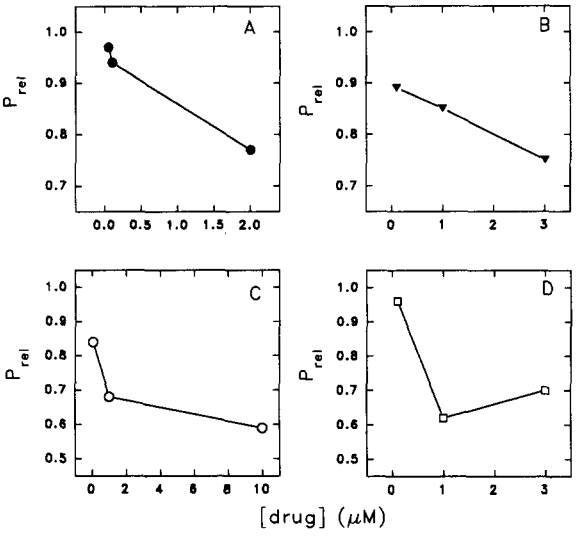

Fig. 2. Relative thermal power (P_{rel}) of CEM cells, as a function of drug concentration. Suspensions of untreated or drug-treated cells (treatment for 24 h with the indicated concentrations of MTX (A), ADR (B), DNR (C), or VCR (D)) were pumped into the calorimeter, using the procedures described in section 2. Values of relative thermal power (P_{rel}) represent the ratio between the heat production rate levels obtained for drug-treated as compared to untreated samples and were calculated from measurements made 10 min after pumping the cells into the calorimeter (zero time point). The results represent the mean of 2–5 independent experiments.

metric index of the effect of each drug – P_{rel} – was calculated. P_{rel} is defined by:

$$P_{rel} = P_d/P_c$$

where P_d and P_c are the thermal power for drug-treated and control samples, respectively (Schön and Wadsö, 1988b). Both values, P_d and P_c, refer to measurements made at the same time point (10 min), after loading the reaction vessels (taken as zero time point).

When values of relative thermal power (P_{rel}) were plotted vs drug concentration, dose-response curves of the type shown in Fig. 2 were obtained. As depicted in Fig. 2, within the range of concentrations analysed, the calorimetrically observed effects of all the compounds tested were a function of drug dose.

In order to analyse the potential value of flow microcalorimetry as an in vitro predictive test for anticancer drugs, results from the calorimetric experiments were correlated with data from cell proliferation assays. For this purpose, values of P_{rel} were compared with corresponding values of ΔC_{rel}, where ΔC_{rel} is defined by:

$$\Delta C_{rel} = \Delta C_d/\Delta C_c$$

ΔC_d represents the change in cell concentration for a 24 h drug-treated sample and ΔC_c denotes the 24 h change in cell concentration for an untreated control sample. Fig. 3 summarizes

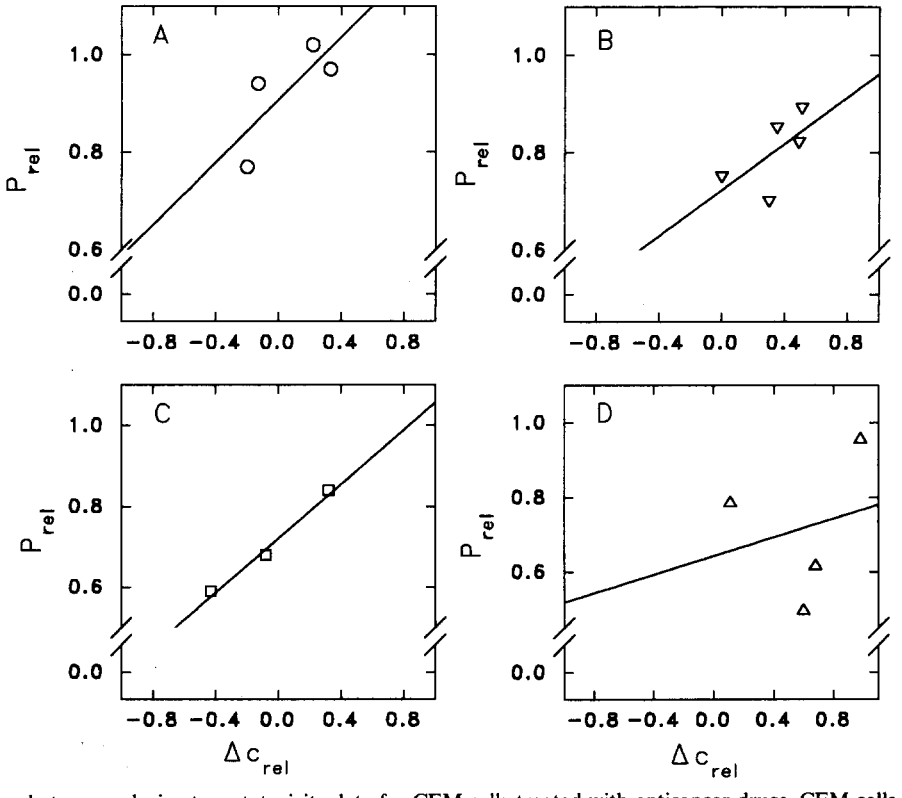

Fig. 3. Correlation between calorimetry-cytotoxicity data for CEM cells treated with anticancer drugs. CEM cells preincubated for 24 h with different concentrations of MTX (A), ADR (B), DNR (C) or VCR (D) were analysed by flow microcalorimetry, as described in section 2. Values of P_{rel} were calculated and compared with the relative changes in cell density (ΔC_{rel}) obtained from cell counting experiments performed in parallel on a second set of cells preincubated under the same drug conditions. Each point represents values obtained from independent experiments.

the results of combined calorimetry-cytotoxicity experiments obtained for the drugs studied in this work. Whereas for MTX, ADR and DNR (Fig. 3A–C) an approximately linear relationship between calorimetric-cytotoxicity results was obtained, such behaviour was clearly not observed for VCR (Fig. 3D). In this regard, it should be noted that, in addition to its growth inhibitory effects, an anticancer drug may interfere with other cellular processes which also contribute to the total heat production rate of a cell suspension, such as the basal maintenance metabolism (Schön and Wadsö, 1988b). Thus, if in addition to an arrest of cell proliferation a drug also induces, for example, changes in the relative rates of different metabolic pathways or activates cellular repair mechanisms, such processes might give rise to novel heat production events which could account for the absence of proportionality between calorimetric and growth inhibition data, as observed in this work for the compound VCR.

Concerning the effects of the other drugs tested, it should be mentioned that, in spite of the tendency to linearity which was found between P_{rel} and ΔC_{rel} (Fig. 3A–C), the results also show a shift from the ideal coordinates one would expect for a direct proportionality between those parameters. In fact, for such a direct proportionality, a straight line between the points $P_{rel} = 1$, $\Delta C_{rel} = 1$ (no drug present) and $P_{rel} = 0$, $\Delta C_{rel} = -1$ (all drug-treated cells dead) would be expected (Schön and Wadsö, 1988b). This would correspond to the ideal situation where every cell affected by a drug would give rise to a certain decrease in the metabolic thermal power, reflecting the probability of cell death, and also that no affected cell should be able to recover. However, as discussed above, the metabolism of a growing cell population is a complex phenomenon and many processes not only related to growth contribute to heat production (Wadsö, 1988; Silva and Lima, 1993). In fact, these considerations are reflected in our results which generally indicated a less pronounced decrease in P_{rel} (small effect on total cell metabolism for drug-treated samples) than expected from the growth inhibition measurements. However, in spite of the fact that no simple quantitative relationship exists between relative thermal power and relative proliferation, it is still interesting that significant linearities between values of these parameters were obtained for MTX, ADR and DNR. Therefore, although less promising for in vitro quantitative assessment of drug-induced cytotoxicity, calorimetric measurements are still judged to be of significant interest in clinical and pharmacological areas as analytical tools, for example, for rapid diagnosis and screening of drug-resistant cells.

Acknowledgements

This work was supported by Junta Nacional de Investigação Científica e Tecnológica (JNICT) and Instituto Nacional de Investigação Científica (INIC), Portugal. We thank Calouste Gulbenkian Foundation for providing the financial support for the acquisition of the LKB microcalorimeter.

References

Adamson, P.C., Balis, F.M., Cole, D. and Poplack, D.G., Comparison of nonclonogenic assays to clonogenic assays for chemosensitivity testing of leukemic lymphoblasts. *Proc. Am. Assoc. Cancer Res.*, 32 (1991) 385–392.

Beck, W.T., The cell biology of multiple drug resistance. *Biochem. Pharmacol.*, 36 (1987) 2879–2887.

Bonadonna, G., Bernardo, G., Gianni, L. and Della Cuna, R., Pharmacology, clinical toxicity, dosage and general indications of growth inhibiting compounds. In Bonadonna, G. and Della Cuna, G.R. (Eds), *Handbook of Medical Oncology*, Masson, Milano, 1988, pp. 335–378.

Bradley, G., Juranka, P.F. and Ling, V., Mechanism of multidrug resistance. *Biochim. Biophys. Acta*, 948 (1988) 87–128.

Brandão, F.O., Silva, C.P., Lima, M.C.P. and Oliveira, C.R., Prediction of the cytotoxic effects of methotrexate on human T-lymphoblastic leukemia cells. *J. Exp. Clin. Cancer Res.*, 11 (1992) 51–62.

Calman, K.C., Smyth, J.F. and Tattersall, M.H.N., *Basic principles of cancer chemotherapy*, Macmillan, London, 1980.

Crabtree, R. and Nicholson, B.A., Thermodynamics of metabolism. In Jones, M.N (Ed.), *Biochemical Thermodynamics*, 2nd Edn, Elsevier, Amsterdam, 1988, pp. 347–390.

Santoro, A. and Gasparini, M., Acute leukemias. In Bonadonna, G. and Della Cuna, G.R. (Eds), *Handbook of Medical Oncology*, Masson, Milano, 1988, pp. 745–764.

Schön, A. and Wadsö, I., The use of microcalorimetry in

studies of mammalian cells. *J. Thermal Anal.*, 33 (1988a) 47–50.

Schön, A. and Wadsö, I., The potential use of microcalorimetry in predictive tests of the action of antineoplastic drugs on mammalian cells. *Cytobios*, 55 (1988b) 33–39.

Silva, C.P. and Lima, M.C.P., Calorimetric methods in Biochemistry and Biology: application of flow microcalorimetry to the study of enzyme activities in crude tissue homogenates. *Ciência Biol.*, (1993) in press.

Wadsö, I., Thermochemistry of living cell systems. In Jones, M.N. (Ed.), *Biochemical Thermodynamics*, 2nd Edn, Elsevier, Amsterdam, 1988, pp. 241–309.