Pergamon

Toxicology in Vitro 11 (1997) 553–556

Measurement of Cytotoxicity by ATP-based Luminescence Assay in Primary Cell Cultures and Cell Lines

I. A. CREE* and P. E. ANDREOTTI†

*Department of Pathology, Institute of Ophthalmology, University College London, Bath Street, London EC1V 9EL, UK and †Atlantic Scientific Development, Inc., 23257 State Road 7, Boca Raton, FL 33428, USA

Abstract—Drug discovery and toxicological safety testing share a need for dependable *in vitro* cellular toxicity tests. Ideally such tests should be objective, quantitative, reproducible and able to lend themselves to automation. A number of assays fulfil these criteria well, but recently it has become clear that the molecular phenotype of the cell tested and the complex interplay between different cell types can radically alter the response to individual agents. The differences observed between primary cell cultures and cell lines make it preferable to use primary cultures for assessment of toxicity, yet the problems of using primary cell cultures are considerable as the number of cells available for testing is often small. Recently, we have developed a short-term cell culture assay based on the detection of ATP by the luciferin–luciferase reaction. Four drugs/agents can be tested in triplicate at seven dilutions in one 96-well microplate with 1000 cells/well in the case of cell lines, or 10,000 cells/well for primary tumour tissue. The small number of cells required is a major advantage of this method. Initially developed as a tumour chemosensitivity assay, the assay has shown considerable promise as a general *in vitro* toxicity assay allowing both cell lines and primary tissue cultures to be tested. Heterogeneity of sensitivity is present in benign tissue biopsies as well as tumours. Molecular alterations within the cell and the interplay of different cell types have been addressed in a number of different model systems using the assay, suggesting that this technology may have more general application. © *1997 Elsevier Science Ltd*

Abbreviations: AUC = area under the concentration–inhibition curve; 5-FU = 5-fluorouracil; TDC = test drug concentration.

Keywords: ATP; luminescence; cell culture; luciferase; chemosensitivity; toxicity.

INTRODUCTION

Drug discovery and toxicological safety testing share a need for dependable *in vitro* cellular toxicity tests. Ideally such tests should be objective, quantitative, reproducible and able to lend themselves to automation. A number of assays fulfil these criteria well, but recently we have developed a short-term cell culture assay based on the detection of adenosine triphosphate (ATP) by the luciferin–luciferase reaction (Andreotti *et al.*, 1995) which shows considerable promise as an *in vitro* toxicity assay as well as a tumour chemosensitivity assay. This microplate ATP assay requires relatively few cells and is therefore suitable for the assessment of toxicity using tissue-derived cells as well as cell lines.

The ATP assay was first developed as a somatic cell viability assay by Lundin *et al.* (1986) and has since been developed by several groups as a tumour chemosensitivity assay (Andreotti *et al.*, 1991 and 1995; Sevin *et al.*, 1993). Four drugs/agents can be tested in triplicate at seven dilutions in one microplate with 1000–5000 cells/well in the case of cell lines, or 10,000 cells/well for primary tumour tissue (Andreotti *et al.*, 1995). Examination of tumour material shows more than 80% tumour cells at the end of the assay due to the action of the selective cell culture medium and use of plates which do not allow cell adherence required by fibroblasts within the tumour (Andreotti *et al.*, 1995). The assay compares favourably with older tests such as tetrazolium reduction assays (e.g. the MTT assay) (Petty *et al.*, 1995) and clonogenic assays (Cree *et al.*, 1995). Intra- and inter-assay variation is generally less than 10% (Andreotti *et al.*, 1995). Cell lines may be used to provide quality control from month to month, using whole plates without drug/agent addition and IC_{50} plates to which a standard concentration of an agent expected to produce 50% inhibition is added (Andreotti *et al.*, 1995). In addition to measurement of effects on cell proliferation/number, ATP assays give information about sublethal cell damage since the production of ATP is a necessary function of cell

*Author for correspondence.

0887-2333/97/$17.00 + 0.00 © 1997 Elsevier Science Ltd. All rights reserved. Printed in Great Britain
SSDI 0887-2333(97)00060–X

metabolism and is transiently depressed by many forms of cell stress. Short-term toxicity is therefore assessable, and both dose- and time-related responses can be examined. Since many of these characteristics are also required in assays used for toxicity testing, we have investigated the suitability of the ATP assay for this purpose.

MATERIALS AND METHODS

The method is illustrated in Fig. 1. Cell suspensions are plated using at 1–20,000 cells per well in a 96-well microplate according to the template shown (Fig. 1). Seeding density is determined by the expected growth characteristics of the cells and the length of the incubation period. Standard polystyrene or polypropylene (for suspension culture) plates with U-wells can be used. Normal or tumour tissue can be dissociated using commercially available dissociation reagents (DCS Innovative Diagnostik Systeme, Hamburg, Germany) to produce a suspension of tissue-derived cells. Alternatively, cell lines can be grown up and the cells harvested by trypsin–EDTA exposure. Ficoll–Hyaque separation is used to obtain pure populations free from large amounts of tissue debris or dead cells which might influence the results of the assay. Following cell counting and viability estimation by simple trypan blue exclusion, the cells to be tested are diluted in the same culture medium used to grow up the cells or in various commercially available media depending on the cell type used.

The assay plate is prepared by adding 100 μl medium to all wells except those to which a maximum inhibitor is added. The drugs/agents to be tested are prepared at 800% test drug concentration (TDC) and 100 μl diluted serially down the plate using triplicate wells. This allows four drugs/agents to be tested in one plate. Finally, 100 μl of the cell suspension is added to each well and the plate incubated in a humidified 5% CO_2 atmosphere for 1–21 days (usually 7 days). The number of plates is determined by the number of agents to be tested and the number of cells available. For the studies summarized here, 5-fluorouracil (5-FU) was obtained as the IV preparation (David Bull Laboratories, Warwick, UK), divided into 500 μl aliquots and frozen at −20°C (Hunter *et al.*, 1994). The 100% TDC for 5-FU was 45 μg/ml in this study.

At the end of the incubation period (1 hr–3 wk) the cells are lysed by a somatic cell extraction reagent (DCS Innovative Diagnostik Systeme) and if desirable frozen at −20°C for later assay. Freezing aids ATP extraction and plates can be re-assayed if necessary. To measure ATP content, 50 μl from each well is added to the wells of a white microplate in the case of a microplate luminometer such as the Berthold LB96P or 3.5 ml plastic tubes in the case of a tube luminometer such as the Berthold LB953. The luminometer is set to inject 50 μl luciferin–luciferase reagent (DCS Innovative Diagnostik Systeme) and loaded with the white plate or tubes as appropriate. Injection is followed by a 10–20-sec integration of chemiluminescence. The count is directly proportional to ATP content and cell number over at least four logs (Andreotti *et al.*, 1995).

Data analysis

The degree of inhibition of ATP is expressed as a percentage of the no drug/agent (MO) control, subtracting the maximum inhibitor values (MI) as follows:

$$\% \; Inhibition = 1 - (Test - MI)/(MO - MI)$$

Further analysis involves calculation of indices such as the IC_{90}, IC_{50}, minimum inhibitory concentration (MIC) and maximum non-toxic concentration (MNTC). For tumour chemosensitivity work, we

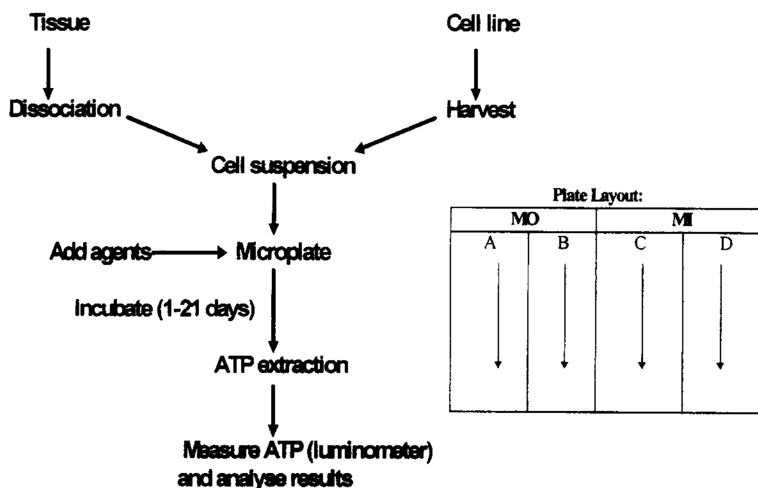

Fig. 1. ATP assay method. Cells derived from fresh tissue by enzymatic dissociation or cell lines are plated with agents at multiple dilutions in 96-well microplates. MO = control wells (no drug), MI = wells with maximum inhibitor added, A–D = individual agents diluted within plate.

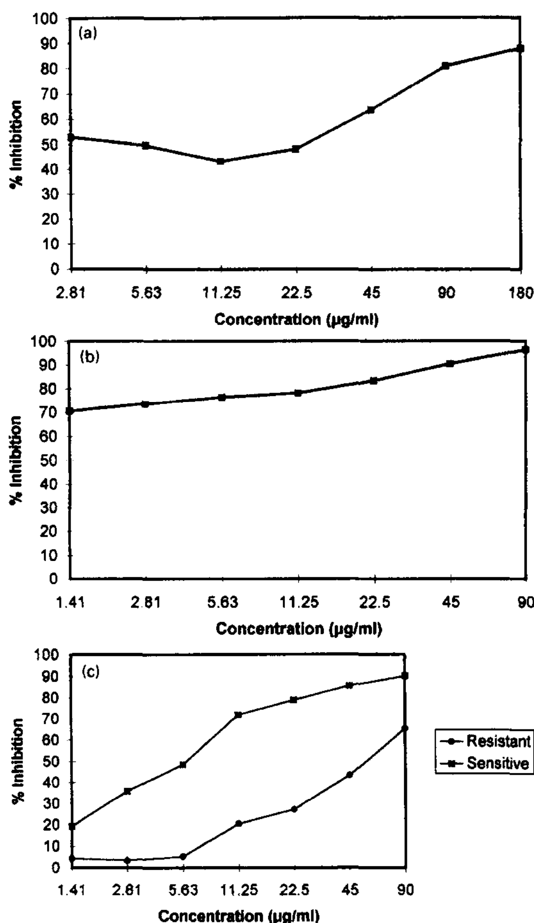

Fig. 2. Typical responses to 5-FU using a 100% test drug concentration (TDC) of 45 μg/ml. (a) normal human conjunctival fibroblasts in RPMI 1640 + 15% FCS at a plating density of 10,000 cells/well. The presence of 15% serum results in rapid cell growth, so that the cells are susceptible to 5-FU treatment with 50% inhibition even at 2.8 μg/ml 5-FU. (b) addition of 5-FU to the rapidly growing CCRM-CEM cell line (10,000 cells/well) results in considerable inhibition with 100% inhibition at 90 μg/ml 5-FU. (c) cells derived from sensitive and resistant breast tumours (both 10,000 cells/well) show very different responses to 5-FU, with no inhibition of the resistant tumour below 5.63 μg/ml.

have used two summary indices: the area under the concentration–inhibition curve (AUC) and a sensitivity index defined as the sum of the inhibition at each concentration (Index).

RESULTS

Typical responses of normal human fibroblasts, a CCRM-CEM cell line, sensitive and resistant cell lines to 5-FU are shown in Fig. 2(a–c), respectively. Like 5-FU, most agents show a sigmoid concentration–inhibition curve, but many plateau before reaching 100% kill (Hunter *et al.*, 1994). Some drugs produce a very shallow curve, approximating to a linear response over the concentration range tested.

There is enormous variation in response between cells of the same histological type from different tumours (Fig. 3). Breast tissue from benign disease also shows heterogeneity of chemosensitivity to 5-FU (Fig. 4).

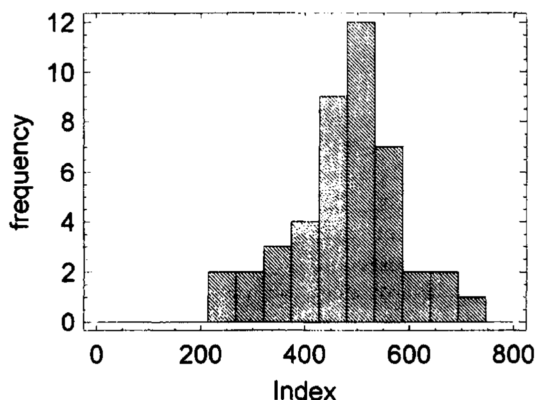

Fig. 3. Variation in sensitivity to 5-FU in primary breast adenocarcinoma (n = 44, each plated at 20,000 cells/well). The sensitivity index is calculated as: Index = 700 − Sum[% inhibition at all seven concentrations tested]. Those tumours with an Index < 700 show inhibition. If the Index is less than 250 they are likely to be sensitive *in vivo* (Hunter *et al.*, 1993).

DISCUSSION

Previous work has shown the ATP-based assay to be a reproducible and reliable assay of cell viability, useful for molecular studies (Petty *et al.*, 1994) as well as the characterization of drug effects on cell lines and tumour samples (Andreotti *et al.*, 1994). In this paper, we show its ability to measure the effects of cytotoxic agents on human cells derived from benign breast disease tissue and cell lines. In earlier work we have found that cell lines tend to be more sensitive than real tumours and that both show greater sensitivity than normal tissue-derived cells (Andreotti *et al.*, 1994). Thus, resistance to toxicity of normal cells is likely to be greater than cell lines. This is often due to the high growth rate conditions in which cell lines are cultured. Use of serum-free media without added growth factors diminishes the growth rate and

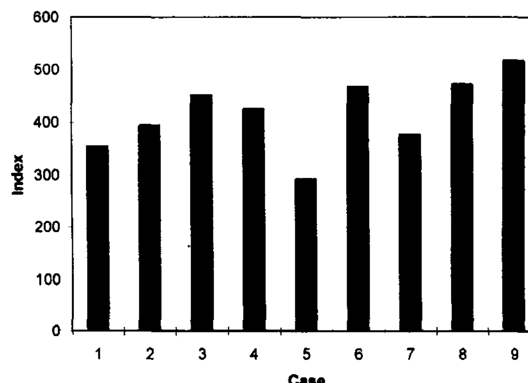

Fig. 4. Benign breast tissue samples (all 20,000 cells/well) also show heterogeneity of 5-FU sensitivity. While these samples do not represent completely normal tissue, there is considerable variation in the sensitivity index.

can alter sensitivity to cytotoxic drugs (Andreotti *et al.*, 1995).

The heterogeneity of chemosensitivity observed in primary human tumours (Hunter *et al.*, 1993) probably reflects heterogeneity of molecular phenotype between tumours of the same type (Cree *et al.*, 1994), but is also likely to occur in normal tissues to a lesser extent due to genetic and phenotypic differences between individuals. However, we believe that the heterogeneity of normal tissues makes it difficult to use anything other than cell lines for routine toxicity testing. It is therefore important to define cell lines suitable for *in vitro* toxicity testing by comparing their toxicity against a defined panel of agents with primary tissue responses *ex vivo* and *in vivo*. Such validation of cell line responses against normal tissue responses should ensure similar sensitivity of cell lines with normal tissue and help to ensure the relevance of *in vitro* toxicity assays (Bruner *et al.*, 1996). Ideally, the concentration–response curves for normal tissue and the cell line should be of similar shape so that predictive accuracy is similar at all concentrations of agents tested.

The ATP assay allows measurements of *in vitro* toxicity to be made with considerable precision and is now undergoing clinical trials for chemosensitivity testing.

Acknowledgements—We are grateful to Dr E.M.M. Hunter, Mrs L.A. McLaughlin and Dr L.H. Bruner for their assistance. This work was performed within the framework of the Preclinical Therapeutic Models Group of the EORTC.

REFERENCES

Andreotti P. E., Cree I. A., Kurbacher C. M., Hartmann D. M., Linder D., Harel G., Gleiberman I., Caruso P. A., Ricks S. H., Untch M., Sartori C. and Bruckner H. W. (1995) Chemosensitivity testing of human tumors using a microplate adenosine triphosphate luminescence assay: clinical correlation for cisplatin resistance of ovarian carcinoma. *Cancer Research* **55**, 5276–5282.

Andreotti P. E., Linder D., Hartmann D. M., Cree I. A., Pazzagli M. and Bruckner H. W. (1994) TCA-100 tumor chemosensitivity assay: differences in sensitivity between cultured tumor cell lines and clinical studies. *Journal of Bioluminescence and Chemiluminescence* **9**, 373–378.

Andreotti P. E., Thornthwaite J. T. and Morse I. S. (1991) ATP tumor chemosensitivity assay. In *Bioluminescence and Chemiluminescence: Current Status*. Edited by P. E. Stanley and L. J. Kricka. pp. 417–420. John Wiley & Sons, Chichester.

Bruner L. H., Carr G. J., Chamberlain M. and Curren R. D. (1996) Validation of alternative methods for toxicity testing. *Toxicology in Vitro* **10**, 479–501.

Cree I. A., Pazzagli M., Mini E., Mazzei T., Hunter E. M. M., Sutherland L. A., Pinzani P., Gerli A. and Andreotti P. E. (1995) Methotrexate chemosensitivity by ATP luminescence in human leukaemia cell lines and in breast cancer primary cultures: comparison of the TCA-100 assay with a clonogenic assay. *Anti-Cancer Drugs* **6**, 398–404.

Cree I. A., Petty R. D., Sutherland L. A., Hunter E. M. M., Subedi A. M. C., James E. A., Andreotti P. E. and Harrison D. J. (1994) Elucidation of molecular determinants of tumour chemosensitivity by ATP-based luminescence assay. In *Chemluminescence and Bioluminescence*. Edited by A. C. Campbell, P. E. Stanley and L. J. Kricka. pp. 407–410. John Wiley & Sons, Chichester.

Hunter E. M., Sutherland L. A., Cree I. A., Dewar J. A., Preece P. E. and Andreotti P. E. (1993) Heterogeneity of chemosensitivity in human breast carcinoma: use of an adenosine triphosphate (ATP) chemiluminescence assay. *European Journal of Surgical Oncology* **19**, 242–249.

Hunter E. M., Sutherland L. A., Cree I. A., Subedi A. M. C., Hartmann D., Linder D. and Andreotti P. E. (1994) The influence of storage on cytotoxic drug activity in an ATP-based chemosensitivity assay. *Anti-Cancer Drugs* **5**, 171–176.

Lundin A., Hasenson M., Persson J. and Pousette A. (1986) Estimation of biomass in growing cell lines by adenosine triphosphate assay. *Methods in Enzymology* **133**, 27–42.

Petty R. D., Cree I. A., Sutherland L. A., Hunter E. M., Lane D. P., Preece P. E. and Andreotti P. E. (1994) Expression of the p53 tumour suppressor gene product is a determinant of chemosensitivity. *Biophysical and Biochemical Research Communications* **199**, 264–270.

Petty R. D., Sutherland L. A., Hunter E. M. and Cree I. A. (1995) Comparison of MTT and ATP-based assays for the measurement of viable cell number. *Journal of Bioluminescence and Chemiluminescence* **10**, 29–34.

Sevin B. U., Perras J. P., Averette H. E., Donato D. M. and Penalver M. (1993) Chemosensitivity testing in ovarian cancer. *Cancer* **71**, 1613–1620.