Anti-Cancer Drugs 1996, 7, pp. 630–635

Correlation of the clinical response to chemotherapy in breast cancer with *ex vivo* chemosensitivity

Ian A Cree, Christian M Kurbacher,[1] Michael Untch,[2] Lesley A Sutherland,[3] Elizabeth M Hunter,[3] Andrea MC Subedi,[3] Elizabeth A James,[3] John A Dewar,[4] Paul E Preece,[5] Peter E Andreotti[6] and Howard W Bruckner[7]

Department of Pathology, Institute of Ophthalmology, University College London, Bath Street, London EC1V 9EL, UK. Tel: (+44) 171 608 6808; Fax: (+44) 171 608 6862. [1]Labor fur Chemosensitivitatestungen, Universitats-Frauenklinik, University of Cologne Medical Centre, Kerpenerstrasse 34, 50931, Cologne, Germany. [2]Department of Obstetrics and Gynaecology, Ludwig-Maximilians Universitat München, Marchioninistrasse 15, 81377 München, Germany. [3]Tumour Chemosensitivity Laboratory, [4]Department of Radiotherapy and Oncology, and [5]Department of Surgery, University of Dundee, Ninewells Hospital and Medical School, Dundee DD1 9SY, UK. [6]Atlantic Scientific Development Inc., 23257 State Road 7, Boca Raton, FL 33428, USA. [7]Mount Sinai Medical Center, One Gustave L Levy Place, Box 1178 New York, NY 10029, USA.

Chemotherapy for breast cancer is given on the basis of empirical information from clinical trials, an approach which fails to take into account the known heterogeneity of chemosensitivity between patients. Previous attempts to determine chemosensitivity *ex vivo* have been disappointing, but in this study results from a newly developed tumor chemosensitivity assay (TCA) have been correlated prospectively with patient response. In this study, we have used heterogeneity data for standard regimens obtained from 116 breast TCAs to set sensitivity/resistance thresholds which were then used to interpret the results from those with known clinical responses. Assay evaluability was 97% in surgical biopsies. Clinical follow-up of stage III/IV assessable disease was obtained from 27 breast tumors which were successfully tested for chemosensitivity, including 13 needle biopsies. The ATP-TCA assay predicted response correctly in 22 out of 29 (76%) tumors with clinically evaluable disease, suggesting that it is capable of predicting outcome in individual patients. Assays were performed in seven patients before and after chemotherapy using residual or recurrent tumor tissue. Four cases with initial sensitivity showed a decrease in sensitivity within 6 months of starting chemotherapy, while two others without clinical resistance were still sensitive by TCA. All nine courses of therapy given on the basis of TCA sensitivity resulted in partial or complete responses. Controlled trials of TCA-directed treatment against standardized empirical therapy should be conducted before this technology is widely adopted to assess its impact on rates of response, survival and the cost of treatment.

Key words: ATP, breast carcinoma, chemiluminescence, chemotherapy, luciferase.

This work was partly supported by BATLE LE Inc.

Correspondence to IA Cree

Introduction

Chemotherapy now plays an important role in the management of breast cancer. It is used in an adjuvant, neo-adjuvant or palliative setting with considerable success. However, tumors of the same type show heterogeneity of chemosensitivity and many patients rapidly develop drug resistance leading to relapse. Any method which allowed treatment to be tailored to each patient would be welcome, but accurate tumor chemosensitivity testing has proved an elusive goal with many false starts.[1,2]

The ATP-tumor chemosensitivity assay (TCA) (DCS Innovative Diagnostik Systeme, Hamburg, Germany) is a commercially available viability assay based on the rapid loss of ATP from dead cells.[3,4] It is an *ex vivo* microplate assay requiring only 1×10^6 cancer cells per plate. Four to six drugs or drug combinations can be tested in each plate at seven different concentrations. Selective tumor cell growth is encouraged during the culture period by a specially formulated culture medium and the use of polypropylene microplates which do not permit cell adherence.[4–7] This leads to selection of tumor cells over the 7 day culture period with more than 85% tumor cells at the end of the assay.[7] The dose–response curves obtained allow both sensitivity and resistance to be observed *ex vivo*.[7]

In this study, we have used the heterogeneity results from 116 primary breast carcinomas[8] in conjunction with known response rates to the combination chemotherapy used in breast cancer patients to establish objective thresholds for the

interpretation of TCA results. The predicted sensitivities have then been correlated with clinical response, in most cases evaluated by the oncologist without knowledge of the TCA results.

Materials and methods

Patients

Small (1–2 cm^2) surgical biopsies ($n = 14$), needle biopsies ($n = 13$) and two pleural effusions from 29 primary or secondary breast tumors (from 27 patients) were obtained on a sequential basis during surgery or work-up in Dundee, Munich or Bonn prior to palliative or neo-adjuvant chemotherapy. Other tumor specimens accepted for assay came from patients who did not receive treatment or did not have assessable disease. These tumors ($n = 116$) were used to provide heterogeneity data[8] which was later used to calibrate the assay (see below). Of the 27 patients with assessable stage III/IV disease, 18 patients were given chemotherapy and their response assessed without the oncologist being aware of the *ex vivo* chemosensitivity results. The remainder were assayed and treated with knowledge of the assay results. Seven patients (from Bonn) were treated according to the assay. A further seven tumor specimens were obtained from mastectomies or other biopsies performed following neo-adjuvant or palliative chemotherapy and their TCA results compared with those from the original tumor. Response was assessed clinically according to standard UICC criteria. The study was approved by local ethics committees in Bonn, Dundee and Munich, and performed in accordance with the Helsinki declaration.

ATP-TCA

The ATP-TCA (DCS) was performed according to the manufacturers' instructions and as previously published.[7] Open or transcutaneous needle biopsies of the tumor were dissociated by enzymes to obtain the constituent cells which were then cultured in polypropylene microplates for 6–7 days in the presence of varying concentrations of cytotoxic agents. In initial assays, the culture medium used was RPMI 1640 + 10% newborn calf serum, but subsequent assays used a proprietary serum-free medium (DCS). At the end of the culture period, the ATP content of surviving cells was measured by a sensitive luminescence assay. The results from cells cultured with drugs were compared with no drug controls (MO) and wells to which a maximum inhibitor of cell growth has been added (MI) to derive a percentage inhibition for each drug concentration tested.[7]

Assay evaluation

On the basis of previous work,[7] assays were regarded as evaluable if three criteria were met: (1) clear evidence of malignant cell involvement of the sample by cytology or histology, (2) MO values greater than 20 000 RLU indicating that there was appreciable cell survival and/or growth within the culture system, and (3) evidence of a dose relationship.

Data analysis

The percent inhibition at each concentration was summed to provide a simple index of chemosensitivity (index = 700 − sum[%inhibition$_{200-6.25}$]) for each tumor.[8] This measurement emphasizes the response at lower drug concentrations and is preferable for discrimination of tumors such as breast carcinomas which generally show good responses to combination chemotherapy at high doses.[8] Heterogeneity data from a large group of primary tumors was then compared with the known results of clinical trials in primary breast cancer to obtain threshold values against which individual patients' results could be

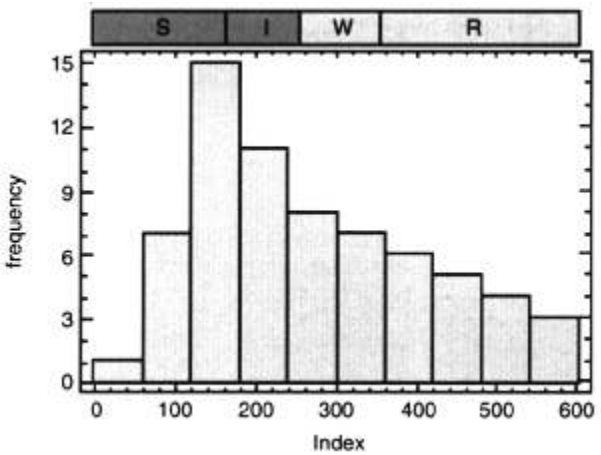

Figure 1. Distribution of chemosensitivity to the CHOP regimen (cyclophosphamide, doxorubicin and vincristine) for 70 tumors assayed with this regimen showing 70th, 50th and 30th percentiles which were used to demarcate strong (S), intermediate (I) and weak (W) sensitivity from resistance (R).

judged sensitive or resistant in the assay.[7,8] An example of this process is shown in Figure 1 in which the chemosensitivity threshold for CHOP (cyclophosphamide, doxorubicin, vincristine and prednisolone) was established by reference to the heterogeneity data.

Results

TCA results for two individual patients are shown in Figure 2. One is predicted to respond to therapy and produced a complete response, while the other appears to be resistant and had progressive disease. Discrimination between these patients is good in the mid-range of concentrations tested, but relatively poor at the highest concentrations used which correspond closely with the peak plasma concentrations achieved *in vivo*.[8,9] A dose response was seen for most drugs tested and was most marked for the anthracyclines in which increased dosage would often have produced enhanced inhibition.

Assay evaluability was 97% (94 out of 96) for surgical biopsies and 72% (13 out of 18) for needle biopsies. Reasons for assay failure in needle and surgical biopsies included low MO (no drug) control results (four tumors), erratic or incomplete results (three tumors) and, in one case, treatment with a drug which was not included on the plate. In all cases, histological assessment of part of the biopsy and cytological assessment of dissociated cells confirmed the presence of breast carcinoma.

The chemosensitivity indices for each tumor are compared against the observed response in Figure 3. This ignores the differences in sensitivity expected between tumors treated with different regimens, but the thresholds for sensitivity calculated according to the method shown in Figure 1 were similar between different regimens. These differences were taken into account for prediction of response, which is compared with clinical response in Table 1. Clearly there is some overlap between tumors which showed clinical evidence of response and those

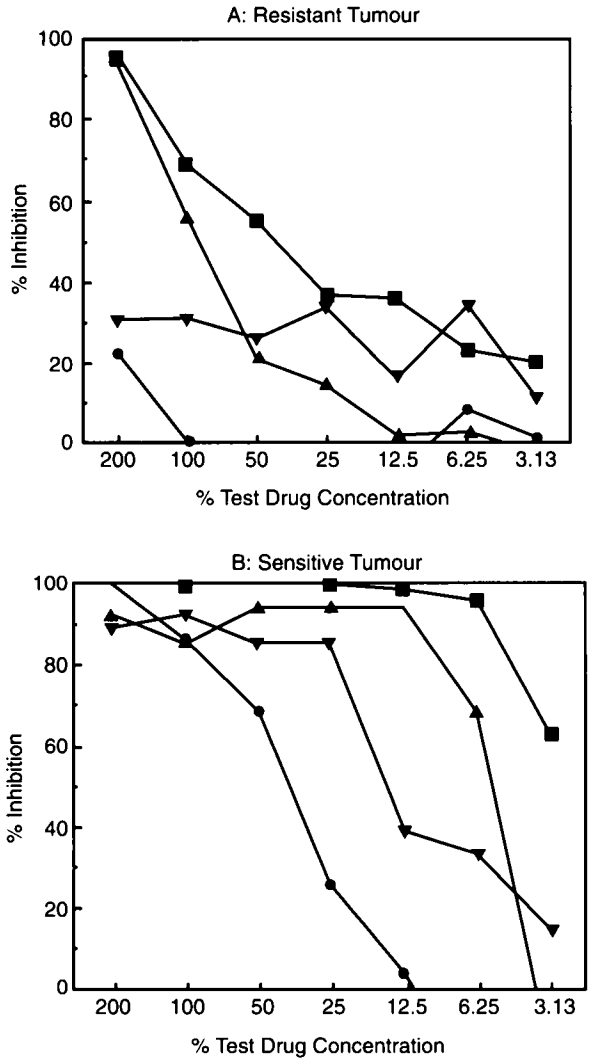

Figure 2. Graphs showing typical results for two patients. Patient A had a resistant tumor and showed no response to CHOP, while patient B responded to CEF (cyclophosphamide, epirubicin and 5-fluorouracil). (A) ●, 4-HC; ▲, doxorubicin; ▼, vincristine; ■, CHOP. (B) ▲, 4-HC; ●, epirubicin; ▼, 5-fluorouracil; ■, CEF.

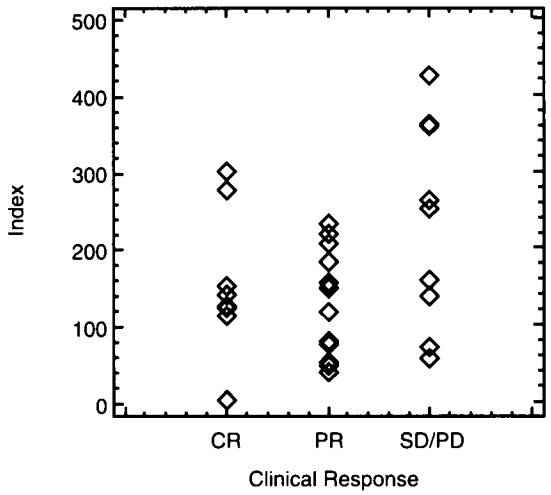

Figure 3. A scattergram showing index versus response for all 29 tumors with clinically assessable disease. There is considerable overlap between those showing complete, partial or stable/progressive disease, consistent with the frequency histogram in Figure 1 which shows a continuous distribution of responsiveness to CHOP.

Table 1. Observed versus TCA-predicted responses for all 29 assay–treatment correlations

	Complete response	Partial response	Stable disease	Progressive Disease	Total
Strong sensitivity	6	11	3	1	21
Intermediate sensitivity	0	1	0	1	2
Weak sensitivity	2	0	0	0	2
Resistance	0	0	3	1	4
Total	8	12	6	3	29

which did not. This is expected. The threshold for treatment could be set at various levels. In practice the assay is used to choose between combinations, rather than to determine whether it is worthwhile treating a patient. If the threshold for treatment is set at 70% as shown in Figure 1, the assay would have indicated treatment in all 20 of those who responded (100% sensitivity), but treatment would also have been given to five clinically resistant patients, two of whom developed progressive disease (44% specificity). Exclusion of the nine tumors (seven patients) in whom TCA was performed and used to choose between different regimens makes no difference to these figures since all nine showed sensitivity to the combination chosen and obtained clinical responses. Furthermore, reinduction of clinical response was obtained with new regimens suggested by the assay in two patients following relapse. Although the patients are not directly comparable and this was not a randomized trial, it is interesting to note that the response rate using the assay was 100% (nine out of nine), but only 56% (10 out of 18) in those treated without knowledge of the TCA results.

TCA results were also obtained from seven specimens following chemotherapy. One patient was resistant both clinically and by TCA on both occasions, four patients showed initial response in their pre-chemotherapy biopsies by TCA with resistance following chemotherapy, while the remaining two patients showed sensitivity and were still sensitive when residual tumor tissue was retested (Figure 4). The time between initial TCA and the second TCA varied from 3 to 11 months, but two patients showed resistance within 3 months of starting chemotherapy.

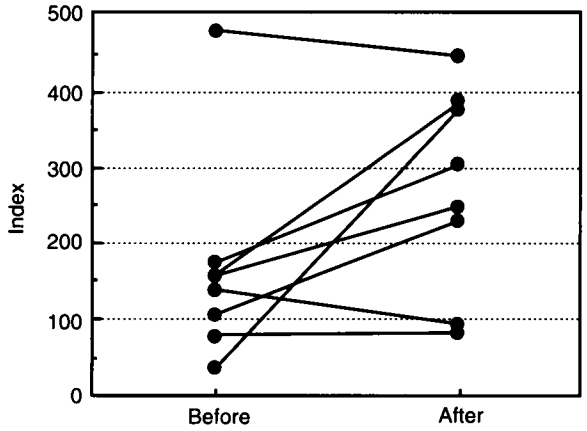

Figure 4. Development of resistance to (a) regimens and (b) doxorubicin in patients treated with doxorubicin-containing regimens. A low index indicates sensitivity: of seven sensitive tumors, five develop resistance in the second ATP-TCA, while two remain sensitive.

Discussion

Prediction of response to chemotherapy on the basis of histology is uncertain, as are nucleotide incorporation assays.[10] Clonogenic assays such as the Hamburger and Salmon assay suffer from poor evaluability rates, take several weeks to report results and demand a high degree of technical competence. Many non-clonogenic assays also require large cell numbers and have poor evaluability rates, but the ATP-TCA provides a particularly sensitive assay of cell viability with excellent reproducibility and evaluability.[7] Both clonogenic and non-clonogenic assays correlate well with the ATP-TCA.[11,12] For surgical breast tumor biopsies, the evaluability rate was 97% in this study. Several TCA methods are in widespread use in the pharmaceutical industry for the evaluation of new anti-cancer agents.[1] The ATP-based assay has recently been shown to be useful for the design of new regimens[13] (Kurbacher *et al.*, unpublished) as a number of possibilities can be tested on each tumor, avoiding the expense of multiple clinical trials.

In this study we have shown that the results of ATP luminescence assays such as the ATP-TCA can

be interpreted objectively on the basis of known drug trial data and that this judgement correlates well with clinical responsiveness, even when the oncologist does not know the results of the assay when treating the patient. Similar results have been reported by Koechli *et al.*,[14] but these authors used a threshold of 70% growth inhibition without reference to heterogeneity data. While in breast cancer the end result may be similar, we believe the use of an arbitrary threshold to be less informative. Good correlation of *in vivo* response rates with predicted values has also been reported for ovarian cancer using this[15] and similar assays.[1,2,16] Use of the assay to direct treatment has been limited to date, but preliminary results in recurrent ovarian cancer indicate a 27% improvement in response rate and enhanced progression-free survival.[17]

Dose dependency of responsiveness is evident in many of the tumors we have studied, a phenomenon which is now being exploited clinically by dose intensification regimens.[18] Chemosensitivity testing could help to identify patients who might benefit from such regimens and aid in the choice of an appropriate regimen, since several agents known to be suitable for this therapeutic approach can be tested on the same tumor sample. It is also possible that the assay could be used to indicate the dose of the agent required.

Two patients showed good responses clinically despite only weak chemosensitivity in the TCA. Both were needle biopsies and it is possible that these samples contained few tumor cells with many benign cells, making them appear more resistant that usual. Needle biopsy results need careful interpretation and rarely produce enough cells to allow several drugs to be tested. We prefer to obtain surgical material whenever possible.

There are probably several reasons for the TCA's failure to predict resistance in several apparently sensitive tumors. The most likely explanation is the development of resistance to the combinations used during therapy. The timing of this is critical as an apparently responsive tumor will fail to respond clinically if it develops resistance within the 4–6 month treatment period. This is supported by the results from post-chemotherapy specimens. Several show the development of resistance quite clearly, two after only 3 months treatment. This may in part be predictable from their p53 status, which promotes initial responsiveness to alkylating agents followed by the early development of resistance.[19] It would be interesting to follow up a larger group of such patients and compare the TCA results with their p53 status.

Resistance is likely to develop if only one drug provides most of the activity of a combination, as illustrated in Figure 2(B) where the cyclophosphamide (4-HC) provides most of the sensitivity achieved by a CEF combination. This is also evident in other tumors.[7,8,20] Regimen selection by TCA therefore depends upon testing of single agents as well as combinations. We believe this to be a major reason for 'false positive' results in this and other TCA studies since resistance to a single agent will develop rapidly, often before clinical response is assessable. The ATP-TCA tests the response of the tumour at the time taken—not 4–6 months later when treatment is completed.

The use of *ex vivo* chemosensitivity testing in clinical practice is controversial, but could be of great benefit to patient care. The number of drugs with proven efficacy in breast cancer is rapidly increasing beyond the capacity of clinical trials to test potential regimens. Tumor heterogeneity makes choice of best agent for individual patients ever more difficult and some method of individualizing chemotherapy is urgently required. The ATP-TCA shows great promise, but we agree with Bellamy[1] that controlled prospective trials of TCA-directed therapy against standard trial-directed treatment are required before this technology is widely adopted. This report shows that the new generation ATP-based assays have the ability to predict solid tumor chemosensitivity. This technology is now ready for much wider evaluation and could be introduced to clinical practice within a few years.

Acknowledgments

We are grateful to Professor JS Beck, Mr RAB Wood, Professor HW Bruckner and Professor A Eremin for their assistance. This work was performed within the framework of the Preclinical Therapeutic Models Group of the EORTC.

References

1. Bellamy WT. Prediction of response to drug therapy of cancer. A review of *in vitro* assays. *Drugs* 1992; **44**: 690–708.
2. Bosanquet AG. *In vitro* drug sensitivity testing for the individual patient: an ideal adjunct to current methods of treatment choice. *Clin Oncol R Coll Radiol* 1993; **5**: 195–7.
3. Lundin A, Hasenson M, Persson J, Pousette A. Estimation of biomass in growing cell lines by adenosine triphosphate assay. *Methods Enzymol* 1986; **133**: 27–42.

4. Andreotti PE, Thornthwaite JT, Morse IS. ATP tumor chemosensitivity assay. In Stanley PE, Kricka LJ, eds. *Bioluminescence and chemiluminescence: current status*. Chichester: John Wiley 1991, 417–20.
5. Andreotti PE, Linder D, Hartmann DM, *et al.* ATP tumor chemosensitivity assay application for solid tumors and leukaemias. In: Szalay A, Kricka LJ, Stanley PE, eds. *Chemiluminescence and bioluminescence: current status*. Chichester: John Wiley 1993: 271–5.
6. Andreotti PE, Linder D, Hartmann DM, Cree IA, Pazzagli M, Bruckner HW. TCA-100 tumor chemosensitivity assay: differences in sensitivity between cultured tumor cell lines and clinical studies. *J Biolum Chemilum* 1994; **9**: 373–8.
7. Andreotti PE, Cree IA, Kurbacher CM, *et al.* Chemosensitivity testing of human tumors using a microplate ATP luminescence assay: clinical correlation for cisplatin resistance of ovarian carcinoma. *Cancer Res* 1995; **55**: 5276–82.
8. Hunter EM, Sutherland LA, Cree IA, Dewar JA, Preece PE, Andreotti PE. Heterogeneity of chemosensitivity in human breast carcinoma: use of an adenosine triphosphate (ATP) chemiluminescence assay. *Eur J Surg Oncol* 1993; **19**: 242–9.
9. Hunter EM, Sutherland LA, Cree IA, *et al.* The influence of storage on cytotoxic drug activity in an ATP-based chemosensitivity assay. *Anti-Cancer Drugs* 1994; **5**: 171–6.
10. Zeigler LD, Connelly JH, Frye D, Smith TL, Hortobagyi GN. Lack of correlation between histologic findings and response to chemotherapy in metastatic breast cancer. *Cancer* 1991; **68**: 628–33.
11. Petty RD, Sutherland LA, Hunter EM, Cree IA. Comparison of MTT and ATP-based assays for the measurement of viable cell number. *J Biolum Chemilum* 1995; **10**: 29–34.
12. Cree IA, Pazzagli M, Mini E, *et al.* 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* 1995; **6**: 398–404.
13. Untch M, Sevin BU, Perras JP, *et al.* Evaluation of paclitaxel (taxol), cisplatin, and the combination paclitaxel–cisplatin in ovarian cancer *in vitro* with the ATP cell viability assay. *Gynecol Oncol* 1994; **53**: 44–9.
14. Koechli OR, Avner BP, Sevin BU, *et al.* Application of the adenosine triphosphate cell viability assay in human breast cancer chemosensitivity testing: a report on the first results. *J Surg Oncol* 1993; **54**: 119–25.
15. Untch M, Crohns C, Konecny G, *et al.* Correlation of *in vitro* tumor chemosensitivity assay with outcome in ovarian cancer patients given carboplatin and cyclophosphamide. *Proc Am Soc Clin Oncol* 1996; **15**: 302 (abstr 841).
16. Sevin BU, Perras JP, Averette HE, Donato DM, Penalver M. Chemosensitivity testing in ovarian cancer. *Cancer* 1993; **71**: 1613–20.
17. Kurbacher CM, Boese S, Mallman P, Krebs D. The ATP tumor chemosensitivity assay direct chemotherapy for recurrent ovarian cancer: first clinical results (abstr 09.07.04). *Proc German Cancer Soc* 1996: 79.
18. Stewart THM, Retsky MW, Tsai SCJ, Verma S. Dose response in the treatment of breast cancer. *Lancet* 1994; **343**: 402–4.
19. Petty RD, Sutherland LA, Hunter EM, *et al.* Expression of the p53 tumour suppressor gene product is a determinant of chemosensitivity. *Biochem Biophys Res Commun* 1994; **199**: 264–70.
20. Kurbacher CM, Cree IA, Brenne U, *et al.* Heterogeneity of *in vitro* chemosensitivity in perioperative breast cancer cells to mitoxantrone versus doxorubicin evaluated by microplate ATP bioluminescence assay. *Breast Cancer Res Treat* 1996; in press.

(Received 5 June 1996; accepted 27 June 1996)