Eur J Cancer, Vol. 29A, No. 11, pp. 1566–1573, 1993.
Printed in Great Britain

0964–1947/93 $6.00 + 0.00

Cytotoxic Effects of Anticancer Agents on Subconfluent and Multilayered Postconfluent Cultures

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The cytotoxic effects of conventional (doxorubicin, 5-fluorouracil, cisplatin) and investigational (2′,2′-difluorodeoxycytidine, hexadecylphosphocholine, EO9, rhizoxin) anticancer drugs were studied in subconfluent and multilayered postconfluent cultures of human colon and ovarian carcinoma cell lines. Chemosensitivity was assessed 4 days after a 24-h drug exposure with the sulphorhodamine B assay. Except for rhizoxin, all drugs tested yielded an EC_{50} (drug concentration producing absorbance readings 50% lower than those of non-treated wells) in postconfluent cultures that were higher than an EC_{50} obtained with subconfluent cultures. Compared with subconfluent cultures, postconfluent cultures showed decreased cellular nucleotide concentrations and ATP/ADP ratios, in addition to an increased percentage of G_0/G_1 cells. The activity of DT-diaphorase, a reductase involved in the bioactivation of EO9, was similar in sub- and postconfluent cultures. These results indicate similarity of the postconfluent model presented with those obtained with *in vivo* models and more complex *in vitro* techniques.
Eur J Cancer, Vol. 29A, No. 11, pp. 1566–1573, 1993.

INTRODUCTION

DURING THE past 40 years, chemotherapy has played an increasingly important role in cancer treatment [1]. However, what determines the success or failure of the drugs now at hand is still poorly understood. The employment of the murine leukaemia models L1210 and P388 for primary screening of new anticancer agents is believed to have been at least partly responsible for the lack of discovery of more efficacious agents against solid tumours [2, 3]. Therefore, alternative screening systems are being tested, focusing on the search for new compounds with some degree of selectivity against solid tumour models [4].

Drug penetration barriers, cell proliferation gradients and microenvironmental conditions (e.g. hypoxia and acid pH) are pointed out as some of the inherent characteristics of the biology of solid tumours determining the outcome of antitumour drug therapy [5]. Therefore, cell culture systems showing some degree of 3-dimensional cell–cell interactions and organisation (e.g. histocultures [6, 7], multicellular spheroids [8] and postconfluent cell cultures [9–11]) have been developed, which have the advantage of mimicking those characteristics better than subconfluent monolayers. Consequently, they should be considered as an alternative to improve the predictive value of *in vitro* chemosensitivity assays for the selection of better anti-solid-tumour drugs.

We have previously characterised the growth patterns and morphological features of postconfluent cultures of colon (HT29, SW620) and ovarian (A2780) adenocarcinoma cell lines in "V"-bottomed, 96-well microtiter plates [12]. Under such conditions, these cell lines grow as multilayered cultures, showing 4–10 layers of cells, depending on the cell type. In addition, postconfluent HT29 formed dome-like structures, consistent with some degree of cellular differentiation. Moreover, the sulphorhodamine B protein-assay proved to be a simple and reliable method to assess chemosensitivity in this cell culture system.

The objective of the present study was to assess the cytotoxic effects of a number of conventional and investigational anticancer drugs with diverse mechanisms of action, using sub- and postconfluent tumour cell cultures. Doxorubicin (DXR), 5-fluorouracil (FU) and cisplatin (CDDP) were selected as representative conventional anticancer drugs. The antimetabolite gemcitabine (2′,2′-difluorodeoxycytidine [dFdC]) [13], the tubulin-interacting agent rhizoxin (RZN) [14], the membrane-interacting ether lipid miltefosine [hexadecylphosphocholine (HPC)] [15], and the bioreductive alkylator EO9 [16], which are all currently undergoing early clinical trials, were also studied. Further characterisation of the postconfluent culture system in relation to the mechanism of action of the drugs tested was performed through the analyses of culture proliferative status, cellular nucleotide pools and DT-diaphorase (DTD) enzyme activity.

MATERIALS AND METHODS

Chemicals and drugs

Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Flow Laboratories (Irvine, Scotland); fetal calf serum (FCS) was from Gibco (New York, U.S.A.); 5-fluorouracil and the sulphorhodamine B dye were from the Sigma Chemical Co. (St. Louis, U.S.A.); doxorubicin was from Farmit-

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Received 23 Feb. 1993; accepted 1 Apr. 1993.

alia Carlo Erba (Nivelles, Belgium) and cisplatin from Bristol-Myers (Brussels, Belgium); miltefosine was a gift from Dr P. Hilgard (Asta Pharma, Bielefeld, Germany); rhizoxin and EO9 were kindly provided by the EORTC New Drug Development Office (Free University Hospital, Amsterdam, The Netherlands). Gemcitabine was supplied by Lilly Research Laboratories (Indianapolis, U.S.A.). All other chemicals were of standard analytical quality.

Cell culture and plating

The human colon adenocarcinoma cell lines, HT29 and SW620, were obtained from the ATCC (Rockville, U.S.A.). The A2780 human ovarian carcinoma cell line was provided by Dr R. F. Ozols (NCI, Bethesda, U.S.A.).

Description of cell culture and plating procedures were published in detail elsewhere [17]. Briefly, mycoplasma-negative cells were maintained in 80 cm^2 culture flasks in DMEM with 5% FCS and 1 mmol/l L-glutamine, incubated at 37°C in a humidified incubator with 95% air and 5% CO_2. Exponentially growing cells were harvested by trypsinisation and resuspended in antibiotic-containing medium (50 μg gentamicin/ml); single cell suspensions (≥ 90% viable cells by trypan blue exclusion assay) were counted and seeded at 15 000 cells/50 μl/well in 96-well plates with "V"-shaped bottoms (Greiner Labortechnik, Solingen, Germany).

Chemosensitivity assays

Chemosensitivity studies were performed using triplicate wells and five 10-fold different concentrations of each drug. Serial dilutions of the stock solutions were prepared in culture medium immediately before drug addition. Microtiter plates were randomly divided into two groups. The drug treatment applied to each group were identical but started either on day 1 (D1) or on day 5 (D5) after plating, when cells were organised as exponentially growing, monolayered subconfluent cultures or as multilayered postconfluent cultures, respectively. In both instances, cultures received 150 μl of medium with or without drugs (controls). After 24 h of drug exposure, wells were rinsed once with culture medium and re-fed. Plates were then incubated for 4 additional days with daily medium renewal until cytotoxicity was assessed with the sulphorhodamine B (SRB) assay. Plates containing A2780 cells had to be centrifuged (1 200 rpm/5 min) prior to medium substitutions to prevent cell detachment. Every plate included control wells incubated with medium and drug-containing medium, but without cells, for background absorbance determination. As part of every experiment, additional plates (non-treated cultures) were assayed for cellular growth control on D1 and D5 after seeding. Thus, by looking at the dose–response curve, it was possible to determine whether the exposure to a certain drug concentration resulted in growth inhibition, cytostasis (O.D. values = control readings) or reduction in cell mass (O.D. values < control readings).

Cellular growth and drug-induced cytotoxicity were assessed with the SRB assay [18] as described elsewhere [12]. Briefly, cells were fixed with 50% trichloroacetic acid (TCA) at 4°C. Plates were then rinsed with tap water and air-dried. Microcultures were stained with 0.4% SRB during 1 h. Wells were washed with 1% acetic acid and the bound dye was solubilised with 10 mmol/l unbuffered Tris. Absorbance was measured with a Titertek Multiskan MCC/340 (Flow Laboratories) at 450 nm. The EC_{50} (drug concentration producing absorbance readings 50% lower than those of non-treated wells) was extrapolated from the resulting dose–response curves.

Cell-cycle distribution analysis by flow cytometry

The preparation of samples for DNA flow cytometry was as described previously [19]. Cells from D1, D5 and D10 plates were harvested with 0.2% EDTA, resuspended in culture medium, washed twice with phosphate buffered saline (PBS), fixed in 100% methanol at 4°C and then kept in the refrigerator until analysis. Cells were then washed with ice-cold PBS, counted (2×10^6 cells/sample) and resuspended in RNase A (0.5 mg/ml Tris-buffer). After 30 min at 37°C, each sample received an equal volume of a solution containing pepsin (1 mg/ml 0.4% HCl) and was further incubated for 5–10 min. Cells were stained with ethidium bromide (20 μg/ml) and Hoechst 33218 (4 μg/ml) stain diluted in PBS containing 1% bovine serum albumin (BSA) at final pH 8. The distribution of DNA per cell content was measured in at least 15 000 cells/ sample, using a fluorescence-activated cell sorter (FACSTAR^{plus}, Becton-Dickinson, Etten-Leur, NL). DNA histograms were analysed using the "DNA Cell-Cycle Analysis Software-Ver.C" (Becton-Dickinson).

[^{3}H]Thymidine autoradiography

The number of cells in S phase was determined by autoradiography detection as previously reported [19]. Briefly, cells from D1, D5 and D10 plates were harvested on a coincidental date, resuspended in [^{3}H]thymidine-containing medium (37 KBq) and incubated at 37°C for 1 h. Cells were subsequently washed with fresh medium and used to make cytospin preparations which were fixed in ethanol:acetic acid, incubated with cold thymidine (0.1 mg/ml) and washed with tap water. Cytospins were then mounted with Kodak AR10 stripping film for a 2-week exposure.

Analysis of cellular nucleotide pools

The measurement of nucleotides in non-treated cells was carried out essentially as previously described [20]. Shortly, cells from D1 and D5 plates were washed with cold PBS, counted and extracted with 5% TCA (final concentration) for 20 min at 4°C. Samples were centrifuged and the supernatant neutralised with 2 vol.alamine-Freon. Ribonucleoside triphosphates were determined with a Partisil-10-SAX column using isocratic elution with 250 mmol/l KH_2PO_4, 500 mmol/l NaCl, pH 4.0. Results were expressed as the concentration of nucleotides per million cells.

DT-diaphorase enzyme activity assay

DTD is a two-electron-donating enzyme that has been shown to reduce EO9, generating bioactivated metabolites which can cause single strand breaks in plasmid DNA [21] and DNA cross-links in DTD-rich rat tumour cells [22]. DTD activity was assessed by the method of Ernster [23] as modified by Benson *et al.* [24] and Prochaska and Talalay [25]. Briefly, the supernatants of cell sonicates were diluted in 1% BSA pH 7.4 for assay purposes. The assay systems contained in a final volume of 3 ml 0.025 mol/l Tris (pH 7.4), 0.2 mmol/l NADH (electron donor) and 0.04 mmol/l 2,6-dichlorophenolindophenol (DCPIP). The reduction of DCPIP by DTD was measured spectrophotometrically (600 nm) at room temperature. Activity attributable to DT-diaphorase was calculated by subtracting the level of activity remaining after the addition of the enzyme inhibitor dicoumarol (100 μmol/l). Protein concentrations were determined by the method of Bradford [26].

RESULTS

Chemosensitivity tests

The cytotoxic effects of a 24-h exposure to dFdC, FU, DXR, EO9, HPC, CDDP and RZN on subconfluent cultures (drug added on D1) were compared with those on postconfluent cultures (drug added on D5). The results obtained with dFdC and HPC were chosen to illustrate the general patterns of chemosensitivity observed (Fig. 1). Overall, there were marked differences between the activity of a drug against D1 or D5 cells. For a given cell line, the shape of both curves was relatively similar in the case of HPC (Fig. 1a–c), as well as for DXR, CDDP and EO9 (not shown). These drugs prompted not only total growth arrest (cytostasis) but also reduction in cell mass, as indicated by a decrease in the absorbance of treated wells to levels which were lower than control readings on D1 and D5. Despite the similarity in shape, D5 cultures treated with these drugs yielded dose–response curves invariably positioned to the right of the curves obtained with D1 cultures. Consequently, EC_{50} corresponding with postconfluent cells (D5-EC_{50}) treated with any one of these four compounds, were higher than those of subconfluent cultures (D1-EC_{50}) (Fig. 2). Such differences were statistically significant ($P < 0.05$; Student's t test) except in the case of HPC in A2780 cells.

In addition to significant disparities between D1-EC_{50} and D5-EC_{50} (Fig. 2), three compounds included in this study, dFdC, FU and RZN, produced dose–response curves with distinct configurations, whether the drug was used on D1 or on D5, dFdC prompted relatively steep dose–response curves against

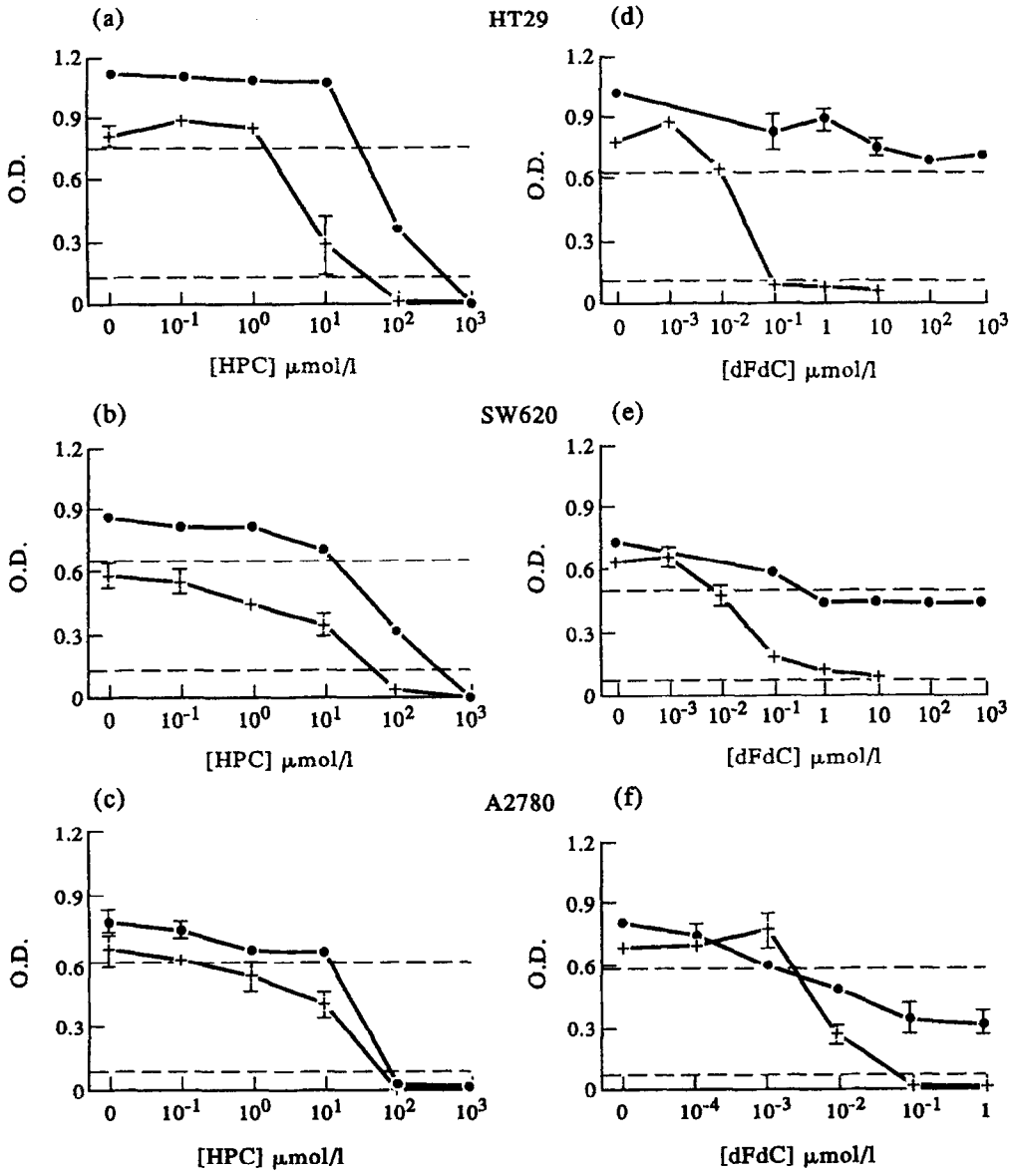

Fig. 1. HPC (a–c) and dFdC (d–f) dose–response curves determined by colorimetric measurements of protein content in HT29, SW620 and A2780 microcultures using the SRB assay (450 nm) as described in Materials and Methods. Cells were exposed to the depicted concentrations of the test compound for 24 h on day 1 (+) or on day 5 (●) after plating, when they were organised as mono- or multilayered cultures, respectively. Points are means ± S.D. of triplicate wells of one representative experiment. In each graph, the horizontal broken lines indicate the mean absorbance levels found in non-treated wells ($n = 12$) of additional plates which were assayed for cellular growth on days 1 (lower line) and 5 (upper line) after seeding. The region of the dose–response curve above these lines represent growth inhibitory effects, while the region below it indicates the presence of cytotoxicity (cell kill). Note: dFdC concentration range for HT29 is the same as shown for SW620, but different for A2780.

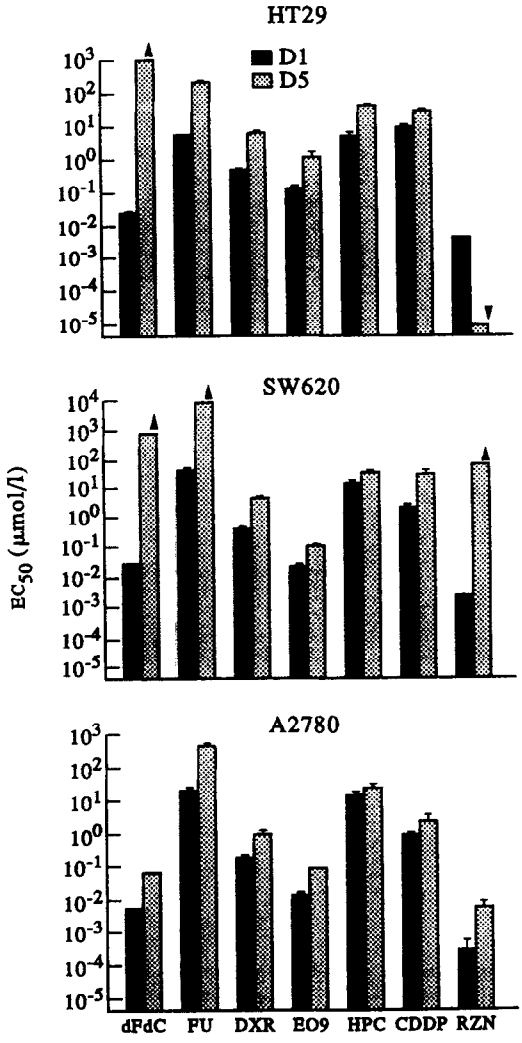

Fig. 2. Comparison between EC_{50} (drug concentrations producing absorbance readings 50% lower than those of non-treated wells) of HT29, SW620 and A2780 cells determined 4 days after a 24-h exposure to the indicated compounds. Results from monolayered subconfluent and multilayered postconfluent cultures, treated, respectively, on days 1 (D1) and 5 (D5), are confronted. Means ± S.D. of at least three independent experiments are depicted. Note: In some instances, the exact EC_{50} could not be determined since they were, respectively, higher (↑) or lower (↓) than the maximum or minimum drug concentrations tested.

subconfluent cultures and inhibited cell growth completely at concentrations ranging from 0.1 to 10 μmol/l (Fig. 1 d–f). Against postconfluent cultures of HT29 and SW620 cells, however, dFdC treatment resulted in very shallow dose–response curves, forming a plateau at O.D. levels that were similar to control readings. For both colon cell lines, dFdC D5-EC_{50} were higher than the maximum drug concentration tested (Fig. 2). Against postconfluent A2780 cells, dFdC was sufficiently cytotoxic to cause reduction in cell mass (Fig. 1c). Moreover, it induced cytostasis at concentrations (1–10 nmol/l) which were significantly lower than those required to cause the same effect on subconfluent cells (60–100 nmol/l). If used on D5, the antimetabolite FU also showed flatter dose–response curves than on D1 (not shown). However, a D5-EC_{50} for FU could not be reached only in the case of the SW620 cell line (Fig. 2). RZN was very potent against subconfluent A2780 cells, but, it also produced higher EC_{50} on D5 as compared with D1 (Fig. 2). It showed a similar profile of cytotoxicity against subconfluent cultures of HT29 and SW620, producing EC_{50} ranging from 3 to 5 nmol/l. However, RZN added to postconfluent cells presented opposite effects depending on which colon cell line was analysed (Fig. 2). It generated shallow dose–response curves with almost insignificant growth inhibitory effects against postconfluent cultures of SW620 cells (not shown), resulting in a D5-EC_{50} > 100 μmol/l. In contrast, RZN yielded a D5-EC_{50} < 0.01 nmol/l with HT29 cells, representing the only example in our study of a drug which was more active against postconfluent than against subconfluent cultures.

In Table 1, the effects of the drugs tested on D1 and D5 are expressed as a function of the D5-EC_{50}:D1-EC_{50} ratios. For a given drug, a small ratio (tending to 1) would result from similar performances against subconfluent and postconfluent cultures. Among the conventional drugs tested, CDDP was the least affected by the culture conditions, producing D5-EC_{50}:D1-EC_{50} ratios equal to 3 in 2/3 lines tested. Considering the investigational drugs, HPC and EO9 showed a more homogenous performance against D1 and D5 cultures, reflected by D5-EC_{50}:D1-EC_{50} ratios ranging from 1.6 to 8.9, contrasting with a much larger variation in RZN and dFdC ratios. Overall, the results showed smaller ratios for A2780 cells than for the two colon cell lines.

Culture proliferative status

Cell-cycle distributions of samples collected from subconfluent and postconfluent cultures were assessed by DNA flow cytometry (Table 2). For the three cell lines tested, there was an increase in the percentage of cells in the G_0/G_1 phase on D5 compared to D1, and a concomitant decrease in the G_2/M compartment. The percentage of S-phase cells in D5 cultures of the ovarian cell line, A2780, was significantly higher than that found in D1 cultures. In contrast, the amount of S-phase cells in D5 samples of HT29 and SW620 was, respectively, 1.6- and 1.7-fold lower than in D1 samples.

HT29 and SW620 cells collected from D1 and D5 cultures were labelled with [^{3}H]thymidine and subjected to autoradiographic detection. For both cell lines, the percentage of labelled cells on D5 (20% ± 5% in HT29 and 30% ± 5% in SW620) was on average 2-fold (range = 1.7–2.3) lower than on D1, indicating a degree of reduction in S-phase cells from D1 to D5 which was in agreement with the DNA flow cytometry results. Within the S-phase fraction, a subpopulation of weakly-labelled cells (3–30

*Table 1. D5-EC_{50}:D1-EC_{50} ratios**

Drug	HT29	SW620	A2780
dFdC	>38,461†	>28,571†	14.2
FU	42.3	>171.5†	25
DXR	14.7	12	5.6
EO9	8.9	4.9	6.8
HPC	8.8	2.4	1.6
CDDP	3	13.4	3
RZN	>1‡	>33,333	25

*Based on mean values presented in Fig. 2.
†D5-EC_{50} was higher than the maximum drug concentration tested. ‡D5-EC_{50} was lower than the minimum drug concentration tested.

grains/nucleus above background), presenting a very low rate of DNA synthesis, was observed in addition to heavily-labelled cells showing almost completely black nuclei. On D5 cultures, weakly-labelled cells were the predominant feature, exceeding the number of heavily-labelled cells at a ratio of approximately 2:1.

The data on [^{3}H]thymidine incorporation and DNA flow cytometry regarding HT29 and SW620 samples from D10 cultures were not significantly different from those described for D5 samples (not shown).

Cellular nucleotide pools

Except for the case of GTP levels in the ovarian tumour line, cellular concentrations of nucleotides on D1 were significantly higher (1.5- to 4.3-fold) than on D5 (Fig. 3). Also in the colon cell lines, the differences in GTP pools between D1 and D5 were smaller than that observed with the other nucleotides. Nucleoside triphosphate:nucleoside diphosphate (NTP:NDP) ratios give an indication of the metabolic status of the cells. High ratios are considered to reflect good metabolic conditions. The ATP:ADP ratios (means of three experiments) of subconfluent HT29, SW620 and A2780 cells were 4.7, 4.6, and 4.5, respectively. The relative decrease in ATP pools on D5 was larger than that for ADP, resulting in significantly lower ATP:ADP ratios in D5 cells (SW620 = 4.2, HT29 = 4.0, A2780 = 3.8) ($P < 0.05$). Together these characteristics reflect a lower energy status of D5 cells.

DTD enzyme activity assay

DTD activity levels (means ± S.D. of at least three experiments) measured in D1 cells were 606 ± 102, 364 ± 56, 216 ± 32 nmol/min/mg protein in HT29, SW620 and A2780 cells, respectively, compared with 822 ± 151, 390 ± 185, and 245 ± 82 in D5 cells. The differences between D1 and D5 results were not statistically significant.

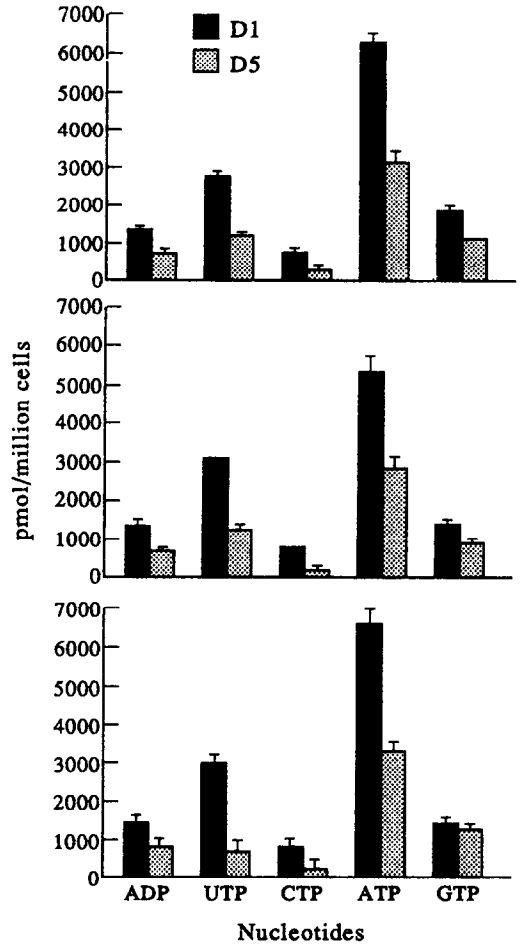

Fig. 3. Concentration of cellular nucleotides in non-treated HT29 (top), SW620 (middle) and A2780 cells (bottom) harvested 1 (D1) or 5 (D5) days after plating. Means ± S.D. of three independent experiments are displayed.

DISCUSSION

We have previously reported that HT29, SW620 and A2780 cells, cultured under the same conditions described in this study, reached confluence 3 days after plating [12]. On D5, cross-sections of culture wells revealed the organisation of multilayered postconfluent cultures and, thereafter, the growth curves of these three cell lines showed a tendency to form a plateau. We have now demonstrated that this plateau in proliferation is preceded by a significant increase in the number of G_0/G_1 cells and an overall decrease in the cellular concentrations of nucleotides on D5 as compared with D1. It has been reported that the activities of key enzymes of purine and pyrimidine biosynthesis peaked during exponential growth and were significantly lower in plateau-phase cultures of hepatoma and hepatocyte-derived cell lines [27]. A similar phenomenon may have limited the rate of nucleotide synthesis in our system of multilayered cultures.

A good agreement was observed between the cell-cycle distribution determined by DNA flow cytometry and the tritiated thymidine incorporation analysis. The latter assay allowed a more subtle discrimination of the S-phase compartment, e.g. the detection of a subpopulation of weakly-labelled, S-phase arrested cells in D5 cultures. Such type of cells was also described in stationary cultures and *in vivo*-grown L1210 leukaemia [19]. Under these conditions, they were most predominant at G_1-S transition, sensitive only to high concentrations of 1-β-arabinofuranosylcytosine, and apparently the product of insufficient nutrient/energy sources. Our findings of decreased cellular ATP pools and ATP:ADP ratios in postconfluent cultures also suggest an association between a relatively low energy status and an increased frequency of weakly-labelled cells. This phenomenon may also be related to the decreased sensitivity of postconfluent

Table 2. Cell-cycle distribution as determined by DNA flow cytometry

	HT29		SW620		A2780	
Phase	D1	D5	D1	D5	D1	D5
G_0/G_1	60*	72	50	66	19	30
S	18	11	19	11	34	43
G_2/M	22	12	31	23	47	27

*Percentage of the total number of cells (15 000/sample) analysed on day 1 (D1) or day 5 (D5) after plating, calculated from the means of at least two experiments. For every cell line and every phase of the cell-cycle, differences between D1 and D5 were statistically significant.

cells to drugs which require ATP-dependent activation, such as dFdC and FU. Also noteworthy is the fact that dFdC [13, 28] and FU [29] exert their antiproliferative effects mainly by inhibition of DNA synthesis in actively proliferating cells. We found that postconfluent cultures of HT29 and SW620 presented a smaller number of cells in S-phase than subconfluent cultures, while the ovarian cells showed the opposite trend. These results may partly explain the plateaus observed in dose–response curves of dFdC and FU, as well as the smaller differences between D1-EC_{50} and D5-EC_{50} obtained for A2780 cells with these two drugs in comparison with HT29 and SW620. Moreover, the degree of sensitivity to dFdC is time- and concentration-dependent and is positively correlated to the amount of the active metabolite, dFdCTP, produced and retained by the cell [13]. In our laboratories, Ruiz van Haperen *et al.* [30] confirmed these results and found that A2780 cells accumulated more dFdCTP than the colon tumour cell line WiDr under standard culture conditions. In addition, A2780 and WiDr cells were grown as xenograft tumours in nude mice and only the ovarian cell line was sensitive to dFdC treatment (manuscript in preparation). Interestingly, preliminary results have also indicated a greater rate of dFdCTP accumulation in postconfluent A2780 cells than in postconfluent HT29 and SW620, incubated for 24 h with 1 μmol/l dFdC (not shown).

The results obtained with DXR, FU and CDDP on postconfluent cultures are in agreement with reports in the literature describing HT29 [31, 32] and other cell lines [33–40] in 3-dimensional cultures used to test these and other clinically active anticancer drugs. Recently, Dimanche-Boitrel *et al.* [11] have demonstrated that confluent HT29 cells were more resistant than non-confluent cells to clinically active drugs, including DXR, FU and CDDP. These authors have also shown that this could be partly explained by a decreased intracellular drug accumulation in confluent cells, coupled to a reduction in plasma membrane fluidity, and an increase in the number of non-cycling cells at confluence with a reduced expression of the enzyme topoisomerase II. Under our experimental conditions, postconfluent HT29 cells displayed features of consistent with some degree of differention in contrast with cells cultured as monolayers [12]. The appearance of these structures are believed to be a consequence of transepithelial fluid transport and entrapment of fluids between the cells and the plastic substratum. Others have reported that subpopulations of HT29 committed to differentiation showed enhanced growth adaptability to methotrexate and FU [41]. In addition, resistance to FU under those conditions was shown to be associated with thymidylate synthase gene amplification [42].

FU and DXR were found to be inactive against HT29 and SW620 xenograft tumours in nude mice, which showed low to moderate sensitivity to CDDP [43]. Note that CDDP was the conventional chemotherapeutic agent least affected by the different culture conditions tested in our experiments. Nevertheless, multilayered postconfluent cultures were less sensitive to FU, DXR and, less strikingly, to CDDP than monolayered subconfluent cultures. Taken together, these data suggest that drugs which have a more similar performance against sub- and postconfluent cultures may have a better chance of a successful performance *in vivo*. In that case, multilayered postconfluent cultures may eventually be useful to make a more stringent selection of drugs which, after a first *in vitro* screening, will undergo further evaluation in animal tumour models.

Although HPC and EO9 were significantly less active against multilayered cultures (except HPC in A2780 cells), they produced small D5-EC_{50}:D1-EC_{50} ratios, suggesting that the performance of these compounds was not so much influenced by the type of culture system employed. The mechanism of action of ether lipids like HPC is still under investigation, however, they seem to exert their effects independently of DNA synthesis/metabolism [44, 45]. This factor could represent an advantage in a system containing a high percentage of non-rproliferating or slowly-proliferating cells like the multilayered cultures described here. Currently, there is no reliable and cost-effective rational approach to discover antitumour agents with novel mechanisms of action. It is likely, though, that other conventional DNA-targeted drugs would show the same pattern of dose–response curves as FU and dFdC against multilayered postconfluent cultures. Thus, this system might pose as an unsophisticated, yet efficient, alternative to select for compounds such as HPC with novel mechanisms of action. It should be emphasised that HPC has produced partial and complete remissions in the topical treatment of skin metastases and local recurrences in breast cancer patients included in phase I studies [46].

In vitro and *in vivo*, EO9 has shown preferential activity against carcinoma-derived cells as compared to leukaemias (personal communication from Dr H.R. Hendriks, EORTC-NDDO, Amsterdam), in addition to preferential cytotoxicity against V79 cells cultured under hypoxia [47], and increased growth inhibitory effects under acidic conditions [48]. It has been suggested that, since EO9 can be reduced to DNA-damaging species by DTD, the activity levels of this enzyme might be correlated to the degree of EO9 sensitivity shown by a given tumour [21]. We found that DTD activity levels on D5 were not significantly different from D1. This feature may have contributed to the relatively small differences between D1-EC_{50} and D5-EC_{50} observed for EO9, while the lower EC_{50} on D5 may have been a consequence of decreased drug penetration in multilayered cultures, as described in multicellular spheroids [49].

RZN is a tubulin-binding agent which has shown activity against vincristine-resistant cell lines *in vitro* and *in vivo* [50]. It has also demonstrated antitumour activity in heavily-pretreated breast cancer patients during phase I trials [51]. Further studies are necessary to define the mechanisms underlying the differences in the effects of RZN on the cell lines described in this report, but such results suggest that the phenomenon of a higher degree of drug resistance displayed by multilayered cultures is not indiscriminately present and seems to be dependent on the cell line and the drug tested. These results also support the concept that chemosensitivity tests using multilayered cultures should be performed in a panel of several cell lines per tumour type.

It appears that multiple factors may have influenced the chemosensitivity in our experiments with multilayered cultures. A more complete validation of this model will necessarily require the testing of additional conventional and investigational agents against a larger panel of cell lines cultured as multilayered postconfluent cultures. Nevertheless, we have shown that, in contrast to monolayers, cells grown as postconfluent cultures in "V"-bottomed, 96-well plates present some phenotypic characteristics and chemosensitivity profiles which resemble those reported in the literature with *in vivo* tests and more time- and labour-consuming *in vitro* techniques, which cannot be easily combined to semiautomated microtiter plate technology. Moreover, our tests identified HPC and EO9, drugs which were inactive in the classical P388 and L1210 screening models according to Hilgard *et al.* [52] and Dr H.R. Hendriks (EORTC-

NDDO, Amsterdam), respectively, as interesting compounds since they had more similar performances against sub- and postconfluent cultures.

1. Bonadonna G. Does chemotherapy fulfil its expectations in cancer treatment? *Ann Oncol* 1990, **1**, 11–21.
2. Boyd MR. Status of the NCI preclinical antitumour drug discovery screen; implications for selection of new agents for clinical trial. In De Vita VT Jr, Hellman S, Rosenberg SA, eds. *Cancer principles and practice of oncology updates* (vol. 3). Philadelphia, Lippincott, 1990, 1–12.
3. Corbett TH, Valeriote FA, Polin L, *et al.* Discovery of solid tumor active agents using a soft-agar-colony-formation disk-diffusion-assay. In Valeriote FA, Corbett TH, Baker L, eds. *Cytotoxic anticancer drugs: models and concepts for drug discovery and development.* Boston, Kluwer Academic Publishers, 1992, 35–87.
4. Grindey GB. Current status of cancer drug development: failure or limited success? *Cancer Cells* 1990, **2**, 163–171.
5. Phillips RM, Bibby MC, Double JA. A critical appraisal of the predictive value of *in vitro* chemosensitivity assays. *J Natl Cancer Inst* 1990, **82**, 1457–1468.
6. Rotman B, Teplitz C, Dickinson K, Cozzolino JP. Individual human tumors in short-term micro-organ cultures: chemosensitivity testing by fluorescent cytoprinting. *In Vitro Cell Dev Biol* 1988, **24**, 1137–1146.
7. Hoffman RM. Three-dimensional histocultures: origins and applications in cancer research. *Cancer Cells* 1991, **3**, 86–92.
8. Sutherland RM. Cell and environment in tumor microregions: The multicell spheroid model. *Science* 1988, **240**, 177–181.
9. Skehan P, Thomas J, Friedman SJ. Postconfluency MDCK monolayers as an *in vitro* model of solid tumor chemosensitivity. *Cell Biol Toxicol* 1986, **2**, 357–368.
10. Pelletier H, Millot JM, Chauffert B, Manfait M, Genne P, Martin F. Mechanisms of resistance of confluent human and rat colon cancer cells to anthracyclines: alteration of drug passive diffusion. *Cancer Res* 1990, **50**, 6626–6631.
11. Dimanche-Boitrel M-T, Pelletier H, Genne P, *et al.* Confluence-dependent resistance in human colon cancer cells: role of reduced drug accumulation and low intrinsic chemosensitivity of resting cells. *Int J Cancer* 1992, **50**, 677–682.
12. Pizao PE, Lyaruu DM, Peters GJ, *et al.* Growth, morphology and chemosensitivity studies on postconfluent cells cultured in "V"-bottomed microtiter plates. *Br J Cancer* 1992, **66**, 660–665.
13. Heinemann V, Hertel LW, Grindey GB, Plunkett W. Comparison of the cellular pharmacokinetics and toxicity of 2′,2′-difluorodeoxycytidine and 1-β-D-arabinofuranosylcytosine. *Cancer Res* 1988, **48**, 4024–4031.
14. Graham MA, Bisset D, Setanoians A, *et al.* Preclinical and phase I studies with rhizoxin to apply a pharmacokinetically guided dose-escalation scheme. *J Natl Cancer Inst* 1992, **84**, 494–500.
15. Eibl H, Unger C. Hexadecylphosphocholine: a new and selective antitumor drug. *Cancer Treat Rev* 1990, **17**, 233–242.
16. Workman P, Binger M, Kooistra KL. Pharmacokinetics, distribution and metabolism of the novel bioreductive alkylating indoloquinone EO9 in rodents. *Int J Radiat Oncol Biol Phys* 1992, **22**, 713–716.
17. Keepers YP, Pizao PE, Peters GJ, Van Ark-Otte J, Winograd B, Pinedo HM. Comparison of the sulforhodamine B protein and the tetrazolium (MTT) assays for *in vitro* chemosensitivity testing. *Eur J Cancer* 1991, **27**, 897–900.
18. Skehan P, Storeng R, Scudiero D, *et al.* New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* 1990, **82**, 1107–1112.
19. Smets LA, Homan-Blok J. S1-phase cells of the leukemic cell cycle sensitive to 1-β-D-arabinofuranosylcytosine at a high-dose level. *Cancer Res* 1988, **45**, 3113–3117.
20. Peters GJ, Nadal JC, Laurensse EJ, De Kant E, Pinedo HM. Retention of *in vivo* antipyrimidine effects of brequinar sodium (DUP-785; NSC 368390) in murine liver, bone marrow and colon cancer. *Biochem Pharmacol* 1990, **39**, 135–144.
21. Walton MI, Smith PJ, Workman P. The role of NAD(P)H: quinone reductase (EC1.6.99.2,DT-diaphorase) in the reductive bioactivation of the novel indoloquinone antitumor agent EO9. *Cancer Commun* 1991, **3**, 199–206.
22. Bailey SM, Friedlos F, Knox RJ, Workman P. Bioreductive activation of indoloquinone EO9: involvement of DT-diaphorase and DNA crosslinking. *Ann Oncol* 1992, **3** (Suppl. 1), 185.
23. Ernster L. DT-diaphorase. *Meth Enzymol* 1967, **10**, 309–317.
24. Benson AM, Hunkelen MJ, Talalay P. Increase of NAD-(P)H:quinone reductase by dietary antioxidants: possible role in protection against chemical carcinogenesis and toxicity. *Proc Natl Acad Sci USA* 1980, **77**, 5216–5220.
25. Prochaska HJ, Talalay P. Purification and characterization of two isofunctional forms of NAD(P)H:quinone reductase from mouse liver. *J Biol Chem* 1986, **261**, 1372–1378.
26. Bradford MM. A rapid and sensitive method for the quantification of microgram quantaties of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976, **72**, 248–254.
27. Mayer D, Natsumeda Y, Ikegami T, *et al.* Expression of key enzymes of purine and pyrimidine metabolism in a hepatocyte-derived cell line at different phases of the growth cycle. *J Cancer Res Clin Oncol* 1990, **116**, 251–258.
28. Huang P, Chubb S, Hertel LW, Grindey GB, Plunkett W. Action of 2′,2′-difluorodeoxycytidine on DNA synthesis. *Cancer Res* 1991, **51**, 6110–6117.
29. Pinedo HM, Peters GJ. Fluorouracil: biochemistry and pharmacology. *J Clin Oncol* 1988, **6**, 1653–1664.
30. Ruiz van Haperen VWT, Veerman G, Noordhuis P, Vermorken JB, Peters GJ. Concentration and time dependent growth inhibition and metabolism *in vitro* by 2′,2′-difluoro-deoxycytidine (Gemcitabine). *Adv Exp Med Biol* 1991, **309 A**, 57–60.
31. Barone RM, Calabro-Jones P, Thomas TN. Surgical adjuvant therapy in colon carcinoma: a human tumor spheroid model for evaluating radiation sensitizing agents. *Cancer* 1981, **47**, 2349–2357.
32. Kupchik HZ, Langer RS, Haberern C, El Deriny S, O'Brien M. A new method for the three-dimensional *in vitro* growth of human cancer cells. *Exp Cell Res* 1983, **147**, 454–460.
33. Erlichman C, Vidgen D. Cytotoxicity of adriamycin in MGH-U1 cells grown as monolayer cultures, spheroids, and xenografts in immune-deprived mice. *Cancer Res* 1984, **44**, 5369–5375.
34. Nederman T. Effects of vinblastine and 5-fluorouracil on human glioma and thyroid cancer cell monolayers and spheroids. *Cancer Res* 1984, **44**, 254–258.
35. Kwok TT, Twentyman PR. The response to cytotoxic drugs of EMT6 cells treated either as intact or disaggregated spheroids. *Br J Cancer* 1985, **51**, 211–218.
36. Kerr DJ, Wheldon TE, Kerr AM, Kaye SB. *In vitro* chemosensitivity testing using the multicellular tumor spheroid model. *Cancer Drug Deliv* 1987, **4**, 63–74.
37. Soranzo C, Ingrosso A. A comparative study of the effects of anthracycline derivatives on a human adenocarcinoma cell line (LoVo) grown as a monolayer and as spheroids. *Anticancer Res* 1988, **8**, 369–374.
38. Durand RE. Distribution and activity of antineoplastic drugs in a tumor model. *J Natl Cancer Inst* 1989, **81**, 146–152.
39. Knuchel R, Hofstadter F, Jenkins WEA, Masters JRW. Sensitivities of monolayers and spheroids of the human bladder cancer cell line MGH-U1 to the drugs for intravesical chemotherapy. *Cancer Res* 1989, **49**, 1397–1401.
40. Donaldson JT, Tucker A, Keane TE, Walther PJ, Webb KS. Characterization of a new model of human prostatic cancer: the multicellular tumor spheroid. *Int J Cancer* 1990, **46**, 238–244.
41. Lesuffleur T, Kornowski A, Luccioni C, *et al.* Adaptation to 5-fluorouracil of the heterogeneous human colon tumor cell line HT-29 results in the selection of cells committed to differentiation. *Int J Cancer* 1991, **49**, 721–730.
42. Lesuffleur T, Kornowski A, Augeron C, *et al.* Increased growth adaptability of 5-fluorouracil and methotrexate of HT-29 subpopulations selected for their commitment to differentiation. *Int J Cancer* 1991, **49**, 731–737.
43. Dykes DJ, Harrison SD, Jr, Abbott BJ, *et al.* Antitumor activity of clinical agents in human tumor xenografts developed from cell lines of the NCI antitumor drug screen. *Proc Am Assoc Cancer Res* 1991, **32**, 381.
44. Tritton TR, Hickman J. How to kill cancer cells: membranes and cell signalling as targets in cancer chemotherapy. *Cancer Cells* 1990, **2**, 95–105.
45. Workman P. The cell membrane and cell signals: new targets for novel anticancer drugs. *Ann Oncol* 1990, **1**, 100–111.
46. Unger C, Peukert M, Sinderman H, *et al.* Hexadecylphosphocholine

in the topical treatment of skin metastases in breast cancer patients. *Cancer Treat Rev* 1990, **17**, 243–246.
47. Stratford IJ, Adams GE, Bremner JC, *et al.* The assessment of bioreductive drug toxicity *in vitro* and in experimental tumors *in vivo*. In Adams GE, Breccia A, Fielden EM, Wardman P, eds. *Selective Activation of Drugs by Redox Processes*. New York, Plenum Press, 1990, 203–212.
48. Phillips RM, Hulbert PB, Bibby MC, Sleigh NR, Double JA. *In vitro* activity of the novel indoloquinone EO9 and the influence of pH on cytotoxicity. *Br J Cancer* 1992, **65**, 359–364.
49. Phillips RM, Bibby MC, Cronin BP, Ekebuisi M. Comparative *in vitro* chemosensitivity of DLD-1 human colon adenocarcinoma monolayers and spheroids to the novel indoloquinone EO9. *Ann Oncol* 1992, **3** (Suppl. 1), 106.
50. Hendriks HR, Plowman J, Berger DP, *et al.* Preclinical antitumor activity and animal toxicology of rhizoxin, a novel tubulin-interacting agent. *Ann Oncol* (in press).
51. Kerr DJ, Bisset D, Graham M, Setanoians A. A phase I and pharmacokinetic study of rhizoxin. *Ann Oncol* 1992, **3** (Suppl. 1), 120.
52. Hilgard P, Stekar J, Voegeli R, *et al.* Characterization of the antitumor activity of hexadecylphosphocholine (D 18506). *Eur J Cancer Clin Oncol* 1988, **24**, 1457–1461.

Eur J Cancer, Vol. 29A, No. 11, pp. 1573–1577, 1993.
Printed in Great Britain

0964–1947/93 $6.00 + 0.00

Genistein Inhibits Tumour Cell Growth *in vitro* but Enhances Mitochondrial Reduction of Tetrazolium Salts: A Further Pitfall in the Use of the MTT Assay for Evaluating Cell Growth and Survival

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The natural isoflavone genistein inhibits the growth of a number of tumour cell lines *in vitro*. During investigations on the antiproliferative effects of genistein we observed that, with respect to direct cell counting, a tetrazolium (MTT) colorimetric assay consistently underestimated the growth inhibitory activity of the substance. Cell proliferation was markedly inhibited by genistein in three tumour cell lines (MCF-7, human breast tumour; Jurkat cells, human T-cell leukaemia; L-929, mouse transformed fibroblasts) when cell number was evaluated by direct counting, whereas a 72-h MTT assay failed to reveal any growth-inhibitory effect. Cell cycle analysis by propidium iodide staining and flow-cytometry revealed a G2/M cell cycle arrest after genistein treatment. Genistein-treated cells displayed an increase in cell volume and in mitochondrial number and/or activity, as revealed by enhanced formazan generation and increased uptake of the vital mitochondrial dye rhodamine 123. These results suggest that alterations in cell cycle phase redistribution of tumour cells by genistein may significantly influence mitochondrial number and/or function and, consequently, MTT reduction to formazan. This may constitute an important bias in analysing the effects of genistein, and possibly other drugs that block the G2/M transition, on growth and viability of cancer cells *in vitro* by MTT assay.
Eur J Cancer, Vol. 29A, No. 11, pp. 1573–1577, 1993.

INTRODUCTION

COLORIMETRIC ASSAYS are extensively used for evaluating the effect of growth factors, hormones and drugs on growth and survival of both normal and tumour cells in culture [1, 2]. Two reagents, tetrazolium (MTT) and formazan (XTT) [3] are commonly employed as indicators of cell number and viability, since they are converted to a coloured formazan derivative via mitochondrial dehydrogenase activity by viable cells. Although a number of factors, such as pH, medium glucose content and age of cultures, influence MTT and XTT reduction by living cells [4, 5], there is, in general, a good concordance between the number of viable cells in the culture and the production of formazan, which can be easily measured by colorimetric methods after solubilisation in dimethylsulphoxide. Because of its simplicity, precision and low-cost, the MTT assay is currently used in cytotoxic drug screening protocols [3, 5, 6]. It has, also, been proposed as a valid alternative to the [^{3}H]thymidine uptake methods for analysing cell proliferation [7]. Our laboratory has successfully applied the test to investigating the growth-inhibitory effect of drugs and cytokines [8, 9].

During the course of experiments aimed at analysing the

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Received 13 Jan. 1993; accepted 15 Feb. 1993.