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Experimental correlations of in vitro drug sensitivity with in vivo responses to ThioTEPA in a panel of murine colon tumours

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Summary. Cell lines derived from three histologically different murine colon tumours (MAC) were used to assess whether or not a tumour colony-forming assay could have retrospectively predicted the wide range of in vivo responses to the alkylating agent, ThioTEPA. Tumour responses ranged from sensitive (MAC 26) to resistant (MAC 15A), with MAC 13 showing only moderate sensitivity. In vitro chemosensitivity studies, in conjunction with pharmacokinetic data, suggest that plasma levels of the drug's primary metabolite, TEPA, should be sufficient to induce significant cell kills in all three tumour lines in vivo. Preliminary studies on the effect of pH on the cytotoxic properties of ThioTEPA in vitro have demonstrated an improved cell kill when cells were exposed to the drug under acidic conditions. As these tumours differ histologically in terms of vascularisation, tumoural pH may play an important part in determining drug efficacy and go some way towards explaining the poor in vitro/in vivo correlation in this model.

Introduction

Clinical trials have shown that the tumour colony-forming assays described by Hamburger and Salmon [12] and Courtney and Mills [8] correctly predict drug resistance (96% true negative) but have a lower true positive rate of 61% for tumours that are sensitive in vitro [17]. However, these trials were carried out on a wide variety of patients, most of whom had been heavily pretreated with cytotoxic drugs, had different physical characteristics (i.e. age, sex, weight etc.) and were at various stages of illness. Pharmacokinetic variations in drug exposure parameters between individual patients in such a population are known to be extreme [6] and may account for the inconsistant response of tumours designated as sensitive in vitro. The aim of this study was to assess the relationship between in vitro and in vivo responses in an experimental model. In this way, a better correlation with tumour sensitivity may be obtained as variations in drug exposure conditions can be minimised and a more objective assessment of tumour response on previously untreated tumours can be made,

The mouse adenocarcinoma of the colon (MAC) series of transplantable tumours in NMRI mice were used in this study. These tumours have been extensively characterised and are similar in terms of histology, cell kinetics and chemosensitivity to tumours of the human colon [9]. A panel of three histologically different solid MAC tumours (grown subcutaneously) were used to assess the anti-tumour activity of triethylenethiophosphoramide (ThioTEPA). ThioTEPA is a polyfunctional alkylating agent that is widely used in installations for superficial bladder carcinoma [18] and is currently employed in combination regimes for advanced ovarian and breast cancers [20]. Chemotherapy and assessment protocols have previously been described [10], and in vivo responses to ThioTEPA, which have also been described elsewhere [5], are summarised in Table 1. Briefly, a broad spectrum of activity was noted, with the well-differentiated, cystic MAC 26 being most responsive, the anaplastic MAC 13 showing only moderate sensitivity and MAC 15A being unresponsive to ThioTEPA at the maximum tolerated dose (20 mg/kg i.p.).

Cell lines derived from these tumours were used to assess whether or not a clonogenic assay could have retro-

Table 1. Comparison of in vitro and in vivo responses to ThioTEPA and TEPA

	Description	In vivo [5] chemosensitivity (% tumor inhibition)	In vitro chemosensitivity (% Reduction in colony formation at experimentally achievable drug c × t and 1 h exposure time)	
		ThioTEPA (20 mg kg^{-1})	ThioTEPA	TEPA
MAC 26	Well-differentiated cystic adenocarcinoma	98%	11%	99%
MAC 13	Poorly differentiated anaplastic adenocarcinoma	58%	30%	95%
MAC 15A (s.c.)	Poorly differentiated anaplastic adenocarcinoma	0%	35%	99%

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spectively predicted in vivo responses to ThioTEPA. The primary metabolite, triethylenephosphoramide (TEPA), was also included in this study as the initial studies describing this subclass of compounds have shown that TEPA is a more potent cytotoxic agent than ThioTEPA [16] and possesses significant anti-tumour activity against a variety of experimental tumours [19]. The plasma clearance of TEPA is slower than that of ThioTEPA [7, 13], and several authors have suggested that pharmacological effects might relate more closely to circulating levels of the metabolite [7, 14]. A range of therapeutically relevant drug and metabolite concentrations for use in vitro were selected on the basis of previous pharmacokinetic studies performed in this laboratory [14]. The plasma clearance of TEPA was slower than that of ThioTEPA, with area under the curve (AUC) values of 41.7 and 14.1 μg·h per milliliter respectively (calculated at 9 h after injection). Finally, a preliminary investigation into the effects of pH on the cytotoxic potency of ThioTEPA is described. Groos et al. [11] have demonstrated a significant reduction in cell survival following exposure of RT 112 bladder carcinoma cells to ThioTEPA in relatively acidic conditions (pH 6.0). Similar results in MAC cell lines would significantly alter chemosensitivity profiles in vitro and, hence, the predictive values of these results.

Materials and methods

Culture conditions. Cell lines were derived by mechanical disaggregation of the solid tumour mass and were routinely maintained as monolayer cultures at 37° C in RPMI 1640 tissue-culture medium supplemented with 10% foetal calf serum (heat inactivated at 56° C for 20 min), penicillin/streptomycin (50 IU/ml, 50 μg/ml), sodium pyruvate (1 m*M*) and buffered using HEPES (25 m*M*). Chemosensitivity studies were restricted to cultures of $\leqslant 10$ passages in age, and cells in the exponential phase of growth were used throughout.

Chemosensitivity studies. The colony-forming ability of tumour cells surviving drug treatment was assessed using a slightly modified version of the Hamburger and Salmon [12] clonogenic assay. In this assay, no soft agar was used, as fibroblastic contamination was minimal. Single-cell suspensions derived from monolayer cultures (Trypsin 0.25%), were exposed to a range of experimentally achievable drug concentrations in "complete" RPMI 1640 and incubated at 37° C at various time intervals. Following drug exposure, the cells were washed twice in Hanks' balanced salt solution and between $2-5 \times 10^4$ viable cells (trypan blue exclusion) were plated into 25 cm^2 plastic culture flasks containing 10 ml complete RPMI 1640. After 5–7 days' incubation at 37° C, colonies of $\geqslant 50$ cells were counted using an inverted microscope and plating efficiencies calculated for each drug concentration. Cytotoxic effects of drug treatment were expressed in terms of percentage survival, taking the control plating efficiencies to represent 100% survival. Triplicate samples for each assay were done.

In vitro sensitivity was defined as a 70% or greater reduction in survival of tumour colony-forming units [21] following a 1-h exposure to drug and metabolite levels that represent the areas under the complete plasma clearance curves for both compounds, i.e. 14.1 μg·h per milliliter and 41.7 μg·h per milliliter, respectively.

pH studies. ThioTEPA concentrations of 5 μg/ml and an exposure time of 1 h were used throughout. Alterations in pH were achieved by adding small aliquots of 1 *N* HCl, NaOH to complete RPMI 1640. Each point was appropriately controlled and chemosensitivity was assessed as described above.

Drug stability in vitro. The extraction, detection and quantification of both compounds in biological fluids has been described elsewhere [14]. Stability studies were performed over a 24-h period in complete RPMI 1640 medium at 37° C.

Results

The relationship between the number of cells plated and the number of colonies produced was linear for all the cell lines tested, and plating efficiencies were below 10% (Fig. 1).

There was no evidence of ThioTEPA or TEPA breakdown in vitro following a 24-h incubation at 37° C in complete RPMI 1640. Both compounds were cytotoxic to all three cell lines, and the extent of cell kill produced was dependent on both drug concentration and the duration of exposure (Figs. 2 and 3). Increasing the duration of drug exposure resulted in increased cytotoxicity for all time points studied. ID_{70} values were consistently lower for TEPA than for ThioTEPA (Table 2), reflecting the greater cytotoxic potency of TEPA over ThioTEPA. However, there were no significant differences in both ID_{70} values or chemosensitivity profiles (Figs. 4 and 5) for each cell line exposed to either compound. No inherent differences in

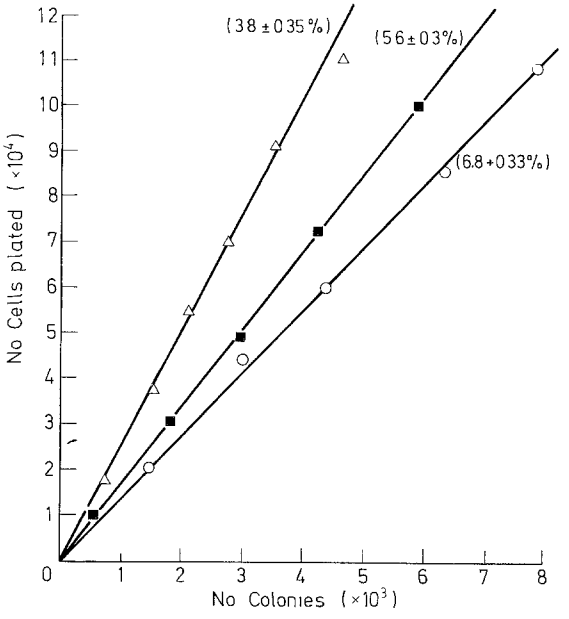

Fig. 1. Relationship between the number of tumour cells plated and the number of colonies produced by MAC 13 (▲——▲), MAC 15A (●——●) and MAC 26 (■——■). Values in parentheses represent efficiency of colony formation ± SD

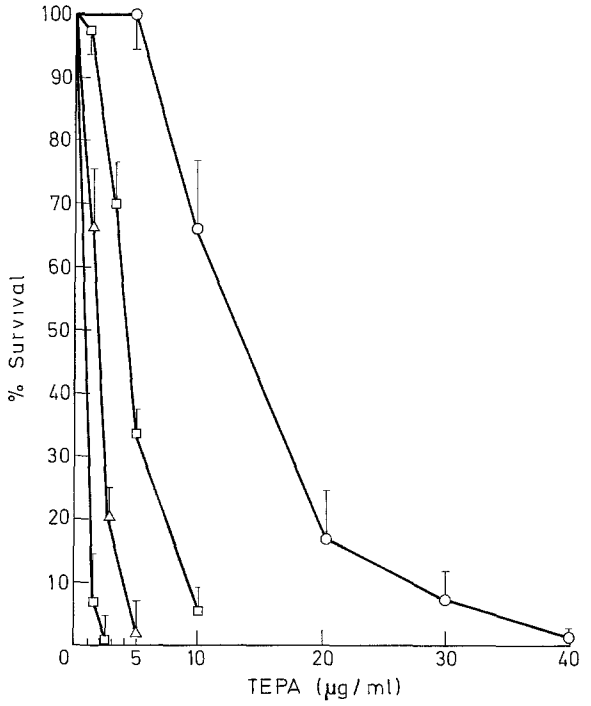

Fig. 2. Dose-response curves for MAC 15A cells treated at various concentrations of TEPA for 1 h (●——●), 3 h (■——■), 6 h (▲——▲) and 24 h (□——□). Values shown are the mean of 3 samples ± SD

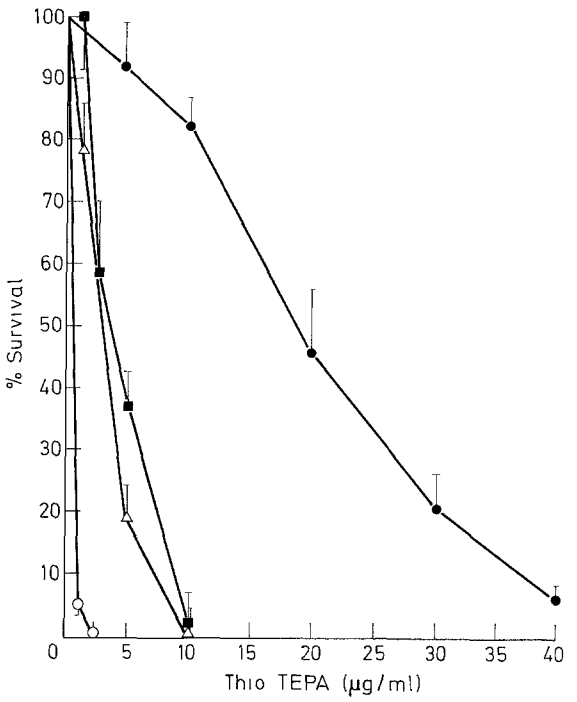

Fig. 3. Dose-response curves for MAC 15A cells treated with Thio-TEPA for 1 h (●——●), 3 h (■——■), 6 h (▲——▲) and 24 h (□——□). Values shown are the mean of 3 samples ± SD

Table 2. ID_{70} values for three MAC cell lines exposed to Thio-TEPA or TEPA for 1 h

	ID_{70} values ($\mu g\ ml^{-1}$)		
	MAC 13	MAC 15A	MAC 26
ThioTEPA	26	26	34
TEPA	20	17	18

the chemosensitivity of these cells to ThioTEPA or TEPA were observed.

The reductions in colony formation following a 1-h exposure (Figs. 4 and 5) to corresponding c × t values for ThioTEPA (14.7 μg·h per milliliter) and TEPA (41.7 μg·h per milliliter) are presented in Table 1. For all three cell lines exposed to ThioTEPA, a maximum cell kill of 35% was produced, which under the conditions previously stated is too low to allow tumour sensitivity to be predicted. Achievable plasma levels of TEPA, however, induced cell kills of greater than 95% in all three cell lines. All three tumour lines should therefore respond to ThioTEPA administration. When compared with in vivo responses (Table 1), these results correlate well for MAC 26 but not for MAC 13, which is only moderately responsive, or MAC 15A, which is unresponsive.

A reduction in cell survival was observed in two cell lines tested against ThioTEPA (5 μg/ml, 1-h exposure) when the pH was decreased during drug exposure (Fig. 6). Cell survival fell from 90% to 35% and from 85% to 50% for MAC 26 and MAC 15A cell lines, respectively, when the pH was reduced from 8.0 to 6.0.

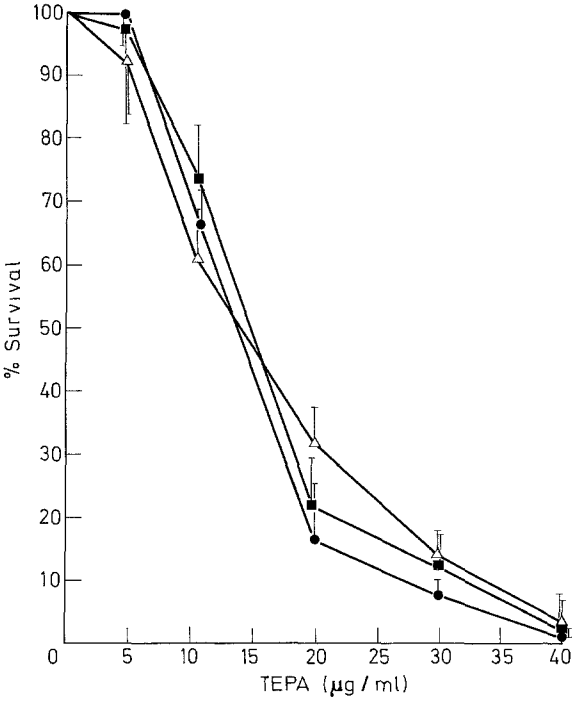

Fig. 4. Dose-response curves for MAC 26 (■——■), MAC 15A (●——●) and MAC 13 (▲——▲), following a 1-h exposure to various concentrations of TEPA. Values shown are the mean of 3 samples ± SD

Discussion

The selection of therapeutically relevant drug concentrations for use in vitro is an essential component in the design of any in vitro test aimed at predicting tumour re-

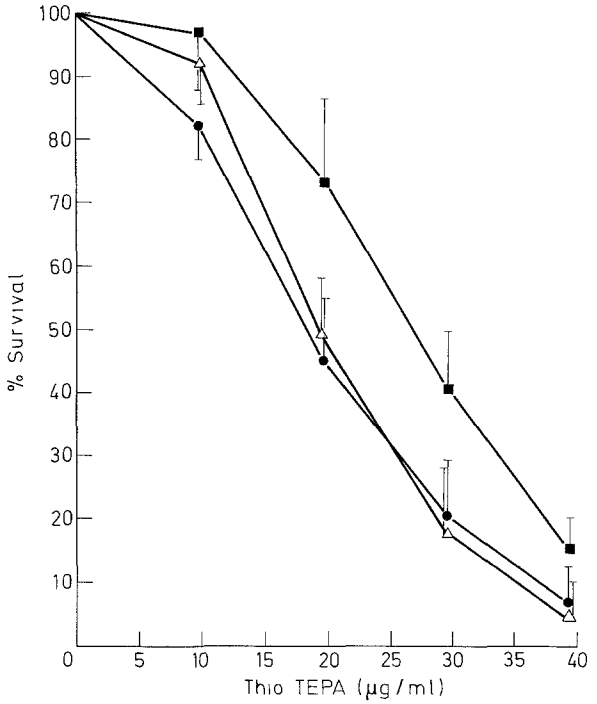

Fig. 5. Dose-response curves for MAC 26 (■———■), MAC 15A (●———●) and MAC 13 (▲———▲), following a 1-h exposure to ThioTEPA. Values shown are the mean of 3 samples ± SD

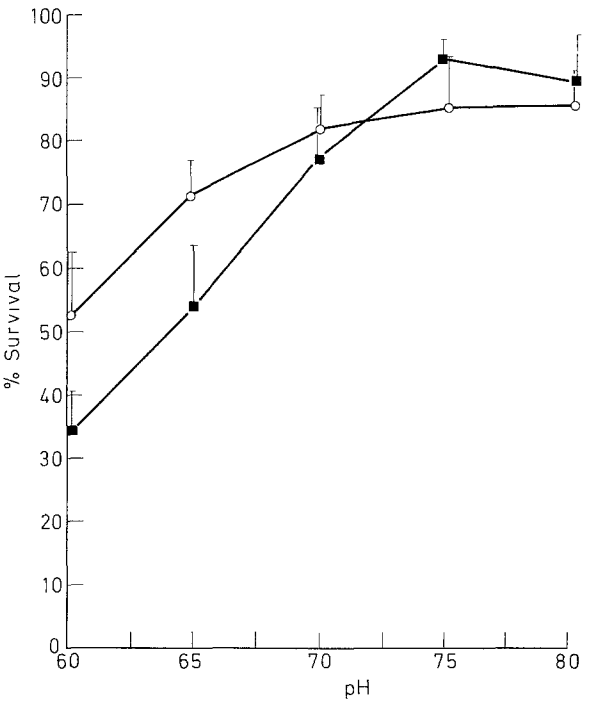

Fig. 6. The effect of pH on the cytotoxic properties of ThioTEPA (5 μg ml^{-1}, 1 h exposure) on MAC 15A (●———●) and MAC 26 (■———■). Values shown are the mean of 3 samples ± SD

sponses in vivo. Plasma drug c × t values and peak plasma concentrations are considered to be the two most relevant parameters for use in vitro [1], and Bateman et al. have demonstrated that these two parameters, when used in vitro, correlated well with cytotoxicity in human xenografts in mice [3, 4]. In this study plasma c × t values, in conjunction with chemosensitivity data, have shown that the metabolite may significantly contribute to anti-tumour effects, as plasma TEPA equivalents induce large cell kills (>95%) in all tumour lines in vitro. The correlation with in vivo responses is, however, only good for one tumour line (MAC 26). Some other factor(s), specific to the individual tumour line, must therefore have a significant bearing on the final outcome of chemotherapy in these tumours, particularly in MAC 15A, which is resistant to ThioTEPA.

The three-dimensional structure of a solid tumour introduces additional factors that may modify the effectiveness of chemotherapy. Problems with drug penetration, proliferation gradients and differences in the microenvironment (i.e., pH, pO_2 nutrients etc.) as a function of distance from a supporting blood vessel exist in a solid tumour. There are many examples in the literature where these factors significantly alter chemosensitivity profiles in vitro and hence the predictive value of these tests.

Differences in drug bioavailability between the tumour lines is unlikely to account for the spectrum of in vivo activity, as previous studies in this laboratory have demonstrated a poor correlation between tumour levels of ThioTEPA or TEPA and chemotherapeutic response [5]. Differences in microenvironmental conditions at the time of chemotherapy, however, could affect chemosensitivity in vivo as preliminary studies have shown that the cytotoxic potency of ThioTEPA is enhanced under acidic conditions. These results are consistent with those reported by Groos et al. [11] and highlight one physiological variable which could have a significant effect on tumour response to these compounds.

Tumour pH can be measured directly by the use of microelectrodes, and several authors have demonstrated that the pH of malignant tumours is considerably lower than the pH of normal tissues in both humans [2] (pH 6.8 to 7.45, respectively) and mice [15] (pH 6.9 to 7.65, respectively). Considerable heterogenity in pH was also found between areas within the tumour mass. Furthermore, these authors have described a further reduction in tumour pH following the i.v. administration of dextrose, and have suggested that a possible therapeutic advantage may be achieved for drugs that are cytotoxically more potent under acidic conditions. As the three tumour lines in this study are histologically distinct with different degrees of vascularisation at the time of chemotherapy (unpublished results), differences in tumour pH may exist, resulting in altered drug potency and chemosensitivity. Further studies on the effects of pH on the cytotoxic potency of TEPA, the measurement of tumour pH in vivo and the response of multi-cellular spheroids (known to contain a central core of low pH) to ThioTEPA are currently being evaluated.

In conclusion, this study has shown that differences in the inherent chemosensitivity of cells derived from three histologically different MAC tumour lines, as assessed by a clonogenic assay, could not account for the broad range of in vivo responses to ThioTEPA, even when pharmacokinetic exposure parameters are taken into consideration. Other factors, i.e. pH, may significantly affect chemosensitivity and would need to be considered in the design of an in vitro test for the assay to be predictive of tumour response.

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References

1. Alberts DS, Chen H-SG, Salmon SE (1980) In vitro drug assay: Pharmacologic considerations. In: Salmon SE (ed) Cloning of human tumour stem cells. Alan R Liss, New York, pp 197–209
2. Ashby BS (1966) pH studies in human malignant tissues. Lancet 2: 312
3. Bateman AE, Peckham MJ, Steel GG (1979) Assay of drug sensitivity for cells from human tumours: in vitro and in vivo tests on a xenografted tumour. Br J Cancer 40: 81–88
4. Bateman AE, Selby PJ, Steel GG, Towse GDW (1980) In vitro chemosensitivity tests on xenografted human melanomas. Br J Cancer 41: 189–198
5. Bibby MC, McDermott BJ, Double JA, Phillips RM, Loadman PM, Burgess L (1987) Influence of the tissue distribution of ThioTEPA and its metabolite, TEPA on the response of murine colon tumours. Cancer Chemother Pharmacol (in press)
6. Chabner BA (1982) The role of drugs in cancer treatment. In: Chabner PA (ed) Pharmacologic principles of cancer treatment. W. B. Saunders, Philadelphia, p 8
7. Cohen BE, Egorin MJ, Kohlhepp EA, Aisner J, Gutierrez PL (1986) Human plasma pharmacokinetics and urinary excretion of ThioTEPA and its metabolites. Cancer Treat Rep 70: 859–863
8. Courtenay VD, Mills J (1978) An in vitro colony assay for human tumours grown in immune suppressed mice and treated in vivo with cytotoxic agents. Br J Cancer 37: 261–262
9. Double JA, Ball CR (1975) Chemotherapy of transplantable adenocarcinomas of the colon in mice. Cancer Chemother Rep 59: 1083–1089
10. Double JA, Bibby MC, Loadman PM (1986) Pharmacokinetics and anti-tumour activity of LM985 in mice bearing transplantable adenocarcinomas of the colon. Br J Cancer 54: 595–600
11. Groos E, Walker L, Masters JWR (1986) The influence of pH on drug cytotoxicity in vitro. Br J Cancer 54: 180
12. Hamburger AW, Salmon SE (1977) Primary bioassay of human tumour stem cells. Science 197: 461–463
13. McDermott BJ, Double JA, Bibby MC, Loadman PM, Turner RL (1985a) Pharmacokinetic studies of ThioTEPA in patients and mice and effect of coadministration of nandrolone decanoate. Br J Cancer 52: 458
14. McDermott BJ, Double JA, Bibby MC, Wilman DEV, Loadman PM, Turner RL (1985b) Gas chromatographic analysis of triethylenethiophosphoramide and triethylenephosphamide in biological specimens. J Chromatogr 338: 335–345
15. Naeslund J, Swenson KE (1953) Investigations on the pH of malignant tumours in mice and humans after administration of glucose. Acta Obstet Gynecol Scand 32: 359–367
16. Personeus G, Halliday SL, McKenzie D, Williams JH, Oleson JJ (1952) Effect of a series of ethylenimine derivatives against metastasizing mammary andenocarcinoma of the rat. Proc Soc Exp Biol Med 81: 614–616
17. Salmon SE (1980) Clinical correlations of in vitro drug sensitivity. In: Salmon SE (ed) Cloning of human tumour stem cells. Alan R Liss, New York, pp 223–247
18. Soloway MS, Ford KS (1983) ThioTEPA induced myelosuppression. Review of 670 bladder installations. J Urol 130: 889–891
19. Sugiura K, Stock CC (1955) Studies in a tumour spectrum: III. The effects of phosphoramides on a variety of mouse and rat tumours. Cancer Res 15: 38–50
20. Turner R (1981) The value of anabolic steroids for patients receiving chemotherapy. Excerpta Med Int Congr Ser 9: 1
21. Von Hoff DD, Casper J, Bradley E, Sanbach J, Jones D, Makuch R (1981) Association between human tumour colony forming assay results and response of an individual patient's tumour to chemotherapy. Am J Med 70: 1027–1032

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