Our data suggest that the detection of the protein oncogene products of c-*myc*, *ras* or c-*jun* by immunochemistry does not give useful prognostic information in advanced carcinoma of cervix and does not predict response to chemotherapy or ultimate outcome.

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Screening of New Anticancer Agents *in vitro* Using Panels of Human Cell Lines Derived from Non-seminomatous Germ Cell Tumours and Transitional Cell Carcinomas of the Bladder

John R.W. Masters, Wendy E.A. Jenkins and Robert H. Shoemaker

Metastatic testis tumours are cured in over 80% of patients using combination chemotherapy, and this hypersensitivity is retained by the cells *in vitro*. To determine whether differential toxicity to testis tumour cells is useful in the screening of novel anticancer agents, we compared the toxicities of 12 compounds against panels of human bladder and testis tumour cell lines using a clonogenic assay. The compounds had screened negative against P388 *in vivo*, and had been retested using the human tumour colony forming assay (HTCFA) and in selected cases against human tumour xenografts. NSC 339004, chloroquinoxaline sulphonamide, was 7-fold more toxic to testis tumour than bladder cancer cells, comparing the mean of the concentrations reducing colony-forming ability by 70%. This was the only one of the compounds selected by the HTCFA shown to have clinical activity. Compound R was selectively toxic to the bladder cancer cells, and might be of value as an intravesical agent. These data indicate that panels of testis and bladder cancer cell lines might be a useful addition to the disease-oriented screening programme.
Eur J Cancer, Vol. 28A, No. 10, pp. 1617–1622, 1992.

INTRODUCTION

ANTICANCER DRUG screening in experimental animals has been conducted by the US National Cancer Institute (NCI) on a large scale for over 30 years. While this programme has discovered or played a key role in the development of nearly all the drugs currently used in cancer treatment [1], the limited activity of these compounds against the common adult solid tumours has led to the development of alternative screening strategies.

The concept of "*in vitro* phase II screening" derives from the testing of new drugs on the colony-forming ability in agar of fresh human tumours of each histological type [2]. Because preliminary data were encouraging, the NCI organised a multicentre analysis of the assay. Approximately 1000 compounds already tested *in vivo* against transplantable P388 leukaemia in mice were entered in the human tumour colony forming assay (HTCFA) screen. Compounds negative against P388 *in vivo*,

Table 1. Origins of the cell lines used in this study

Cell line designation	Histopathological type	Previous treatment	Cell no. plated	PDT‡ (h)	Reference
SuSa	NSGCT	None	4000	28	15
833K	NSGCT	Chemotherapy*	2000	23	16
GH	NSGCT	None	4000	25	17
1618K	NSGCT	Chemotherapy†	2400	32	18
RT112	TCC	None	500	22	19
RT4	TCC	^{198}Au grains	4000	37	20
T24	TCC	None	400	21	21
HT1376	TCC	None	4000	31	22
HT1197	TCC	None	4000	61	22

NSGCT = Non-seminomatous germ cell tumour; TCC = Transitional cell carcinoma of the bladder.
*Methotrexate, cyclophosphamide, actinomycin D.
†Cisplatin, bleomycin, vinblastine, etoposide, doxorubicin.
‡Population doubling time.

but positive in the HTCFA screen, were also tested against P388 *in vitro*, to determine whether the lack of antitumour activity *in vivo* was due to metabolic inactivation. The HTCFA screen selected approximately 40 of the 1000 compounds for further testing in an *in vivo* tumour panel, which included human tumour xenografts. Two of these 40 compounds, dihydrolenperone and chloroquinoxaline sulphonamide, were selected for development towards clinical trial [3]. However, the technical limitations of the HTCFA [4, 5] and, in particular, the limited capacity possible with this technology, led NCI investigators to consider alternative approaches for large scale *in vitro* primary screening. Panels of continuous cell lines derived from tumours at different sites have been collected to provide a "disease-oriented screen", aiming to identify compounds with differential or tumour-selective growth inhibitory properties [6].

Consistent with the concept of the "disease-oriented screen" [6], we have shown that the drug sensitivities of continuous cell lines derived from human testis tumours reflect the responsiveness of the tumours of origin. Metastatic testicular germ cell tumours are cured in over 80% of men using combination chemotherapy [7], whereas most other disseminated solid cancers in adults are incurable. Bladder cancer was used for comparison, because cisplatin is one of the most effective single agents and the cornerstone of combination chemotherapy for both diseases [7, 8]. Bladder cancer responds to combination chemotherapy in 40–50% of patients, but responses are usually of short duration and long-term survival is rare [8]. Compared with bladder cancer cell lines, those derived from testis tumours were hypersensitive to cisplatin [9–11], gamma radiation [12] and other chemotherapeutic drugs [13], apparently reflecting the high cure rate in patients. Extrapolating the dose–response curves, a dose of cisplatin that killed 10^{12} testis tumour cells (equivalent to a large tumour in a patient), reduced bladder cancer cell survival by a magnitude of only 3–4, leaving 10^8–10^9 cells to regrow.

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In this study we investigated whether differential toxicity to testis tumour cell lines might be useful in the evaluation of new drugs. The toxicities of 12 of the compounds negative in the P388 *in vivo* screen and tested in the HTCFA were measured using panels of testis and bladder cancer cell lines. The 12 drugs showed a range of activity in the HTCFA and included the two selected by the HTCFA for clinical evaluation.

MATERIALS AND METHODS

The origins of the nine cell lines used in this study are shown in Table 1. All cell lines were maintained routinely under identical conditions as monolayers in 25 cm^2 tissue culture flasks (Nunc) using RPMI 1640 medium supplemented with a single batch of 5% heat-inactivated fetal calf serum (Tissue Culture Services, Slough, U.K.) and 2 mmol/l L-glutamine (Flow) in a humidified atmosphere of 5% CO_2 in air at 36.5°C. The cells were used over a restricted range of 10 passages to minimise changes that might occur during long-term culture.

Cytotoxicity assay

Exponentially-growing cells were detached using 0.05% trypsin (Difco, 1:250) in an aqueous solution of 0.016% EDTA (BDH). Cells were plated in 5 cm dishes containing 5 ml of prewarmed and gassed medium at an appropriate density (see Table 1) and incubated for 48 h to permit attachment and the resumption of exponential growth. Then the medium was replaced either with fresh medium alone (in quintuplicate) or containing 0.5% dimethyl sulphoxide (DMSO, Sigma) or a range of drug concentrations (in triplicate for each concentration). Preliminary experiments identified the range of cytotoxic concentrations and at least three concentrations which fell on the exponential region of the dose–response curve were selected. After a further 6–19 days culture, colonies were fixed in methanol (BDH) and stained with 10% Giemsa (BDH). Colonies containing a minimum of 50 cells were counted using a binocular dissecting microscope. The mean colony-forming ability was expressed as a percentage of the untreated controls and computed using least squares regression analysis on the straight portion of the dose–response plot. Each drug was tested against each cell line in at least three further experiments.

Drugs

Compounds tested in this study were supplied by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics

Table 2. Summary details of the agents used in this study, showing the solvents, the mean IC_{70}s of the four testis and five bladder cancer cell lines and the response rates in the HTCFA

NSC number	Solvent	Mean IC_{70} (μg/ml)	HTCFA No. of tumours responding (%)
339004	DMSO	1.8	23/67 (34)
339125	DMSO	4.7	0/7 (0)
339324	Water	>40	0/3 (0)
339675	DMSO	3.0	1/33 (3)
Compound R	DMSO	0.08	31/38 (82)
341201	DMSO	5.4	1/7 (14)
341346	DMSO	2.7	1/7 (14)
341964	Water	>40	3/37 (8)
341982	DMSO	5.1	1/5 (20)
343025	Water	>40	0/12 (0)
343513	DMSO	4.2	10/89 (11)
343529	Water	3.5	6/35 (17)

Program, NCI. The NCI accession numbers (NSC numbers) of the compounds are shown in Table 2, and their structures are depicted in Fig. 1 (except compound R). The drugs were dissolved in distilled water or DMSO (as shown in Table 2), aliquoted in suitable volumes and stored at −20°C until the day of use.

Other screening methods

The techniques used for evaluating compounds against P388 *in vivo* and *in vitro* have been described [1], as have those for the HTCFA [5].

RESULTS

The mean IC_{70}s (concentrations reducing colony-forming ability by 70%) are shown in Table 3 for the testis and bladder cancer cell lines. Three agents (339324, 341964, 343025) had no effect on colony-forming ability at concentrations up to a maximum of 40 μg/ml. Compound R was the most potent agent, approximately 100-fold more toxic than any other compound tested, with IC_{70}s in the range 0.04–0.173 μg/ml. Six compounds (339675, 341201, 341346, 341982, 343513, 343529) were similar in their toxicity and had mean IC_{70}s within the range 1.1–8.2 μg/ml. 339125 was 15 × and 30 × more toxic to two of the four testis lines, comparing the IC_{70}s with those of the other two testis and the bladder cancer lines. Similarly, 339004 was differentially toxic to one of the bladder lines, RT4.

All 12 compounds were negative in the P388 *in vivo* prescreen, but two were positive in the HTCFA, 339004 (chloroquinoxaline sulphonamide) and 343513 (dihydrolenperone). 339004 was retested against P388, because it is insoluble in water and had originally been tested in suspension. On reformulation in DMSO the compound was active, but borderline, with a maximum T/C of 130. Chloroquinoxaline sulphonamide was differentially toxic to the testis tumour cells ($P < 0.05$ comparing mean IC_{70}s using the Student's *t*-test)(see Fig. 2). The testis lines were also significantly more sensitive to 341346 ($P < 0.05$ comparing mean IC_{70}s using Student's *t*-test), but the difference was small, with mean IC_{70}s of 2.0 and 3.4 μg/ml. Two of the four testis tumour lines were differentially sensitive to 339125, but this compound was negative in two other *in vivo* test systems and had no activity in the HTCFA. Compound R was significantly more toxic to the bladder cancer cells ($P < 0.02$ comparing mean IC_{70}s using the Student's *t*-test)(see Fig. 3), but lacked activity in a variety of *in vivo* screens. The other compound selected by the HTCFA, dihydrolenperone (343513), was not differentially toxic to the testis tumour cells.

DISCUSSION

The cytotoxicities of a structurally diverse group of compounds, previously screened against P388 *in vitro* and *in vivo* and in the HTCFA, were measured using human bladder and testis cancer cell lines. Only one agent, chloroquinoxaline sulphonamide (339004), showed the level of differential toxicity to the testis cancer cells we had previously observed using established anticancer agents such as cisplatin, bleomycin and etoposide [11–14]. Comparing mean IC_{70}s, 339004 was seven times more toxic to the testis tumour cells. 339004 showed activity in human tumour xenografts and progressed to clinical trial. Objective responses to 339004 were seen in a phase I trial in 8/73 patients, including 4/40 with previously-treated non-small cell lung cancer [25]. Only mild toxicity was seen and the maximum tolerated dose is yet to be reached [25].

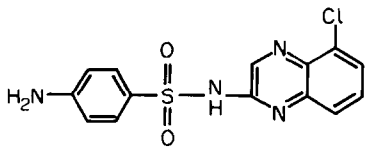
Five agents were not differentially toxic, comparing testis and bladder cancer cells. However, one of these compounds, 343513 (dihydrolenperone, DHLP), was selectively toxic to human non-small cell lung carcinomas in the HTCFA, and entered phase I clinical trials in this disease. Only a single minor clinical response was observed, and plasma concentrations at the maximum tolerated dose were far below the concentrations associated with growth inhibition *in vitro* [26].

Three agents did not kill cells at concentrations up to the maximum tested, 40 μg/ml. Lack of toxicity *in vitro* does not preclude activity *in vivo* because some drugs require metabolic activation. The inability of cells *in vitro* to metabolically activate some prodrugs is considered to be a major limitation of *in vitro* screening systems [27]. This limitation can be overcome with the use of hepatocyte co-cultures or microsomal fractions [28], but such refinements make *in vitro* screening more complicated, time-consuming and expensive.

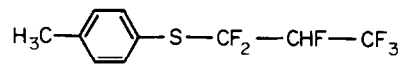
While some agents are metabolically activated *in vivo*, others are rapidly detoxified. For example, Compound R was rapidly inactivated by hepatocyte co-cultures *in vitro* and lacked anticancer activity in several xenograft models *in vivo* [29]. This compound was the most cytotoxic agent we tested, but because of its rapid detoxification it would be unlikely to show anticancer activity in patients following systemic administration. Nevertheless, there may be a clinical role for Compound R, because it was the one drug with selective activity against bladder cancer cells. Superficial bladder cancer can be treated by instillation of drugs directly into the bladder, where detoxification is unlikely to occur before the drug has reached the tumour cells. Furthermore, high doses of compound R could be instilled into the bladder with minimal risk of systemic toxicity, because the drug would be rapidly detoxified if absorbed systemically.

Two further agents showed evidence of differential toxicity, 341346 and 339125. The testis tumour cells were consistently more sensitive than the bladder cancer cells to 341346, but comparing the mean IC_{70}s, the difference was small, less than 2-fold. Two of the four testis tumour cell lines were hypersensitive to 339125. While the purpose of the disease-oriented screen is to identify such compounds, it is questionable whether compounds with limited differential toxicity are worth developing further.

Our aim was to investigate whether differential toxicity to testis tumour cells might be an aid to the screening of new



339004



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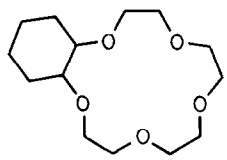
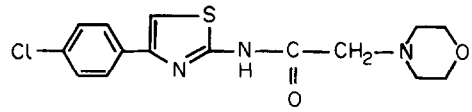
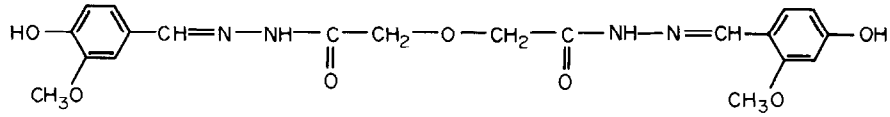


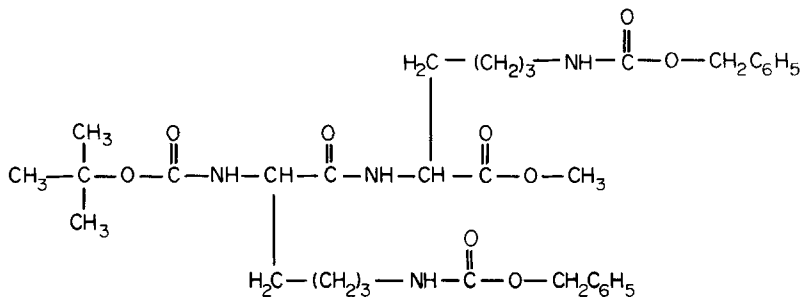
339324



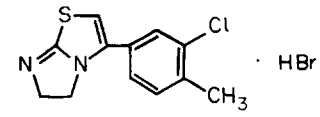
339675



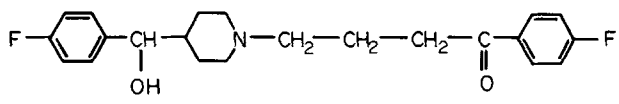
341201



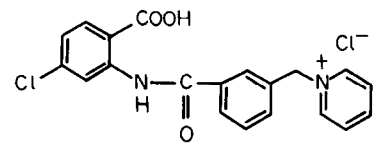
341346



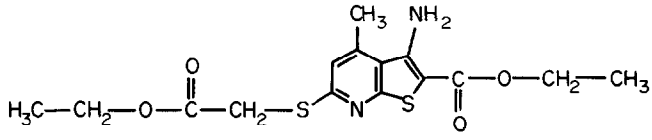
343529



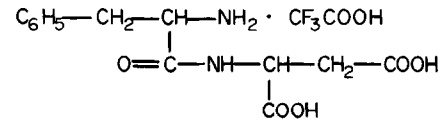
343513



341964



341982



343025

Fig. 1. Structures of 11 of the chemicals tested.

Table 3. Concentrations of compounds in μg/ml reducing colony-forming ability by 70%, showing the mean and standard error. The compounds 339324, 341964 and 343025 are not included as no cell kill was observed at concentrations up to the maximum tested, 40 μg/ml

Cell line and origin	NSC number								
	339004	339125	339675	Compound R	341201	341346	341982	343513	343529
NSGCT									
SuSa	0.24(0.02)	0.37(0.04)	2.0(0.3)	0.09(<0.01)	6.9(0.1)	2.2(<0.1)	5.4(0.2)	2.1(<0.1)	4.1(0.1)
833K	0.38(0.02)	6.0(0.1)	2.5(0.2)	0.10(0.01)	6.3(0.2)	2.3(0.1)	5.3(0.3)	4.9(0.4)	5.9(0.1)
GH	0.39(0.08)	0.19(<0.01)	1.9(0.1)	0.09(0.01)	3.0(<0.1)	1.1(0.2)	2.8(0.2)	3.8(0.1)	2.9(0.2)
1618K	0.50(0.08)	5.8(0.7)	3.9(0.2)	0.17(0.03)	7.9(0.6)	2.2(0.6)	6.2(0.1)	4.9(<0.1)	2.3(0.2)
Mean	0.38	3.1	2.6	0.11	6.0	2.0	4.9	3.9	3.8
Bladder									
RT112	3.7(0.1)	6.2(<0.1)	2.9(0.1)	0.05(<0.001)	3.8(0.2)	4.4(0.1)	5.4(0.1)	4.7(<0.1)	1.7(0.1)
RT4	0.4(0.01)	4.9(0.2)	2.3(0.3)	0.04(0.004)	3.1(0.2)	2.5(0.4)	2.6(0.3)	3.0(0.2)	4.3(0.5)
T24	5.2(0.7)	6.6(0.4)	4.6(0.2)	0.06(0.001)	7.7(0.3)	3.9(0.2)	8.2(0.1)	6.1(0.4)	6.0(<0.1)
HT1376	3.3(0.6)	6.5(0.8)	2.8(0.1)	0.07(0.004)	5.7(0.2)	2.3(0.2)	6.1(0.2)	5.2(0.5)	2.3(<0.1)
HT1197	2.0(0.5)	5.7(0.2)	4.2(0.2)	0.06(0.011)	4.3(0.2)	3.8(0.6)	3.8(0.3)	3.4(0.2)	2.1(0.1)
Mean	2.9	6.0	3.4	0.054	4.9	3.4	5.2	4.5	3.3

Mean (S.E.).

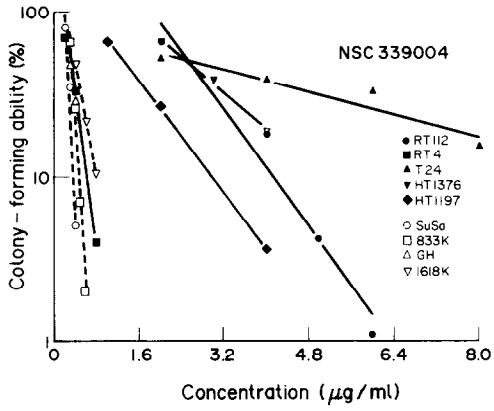


Fig. 2. Dose–response curves of the four testis cancer cell lines (dashed lines and open symbols) and the five bladder cancer cell lines (solid lines and closed symbols) to NSC 339004. The testis cancer cells are significantly more sensitive than the bladder cancer cells, comparing mean IC_{70}s ($P < 0.05$).

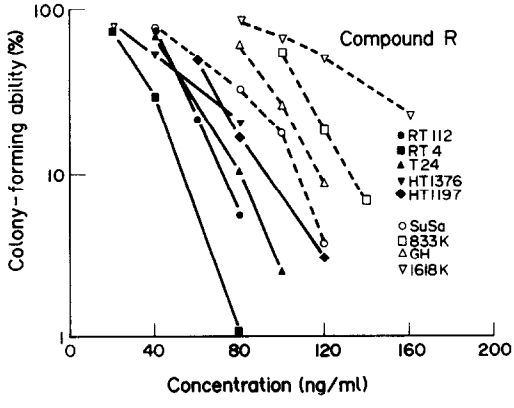


Fig. 3. Dose–response curves of the four testis cancer cell lines (dashed lines and open symbols) and the five bladder cancer cell lines (solid lines and closed symbols) to Compound R. This is the only drug in this study significantly more toxic to bladder than testis cancer cell lines, comparing mean IC_{70}s ($P < 0.02$).

anticancer agents. A high level of differential toxicity to testis tumour cells was observed with only one agent, 339004. This was one of two drugs selected by the HTCFA and xenograft screening systems, and is the one drug to have shown activity in the clinic. In our studies of established anticancer agents, testis tumour cells are more sensitive than bladder cancer cells. Surprisingly, one compound in this study was differentially toxic to the bladder cancer cell lines, and might be an effective intravesical agent. In conclusion, these data indicate that it would be valuable to include panels of bladder and testis tumour cell lines in the disease-oriented screen.

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Downmodulation of c-*myc* Expression by Interferon γ and Tumour Necrosis Factor α Precedes Growth Arrest in Human Melanoma Cells

Susanne Osanto, Rumo Jansen and Monica Vloemans

After *in vitro* incubation of melanoma tumour cells Cmel453A with either recombinant interferon gamma (rIFN-γ) or tumour necrosis factor alpha (rTNF-α) a dose-dependent inhibition of cell growth occurred; when both cytokines were added, a synergistic action was observed. Inhibition of DNA synthesis, as measured by [^{3}H] thymidine incorporation, occurred after 6 h of incubation with rIFN-γ or rTNF-α, and this action was potentiated when the two cytokines were applied simultaneously. Within 1 h, the level of c-*myc* mRNA in tumour cells had already decreased by, respectively, 60% (S.D. 7) and 25% (S.D. 7); the combined addition of the cytokines resulted in a greater reduction of c-*myc* mRNA than by each cytokine alone. Downregulation of c-*myc* expression is an early event, occurring hours before the actual inhibition of outgrowth. Thus, in melanoma cells like Cmel with a high constitutive expression of the c-*myc* oncogene, the antiproliferative action of rIFN-γ and rTNF-α may be mediated by an inhibition of the expression of c-*myc*.
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INTRODUCTION

INTERFERON GAMMA (IFN-γ) and tumor necrosis factor alpha (TNF-α) are regulatory cytokines with pleiotropic biological activities in addition to antitumour actions. Both cytokines exert a potent antiproliferative action, *in vitro* as well as *in vivo*, on the growth of various tumour cells [1, 2].

The mechanism of the antiproliferative action of these cytokines is uncertain. Recent evidence suggests that the growth-inhibiting action of cytokines in tumour cells can be mediated by a suppression of the expression of oncogenes [3, 4]. In this context, attention is focused on the c-*myc* oncogene that is thought to play a key role in the control of cell proliferation [5,