Ex vivo therapeutic index by drug sensitivity assay using fresh human normal and tumor cells*

Andrew G Bosanquet and Philip B Bell

Bath Cancer Research, Royal United Hospital, Bath BA1 3NG, UK

Correspondence to: Dr Andrew Bosanquet, Bath Cancer Research, Wolfson Centre, Royal United Hospital, Bath BA1 3NG, United Kingdom. Telephone: 011 44 1225 825 425. Fax: 011 44 1225 824 114. E-mail: agb@caltri.org

Toxicity is a major deterrent to achieving substantial improvements in cancer management, since most anticancer drugs inadequately distinguish normal and neoplastic tissues. Improving the differential between beneficial and toxic effects of therapy - therapeutic index - is a major clinical objective, but therapeutic index for cytotoxic drugs is narrow. Fresh tumor and normal cells from 59 patients with acute myeloid leukemia, non-Hodgkin's lymphoma, ovarian cancer and cancers of unknown origin were tested for *ex vivo* drug sensitivity using apoptosis by morphology assays. Drugs tested included carboplatin, doxorubicin, vincristine, cytarabine, fludarabine, mafosfamide and etoposide. Therapeutic index was derived from the ratio of normal and tumor cell LC_{90}s. Individual patient therapeutic index varied markedly for different drugs and drug therapeutic index varied from patient to patient ranging from extremely unfavourable (<0.001) through excellent (>1000) reflecting patient heterogeneity. Therapeutic index for each drug was consistent with clinical expectations. Significantly, there was no relationship between normal and tumor cell LC_{90}s. We conclude that further laboratory and clinical evaluation is required but the derived *ex vivo* therapeutic index could enhance choice of chemotherapy by reducing toxicity and/or improving efficacy.

Key words: toxicity, efficacy, therapeutic index, ex vivo drug sensitivity assay, Apoptosis by Morphology assay

INTRODUCTION

Choosing optimum anticancer therapy for an individual within the heterogeneous patient population is difficult. The clinician must balance the cytotoxic effect of treatment on tumor cells with the toxicity experienced by the patient (i.e. effect on normal cells). Commonly termed a therapeutic index, this has been defined as 'toxicity to the tumor divided by toxicity to the host' (1). Improving the therapeutic index by increasing the window between the beneficial and adverse effects of therapy is a major research goal (2). However, the therapeutic index of cytotoxic drugs is narrow (3) compared with other drug therapy and may be associated with potentially severe or life-threatening side effects. Patient heterogeneity also ensures unpredictable variations in response.

Many studies undertaken in an attempt to improve therapeutic index, by either decreasing treatment complications or increasing treatment efficacy, have relied on animal models, cell lines or clinical trials (2). As a result, most cytotoxic chemotherapy is given at or near the phase I-determined maximum tolerated dose but, whilst tumor cells are killed, normal cells are also adversely affected. The consequent morbidity, ranging from minimal to life threatening and for some aggressive regimens resulting in a significant treatment-related mortality (4), highlights the unpredictable variations in therapeutic index that occur. However, until a drug is administered, a patient's therapeutic index cannot be assessed, and then only qualitatively. Combination chemotherapy further complicates therapy decisions.

Table 1. Details of specimens tested.

Tumor	No. tested	Previously treated (%)	Age range (yr)	Source of tumor cells (No.)	Source of normal cells[b]	Predominant cell lineage of normal cells[a]			
						lym	mye	mac	mix
AML[c]	21	16 (76%)	2.3 – 83.1	Blood (10)	co-cultured		4		6
				Bone marrow (11)	co-cultured	1	5		5
NHL[d]	15	12 (80%)	41.7 – 75.9	Blood (5)	co-cultured		1	3	1
				Bone marrow (5)	co-cultured		4	1	
				Lymph node (1)	co-cultured	1			
				Ascites (3)	co-cultured	1		2	
				Pleural fluid (1)	co-cultured	1			
Ovarian	18	11 (61%)	49.1- 76.2	Lymph node (1)	Blood				1
				Ascites (5)	co-cultured	1		1	3
				Ascites (4)	Blood	2		2	
				Pleural fluid (1)	co-cultured	1			
				Tumor (7)	Blood	2			5
UKP	5	3 (60%)	65.6 - 77.0	Ascites (4)	co-cultured	3		1	
				Pleural fluid (1)	co-cultured	1			

[a] Defined as ≥ 65% of normal cells present. Otherwise called 'mix' (mixed). lym, lymphoid; mye, myeloid; mac, macrophages. Mesothelial cells did not survive the 4-day incubation period

[b] In co-cultures, no cell separation was performed. Cells were isolated together and incubated together

[c] Acute Myeloid Leukemias: 2 AML1, 2 AML2, 1 AML3, 2 AML4, 1 AML6, 3 AML7, 3 secondary AML, 7 classification unknown

[d] Non Hodgkin's Lymphomas: 4 mantle cell, 2 'low-grade', 2 'high-grade', 1 lymphoplasmacytoid and 2 follicular lymphomas, and 4 chronic lymphocytic leukemias

Although it has been recognised that 'chemosensitivity testing of normal tissues should predict for the toxic effects of antitumor drugs' (5,6), only anecdotal evidence has been presented for individual patients. In this paper, we describe the testing of therapeutic index in primary co-cultures of normal and tumor tissue. We have used an *ex vivo* drug sensitivity test that assesses apoptosis by morphology - the Differential Staining Cytotoxicity assay (7,8). In contrast to assays with machine-read endpoints, assays that assess apoptosis by morphology as the endpoint are able to determine therapeutic index in co-cultures of normal and tumor cells. We suggest that further work should be undertaken so that, where applicable, therapeutic index could be added to *ex vivo* drug sensitivity data with the aim of improving choice of chemotherapy for individuals.

MATERIALS AND METHODS

Specimens

Tumor specimens sent for drug sensitivity testing by published methods (7-9) were processed and incubated with a panel of drugs relevant to the diagnosis within 24 hours of phlebotomy or biopsy. Where mixed tumor and normal cells were isolated from a specimen, these were co-cultured; no attempt at cell separation was performed as normal and tumor cells were to be

identified morphologically at the end of incubation. Where cell isolations yielded >90% tumor cells, normal cells were obtained from other sources, usually blood (see Table 1), and cultured separately under identical conditions. We chose four diagnoses – a drug-sensitive (ovarian) and a drug-resistant (unknown primary (UKP)) solid tumor and two haematological malignancies (acute myeloid leukemia and non-Hodgkin's lymphoma) – to investigate tumor and normal cell sensitivity. All results tested over a 6-year period that gave results in both normal and tumor cells are included in this report. Co-incubation was performed in 43 specimens (73%); tumor and normal cells were incubated separately in the remainder.

Drug sensitivity testing

The *ex vivo* apoptotic drug sensitivity assay methodology used has been described in detail (7,10). Briefly, mononuclear cells were isolated: from blood and bone marrow using density gradient centrifugation; from lymph nodes and tumors by chopping and enzyme disaggregation (8); from ascites and pleural fluid by centrifugation. The cells were washed, counted and incubated at 37°C in RPMI 1640 medium containing 10% fetal bovine serum (Gibco, Paisley, Scotland) or Ultraculture (BioWhittaker, Wokingham, UK) for 94 hours in five concentrations of drug in duplicate. Polypropylene tubes were used to prevent cell adhesion
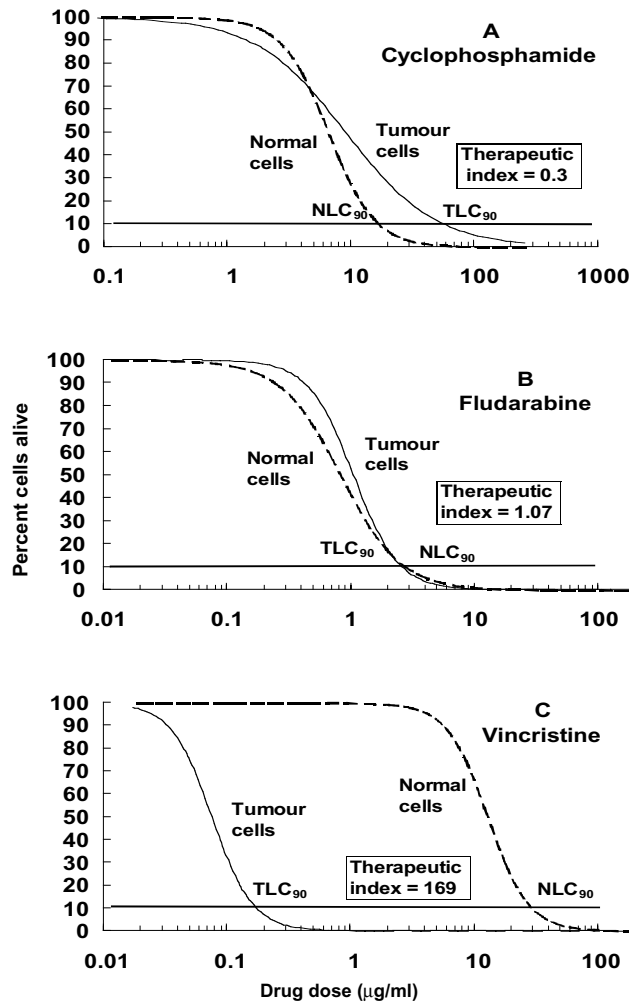
Figure 1. Cell survival and calculation of therapeutic index in a previously treated patient with mantle cell non-Hodgkin's lymphoma. A, unfavourable therapeutic index for cyclophosphamide (mafosfamide *in vitro*); B, therapeutic index of approximately 1 for fludarabine; and C, a favourable therapeutic index for vincristine. Cell survival curves were determined by fitting logistic regression curves to the cell count survival data as previously described (12). ——, tumor cell survival; - - - -, normal cell survival; TLC_{90}, tumor cell LC_{90}; NLC_{90}, normal cell LC_{90}.

during incubation. Drug makeup and storage has been detailed (7). Specimens were tested against a panel of drugs chosen for their differing modes of action at concentrations encompassing the range of clinically relevant plasma levels (7). Fixed duck erythrocytes (as an internal standard) and fast green/ nigrosin (Sigma) (to stain dead cells black) were then added and the cells

cytocentrifuged onto microscope slides. The slides were air-dried and counter-stained with a Romanowsky stain to facilitate identification of remaining live cells. Normal and tumor cells were identified morphologically and cell survival scored. In co-cultures, slides were scored once to determine tumor LC_{90}s (lethal concentration of drug to kill 90% cells) and a second time to give normal cell LC_{90}s.

Data handling and statistical analysis

LC_{90}s were calculated by fitting logistic regression curves to the cell count survival data (the logit of survival was taken to be linear with respect to the logarithm of the drug concentration) and calculating the log dose at which the fitted survival probability was equal to 0.1 (11,12). *Ex vivo* therapeutic index was calculated for each drug thus:

Therapeutic index = normal cell LC_{90} / tumor cell LC_{90}

A neutral therapeutic index where normal and tumor cell sensitivities are equal has a value of 1.0. Adverse therapeutic indices of <1.0 occur when normal cells are more sensitive to drug than tumor cells; a beneficial therapeutic index of >1.0 indicates a drug with efficacy against tumor cells greater than toxicity against normal cells. Distributions of LC_{90} and therapeutic index are approximately log-normal i.e. normally distributed when drug concentration is plotted on a log scale. All data analysis was therefore performed on $\log(LC_{90})$ or $\log$(therapeutic index) values (13): mean, standard deviations and 95% confidence intervals (CI) were calculated. Calculations of Pearson's linear correlation coefficients (r) and significance (p) were performed using Fig P for Windows version 2.98. Bonferroni correction of significance was used where multiple correlation coefficients were calculated.

RESULTS

For 59 patients, both normal and tumor cell LC_{90}s were available and the *ex vivo* therapeutic index was calculated; 41 (71%) of patients were previously treated. This study presents all results produced in the four diagnoses using the seven chosen drugs: carboplatin, doxorubicin, vincristine, cytarabine, fludarabine, cyclophosphamide (mafosfamide *in vitro*) and etoposide. Details of specimens are given in Table 1. No distinction was made for normal cell source when analysing results since, as previously reported, source or lineage of cells made little difference to normal cell drug sensitivity (12).

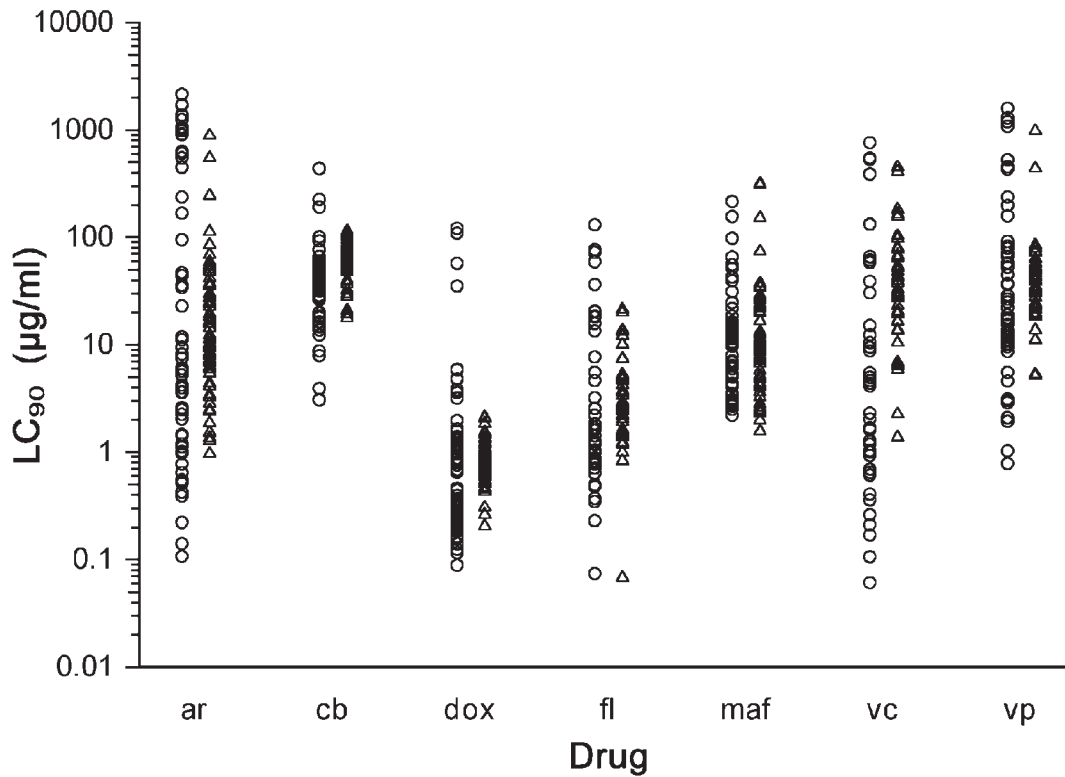Figure 1 illustrates *ex vivo* therapeutic indices for

Figure 2. Comparison of the scatter of LC_{90}s for tumor and normal cells. ○, tumor cell LC_{90}s; △, normal cell LC_{90}s; ar, cytarabine; cb, carboplatin; dox, doxorubicin; fl, fludarabine; maf, cyclophosphamide (mafosfamide *in vitro*); vc, vincristine; vp, etoposide.

cyclophosphamide, fludarabine and vincristine from a previously treated mantle cell lymphoma patient. Tumor cells were more resistant than normal cells to cyclophosphamide, giving an unfavourable therapeutic index <1.0 (Figure 1A). Normal and tumor cells had similar sensitivity for fludarabine with an *ex vivo* therapeutic index of ~1.0 (Figure 1B). However, tumor cells were more sensitive than normal cells to vincristine and *ex vivo* therapeutic index was 169 (Figure 1C). These results are typical of the large intra-patient variation from drug to drug elucidated when *ex vivo* therapeutic index is calculated.

The LC_{90}s derived from these sigmoidal curves for both normal and tumor cells were plotted for all tumor types (Figure 2). There was a large inter-patient variability in tumor cell LC_{90} values, reflecting the heterogeneity of response to therapy both between diagnoses and within any diagnosis. Cytarabine had the greatest range of tumor cell sensitivity with LC_{90}s, from 0.109-2186 µg/ml, a 20,000-fold difference reflecting the sensitivity of acute myeloid leukemia and non-Hodgkin's lymphoma and the resistance of solid tumors, including unknown primary carcinomas, to this drug. The greatest range within one tumor type was 3000-fold, for unknown primary. In contrast,

carboplatin had less variation in tumor cell sensitivity with a 142-fold difference between the most sensitive, an acute myeloid leukemia, and the most resistant, an ovarian specimen. Normal cell LC_{90}s generally varied to a lesser extent (12,14); for carboplatin, they showed a 6.5-fold variation, with the most sensitive cells from an ovarian and the most resistant from an acute myeloid leukemia patient. These results illustrate how, compared with its effect on tumor cell sensitivity, diagnosis has little effect on normal cell sensitivity.

By assessing the mantle cell lymphoma patient's normal cell sensitivity results in Figure 1 in the context of all 59 patients in Figure 2, they are found to be close to the mean normal cell LC_{90} for the three drugs presented. However, the variability in the patient's tumor cell sensitivity produces very different therapeutic indices for the three drugs.

When tumor cell LC_{90} versus normal cell LC_{90} for all diagnoses was plotted (for doxorubicin and cyclophosphamide as examples, Figure 3), **no** relationship was found between the two parameters. This pattern was confirmed with other drugs, with the correlation coefficients ranging from -0.14 to 0.39 (all p = not significant). In contrast, there was a strong negative relationship between tumor cell LC_{90} and
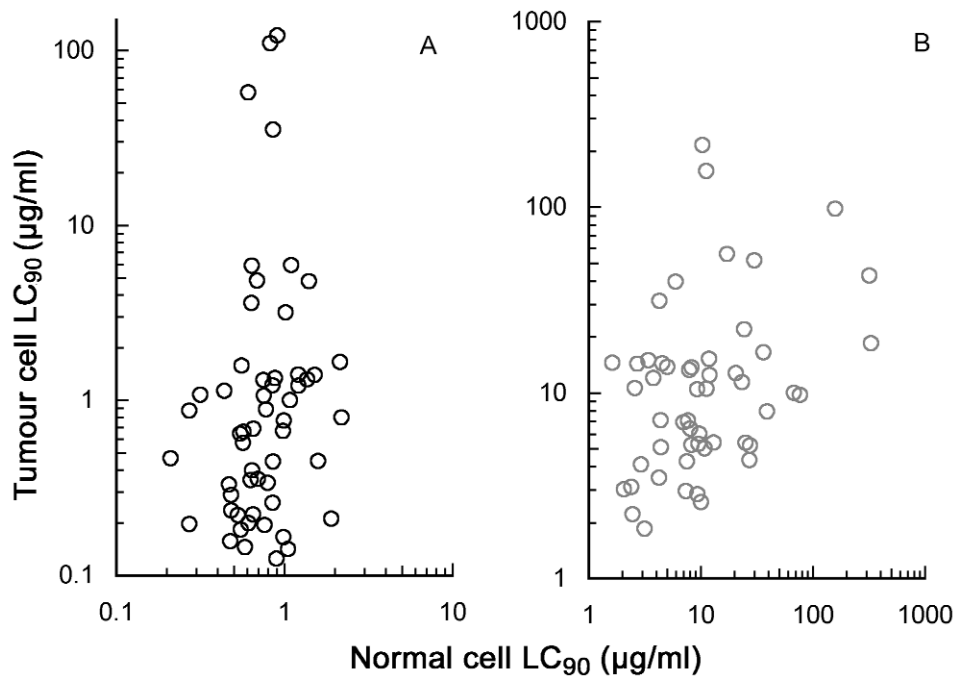
Figure 3. Relationship between tumor LC_{90} and normal LC_{90}. A, doxorubicin (r = 0.173; p >0.05; Bonferroni corrected); B, mafosfamide (r = 0.364; p >0.05).

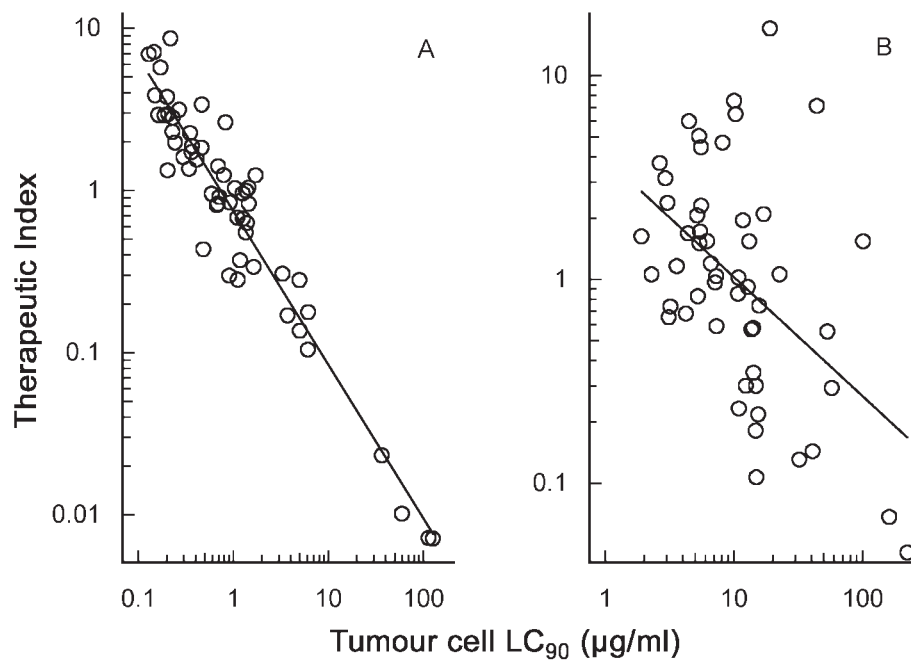**Figure 4.** Relationship between tumor LC_{90} and therapeutic index. A, doxorubicin (r = -0.954; p <<0.0001); B, mafosfamide (r = -0.641; p <0.0001).

therapeutic index for all drugs (range of r: -0.48 to –0.95, all p<0.01 and most p<<0.0001, Bonferroni corrected) as illustrated by doxorubicin and cyclophosphamide (Figure 4). For most drugs (excepting fludarabine and cyclophosphamide), there

was no significant relationship between normal cell LC_{90} and therapeutic index (data not shown). Thus, in most cases drug therapeutic index was more influenced by tumor rather than normal cell sensitivity. This may mean that, for some drugs, therapeutic index is no
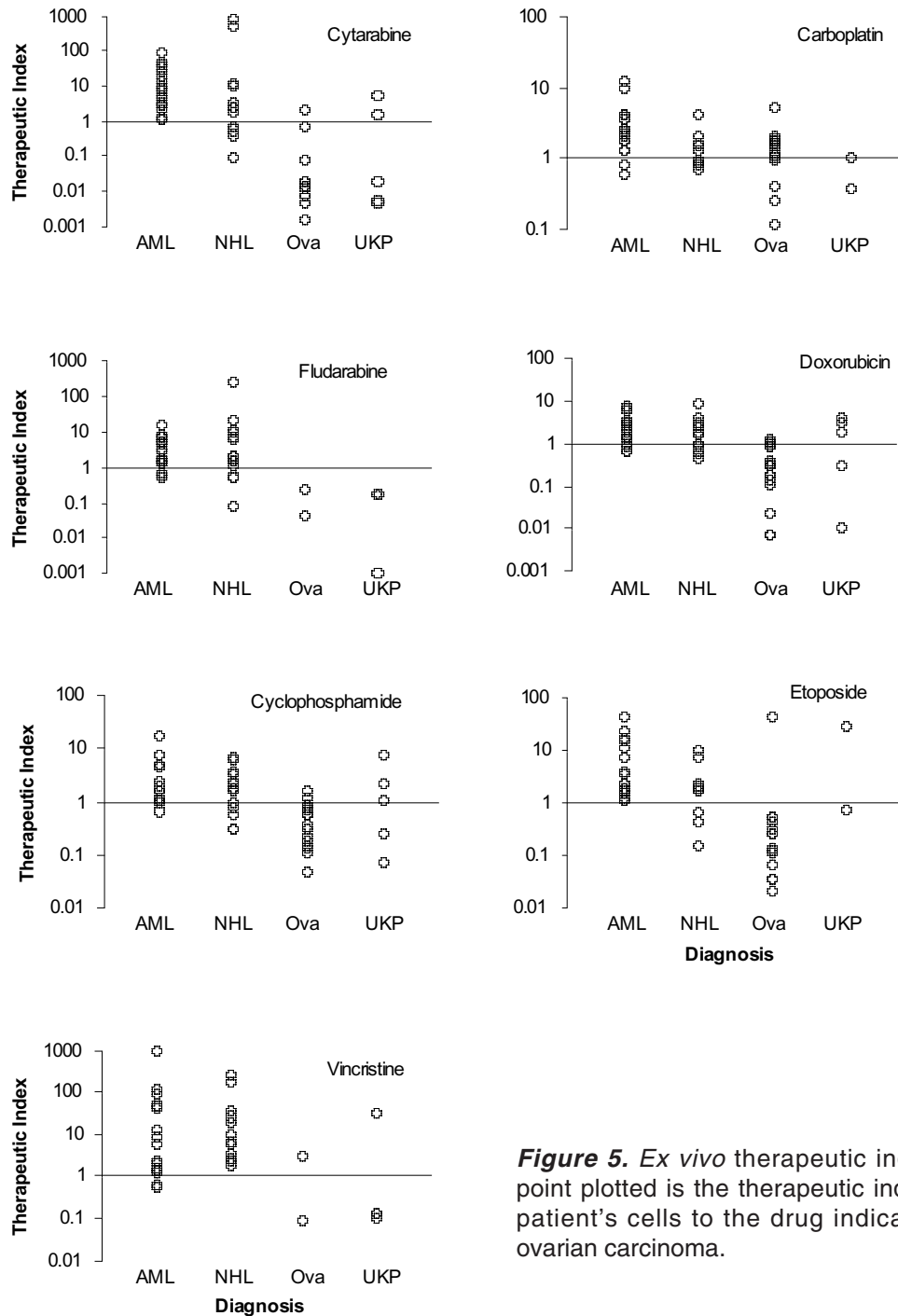
Figure 5. *Ex vivo* therapeutic index. Each point plotted is the therapeutic index of one patient's cells to the drug indicated. Ova, ovarian carcinoma.

better than LC$_{90}$ but further work would be required to ascertain this. However, similar drug sensitivities can produce very different therapeutic indices: for instance with cyclophosphamide, for tumor cell LC$_{90}$s around the median of approximately 15 µg/ml, therapeutic index varies by more than 100-fold – from 0.1 to 20 (Figure 4B).

When drug *ex vivo* therapeutic index is plotted by

diagnosis, there is a large inter-patient variation (Figure 5). Cytarabine therapeutic index ranges over 1,000,000-fold and even within a particular diagnosis, values can range up to 10,000-fold e.g. cytarabine in non-Hodgkin's lymphoma. In general, the range for therapeutic index is consistent with clinical expectation. Thus, cytarabine and fludarabine are clinically active (and generally have a favourable
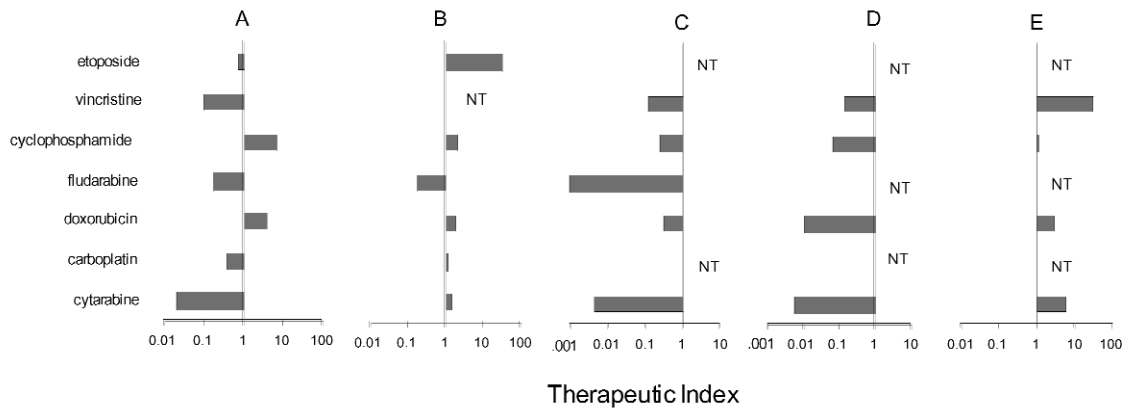
Figure 6. Therapeutic index charts for the five UKP patients included in this study. Patients A, B and E had received cytotoxic therapy prior to assay; patients C and D had not. NT, not tested.

therapeutic index of >1.0) in acute myeloid leukemia and non-Hodgkin's lymphoma and less active in solid tumors (equating with an adverse therapeutic index <1.0). Carboplatin, on the other hand, shows comparable activity in mostly pre-treated ovarian cancers and the haematological malignancies, and is used in both haematological and solid tumor regimens. In all solid tumor specimens tested, fludarabine had a therapeutic index <1.0, reflecting its inactivity in these diagnoses. Vincristine has a better than expected therapeutic index probably because its dose limiting neuro-toxicity is clearly not represented by the normal cells tested.

Four of 18 specimens from ovarian patients had a therapeutic index of <1.0 for carboplatin (Figure 5). The most favourable therapeutic index for all four patients was found with cyclophosphamide (mafosfamide *in vitro*). Untreated ovarian carcinoma is a drug sensitive tumor, but treatment induces a pleiotropic drug resistance, reflected in the resistance of these pre-treated patients.

Only occasionally do patients with similar stage of the same disease have approximately similar patterns of therapeutic index. Whilst Figure 5 illustrates trends, individual patient data in any diagnosis exhibit greater heterogeneity, as in the results for cancers of unknown origin (Figure 6) and mantle cell lymphomas (Figure 7). Of the five unknown primary tumors tested, two (Figure 6C & 6D) showed the expected adverse therapeutic index to all drugs tested, despite having had no prior chemotherapy. The other three (from previously treated patients) had a favourable therapeutic index for some drugs: one for etoposide and a vinca alkaloid (Figure 6B) and another to vincristine and cytarabine but to no other drugs (Figure 6E). In contrast, a third had an unfavourable therapeutic index for etoposide, vincristine and cytarabine but a favourable therapeutic index to cyclophosphamide and doxorubicin (Figure 6A). Thus, therapeutic index reflects known patient heterogeneity; it might also reflect site of primary tumor - drug sensitivity results sometimes 'suggest' or clarify a diagnosis that is difficult to ascertain. In Figure 7, the therapeutic indices for the four patients with mantle cell lymphoma are presented. As a group, they show less variation than the unknown primary cancers, but each patient's cells has a unique drug sensitivity profile.

DISCUSSION

For many years, researchers have recognised that selecting compounds for clinical trials should be based not only on cytotoxicity but also on therapeutic index (15). The latter representing the margin between antitumor activity and toxicity to normal cells was understood to relate to factors responsible for efficacy and toxicity (16). One of the greatest challenges for chemotherapy is the relative inability of anticancer drugs to distinguish between normal and neoplastic tissue and consequently a broad range of toxicities is experienced (17). This results in dose-limiting toxicity and, therefore, reduced antitumor efficacy. Any agent that selectively kills tumor cells more efficiently than normal cells is potentially a useful anticancer drug but this therapeutic index is not easily determined for individual patients prior to treatment.

Therapeutic index has been defined as 'toxicity to the tumor divided by toxicity to the host' (1) or 'ratio of the dose that brings about an anticancer effect to the dose that brings about a toxic effect' (18). However, these ratios are difficult to quantify in humans – the former because values are not available, the latter because individual patients are not treated with a series of drug doses. Using fresh human cells cultured with
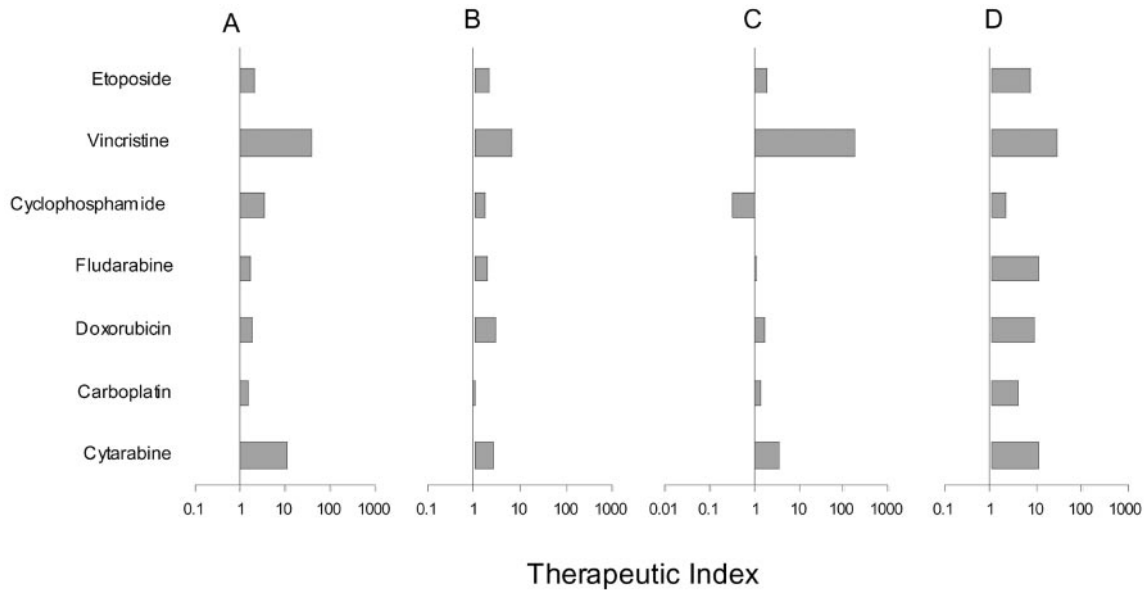
Figure 7. Therapeutic index charts for the four previously treated mantle cell non-Hodgkin's lymphoma patients included in this study. Patient C is the same as illustrated in Figure 1.

anticancer drugs, we have identified selective cell kill morphologically by measuring tumor cell kill and comparing this with normal cell kill, thereby deriving a quantifiable *ex vivo* therapeutic index. For those drugs where haematopoietic toxicity is not great (for instance vincristine), these data with haematopoietic cell sensitivities may not be so clinically relevant. However, we propose further laboratory and clinical studies should be undertaken to determine whether, for other drugs tested, *ex vivo* therapeutic index may sometimes add useful information to guide the clinician in treatment choice and reflect potential efficacy of the drug in relationship to its toxicity.

For three decades, it has been suggested that chemosensitivity testing of normal tissues should be meaningful in predicting the toxic effects of antitumor drugs' (5,15), but to date, a routine method for assessing comparative toxicity of therapy pre-administration in order to tailor treatment to the individual has been lacking. Toxicity has been one of the main deterrents to substantial improvements in cancer management (19): clinicians are limited to using drug at or slightly below the maximum tolerated dose of a selected cohort of patients, and by extension, 'the average patient', to provide efficacy with acceptable or minimal toxicity. However, even for leukemia and lymphoma, the success stories of modern cancer therapy, 'treatments are toxic, expensive and ineffective for many patients' (20). For the majority, treatment based on drug concentrations and doses designed for the average patient is sub-optimal and

may be positively harmful. For patients who experience drug-induced mortality, adjustment of therapy based on response and toxicity (21) is inappropriately late! In acute myeloid leukemia, improved response and survival rates largely resulting from intensification of chemotherapy combined with good supportive care have been associated with a treatment-related mortality rate of up to 15% (4). It is unclear whether further advances are possible, given the high intensity of current regimens. However, for some drugs, where an experiment is performed on fresh cells from the patient, both drug sensitivity and *ex vivo* therapeutic index can be determined before unacceptable morbidity or even mortality are induced.

Normal cell sensitivity, as a surrogate indication for toxicity and especially myelosuppression, enables the antitumor activity to be assessed 'in context'. The normal cells tested mostly originate in the bone marrow and so their drug sensitivity is likely to resemble that of bone marrow cells. In general the xenobiotic tolerance in other organ systems of the body is the same or greater than marrow, so that myelosuppression is usually the main clinical consequence of exposure to cytotoxic drugs (6). Hence, it is reasonable to suppose that *ex vivo* normal cell drug sensitivity is related to clinical myelosuppression, the most common dose-limiting toxicity.

Heterogeneity of response to therapy and toxicity challenges the orthodoxy of reliance on cohort-selected therapy: an alternative approach with treatment choice refined by patient-tailored rather than cohort-specific

regimens (22) could improve response and survival for some patients. Increasingly, traditional approaches are no longer sufficient to meet contemporary expectations (18). Adapting therapy to the individual by *ex vivo* drug sensitivity in conjunction with appropriate use of ex vivo therapeutic index could greatly assist the choice of a drug for dose escalation (or reduction (23)), the best drugs for bone marrow ablation, a drug for palliation, or a novel agent. This might well produce significant improvements in patient outcome – expectations of both clinicians and patients could more often be realised. Whilst tailor-made therapies place greater demands on clinicians and scientists, enhanced treatment outcomes should result.

Both in the laboratory and the clinic, further research is required to ascertain how useful this approach will be in clinical terms. Where large therapeutic indices are obtained (good or bad), escalation of drug dose, or conversely avoidance of toxic drugs could be possible. The potential rewards of using ex vivo drug sensitivity assays in terms of increased response and survival could provide the incentive necessary to achieve this paradigm shift in approach. They can be financially beneficial in terms of quality life years saved (24). The use of apoptosis by morphology drug sensitivity assays can significantly improve choice of effective chemotherapy (25). A randomised clinical trial in relapsed/resistant chronic lymphocytic leukemia is being used to quantify response and survival using this methodology (26).

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