Ex vivo effects of the dual topoisomerase inhibitor tafluposide (F 11782) on cells isolated from fresh tumor samples taken from patients with cancer

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Tafluposide (F 11782), a novel epipodophylloid with a unique mechanism of interaction with both topoisomerase I and II, has shown outstanding antitumor activity *in vivo* against a panel of experimental human tumor xenografts. The aim of this study was to evaluate its cytotoxicity against fresh tumor cells taken from patients. Cells derived from bone marrow, peripheral blood, malignant effusions or solid biopsies from 84 patients with either hematological or solid tumors were exposed continuously to 0.8–100 μM tafluposide for 48 h, 96 h or 7 days. Cell survival was measured using an MTT assay or the ATP assay and LC_{50} values (drug concentration required for 50% cell kill) were calculated. Tafluposide showed significant cytotoxicity against cells derived from either hematological or solid tumors, with a marked inter-patient variation. There was no significant difference between the effect of tafluposide in samples from untreated or previously treated patients ($p > 0.05$ for all cancer types). Whilst tafluposide appeared to show weak ($p < 0.05$) cross-resistance with the topoisomerase II inhibitor etoposide in acute myeloid leukemia (AML), there did not appear to be any correlation with the effect of the topoisomerase I inhibitor topotecan ($p > 0.05$) in either hematological or solid malignancies. True synergism was identified when combining tafluposide with cisplatin in ovarian cancer [combination index (CI) = 0.14, 0.79] and with etoposide in AML (CI = 0.49, 0.63 and 0.78). Our results suggest that tafluposide is a strong candidate for inclusion in clinical trials, particularly in hematological malignancies. *Anti-Cancer Drugs* 14:467–473 © 2003 Lippincott Williams & Wilkins.

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Introduction

Tafluposide (F 11782; 2″,3″-bis pentafluorophenoxyacetyl-4″,6″-ethylidene-β-D-glucoside of 4′-phosphate-4′-demethylepipodophyllotoxin, 2*N*-methyl glucamine salt) represents the first of a new class of topoisomerase-interacting drugs. Tafluposide is a dual inhibitor of topoisomerase I and II, but unlike other classical topoisomerase inhibitors it neither stabilizes cleavable complexes nor intercalates DNA, appearing to act via a previously undescribed mechanism. It is a catalytic inhibitor of topoisomerase I and II which completely impairs the binding of these nuclear proteins to DNA, most probably through a direct interaction between tafluposide and the topoisomerase enzymes themselves [1].

Tafluposide has proven cytotoxic activity *in vitro* in established cell lines [2]. It appears to be a promising candidate for combination chemotherapy as, unlike many combinations used in the clinic today, it displays true synergism with other DNA-damaging agents [3]. Synergism could be due to the fact that this agent is a potent inhibitor of nucleotide excision repair, specifically affecting the incision step [4]. Also, tafluposide has been shown to induce both single- and double-strand breaks, with DNA damage increasing linearly with time of incubation [5,6]. However, the nature of the damage induced appeared distinctive from that resulting from exposure to either etoposide or camptothecin.

The *in vivo* antitumor activity of tafluposide has been shown to be markedly superior to that of other dual topoisomerase inhibitors [2,7] as well as to other catalytic topoisomerase inhibitors [8], which could be due in part to the high level of stability of the DNA damage induced. The marked *in vivo* activity of tafluposide was a major contributory factor to this agent being selected for clinical development and phase I clinical trials in Europe are now scheduled.

The purpose of the present study was to investigate whether cytotoxicity to tafluposide could be demonstrated

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Table 1 Tumor types and patient samples

Tumor type	Number tested	Sample type	Tumor cells [median % (25–75th percentile]	Number previously treated
Hematological tumors				
AML	25	12 BM, 13 PB	87 (71–91)	12
ALL	13	7 BM, 6 PB	96 (87–99)	5
CLL[a]	15	15 PB	99 (75–100)	9
CML[b]	1	1 PB	70	1
NHL[c]	5	2 PB, 3 lymph node	95 (95–95)	0
Solid tumors				
Ca ovary	28	17 malignant effusions		7
		11 solid biopsies	80 (50–90)	
melanoma	1	1 lymph node	80	0
Total	88			34

[a]Chronic lymphocytic leukemia.
[b]Chronic myeloid leukemia.
[c]Non-Hodgkin's lymphoma.

in fresh tumor cells taken directly from patients with cancer and also to see if synergism with DNA-damaging agents, as found in experimental tumor models, translates to these clinical samples. Chemosensitivity testing using patients' samples has been shown to give an accurate prediction of clinical response to cytotoxic therapy [9,10]. Also, we have shown that ovarian cancer patients, when treated with cytotoxic drugs found sensitive *ex vivo*, survive significantly longer overall [11]. Furthermore, chemosensitivity testing can help identify specific tumor types responsive to novel agents such as tafluposide whilst providing the ideal environment in which to study different combinations of cytotoxic agents using a technique that may give a more factual representation of the heterogeneity of response found in the clinic. The data presented here clearly demonstrate that tafluposide is an exciting addition to the existing repertoire of anticancer agents, particularly for hematological tumors, and should be considered for evaluation in future clinical trials.

Methods

Patients and samples

After informed consent, 88 samples were taken from 84 patients. Table 1 lists the tumor and sample types involved. Samples of bone marrow or peripheral blood were collected aseptically into citrate phosphate dextrose; solid biopsies and lymph nodes were collected at operation into RPMI 1640 medium plus antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin) and antimycotics (1.25 μg/ml amphotericin B and 100 μg/ml kanamycin; medium and supplements being purchased from Sigma Aldrich, Poole, UK). Samples of malignant effusions were collected into dry sterile containers. Thirty-four of these samples were from patients who had received previous chemotherapy and now had relapsed or refractory disease.

Cell preparation

Cells were harvested from bone marrow (BM), peripheral blood (PB) or malignant effusions using density gradient centrifugation (Histopaque; Sigma Aldrich). Mechanical disaggregation was used to isolate tumor cells from solid biopsies, and these cells were also subjected to density gradient centrifugation to remove red blood cells and necrotic cells. After washing, cells were resuspended in RPMI 1640 medium plus 10% FCS, 100 IU/ml penicillin and 100 μg/ml streptomycin. Cytospin preparations were made of this final cell suspension and cell morphology was checked using May Grünwald Giemsa staining.

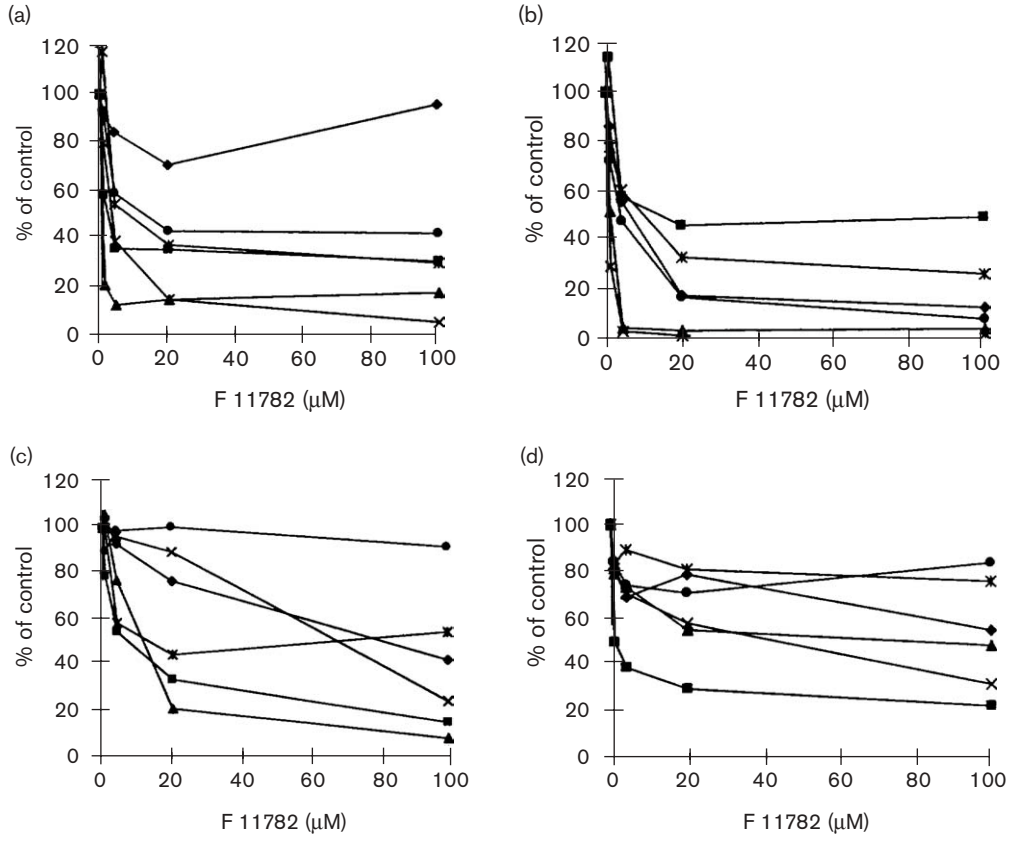
Chemosensitivity assay

Drug exposure was carried out as previously described [9,11]. Cells were exposed continuously, in triplicate, to four concentrations of tafluposide (0.8–100 μM) for 48 and 96 h or, in the case of samples from patients with ovarian cancer for 7 days, at 37°C, 5% CO_2. For cross-resistance studies, cells were also exposed to the topoisomerase inhibitors etoposide (10–85 μM) or topotecan (1.25–10 μM; kind gift of GlaxoSmithKline). Cell survival was measured using the MTT assay as previously described [9,11] or, in the case of acute lymphocytic leukemia (ALL) samples, the ATP assay (Vialight HS kit; Cambrex Biosciences, Wokingham, UK) according to manufacturer's instructions [12]. Results were expressed as the mean percentage of the triplicate experiments of untreated control cells, dose–response curves were drawn and the LC_{50} (drug concentration lethal to 50% of cells) calculated using our own customized software.

Drug combinations

In order to study the effect of combining tafluposide with other cytotoxic agents, blast cells from patients with acute myeloid leukemia (AML) were exposed to tafluposide (0.8–100 μM). and cytosine arabinoside (ara-C; 2.5–20 μM) or to tafluposide and etoposide (10–85 μM). Cells from patients with ovarian cancer were exposed to tafluposide and cisplatin (2–16 μM). These drugs were tested both singly and in combination at a constant ratio. CalcuSyn software (Biosoft, Cambridge, UK) was used to carry out median effect analysis. Results were expressed as mutually non-exclusive combination index (CI) values,

Fig. 1



The effects of tafluposide on cells derived from clinical samples: (a) AML, (b) ALL, (c) CLL and (d) ovarian cancer. Results for samples from six patients from each tumor type are shown. Distinct symbols are used for each patient. Each point represents the mean of triplicate experiments carried out on the same occasion. SE typically <10%.

a quantitative measure of the degree of drug interaction. CI was calculated for three different fractions, ED_{50}, ED_{75} and ED_{90}, and the mean of these values was used to identify synergism (CI < 1), additivity (CI = 1) or antagonism (CI > 1) for the different combinations tested.

Statistics

Non-parametric statistical methods were used. LC_{50} values for tafluposide were compared to those for other drugs using Spearman's rank correlation coefficient. The effect of time on sensitivity to tafluposide was assessed using a matched-pair Wilcoxon signed-rank test. Differences between groups of patients were determined using the Mann–Whitney *U*-test.

Results

Ex vivo effect of tafluposide

The effect of tafluposide varied both between tumor types and markedly between patients within the same tumor type (Fig. 1). The median LC_{50} values found for each tumor type are shown in Table 2 in descending sensitivity. The hematological tumors showed the greatest sensitivity to tafluposide, particularly the acute leukemias. In Fig. 1, the incubation time with drug was 96 h for all tumor types except ovarian cancer where, since only a minimal effect was noted at 96 h, the incubation time was extended to 7 days. Indeed, increasing the incubation time led generally to increased sensitivity in the tumors tested. (AML $n = 12$, $p < 0.01$; ALL $n = 5$ $p < 0.05$; ovarian cancer $n = 15$, $p < 0.05$; Wilcoxon signed-rank test, Fig. 2).

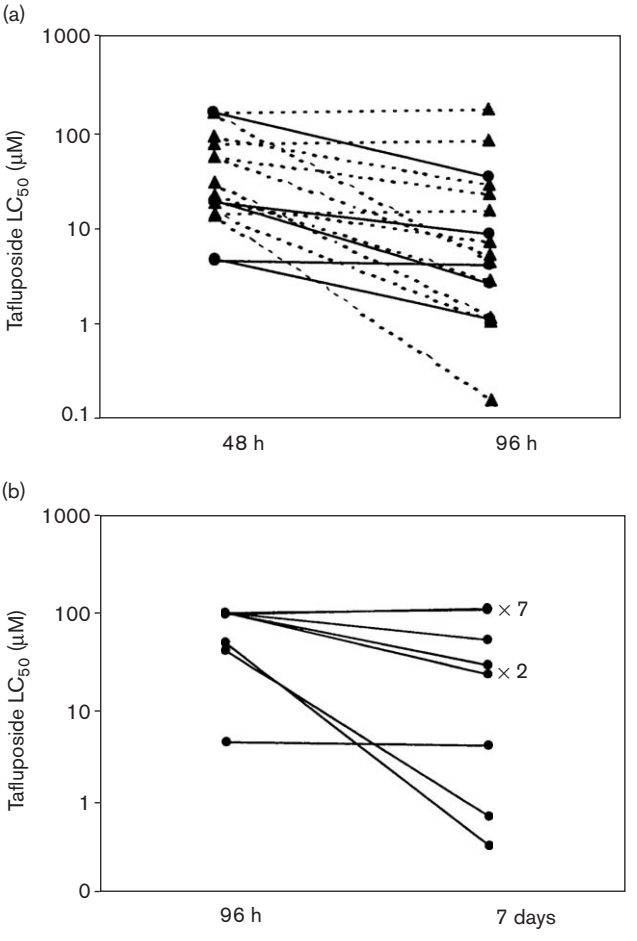
In ALL, AML and ovarian cancer there was no significant difference in tafluposide LC_{50} values for patients who had received previous chemotherapy compared to their untreated counterparts (all $p > 0.05$, Mann–Whitney *U*-test, Fig. 3). Indeed, one patient (3211) with ovarian cancer who had been heavily pretreated with a platinum, a taxane and alkylating agents, and was now resistant both clinically and *ex vivo* to these agents, remained very sensitive to tafluposide with an LC_{50} value of 0.62 μM, suggesting that this patient may well have benefited from receiving tafluposide as third-line therapy.

Table 2 ***Ex vivo*** **effect of tafluposide on different tumor types**

Tumor type	Drug exposure time	Number LC_{50} < 100 μM/number tested	Median LC_{50} (μM)	Range of LC_{50} (μM)
Hematological tumors				
ALL	48 h	6/7	4.9	0.4–>100
	96 h	11/11	1.8	0.05–21.0
AML	48 h	9/13	19.0	9.4–>100
	96 h	17/24	8.0	0.1–>100
CLL[a]	48 h	9/15	80.0	5.6–>100
	96 h	13/14	18.9	2.9–>100
CML[b]	96 h	0/1	>100	
NHL[c]	48 h	0/2	>100	
	96 h	3/5	37	7.3–>100
Solid tumors				
Ca ovary	48 h	1/9	>100	45–>100
	96 h	5/27	>100	4.2–>100
	7 days	7/15	105	0.3–>100
melanoma	48 h	0/1	>100	

[a]Chronic lymphocytic leukemia.
[b]Chronic myeloid leukemia.
[c]Non-Hodgkin's lymphoma.

Fig. 2



The effect of increasing incubation time with tafluposide in (a) hematological tumors, 48 versus 96 h (ALL: $n=5$, circles; AML: $n=12$, triangles) and (b) ovarian cancer, 96 h versus 7 days. Each line represents the change in LC_{50} values for an individual patient.

Comparison with other topoisomerase inhibitors

Cross-resistance patterns between the dual topoisomerase inhibitor tafluposide, the topoisomerase I inhibitor topotecan and the topoisomerase II inhibitor etoposide were investigated. In the hematological tumor AML, some cross-resistance was identified with etoposide, but not with topotecan (Table 3). The effect of tafluposide was also compared with topotecan in ovarian cancer samples and, as with AML, there did not appear to be any cross-resistance.

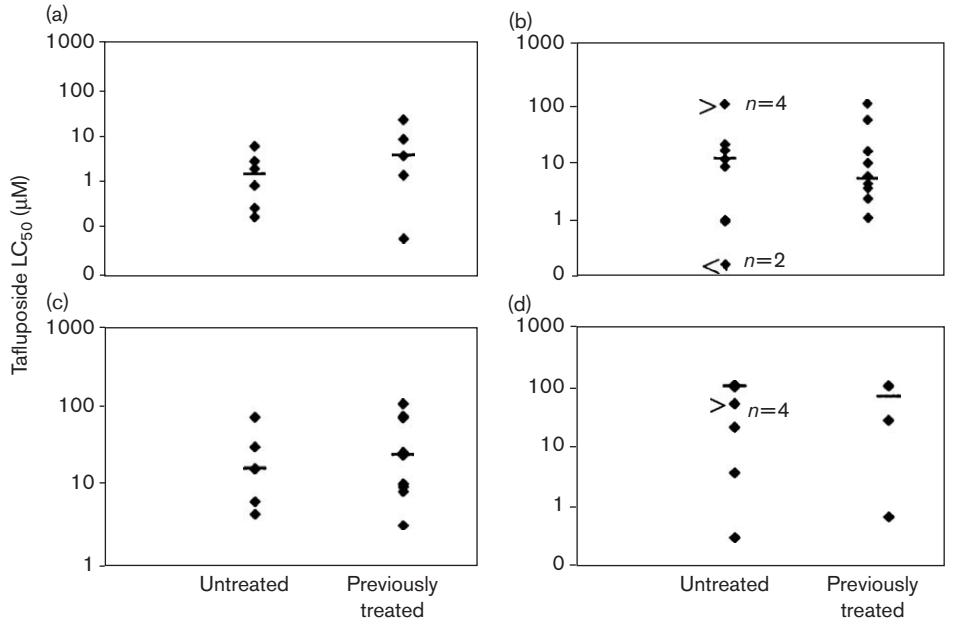
Tafluposide in combination with other anticancer agents

Tafluposide was tested in combination with other DNA-damaging agents. Seventeen samples from patients with ovarian cancer were tested against tafluposide ± cisplatin and eight samples from patients with AML were tested against tafluposide ± ara-C and tafluposide ± etoposide. The results of median effect analysis are shown in Table 4. Cases of true synergism were identified in ovarian cancer for tafluposide + cisplatin, with CIs of < 0.1 and 0.4 after a 96-h incubation and 0.14 and 0.79 after a 7-day incubation, and with tafluposide plus etoposide in AML, CIs of 0.49, 0.63 and 0.78 were recorded.

Discussion

Despite considerable advances in cancer chemotherapy over the past few decades, there remains a major need for newer cytotoxic drugs, particularly those exhibiting different and novel modes of action. Experimental results indicate that the dual catalytic topoisomerase inhibitor, tafluposide, is one such agent. Its superior preclinical activity led us to test the effect of tafluposide on tumor cells derived from samples taken from individual patients with cancer, in an attempt to accelerate its clinical development. We found significant activity in

Fig. 3



The effect of previous cytotoxic therapy on sensitivity to tafluposide in (a) ALL, (b) AML, (c) CLL and (d) ovarian cancer. Each dot represents the LC_{50} value for an individual patient. The lines represent median values.

Table 3 Cross-resistance patterns between tafluposide and other topoisomerase inhibitors

Tumor	Drug comparisons	Number tested	Spearman's correlation coefficient r_s	p
AML	Tafluposide (48 h) versus etoposide[a]	12	0.622	<0.05
AML	Tafluposide (96 h) versus etoposide	22	0.475	<0.05
AML	Tafluposide (48 h) versus topotecan[a]	7	0.536	>0.05
AML	Tafluposide (96 h) versus topotecan	16	0.359	>0.05
Ca ovary	Tafluposide (7 days) versus topotecan	15	0.385	>0.05

[a]Etoposide and topotecan were both tested after 48 h incubation throughout.

Table 4 Effect of combining tafluposide with other DNA-damaging agents

Tumor type	Tafluposide +	Incubation time	Number tested	Combination index (n)		
				<1	1	>1
AML	ara-C	96 h	7	0	1	6
	etoposide	96 h	7	3	0	4
Ca ovary	cisplatin	96 h	18	2	0	16
	cisplatin	7 days	14	2	1	11

hematological tumors particularly, the acute leukemias and also in solid tumors such as ovarian cancer. This effect varied considerably between patients, which agrees both with the situation found in the clinic and also our previous findings testing conventional cytotoxic drugs [9,13]. Indeed, we have found that interpatient variation is an essential prerequisite for successful chemosensitivity testing as, without this, effects measured could be either non-specific or outside of the appropriate drug concentration range. Overall, tafluposide appeared more active against the leukemias than ovarian cancer, but our results would suggest that it may be beneficial for certain individuals with ovarian malignancies.

The effect of tafluposide was very dependent on the length of drug exposure. The LC_{50} values for nearly every tumor tested decreased significantly with time. This agrees with previous studies of this agent showing that damage incurred after exposure to tafluposide is particu-

larly stable [14]. Furthermore, tafluposide is known to induce a low level of DNA strand breaks, including double-strand breaks. It is possible that these are then detected with difficulty by DNA repair processes and thus persist with time [6]. We would recommend that at least a 96-h drug exposure for hematological tumors and a 7-day exposure for solid tumors be employed when testing the effect of this agent in fresh tumor cells

Interestingly, there was no significant difference in the overall effect of tafluposide in cells derived from patients who had relapsed or refractory disease, and had therefore received previous chemotherapy, compared to cells from untreated patients. Of course, none of the treated patients had received treatment with tafluposide, but our overall experience of chemosensitivity testing in general has shown that cross-resistance with other cytotoxic agents is a common feature. The fact that cells from patients who have previously been treated with chemotherapy, and therefore have suffered prior DNA damage, do not appear to be resistant to tafluposide augurs well for its use as second-line chemotherapy in a variety of tumor types. Indeed in our study we found that one of the patients with ovarian cancer had been heavily pretreated yet her tumor cells remained sensitive to tafluposide. This would be consistent with earlier experimental studies in which a tafluposide-resistant P388 leukemic subline selected for resistance *in vivo* retained definite sensitivity to all the standard cytotoxic agents tested, including etoposide, with the single exception of doxorubicin [15].

The cross-resistance pattern of tafluposide with other topoisomerase inhibitors was studied *ex vivo* and whilst there appeared to be a weak correlation with the effect of the structurally related compound, etoposide, none was found for the topoisomerase I inhibitor, topotecan. Interestingly, tafluposide has been found to be less mutagenic than etoposide [6]. The DNA damage incurred using tafluposide is far less reversible compared to that found in etoposide-treated cells, suggesting that cell death would be the major outcome rather than cell cycle arrest followed by incomplete repair, then cell division leading to possible mutation. Tafluposide therefore may present an exciting alternative to etoposide in the cancer chemotherapy armament.

It has been shown previously that enhanced cell kill may be achieved by combining tafluposide with other DNA-damaging agents [2,3]. We tested the effect of combining tafluposide with cisplatin in ovarian cancer and with etoposide or ara-C in AML. Whilst an increased effect was not found for all samples tested, individual cases that displayed true synergism between tafluposide and cisplatin and tafluposide and etoposide were apparent. We have previously studied synergism between drugs in the conventional combinations used routinely to treat AML yet have rarely found true synergism, mainly additivity [16]. Therefore the fact that we have demonstrated synergism in this relatively small cohort of patients, suggests wide-ranging possibilities for including tafluposide in combination chemotherapy regimens. Our findings are consistent with previous reports combining tafluposide with cross-linking agents including cisplatin in established cell lines, both *in vitro* and *in vivo* [4].

One mechanism of drug resistance common to many different types of cytotoxic agents is the increased ability of resistant cancer cells to repair the DNA damage incurred. Several different categories of DNA repair have been identified. Tafluposide is known to be a potent inhibitor of one of these mechanisms, nucleotide excision repair (NER), and damage caused by cross-linking agents is thought to be repaired predominantly by NER [4]. In a further study by this group, clear synergism was found for a combination of tafluposide with etoposide in the A549 human non-small cell lung cancer line [3]. We found synergistic activity between the two topoisomerase inhibitors, tafluposide and etoposide, lending weight to the fact that these two epipodophylloid derivatives have a quite different mode of action. We found no synergism between tafluposide and ara-C, which is consistent with previous studies where tafluposide in combination with 5-fluorouracil led to either additivity or antagonism in human tumor cell lines [3].

Chemosensitivity testing using fresh tumor cells taken directly from patients is not used conventionally for preclinical studies at present. We believe, nevertheless, that it provides added information on the cellular sensitivity of novel agents by getting nearer to the clinical situation. Testing patients' tumor cells in short-term culture (48–96 h) does not allow mutation from the original phenotype. Moreover, chemosensitivity testing has been repeatedly shown to predict clinical outcome. For example, we have found that ovarian cancer patients treated with drugs in which *ex vivo* sensitivity was identified live twice as long as patients given drugs to which resistance was evident in laboratory tests [11]. The case for inclusion of chemosensitivity testing in preclinical drug development is strengthening rapidly and the addition of this technique could help to identify ineffective agents *before* they follow the expensive route of phase I/II clinical trials. Chemosensitivity testing lends itself to evaluating drugs *ex vivo* both singly and in combination, meaning that many permutations can be studied simultaneously in an individual patient without the need for any clinical intervention.

In conclusion, we have found that the novel epipodophylloid, tafluposide, shows significant anticancer activity in a variety of tumor types. It is particularly effective in

hematological malignancies and therefore is a strong candidate for inclusion in future clinical trials, both as a single agent and in combination with conventional cytotoxic drugs, including DNA-damaging agents.

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