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In vitro toxicity of ET-743 and aplidine, two marine-derived antineoplastics, on human bone marrow haematopoietic progenitors: comparison with the clinical results

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Abstract

Ecteinascidine-743 (ET-743) and aplidine are two marine-derived antineoplastics currently in phase II development. With the aim of evaluating whether *in vitro* haematopoietic studies can predict the toxicity of these two drugs in patients, human bone marrow (BM) samples were incubated with these drugs under conditions which mimicked the administration exposures used in the clinics. As it was observed in different cancer cell lines, ET-743 was more toxic on an equimolar basis in human hematopoietic progenitors (inhibitory concentration reducing the viability to 50% after 24 h exposures; $IC50_{24h}$: 10–50 nM) compared with doxorubicin ($IC50_{24h}$ values: 280–460 nM), used as a control anticancer drug. In contrast to the high haematotoxic effects observed for ET-743, similar IC values were obtained for aplidine ($IC50_{24h}$: 150–530 nM) compared with doxorubicin. For both ET-743 and aplidine, the megakaryocytic progenitor was the most sensitive, compared with the other haematopoietic progenitors (IC50 values were 3- to 5-fold lower in the CFU-Megs compared with the CFU-GMs). The observation that the Cmax observed in patients treated with the aplidine maximum tolerated dose (MTD) (7.1 nM) was 21–75 fold lower than the $IC50_{24h}$ value observed for the different haematopoietic progenitors is highly consistent with the lack of haematotoxicity observed in patients treated with this drug. In the case of ET-743, differences between the Cmax value corresponding to the MTD (2.6 nM) and the *in vitro* IC50 values corresponding to the different progenitors were much lower (4–19-fold), also consistent with the haematotoxicity that was observed in patients treated at recommended doses (RDs) and MTDs. Although CFU-Megs were more sensitive than CFU-GM progenitors to ET-743 *in vitro*, clinical data showed that neutropenic events were more frequent than thrombocytopenic episodes. Aiming to further improve the predictive value of *in vitro* IC values corresponding to the different haematopoietic progenitors, additional refinement parameters derived from pharmacokinetic and animal studies are proposed.

Keywords: ET-743; Aplidine; Toxicity

1. Introduction

Ecteinascidin-743 (ET-743) is a tetrahydroisoquinoline alkaloid that was isolated from the ascidian, *Ecteinascidia turbinata* [1]. Mechanistic studies have identified ET-743 as a guanine-specific alkylating agent that non-covalently binds to minor groove of duplex DNA [1,2]. ET-743 induces non-p53-dependent apoptosis and produces a cell cycle block in late S and G_2M phases [3]. ET-743 inhibits the binding of the CCAAT box factor NF-Y at pharmacologically relevant concentrations [4] and abrogates the transcriptional activation of the *MDR-1* gene by histone deacetylase inhibitors and ultraviolet (UV) light, having minimal effects on constitutive *MDR-1* transcription. [5]. Aplidine is a cyclic depsipeptide originally isolated from the Mediterranean tunicate, *Aplidium albicans* [6]. It causes cytotoxicity and G1 block independently of cellular p53 status and decreases the expression of the flt-1 receptor for the vascular endothelial growth factor (VEGF) [7,8].

ET-743 and aplidine are active *in vitro* against different solid tumour cell lines including melanoma, ovarian, renal, prostate and breast cancers. In xenograft models, significant activity was also shown in melanoma,

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non-small cell lung (NSCL), ovarian and breast cancers for ET-743 and in melanoma, non-Hodgkin's lymphoma, prostate, bladder and gastric carcinoma for aplidine [9–12]. Both ET-743 and aplidine, are currently being evaluated in phase II clinical trials.

Previous toxicology studies showed that haematological toxicity was dose-limiting in the case of ET-743 [10]. In particular, severe neutro- and thrombocytopenia were the dose-limiting toxicities (DLT) in patients treated during the phase I studies [11–17]. In contrast to this, aplidine showed minimal or no myelotoxicity in rodents and dogs (Pharmamar data on file). Moreover, patients included in phase I studies with this drug had no myelotoxicity, even at the maximum tolerated doses (MTD) [18–21]. The DLTs for aplidine were skeletal muscle toxicity characterised by cramps, pain, weakness and increased creatine-kinase with normal MB fraction, liver toxicity characterised by reversible increases in transaminases and bilirubin, flu-like syndrome and prolonged emesis.

Regarding the antitumoral activity of these drugs, objective activity was reported during the phase I development of ET-743 in breast cancer, soft-tissue carcinoma, melanoma, mesothelioma, ovarian and colorectal cancer [11–14]. More recent results corresponding to phase II studies confirmed the activity of ET-743 on breast cancer [15] and soft-tissue sarcoma [16,17]. In the case of aplidine, activity has been reported in melanoma, renal cancer, non-Hodgkin's lymphoma, colorectal and gastric cancer [18–21].

Previous *in vitro* studies with ET-743 and aplidine showed the high cytotoxicity of these compounds on a number of cancer cell lines, when compared with haematopoietic progenitors from cord blood or mobilised peripheral blood [22–24]. Aiming to evaluate the capacity of haematopoietic culture assays to predict acute neutropenia, thrombocytopenia and anaemia following administration of these marine anticancer drugs, human bone marrow (BM) samples were exposed *in vitro* to ET-743 and aplidine under conditions which mimicked the clinical administration schedules. Thereafter, the sensitivity of the granulo-macrophage (CFU-GM), erythroid (BFU-E) and megakaryocytic (CFU-Meg) progenitors was assessed after each of the incubation protocols. The comparison of these experimental results with the haematotoxicity data generated during the phase I programme demonstrates the potential, as well as current limitations, of haematopoietic culture assays to predict haematological toxicity in patients treated with anticancer drugs.

2. Materials and methods

2.1. Preparation of drugs

ET-743 and aplidine were provided by PharmaMar (Madrid, Spain) as a dry powder to be reconstituted with dimethyl sulphoxide (DMSO, Sigma Chemical Co, St. Louis, MO, USA) and diluted to a concentrated stock solution and kept in aliquots at −80 °C. Both drugs were prepared fresh before each experiment. Doxorubicin was purchased from Sigma. The source of aplidine was semi-synthetic, being synthesised in three steps from natural didemnin A [6].

2.2. Human bone marrow cells

Frozen mononuclear human bone marrow cells from four different donors (Poietic Technologies, Gaithersburg, MD, USA) were thawed and diluted 1:1 with 2.5% human albumin (Behring, Hoechst Iberica, Spain) and 5% dextran 40 (Rheomacrodex 10%; Pharmacia Biotech, Uppsala, Sweden), and maintained at room temperature for 10 min. Cells were then diluted with Iscove's modified Dulbecco's medium (IMDM;GIBCO, Grand Island, NY, USA) and centrifuged at 800*g* for 15 min. After washing, cells were dispersed in IMDM supplemented with 10% fetal bovine serum (FBS;GIBCO).

2.3. Drug exposures

After thawing, cells were washed and then exposed to ET-743 for 1, 3, 6, 8 or 24 h and to aplidine for 1 h, 24 h, 5 days (5 d) or 1 h daily for 5 days (1 h×5 d). These exposure times were chosen to mimic the clinically used infusion schedules. After incubation in IMDM plus 10% FBS at 37 °C, cells were washed, viability determined by trypan blue exclusion, and an appropriate number of cells plated on semi-solid medium for conducting clonogenic assays. For the 1 h×5 d daily experiments with aplidine, cells were incubated each day with the drug for 1 h, then washed and subsequently maintained at 37°C in fresh IMDM containing 10% FBS (GIBCO) until the next exposure 23 h later. Controls were treated in the same way, but without the addition of Aplidine. For 5 day exposures of aplidine, cultures with and without low concentrations of Haematopoietic Growth Factors (HGFs) (10 ng/ml of interleukin (IL)-3, IL-6 and scatter cell factor (SCF) and 2 U/ml of erythropoietin (EPO)) were performed. Because a similar toxicity of aplidine was observed in the presence and absence of HGFs (data not shown), experiments were routinely conducted in the absence of HGFs. In all instances, a first set of experiments was set up following a half-log drop schema in the range of 10–10 000 nM. In a second set, doses were chosen within the IC30–IC90 range in order to fine-tune the dose–response curve between critical concentrations. Doxorubicin was tested at concentrations of 1.0-, 0.1- and 0.01-fold the relevant clinical peak plasma concentrations observed (i.e. 700, 70 and 7 nM).

2.4. Clonogenic assay

After drug exposures, cells were washed and viability determined by trypan blue exclusion. An appropriate number of viable cells (50 000–100 000 cells per dish) were seeded in dishes containing MethoCult GF H4434 culture media (StemCell Technologies, Vancouver, BC, Canada) for evaluating the number of CFU-GM (myeloid progenitors), BFU-E (erythroid progenitors) and CFU-Mix (multilineage progenitors) after 14 days of incubation. For the determination of CFU-Meg (megakaryocytic progenitors), cells were grown for 11 days with Megacult C (StemCell Technologies). In all instances, cultures were maintained at 37 °C in 5% CO_2 and fully humidified air. The number of colonies (mean ± standard error (S.E.)) of untreated mononuclear BM cells was 456 ± 33 CFU-GM, 178 ± 52 BFU-E, 132 ± 4 CFU-Meg and 25 ± 3 CFU-Mix per 10^5 seeded cells. Samples giving rise to less than 100 CFU-GM per 10^5 cells were not considered for the haematotoxicity experiments.

2.5. Comparison with clinical data

The *in vitro* results were compared with the plasma concentrations and correlative haematological toxicity determined in patients during phase I clinical studies. Plasma concentrations of aplidine and ET-743 were determined using previously described methods [25,26]. Haematological toxicity was graded using standard National Cancer Institute-common toxicity criteria (NCI-CTC) criteria. Both drugs were given by intravenous (i.v.) infusion. Although several infusion schedules of ET-743 were considered in the clinical trials (1 h, 3 h [11], 24 h [12], 1 h infusion during 5 consecutive days (1 h × 5 d) [13] and 72 h [14] every 3 weeks), comparisons with *in vitro* data have been established with the 24 h infusion protocol. Aplidine was administered either 1 or 24 h weekly [21], 24 h every 2 weeks [20], or 1 h during 5 consecutive days (1 h × 5 days) every 3 weeks. Haematological dose-limiting toxicities (DLTs) were defined as grade (G) 3 or 4 anaemia, G4 thrombocytopenia, G4 neutropenia lasting for more than 5 days or complicated neutropenia. The maximum observed plasma concentration (Cmax) was derived directly from the experimental data. The exposure of patients to the drugs was assessed by calculating the area under the plasma concentration–time profile (AUC) using the linear log-trapezoidal method. The volume of distribution at steady state (Vss) and the terminal half-life were calculated using standard non-compartmental methods [27].

2.6. Statistical methods

All the results were calculated as survival percentages with respect to control cultures exposed to 0.1% DMSO (final concentration of drug vehicle in cultures). The survival data were fitted by least squares only for experiments with at least three available data points. The IC50, IC70 and IC90 were obtained algebraically, solving the fitted quadratic equation for the value of dose where the estimated percentage of surviving cells would equal 50, 30 and 10%, respectively. The estimated IC50, IC70 or IC90 (one for each experiment) were considered as dependent variables in an analysis of variance (ANOVA) model where each experiment was considered as an independent replicate. Data in Tables 1–3 show the mean ± standard error (S.E.) of ICs. Correlations between Cmax and AUC and the CTC grade of neutropenia and thrombocytopenia were assessed using Spearman's rank correlation test. The influence of ET-743 AUC categorised in 3 regions (<40, 40–70 and >70 h*mcg/l) on the incidence of G4 thrombocytopenia or long (>5 days) G4 neutropenia was assessed using the Fisher's Exact test.

3. Results

3.1. Haematotoxicity of ET-743 and aplidine versus doxorubicin in 24 h exposures

Survival data in Fig. 1 and determinations of IC values shown in Table 1 firstly evidence that ET-743 is significantly more toxic than aplidine or doxorubicin in all the haematopoietic progenitors studied ($P < 0.05$). When comparing the toxicity of these latter two drugs in the different haematopoietic progenitors, no significant differences in any of the IC values shown in Table 1 were found. When the sensitivity of the different progenitors to these compounds was compared, we observed that CFU-Meg and CFU-Mix were significantly more sensitive to aplidine compared with the CFU-GM and BFU-E progenitors ($P < 0.05$) and that CFU-Meg progenitors were particularly sensitive to ET-743 when compared with the other progenitors studied (IC50 was 3 to 5 times lower in the CFU-Meg compared with the other progenitors).

3.2. Influence of the treatment schedule of ET-743 and aplidine on the survival of the haematopoietic precursors

Data in Fig. 2 and Table 2 show the clonogenic survival data and the IC values following 1, 3, 6, 8 and 24 h incubation with increasing concentrations of ET-743. These data show that the sensitivity of CFU-GM and BFU-E progenitors to ET-743 followed a similar behaviour in most of the incubation protocols tested. In this respect, the sensitivity of both progenitors increased significantly at incubation periods of 3 h or longer with respect to the 1 h exposures, while no significant increase of sensitivity was observed between the 6 and

Table 1
IC50, IC70 and IC90 values after 24 h exposures of doxorubicin, aplidine and ET-743: comparison with clinically relevant concentrations

In vitro toxicity results	Doxorubicin	ET-743	Aplidine
IC50 (nM)			
CFU-GM	460±61	50±10[a]	530±63
BFU-E	380±22	30±5[a]	360±40
CFU-Mix	270±30	40±15[a]	150±71[b]
CFU-Meg	280±72	10±1[a,b]	150±60[b]
IC70 (nM)			
CFU-GM	684±91	80±10[a]	810±83
BFU-E	534±15	50±10[a]	563±172
CFU-Mix	493±58	72±10[a]	360±93[b]
CFU-Meg	450±20	20±7[a,b]	370±40[b]
IC90 (nM)			
CFU-GM	902±130	110±32[a]	1,086±123
BFU-E	690±17	70±15[a]	760±204
CFU-Mix	720±45	100±7[a]	580±115[b]
CFU-Meg	630±5	30±3[a,b]	730±100
Clinical results			
Administration dose (24 h infusions)	1500 μg/m^2 (RD)[c]	1800 μg/m^2 (MTD)[c]	4500 μg/m^2 (MTD)[d]
Cmax after 24 h infusion (nM)	414[c] 1379–5600[c]	2.2±1.6 (RD)[d] 2.6±1.7 (MTD)[d]	7.1±1.5 nM (MTD)[e]
% Patients with G3 neutropenia	20.0	25.0	0
% Patients with G4 neutropenia	17.8	75.0	0
% Patients with G3 thrombocytopenia	8.9	50.0	0
% Patients with G4 thrombocytopenia	4.4	50.0	0

ET-743, Ecteinascidin-743; IC50, IC70, IC90: dose-inhibiting the growth of 50, 70 and 90% of progenitors (nM), respectively; CFU-GM, granulomacrophage progenitor; BFU-E, erythroid progenitor; CFU-Mix, multilineage progenitor; CFU-Meg, megakarcyocytic progenitor; Cmax, the maximum observed plasma concentrations; G, grade.
To convert doxorubicin concentrations from nM to μg/ml multiply by 0.580. To convert ET-743 concentrations from nM to ng/ml multiply by 0.762. To convert aplidine concentrations to ng/ml multiply by 1.112.

[a] $P<0.05$ for ET-743 versus aplidine and doxorubicin.

[b] $P<0.05$ for CFU-Meg and CFU-Mix versus CFU-GM and BFU-E.

[c] After an unusual dose and schedule, 30 mg/m^2 in 24 h, included because of the easier comparison of schedules. More usual dosing (60 mg/m^2 bolus) produces a Cmax of 1379 to 5600 nM. Ref 37.

[d] Patients treated with ET-743 at clinically relevant doses (1500 or 1800 μg/m^2) of the 24 h schedule every 3 weeks (nM in plasma).

[e] Patients treated with aplidine at the maximum tolerated dose (4500 μg/m^2) of the 24 h infusion schedule every week (nM in plasma).

24 h exposures. When the CFU-Mix progenitor was considered, similar IC50 values were obtained at the different drug exposure times. However, the IC70 and IC90 after 3 h exposures were significantly higher than those after 6–24 h exposures. When the CFU-Megs were considered, it was observed that the incubation period markedly affected the survival of these progenitors. A comparison of the IC values showed significant differences ($P<0.05$) among 1, 3–6 and 8–24 h exposures, respectively.

Data in Fig. 3 and Table 3 show the clonogenic survival data and the IC values obtained after incubations ranging from 1 h to 5 days with increasing concentrations of aplidine (Table 3 and Fig. 3). When the sensitivity of the different progenitors was compared, a similar behaviour was observed between the CFU-GM and the BFU-E progenitors in most of the treatment schedules (the only significant difference between these two progenitors was observed in the IC90 value corresponding to the 5 day exposure). In the case of 1 h exposures, the sensitivity of CFU-Mix to aplidine was, similar to that observed for CFU-GM and BFU-E progenitors. However, at longer incubation periods (24 h and 5 days), CFU-Mix were more sensitive when compared with these progenitors. A significant increase in the sensitivity of all the progenitors studied was observed as a result of the increase in the incubation period from 1 h to 24 h and also from 24 h to 5 day exposures (only one exception was observed in the CFU-Meg progenitors incubated for 24 h). When 5 day exposures were compared with 1 h exposures for 5 days (1 h×5 d in Table 3), no significant differences were obtained in any case, even though the mean IC values were increased up to approximately 3-fold as a result of the fractionation of the dose.

3.3. Haematological toxicity in patients treated with ET-743 and aplidine during phase I trials

As indicated in previous reports, haematological toxicity was the DLT in all administration schedules of ET-743 [11–14]. While no severe or dose-limiting

anaemia was reported, neutropenia as well as thrombocytopenia were the main toxicities of these patients. A clear dose–response relationship for haematological toxicity was observed in these patients. In the phase I study incorporating the 24 h i.v. infusion schedule, the maximum tolerated dose (MTD) was 1800 μg/m^2 and the recommended phase II dose (RD) was 1500 μg/m^2. At the dose level below the RD (1200 μg/m^2), no cases of neutropenia greater than G2 and no thrombocytopenia were observed. At the RD, 20% of treatment courses were complicated by G3 neutropenia and 8.9% by G3 thrombocytopenia. At this dose, 17.8 and 4.4% of the courses were complicated by G4 neutropenia and G4 thrombocytopenia, respectively. When MTDs were used, 25 and 50% of treatment courses were complicated by G3 neutropenia and thrombocytopenia, respectively, and 75 and 50% of the courses were complicated by G4 neutropenia and thrombocytopenia (Table 1).

The mean plasma Cmax value found at the MTD of ET-743 was 2.6 nM. This value was close to the $IC50_{24h}$ value observed for the CFU-Meg progenitor (10 nM). When the Cmax was compared with the IC value corresponding to the CFU-GM progenitor, a larger difference between both parameters was observed (19.2-fold). A detailed comparison of patients' concentrations with *in vitro* inhibiting concentrations is provided in Table 1.

Table 2
Effect of ET-743 treatment schedule on haemopoietic precursors

Time of drug exposure (h)	CFU-GM	BFU-E	CFU-Mix	CFU-Meg
IC50: dose inhibiting the growth of 50% of progenitors (nM)				
1	220±30	120±30	40±20[c]	50±20[c]
3	120±20	40±10[a,c]	60±20	30±10[a,b,c]
6	80±20[a]	41±9[a]	50±10	30±9[a,b,c]
8	82±20[a]	40±12[a]	60±20	20±2[a,c]
24	50±10[a]	30±5[a]	40±15	10±1[a,c]
IC70: dose inhibiting the growth of 70% of progenitors (nM)				
1	360±50	210±50	190±50[c]	50±15[c]
3	170±30	90±30[a,c]	100±30[c]	40±10[a,b,c]
6	110±20[a]	70±20[a]	70±20[a]	33±11[a,c]
8	120±30[a]	80±30[a]	90±25[a]	23±8[a,c]
24	80±10[a]	50±10[a]	72±10[a]	20±7[a,c]
IC90: dose inhibiting the growth of 90% of progenitors (nM).				
1	510±133	310±15	320±40[c]	90±3[c]
3	250±66[a]	150±42[a]	143±64[a]	60±3[a,b,c]
6	150±49[a]	100±18[a]	90±18[a]	43±6[a,c]
8	163±78[a]	123±58[a]	130±56[a]	36±6[a,c]
24	110±32[a]	70±15[a]	100±7[a]	30±3[a,c]

ET-743, Ecteinascidin-743; IC50, IC70, IC90: dose-inhibiting the growth of 50, 70 and 90% of progenitors (nM), respectively; CFU-GM, granulo-macrophage progenitor; BFU-E, erythroid progenitor; CFU-Mix, multilineage progenitor; CFU-Meg, megakarcyocytic progenitor

[a] $P<0.05$ for 3–24 h versus 1 h.
[b] $P<0.05$ for 3–8 h exposure versus 24 h.
[c] $P<0.05$ for BFU-E, CFU-Meg or CFU-Mix versus CFU-GM.

In contrast to observations made in patients treated with ET-743, haematological toxicity has not been reported in aplidine phase I trials. In the study with the weekly 24 h i.v. infusion, 4500 μg/m^2/week has been defined as the MTD, muscular and liver toxicity being the dose-limiting factors. In the study with the bi-weekly 24 h i.v. infusion, a MTD of 6000 μg/m^2/2 weeks has been defined due to muscular toxicity, although further dose escalation under L-carnitine protection proved to be feasible with a new MTD of 8000 μg/m^2. In the trial involving weekly, 24 h infusions, the Cmax observed in patients exposed to the MTD was 7.1 nM; a value which is 21–75-fold lower than the $IC\ 50_{24h}$ values observed for the different haematopoietic progenitors tested in this study.

4. Discussion

The aim of this study was to evaluate the capacity of *in vitro* haematopoietic cultures to predict the human haematotoxicity of ET-743 and aplidine, two different marine-derived anticancer drugs. Aiming to amplify the scope of application of haematopoietic culture assays in human haematotoxicity, cultures allowing the growth of granulo-macrophage, erythroid and megakaryocytic lineages were set up to determine the sensitivity of their corresponding progenitors to these compounds.

Table 3
Effect of aplidine treatment schedule on haemopoietic precursors

Time of drug exposure	CFU-GM	BFU-E	CFU-Mix	CFU-Meg
IC50: dose inhibiting the growth of 50% of progenitors (nM)				
1 h	1180±285	960±223	496±44	310±55[c]
24 h	530±63[a]	360±40[a]	150±71[a,c]	150±60[c]
5 days	120±8[a,b]	80±25[a,b]	50±20[a,b,c]	70±20[a,b,c]
1 h×5 days	130±38[a,b]	170±35[a,b]	160±52[a]	60±18[a,b,c]
IC70: dose inhibiting the growth of 70% of progenitors(nM)				
1 h	1690±325	1420±200	1050±186	655±135[c]
24 h	810±83[a]	563±172[a]	360±93[a,c]	370±40[a,c]
5 days	183±13[a,b]	105±15[a,b]	75±25[a,b,c]	95±35[a,b,c]
1 h×5 days	260±37[a,b]	260±40[a,b]	230±45[a]	110±5[a,b,c]
IC90: dose inhibiting the growth of 90% of progenitors (nM)				
1 h	2210±371	1890±190	1620±407	1510±15[c]
24 h	1,086±123[a]	760±204[a]	580±115[a,c]	730±100[a]
5 days	250±20[a,b]	140±5[a,b,c]	120±15[a,b,c]	130±40[a,b,c]
1 h ×5 days	400±53[a,b]	355±45[a]	303±38[a,b]	220±5[a,b,c]

ET-743, Ecteinascidin-743; IC50, IC70, IC90: dose-inhibiting the growth of 50, 70 and 90% of progenitors (nM), respectively; CFU-GM, granulo-macrophage progenitor; BFU-E, erythroid progenitor; CFU-Mix, multilineage progenitor; CFU-Meg, megakarcyocytic progenitor

[a] $P<0.05$ for 24 h, 5 days or 1 h×5 days versus 1 h exposure.
[b] $P<0.05$ for 5 days and 1 h×5 days versus 24 h exposure.
[c] $P<0.05$ for BFU-E, CFU-Meg or CFU-Mix versus CFU-GM.

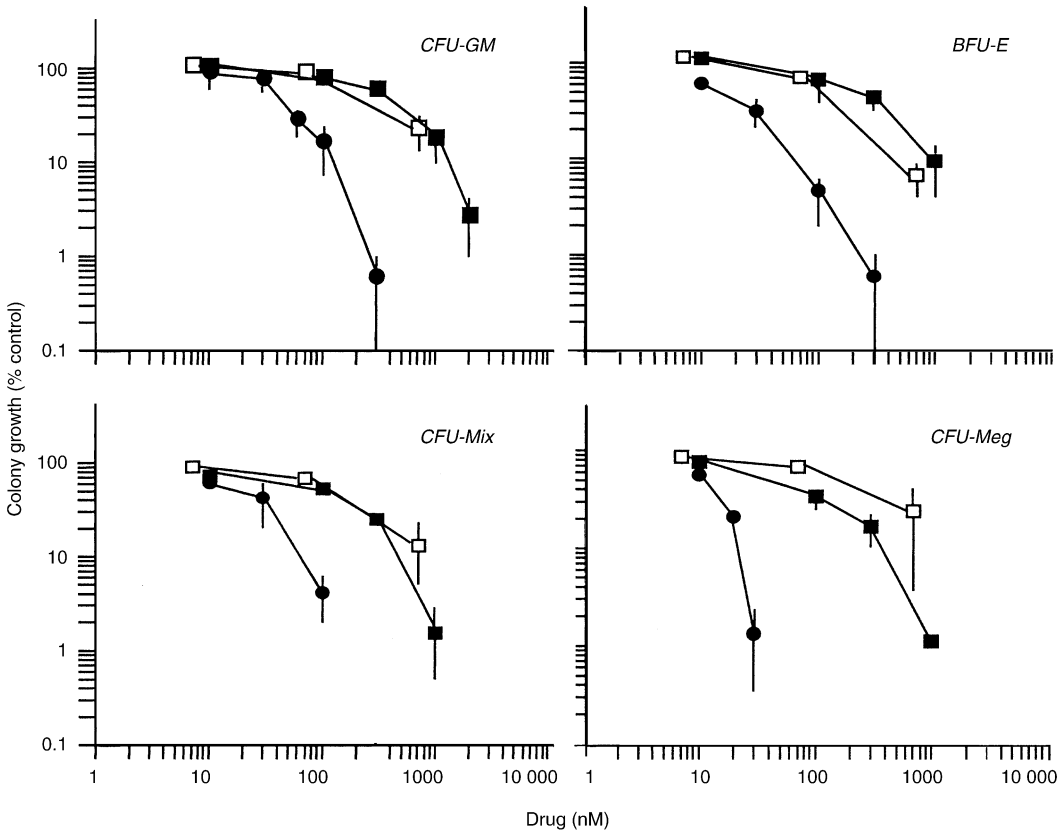

Fig. 1. *In vitro* colony growth inhibition of haematopoietic progenitors after 24 h exposure to Ecteinascidin-743 (ET-743) (●), aplidine (■) and doxorubicin (□).

Although it is traditional to use IC50 values to report the results of toxicity studies, there are data indicating that the IC50 does not predict clinically important reductions in the haematopoietic function of patients [28,29]. Some *in vitro* studies have proposed that IC70–IC75 values predict more closely the appearance of G3 neutropenia. In this respect, IC70 was a predictive endpoint for clinical neutropenia for carzelesin, tallimustine and melphalan [30]. However, from the carzelestin study in humans [28], it was apparent that the exposure level that inhibited CFU-GM progenitors by 90% *in vitro* was similar to the dose that decreased the absolute neutrophil count (ANC) by 90% *in vivo*. The importance of the IC90 value has been confirmed in studies related to the camptothecin family of anticancer drugs [29] and to a thichothecene-derived mycotoxin called DAS [31]. Hence, the present work reports IC50, IC70 and IC90 values to facilitate the prediction of the haematotoxic effects of ET-743 and aplidine in patients.

When comparing ET-743 IC50 values corresponding to CFU-GM, BFU-E and CFU-Mix progenitors (range: 30–220 nM at 1–24 h exposures) with a number of cancer cell lines, the results showed that normal haematopoietic progenitors are more resistant to this drug. This can be applied to primary cells from head and neck cancer, mesothelioma, ovarian and sarcoma tumour cells, and also for a number of human cancer cell lines, whose IC50s are in the range 0.1–10 nM, after equivalent exposure periods [32,33]. In contrast to the sensitivity of the above mentioned progenitors, IC50 values corresponding to CFU-Meg progenitors (10–50 nM at 1–24 h exposures) are in the same range of those corresponding to some human cancers, suggesting that recommended anticancer doses of this drug may be associated with thrombocytopenic events.

Compared with other anticancer drugs, the toxicity of ET-743 on different human cancer cell lines showed that ET-743 reached a similar antiproliferative activity at molar concentrations which were 1–3 orders of magnitude lower than those corresponding to paclitaxel, camptothecin, doxorubicin, mitomycin C, cisplatin, bleomycin and etoposide [15]. When comparisons of toxicity on human haematopoietic progenitors are made, ET-743 showed a similar toxicity compared with paclitaxel [34,35], and only about 1 order of magnitude higher compared with doxorubicin and melphalan. Thus, according to this *in vitro* data, a positive therapeutic index can be predicted for ET-743.

Regarding the influence that the exposure schedule of ET-743 has on the target cells, earlier studies have suggested the relevance of long tissue exposures for improving the toxic effects of the drug in cancer cells

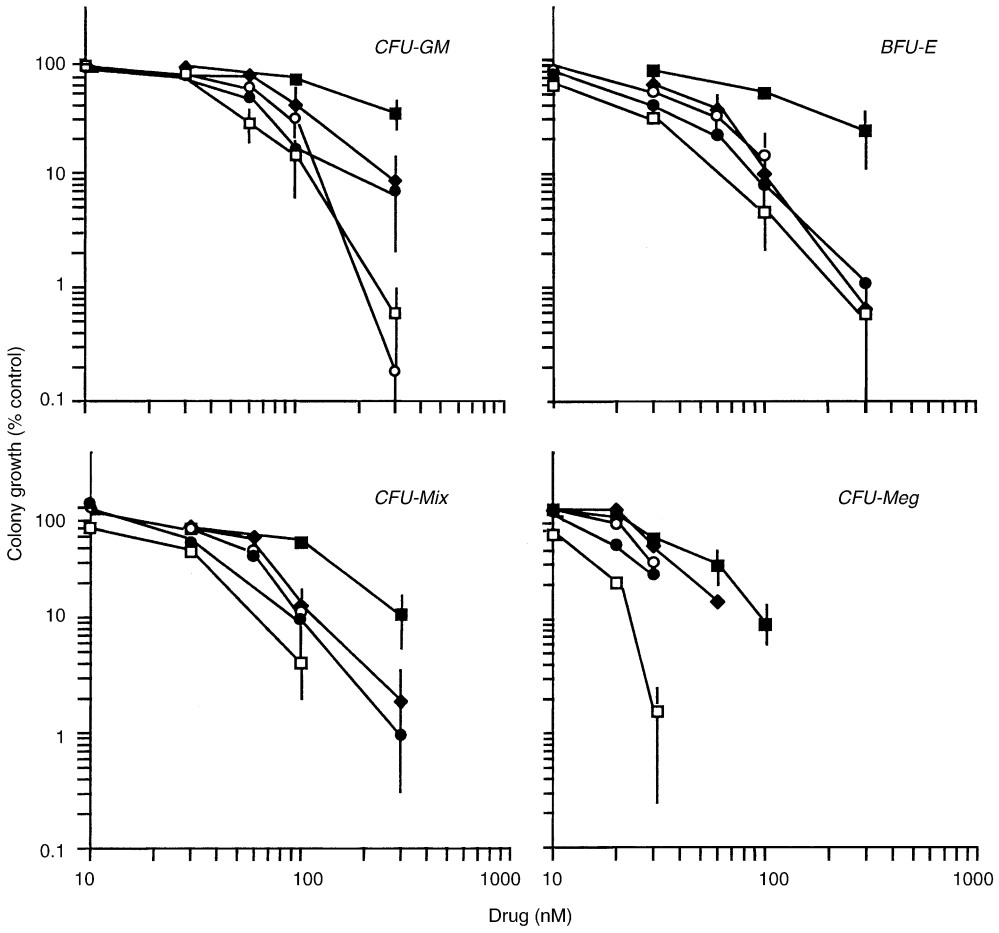

Fig. 2. *In vitro* colony growth inhibition of hematopoietic progenitors after different times of exposure to Ecteinascidin-743 (ET-743) (1 h: ■; 3h: ◆; 6 h: ○; 8 h: ●; 24 h: □).

[36]. According to our data, a similar effect was observed in the BM progenitors, mainly for exposures longer than 3 h when compared with 1 h exposures. This observation is consistent with earlier results obtained in granulomacrophage and erythroid progenitors from cord blood [24], suggesting a generalised increased toxicity of the drug when prolonged exposures are considered.

When *in vitro* data corresponding to aplidine are considered, a first observation is deduced from the comparison of the sensitivity of the haematopoietic progenitors with respect to a number of cancer cell lines. Exposures to concentrations of aplidine $\geqslant 500$ nM for 1 h were capable of completely inhibiting colony formation of cancer cells [23], whereas this concentration was below the $IC50_{24h}$ values corresponding to CFU-GM and BFU-E progenitors (1180 and 960 nM, respectively) and was between the IC50 and IC70 values corresponding to the CFU-Meg progenitor (310 and 655 nM for 1 h exposures). From the analysis of median reductions of tumour colony formation, it was inferred that aplidine is active at concentrations >10 nM [23]; a concentration that was almost not toxic to the CFU-Meg progenitors. These observations suggest that if such differences in sensitivity between the cancer cells and normal BM progenitors are maintained *in vivo*, no clinically relevant haematotoxic effects would be predicted for aplidine used at the recommended doses.

As was observed for ET-743, increasing the exposure of aplidine resulted in a generalised increase in the haematotoxic effects of the drug. However, modifying the 5 day incubation period for five repetitive short exposures of the drug implied a generalised reduction of the drug toxicity, which, however, could not be considered significant in any tested progenitor.

When *in vitro* IC values corresponding to the different haematopoietic progenitors are compared with plasma Cmax values corresponding to the MTD in patients treated with these anticancer drugs, a number of conclusions are raised. Firstly, our *in vitro* data clearly show a marked difference on the haematotoxicity of Aplidine and ET-743; differences which were essentially reproduced in the clinics (see Table 1). Regarding aplidine haematotoxicity, it is of significance that the plasma Cmax value corresponding to the MTD was 7.1 nM (24 h infusions); a dose that was incapable of inducing

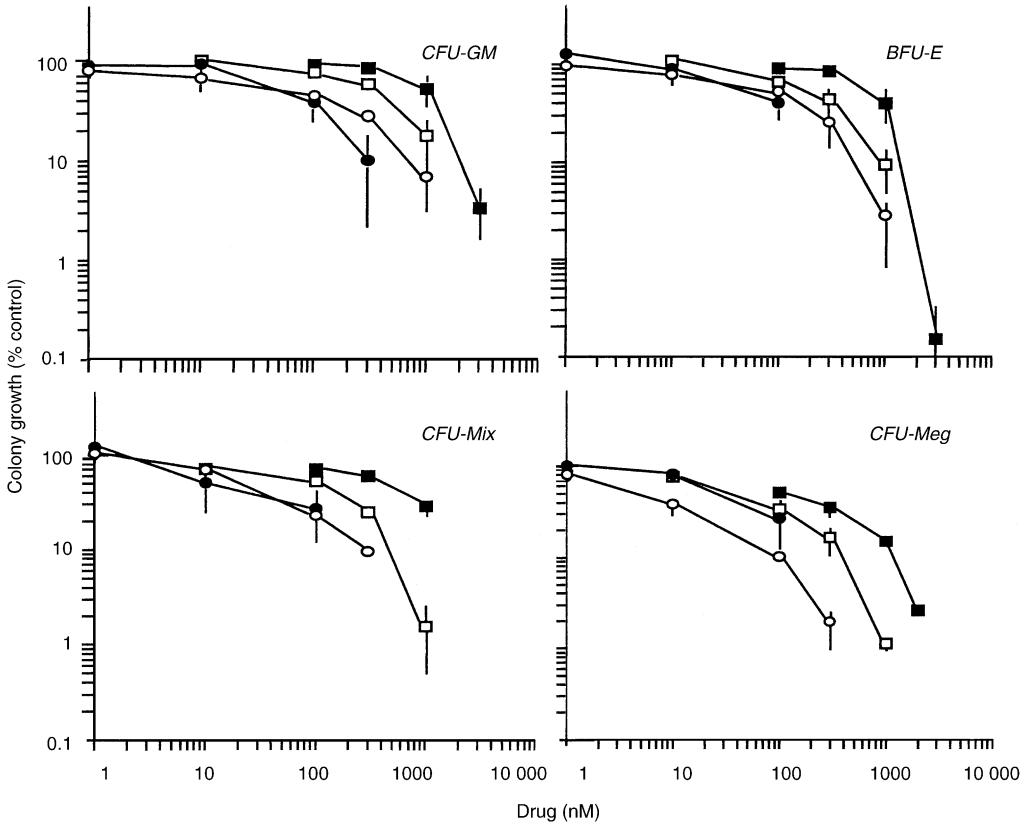

Fig. 3. *In vitro* colony growth inhibition of haematopoietic progenitors after different times of exposure to aplidine (1 h: ■; 24 h: □; 5 days: ●; 1 h daily for 5 days: ○).

significant inhibition in the tested haematopoietic progenitors (IC50 values ranged from 150 to 530 nM; see Tables 1 and 3, and Fig. 3). This observation is highly consistent with the clinical observation that the MTD of aplidine did not induce any G3 neutropenia nor G3 thrombocytopenia in patients in the phase I trial.

The mean plasma Cmax value corresponding to patients treated with the MTD of ET-743 was 2.6 nM after 24 h infusions. As was shown in Table 1, a very similar Cmax (2.2 nM) was observed for the RD of ET-743, indicating that very small changes in the Cmax resulted in marked differences in toxicity *in vivo*, it was also the case *in vitro* (see Figs. 1 and 2). Given that these Cmax values are similar to the one corresponding at the Aplidine MTD—while much lower IC values characterised ET-743 versus aplidine *in vitro*—then, it may be forecast that there is a risk of neutropenia after treatment with the recommended doses of ET-743. It is of interest, however, that if the CFU-GM IC50 value (50 nM at 24 h exposure) is considered for neutropenia prediction, clinically relevant neutropenias would have been expected at Cmax values about 10 times higher compared with the actual value observed in the patients. Regarding the megakaryocytic lineage, the *in vitro* CFU-Meg IC50 value (10 nM) was close to the Cmax corresponding to the ET-743 MTD. Since significant thrombocytopenic events were observed in these patients, it can be proposed that the CFU-Meg $IC50_{24h}$ value can be considered a good predictor for the occurrence of thrombocytopenias in patients.

In this context, however, it should be considered that ET-743 is characterized by a very high distribution volume (mean at the MTD of the 24 h infusion 5139 l/m^2) suggesting higher extravascular concentrations compared to plasma values. Although IC values corresponding to ET-743 were significantly lower than those corresponding to doxorubicin, much higher concentrations of doxorubicin should be reached in patients (Cmax about 414 nM after 30 mg/m^2 in 24 h infusion, 1379 to 5600 nM after 60 mg/m^2 in bolus [37]. As was observed for the Cmax concentrations, the correlation between the ET-743 AUC values and the grade of thrombocytopenia was better than those for neutropenia (data not shown), as expected from the sharp concentration–response relationship for CFU-Meg inhibition. However, from the *in vitro* results, thrombocytopenia would be expected to be more important than neutropenia, and human results showed the opposite. This is probably due to the different bloodstream entry and exit kinetics of neutrophils and platelets. The half-life of leucocytes in peripheral blood has been reported to be 7 h [38] compared with reported half-lives for platelets in normal subjects of 5.1 ± 0.5 days [39]. The kinetics of cell entry and exit in the bloodstream has

been shown to be important for the successful modelling of leucopenia and neutropenia induced by paclitaxel or etoposide by Minami and colleagues [38]. Differences in the kinetics of generation of white and red blood cells should also account for the absence of significant anaemia in patients treated with ET-743, even though the CFU-GM and BFU-E sensitivity to this drug was similar *in vitro* (see Tables 1 and 2).

Taken together, our results indicate the potential of *in vitro* haematopoietic assays to predict the appearance of clinical BM depression in patients treated with therapeutic doses of anticancer drugs, in particular of marine anticancer drugs, and specifically of marine anticancer drugs like ET-743 and aplidine. The *in vitro* analyses predicted the lack of clinical haematotoxicity of aplidine, even at the MTD and suggested occurrence of haematotoxic events as a result of the administration of therapeutic doses of ET-743. Of significance, however, is also the observation that according to our *in vitro* studies, thrombocytopenias would have been the main limiting event for ET-743 exposures, while episodes of neutropenia were more frequent than thrombocytopenic events in ET-743-treated patients. This observation indicates the relevance of conducting additional studies to further improve the predictive value of simple IC determinations *in vitro*. Either the inclusion of *in vivo* MTD data from experimental animals [40] or the introduction of IC correction values derived from pharmacokinetic analyses—like AUC determinations or analyses of tissue distribution and protein binding—should be considered in this respect.

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