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Cytotoxicity and neurocytotoxicity of new marine anticancer agents evaluated using in vitro assays

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Abstract *Purpose*: New classes of anticancer drugs, isolated from marine organisms, have been shown to possess cytotoxic activity against multiple tumor types. Aplidine, didemnin B, and isohomohalichondrin B (IHB), among the more promising antitumor candidates, have been evaluated in the present study on a comparative basis in terms of their antiproliferative activity and neurotoxic effects in vitro. *Methods*: Using a panel of different human, prostatic cancer cell lines (DU 145, PC-3 and LNCaP-FGC) the effects of Aplidine, didemnin B, and IHB on tumor cell proliferation were tested in a colorimetric (XTT) assay and compared with the effects of vincristine, vinorelbine, and Taxol. Under analogous in vitro conditions these drugs were also monitored for neurocytotoxic effects using a PC 12 cell line based model. *Results*: Didemnin B and – especially – Aplidine were more effective in the inhibition of prostate cancer cell proliferation than vincristine, vinorelbine or Taxol at concentration levels between 5 and 50 pmol/ml. At these same concentrations, however, Didemnin B and Aplidine were also most potent in the in vitro neurotoxicity assays. IHB was found to exert even more potent antiproliferative activity (at concentration levels between 0.05 and 0.1 pmol/ml). However, neurotoxic effects were also found to be present at these levels. After drug withdrawal, the neurotoxic damage, inflicted by aplidine or IHB appeared to be more long lasting than after vincristine or vinorelbine exposure. *Conclusions*: These results point to high antiproliferative activity of aplidine and IHB in prostate cancer. At the same time, the data urge some caution in the clinical use of these agents because of potential neurotoxic side-effects. The use of a newly formulated Aplidine may involve a more favorable therapeutic profile.

Key words Aplidine · Didemnin B · Isohomohalichondrin B (IHB) · Neurocytotoxicity · Cytotoxicity

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Introduction

A family of naturally occurring cyclodepsipeptides called didemnins has been isolated from marine tunicates and identified as a class of highly potent anticancer agents [17]. From this family, didemnin B has been tested in phase I and phase II studies for a number of tumors including prostate cancer [29], non-small cell lung cancer [22], myeloma [28], and melanoma [22]. Considerable neuromuscular toxicity has been described as a side-effect of didemnin B in the clinical setting [22, 23]. The mechanism of action of this group of compounds appears to be related to their inhibition of protein synthesis, and to a lesser extent also of DNA and RNA synthesis [13, 25, 27]. More recently, a depsipeptide was isolated from the Mediterranean tunicate, *Aplidium albicans*, chemically classified as dehydrodidemnin B and later called Aplidine. The preclinical findings for Aplidine point to a potentially high anticancer activity against an array of different, rapidly proliferating tumor types [3, 14, 19, 27]. The possible occurrence of neurotoxic side effects is not yet known.

Another marine organism, the sponge *Lissodendoryux* spp. was reported to contain the potent isohomohalichondrin B (IHB), with activity against lung cancer and melanoma cell lines [11]. This compound

appeared to disorganize the microtubule network by preventing microtubule assembly and glutamic pyruvic transaminase (GTP) binding to tubulin [5]. On the basis of this microtubule interaction, neurotoxicity as a potential side-effect can be anticipated. Therefore, a careful establishment of cytotoxicity and neurocytotoxicity using standardized endpoints under well-controlled conditions seems to be warranted to evaluate the therapeutic potential versus side-effects of both Aplidine and IHB.

We have previously established an in vitro assay for monitoring neurotoxicity of a variety of cytostatic compounds using a pheochromocytoma cell line [6]. This assay has been used to show the differential effects of the various clinically used vinca alkaloids and the possibility of reversibility of neurotoxic effects [9]. The present paper aims at determining in vitro antitumor cytotoxicity using a panel of different human prostate tumor cell lines and comparing antitumor activity with in vitro neurocytotoxicity under identical, standardized in vitro conditions. The experimental drugs are compared with a set of antitumor agents known to display a range of neurotoxic activity in clinical practice.

Materials and methods

Tumor cell lines used and culture conditions

Prostate tumor cell lines (three human derived lines) were used to measure the antitumor cytotoxicity of the drugs. To establish in vitro neurotoxicity a rat pheochromocytoma cell line (PC12) was used. The following three human prostatic cancer cell lines were obtained from the American Type Culture Collection, Rockville, Md., USA:

1. LNCaP-FGC (ATCC# CRL 1740), used: passage 21–80
2. DU-145 (ATCC# HTB 81), used: passage 62–110
3. PC-3 (ATCC# CRL 1435), used: passage 28–75

PC12 rat pheochromocytoma cells were obtained from American Tissue Type Culture Collection (ATCC# CRL 1721). Cell culture conditions have been described earlier [7]. In short, all the tumor cells mentioned above were cultured using RPMI 1640 medium (Gibco BRL, Life Technologies, Gaithersburg, Md., USA), supplemented with 10% fetal calf serum (Integro, Zaandam, The Netherlands), 100 U/ml penicillin/streptomycin (Gibco BRL), 1 m*M* sodium pyruvate (Gibco BRL), and insulin/transferrin/selenite medium supplement (Sigma Chemicals, St. Louis, Mo., USA), at 37 °C in a humidified atmosphere of 5% CO_2/95% air.

Cytostatic drugs

- Aplidine (Dehydrodidemnin B), Didemnin B, and IHB were kindly provided by PharmaMar through the New Drug Development Office (NDDO) of the EORTC. Aplidine (Dehydrodidemnin B) and Didemnin B were dissolved in ethanol absolute (1 mg/ml) and stored in the dark at 4 °C until use. Further dilution was performed in phosphate-buffered salt solution (PBS: 0.12 *M* NaCl/10 m*M* Na_2HPO_4/3 m*M* KH_2PO_4 at pH = 7.4) before addition to the culture medium. Unless otherwise specified, this batch was used as the Aplidine source in the experiments described here under. In two experiments a newly formulated form of Aplidine* [16] was used: 1 mg dissolved in the accompanying 2-ml solution (containing cremophor/ethanol/water for injection). Further dilutions were prepared in PBS immediately before the experiments. IHB was dissolved in ethanol absolute (2 mg/ml) and stored in the dark at 4 °C until use.
- Paclitaxel: Taxol: 2 mg/ml ethanol (Bristol-Myers Squibb, New York, NY, USA)
- Vincristine: Vincristine-sulfate 1 mg/ml (TEVA Pharmaceutical Industries, Netanya, Israel)
- Vinorelbine: Navelbine 50 mg/5 ml (Pierre Fabre Oncologie Laboratories, Boulogne, France)

Drug effect on in vitro prostate tumor cell proliferation: XTT proliferation assay

The effect of the drugs on human prostate tumor cell proliferation was investigated using a commercially available proliferation kit (XTT II, Boehringer Mannheim, Mannheim, Germany). Briefly, the cells were plated in 96-well culture plates (Greiner) at a density of 10 000 cells (LNCaP-FGC); 750 cells (PC-3) or 500 cells (DU-145), respectively, per well in 100-µl RPMI culture medium and allowed to attach for 2 h. Drugs were added to various final concentrations (and control: 0 pmol/ml) in triplicates. After 72 h of culture, 50 µl of XTT reaction solution (sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate and N-methyl dibenzopyrazine methyl sulfate; mixed in proportion 50:1) was added to the wells. The optical density was read at 450 nm wavelength in an ELISA plate reader after 4 h incubation of the plates with XTT in an incubator (37 °C and 5% CO_2 + 95% air). All determinations were confirmed using replication in at least three identical experiments. The data shown are for only one experiment, but representative for all replications. Inter-assay variation (CV), for measurement of control culture wells without drug addition, was 4.90% for DU-145 cells, 1.86% for PC-3 cells, and 2.41% for LNCaP-FGC cells, respectively.

In vitro neurotoxicity effects: nerve growth factor-induced neurite outgrowth assay

The nerve growth factor (NGF)-induced neurite outgrowth assay was used as described before [6]. In short, PC12 cells are pretreated for 8 days by adding β-NGF, 2.5S (murine, #G5141, Promega, Madison, Wis., USA) to a final concentration of 50 ng/ml, trypsinized, washed using RPMI 1640 medium and plated in 12-well culture chambers (Costar, Nuclepore). Optimal adherence and neurite formation required pre-coating the culture well plastic using a 0.5-mg/ml water solution of polylysine-hydrobromide (P2636, Sigma, St Louis), followed by washing in saline [6]. After 3 days of culture in the presence of NGF (10 ng/ml), the percentage of neurite-forming cells (neurite length > 1 × cell body length) is scored in triplicate wells using a phase contrast microscope.

Interference by cytostatic drug with neurite outgrowth was measured by adding the drugs to various final concentrations. To control wells (“0 pmol/ml”) PBS solution was added. All incubations were performed in triplicate. After 72 h of culture, in each well 100 cells were randomly chosen and scored for both short (< 2 × cell body) and for long neurites (> 2 × cell body). The results are given as percentage of cells expressing neurites (both short and long) or as percentage of cells expressing long neurites only (> 2 × cell body). All determinations were confirmed using replication in at least three identical experiments. The data shown are for only one experiment, but representative for all replications. In order to compare drug effects on established neurites with effects on developing neurites, series of experiments were performed after neurite establishment (48 h culture in presence of NGF followed by 48 h incubation with drugs). The pattern of drug-induced neurite reduction (data not shown) was completely comparable with the effects shown in the present experiments. Inter-assay variation (CV), for determination of neurite-forming cell numbers in control wells without drug addition, was 2.45% for all neurites and 1.89% for long neurites only.

Reversibility of in vitro neurotoxicity: drug withdrawal assay

The procedure described above was adapted to measure recovery of neurite formation after drug treatment and has been described before [9]. In short, (pretreated) PC12 cells were plated in the presence of 10 ng NGF/ml in polylysine-hydrobromide-coated culture wells and incubated with various drug concentrations (and control: "0 pmol/ml") for 48 h. After the incubation, the drug-containing medium (respectively the control medium) was removed and the cells were washed by a 10-min incubation at room temperature in fresh RPMI 1640 culture medium. After removal of this medium shift, new RPMI 1640 medium was added, containing NGF (10 ng/ml) and the culture were incubated at 37 °C (5% CO_2) for another 2 days. The percentage of neurite-forming cells was determined before changing the medium, 24 h and 48 h later.

Statistical analysis

Experimental results from XTT proliferation assays were analyzed using one-way Analysis of Variance at $P < 0.05$ (Bonferroni-corrected). The results of drug effects in neurite outgrowth assays were analyzed using (χ^2 test (two-tailed) at a $P = 0.05$ significance level.

Results

In vitro general cytotoxicity

The cytotoxic effects of Aplidine, Didemnin B, vincristine, vinorelbine, and Taxol at various concentrations were evaluated in a standardized in vitro cytotoxicity assay against the following human prostate cancer cell lines: PC-3; DU-145 and LNCaP-FGC (cultured in RPMI-1640 culture medium). Drug concentrations of 0, 5, 25 pmol/ml, and 50 pmol/ml were incubated for 72 h in cultures in microtiter culture plates in six-fold (volume 100 µl/well). After 72 h the number of cells surviving was determined quantitatively using a colorimetric XTT assay in a microtiter plate reader (Fig. 1).

Potent antiproliferative activity of Aplidine and Didemnin B was recorded against all three prostate cancer cell lines tested. Analysis using one-way Analysis of Variance (ANOVA) showed that overall treatment effect by different concentrations of all cytostatic drugs was extremely significant for all three human prostate cancer cell lines tested ($P < 0.0001$). Analysis using ANOVA post-tests showed that at 5 pmol Aplidine/ml, the OD450 values (reflecting cell survival after drug incubation) were significantly lower than after incubation with 5 pmol/ml Didemnin B for DU-145 cells ($P < 0.001$) and for PC-3 cells ($P < 0.05$). Only in the LNCaP-FGC cell line there was no significant difference in effect at this concentration between Aplidine and Didemnin B. For the other drugs tested, the prostate tumor cell survival was significantly higher than after Aplidine incubation at this concentration ($P < 0.001$). IHB cytotoxic effect at various concentrations was evaluated in the standardized in vitro cytotoxicity assay against the human prostate cancer cell lines PC-3, DU-145, and LNCaP-FGC. Drug concentrations of 0, 0.045, 0.09, 0.14, 0.18, 0.22, 0.45 pmol/ml and 0.9 pmol/ml were included for 72 h in cultures in microtiter culture plates (volume 100 µl/well). After 72 h the cell survival was determined quantitatively using a colorimetric XTT assay in a microtiter plate reader ($n = 6$; see Fig. 2).

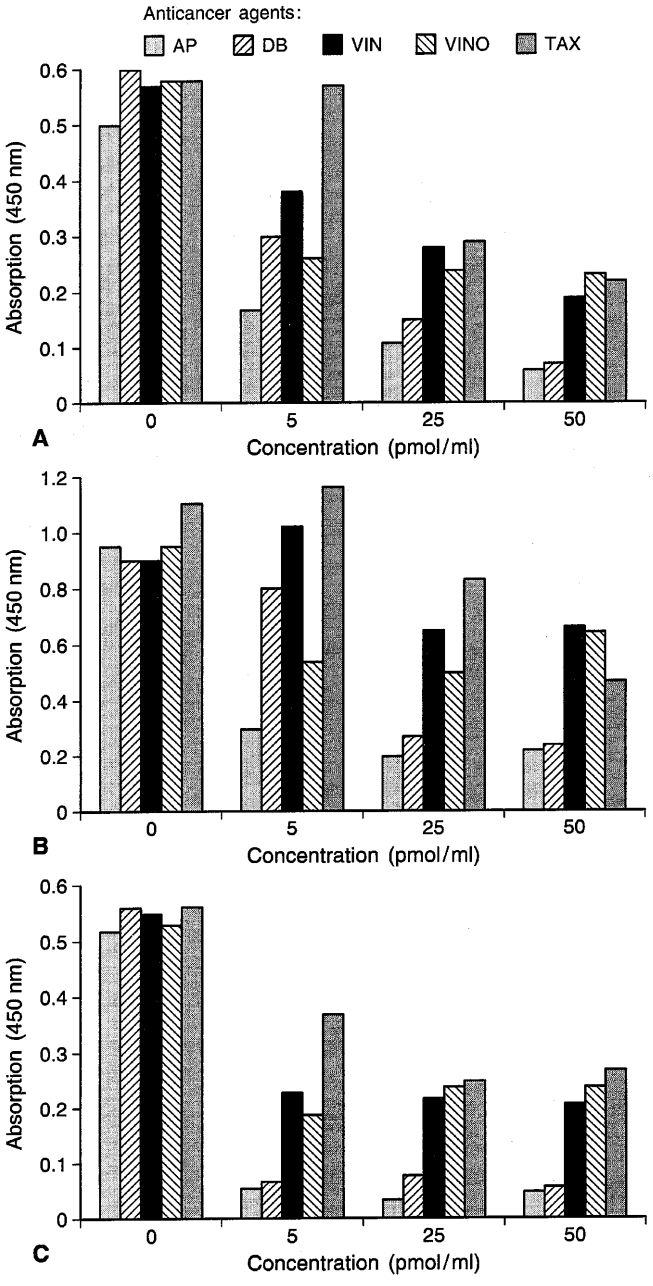

Fig. 1 Inhibition of proliferative activity by Aplidine (*AP*) and Didemnin B (*DB*) was determined against three prostate cancer cell lines: PC-3 (**A**), DU-145 (**B**) and LNCaP-FGC (**C**) using colorimetric XTT assay. *VIN* vincristine, *VINO* vinorelbine, *TAX* taxol

ANOVA overall analysis of these results shows significant treatment effects of increasing doses of IHB for DU-145 and LNCaP-FGC prostate tumor cell lines ($P < 0.0001$) and for PC-3 prostate tumor cells ($P = 0.0033$). Extremely significant (ANOVA post-test; Bonferroni P value < 0.001) effects of doses of IHB as

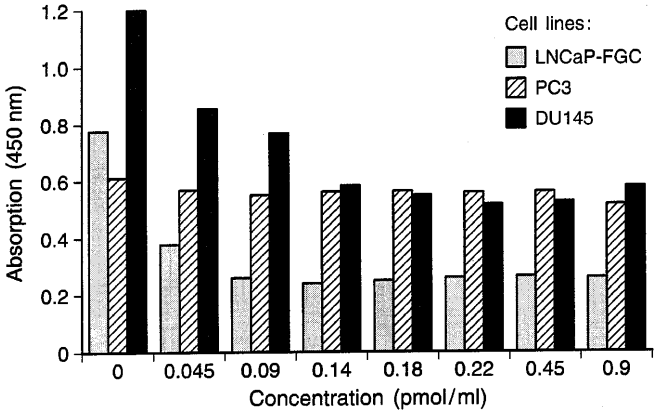

Fig. 2 Effect of isohomohalichondrin B on proliferation of human prostate tumor cell lines PC-3, DU-145 and LNCaP-FGC, measured using colorimetric XTT assay

small as 0.22 pmol/ml were observed for the DU-145 and LNCaP-FGC human prostate cancer cell lines tested.

In vitro neurotoxicity

An in vitro neurotoxicity assay measuring NGF-dependent neurite-outgrowth from the PC12 pheochromocytoma cell line was used to predict neurotoxicity after 72 h incubation with Aplidine, Didemnin B, vincristine, vinorelbine, and Taxol at various concentrations (0, 5, 25 pmol/ml; and 50 pmol/ml) in three-fold. Potent in vitro neurotoxicity of Aplidine and Didemnin B was demonstrated (Fig. 3). Analysis using the χ^2 test showed extremely significant (two-tailed $P < 0.00001$) overall treatment effect by the different drug concentrations. Analysis using Fisher's exact test for 2×2 tables (χ^2) for neurites of all sizes showed that the difference between control treatment and drug treatment at 5 pmol/ml was significant for Aplidine only (two-tailed P value 0.00089). For the other drugs no significant effects at this concentration were shown. For long neurites only, treatment effects were significant for all drugs tested at 5 pmol/ml concentration (Aplidine: $P = 0.00001$; Didemnin B: $P = 0.00002$; vincristine: $P = 0.00021$; vinorelbine: $P = 0.04555$; Taxol: $P = 0.14595$). At 5 pmol/ml and 25 pmol/ml the aplidine effect was significantly stronger than the Didemnin B effect $P = 0.03785$ and $P = 0.02456$, respectively).

Effects of IHB were also measured in the PC12 neurite outgrowth assay at drug doses 0 (control), 0.045, 0.09, 0.14, 0.18, 0.22, 0.45 pmol/ml, and 0.9 pmol/ml ($n = 3$). Results after 72 h incubation indicated potent in vitro neurotoxicity of IHB (Fig. 4). Analysis using Fisher's exact test for 2×2 tables (χ^2 test) indicated a significant treatment effect of IHB on all "neurites" ($P < 0.00001$) at a concentration of 0.045 ng/ml. This IHB concentration also yielded an extremely significant effect ($P < 0.00001$) on "long neurites".

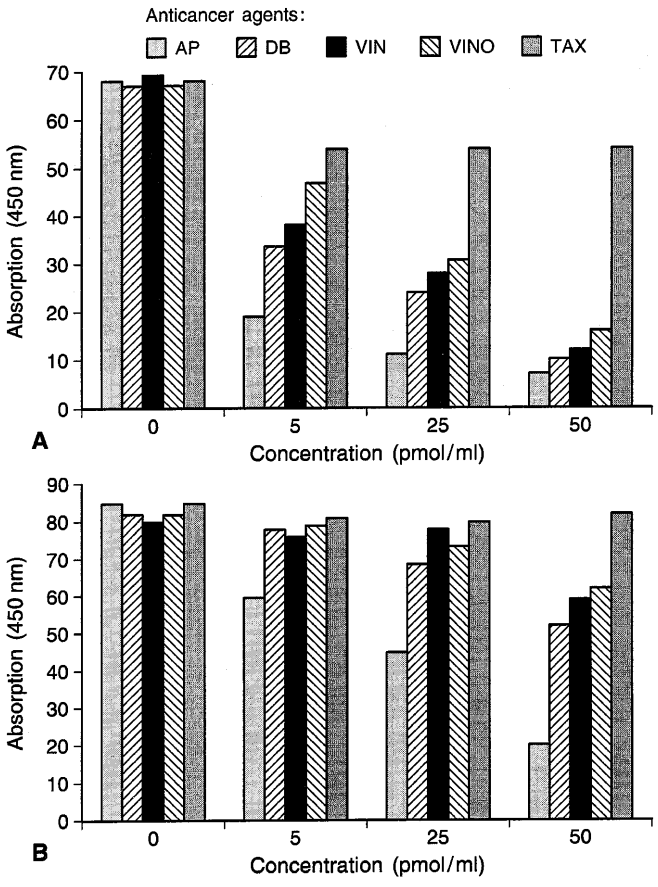

Fig. 3 In vitro neurotoxicity of Aplidine (*AP*) and Didemnin B (*DB*) was determined using PC12 neurite outgrowth assay (see also legend to Fig. 1). Effects on long neurites only (> 2 times cell body) (**A**) and on total number of neurites (**B**), respectively. *VIN* vincristine, *VINO* vinorelbine, *TAX* Taxol

Reversibility of in vitro neurotoxicity (drug withdrawal assay)

To evaluate the potential reversibility of in vitro neurocytotoxic damage, the temporal effects were investigated of withdrawal of cytostatics from the culture medium of neurite-forming PC12 cells after 48 h incubation ($n = 3$). Aplidine effects (concentrations 0, 5, 25 pmol/ml, and 50 pmol/ml) and IHB effects (concentrations 0, 0.09, 0.18 pmol/ml, and 0.36 pmol/ml) were compared with vincristine and vinorelbine (concentrations 0, 1, 10 pmol/ml, and 100 pmol/ml; Figs. 5, 6, and 7).

Withdrawal of vincristine or vinorelbine resulted in total restoration (long neurites as well as neurites of all sizes) within 24 h, even after high concentrations, to neurite scores indiscernible from control values (at $P = 0.05$). Withdrawal of Aplidine from the vitro cell culture resulted in some restoration of in vitro neurotoxic effects but to a lesser degree than that seen after withdrawal of vincristine or vinorelbine and the numbers of neurites (both long and of all sizes) at 48 h after withdrawal were still significantly different from un-

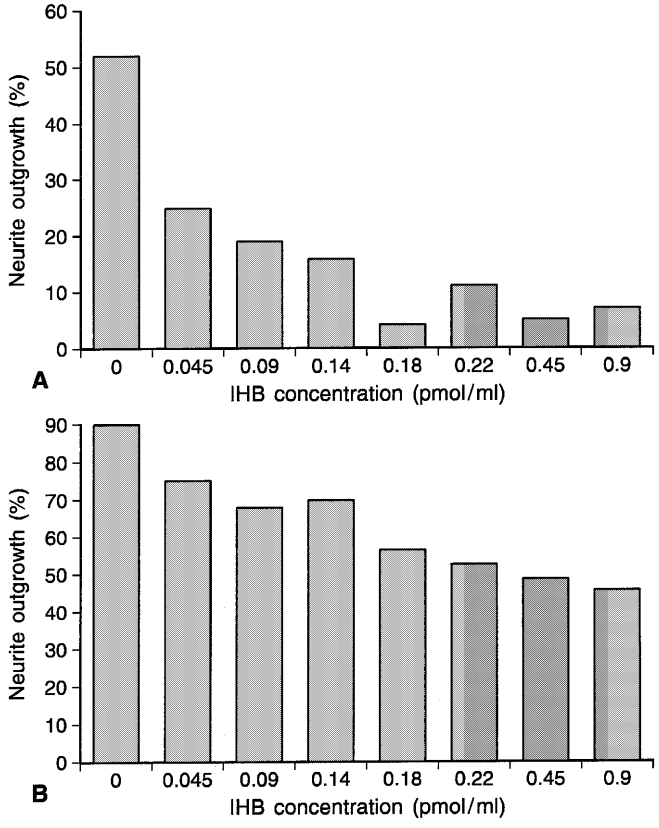

Fig. 4 In vitro neurotoxicity of isohomohalichondrin B (*IHB*) was determined using PC12 neurite outgrowth assay. Effects on long neurites (>2 times cell body) only (**A**) and on total number of neurites (**B**), respectively

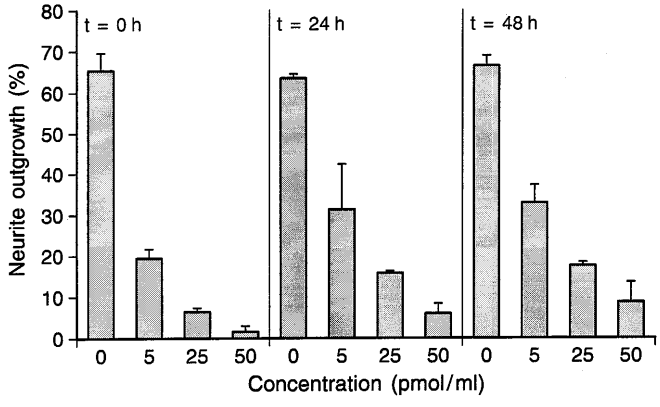

Fig. 5 Restoration of in vitro neurotoxic effects was determined after withdrawal of aplidine from the in vitro cell culture. Percentage of cells forming long neurites (>2 times cell body), before drug withdrawal and 24 h and 48 h after withdrawal of aplidine

treated control ($P < 0.00005$). Withdrawal of IHB from the in vitro cell culture also resulted in some restoration of in vitro neurotoxic effects but to a lesser degree than after withdrawal of vincristine or vinorelbine and the numbers of neurites (both long and of all sizes) at 48 h

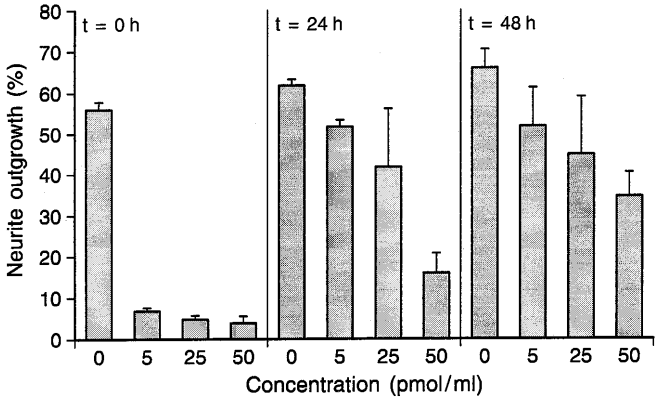

Fig. 6 Restoration of in vitro neurotoxic effects was determined after withdrawal of isohomohalichondrin B from the cell culture. Percentage of cells forming long neurites (>2 times cell body), before drug withdrawal and 24 h and 48 h after withdrawal of IHB

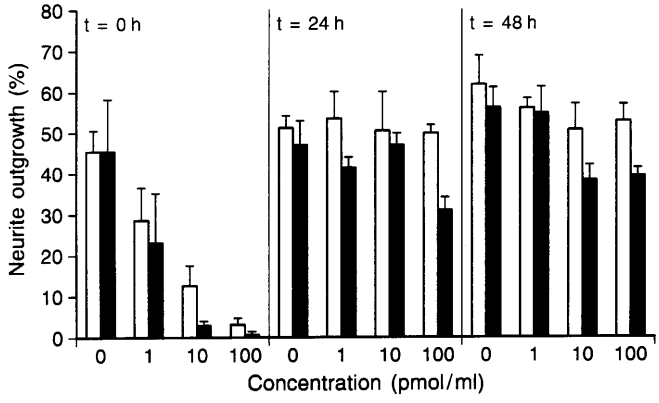

Fig. 7 Restoration of in vitro neurotoxic effects was determined after withdrawal of vincristine (*white bars*) and vinorelbine (*shaded bars*) from the cell culture. Percentage of cells forming long neurites (>2 times cell body), before drug withdrawal and 24 h and 48 h after withdrawal of vincristine

after withdrawal were still significantly different from untreated control ($P < 0.00005$).

Newly formulated aplidine batch

A newly formulated batch of aplidine was compared with the original one for general cytotoxicity using three human prostate cancer cell lines and compared with vincristine using colorimetric XTT assay ($n = 6$). Results (Table 1) show that cytotoxicity of both batches of aplidine do not differ significantly (at $P = 0.05$). A similar comparison was also performed for effect on in vitro neurite formation ($n = 3$). In this case, differences were recorded between aplidine and the newly formulated aplidine (Table 2). Analysis using Fisher's exact test for 2×2 tables (χ^2) shows a significantly stronger effect for neurites of all sizes with aplidine compared with new formulation aplidine at 1 pmol/ml

Table 1 Optical density readings (450 nm) from XTT test after 72 h culture in the presence of Aplidine, new formulation Aplidine, and vincristine. Mean values ± SD ($n = 6$). *SD* standard deviation

	Concentration	DU-145	PC-3	LNCaP-FGC
Aplidine	0 pmol/ml	1.10 ± 0.10	0.97 ± 0.08	0.94 ± 0.15
	5 pmol/ml	0.50 ± 0.05	0.25 ± 0.04	0.25 ± 0.07
	25 pmol/ml	0.31 ± 0.03	0.17 ± 0.02	0.12 ± 0.03
	50 pmol/ml	0.27 ± 0.04	0.14 ± 0.02	0.10 ± 0.02
New formulation Aplidine	0 pmol/ml	1.10 ± 0.10	0.97 ± 0.08	0.94 ± 0.15
	5 pmol/ml	0.57 ± 0.03	0.24 ± 0.04	0.29 ± 0.06
	25 pmol/ml	0.29 ± 0.05	0.16 ± 0.02	0.16 ± 0.04
	50 pmol/ml	0.25 ± 0.02	0.16 ± 0.01	0.07 ± 0.02
Vincristine	0 pmol/ml	1.10 ± 0.10	0.97 ± 0.08	0.94 ± 0.15
	5 pmol/ml	0.77 ± 0.04	0.61 ± 0.06	0.20 ± 0.02
	25 pmol/ml	0.48 ± 0.04	0.33 ± 0.02	0.09 ± 0.02
	50 pmol/ml	0.31 ± 0.05	0.31 ± 0.04	0.10 ± 0.01

Table 2 Percentage of PC12 cells showing neurites, after 72 h incubation with drugs

	Concentration (pmol/ml)	Percentage of cells with neurites (all)	Percentage of cells with long neurites
Aplidine	0	98.0	87.0
	1	77.0	67.5
	10	85.5	46.0
	100	18.5	6.0
New formulation Aplidine	0	98.0	88.5
	1	99.0	84.0
	10	94.0	64.5
	100	61.5	8.0

($P < 0.00001$) and at 100 pmol/ml ($P < 0.00001$). For long neurites only, a significantly stronger effect was observed with aplidine compared with new formulation aplidine at 1 pmol/ml ($P = 0.00813$) and at 10 pmol/ml ($P = 0.01025$).

Discussion

In conjunction with the potent cytotoxic activity observed in preclinical studies, considerable toxic side-effects of Didemnin B have been shown in phase I and phase II studies [10, 12, 15, 23, 25, 28, 29]. These reports have caused some disappointment in terms of the outlooks for this compound for application as a clinical cancer therapeutic. However, the results of comparative, preclinical studies with the new derivative, Dehydrodidemnin B (Aplidine) have fostered now hope [19]. Screening of Aplidine activity profile should also encompass possible neurotoxicity effects at drug levels with antiproliferative potency. The present paper describes the simultaneous monitoring of anticancer cytotoxicity and neurotoxicity of Aplidine under identical and standardized in vitro conditions. Additionally, another marine antitumor candidate, IHB, was also screened for both cytotoxicity and neurotoxicity in vitro.

Neurotoxicity was measured using a previously described in vitro assay [6] and antitumor cytotoxicity was measured using an in vitro panel of prostate tumor cell lines followed by the colorimetric determination of cell survival. This side-by-side approach enabled us to determine antitumor cytotoxic effect and neurotoxic "side-effects" in a comparative way under identical and standardized conditions. The validity of the PC12 model has been shown before by comparing in vitro neurotoxicity and clinical neurotoxicity profiles induced by vinca alkaloids and taxol [8, 9].

From the present results it can be concluded that Aplidine and IHB do show an effective and very potent antiproliferative response against human prostate cancer cell lines. However, moderate to severe in vitro neurotoxicity was observed at the same time at cytotoxic concentrations. A potential reversibility of in vitro neurotoxic effects by Aplidine and IHB was recorded, but to a lower level than after vincristine. The difference in neurotoxic effects between the original and a new batch of aplidine may be caused by impurities in the original batch, which contributed to the damage inflicted to the neurites. The presence of cremophor in the new formulation is hard to understand as a modulating factor. At this point, no clues are known as to the chemical nature of eventual impurities.

The differential effects of IHB compared with the vinca alkaloids could be related to differences in mode of action at the cellular and subcellular level. Like the vinca alkaloids and taxol, IHB has been shown to bind to and interfere with tubulin [1]. Such interference will conceivably result in inhibition of mitotic spindle formation and ultimately in prevention of cell division, making these so-called microtubules poisons important targets for anticancer research. This interaction with microtubules is also considered to be instrumental in side-effects of anticancer agents like neurotoxicity. There are, however, important differences in their mode of action, which may be implicated in the differential patterns of side-effects of these drugs. Taxol has been described to affect the dynamic equilibrium of microtubule polymerization and depolymerization and lead to increased stability [20, 21]. The vinca alkaloids are thought to bind to tubulin and induce microtubule disassembly by induction of tubulin aggregation [4, 18, 26]. IHB could disorganize the microtubule network by direct interaction with tubulin, resulting in a decreased binding of

GTP to the protein, essential for tubulin polymerization [1, 5]. This latter fact could be responsible for the long-term effects on neurites observed in the present study.

For aplidine, an inhibitory effect on protein synthesis (and to lesser extent on DNA and RNA synthesis) has been described [13, 24]. It is not known whether aplidine exerts any interaction with tubulin. Interestingly, the PC12 cells used in the present study to predict in vitro neurotoxicity have been used as a cellular tool to investigate drug interaction with cellular tubulin [2]. The PC12 model is believed to be a potent instrument in elucidating mechanistic details of neurotoxic effects of cytostatic drugs. Such information may prove valuable for developing approaches to counteract these effects in clinical practice. In conclusion, it can be said that the in vitro assays used have the potential to elucidate the comparative therapeutic potency of new drug formulations and to indicate candidate drugs for further testing.

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