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Antitumor Activity of Didemnin B in the Human Tumor Stem Cell Assay

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Summary. *In vitro studies of the schedule dependency and cytotoxicity of didemnin B, a novel depsipeptide isolated from a Carribean tunicate (Didemnidae), were carried out in fresh tumor cells obtained from biopsies from 39 cancer patients using the human tumor stem cell assay. Two schedules of drug exposure were examined (1 h and continuous exposure in the agar). Tumor cells from nine of 26 patients (34.6%) showed reduced survival of tumor-colony forming units to 30% of control or less at the 0.01 μg level in the continuous exposure studies. Cells from eight of 17 patients (47%) showed a similar degree of sensitivity to didemnin after a 1-h exposure to 0.1 μg prior to plating. The median ID_{50} values were 4.2×10^{-3} μg/ml and 46×10^{-3} μg/ml for continuous and 1-h exposures, respectively. A clear dose-response relationship was observed with both dosage schedules. Comparison of the slopes of the continuous and 1-h exposures and of the ID_{50} of the drug schedules suggests that didemnin is a cell-cycle-non-specific cytotoxic agent. Significant in vitro antitumor activity was observed at low concentrations against carcinomas of the breast, ovary, and kidney, and also mesothelioma and sarcoma. These results can provide pharmacologic goals to be achieved in phase I clinical trial. Further in vitro testing should help select tumor types for study in phase II trials of this very promising new anticancer drug.*

Introduction

Didemnins, a new class of depsipeptide agents, were isolated from Carribean tunicates (*Didemnidae*) (ascidian, sea squirt) [5]. The depsipeptide didemnins represent novel ring-peptide structures containing hydroxyisovalerylpropionate and a new stereoisomer of the highly unusual amino acid statine (Fig. 1) [7]. Recently, Rinehart et al. [7, 8] and Weinheimer et al. (personal communication) found that didemnins, especially didemnin B (NSC 325319), could inhibit the growth of KB cells, L1210 and P388 leukemias, and B16 melanoma in vitro and in vivo. The LD_{10} of didemnin B is in the range of 160 μg/kg, given IP, with significant antitumor activity observed in B16 melanomas in vivo at that dosage when administered on days 1–9 (lifespan extended to 160% of control) (J. Venditti, personal communication). Didemnins also inhibit replication of several DNA and RNA viruses in vitro. We thought it would be of interest to test this agent on clonogenic human tumor cells, as this novel agent has recently been approved by the NCI's decision network for toxicology testing and subsequent entry into phase I clinical trials (J. Venditti, personal communication).

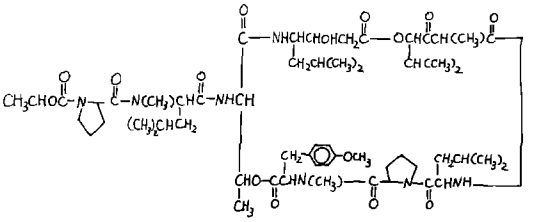

Fig. 1. Structure of didemnin B (NSC 325319)

The in vitro human tumor stem cell assay developed by Hamburger and Salmon [3] has proven useful for testing new anticancer drugs for potential phase II activity [12] and for predicting clinical response to individual chemotherapeutic drugs in individual patients [9, 10, 13]. Therefore, it might prove to be a very effective tool for new drug screening and development. In this paper the results of our initial antitumor studies of didemnin B in the human tumor stem cell assay are presented. Our data may prove useful for further experimental studies and in relation to future phase I–II clinical trials of this agent.

Materials and Methods

Drugs. Purified didemnin B (NSC 325319) was kindly provided by the Natural Products Branch, Division of Cancer Treatment, NCI and Dr A. J. Weinheimer, University of Houston, Texas, USA. Stock solutions of didemnin B were prepared in 100% ethanol, then diluted with 0.9% sterile NaCl, and stored at −80° C in aliquots sufficient for individual assays. Subsequent dilutions for incubation with cells were made with saline. The final concentration of ethanol in the incubation was 0.4% for 1-h exposure and 0.16% for continuous exposure.

Preparation of Tumor Cell Samples. A variety of types of malignant tumors from 39 patients were used for evaluating anticancer activity of didemnin B in these studies. The tumor types were as follows: ovarian, 11; breast, five; lung, three; cervical, three; endometrial, three; sarcoma, three; mesothelioma, two; malignant peritoneal or pleural effusion with

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unknown original sites, four; and cancer of kidney, skin, stomach, pancreas, and melanoma, one each.

Cell suspensions were prepared mechanically according to techniques reported previously [9]. Solid tumors were minced into small pieces with scissors, then enzymatically disaggregated with 0.8% collagenase and 0.002% DNase on a magnetic stirrer at room temperature for 30 min. After the suspension had been passed through a gauze to remove clumps, the single cell suspension was washed twice with serum-free medium and adjusted to a final concentration of 3×10^6 cells/ml in McCoy's 5A medium with 10% FCS.

In vitro Exposure to Drug. Two procedures were used for exposure of human tumor cells to drugs. One-hour exposure: Tumor cells incubated with didemnin in 0.4% ethanol or 0.4% ethanol alone for 1 h at 37° C in McCoy's 5A with 10% FCS. The cells were then washed twice and prepared for culture. Continuous contact: Didemnin was incorporated into the upper layer of the culture system for the duration of the culture.

For the initial dose finding, in each of the first 14 tumors tested didemnin B was tested at a single concentration (2.5, 1.0, or 0.1 μg/ml by continuous exposure). Subsequently, tumors were tested with three concentrations of the drug (0.1, 0.01, 0.001 μg/ml by continuous exposure or 1.0, 0.1, 0.01 μg/ml for 1 h).

Tumor Stem Cell Assay. The clonogenic assay system utilized was the method of Hamburger and Salmon [3]. In brief, cells to be tested were suspended in 0.3% agar in enriched CMRL 1066 medium to yield a final concentration of 0.5×10^6 cells/ml. Of this mixture, 1 ml was pipetted into each of three 35-mm petri dishes containing 1 ml 0.5% agar in enriched McCoy's 5A medium, but without conditioned medium. All drug assays were done in triplicate along with six simultaneous controls incorporating the ethanol solvents.

Plates were incubated at 37° C in a humidified atmosphere containing 6% CO_2 for 7–30 days (average: 18 days). Only specimens with good in vitro growth are reported. The number of colonies on control and drug-treated plates was determined with an automated image analysis system (Bausch and Lomb Omnicon Fas II). At least 30 tumor colonies per control plate, each with at least 60 μm diameter, were required for a drug experiment to be considered evaluable for measurement of drug effect. As it was set up, the FAS II counted 52% of the total colony growth area in the agar, so that in the reported experiments the minimum number of colonies per plate in controls was approximately 60. In this study, the median number of colonies counted by the FAS II was 64 per control plate.

Data Analysis. Colony counts for the triplicate plates for a particular drug concentration were averaged to obtain each data point and the standard error was calculated. The percent survival of tumor colony-forming units (TCFU) in the treated plates was calculated from the formula:

$$\% \text{ Survival} = \frac{\text{No. of colonies in treated plates}}{\text{No. of colonies in control plates}} \times 100 .$$

Reduction in survival to 30% of control or less was applied as the criterion of in vitro sensitivity to didemnin. To compare the activity between different drugs or different exposure procedures, the concentration of didemnin required to reduce survival to 50% of the control TCFU (ID_{50}) was determined from dose-response curves with the Lagrange interpolation function and the Newtonian iteration method.

Results

Effects of Didemnin B on Clonogenic Human Tumor Cells

Continuous Exposure

Table 1 and Fig. 2 summarize the in vitro response to didemnin B of more than 12 different types of tumors from 39 patients. In the initial experiments with drug concentrations of 1.0 and 2.5 μg/ml in continuous contacts, the TCFU survival was less than 15% in all samples tested, with means of 5.8 ± 2.9% and 5.1 ± 2.3%, respectively (means ± SE). There was less than 1% survival of TCFU in three tumors. The mean percent survival rose progressively with decreasing doses of didemnin. Thus, the mean survival of TCFU was 30.6 ± 5.8%

Table 1. Sensitivity of human tumor stem cells to didemnin B in 1-h and continuous exposure experiments

	Continuous contact concentration (μg/ml)					1-h exposure concentration (μg/ml)		
	2.5	1.0	0.1	0.01	0.001	1.0	0.1	0.01
Ovarian			6/9[a]	2/10	0/10	3/8	3/8	0/8
Breast	1/1	2/2	2/2	1/1	0/1	1/1	1/1	0/1
Lung			1/3	1/3	1/3	1/2	0/2	0/2
Cervical		1/1		0/2	1/2			
Endometric	3/3							
Sarcoma			3/3	2/3	2/3	2/3	2/3	2/3
Mesothelioma			0/1	0/1	0/1	1/1	1/1	1/1
Renal			1/1	1/1	0/1	1/1	1/1	0/1
Skin			0/1	0/1	0/1			
Stomach	1/1							
Pancreatic		1/1						
Melanoma		1/1						
Others			0/1	2/4	0/3	0/1	0/1	0/1
Total	5/5	5/5	13/21	9/26	4/25	9/17	8/17	3/17

[a] No. of sensitive cultures/no. tested. Sensitivity = survival ≤ 30% of control TCFU (see *Methods*)

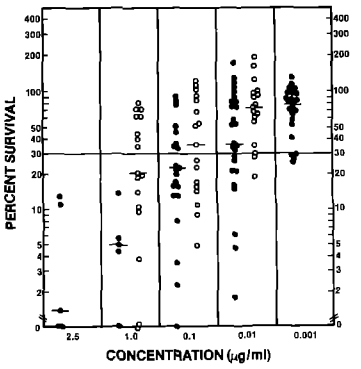

Fig. 2. In vitro activity of didemnin B against tumor colony-forming cells from 39 patients. Each *point* (● continuous exposure; ○ 1-h exposure) represents an individual tumor sample tested. The *horizontal bars* represent the median percent survival for all tumors at the concentration tested

at 0.1 μg/ml, 58.0 ± 8,4% at 0.01 μg/ml, and 75.0 ± 5.9% at 0.001 μg/ml. Nevertheless, there were four instances in which the percent survival was less than 10% at 0.1 μg/ml, and four additional cases (2 sarcoma, 1 lung, 1 renal carcinoma) where survival was less than 20% of control at the 0.01 μg/ml concentration. The survival curves for tumors tested at multiple doses were similar and showed a clear dose-response relationship. In the continuous exposure studies, approximately 62% of tumor specimens tested were sensitive to didemnin at the concentration of 0.1 μg/ml, 34% at 0.01 μg/ml, and 16% at 0.001 μg/ml.

One-Hour Exposure

In the 1-h exposure experiments 17 tumors were tested against didemnin B at three concentrations. A clear dose-response relationship was observed. The mean percent survival was 33.2 ± 6.6% at 1.0 μg/ml, 50.6 ± 9.8% at 0.1 μg/ml, and 80.7 ± 11.2% at the 0.01 μg/ml concentration. Of the tumors tested, 53% were sensitive to the drug at the concentration of 1.0 μg/ml, 47% at 0.1 μg/ml, and 18% at 0.01 μg/ml, respectively. Less than 10% survival was observed with one sarcoma and renal carcinoma at the concentration of 0.1 μg/ml. Three ovarian carcinomas and one additional sarcoma had survival reduced to less than 20% at that dose level.

The ID_{50} of didemnin B in the 1-h and continuous exposure studies was calculated for 11 tumors which provided sufficient cells to carry out both types of drug exposure simultaneously on replicate aliquots. The mean ID_{50} was 0.0042 ± 0.0010 μg/ml for continuous exposure and 0.0462 ± 0.0130 μg/ml for the 1-h exposure. However, the median ID_{50}s were 0.0035 μg/ml and 0.0168 μg/ml, respectively. The ID_{50} ratio (1 h/continuous) had a mean rate of 11 and a median of 4.8.

Discussion

In these studies of the new antitumor agent didemnin B, we have used the soft agar human tumor stem cell assay to study its potential antineoplastic potency and spectrum against clonogenic human tumor cells. Our results in 39 fresh human tumors indicate that low doses of didemnin B can significantly reduce the ability of a variety of human tumor types to form colonies in soft agar. The human tumor stem cell assay has previously been applied to testing clonogenic human tumor cells against a variety of standard and new anticancer drugs [2, 6, 10–12, 14, 15]. Correlative clinical trials from several centers have indicated that in vitro sensitivity in this assay system can predict clinical response with 60%–70% accuracy and failure of response to therapy with 95% accuracy [9, 10, 13]. True positive clinical correlations (observe clinical response) have been obtained when standard cytotoxic drugs reduce survival of TCFU to 30% of control or less after a 1-h exposure to doses that are 10% of the peak plasma concentration or concentration-time product or less [1]. True negative clinical correlations (failure of response) have been observed when survival of TCFU is greater than 30% at such doses, and particularly when greater than 50% survival is observed with the agent tested in vitro. Available data on various new agents tested in this assay system suggest that in vitro analysis can pinpoint tumor types that may prove sensitive to the agent, and thereby serve as a type of 'in vitro phase II trial' [12, 14, 15]. In view of the positive results observed with various murine tumors in vivo, and with our in vitro observations in human tumors, it is reasonable to project that didemnin will have significant antitumor activity in clinical practice, assuming that excessive toxicity is not observed with didemnin in phase I clinical trials, and that clinically achievable plasma concentrations are at least ten times higher than the in vitro concentrations utilized in the 1-h exposure studies in which sensitivity (survival ≤ 30% of control) was observed. Thus we predict that this agent will prove useful if peak plasma concentrations or concentration-time products can be obtained in vivo in the range of 0.1–1.0 μg · h/ml. Inasmuch as pharmacokinetic and toxicologic data have yet to be obtained in man, these projections remain conjectural, but nonetheless they should provide a useful level to set as a pharmacokinetic goal for phase I studies of didemnin. We have previously used the comparison of patterns of dose-survival curves and ID_{50} values in 1-h and continuous exposure experiments to gain some knowledge of the mechanism of action and schedule dependency of anticancer drugs [1, 4]. When the slope of the survival-concentration curves is much steeper in the continuous exposure studies than the 1-h studies, and the ratio of ID_{50} values (1 h/continuous) is very high (e.g., 200–300), then the drug tested has a high likelihood of being schedule-dependent or cell-cycle-specific. Conversely, when 1-h and continuous exposure curves are parallel, and the ratio of ID_{50} values is in the range of 10 or lower, then it is likely that the agent is a cell-cycle-non-specific drug. We recognize that our approach to assessing cell cycle activity or schedule dependency of a new drug (by comparing 1-h and continuous exposure data) is only a first approximation. It is generally recognized that even cycle-non-specific drugs often have higher lethality against cycling than against resting cells, but the differential effect is of far lesser magnitude than is observed with drugs that kill only cycling cells. Thus, in our prior studies of doxorubicin and camptothecin (two cycle-non-specific agents), the ratio of ID_{50} values was in the range of 10 [4], whereas for drugs such as bleomycin [1] and VP-16, schedule-dependent in vitro toxicity is observed in the clonogenic assay, effective doses in the continuous exposure experiments being less than 1% of those required in the 1-h studies. The results of such comparisons for didemnin suggest that it is a cell-cycle-non-specific cytotoxic agent, as its ID_{50} ratio was in the range of 10. The molecular mechanism of action remains to be determined, but is likely to be novel, as this agent does not bear any apparent structural similarities to alkylating agents or other cycle-non-specific agents.

As the major objective of this in vitro study was to define dosage exposures and schedule dependency of didemnin, our experience with regard to its antitumor spectrum is still preliminary. The data summarized in Table 1 lends encouragement to the view that this agent will have a broad spectrum of action, as significant antitumor activity was observed at relatively low doses (0.1 μg/ml for 1 h) in ovarian, breast, and renal carcinomas, as well as in mesothelioma and sarcoma. Additional in vitro investigation (with 1-h exposure) against a larger spectrum and number of tumors is clearly warranted for didemnin, so that additional tumor types with a high likelihood of clinical response can be more definitively identified and thereby help provide goals for phase II clinical trials with this promising new anticancer drug. It is important to point out that didemnins have a molar potency similar to that of actinomycins; therefore, even 1-h exposure dosages of 0.1 μg/ml may overestimate potential clinical activity for didemnins, and in vitro studies with even lower dosage levels may prove necessary.

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