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Anticancer Activity of Zoanthids and the Associated Toxin, Palytoxin, against Ehrlich Ascites Tumor and P-388 Lymphocytic Leukemia in Mice

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Abstract ☐ Crude alcoholic extracts of zoanthids, indigenous to Hawaii, have been shown to have general antitumor activity against Ehrlich ascites tumor in mice. The toxic component, palytoxin, was effective in the control and cure of Ehrlich ascites at extremely low dosages using several different methods of administration and displayed marginal activity against P-388 lymphocytic leukemia.

Keyphrases ☐ Zoanthids—antitumor activity of crude extracts, palytoxin as potent antitumor component ☐ Anticancer agents, potential—pharmacological testing of zoanthids and palytoxin against Ehrlich ascites tumor and P-388 leukemia ☐ Palytoxin—pharmacological studies as antitumor agent

Observations of antitumor activity in extracts of marine invertebrates (1–3), particularly the phylum Coelenterata (4, 5), have now been extended to extracts of a number of species belonging to the family Zoanthidae, order Zoantharia. The water-soluble materials, from ethanolic extracts of eight Hawaiian species representing three genera of zoanthids, have been found to inhibit growth of Ehrlich ascites carcinoma in Swiss Webster mice.

The specific Ehrlich ascites activity increased with increasing toxicity of the extracts, suggesting that the toxic component itself may be responsible for the antitumor activity. Palytoxin, the toxic principle of "limu-make-o-Hana," a zoanthid from Maui, Hawaii (6), which has been identified as *Palythoa toxica* Walsh and Bowers (7), was found to be a potent antitumor agent that completely controlled Ehrlich ascites carcinoma in mice at doses as low as 84 ng/kg and exhibited an inhibitory effect on the tumor in doses as low as 5.25 ng/kg. Several different modes of administration and injection schedules were used to evaluate its activity against Ehrlich ascites tumor. Palytoxin was tested also against P-388 lymphocytic leukemia in mice but showed only marginal activity.

RESULTS AND DISCUSSION

The activities of the crude extracts against Ehrlich ascites tumor are given in Table I. In this laboratory, during the past 5 years, more than 2500 mice were used as infected controls and none survived for as long as 30 days. Consequently, the presence of any survivors in the treated group at 30 days indicates significant activity. The absence of observable abdominal distension from ascitic fluid was the basis for describing survivors as nonascitic (8).

Since the specific Ehrlich ascites activity generally increased with increasing toxicity of the extracts, the antitumor activity may be primarily due to palytoxin. In this study, the most toxic and Ehrlich ascites-active sample was obtained from an unidentified zoanthid from Haleiwa, Oahu; nontoxic extracts, at least at the levels tested, with low Ehrlich ascites activity were obtained from *Zoanthus pacificus*, *Palythoa psammophilia*, and an unidentified zoanthid from a tidepool adjacent to Makapuu Beach, Oahu. The palytoxins from *Palythoa tuberculosa*, *Palythoa vestitus*, and the zoanthid from Haleiwa were found to have UV spectra identical with that of palytoxin from *P. toxica*. Whether the palytoxins from these various sources are all structurally identical or subtly different remains to be demonstrated. Recently, it was found that the toxicity of *P. tuberculosa* is seasonally dependent and, more importantly, is linked with the reproductive cycle of the zoanthid because the toxin appears to be associated with the bisexual and female polyps and is located in the eggs of the female polyps (9). This finding might explain the low toxicity of the specimen of *P. toxica* that was collected in September at the Halona Blowhole, Oahu, since all previous specimens of this organism had shown toxicities comparable to that of *P. toxica* from Muolea, Maui.

Only a few Hawaiian zoanthids have been adequately described in the literature (7), and all have been classified in the three genera *Palythoa*, *Zoanthus*, and *Isaurus*. In this work, six known and two new species of zoanthids[1] were collected and evaluated for anticancer activity. For a preliminary chemotaxonomic identification of each organism, the sterol complement of the zoanthid was isolated, analyzed by GLC and mass spectrometry, and compared with those from known zoanthids. The two unidentified zoanthids from Haleiwa and Makapuu, as well as *P. toxica*, contained essentially a single sterol (24-methylenecholesterol), the

[1] The authors thank Dr. R. E. Bowers, Leeward Community College, Pearl City, HI 96782, for his help in identifying the zoanthids.

Table I—Activity of Crude Extracts against Ehrlich Ascites Tumor

Species	Month of Collection	Dose, mg/kg	Number of Mice	Deaths due to Toxicity	Survivors at 30 Days	
					% Alive	% Nonascitic
P. psammophilia Walsh and Bowers	March	80	5	1	25	25
		40	5	—	60	40
		22.7	5	—	0	0
		11.4	5	—	40	20
		5.7	5	—	0	0
P. tuberculosa Esper	January	22.3	5	5	—	—
		4.54	5	—	100	60
		4.48	5	—	100	100
		2.27	5	—	20	0
		1.14	5	—	0	0
P. vestitus Verrill	December	22.9	5	1	100	100
		11.6	5	—	100	100
		4.64	5	—	40	0
P. toxica Walsh and Bowers	September	3.3	5	—	100	80
		1.67	5	—	0	0
		0.83	5	—	0	0
Z. pacificus Walsh and Bowers	March	160.4	5	—	60	40
		79.3	5	—	20	0
		59.4	5	—	20	20
		29.7	5	—	0	0
		17.4	5	—	0	0
Zoanthid from Haleiwa	July	1.78	5	5 (15 min)	—	—
		0.36	5	4 (chronic, Days 6–9)	100	100
		0.33	5	—	100	100
		0.165	5	—	100	100
		0.064	6	—	100	0
Zoanthid from Makapuu	January	45.4	5	—	20	0
		40	5	—	0	0
		21.7	5	—	40	40
		20	5	—	20	20
		11.4	5	1	50	25
		10	5	—	0	0
I. elongatus Verill	October	13	5	2	25	25
		6.7	5	1	0	0

same as reported previously for a Tahitian *Palythoa* species (10). In contrast, *P. tuberculosa* (10), *P. psammophilia*, and *P. vestitus* elaborate an identical complex mixture of sterols containing no detectable amounts of 24-methylenecholesterol while *Z. pacificus* [formerly *Z. confertus* (10)] and *Isaurus elongatus* contain sterol mixtures different both from each other and from the *Palythoa* species[2]. The genus *Palythoa* might well be comprised of two different genera.

The behavior of palytoxin in the standard screening procedure is shown in Table II. Palytoxin completely inhibited the growth of Ehrlich ascites tumor in 100% of mice treated with doses as low as 84 ng/kg and gave some nonascitic survivors at 30 days with doses down to the 5.25-ng/kg level. The mice from two experiments were kept for 3 months to check the long-term effects. At 3.37×10^{-1} μg/kg, given twice, eight out of the 10 mice (two deaths due to toxicity) survived 30 days and were nonascitic and all of these mice were alive and nonascitic at 90 days. At 8.44×10^{-2} μg/kg, given 20 times, nine out of the 10 mice (one death due to toxicity) survived 30 days and were nonascitic and eight out of these nine mice survived the 90-day period in good health. These results would indicate that all tumor cells were killed in 16 of the 17 mice (94%) examined and that the disease was cured.

Palytoxin gave some life extension using the P-388 lymphocytic leukemia screen; however, the best figure obtained was only a 32% life extension and does not appear sufficient to warrant further work with this system. Some crude extracts were examined by the National Cancer Institute's screening service against P-388 and human epidermoid carcinoma of the nasopharynx (KB cell culture). The zoanthid from Haleiwa was active against KB with an ED_{50} of 3.0×10^{-2} μg/ml, while both *P. psammophilia* and *Z. pacificus* were inactive, having ED_{50}'s greater than 100 μg/ml. In the P-388 screen, however, *P. psammophilia* was active, having now reached a confirmed active status, while both *Z. pacificus* and the zoanthid from Haleiwa were inactive, suggesting that the antileukemia agent in *P. psammophilia* is not palytoxin.

The correlation between the antitumor properties of palytoxin and its occurrence in the eggs of female *P. tuberculosa*[3] is a fascinating one. The presence of highly toxic substances in the reproductive organs of animals is not unusual. Tetrodotoxin is associated with the ovaries of the puffer fish and has its highest concentration just before spawning. It has been isolated (under the name tarichatoxin) from the eggs of western American newts of the genus *Taricha* (11). Palytoxin might be expected, therefore, to have some function in cell growth and cell differentiation and its antitumor activity could be a consequence of this biological function.

The experimental data from a study of different modes of drug administration in the control of Ehrlich ascites tumor with palytoxin are given in Table III. Palytoxin appears to be systemic in action; not only is it effective in the screening procedure where Ehrlich ascites cells and drug are administered intraperitoneally, but it is effective also when Ehrlich ascites cells are injected subcutaneously, resulting in the formation of a solid tumor, and the drug is administered intraperitoneally. Some effectiveness is displayed when the tumor is peritoneal and the drug is administered subcutaneously. In the latter case, however, the skin around the injection site became calloused and it appeared that drug distribution was poor. Delay of treatment until 4 days after tumor inoculation resulted in some control; however, the survival rate was not 100% as observed when palytoxin was administered 20 hr after inoculation. The results indicate, however, that palytoxin can control, to some degree, well-established tumor proliferation. In one experiment, two doses, administered 20 and 27 hr after in-

[2] R. E. Moore, K. Gupta, and P. J. Scheuer, unpublished work.

[3] Dr. Y. Hashimoto, Laboratory of Marine Biochemistry, Faculty of Agriculture, University of Tokyo, Tokyo, Japan, personal communication.

Table II—Activity of Palytoxin against Ehrlich Ascites and P-388 Leukemia

		Ehrlich Ascites Carcinoma				P-388 Lymphocytic Leukemia		
				Survivors at 30 Days				
Dose, μg/kg	Number of Doses	Number of Mice	Deaths due to Toxicity	% Alive	% Nonascitic	Number of Mice	Deaths due to Toxicity	T/C, %
2.7	1	10	10[a]	—	—	—	—	—
1.35	1	10	10[a]	—	—	—	—	—
6.75×10^{-1}	1	10	0[a]	—	—	—	—	—
4×10^{-1}	20	—	—	—	—	5	1	125
3.37×10^{-1}	2	10	2[b]	100	100[c]	—	—	—
2×10^{-1}	20	—	—	—	—	5	0	123
1×10^{-1}	20	—	—	—	—	5	0	132
8.44×10^{-2}	20	10	1[b]	100	100[d]	5	0	128
2.11×10^{-2}	20	10	0	90	80	5	0	122
5.25×13^{-3}	20	10	0	20	20	5	0	118
2.65×10^{-3}	20	10	0	0	0	5	0	110

[a] Toxicity tests, no Ehrlich ascites cells. [b] Observed toxicity after Ehrlich ascites cell inoculation. [c] One hundred percent alive and nonascitic at 90 days. [d] Eighty-nine percent alive and nonascitic at 90 days.

Table III—Effectiveness of Palytoxin Using Different Methods of Administration

				Survivors at 30 Days	
Dose, μg/kg	Number of Doses	Number of Mice	Deaths due to Toxicity	% Alive	% Nonascitic
Different routes of administration:					
Ehrlich ascites cells intraperitoneally, drug intraperitoneally					
1.08×10^{-1}	20	5	—	100	100
8.64×10^{-3}	20	5	—	100	80
4.32×10^{-3}	20	5	—	20	20
1.73×10^{-3}	20	5	—	0	0
Ehrlich ascites cells intraperitoneally, drug subcutaneously					
1.3×10^{-1}	20	10	—	40	30
Ehrlich ascites cells subcutaneously, drug intraperitoneally					
1.73×10^{-1}	8 (1/day)	10	1	Average tumor weight on 9th day 84 mg (range: 39–119 mg)	
Control		10	—	Average tumor weight on 9th day 446 mg (range: 262–824 mg)	
Delayed treatment: drug treatment commenced 4 days after Ehrlich ascites cell inoculation					
3.46×10^{-1}	20	8	—	75	75
1.73×10^{-1}	20	8	1	28.6	14.3
Minimum number of doses required:					
5.4×10^{-1}	1	8	—	37.5	25
5.4×10^{-1}	4(2/day)	8	—	62.5	62.5
3.37×10^{-1}	2(2/day)	10	2	100	100

oculation, resulted in complete tumor control; however, the survival rate was not as good in other experiments. Palytoxin displays a very strong kill of tumor cells as it approaches the toxic level.

In Table IV the Ehrlich ascites activities of palytoxins from three different sources are compared. This table illustrates one difficulty associated with cancer chemotherapy. The results in Tables I-III were obtained when using mice from a different source than those recorded in Table IV. The mean survival times for control animals in the three runs prior to the change were 18, 22, and 19 days as opposed to 12, 9, and 11 days after the change. The latter mice appear more susceptible to Ehrlich ascites tumor, and the established tumor appears more resistant to the action of drugs.

From the data, however, there do not appear to be any significant differences among the three samples of palytoxin. Although palytoxin was not as effective in this study as in the prior studies, it still gave essentially cures, albeit not 100% of the treated group.

Further work is continuing on the isolation of the P-388 leukemia active material from *P. psammophilia*.

EXPERIMENTAL

Extraction Procedure—Extraction was generally carried out by allowing the animals to soak in 95% ethanol at room temperature. The solution was decanted, and the process was repeated until no further material was extracted. The combined extract was evaporated under reduced pressure to remove ethanol, and the aqueous suspension was extracted with chloroform. The aqueous suspension was filtered or centrifuged as necessary to remove insoluble material and either evaporated under reduced pressure or freeze dried.

P. psammophilia was extracted by a different procedure. The collected samples were stored in a small volume of 95% ethanol. The animals were removed from the storage liquid, and the polyps were freed of coral substrate. The wet zoanthids (323 g) were homogenized in a blender, in two batches, with 28.5% ethanol (1.6 liters) for 10 min at highest speed; they were then combined with the storage liquid (490 ml) and allowed to stand at room temperature for 4 days. The supernate was decanted and the residue centrifuged (2000 rpm for 5 min). The combined supernate (1.74 liters) was evaporated under reduced pressure (bath temperature

Table IV—Comparison of Various Palytoxin Preparations against Ehrlich Ascites Tumor

Source of Palytoxin[a]	Dose, μg/kg	Number of Mice	Deaths due to Toxicity	Survivors at 30 Days	
				% Alive	% Non-ascitic
P. toxica from Muolea, Maui	1.28	5	5	—	—
	6.4×10^{-1}	5	5	—	—
	3.2×10^{-1}	5	0	100	100
	3.2×10^{-1}	5	0	80	60
	1.6×10^{-1}	5	0	80	80
	8×10^{-2}	5	0	0	0
	2×10^{-2}	5	0	0	0
	5×13^{-3}	5	0	0	0
P. mammilosa from Jamaica	1.28	5	5	—	—
	6.4×10^{-1}	5	5	—	—
	3.2×10^{-1}	5	1	100	100
	3.2×10^{-1}	5	1	75	50
	1.6×10^{-1}	5	0	80	80
	8×10^{-2}	5	0	0	0
	2×10^{-2}	5	0	0	0
	5×13^{-3}	5	0	0	0
P. spp. from Tahiti	1.28	5	5	—	—
	6.4×10^{-1}	5	5	—	—
	3.2×10^{-1}	5	4	100	100
	3.2×10^{-1}	5	0	80	80
	1.6×10^{-1}	5	0	0	0
	8×10^{-2}	5	0	40	40
	2×10^{-2}	5	0	60	60
	5×10^{-3}	5	0	0	0

[a] The authors thank Dr. Tatsuo Higa and Professor Paul J. Scheuer for samples of palytoxin from *P. mammilosa* and the Tahitian *P.* spp.

45°) to 330 ml of aqueous suspension and centrifuged (20,000×*g*, 10 min), and the aqueous solution was freeze dried to give a solid (14.24 g) which was used for the tests. Soaking the residue with an additional 1 liter of 28.5% alcohol yielded 2.7 g solid material.

Isolation of Palytoxin from Toxic Zoanthids—The palytoxins were isolated from the extracts of *P. tuberculosa, P. vestitus*, and the unidentified zoanthid from Haleiwa as previously described (6). All three toxins showed UV spectra indistinguishable from that of palytoxin from *P. toxica*.

Bioassay Method Using Ehrlich Ascites Tumor[4]—*Method 1 (Standard Screening Procedure)*—This method consisted of intraperitoneal injection of Ehrlich ascites cells and intraperitoneal injection of drug. The method used was previously described (4).

Method 2—This method consisted of intraperitoneal injection of Ehrlich ascites cells and subcutaneous injection of drug. The method was the same as Method 1 except that drug was administered subcutaneously on the back of the mouse.

Method 3—This method consisted of subcutaneous injection of Ehrlich ascites cells and intraperitoneal injection of drug. Ehrlich ascites cells (10^6) in 0.2 ml volume (Hank's solution) were inoculated subcutaneously at slightly above the midabdominal region of the mouse. The mice developed a solid tumor at the site of inoculation in about 3-5 days. Drug injection was initiated 18–20 hr after Ehrlich ascites cell inoculation, and daily injections of 0.1 ml volume of drug were given intraperitoneally for up to 10 days. At the end of 9-14 days, before the first sign of ulceration of any solid tumor, the animals were sacrificed and their tumor weights were taken.

Bioassay Method Using Lymphocytic Leukemia (Standard Screening Procedure)—P-388[5] was transplanted in male or female DBA/2 mice[6] every 7 days.

Ascitic fluid was drawn from the intraperitoneal cavity of the DBA/2 mice 7 days after the previous transfer. The ascitic fluid was diluted with Hank's saline solution to 10^6 cells/0.1 ml. Then cells (10^6) were implanted intraperitoneally into female BDF_1 mice (20–23 g)[6]. Drug treatment was commenced 18–20 hr after inoculation and was administered twice daily for 10 days. Mean survival time was used as the activity parameter, and the results were expressed as the percentage of the mean survival time of the controls.

[4] Data in Tables I-III obtained using Swiss Webster mice from University of Hawaii Animal Colony and that in Table IV using Swiss Webster mice from Simonsen Labs., Gilroy, Calif.

[5] The authors thank Dr. Jonathan L. Hartwell, Natural Products Section, Cancer Chemotherapy, National Cancer Institute, Bethesda, Md., for arranging to supply the P-388 system.

[6] From Simonsen Labs., Gilroy, Calif.

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