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# EFFECT OF HOLOTHURIN ON SARCOMA 180 AND B-16 MELANOMA TUMORS IN MICE<sup>1</sup>

by

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## ABSTRACT

Holothurin, a biotoxic principle from the Cuvierian glands of the Bahamian sea-cucumber, *Actinopyga agassizi*, was studied as an anti-tumor agent capable of retarding tumor growth and prolonging the life of tumor-bearing mice. White Swiss mice injected with Sarcoma 180 had a mean survival time of 18.1 days with an average weight gain representing tumor and ascites fluid accumulation amounting to 25.1 grams in 15 days. Of five white Swiss mice with Sarcoma 180 receiving 0.15 mg Holothurin every other day, one was alive at 57 days and the average weight gain of the group was 10.4 grams in 15 days. White Swiss mice with Sarcoma 180 which survived the lethal effects of slightly higher doses of Holothurin also had prolonged survival times and negligible tumor growth. C57-B1-6J mice with B-16 melanoma tumors did not show increased survival time using the same doses of Holothurin that were effective in white Swiss mice. Toxicity tests indicated that the safe upper limit for intraperitoneal Holothurin injection in white Swiss mice was 0.10 mg/day, 0.15 mg given every other day, and up to 0.30 mg given in a single injection. Similar tests with C57 black mice showed survival with as much as 0.60 mg Holothurin in a single subcutaneous injection. Holothurin was found to be 250 to 500 times more effective in causing red blood cell hemolysis than saponin and thus appears to have some action on living cells in addition to its surfactant action.

## INTRODUCTION

Cancer chemotherapy began in 1946 when nitrogen mustard was used in treating leukemia patients. During the next 15 years only some thirty drugs were used in cancer chemotherapy but screening of many thousands of compounds was carried out each year (Clark 1961). Although marine invertebrates provide a particularly rich source of compounds with biological activity in mammalian species, less than

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1% of all marine invertebrates thought to possess biotoxic substances have been investigated and of those, only a dozen have been thoroughly investigated pharmacologically (Halstead 1965).

Small concentrations of a crude water extract made from the whole body of the Bahamian sea-cucumber, *Actinopyga agassizi*, was found to be lethal to mice and fish and to have some tumor cell inhibitory action *in vitro* (Nigrelli and Zahl 1952). The active principle of this crude extract was found to be concentrated in the Cuvierian tubules located in the sea-cucumber respiratory tree and was named Holothurin (Chanley et al. 1955). Holothurin was the first known steroid saponin of animal origin (Nigrelli et al. 1959). Chemical analysis of Holothurin indicates that it is highly soluble in water, non-volatile, heat stable, and exhibits surface-active properties (Nigrelli and Jakowska 1960). Holothurin appears to consist of a few steroid aglycones that are bound individually to four monosaccharide molecules. A provisional formula has been proposed (Alender and Russell 1966). See Fig. 1.

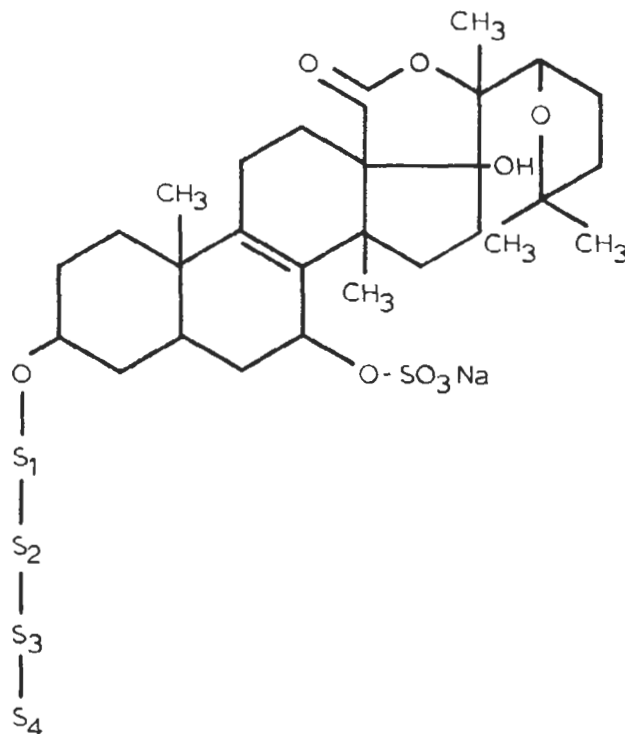


Figure 1. Holothurin formula. Proposed structure for Holothurin molecule, a steroid saponin of animal origin; the monosaccharide groups represented by S<sub>1</sub>-S<sub>4</sub> are, respectively, D-glucose, D-xylose, D-quinovose, and 3-o-methyl glucose.

Holothurin is highly toxic to many types of organisms in very small concentrations. It retards onion root tip development (Nigrelli and Jakowska 1960) and fruit fly pupation (Goldsmith, Osburg and Nigrelli 1958) at 1000 ppm, alters regeneration of planarians at 100 ppm (Quaglio et al. 1957), is lethal to the "pearl" fish *Carapus* at 1 ppm and affects sea urchin development at 0.01 ppm (Ruggieri and Nigrelli 1960). Holothurin exhibits a hemolytic effect on red blood cells and reduces the size of a subcutaneously injected Sarcoma 180 tumor in white Swiss mice (Nigrelli 1952). Krebs-2 ascites tumors in white Swiss mice are also inhibited by Holothurin (Sullivan, Ladue and Nigrelli 1955).

The present study demonstrates inhibition of Sarcoma 180 tumors in white Swiss mice but not of B-16 melanoma tumors in C57 Black 6J mice. Toxicity of Holothurin was estimated by measuring its effects on red blood cell hemolysis and by lethal-dose measurements in mice. It appears that the anti-tumor activity of Holothurin is not only dose-dependent but that the effective dose is determined by the sensitivity of the species to the lethal effects of Holothurin.

#### MATERIALS AND METHODS

*Holothurin*: The Holothurin referred to in this paper was a "crude extract" obtained from Dr. Ross Nigrelli and was made from the sun-dried, powdered Cuvierian glands of Bahamian sea-cucumbers, *Actinopyga agassizi*.

*Red Blood Cell Hemolysis Tests Using Holothurin and Saponin*: Fresh red blood cells were obtained from white Swiss mice just prior to testing the hemolytic effects of Holothurin and saponin of plant origin. The blood was obtained by cardiac puncture while the mice were under light ether anesthesia. Approximately 5 ml of blood was diluted in 100 ml of balanced-saline solution buffered at pH 7.4 (Olmsted 1967) containing calcium oxalate (0.1%) to prevent coagulation. A serial dilution of Holothurin was prepared ranging from 2 mg/ml to 0.001 mg/ml and a similar series of dilutions of saponin was made for comparison.

One ml of diluted blood was added to 5 ml of each of several concentrations of both Holothurin and saponin. The test tube was centrifuged 1 minute after mixing the contents and the optical density of the supernatant was measured using a spectrophotometer set at 540 mu. The degree of hemolysis was estimated by the concentration of hemoglobin in the supernatant. These values were compared to 100% hemolysis values obtained either by using water only as the diluent or by using a high concentration of Holothurin or saponin.

*Holothurin Toxicity in Mice*: All Holothurin injections were given at dosages determined by previous tests done in this laboratory

on normal, non-tumor-bearing mice. Tests to determine the lethal and sub-lethal intraperitoneal doses of Holothurin in white Swiss mice were carried out using several concentrations given daily, given on alternate days, and for several concentrations given in a single injection. The dosages of Holothurin given to the white Swiss mice bearing Sarcoma 180 tumors were based on these data. The dosages of Holothurin given to the C57 black mice bearing B-16 melanoma tumors was based on the effective doses of Holothurin given to tumor-bearing white Swiss mice. In addition, a test of toxicity of a single subcutaneous injection of Holothurin was carried out using several concentrations of Holothurin given to normal, non-tumor-bearing C57-B1-6J mice.

*Holothurin Administration to Mice Bearing Sarcoma 180:* Sarcoma 180 cells and the white Swiss mice were obtained from the Gulf South Research Institute in New Orleans. The mice were inoculated intraperitoneally with 0.5 ml of Sarcoma 180 ascites fluid containing  $11.6 \times 10^6$  cells/ml. Groups of five mice received daily injections of Holothurin at 0.01, 0.05, 0.10, and 0.15 mg each, and three groups received injections of Holothurin every other day in the amounts of 0.10, 0.15 and 0.20 mg each. Twelve mice received Sarcoma 180 inoculations, but no Holothurin. The survival time was recorded for each mouse and the mice were weighed individually before inoculation with tumor cells and at 5-day intervals during the course of the experiments.

*Holothurin Administration to Mice Bearing B-16 Melanoma:* A single C57-B1-6J mouse with a B-16 melanoma tumor was used as the source for all of the B-16 melanoma used in this study. The tumor-bearing donor mouse was obtained from the Department of Surgery at Tulane Medical School. The C57-B1-6J mice used in the experiments here were obtained from the Roscoe B. Jackson Memorial Laboratory in Bar Harbor, Maine. The solid tumor was taken from the donor mouse under light ether anesthesia and was minced with scissors in a tissue culture nutrient medium containing serum (Olmsted 1967). Several small pieces of the minced tumor were trochared subcutaneously into the left side of thirty-five recipient male mice. Thirty of these mice were used for the experiments reported here. Daily injections of 0.05, 0.10, and 0.15 mg Holothurin were given to each of the five tumor-bearing mice in three groups and five tumor-bearing mice in another group were each given 0.20 mg Holothurin every other day. Five mice without melanoma, and ten mice with melanoma tumors, were also maintained but not given Holothurin. All Holothurin injections were given subcutaneously near the region of tumor implantation in 0.10 ml volumes, and were given over a 13-day period. The survival time was recorded for each mouse, and the mice were weighed in groups of five before tumor implantation and at 5-day intervals during the course of the experiments.

## RESULTS

*Hemolysis Tests:* Minimum concentrations of Holothurin and saponin that produced complete hemolysis were found to be 0.001 mg/ml of Holothurin and 0.25–0.50 mg/ml of saponin indicating that Holothurin was 250 to 500 times more active as a hemolytic agent than saponin.

*Holothurin Toxicity in Mice:* Holothurin treatment of tumor-bearing white Swiss mice at the level of 0.15 mg/day was lethal at 3 to 8 days with a mean of 4.2 days. See Table 1. Toxicity tests on other

Table 1.  
Effects of Holothurin on White Swiss Mice with Sarcoma 180

No. of Mice	Sex	Dose (mg)	Frequency of injection	Survival Time (days)	Body Weight Change		
					5th day (g)	10th day (g)	15th day (g)
12	F	0	0	18.1 (15–21)	+2.3	+13.0	+25.1
5	F	0.01	daily	19.2 (15–22)	+2.0	+12.7	+27.6
5	F	0.05	daily	17.8 (16–23)	0	+13.8	+33.0
5	F	0.10	daily	16.3 (14–19)	-1.4	+ 8.2	+30.7
5	F	0.10	alternate days	16.8 (15–20)	-1.1	+10.1	+29.4
5	F	0.15	alternate days	26.4 (16–58)	-1.0	+ 2.9	+10.4
5	F	0.15	daily	4.2 (3–8)	died	--	--
5	M	0.20	alternate days	22.2 (10–47)	-1.8	0	0

groups of normal, non-tumor-bearing white Swiss mice indicated that either 0.10 mg/day or 0.15 mg given on alternate days was a safe upper limit for intraperitoneal Holothurin administration. Additionally, 0.30 mg Holothurin in a single injection could be tolerated, but more than this amount in a single injection was lethal. Toxicity tests

on C57-B1-6J mice without B-16 melanoma tumors indicated that these mice could survive single subcutaneous injections of Holothurin as high as 0.60 mg.

*Effect of Holothurin on White Swiss Mice with Sarcoma 180:* Twelve tumor-bearing mice that were not treated with Holothurin lived for an average of 18.1 days after tumor cell inoculation. These mice gained an average of 25.1 g by the fifteenth day after tumor cell inoculation. This increase in body weight represents the extent of increase in tumor size and ascites fluid accumulation since body weight increase due to growth of the animal during this time would have been negligible. The tumor-bearing animals receiving 0.15 mg Holothurin on alternate days had a mean survival time of 26.4 days representing an increase in survival time of 46%, and a mean weight gain at 15 days of 10.4 g representing a 60 to 70% decrease in tumor growth and ascites fluid accumulation. One of the mice in this high Holothurin dose group had a weight gain of only 5.1 g and was still alive after 58 days. Three of the mice receiving 0.20 mg Holothurin every other day died in less than 12 days probably as a result of Holothurin overdose. Of the other two mice in the 0.20 mg group, one lived for 30 days and the other for more than 47 days and both had negligible weight gain during this period of time indicating complete tumor suppression. The four groups of tumor-bearing mice receiving less than 0.15 mg of Holothurin per injection did not show any significant increase in survival time or decrease in weight gain. See Table 1.

*Effects of Holothurin on C57-B1-6J Mice with B-16 Melanoma:* Ten C57-B1-6J mice not injected with Holothurin lived for an average time of 29.4 days after B-16 melanoma inoculation with a range of 18 to 43 days. The animals with malignant melanoma tumors decreased in total body weight. Normal, non-tumor-bearing C57-B1-6J mice of comparable age to the tumor-bearing mice all gained weight on the diet and the conditions of this experiment. Measurement of body weight was not a valid indicator of tumor growth or of Holothurin effectiveness, as in the case of mice with Sarcoma 180, because the melanoma tumor grows in a small solid mass and does not induce ascites fluid accumulation. As the Holothurin dosages used here were comparable to the effective doses for white Swiss mice bearing Sarcoma 180 tumors, and were well below the toxic levels of Holothurin for C57-B1-6J mice, none of the animals in this experiment showed any increase in the survival time. See Table 2.

## DISCUSSION

The crude extract of Holothurin used here was probably more potent than purified preparations would be. Upon purification, the anti-tumor principle is apparently lost or considerably reduced in con-

Table 2.  
Effects of Holothurin on C57-B1-6J Mice with Melanoma

No. of Mice	Sex	Dose (mg)	Frequency of injection	Survival Time (days)	Body Weight Change			
					5th day (g)	10th day (g)	15th day (g)	20th day (g)
10	M	0	0	29.4 (18-43)	+0.4	-0.7	-0.8	-0.3
5	M	0.05	daily	25.6 (20-42)	-1.1	-3.4	-3.5	-2.5
5	M	0.10	daily	22.7 (19-26)	-4.1	-4.8	-5.4	-4.4
5	M	0.15	daily	24.4 (20-35)	-3.1	-3.5	-4.3	-4.5
5	M	0.20	alternate days	29.6 (25-33)	-2.6	-3.5	-3.6	-3.3

centration (Chanley et al. 1960). One sample of purified Holothurin showed a tenfold-loss in hemolytic activity, and no anti-tumor activity (Nigrelli and Jakowska 1960).

Although the crude extract of Holothurin is effective against various tumors (Nigrelli 1952, and Sullivan, Ladue and Nigrelli 1955), the present study demonstrates that Holothurin is apparently not effective at the same dose levels in differing species of mice. Larionov (1967) suggests that a natural biological product should be more promising as a chemotherapeutic agent than a synthetic compound. The same author has stated that the maximum effective acceptable dosage of an anti-tumor compound is close to the dosage which kills the animal, whereas a decrease in dosage, even slight, leads to a sharp drop in effectiveness. The narrow threshold is seen here in regard to the ineffectiveness of the lower concentrations of Holothurin on the Sarcoma 180 tumors, and the lethal effect of 0.20 mg given over a period of several alternate days to the tumor-bearing mice. Due to the narrow range of effective concentrations of Holothurin in relation to its lethal dose, it is likely that the ineffectiveness of Holothurin in the C57-B1-6J mice bearing B-16 melanomas was a reflection of the tolerance to higher doses of Holothurin in these animals. There is no indication that Holothurin, or any other chemotherapeutic agent, would have differing effects in male and female tumor-bearing mice although profound metabolic differences between male and female rats and mice are well-known (Olmsted 1969). However, the differences seen



in this study could also be due to differences in the injection site or in the types of tumor. While this consideration presents many variables, the Sarcoma 180 tumor grows best in white Swiss mice and the B-16 melanoma grows best in C57-B1-6J mice. Further, it should be noted that the mean survival time of the untreated mice with the two different types of tumor was about the same, i.e., about 3 to 4 weeks after tumor inoculation. The hypothesis is further supported by the finding that Holothurin is many times more effective in causing hemolysis than saponin when living red blood cells are used in the test system.

Other hemolysis experiments similar to those described here have shown that between 0.04 and 0.10 mg of the crude extract of Holothurin would cause complete hemolysis, while 0.08 to 0.10 mg of saponin was required for the same effect (Nigrelli and Jakowska 1960). The apparent 40 to 100-fold discrepancy between the findings of Nigrelli and Jakowska and the findings of the present study could be accounted for by the use of different dilutions of red blood cells, or by differences in the age of red blood cells used, or by species effects. Nigrelli and Jakowska's data suggest that Holothurin is only slightly more effective in causing hemolysis than saponin, while in the present study using red blood cells that were freshly obtained from mice just prior to testing it would appear that Holothurin is some 250 to 500 times more effective than saponin in causing hemolysis. Since a number of studies have shown Holothurin to have profound biological effects in concentrations as low as 0.01 ppm it is not surprising that this biotoxic principle is much more effective than saponin on fresh red blood cells. It would therefore appear that the action of Holothurin involves more than a surface tension lowering effect on living cells.

Because Holothurin is an effective anti-tumor agent at concentrations only slightly lower than the lethal dose, it might be tentatively hypothesized that Holothurin is acting on some process common to all cells, not just tumor cells. However, no studies have been done on the metabolic effects of Holothurin. The studies reported here indicate that the mode of action of Holothurin in retarding tumor growth and prolonging life of tumor-bearing mice is probably different from the action of a surfactant such as saponin. It is likely that the energy requirements of the white Swiss mice with Sarcoma 180 differed from those of the C57-B1-6J mice bearing B-16 melanoma tumors. Other studies from this laboratory on the C57-B1-6J mice with B-16 melanoma tumors have shown that the growth of melanoma tumor occurs at the expense of the anabolic processes of the host mouse. Structural phospholipids synthesized by the tumor were shown to be derived from synthesis in the liver (Terranova, Hardy and Olmsted 1970). Although several experiments have been reported in the literature regarding saponin, none have shown saponin to have any anti-tumor activity *in vivo*. It is likely that saponin destroys cells simply by its

action to lower surface tension starting with the plasma membrane and continuing its action on membranes of other cellular constituents in proportion to the concentration of saponin used. While the action of Holothurin has some yet unknown effect other than, or in addition to, its surfactant effect, it is likely that it causes an inhibition of an active, or energy-dependent, physiological process.

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