

ORIGINAL ARTICLE

ANTITUMOR ACTIVITY OF *Vibrio sp.* ISOLATED BIOPOLYMERS IN INDUCED BREAST CANCER IN RATS

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ABSTRACT

Objective: To evaluate the antitumor activity of the raw extract from biopolymers isolated from the *Vibrio sp.* marine bacteria in breast cancer induced by N-Methyl-N-nitrosourea (MNU) in rats. **Materials and methods:** The *Vibrio sp.* marine bacteria was cultured for seven days, then the raw supernatant was filtered, precipitated and concentrated. MNU was administered in a single dose of 50 mg/kg to 39 Holtzman rats and were daily treated for 9 weeks orally: G1 (n = 13): 0.1 mL/100 g of saline solution; G2 (n = 13): 20 mg/kg of raw extract from *Vibrio sp.* biopolymers; G3 (n = 13): 100 mg/kg of tamoxifen; G4 (n = 11) received no MNU and only 0.1 mL/100 g of saline solution. Body weight and the appearance of breast tumors identified by palpation were assessed weekly, as well as histopathological examination at the end of treatment. **Results:** Seventy-seven percent of the rats in the G1 group developed tumors from week 7 onwards in an average of 2.2 tumors per animal; in contrast to the group treated with the raw biopolymer extract and tamoxifen; where only one rat (8%) in each group developed tumors after week nine of induction (p = 0.001). The histopathological results support that all the removed tumors correspond to breast ductal adenocarcinoma with different patterns: solid, papillary and cystic. Likewise, necrotic foci were evidenced in 30% of the tumors of the G1 group. **Conclusion:** The raw extract of biopolymers isolated from *Vibrio sp.* present antitumor effect in breast cancer induced in rats.

Keywords: Biopolymers; *Vibrio*; Methylnitrosourea; Breast Cancer (source: MeSH NLM).

INTRODUCTION

Breast cancer is a social health problem due to its high incidence, the mortality rate, therapeutic complexity and the high cost of treatment^(1,2). Among the therapeutic options, chemotherapy is linked to potential multisystemic adverse effects that result in the lack of adherence to treatment and an increase in morbidity and mortality. This has led to an increased search in recent decades for new substances with anti-tumor potential as alternative treatments, such as natural products derived from marine organisms, which account for more than 60% of the antineoplastic agents currently used⁽³⁻⁵⁾.

Various peptides, produced by marine species and their host bacteria, present a wide range of biological activity with antimicrobial, antitumor and antiviral action. Among the mechanisms that involve antineoplastic effects, the blocking of cell division, which mainly affects tubulin, is similar to the effect of alkaloid drugs like vinca and taxanes⁽⁶⁾. Drugs derived from marine peptides such as trabectedin, cytarabine, vidarabine, and ziconotide have been approved by the European Union and the Food and Drug Administration (FDA) for treating advanced stages of soft tissue sarcoma and ovarian cancer⁽⁷⁻¹⁰⁾. An estimated 118 natural marine products (NMP) are in pre-clinical trials; 22 NMP are in clinical trials; and 4 NMP are in the pharmaceutical market⁽¹¹⁾.

Bacteria of the genus *Vibrio* are known to be indigenous members of the bacterial biota of the seas and estuaries, and constitute 0.1 to 60% of the total heterotrophic bacteria. There are

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studies that show that the crude extract produced by some species of this genus present antibacterial activity against *Staphylococcus aureus*, resistant to methicillin, and cytotoxic activity against some cell lines⁽¹²⁾. Other studies indicate that peptides obtained from the genus *Vibrio* present cytotoxic action against the murine leukemia P388 cell line⁽¹³⁾. Also, one of the compounds produced by a marine member of Vibrionaceae is in a clinical trial for the treatment of prostate, lung and liver cancer and possibly these metabolites will sensitize the tumor cells to macrophage-mediated cytotoxicity⁽¹⁴⁾.

Consequently, the evaluation of the anti-tumor activity of peptides derived from marine *Vibrio* sp. constitutes an important line of biotechnological and pharmacological research; however, further studies are required to determine the efficacy of their biological activity in solid tumors such as breast cancer. This study aims to evaluate the anti-tumor effect of biopolymers isolated from the marine bacterium *Vibrio* sp. on N-Methyl-N-nitrosourea (MNU)-induced breast cancer in rats.

MATERIALS AND METHODS

Population and sample

Experimental study conducted with two comparable groups. One group (n = 13) with MNU-induced breast cancer and a second group (n = 11) with animals without the disease. The assignment of the experimental subjects was randomized. All pharmacological trials were conducted at the Pharmacology Research Laboratory of the Faculty of Medicine, Universidad Nacional Mayor de San Marcos (UNMSM).

Animals

A total of 50 female 21-day-old rats of the Holtzman strain acquired from the Peruvian Instituto Nacional de Salud (INS) were used. They were conditioned in the Bioherium of the UNMSM School of Medicine, in stainless steel cages with a light-dark cycle (12-12 hours), at an approximate ambient temperature of 23 °C. They also received a balanced diet of pellets and water *ad libitum*. The conditioning time prior to the start of the experiment was seven days. All animals were treated according to INS guidelines for the care and use of experimental animals⁽¹⁵⁾.

Procedure

Micro-organisms and culture conditions

The marine bacterium *Vibrio* sp. was provided by the strain depository of the Instituto del Mar del Peru (IMARPE) (Station E14-Pisco). The genus *Vibrio* was selected because it

KEY MESSAGES

Motivation for the study: Glycoproteins from marine species are an alternative for treating cancer. The aim is to contribute to scientific progress in oncological pharmacology with the search for new drugs derived from marine bacteria that offer preventive or therapeutic efficacy, with a good safety profile for the treatment of breast cancer.

Main findings: The crude extract of biopolymers isolated from *Vibrio* sp. presents antitumor effect in breast cancer induced in rats.

Implications: To promote the anti-tumor potential of biopolymers isolated from *Vibrio* sp. in mammary adenocarcinoma as an effective and safe adjuvant alternative.

has been reported that it produces bioactive metabolites and that the crude extract has shown antibacterial and cytotoxic activity against some cell lines^(12,16); the inhibitory activity of this genus indicates that it would also have antitumor activity.

The preparation of the bacteria culture medium was carried out in four liters of sterile seawater enriched with D-glucose (5 g/L) and meat peptone (3 g/L) where the bacteria were inoculated according to standard protocol and shaken manually and daily during five minutes for seven days at room temperature.

Once the fermentation was completed, the culture medium was centrifuged for 30 minutes, thus removing the remaining cells and solids. The supernatant was filtered through a Microfil support system (Merck Millipore) with a 0.2 µm bacterial filter to remove bacteria present in the supernatant.

Extraction of the crude biopolymer through fermentation

The concentrated (30 mL) supernatant filtrate was precipitated with ammonium sulfate ($[(\text{NH}_4)_2\text{SO}_4]$ saturated solution to a final concentration of 70%. It was left to stand for 24 hours at 4 °C, then 400 g were centrifuged for 30 minutes, obtaining a yellowish-white precipitate that was dissolved in the minimum possible volume of distilled water. The resulting product was taken to an oven to dry at a maximum temperature of 40 °C. The final product was dissolved in distilled water at a concentration of 20 mg/mL.

Evaluation of anti-tumor activity of biopolymers

A total of 39 rats were used for cancer induction with a single dose of MNU (Sigma, St. Louis, MO, USA) 50 mg/kg

intraperitoneally (IP). According to this model, MNU-induced tumors exhibit estrogen-dependent hormone regulation, subject to growth factors similar to that described for human breast cancer⁽¹⁷⁾.

The animals were then randomly distributed into three groups and treated orally (PO): G1 (n = 13): saline 0.1 mL/100 g; G2 (n = 13): crude extract of biopolymers isolated from *Vibrio sp.* 20 mg/kg; G3 (n = 13): tamoxifen 100 mg/kg. A final group received no MNU and was treated with 0.1 mL/100 g, it was named G4 (n = 11). Treatments were administered daily, six days per week, at the same time for nine weeks.

Assessment of anti-tumor activity

The animals' body weight and diet consumption were evaluated every week. The rats were examined by the same evaluator, who palpated the mammary line for evidence of tumors and recorded their time of appearance. At the end of the experiment, the animals were euthanized with 100 mg/kg of sodium pentobarbital. The number, weight, size and location of each palpable tumor was recorded, and the visible presence of metastases in the lymphatic chain and other organs was evaluated.

All tumors were excised, fixed in 10% neutral formalin and then processed by a pathology technician using routine methods, under uniform fixation and paraffin inclusion conditions, and stained in 6 mm sections with hematoxylin and eosin. The following histopathological criteria were considered to determine the malignancy of the breast tissue samples: loss of tubular-alveolar pattern of the normal mammary gland, presence of large epithelial cells with an increased nuclear cytoplasmic ratio, stromal response by fibrosis and infiltration of inflammatory cells, necrosis and hemorrhage⁽¹⁸⁾. The reading of the slides was performed by a specialized pathologist at the Institute of Pathology of the Faculty of Medicine of the UNMSM.

Statistical analysis

Numerical variables were expressed as mean and standard deviation (SD), while categorical variables were expressed as relative frequencies. Significant differences between the three treatment groups were determined using the Kruskal-Wallis test. When intra-group differences were found, the Mann-Whitney U test was used for peer-to-peer comparisons between treatment groups. For qualitative variables regarding the presence or absence of tumor, linear-by-linear association was used. $p < 0.05$ was considered statistically significant.

Ethical aspects

The animal care and handling was conducted according to the INS institutional guide for rodent care. For the purpose

of this experiment, the pentobarbital-induced euthanasia method was chosen: 100 mg/kg. This procedure is considered an acceptable method for rodents according to the publications "Report of the AVMA Panel on Euthanasia" of the American Veterinary Medical Association (1993) and "Euthanasia of Experimental Animals" of the European Union (1995)⁽¹⁹⁾.

RESULTS

One of the parameters observed to assess disease severity was body weight gain at the end of treatment. The group treated with only the cancer inducer (MNU) presented a mean weight gain of 115 g; while animals treated with MNU + crude extract of biopolymers isolated from *Vibrio sp.* showed a 141 g mean weight gain, this difference being significantly higher ($p = 0.03$) to the MNU group and close to the control group (133 g) (Figure 1).

Figure 2 shows the average number of animals that developed tumors and shows that 76.9% of the rats in the control group developed tumors at the end of the experiment. Only 7.7% of the animals in the MNU + crude biopolymer of the marine bacteria *Vibrio sp.* groups and MNU + tamoxifen developed tumors ($p = 0.001$). The most frequent locations were axillary, suprainguinal and suprapubic on both sides of the mammary chain. The latency period for tumor occurrence was 7 weeks for the control MNU group and 9 weeks for the MNU + biopolymer group.

The total number of tumors observed in the control group was 22 with an average of 2.2 tumors per animal, while only one tumor was evident in each of the MNU + crude biopolymer extract of *Vibrio sp.* groups and MNU + tamoxifen, as shown in Table 1. In addition, it was observed that the average weight of the tumor in the group treated with crude biopolymer extract was greater than that treated with MNU alone, however, for the tamoxifen treated group, the tumor developed, was significantly smaller than the control.

According to the histopathological results, all the removed tumors correspond to breast ductal adenocarcinoma with different patterns: solid, papillary and cystic. Likewise, necrotic foci were observed in 30% of the tumors found in the MNU group, suggesting a greater invasive capacity of the tumor (Table 2).

DISCUSSION

Marine organisms and their metabolites represent an enormous potential of unexplored natural resources and therapeutic products. Bacterial proteins and peptides derived

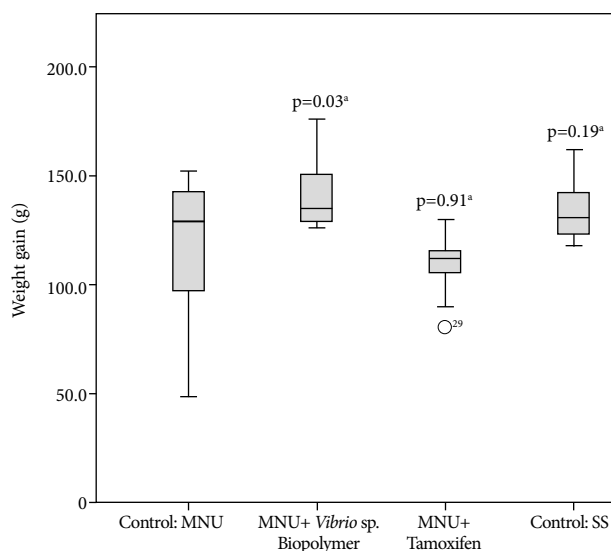


Figure 1. Weight gain at the end of the study in animals treated with marine bacteria *Vibrio* sp. biopolymers.

MNU: methyl-nitrosurea; SS: saline solution

^a p-value of the U Mann-Whitney test for paired comparisons in relation to the MNU control.

from marine organisms are a promising group of bioactive compounds and possible anti-cancer drugs; these include anti-cancer antibiotics (actinomycin D, bleomycin, doxorubicin, mitomycin C) and bacterial toxins used in cancer treatment, while other substances are in clinical trials or have been tested in *in-vitro* research^(20,21).

Using a standard model induced by MNU, it has been possible to reproduce breast cancer in rats; this has allowed us to explore *in-vivo* the anti-tumor activity of biopolymers extracted from the marine bacterium *Vibrio* sp. culture.

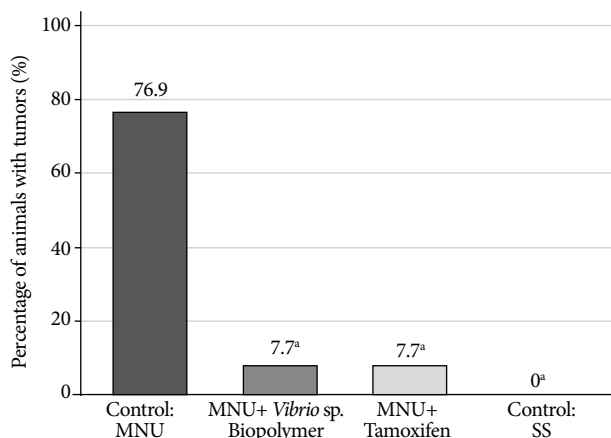


Figure 2. Percentage of palpable tumors in animals treated with marine bacteria *Vibrio* sp. Biopolymers.

MNU: methyl-nitrosurea; SS: saline solution.

^a Value of $p < 0.001$ for linear by linear association in relation to MNU control.

According to the results, the latency period for tumor occurrence in the control group was 7 weeks post-induction; while in the MNU + biopolymer treated group, it was 9 weeks, similar to the tamoxifen treated group, which would demonstrate that the biopolymers would delay the occurrence of MNU-induced tumors. In addition, we note that in the biopolymer-treated groups only 7.7% of the animals developed tumors in contrast to the control group where 77.9% of the animals developed tumors with an average of 2.93 per animal.

Although the protection mechanism is not known, other authors postulate that in solid tumors, biopolymers extracted from marine bacteria would have an immunomodulatory effect, activating the mechanisms against tumor cells. This would produce some tumor regression and, consequently, the latency period for tumor appearance and the life of the animals would be extended⁽²²⁾.

Studies with the *Vibrio* genus isolated from marine species show that it produces substances with antagonistic activity against other pathogenic bacteria⁽¹⁶⁾. Conde, *et al.*⁽¹²⁾ evaluated the cytotoxic and dereplication activity of the fractions extracted from the culture medium of *Vibrio diabollicus*; the authors showed that the F4 and F6 fractions were cytotoxic against the human cervical epithelial cancer cell line, SiHa, with an IC50 of >100 µg/mL and 80 µg/mL, respectively. On the other hand, the F5 fraction showed higher cytotoxic activity with an IC50 of 28 µg/mL against the same cell line. Furthermore, none of these fractions showed inhibition of the non-tumor cell line (CI50 >100 µg/mL).

Cao, *et al.*⁽²³⁾ investigated the molecular mechanisms of the polysaccharide EPS11, of marine bacterial origin (*Bacillus* sp.), on cytotoxicity in small-cell lung cancer. The authors found that EPS11 significantly affects cell proliferation and blocks adhesion in tumor cells. In addition, the expression of several proteins associated with cell adhesion is negatively regulated and the filiform structures of lung cancer cells are destroyed after treatment with EPS11. This same biopolymer has also been shown to inhibit liver cancer cell growth by blocking cell adhesion and attenuating the formation of filiform structure, and stopping cancer cell metastasis⁽²⁴⁾.

Studies about the action of biopolymers derived from marine bacteria on breast cancer have only been conducted in *in-vitro* models of breast carcinoma cell lines. Mahgoub *et al.*⁽²⁵⁾ evaluated the effect of a biopolymer isolated from the marine bacterial strain, *Bacillus velezensis*, on breast cancer cell lines MCF-7. According to published results, this metabolite increases apoptosis and hinders the proliferation of MCF-7 cells by 5-80 µg/mL compared to the control group. In addition, induced apoptosis is associated with activation of caspase-3 which is dose-dependent and does not have cytotoxicity against normal cells; similar results were reported by Sirpu *et al.*⁽²⁶⁾ with the partially purified

Table 1. Number of palpable tumors and average weight of tumors in rats treated with marine bacteria *Vibrio* sp. biopolymers.

Variable	Control: MNU	MNU + biopolymer	MNU + tamoxifen	Control: SS	p-value ^a
Total number of tumors	22	1 ^b	1 ^b	0 ^b	<0.001
Tumors mean weight (g)	2.02	2.98	0,26	0	-

MNU: methyl-nitrosurea; SS: saline solution

^a p-value obtained with the Kruskal Wallis test.^b Mann-Whitney U test for paired comparisons in relation to the MNU control: p < 0.01.

crude extract of marine NMK17 *Bacillus subtilis* (PPCEBS) in the human MCF-7 breast cancer cell line. The findings demonstrated that PPCEBS significantly induces apoptosis in the studied cell line and would result in increased expression of caspase-3 and Bax revealing the possible mechanism of the apoptosis-inducing property. Similarly, PPCEBS also presented antibacterial and antioxidant activities.

These results suggested that the compounds present in PPCEBS of marine bacteria *B. subtilis* NMK17 would be cytotoxic metabolites that could be candidates for developing a drug specific to breast line tumor cell apoptosis, with minimal toxicity. However, other authors observe that two new cyclic hexapeptides, venturamide A and B obtained from the marine cyanobacterium *Oscillatoria* sp. have only mild activity when tested against breast cancer cells MCF-7 with a CI50 value of 13.1 and >54 μ M, respectively⁽²⁷⁾. Likewise, when pitiprolamide, a cyclic depsipeptide derived from the marine cyanobacterium *Lyngbya majuscula*, was isolated, only weak cytotoxic activity against the colorectal carcinoma HCT116 and the breast adenocarcinoma cell lines MCF7 was noted (CI50 33 μ M for both)⁽²⁸⁾.

Besides the anti-tumor effect, it was observed that weight gain was significantly higher for the group treated with crude biopolymer extract derived from *Vibrio* sp. bacteria in relation to the control group (Figure 2). These results have a positive anti-tumor implication since the weight loss in cancer is due to an increased caloric demand due to the tumors' presence (with the corresponding competition for nutrients between the patient's cells and those of the tumor) and malnutrition due to anorexia caused by pro-emaciation cytokines released during the progression of the disease⁽²⁹⁾.

On the other hand, only one animal in the biopolymer-treated group presented a tumor, the tumors' weight

was higher than the average weight of the control. However, this was only observed in one tumor and in one animal in a ratio of 1/13 in contrast to the control group where there was an average of 2.2 tumors per animal and in a ratio of 10/13; therefore, it could be considered as an isolated event.

Sample size extension and biopolymer dosing are required to obtain a dose-response curve. In addition, the observation time needs to be extended to assess the effect of biopolymers on the natural course of the disease. Although the MNU-induced breast cancer model replicates many pathophysiological features of what is observed in human disease, an in-vivo model with a human tumor cell line infiltration in experimental animals would contribute greatly. Some mechanisms of action of the anti-tumor effect suggested could be related to the activation of tumor cell apoptosis and activation of the immune system, as has been observed in cultures with other bacteria^(24,26).

The main limitation of this study is that it has not been possible to isolate, identify or perform chemical characterization of the biopolymers obtained and only their crude extract has been evaluated. It would be necessary to perform fractionation and pharmacological tests to identify the fraction with the best antitumor activity. Although it is true that three groups were included (a control group of healthy animals, a group with the disease and another group treated with a standard drug for the treatment of breast cancer), our limitation was not to include an experimental group treated with the biopolymer-free culture medium, and we did not perform acute and chronic toxicity tests on the biopolymers.

Results show that the crude extract of the bacteria *Vibrio* sp. can constitute a source of biopolymers with anti-tumor activity. Therefore, it is suggested to deepen research about breast cancer, as well as its possible mechanism of action and

Table 2. Histopathological findings grouped according to treatment, in the induction of breast cancer by methyl-nitrosurea.

Group	Main histopathological findings in resected tumors	Number of cases
Control: MNU	Breast ductal adenocarcinoma with different patterns: solid, papillary and cystic, stromal response by fibrosis and infiltration of inflammatory cells with necrotic foci.	10
MNU + biopolymer	Breast ductal adenocarcinoma with papillary pattern.	1
MNU + tamoxifen	Breast ductal adenocarcinoma with different patterns: solid, papillary and cystic.	1
Control: SS	Mammary gland with normal characteristics without histopathological alterations.	0

MNU: metilnitrosurea; SF: suero fisiológico

the active metabolite. The extrapolation of the expected benefits with the application of special isolation techniques of the active principle would allow to be a potential candidate in the prevention and treatment of neoplastic diseases.

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of the article. DFC, EVP, MPY and JRTD performed the critical review of the article. DCR prepared the isolation process and the biopolymers of marine bacteria *Vibrio* sp. All authors participated in the evaluation of results, their interpretation, the approval of the final version of the manuscript and assume responsibility for the contents of this article.

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