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REVIEW ARTICLE

Parasporins as new natural anticancer agents: a review

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Summary

In 1999 Mizuki and co-authors studied for the first time the parasporal inclusion proteins extracted from B. thuringiensis strains (a Gram-positive, soil-dwelling bacterium) for cytotoxic activity against human leukaemia T-cells. Later some other proteins with this unusual property to recognize human leukemic cells were isolated from this strain of bacteria and named parasporins. At present 6 types of parasporins are identified and characterized. This review summarizes the properties of these new potentially useful antitumor agents of natural origin. Various types of parasporins possess unique cytotoxic mechanisms against cancer cells. The cytotoxic activity for cancer cells makes parasporins possible candidates for anticancer agents in clinical oncology. Recently, genetic engineering was applied for the production of parasporins and the gene responsible for the production of the proteins was expressed in E. coli. However, there are virtually no data regarding the cytotoxic (antitumor) activity of parasporins in vivo. These relatively new cytotoxic proteins warrant further investigation, especially in rodents, for possible application in clinical oncology.

Key words: *B. thuringiensis, cytotoxicity, human cancer cell lines, parasporins*

Introduction

In 1911 Berliner described for the first time a new strain of bacillus, *Bacillus thuringiensis*, isolated from the Mediterranean flour moth *Anagastakuehniella* [1]. He named it after the province Thuringia in Germany where the infected moth was found. Roh et al. [2] noted that the strain firstly appeared under this name but it was isolated in 1901 by the Japanese scientist Ishiwata Shigetane as the causative agent of a disease afflicting silkworms.

The bacterial strain was first thought as a threat for silkworms but later it was used as insecticidal agent. The first commercial production of the agent as pesticide was in France in 1938 under the name "Sporeine" [3]. Then, in 1956 Canadian microbiologist Angus demonstrated that the crystalline protein inclusions formed in the course of sporulation were responsible for the insecticidal action [4].

Afterwards, American scientists Gonzales et al. [5] found that the genes coding for crystal (Cry) proteins were localized on transmissible plasmids, using a plasmid curing technique.

Schnepf and Whiteley [6] first cloned and characterized the genes coding for Cry proteins that had toxicity to larvae of the tobacco hornworm, from plasmid DNA of *B. thuringiensis*, subspp. *kurstaki* HD-1. This first cloning was followed quickly by the cloning of many other *Cry* genes and eventually led to the development of *B. thuringiensis* transgenic plants. In the 1980s, several scientists successively demonstrated that plants can be genetically engineered, and finally, *thuringiensis* cotton reached the market in 1996 [2,7].

The insecticidal bacterium *B. thuringiensis* is an aerobic Gram-positive sporeforming bacterium that produces proteinaceous inclusions during sporulation [2]. These inclusions can be distinguished as distinctively shaped crystals by phase-contrast microscopy. The inclusions are composed of proteins known as Cry proteins, or δ-endotoxins, which are highly toxic to a wide va-

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riety of important agricultural and health-related insect pests as well as other invertebrates (mostly specific for insects and nematodes) [2]. Because of their high specificity and their safety for the environment, Cry proteins are a valuable alternative to chemical pesticides for control of insect pests in agriculture and forestry and in the home. It has been proposed that the rational use of Bt toxins will provide a variety of alternatives for insect control and for overcoming the problem of insect resistance to pesticides [2]. It is well known now that the insecticidal activity of Cry proteins is induced by its specific binding to the receptor located on the plasma membrane of the mid-gut epithelial cells of susceptible insects [8]. This property makes *B. thuringiensis* an environmentally safe and ecologically sound microbial agent in controlling agricultural insect pests [8].

B. thuringiensis seems to be indigenous to diverse environments. Strains of this bacillus were isolated worldwide from soil, insects, stored-product dust, and deciduous and coniferous leaves [2,9]. *B. thuringiensis* belongs to the Bacillaceae family and is closely related to *B. cereus* [9]. The only notable phenotypic difference between these two species of bacilli is the production of one or more insecticidal crystals. However, there are many crystal-producing *B. thuringiensis* strains without known insecticidal activities. In addition, many acrystalliferous strains have been described and can be easily obtained by chemical mutagenesis or by plasmid curing, resulting in cells phenotypically indistinguishable from *B. cereus*. Furthermore, recent studies of a series of molecular markers in a large set of strains have indicated that *B. thuringiensis* and *B. cereus* should be considered as one species [2].

The bacillus has also been used successfully to suppress the population levels of medically important dipteran pests. Examples include the mosquito vectors of malaria, virus diseases (including dengue hemorrhagic fever and West Nile fever) and lymphatic filariasis, and the black-fly that transmits onchocerciasis [8]. Of particular interest in the recent findings is that a therapeutic activity against the human and animal hookworm parasite is associated with a unique nematode-killing *B. thuringiensis* Cry protein [10].

Since the bacillus can kill parasites, it was studied for its activity against *Trichomonas vaginalis* [11], a sexually transmitted infection which affects 170 million persons worldwide [12]. Kondo et al. [11] studied parasporal inclusion proteins from a total of 816 *B. thuringiensis* strains isolated in Japan for antitrichomonal activity against *T. vaginalis*. Ten strains of *B. thuringiensis* inhibited the growth of *T. vaginalis* in 48-h cultures. Moreover, two strains, B622 and B626, clearly showed trichomonacidal effects against *T. vaginalis*. The H antigen serotypes of both strains were identified as H13/29 (pakistani/amagiensis). The parasporal inclusion protein from both strains consisted of 3 major polypeptides of 77, 45 and 25 kDa [11].

The U.S. Food Quality Protection Act and the European Economic Council directives aimed at reducing the use of carbamate and organophosphate insecticides were expected to increase the use of narrowly targeted "soft" compounds like *B. thuringiensis* [13]. Proteins obtained from this bacterial strain are highly active *in vitro* and *in vivo* against a globally significant nematode parasite [10]. These proteins (parasporins) warrant further clinical development for human and veterinary use [10].

Discovery of parasporins

In 1999 Mizuki et al. [14] studied for the first time parasporal inclusion proteins from a total of 1744 *B. thuringiensis* strains for cytocidal activity against human leukaemia T-cells and haemolytic activity against sheep erythrocytes. Mostly B. thuringiensis strains had no haemolytic activity (1684 strains), but 42 exhibited *in vitro* cytotoxicity against leukaemia T- cells. These non-haemolytic but leukaemia cell-toxic strains belonged to several H-serovars including *dakota, neoleonensis, shandongiensis, coreanensis* and other unidentified serogroups. Purified parasporal inclusions of the 3 selected strains, designated 84-HS-1-11, 89-T-26-17 and 90-F-45-14, exhibited no haemolytic activity and no insecticidal activity against dipteran and lepidopteran insects, but were highly cytocidal against leukaemia T-cells and other human cancer cells, showing different toxicity spectra and varied activity levels. Furthermore, the proteins from 84-HS-1-11 and 89-T-26-17 were able to discriminate between leukaemia and normal-T cells, specifically killing the leukaemic cells. The scientists concluded that these findings may lead to the use of *B. thuringiensis* inclusion proteins for medical purposes [14].

Mizuki et al. [15] investigated further an unusual property of human leukemic cell-recognizing activity, associated with parasporal inclusions of *B. thuringiensis* and found a protein (named parasporin) responsible for the activity. Afterwards they cloned it [15]. The parasporin, encoded by a gene 2,169 bp long, was a polypeptide of 723 amino acid residues with a predicted molecular

weight of 81,045. The sequence of parasporin contained the 5 conserved blocks commonly found in *B. thuringiensis* Cry proteins; however, only very low homologies (<25%) between parasporin and the existing classes of Cry and Cyt proteins were detected. Parasporin exhibited cytocidal activity only when degraded by proteases into smaller molecules of 40 to 60 kDa. Trypsin and proteinase K activated parasporin, while chymotrypsin did not. The activated parasporin showed strong cytocidal activity against human leukemic T-cells (MOLT-4) and human uterus cervix cancer cells (HeLa) but not against normal T-cells [15].

Types of parasporins

According to the Committee of Parasporin Classification and Nomenclature (http://parasporin.fitc.pref.fukuoka.jp/), the 81-kDa protein constitutes the parasporin family -1Aa (PS1Aa) in the current classification scheme (it contains three-domain Cry protein, activated by *N*-terminal digestion) [8]. This PS1Aa1 protein, corresponding to Cry31Aa1, is a polypeptide of 723 amino acid residues with a predicted molecular weight of 81,045. The gene is 2,169 bp long. This protein has a structure of the three-domain type, whose amino acid sequence contains five conserved blocks commonly retained in insecticidal Cry proteins [8,14,15].

Subsequent investigations have established three additional families of parasporin: parasporin-2 (PS2, contains non-three-domain type, activated by *N*- and *C*-terminal digestion) [16]. This protein PS2Aa1 (Cry46Aa1) is a polypeptide of 338 amino acid residues with a predicted molecular weight of 37,446. The gene is 1,014 bp long. Unlike PS1Aa1, PS2Aa1 is not a protein with three domains, lacking the block sequences conserved in insecticidal Cry proteins. This protein shares a low sequence homology only with Cry15Aa among the existing classes of Cry and Cyt proteins of *B. thuringiensis* [8,17].

Rarasporin-3 (PS3) contains three-domain Cry protein, activated by *N*- and *C*-terminal digestion [8]. This PS3Aa1 protein, corresponding to Cry41Aa1, has a typical three-domain structure with five block sequences commonly conserved in the known insecticidal Cry proteins. It consists of 825 amino acid residues with a deduced molecular weight of 93,689, sharing low homologies with insecticidal Cry proteins. It is of interest to note that the *C*-terminal sequence of PS3Aa1 is similar to that of *Clostridium botulinum hemagglutinin* HA-33 [18]. Proteolytic digestion is required for activation of PS3Aa1; the 81-kDa precursor is converted to the 64-kDa toxic moiety by proteolytic processing of both *N*- and *C*-terminal regions [8].

Parasporin-4 (PS4) contains non-three-domain type, activated by *C*-terminal digestion [19]. This PS4Aa1 protein (Cry45Aa1) comprises 275 amino acid residues, with a predicted molecular weight of 30,078. The gene is 828 bp long. None of the five block sequences, commonly conserved in most of the Cry proteins (including PS1Aa1 and PS3Aa1), is retained in PS4Aa1. The three-domain structure is not associated with this protein [8].

Parasporin-5 (Cry64Aa) has been isolated from the *B. thuringiensis* strain A1100 by Ekino and Shin in 2009 [20]. Not much has been reported for this parasporin. Sequence analysis reveals that parasporin-5 (Cry64Aa) is an Epsilon/Mtx like toxin [21].

Parasporin-6 (Cry63Aa) has been isolated from the *B. thuringiensis* strains M109 and CP84 [22]. Sequence analysis suggests that it is a three-domain Cry toxin. The proteolytically active toxin was cytotoxic against human hepatocellular cancer HepG2 cells and uterine cervix cancer HeLa cells. The treatment was accompanied by swelling of the cells and formation of vacuoles in the cytoplasm of the sensitive cells [22]. Not much work has been reported on this mammalian Cry toxin.

A total of 13 Parasporin proteins have been isolated from 11 strains of *B. thuringiensis*. Of these, 8 proteins are allied to PS1, 2 to PS2, 2 to PS3 and 1 to PS4 [8].

Some recent studies have provided evidence that the organisms with parasporin activities are among the common members in *B. thuringiensis* natural populations occurring in many countries [23,24].

Mizuki et al. [14,15] have reported that some *B. thuringiensis* strains do not have insecticidal or hemolytic activities but they do have mammalian cell recognition and killing activity. They showed that some proteins extracted from these strains of *B. thuringiensis* are very active against human cervix cancer (HeLa) and human acute T-leukemia (MOLT-4) cells.

These activities are mediated by parasporins that have been classified by quaternary rank based on amino acid homology.

Recently Poornima et al. [25] found that the *B. thuringiensis* strain *B.t.* LDC-391 produces cytocidal proteins against the human colon cancer cell line HCT-116. Possibly, these proteins are new parasporins. The *B. thuringiensis B.t.*LDC-391

strain lacks β–hemolysis on human erythrocytes which is a common feature of other parasporin producers.

Anticancer (cytotoxic) activity of parasporins

PS1Aa1 was very cytotoxic for HeLa (uterine cervical cancer), MOLT-4 (acute T-cell lymphoblastic leukemia), HL-60 (promyelocytic leukemia) and HepG2 (hepatocellular carcinoma) cells [16,26,27]. Recently Kuroda et al. [28] isolated a new Cry protein, PS1Ac2, from *B. thuringiensis* B0462 strain which was highly cytotoxic for HeLa, but not for MOLT-4 cells.

CryIAa toxin bound the cadherin repeat adjacent to the membrane (cadherin-like protein BtR175) and made a pore that passed inorganic ions, causing the cell to swell and burst [29]. This was not observed with a BtR175 variant, lacking the toxin-binding site. This *in vitro* experiment mimicked the specific insecticidal action of the toxin *in vivo* well [29].

Nagamatsu et al. [22,30] studied a mode of action of CryIA(a) toxin. Histological examination showed that this toxin acted on the brush-border membrane of the midgut columnar cells of lepidopteran insects and broke it in folding structure, causing cell lysis. The membrane vesicles were purified, and a 175-kDa protein binding the toxin was found that accounted for some 0.015% of membrane proteins. The protein, designated BtR175, was a glycoprotein that reacted with concanavalin A. Anti-BtR antibodies inhibited the binding of toxin to membrane vesicles *in vitro* and decreased the effect of the toxin to silkworms *in vivo*. BtR175, although found in the gut, was not found in fat bodies, integumentary structures, or silk glands [30]. These results indicated that BtR175 was the receptor protein for the insecticidal toxin. Proteins (137 and 107 kDa) binding the CryIA(a) toxin also were found in the gut membranes of *Tenebriomoritor* larvae, a coleopteran not sensitive to the toxin. The specificity of the toxin could not be explained only in terms of the existence of its binding protein [30].

In 2006 Kitada et al. [27] evaluated the cytotoxicity of parasporin 2 in 12 human cell lines. The highest activity was found in endometrial adenocarcinoma cells Sawano – 0.002 μg/ml. The activity of the protein was similar in HepG2, Caco-2, MOLT-4, Jurcat and HL-60 cells (range between 0.013 and 0.022 μg/ml). The least effectiveness was noticed in TCS (uterine cervical cancer) cells 7.8 μg/ml [27].

Kitada et al. [31] summarized the antitumor activity of 4 parasporins in a variety of human cancer cell lines (N=8) and normal human cell lines. Parasporin 1 was mostly effective in HeLa cells (human uterine cervix cancer cells) at a concentration of 0.12 μg/ml. Parasporin 2 was extremely effective in Sawano cells (endometrial cancer) at a concentration of 0.002 μg/ml. Parasporin 2 was quite active in HepG2 cells (hepatocellular carcinoma), HL-60 cells (promyelocytic leukemia) and MOLT-4 cells (acute T-cell lymphoblastic leukemia) at concentrations ranging from 0.019 to 0.022 μg/ml. Parasporin 3 was much less active than the previous 2 proteins. It inhibited the growth of HL-60 cells at high concentration (1.32 μg/ml). Parasporin 4 was mostly effective in Caco-2 (colorectal carcinoma) cells at a concentration of 0.124 μg/ml [31].

PS2Aa1 was highly active for Sawano (endometrial adenocarcinoma), TCS (uterine cervical cancer), MOLT-4 (acute T-cell lymphoblastic leukemia), HL-60 (promyelocytic leukemia), Jurkat (T-cell leukemia), HepG2 (hepatocellular carcinoma), A549 (lung adenocarcinoma) and Caco-2 (colorectal carcinoma) cells [16,26,27]. The 37 kDa inactive protein of this parasporin is proteolytically activated to a 30 kDa active form. This parasporin induced marked cytopathy in HeLa cells (human uterine cervix cancer cells) upon proteolytic activation [32]. The cytopathy was characterized by cell-ballooning, followed by gradual cell shrinking. Unlike HeLa cells, non-cancer UtSMC cells (human uterine smooth muscle cells) were not susceptible to PS1Ab1 [32].

The active form of the recombinant protein toxin was crystallized in the presence of ethylene glycol and polyethylene glycol 8000 at neutral pH [33]. Hayakawa et al. [34] reported about a novel Cry protein that exhibited potent cytotoxicity against human leukemic T-cells and which was cloned from the *B. thuringiensis* TK-E6 strain. The protein, designated as parasporin-2Ab (PS2Ab), was a polypeptide of 304 amino acid residues with a predicted molecular weight of 33,017. The deduced amino acid sequence of PS2Ab showed significant homology (84% identity) to parasporin-2Aa (PS2Aa) from the *B. thuringiensis* A1547 strain. Upon processing of PS2Ab with proteinase K, the active form of 29 kDa was produced. The activated PS2Ab showed potent cytotoxicity against MOLT-4 and Jurkat cells and the EC_{50} values were estimated as 0.545 and 0.745 ng/mL, respectively. The cytotoxicity of PS2Ab was significantly higher than that of PS2Aa. Although both cytotoxins

were structurally related, it was thought that the minor differences found were responsible for the different cytotoxicities of PS2Ab and PS2Aa [34]. Cytological and biochemical observations on PS2Aa showed that the protein is a pore-forming toxin. To confirm this hypothesis, Akiba et al. [35] have determined the crystal structure of its active form at a resolution of 2.38 Å. The protein was unusually elongated and comprised mainly long β-strands aligned with its long axis. It is similar to aerolysin-type-β-pore-forming toxins, which strongly reinforce the pore-forming hypothesis. The molecule can be divided into three domains. Domain 1, comprising a small β-sheet sandwiched by short α-helices, is probably the target-binding module. Two other domains are both β-sandwiches, thought to be involved in oligomerization and pore formation. Domain 2 has a putative channel-forming β-hairpin characteristic of aerolysin-type toxins. The surface of the protein has an extensive track of exposed side chains of serine and threonine residues. The track might orient the molecule on the cell membrane when domain 1 binds to the target until oligomerization and pore formation are initiated. The β-hairpin has such a tight structure that it seems unlikely to reform as postulated in a recent model of pore formation developed for aerolysin-type toxins. A safety lock model is proposed as an inactivation mechanism by the N-terminal inhibitory segment [35].

PS2Aa is thought to assemble on the cell surface via binding to lipid rafts, leading to cell lysis with typical blebs around peripheral cells. Kitada et al. [36] found that glycosylphosphatidylinositol (GPI)-anchored proteins are involved in the cytocidal actions. Depletion of the cellular cholesterol and loss of sphingolipid in lipid rafts slightly decreased cytolysis by PS2. Beyond those, the cells temporally resisted PS2Aa with reduction of the toxin binding after GPI-anchored proteins were cleaved off by phosphatidylinositol-specific phospholipase C. PS2Aa and aerolysin showed individual cytocidal specificity while aerolysin's receptor is GPI-anchored proteins. When Kitada et al. [36] confirmed the expression of GPI-anchored proteins on four cell lines, showing different cytotoxicity by PS2Aa, GPI-anchored proteins were evenly expressed on the cells. Therefore, PS2Aa requires a kind of GPI-anchored proteins for effective cytolysis [36].

PS3Aa1 was active only in HL-60 (promyelocytic leukemia) and HepG2 (hepatocellular carcinoma) cells [16,26,27].

In 2004 Okumura et al. [37] reported that *B. thuringiensis* strain A1470 (previously designated 89-T-34-22) exhibits cytotoxicity against MOLT-4 cells, and that the strain produces at least two cytocidal proteins. One of these cytocidal proteins was identified and named parasporin-4 (PS4). Later it was found that it is a unique β-pore-forming toxin with cholesterol-independent activity [38]. It was also shown that human leukemic T (MOLT-4) cells were >100 times more susceptible than HeLa and normal T-cells to the proteins of 89-T-34-22. The cytotoxicity was dose-dependent and the median effective concentration for the MOLT-4 was 3.5 μg/ml [39].

PS4Aa1 was active against Sawano (endometrial adenocarcinoma), TCS (uterine cervical cancer), MOLT-4 (acute T-cell lymphoblastic leukemia), HL-60 (promyelocytic leukemia), HepG2 (hepatocellular carcinoma) and Caco-2 (colorectal carcinoma) cells [16,26,27]. In another study cytotoxicity of PS4 was tested in 20 cultured mammalian cells (11 human tumor and 1 rat tumor cells, and 8 normal rodent tissue cells) [40]. The most active PS4 was against human colon cancer cells Caco-2 (EC_{50}=0.124 μg/ml) and human endometrial cancer cells Sawano (EC_{50} = 0.245 μg/ml) [40].

Careful analyses of these data show that PS2Aa1 and PS4Aa1 have higher cytotoxicity spectra than others. It is noteworthy that PS2Aa1 and PS4Aa1 each induces cell death in 6 out of 9 human cancer cell lines. Also, all of the three leukemic cell lines (MOLT-4, HL60 and Jurkat) are highly sensitive to PS2Aa1. PS3Aa1, a typical three domain-type Cry protein, exhibits moderate cytotoxicity against only 2 cancer cell lines, i.e. HL-60 (leukemic) and HepG2 (liver cancer). Hence, PS3Aa1 has the narrowest activity spectrum among the four parasporins. It is also noteworthy that the 3 cancer cell lines (HeLa, TCS and Jurkat) are monosensitive to one of the three proteins, PS1Aa1, PS2Aa1 or PS4Aa1 [8].

Some strains of *B. thuringiensis* produce more than one parasporin [22]. For example, *B. thuringiensis* (strain M019) which is non-pathogenic to lepidopteran and dipteran insects, produces three types of parasporal inclusion that consist of three 84-kDa Cry proteins. CP78A and CP78B, which exhibit 83.5% amino acid identity, were new variants of the previously reported HeLa cell-killing protein (parasporin-1). CP84 was a novel CP showing low-level homology of 21.9% (56.4% similarity) with the insecticidal Cry2 toxin. *In vitro* solubilization with dithiothreitol at pH 10.2 and limited hydrolysis with trypsin resulted in removal of N-terminal portions of the Cry proteins and

their activation. The 70-kDa proteins (15- and 55-kDa fragments) from CP78A and CP78B and the 73-kDa protein (14- and 59-kDa fragments) from CP84 exhibited varying degrees of cytocidal activity, preferentially against human hepatocyte cancer HepG2 cells and uterine cervix cancer HeLa, causing swelling or the formation of vacuoles in the cytoplasm. These toxins appeared to attack an identical target on human cells [22].

Okamura et al. [38] confirmed recently that the A1470 strain of *B.thuringiensis* simultaneously produces parasporin-2 and parasporin-4.

It is noteworthy that new parasporins are introduced. Poornima et al. [41] reported that activated inclusion protein from one of the isolates, *B. thuringiensis* LDC-391, was found to be highly cytotoxic to HCT-250 (adherent human colon cancer cells) and moderately toxic to U-937 (human histiocytic lymphoma cells) but nontoxic to normal lymphocytes. This strain did not show any insecticidal activity against the lepidopteran and dipteran larvae tested, as well as it was nonhaemolytic on human erythrocytes [41].

Namba et al. [42] isolated from the crystal produced by *B. thuringiensis* subspp. *coreanensis* A1519 strain a novel cytotoxic protein. Upon treatment of the Cry proteins by proteinase K, significant cytotoxicity against the leukemic T cell, MOLT-4, was exhibited.

The 29-kDa polypeptide proved to be an active component of the proteinase K-digested A1519 Cry proteins. EC_{50} of the purified 29-kDa polypeptide was 0.078 μg/ml.

A number of research groups reported about isolation of new parasporins. Uemori et al. [43] reported that they isolated from *B. thuringiensis* strains A1965, B0186, and B0195 new parasporal proteins. They were recovered from natural environments in three locations in Fukuoka Prefecture, Kyushu, Japan. When observed with a phase-contrast microscope, the three isolates produced large spherical parasporal inclusions that were firmly attached to spores.

These parasporins showed strong and rapid cytocidal activities against human uterine cervix cancer cells (HeLa), while showing no cytotoxicity on non-cancer uterine smooth muscle cells (Ut-SMC). The proteins of the three strains exhibited no insecticidal activities against three lepidopterous and one dipterous insect species. Furthermore, they had no hemolytic activity against sheep erythrocytes. Ouchterlony immunodiffusion tests revealed that the proteins of these strains were immunologically closely related to the group parasporin-1, Cry31Aa [43].

Yasutake et al. [44] studied 63 *B. thuringiensis* isolates obtained from urban soils in Vietnam. None of these isolates showed any larvicidal activity against lepidopterans. Noninsecticidal Cry proteins of 4 isolates had cytocidal activities against human cervix cancer HeLa cells. They also have hemolytic activities. These cancer cell-killing proteins proved closely related to parasporin-1 (Cry31Aa), not only immunologically but also phylogenetically [44].

In Table 1 are presented some data concerning the cytotoxicity of parasporins.

Mode of anticancer (cytotoxic) action of parasporins

Parasporins PS2Aa1, PS3Aa1 and PS4Aa1 induced cytotoxicity in cancer cells during 1h [16], unlike PS1Aa1 which induces cytotoxicity dur-

Table 1. Cytotoxic activities of parasporins in various human tumor and normal cells

Cells	*Characteristics of cells*	LC_{50} (μg/ml)			
		PS-1	*PS -2*	*PS-3*	*PS-4*
MOLT-4	Leukemic T cell	2.2	**0.022**	>10	0.472
Jurkat	Leukemic T cell	>10	**0.018**	>10	>2
HL-60	Leukemic T cell	0.32	**0.019**	**1.32**	0.725
T cell	Normal T cell	>10	- [a]	>10	>2
HepG2	Hepatocellular cancer	3.0	**0.019**	2.8	1.90
HC	Normal hepatocyte	>10	1.1	>10	>2
HeLa	Uterus (cervix) cancer	**0.12**	2.5	>10	>2
Sawano	Uterus cancer	>10	**0.0017**	>10	**0.245**
TCS	Uterus (cervix) cancer	-	7.8	>10	0.719
UtSMC	Normal uterus	>10	2.5	>10	>2
Caco-2	Colon cancer	>10	**0.013**	>10	**0.124**

[a] not studied; the most effective concentrations are shown in bold

ing 8–10 hrs [14,15]. This could be due to various mechanism of action of these proteins [8].

It was shown that PS1Aa1-treated HeLa cells show decrease in the levels of cellular protein and DNA synthesis. An increase of the intracellular Ca^{2+} concentration within 1-3 min after treatment of HeLa cells with this protein can also play a role in cytotoxicty. In this regard, it should be noted that suramin, an inhibitor of the trimeric G-protein signaling, suppresses both Ca^{2+} influx and cytocidal activity of PS1Aa1 [8]. There is also strong evidence that PS1Aa1 induces apoptosis in HeLa cells [8] because the cytotoxic activity of PS1Aa1 is suppressed by synthetic caspase inhibitors. Secondly, PS1Aa1 treatment leads to degradation of the apoptosis-related proteins procaspase-3 and poly (ADP-ribose) polymerase in HeLa cells [8].

Investigations on the cytotoxic action of PS2Aa1 show that, unlike PS1Aa1, it increases plasma membrane permeability of tumor cells [8,45]. Cytoplasmic lactate hydrogenase leaks from the intoxicated HepG2 cells, while the extracellular propidium iodide enters the cytoplasm. The initial step in the cytotoxic action of PS2Aa1 is the specific binding of the toxin to a putative receptor protein, as yet unidentified, located in the lipid raft of plasma membrane of tumor cells susceptible to this protein. This is followed by the formation of oligomers of PS2Aa1 in plasma membranes, leading to pore formation and cell lysis [45]. The oligomerization occurs in the presence of membrane proteins, lipid bilayer and cholesterols. It is noteworthy that a substantial homology exists in amino acid sequences between PS2Aa1 and *Clostridium perfringens* epsilon toxin whose cell-killing mechanism involves the toxin oligomerization in lipid rafts and pore formation in plasma membrane [45].

Abe et al. [46] studied the mode of action of PS2. They found that the toxin binds to the surface of target cells and increases the permeability of plasma membrane. Subcellular fractionation and immunoblot analysis of the toxin-treated cells revealed that the toxin bound to lipid rafts and formed SDS-resistant oligomers. The binding and oligomerization of the toxin were inhibited by treating the cells with phosphatidylinositol-specific phospholipase C. Thus, the interaction of parasporin-2 with glycosyl phosphatidylinositol-anchored proteins was required for the formation of oligomeric toxin that could permeabilize the plasma membrane [46]. Abe et al. [47] investigated the mode of action of parasporin-2 in human HepG2 cell line (hepatoma) and showed that this Cry toxin targets lipid rafts and assembles into oligomeric complexes in the membrane of HepG2 cells. The authors concluded that this protein is pore-forming toxin that accumulates in lipid rafts of tumor cells. Recently Bokori-Brown et al. [48] showed that ε-toxin produced by *Clostridium perfringens* (the aetiological agent of dysentery in newborn lambs, enteritis and enterotoxaemia in goats, calves and foals) forms heptameric pores in target cell membranes exactly like parasporin-2.

The less investigated parasporin in regard to cytotocity is PS3Aa1, typical three-domain-type Cry protein closely related to the insecticidal Cry proteins. This may lead to the hypothesis that, by analogy to insecticidal Cry proteins, PS3Aa1 acts as a pore-forming toxin on the plasma membrane of cancer cells. This is partly supported by the fact that PS3Aa1 increases plasma membrane permeability of target cells [8]. It is now well accepted that the insecticidal Cry1A proteins induce cell death through pore formation in plasma membrane after initial binding to the GPI-anchored receptors, aminopeptidases and cadherin-like proteins in the mid-gut epithelial cells of the susceptible lepidopteran insects [49].

Mahalakshmi et al. [50] investigated the mode of action of PS2Aa1. The initial step in cytocidal action of PS2Aa1 is the specific binding of this cytotoxin to a putative receptor located in the lipid rafts, followed by its oligomerization and pore formation in plasma membrane. Secondary structures observed in the model (Accession No: BAD 35170) are organized virtually in the same way as the experimentally determined parasporin-2 protein (PDb id: 2ZTB). Even though 2ZTB and 2D42 belong to the parasporin type 2 protein sequence, BAD35170 bears greater similarity to 2ZTB in its domain organization [50].

Nadarajah et al. [51] studied the mechanism of action of parasporins Cry 15Aa, Cry 24Aa, Cry 25Aa in human leukemic cell line CEM-SS and showed that the mechanism of tumor cells killing is apoptosis.

Phonnok et al. [52] studied the anticancer and apoptosis-inducing activities of microbial metabolites. They found that chromosomal DNA cleavage detected as DNA ladder pattern by gel electrophoresis including activation of cellular caspase-3 activity, a hallmark of apoptosis, were observed in all parasporin-treated cancer cell lines. These characteristics suggested the mechanism of apoptosis is induced by most of the parasporins [52].

As mentioned above, PS4Aa1 is different from the three other parasporins in many aspects. Thus, it is conceivable that the mode of action of

this protein also differs from those of the others. At present, however, no information is available for understanding the mechanism of preferential activity associated with PS4Aa1 [8]. This needs clarification.

Lee at al. studied the antitumor action of the 89-T-34-22 proteins (PS4) and found that cytopathological changes induced by these proteins were characterized by remarkable condensation of the nucleus and cell ballooning [39].

Since quite little is known about the receptor molecules that bind parasporins and the mechanism of anticancer activity, Krishnan et al. [53] carried out a very interesting investigation using a Malaysian *B. thuringiensis* isolate, designated *B. thuringiensis* 18. It produces parasporal protein that exhibits preferential cytotoxic activity for human leukaemic T-cells (CEM-SS) but is non-cytotoxic to normal T lymphocytes or other cancer cell lines such as human cervical cancer (HeLa), human breast cancer (MCF-7) and colon cancer (HT-29), suggesting properties similar to parasporin. In this study Krishnan et al. [53] aimed to identify the binding protein for *B. thuringiensis* 18 in human leukaemic T-cells. The protein was separated using Mono Q anion exchange column attached to a HPLC system and antibody was raised against the purified 68-kDa parasporal protein. Receptor binding assay was used to detect the binding protein for Bt18 parasporal protein in CEM-SS cells and the identified protein was sent for N-terminal sequencing. Double immunofluorescence staining techniques were applied to localize *B. thuringiensis* 18 and binding protein on CEM-SS cells. The results of this study showed that anion exchange separation of Bt18 parasporal protein yielded a 68-kDa parasporal protein with specific cytotoxic activity. Polyclonal IgG (anti-*B. thuringiensis* 18) for the 68-kDa parasporal protein was successfully raised and purified. Receptor binding assay showed that *B. thuringiensis* 18 parasporal protein bound to a 36-kDa protein from the CEM-SS cells lysate. N-terminal amino acid sequence of the 36-kDa protein was GKVKVGVNGFGRIGG and it has been revealed that the binding protein was Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Double immunofluorescence staining showed co-localization of *B. thuringiensis* 18 and GAPDH on the plasma membrane of the CEM-SS cells [53]. Hence, GAPDH has been well known as a glycolytic enzyme, but recently GAPDH was discovered to have roles in apoptosis and carcinogenesis. Pre-incubation of anti-GAPDH antibody with CEM-SS cells decreases binding of Bt18 to the susceptible cells. Based on a qualitative analysis of the immunoblot and immunofluorescence results, GAPDH was identified as a binding protein on the plasma membrane of CEM-SS cells for Bt18 parasporal protein [53].

Recently, Okumura et al. [54] studied the mode of anticancer action of parasporin-4 (PS4) produced by *B. thuringiensis* strain A1470 which exhibits specific cytotoxicity against the human cancer cell lines CACO-2, Sawano, and MOLT-4. When cells were administered PS4, cell swelling and nuclear shrinkage were induced, and the ballooned cells bursted within 24 hrs. PSI-BLAST search showed that the protein shared homology not only with *B. thuringiensis* Cry toxins but also with aerolysin-type β-pore-forming toxins. Circular dichroism measurements suggested that PS4 was a β-sheet-rich protein. PS4 aggregated into oligomers on the plasma membrane of PS4-susceptible CACO-2 cells, but not on that of PS4-resistant HeLa cells. Leakage of lactate dehydrogenase and influx of extracellular FITC-dextrans were observed only in susceptible cells. Activation of effectors caspase 3 and/or 7 was not observed in PS4-treated CACO-2 cells. It was shown that cytotoxicity of the PS4 against CACO-2 cells was obvious when treated by cyclodextrin which induces cholesterol depletion. These results suggest that PS4 is a unique β-pore-forming toxin with a cholesterol-independent activity.

Lee et al. [55] purified from proteinase K-digested parasporal inclusion of a *B. thuringiensis* serovar *shandongiensis* isolate a 28 kDa protein which exhibits cytocidal activity specific for human leukemic T (MOLT-4) cells. The N-terminal sequence of the protein was identical with that of the 32 kDa protein, regarded as a protoxin, of the inclusion proteins. The median effective concentration of this protein was 0.23 μg/ml against MOLT-4 cells and its specific activity was 7.9-fold higher than that of the whole inclusion proteins. The 28 kDa protein induced necrosis-like cytotoxicity against MOLT-4 cells and the cytopathic effect with the pass of time was characterized by cell swelling, nuclear membrane isolation and chromatin condensation [55].

Wong et al. [56] compared the mode of antitumor action of parasporin (purified Bt18 toxin) with the action of drugs widely used in clinical oncology (cisplatin, doxorubicin, etoposide, navelbine and methotrexate) in CEM-SS (T-lymphoblastic leukaemia cell line). They showed that the mechanisms of tumor cell death may differ from that of antitumor drugs, since it did not sig-

nificantly compete with these compounds for the same binding site [56].

Krishnan in his recent report [21] tried to summarize the mode of action of all parasporins. He concluded that each parasporin studied so far is endowed with its own mechanism for inducing cell death. PS1 activates the apoptotic signaling due to increased levels of calcium ions. PS2 acts as a cytolysin which causes permeabilization of the plasma membrane with target cell specificity. PS4 is a cholesterol-independent pore-forming toxin. The mode of action of PS3, PS5 and PS6 still needs investigation. Although each of the parasporins differed in their specificity to different tumor cell types, human HepG2 liver carcinoma cell line is susceptible to all of them [21].

A cytotoxic protein from the Cry produced by *B. thuringiensis* subspp. *coreanensis* A1519 strain after treatment by proteinase K was extremely cytotoxic to MOLT-4 cells [42]. Microscopic observation indicated that the cell death was accompanied by no extensive rupture of the cell membrane. It was, therefore, suggested that the cell death of MOLT-4 was induced through a mechanism other than the colloid-osmotic swelling and cell lysis as caused by hitherto known *B. thuringiensis* Cry proteins [42].

Safety of parasporins

Acute toxicity and genotoxicity of parasporins have not been adequately investigated. Only scarce data are found in the literature. Recently the results of toxicity study in mice of one of the parasporins, namely PS4, was published [57]. The LD_{50} of PS4 was 160 μg/kg in male ICR mice after subcutaneous injection. Although it is less toxic than the vast majority of bacterial toxins [58], it can be considered as highly toxic substance according to classification of Gosseline et al. [59], i.e. the LD_{50} value is less than 1.0 mg/kg. Moreover, concentrations of cations, creatinine and urea nitrogen in the urine and serum indicate that PS4 impairs kidney function in mice. Histological evaluation of kidney of mice treated with PS4 show that the protein may induce damage of the proximal renal tubule of the organ [57]. It should be also mentioned that recently the LD_{50} of PS2 was found to be 0.42 mg/kg after intraperitoneal injection in mice (Kyushu Institute of Technology, Japan; not published).

Bhat at al. [60] evaluated the cytotoxic and genotoxic effects of crude *Cry1Ac* toxin extract (highly similar to parasporins) in human lymphocytes using MTT assay, cytokinesis blocked micronucleus (MN) assay and erythrolysis tests. The exposure of human lymphocytes with increasing doses of toxin (from 0.2 to 1.0 mg/ml) showed decreased cell survival (47.08 %) at 72-h incubation. The number of MN formed was 2.52 %, which was significantly higher than in control (1.11%). The *Cry1Ac* toxin showed haemolytic activity on human lymphocytes. It was concluded that the crude *Cry1Ac* toxin has possible cytotoxic and genotoxic potential on human lymphocytes with increased dosage of toxin and time of exposure [60]. Since parasporins are expected to be used in clinical oncology, their toxicity profiles should be investigated in depth to ensure their safety in humans.

Grisolia et al. [61] evaluated the toxic and genotoxic properties of four δ-endotoxins (cry 1Aa, cry 1Ab, cry 1Ac, and cry 2A) from *B. thuringiensis* which are closely related in regard to the chemical structure to parasporins on zebrafish Danio rerio. Genomic changes and also their effects on embryos was studied. Cry 1Aa increased MN rates in peripheral erythrocytes of adult *D. rerio* 7.3-fold, while cry 1Ab, cry 1Ac and cry 2A had no genotoxic activity after 96-h exposure at concentration of 100 mg/L. Exposures to mixtures of toxins (cry1Aa+cry 1Ac, 50:50 mg/L and cry 1 Aa+cry 2A, 50:50 mg/L) for 96 hrs showed also significant increased MN frequencies (7.1-fold in both cases). Other combinations of toxins did not show genotoxicity. Activity of glutathione S-transferase measured in the gills did not alter after exposure to all tested toxins and combinations of toxins. In the embryo-larval study, all tested δ-endotoxins showed toxicity and developmental delay after exposure to concentrations of 25, 50, 100 and 150 mg/L for 96 hrs. It is noteworthy that cyclophosphamide, which was used as a positive control in this study, increased MN level by 23-fold. This means that these toxins possess slight genotoxic activity [61].

Milestones for future investigations

We failed to find any investigation in the literature concerning antitumor activity of parasporins in *in vivo* test-systems, e.g. in mice and rats. This is a serious shortcoming since many agents which are active in cancer cells in culture are inactive in rodents [62].

In their review paper Ohba et al. [8] mentioned that parasporal inclusion-forming organisms occur in a variety of aerobic and anaerobic microorganism species which belong to some genera e.g. *Bacillus, Paenibacillus, Brevibacillus* and *Clostridium,* and future work should include screening tests for novel anticancer parasporal pro-

teins from these species.

But, in our opinion, the most important point at present is to know whether parasporins are active in experimental rodents with different tumors. According to the results of the study by Kaneno et al. (Institute of Bioscience, Botucatu, Brazil) one of the parasporins which shows high cytotoxicity *in vitro* was not active in BALB/c mice with grafted CT-26 colorectal cancer cells (personal communication of Dr. Kaneno, unpublished results). Moreover, parasporin was highly toxic, affecting the liver and spleen of mice along with significant decrease of their lifespan.

Conclusion

The cytotoxic activity for cancer cells makes parasporins possible candidate anticancer agents in clinical oncology. It is expected, however, that their application will induce undesired immunological responses in patients. The strategy to solve this problem includes identification of parasporin-specific cell receptors in cancer cells. This may lead to the creation of very effective antitumor drugs targeting the receptors.

Parasporal inclusion-forming organisms occur in a variety of aerobic and anaerobic species belonging to several genera, e.g. *Bacillus, Paenibacillus, Brevibacillus* and *Clostridium*. Thus, future work also includes screening tests for novel anticancer parasporal proteins from non-*B.thuringiensis* spore-forming bacteria [8].

Okumura et al. [38] recently applied genetic engineering for the production of parasporin. The gene responsible for production of the protein was expressed in *E. coli,* and the cytotoxic activities of the recombinant protein against four human cell lines (MOLT-4, Jurkat, HeLa, and HepG2) were similar to those of parasporin-2. No one parasporin was studied in *in vivo* test system, and at present the ability of these proteins to induce antitumor effect in a whole organism is unknown. Further *in vivo* investigations of parasporins are certainly warranted.

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