Bacillus Thuringiensis Parasporal Proteins And Their Effect On Human Cancer Cells : An Overview

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Bacillus thuringiensis is an anaerobic, spore forming bacterium that produces various toxic proteins both during its vegetative stage and sporulative stage. During its sporulative stage, it produces parasporal proteins that have long been used in the agriculture fields as insecticides. Although anticancer effect of *Bacillus thuringiensis* parasporal proteins can be dated back to the 1970s, research in this area went through a giant leap in the late 1990s, with much of the work being done in Japan. It has been found that some strains of non-insecticidal *Bacillus thuringiensis* produce parasporal proteins that exhibit anticancer activity. Due to their selectivity against human cancer cells but not normal cells, some of these proteins have been extensively studied for their anticancer effect and the mechanism of action by which these proteins kill cancer cells have also been widely explored in Japan and Malaysia with sporadic reports from other parts of the world. The abundance of these bacilli in nature and their selectivity have made them potential candidates for cancer treatment. However, literature on the *in vivo* effect of these proteins is scarce. Since different *Bacillus thuringiensis* strains produce different cytotoxic proteins with wide variations in their anticancer effect and mechanism of action, further investigations are necessary and their effect *in vivo* must be well established before they can be used in human subjects.

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Introduction

Bacillus thuringiensis (Bt) is an insecticidal bacterium producing toxins which are used commercially in insect control in the agricultural field. For many years, researchers have studied numerous toxins isolated from this facultative aerobic, motile, gram-positive, spore-forming bacterium.[1] Besides being used as an insecticide, Bt has been found to be medically useful. Bt has been shown to suppress medically important dipteran pests such as mosquito vectors of malaria, viral diseases like dengue and West Nile fever and filariasis, as well as black flies that transmit onchocerciasis.[2] Bt has also been shown to have nematode-killing activity against human and animal hookworms.[3] Recently, studies have also shown that Bt toxins are also capable of inducing cell death in human cells. It has been found that various Bt strains are capable of producing parasporal proteins that have selective cytocidal effects on different cancer cell lines. These studies have been carried out in various parts of the world such as Japan[4-10], Malaysia[11-13], Vietnam[14] and Canada[15]. Although much work has been done, a thorough review is lacking and this is especially true for the Malaysian isolates of *Bacillus thuringiensis*. Therefore, this article aims to give an overview of both non-Malaysian and Malaysian *Bacillus thuringiensis* isolates which produce cytotoxic parasporal proteins against human cancer cells with an emphasis on the mechanism of cell death these proteins induce on human cancer cells.

The discovery of *Bacillus thuringiensis* (Bt)

Bt was first discovered by Ishiwata in a diseased silk moth population at the beginning of the 20th century. It was later re-isolated by Berliner in 1909 from a diseased Mediterranean flour moth population in Germany.[16] Berliner described the presence of an inclusion body or crystal, which is now used as a phenotypic characteristic to discriminate *Bacillus thuringiensis* from *Bacillus cereus*. He also suggested that *Bacillus thuringiensis* can be used as an insecticide to control insect populations. In 1938, Bt first became available as a commercial insecticide in France and in the 1950s entered commercial use in the United States.[17]

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Various toxins produced by Bt

Bt is a member of the genus *Bacillus*, a diverse group of gram-positive, anaerobic, spore-forming bacteria and is closely related to two other important spore-forming Bacilli, *Bacillus cereus* and *Bacillus anthracis*.[18] Bt occurs naturally in the environment and has been isolated from soil, insects and plant surfaces. Its occurrence in nature is predominantly as spores that can disseminate widely throughout the environment.[19] The insecticidal activity of *Bacillus thuringiensis* is due to its ability to produce large amounts of proteins that are found in a parasporal inclusion during the sporulation phase. These crystalline proteins are also known as delta-end toxins.[20] There are two types of delta-endotoxins, namely Cry (crystal delta-endotoxins) and Cyt (cytolysins).[21] Hofte and Whiteley defined four classes of Cry genes and two classes of Cyt genes in 1998.[22] The Cry toxins bind to specific receptors while the Cyt toxins have no specific receptors. CryI and CryII toxins are active against lepidopterans, Cry II and CryIV against dipterans and CryIII against coleopterans. On the other hand, Cyt toxins are active against dipteran and coleopteran pests.[22] However, their mosquitocidal activity is lower than that of the Cry toxins.[23]

The delta-endotoxins exist as protoxins in the parasporal inclusion. When ingested by insects, the parasporal inclusion is solubilised in the midgut, releasing the delta-endotoxins. These protoxins are then activated by midgut proteases. The activated toxins specifically bind to protein receptors in the epithelium of the insect midgut[24] and produce pores, causing loss of normal membrane function.[25] It is this increase in membrane permeability that leads to epithelial cell lysis and paralysis of the feeding activity. The insect dies of starvation, septicaemia or both as a result. Many studies have been carried out to identify Bt toxin receptors. For instance, in 1986 Knowles and Ellar proposed that it could be a glycoprotein.[26] In 1997, Keeton and Bulla related the receptor for Cry1A to cadherin-like proteins.[27] Two years later, Garner *et al*. suggested that the receptor for CryA1 were aminopeptidases[28] and in 2002, Rajagopal *et al*. suggested that the receptors for Cry1C were aminopeptidases.[29]

Other than insecticidal proteins, Bt also produces a) several enzymes, b) some compounds that lyse erythrocytes, and c) some compounds that are enterotoxic to vertebrates.[30] *Bacillus thuringiensis* also produces proteins during its vegetative state. Some of these are insecticidal and are called vegetative insecticidal proteins (VIPs).[31]

The discovery of the anticancer effect of *Bacillus thuringiensis*.

As early as the 1970s, Prasad and Shethna had carried out research on the antitumour effects of Bt toxins.[32-34] Later in the 1970s, 1980s and 1990s few studies were carried out on the effect of various Bt toxins on cancer cells.[35-37] However, in the 21st century, much work has been done on the antitumour effects of Bt toxin from different strains or subspecies of *Bacillus thuringiensis* in different parts the world.[4-15] For example, parasporal proteins of *Bacillus thuringiensis* serovar *shandongiensis* were found to be cytotoxic to human leukaemic T cells.[38,39] A soil isolate designated 90-F-45-14, belonging to *Bacillus thuringiensis* serovar *dakota* (H15) was found to produce non-cyt inclusion proteins that are highly cytotoxic against human leukaemic T cells (MOLT-4) and moderately cytotoxic to human cervical cancer cells (HeLa).[40]

Much of the ground breaking work was initiated by Mizuki *et al* in 1999, when a massive screening was done on a large number of Bt strains in Japan.[4] One thousand seven hundred and forty four Bt strains were investigated for their cytocidal activity against human leukaemic T cells and their haemolytic activity against sheep erythrocytes. Out of these 1744 strains, 1700 were Japanese strains and 44 were reference type strains of existing H serovars of Bt. It was found that 1684 strains were non-haemolytic and 42 strains demonstrated *in vitro* cytotoxicity against leukaemic T cells. These strains belong to serovar *dakota*, *neolensis*, *shangongiensis* and *coreanensis*. Out of these serovars,

3 selected strains, namely 84-HS-11, 89-T-26-17 and 90-F45-14 were non-insecticidal, non-haemolytic and were found to be cytocidal to leukemia T cells and other human cancer cells and the first two strains able to discriminate between leukaemic T cells and normal human T cells.

The parasporins and their mechanism of action

In the year 2000, the word "parasporin" was first used by Mizuki *et al* to describe a novel protein with a unique cytotoxicity.[5] Today the word "parasporin" is defined as "*Bacillus thuringiensis* and related bacterial parasporal proteins that are non-haemolytic but capable of preferentially killing cancer cells".[41] Since Mizuki's discovery, other parasporins have been discovered, all of which display non-haemolytic and anticancer properties. Therefore, the huge reservoir of Bt toxins in nature remains a target of constant research because they are good potential candidates for cancer treatment. In 2006, the Committee of Parasporin Classification and Nomenclature was organized to construct a taxonomically sound classification system based on the amino acid identity [42].The parasporins are broadly divided into 6 main classes, namely parasporin 1 (PS1), parasporin 2 (PS2), parasporin 3 (PS3), parasporin 4 (PS4), parasporin 5 (PS5) and parasporin 6 (PS6). Among these 6 classes, the mechanism of action of PS1 and PS2 has been well studied and much less is known about PS3, PS4, PS5 and PS6. To date 18 parasporins have been discovered and have been placed on the list of parasporins by the committee.[42]

Parasporin 1

Several studies have looked at the anticancer effect of PS1 [5, 8, 9, 14]. The mechanism of action of PS1 produced by Bt A1190 (formerly known as 84-HS-1-11) was well elucidated in a study done by Katayama *et al*.[8] The protein exists as 81 kDa protein, Pro-PS1. Upon activation, PS1 exists as a 15 kDa and 56 kDa heterodimer. In the study, Katayama *et al* investigated the effect of the protein on membrane permeability, calcium homeostasis and the mode of cell death. Several conclusions were drawn from the study regarding PS1: 1)PS1 was cytotoxic against HeLa and MOLT-4 cells, 2) PS1 caused an increase in Ca^{2+} influx and that the influx was not related to Ca^{2+} channels and not due to intracellular Ca^{2+} but extracellular Ca^{2+}, 3) heterotrimetric G-proteins or G-protein coupled receptors were involved in parasporin-1 induced Ca^{2+} influx, 4) PS1 was not a pore forming toxin and 5) the mode of cell death was most likely apoptotic.

Parasporin 2

The mechanism of action of PS2 has also been widely explored [43-46]. In a study done by Kitada *et al* on PS2 produced by Bt A1547 (formerly known as 94-F-45-14) the protein was shown to be cytotoxic to HepG2 and MOLT-4 cells. In contrary to PS1, the 30kDa protein was found to be a pore forming toxin which caused increased permeability to the susceptible cells. Kitada *et al* also showed that the mode of cell death was unlikely to be apoptotic in contrary to PS1.[44] It was also found that PS2 was distributed at the cell periphery and the immunostaining pattern was the same as the native distribution of cadherin, a cell-cell adhesion protein in the plasma membrane. This finding corresponded with that by Wong *et al*, who demonstrated that the parasporal protein of Malaysian isolate, Bt 18, also bound to a binding site on the cell surface, suggesting the possibility of a specific cell-surface receptor.[13] Further investigations by Kitada *et al* revealed that PS2 oligomerised at the cell surface via binding to lipid rafts which led to cell lysis and that glycosylphophatidylinositol (GPI)-anchored proteins were involved in such cytocidal activity.[45] Investigations on the crystal structure of PS2 by Akiba *et al* suggested that the protein showed close resemblance to the aerolysin-type-beta-pore-forming toxins, which supported earlier finding by Kitade *et al* that PS2 is a pore-forming protein.[10]

Parasporins 3, 4, 5, and 6

Comparing to PS1 and PS1, literature on PS3 [47], PS4 [6, 48], PS5 and PS6 [49] is scarce. Mechanism of action these parasoporins on human cancer cells is not well studied. PS3 was thought to be a pore forming toxin which increased cell membrane permeability of cancer cells.[47] The mechanism of action of PS4 was thought to be different from PS1, PS2 and PS3 and awaits further exploration.[50] Very little is known about PS5 in existing literature while PS6 produced by Bt strain M019 with anticancer activity against human hepatocyte cancer cells and cervical cancer cells. It was speculated that PS6 could be a pore-forming protein.[49]

The Malaysian Bt isolates and their anticancer effect

Several studies have been carried out on various strains of the Malaysian isolates of *Bacillus thuringiensis*.[11-13] Works on the Malaysian isolates of Bt can be dated back to the 1980's during which there was a national screening program for microbial control agents for mosquitoes. Twenty Bt strains were obtained from this screening program with the discovery of a new strain named Bt subspecies *malaysianniensis*.[51] Several years later, Bt *jegathesan*, another new subspecies was added to the pool of Malaysian Bt isolates.[52]

Haemolytic and cytotoxic activity

More recently, Nadarajah *et al* carried out a study on the Malaysian mosquitacidal *Bacillus thuringiensis* to investigate the haemolytic and lectin activity of these Bt strains against human and rat erythrocytes.[11] Twelve Bt strains (Bt1, Bt 2, Bt 4, Bt 7, Bt 8, Bt 9, Bt 10, Bt 18, Bt 19, Bt 20, Btj and IPS-82) were screened for their haemolytic activity and it was found that parasporal proteins of 4 Bt strains (Bt 1, Bt 4, Bt 7 and Btj) were haemolytic to both human and rat erythrocytes and parasporal proteins of 6 Bt strains were haemolytic to either type of erythrocytes (Bt 2, Bt 9, Bt 10, Bt 19, Bt 20, IPS-82). Bt 8 was the only strain being non-haemolytic to both types of erythrocytes whereas Bt 18 demonstrated weak haemolytic activity against rat erythrocytes. In the same study, it was also observed that parasporal inclusions from the Malaysian Bt strains demonstrated lectin activity. In another study, Nadarajah *et al* screened the anticancer activity of the 12 mosquitocidal Malaysian isolates against CEM-SS cells and HeLa cells [12]. Several conclusions were drawn from this study: 1) All 12 strains tested demonstrated weak to strong cytotoxic activity against CEM-SS cells and Hela cells, 2) Bt 18, a non-haemolytic strain, was most cytotoxic against CEM-SS and least cytotoxic against HeLa cells, 3) Bt 8, another non-haemolytic strain demonstrated selective cytotoxicity against CEM-SS cells. It was also demonstrated that Bt 18 parasporal proteins was not only non-haemolytic, but was also non-cytotoxic to normal human T lymphocytes.[53]

Binding behaviour and mechanism of cell death

Wong *et al* later carried out a study on the binding behaviour of purified Bt 18 parasporal protein.[13] The binding affinity of parasporal proteins of three Bt strains (Btj, Bt 18 and Bt 22) was investigated. It was found that Bt 18 demonstrated the strongest binding affinity for CEM-SS cells among the three. Heterologous competitive binding assays showed that the parasporal proteins of the three strains did not compete for binding sites on CEM-SS cells, suggesting the possibility of different binding sites for each strain and hence a probable different mechanism of action. The study also demonstrated that purified Bt 18 parasporal protein did not compete with commercial anticancer drugs (cisplatin, doxorubicin, etoposide, navelbine and methotrexate) for binding sites on CEM-SS cells, suggesting a probable different mechanism of cell death from these drugs. The binding site of purified Bt 18 parasporal protein was postulated to be on the cell-surface. However, literature on the mode and mechanism of cell death is still lacking for purified Bt 18 parasporal protein to date.

Conclusion

The abundance of literature with promising findings suggests that *Bacillus thuringiensis* parasoral proteins

may be useful in the treatment of cancer. Although the mechanism of action of some of these proteins have been studied *in vitro*, suffice to say that more exploration is needed before these proteins can used as anticancer agents in human subjects. This is especially true for parasporins and parasporal proteins of various Bt strains whose mechanism are still less well understood. Future directions of Bt work should therefore, focus on in-depth study of the mechanistic aspects of Bt parasporal proteins and parasporins and the *in vivo* effect of these proteins.

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