

Use of Tumor Targeting Bacteria as Cancer Therapeutic Agents and Drug Delivery Vehicles: A Conceptual Approach

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Abstract

Cancer is an uncontrolled and non-regulated cell proliferation disorder. It remains a life-threatening disease around the globe. Unavailability of effective cancer treatment causes hundreds of thousands deaths every year throughout the world. More effective and less harmful therapies are needed to eradicate this deadly disease. The advent of new technologies in the fields of microbiology, biotechnology and genomics has paved the path for bacteria to be considered in the anti-cancer strategies. Hypoxia is the unique feature of all kinds of solid tumors where oxygen level drops to < 1%. Exploiting the hypoxic phenomenon of solid tumors, obligate and facultative anaerobic bacteria such as *Escherichia coli*, *Bifidobacterium longum*, *Clostridium sporogenes* M-55 and *Salmonella typhimurium* are being used as oncolytic and anti-cancer drug delivery agents. Genetically modified bacteria have more efficient and safety profile in terms of mouse model trials; however, more enhancement needs to be brought about to make this novel anti-cancer strategy applicable for human use. This article aims to bring forth a conceptual approach that could provide a way forward for the scientific investigations done on bacteria as anti-cancer agent.

Keywords: Anti-Cancer Agent; Oncolytic Bacteria; Hypoxia; Microbiology; Therapeutics

Abbreviations

DNA: Deoxyribonucleic Acid; NT: None Toxic; C-DEPT: Clostridial-Directed Enzyme Prodrug Therapy; PCEs: Prodrug Converting Enzymes; PAMPs: Pathogen Associated Molecular Patterns; PRRs: Pattern Recognition Receptors; COBALT: Combination Bacteriolytic Therapy

Introduction

Cancer is putting devastating impacts on human life as it is responsible for the large number of deaths every year throughout the globe. Around 600,000 cancer caused deaths occurred in United States in 2016 and recorded newly emerged cancer cases [1]. Cancer caused almost 3 million deaths alone in China in the year 2015 out of 4 million reported cancer cases [2]. The only available cancer therapies are, radio therapy and chemotherapy, which are having no safety profiles and hence toxic to the normal cells.

One other drawback of the radio and chemotherapy is the cell's DNA repair mechanism as cells become resistant when repeatedly exposed to the anti-cancer drugs and radiations intended for the cancer cure. The cellular defense system often makes interference in the

apoptosis process. Anti-cancer drugs when applied, expedite the production of detoxifying enzymes against anti-cancer drugs and also the drug delivery system [3]. Patients are always at risk because current cancer therapies put ill effects on their health causing impotence, nausea, and high risks of other kinds of cancer development [4].

It is therefore a dire need of new anti-cancer strategies development that could better eliminate cancer and stand as an ideal method of cure. With reference to the latest techniques and development in Microbiology, Genomics and Biotechnology, gene therapy is being considered as a hope for the cancer treatment as to specifically target the cancer genes. The major matter of concern is the specific delivery vehicles which could manage gene transfer as well as anti-cancer drugs at a targeted site e.g. tumor. Bacteria have been the centre of focus for the scientists since the last few decades to be used as anti-cancer therapeutic agent and anti-cancer drug and gene delivery vehicles.

Solid tumors have some shared universal features including compacted mass of cells, hypoxic and necrotic regions [5,6]. These features of tumors are somehow exploited by bacteria of obligate and facultative anaerobic respiration nature. They specifically colonize and proliferate in the hypoxic regions and generate nutrition deficient environment for the tumor cells. Bacteria that are being used in cancer therapies include; *Escherichia coli* [7], *Bifidobacterium* [8], *Clostridium* [9] and *Salmonella* [10].

Use of *Clostridium* spp in cancer therapy

Clostridium is an anaerobic bacterium that preferably colonize and proliferate in hypoxic environment, such as in the solid tumor where oxygen level drops to < 1%. Using *Clostridium* as an anti-cancer tool dates back to 19th century when cancer patients suffered from gangrene a clostridial infection, and dramatic regression of tumors were recorded [11,12]. It was later investigated and clostridia stood to be used in cancer treatment. In 1935, Connell prepared sterile filtrates of Proteolytic enzymes containing *Clostridium histolyticum* and applied directly to the tumors through injection [13]. After further improvements parker and coworkers discovered that *C. histolyticum* and its enzymes could result in tumor lysis when studied in transplanted mice sarcomas.

Conventional ways of treatment of cancer often fail because of necrotic and hypoxic features of solid tumors. *Clostridium novyi*-NT is a broadly studied organism as it extensively colonizes in the tumor tissues; additionally, it has been made a better candidate in cancer treatment with the non-toxinogenic properties after deletion of α -toxin gene [14]. *Clostridium novyi* was used in some cancer types during trial phases, such cancer types included: renal carcinoma [15], colorectal cancer, sarcomas [16] and gliomas [17], the experiments were intended to monitor anti-cancer properties of the organism in regards with specific colonization, immunity boost up and production and release of chemokines followed by oncolysis. Mose and Mose in 1959 investigated a non-pathogenic *Clostridium butyricum* M-55 strain which had tremendous tumor necrotic effects after being injected its spores in animal models and later in human subjects [18]. Proteolytic activities of *C. novyi*-NT and *C. sporogenes* make them an aggressive colonizers and a better choice of cancer treatment. The most promising evidence about the specificity and efficacy of *Clostridium* in tumor targeting was provided when animals with having tumors were infected intravenously with *Clostridium tetani* spores. The consequence of infection was death of animals within 48 hours in response to tetanus lethal toxin production in the tumors; whereas other animals with no tumors survived because spores could not find a place to germinate and proliferate [19].

Role of *Clostridium* spp in directed enzyme prodrug therapy-CDEPT

The best cancer therapy is when if it specifically targets tumor cells and does not harm normal healthy cells. A technique named as Directed Enzyme prodrug therapy or DEPT, utilizes the specific enzymes called Prodrug Converting Enzymes (PCEs). Prodrugs are converted from non-functional form to highly toxic form in the tumor vicinity harming only cancerous cells and this is the best solution of selectively targeting cancer tissues [20,21]. Delivery and expression of PCEs at specific site was a major issue of concern. In a related study design *Clostridium sporogenes* was used to deliver and express PCEs in tumor tissues as *C. sporogenes* colonizes in hypoxic regions. This type of Directed Enzyme Prodrug Therapy is known as Clostridial-Directed Enzyme Prodrug Therapy (CDEPT) (Figure 1) [22].

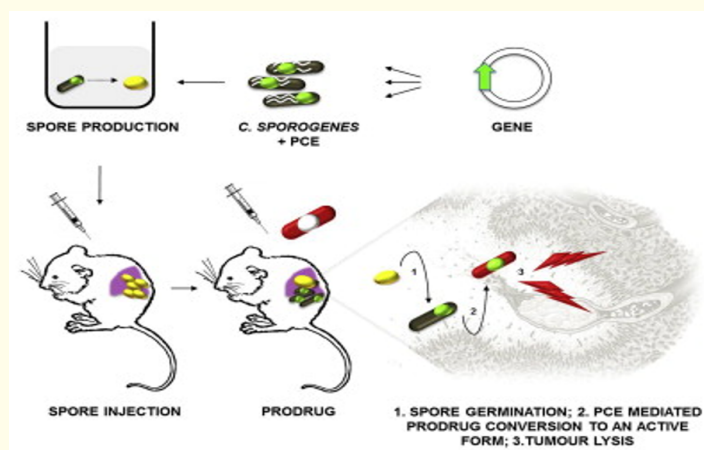


Figure 1: Depiction of Clostridial-Directed Enzyme Prodrug Therapy (CDEPT). Derived from Kubiak AM and Minton NP 2015 [22].

Use of invasive *E. coli* as anti-cancer therapeutic protein delivery vehicle

Gene therapy has gained so much attention among the researchers, it has made it easy to manipulate genome of certain organisms and desired output can be obtained. Keeping in view the strategies in anti-cancer treatments, non-pathogenic and non-replicating strains along with some important components such as therapeutic drugs, toxins to kill cancerous cells, are being delivered to the affected tissues. Since *E. coli* has been used in genetic engineering so non-pathogenic *E. coli* strains have been engineered to express some proteins called invasins, which are normally utilized by *Yersinia pseudotuberculosis* for using it as entry pass into the host cell during invasion. Epithelial cells and cancerous cells have β 1-integrin on their surfaces so invasins bind to the β 1-integrin and get entry into the host cells this receptor-mediated endocytosis can be exploited in anti-cancer strategies. Invasin genes were cloned in *E. coli* and upon expression *E. coli* could get into the host cell [23]. Evading from lysosomes in the host cell *E. coli* could be armed with listeriolysin O (LLO) which forms pores into the lysosomes [23]. Expressing anti-cancer toxins into the cytosol, results in cancer cell death. Tumors are well known to suppress host immune system and hence make them difficult to be treated, so they carry on unchecked proliferation. Apart from being involved in therapeutic activities, *E. coli* also boosts host immune system both at the site of infection and systemically. *E. coli* with Pathogen Associated Molecular Patterns (PAMPs) expressed are recognized by Pattern Recognition Receptors (PRRs) on the immune cells. Reactive oxygen and nitrogen species are released after the immune cells interact with PAMPs. This interaction also activates T lymphocytes such as CD8+ T cells and CD4+ T cells. These lymphocytes are responsible for the clearance of tumor cells [24]. In a same way CD8+ T cells and CD4+ T cells have the ability to halt the further proliferation of cancer cells.

Experimental model trials of *E. coli* in tumor therapy

Population of 100 *E. coli* per cell was observed to have non-toxic effects and outstanding delivery of proteins achieved after expressing β 1-integrin. In the experiments of *in vitro* trials, functional and intact proteins were efficiently delivered using 100 *E. coli* per cell in the vast population of cells. Gene expression was also monitored in these trials and resulted 5% of the delivered genes were expressed in the cells *in vitro*.

Use of *Bifidobacterium* in anti-cancer gene therapy

As hypoxic regions in the tumor tissues are the best place of colonization for the anaerobic bacteria such as *Bifidobacterium* so if injected intravenously, *Bifidobacterium* can colonize the hypoxic regions of solid masses of cancerous cells [26]. Endogenous microorganism like *Bifidobacterium* is a normal resident of small and large intestine in humans so they protect the host from carcinogenesis and promote good health. It is also known that these normal residents guarantee prompt immunity to fight against diseases [27-29].

In an experimental study, wild and domestic strains of non-pathogenic *Bifidobacterium longum* were investigated for the ability of targeted colonization. Both the tumor hypoxic environments as well as normal organs of animal models were subjected to the inoculation

of Bifidobacterium. Both wild and engineered strains were found to have proliferated in the tumor tissues but not in the normal tissues of mouse e.g. spleen, liver, and kidney. The results were obtained after 168 hours of the incubation period of Bifidobacterium in the mice. Two types cancer bearing mice were studied, one with Lewis lung cancer and other with B16-F10 melanoma. The number of bacteria per mice was $5 - 6 \times 10^6$ containing pBLES100 plasmids with spectinomycin adenyl transferase encoding gene. After incubation period it was concluded that transformed *B. longum* 105-A, strains were found at slightly different ratios in both types of tumor tissues but not in the normal tissues (Figure 2). This was demonstrated by cultivated the organisms on spectinomycin containing agar plates, tumor tissues were found to have 400 cfu resistant to spectinomycin which confers that transformed bacteria were proliferated only in the tumor tissues. Bifidobacterium is good choice of gene delivery vehicle as it was earlier shown in the study that it carried pBLES100 with spectinomycin resistance gene. It suggests that this organism is better candidate in gene therapy the plasmid it carry can have any gene that is beneficial in the treatment of cancer [25].

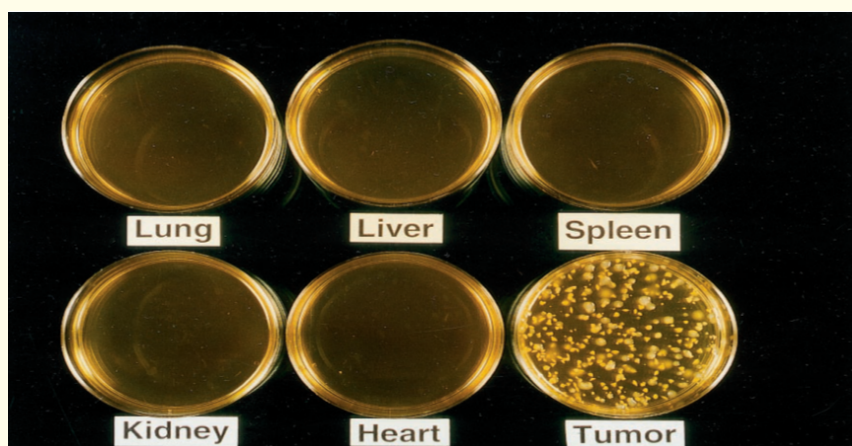


Figure 2: Tissue samples were taken and homogenized after 168 hours of incubation in mice and cultivated 100 μ L of it in spectinomycin containing Petri plates, results after 3 days of incubation revealed that around 400 colonies of *B. longum* were counted on plates with tumor sample but not on other plates with normal tissue samples. Derived from Yazawa, et al. 2000 [25].

Cancer therapy with genetically modified *Salmonella typhimurium*

Salmonella typhimurium is a Gram negative, rod shaped, anaerobic bacteria, which is extensively studied for the purpose of using it in cancer treatment. *S. typhimurium* is used either used as therapeutic agent or as a drug delivery vehicle. Various engineering techniques are being used to make this organism much suitable for use in tumor treatment like making it less harmful by inhibiting the expression of virulent genes. *S. typhimurium* mutant strain e.g. VNP20009 harboring a modified sequence (msbB-, pur I-) in lipid A and purine auxotrophic mutation cause significantly less septic shock than their non-modified counterparts. Mutant *S. typhimurium* has some safety profiles when administered to patients with metastatic melanoma and renal carcinoma in phase I clinical studies [30-32]. This Gram negative organism has the ability to grow both in aerobic and anaerobic conditions so they can colonize in small and large solid tumors. Combination of radio therapy and chemotherapy is found to increase the efficacy of *S. typhimurium* therapeutic activity [33,34].

Role of *S. typhimurium* in cancer therapy

In the past era radio and Chemotherapies were the only available means of treating cancer but with the advent of new techniques in the fields of Microbiology, Biotechnology and Genetics, some other means have been tested and found affective in the treatment of this dreadful disease. Radio and chemotherapies are somehow affective in cancer treatment but cannot eradicate complete cancer cells and also are toxic to normal tissues. Following reasons make these two means of treatment less affective: (a) incomplete tumor targeting (b) failure to penetrate in tumor tissues (c) limited toxicity to all tumor cells. These drawbacks cause increased risk of morbidity and mortality [35].

Recognition of tumor micro environment

The prime universal feature of tumor microenvironment is the hypoxia which means tumors contain less than 1% of oxygen therefore radio and chemotherapy fail to be effective [6]. Less amount of oxygen in tumor tissues is because of high rate of multiplication of cells and premature blood vessels which in return supply less amount of oxygen thus make it oxygen deficient environment. This hypoxic micro environment is an important signal for the anaerobic bacteria, like *S. typhimurium*, to sense and specifically colonize there. Once the mutant strains have reached the targeted site they start expression of desired genes intended for cancer therapy e.g. asd (aspartate-semialdehyde dehydrogenase) gene under hypoxic conditions [36] or delivery of therapeutic drugs under the control of hypoxia-inducible promoter-1 (HIP-1) [37]. Microenvironment of tumors have ribose and amino acids which are released from dying cancer cells, hence these small nutrients become a source of attraction for the engineered bacteria, a process called chemotaxis.

Bacteria	Attenuated strain	Description	References
<i>E. coli</i>	<i>E. coli</i> (BM2710)	This strain contains a plasmid with the <i>inv</i> gene derived from <i>Yersinia pseudotuberculosis</i> and produce invasin which helps the cell to be phagocytosed by mammalian cell.	[23,38,39]
<i>Salmonella typhimurium</i>	VNP20009	This modified strain has purine dependent way of colonization. It has been made to have reduced septic shock causing property. This strain has relatively better safety profile.	[33,40]
	A1-R	It has a leucine and arginine dependent way of colonization; it inhibited the growth of various cancer types in mouse models. This strain is also found to be helpful in cell cycle alteration.	[41,42]
	ΔppGpp	This strain has been modified to have a property of down regulation of endotoxin genes. It is avirulent to mouse after injected systematically. It is a good vector for targeted delivery of anti-cancer molecules.	[43]
Clostridium	<i>C. beijerinckii</i> NCIMB 8052	This strain expresses dihydropteridine gene that later catalyze the prodrug CB1955, 22 fold increase in tumor eradication has been observed <i>in vitro</i> .	[44]
	<i>C. sporogenes</i> NCIMB 10696	Attenuated strain with a Cod A gene from <i>E. coli</i> that codes for cytosine deaminase. When colonized in SCCVII-tumor bearing mice followed by 5-fluorocytosine injection it showed remarkable anti-tumor effects.	[45]
	<i>C. novyi</i> -NT	This strain has vigorous tumor specific colonization property and proteolytic. COBALT technique resulted in tumor regression and cured 50% HTC116 xenografts.	[14]
<i>Bifidobacterium</i>	<i>B. longum</i> 105-A	The strain is studied to have delivered spectinomycin resistance gene effectively.	[25]

Table 1: Description of genetically modified bacterial strains used in cancer therapy.

It therefore helps in specific accumulation at tumor site [46,47].

Penetration and multiplication in Tumor tissues

Among the reasons of failure of biological and chemical drugs, low penetration in the tumor tissues is a major obstacle. *S. typhimurium* has features like locomotion in swimming manner that can actively penetrate in the tumor tissues [18]. It can effectively deliver therapeutic drugs when these bacteria have higher accumulation opportunity in the tumor tissues. Reduced activity of host macrophages resulted in the increased tumor and spleen colonization. It has been investigated that targeted colonization of *S. typhimurium* is 1000 times greater in the tumor as compared to its growth in normal tissues e.g. spleen and liver [10,47]. Cancerous cells confront with the worst nutrition deficient environment after the massive bacterial growth and this phenomenon leads tumor cell to death [48].

Immunization

Tumors have the capability to escape host defense systems by suppressing the immune cells maturation and infiltration [48,49]. Host immunity can be provoked by the administration of genetically modified *Salmonella typhimurium*. As some conserved parts of bacteria like Pathogen Associated Molecular Patterns (PAMPs) are recognized by receptors called pattern recognition receptors (PRRs) on the tumor cells. Strong agonists to PRRs are those present on the bacterial surface such as lipopolysaccharide, flagellin, or cpG sites and activate innate and adaptive immunity. At the early stage of infection with *S. typhimurium*, bacterial flagellin are identified by IPAF inflammasomes within the cell [54]. NLRP3 inflammasome get activated by the internal danger signaling (damage-associated molecular pattern molecules) and also increase in K efflux occurs [50]. Inflammasomes activate caspase-1 which cleave pro-IL-1 β and pro-IL-18 to yield active IL-1 β and IL-18 [51,52]. These activated chemokines along with TNF- α and IFN- γ , promote the migration of immune cells such as CD8+ cells, NK cells and macrophages that finally result in tumor regression (Figure 3).

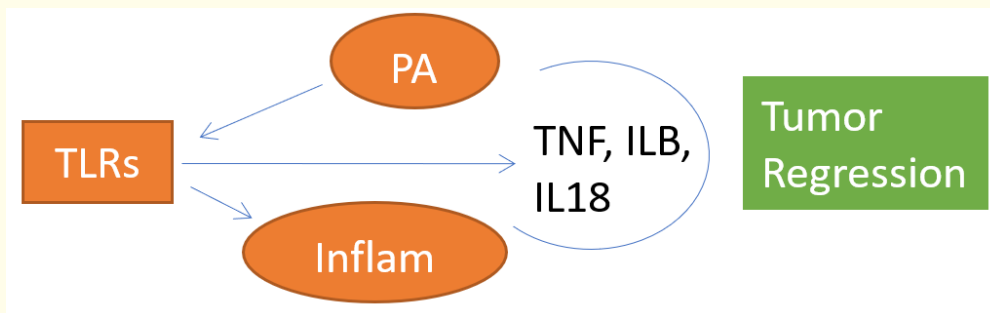


Figure 3: Schematic depiction of immune stimulation by *S. typhimurium*.

Programmability

Apart from being used as therapeutic agents, bacteria are being utilized to make delivery of some important cargo molecules such as cytokines [55-57], cytotoxic molecules [37,53,54], RNA interference [60-62] and prodrug enzymes [32,58,59] to the tumor tissues and hence help in increasing the efficacy of bacterial cancer therapy. Regarding the regulation of cargo molecules, they could better be expressed by external inducible signals and it would be helpful to avoid the genes from being expressed in normal tissues. Two types of external signals have been investigated till now, (1) L-arginose-inducible pBAD promoter [63,64], tetracycline or doxycycline-inducible pTet promoter [53] and (2) hypoxia-inducible fumarate and nitrate reduction regulator [37,65] and the quorum-sensing system, which turns transgenes expression at large population of bacteria in tumor tissues [66].

Conclusion

Bacterial cancer therapy has been used since very long but efforts seem fruitful since the last decades. Advances in new technologies have turned the attention of researchers towards the bacterial cancer therapy again as it is self-propagating, convenient in gene transfer and specific targeting. There are so many strategies to treat cancer and they are somehow effective but meanwhile toxic to normal tissues. Bacteria are being genetically modified to make them efficiently target cancer cells and also provide a safer way of treatment to the patients. Engineered bacteria have been used as therapeutic and drug delivery vehicles to specifically target the tumor tissues and avoid harming of normal cells. Bacterial cancer therapy is considered a better step towards tumor therapy but this technique is found to be successful only in animal models.

Bacterial cancer therapy is good way of treatment and it can suggested that accurately engineered bacterial strains be used according to the strategy like if immunity boost up is intended, surface marker are targeted to be focused and if gene delivery or gene silencing is intended, its needed to extensively study the genetic makeup. Similarly, if therapeutic purposes are to be achieved, bacterial strains need to be made accurately made a virulent to avoid harmful effects on normal cells. Further improvements need to be made in cancer treatment

strategies especially in terms of bacterial mediated cancer therapy. Such strategies need to be approved so that bacterial cancer therapy could be useful for human use and make it available for safe use in cancer treatment in future on large scale.

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