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Bacterial Therapies: Completing the Cancer Treatment Toolbox

Adam T. St. Jean^{*}, Miaomin Zhang^{*}, and Neil S. Forbes

Department of Chemical Engineering, University of Massachusetts, Amherst, Amherst, MA 01003-9303

Summary of Recent Advances

Current cancer therapies have limited efficacy because they are highly toxic, ineffectively target tumors, and poorly penetrate tumor tissue. Engineered bacteria have the unique potential to overcome these limitations by actively targeting all tumor regions and delivering therapeutic payloads. Examples of transport mechanisms include specialized chemotaxis, preferred growth, and hypoxic germination. Deleting the ribose/galactose chemoreceptor has been shown to cause bacterial accumulation in therapeutically resistant tumor regions. Recent advances in engineered therapeutic delivery include temporal control of cytotoxin release, enzymatic activation of pro-drugs, and secretion of physiologically active biomolecules. Bacteria have been engineered to express tumor-necrosis-factor- α , hypoxia-inducible-factor-1- α antibodies, interleukin-2, and cytosine deaminase. Combining these emerging targeting and therapeutic delivery mechanisms will yield a complete treatment toolbox and increase patient survival.

Introduction

To cure cancer completely many of the deficiencies in current therapy must be overcome. Many standard therapies do not effectively target tumors over normal tissue and do not efficiently penetrate tumor tissue [1–3]. Engineered bacteria possess unique abilities to overcome both of these deficiencies [4,5]. Poor targeting limits the dosage of standard therapies that can be administered because high doses would induce systemic toxicity. Poor penetration reduces the dosage present throughout tumors. Both of these limitations prevent complete eradication of all cancer cells following individual treatments. Between courses of chemotherapy remaining cancer cells repopulate tumors, which leads to loss of local control and recurrence [6,7]. In addition, time between courses of chemotherapy allows individual cancer cell intravasation into blood vessels and increases the chance of metastasis. Tumor recurrence and metastatic disease are the primary cause of mortality from cancer [8].

To be effective bacterial therapies need to 1) target tumors over normal tissue, 2) be genetically modifiable, 3) be non-toxic, 4) target therapeutically resistant regions of tumors, and 5) deliver an effect anticancer therapeutic [4]. Many different genera of bacteria have been shown to accumulate specifically in tumors over normal tissue in mice including *Clostridium* [9–13], *Salmonella* [14,15], *Bifidobacterium* [16,17], and *Escherichia* [18]. In clinical trials, however, efficacy has been limited by insufficient colonization [19,20]. The next two requirements,

Corresponding Author: Neil S. Forbes, Ph.D., Department of Chemical Engineering, University of Massachusetts Amherst, 159 Goessmann Laboratory, 686 North Pleasant Street, Amherst, MA 01003, 413-577-0132 (office), 413-545-1647 (FAX), forbes@ecs.umass.edu.

^{*}These authors contributed equally to this work.

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toxicity and genetic manipulability, have mostly been addressed [14,21–24]. The last two challenges, effective intratumoral targeting and controlled delivery of therapeutics, are the focus of most current research. Enhancing the ability to target all regions of tumors and deliver potent therapeutics would greatly improve efficacy and tumor colonization in human tumors. Many creative approaches are exploiting bacterial processes to tailor bacteria into therapy vectors and cancer cell destroyers. Once these problems have been solved, we believe that bacteria will be a key step towards completing the cancer therapy toolbox.

Genetic Modification and Toxicity

To make bacteria into effective anti-cancer vectors, considerable research has been performed over the past decade to produce bacteria that are non-toxic and genetically modifiable. Genetic modification is necessary to design bacteria to exhibit desired therapeutic properties. Of the three genera being researched, *Escherichia coli* is the standard organism for genetic engineering, allowing for robust and facile manipulation, and *Salmonella* has similar genetic modification capabilities. Species of *Clostridium* have traditionally posed significant challenges because they are not easily transformable using standard plasmid vectors and electrotransformation techniques [25]. However, a technique utilizing conjugative transfer from *E. coli* was recently developed [23,24], that will greatly expand the breadth of genetic modification possible in *Clostridium*.

Bacterial toxicity must be eliminated for many of these strains that are intrinsically pathogenic. A non-toxic (NT) strain of *Clostridium novyi* strain was created by deleting the virulence gene by heat treatment [21]. A non-pathogenic *msbB*[−] and *purT*[−] mutant of *Salmonella typhimurium* has been created [14,22] and found to be non-toxic in clinical trials [19,20]. It has also been shown that non-pathogenic, pro-biotic *E. coli* Nissle 1917 can be utilized in cancer therapies [26].

Intratumoral Targeting

From an engineering perspective, the limited penetration of chemotherapeutics into in all tumor microenvironments is a provocative problem for which bacteria are ideal solutions. Currently no therapies have been designed to target explicitly the therapeutically resistant regions in tumors. Three of the mechanisms by which bacteria target different intratumoral regions are specific chemotaxis, preferential growth, and hypoxic germination (Figure 1). Specific chemotaxis and preferential growth are employed by facultative anaerobes *Salmonella* and *Escherichia*, while hypoxic germination is employed primarily by obligate anaerobes *Clostridium* and *Bifidobacterium*. These mechanisms, which enable bacteria to target specific tumor regions, also enable bacteria to target tumors over normal tissue. This is the primary reason for the exceptional targeting ability of bacteria: tumors contain unique microenvironments, which are not present in most normal tissue and are attractive to bacteria.

Microenvironments and blood vessel structures are vastly different between normal and tumor tissue. Normal tissue receives oxygen, nutrients, and drug molecules via an organized vasculature system. All cells in normal tissue have access to oxygen and nutrients. In tumors this balance is disrupted by disorganized and variable blood flow [27], which creates heterogeneous microenvironments, including gradients in cell growth rate and drug concentration, as well as regions of hypoxia and acidity (Figure 2) [28].

Gradients in drug concentration expose some cancer cells to drug levels that are not sufficiently cytotoxic, which prevents complete cancer clearance [28,29]. To overcome these diffusion limitations a method of active drug transport is needed. Because active transport requires energy storage or recruitment from the local environment, not many suitable systems are available. Most cutting-edge therapeutics, including viruses, liposomes, and antibodies rely on

passive diffusion and do not actively transport. These modalities can be highly specific to cancer cells but, because of diffusion limitations, cannot penetrate deep into tumor tissue [30,31]. To illustrate the importance of active transport consider a hypothetical therapeutic that kills cancer cells on contact. This agent would be effective at killing cancer cells lining the tumor vasculature, but would leave surviving cells and not completely eliminate tumors. We call the approach of actively targeting specific therapeutically-resistant regions of tumors *targeted intratumoral delivery*.

Salmonella typhimurium, a facultative anaerobe, has been shown to be specifically attracted by chemotaxis toward chemicals excreted by distinct microenvironments in tumors (Figure 1-top) [5,32]. This ability makes motile bacteria an attractive system because they are able to actively transport, overcome diffusion limitations, and penetrate into therapeutically resistant regions of tumors. To the best of our knowledge, motile bacteria are the only treatment modality that can actively transport and are therefore the only means of effectively treating all regions within a tumor. Experiments with cylindroids, an *in vitro* model that mimics the heterogeneous microenvironment of tumors, showed that *S. typhimurium* bacteria are preferentially attracted to dying cancer cells in therapeutically resistant tumor regions [32]. Further investigations led to a discovery that the aspartate receptor controls migration toward tumors, the serine receptor initiates penetration, and the ribose/galactose receptor directs *Salmonella* into necrotic regions [5]. Knocking out the gene for the ribose/galactose receptor causes *S. typhimurium* to accumulate in therapeutic-resistant regions where they induce cancer cell apoptosis [5]. These findings suggest that *Salmonella* can be directed into any tumor region by manipulating the expression of specific chemoreceptors.

Another mechanism used by facultative anaerobes to target tumors is preferential growth (Figure 1-middle). In cylindroids wild-type *S. typhimurium* was shown to preferentially grow in dying tissue and not in regions of actively growing cells [32]. Targeting can be controlled by creating auxotrophic bacteria that cannot survive without nutrients from specific microenvironments. When injected intravenously, auxotrophs migrate towards regions of tumors that have high concentrations of required nutrients where they preferentially proliferate. Auxotrophs would not be able to grow in normal tissue. To demonstrate this principle an auxotrophic *S. typhimurium* mutant for leucine and arginine has been shown to accumulate throughout tumors in mice bearing metastatic PC-3 human prostate tumors [33–35].

Salmonella bacteria have also been shown to target non-hypoxic regions and metastases. Because early metastases and viable tumor cells outside necrotic regions are well or partially oxygenated, they are inaccessible to obligate anaerobic bacteria, which cannot tolerate the slightest amount of oxygen [36]. Non-pathogenic strains of *S. typhimurium* have been shown to preferentially accumulate in subcutaneous mouse tumors 2000-fold more than in the liver and spleen, retard tumor growth, and prolong survival [14,15,22,37,38]. It has been shown that, in addition to chemotaxis and preferential growth, facultative anaerobes accumulate in the immune privileged environments of tumors because of limited immune clearance by macrophages and neutrophils [39,40]. In addition to primary tumors, *Salmonella choleraesuis* has been shown to accumulate in lungs metastases and not in the healthy surrounding parenchymal tissue [41]. In orthotopic tumors this strain was shown to increased neutrophil and T-cell infiltration and have distinct anti-tumor effects, when administered with cisplatin [42,43].

Recently, *Escherichia coli* has been shown to accumulate in tumors [18,26,44]. Colonization levels of *E. coli* strains in the spleen and liver were very low compared to tumor tissue [26]. Because it is a facultative anaerobe, *E. coli* most likely employs the same tumor targeting mechanisms as *Salmonella*. Administration of *E. coli* K-12 to mice bearing murine 4T1 breast

carcinomas effectively stimulated an anti-tumor immune response and result in major reduction of pulmonary metastatic events [44].

The best understood mechanism of bacterial tumor targeting is hypoxic germination (Figure 1-bottom). When intravenously injected as spores, obligate anaerobes selectively colonize hypoxic tumor regions because they only germinate in poorly oxygenated environments. *Clostridium* is the primary obligate anaerobe investigated as an anti-cancer agent. In the 1960's several researchers demonstrated that *Clostridium* accumulates in necrotic tumor regions of mice [45–48] and humans [49]. Administration of *C. novyi NT* spores effectively target the avascular/necrotic tumor regions in mice [21] and induced substantial tumor regression in combination with chemotherapeutic drugs and anti-vascular agents [50]. Administration of *C. novyi NT* in combination with radiation therapy greatly increased the efficacy of the radiation [51]. The complete genomic sequence of *Clostridium novyi-NT* shows that the spores contain mRNA for redox proteins and that vegetative *C. novyi* produce lipases that enable them to thrive and proliferate in the environments in tumors [52]. Spores of nonpathogenic *Clostridium acetobutylicum* have been tested with the vascular targeting agent combretastatin A-4 phosphate on tumor bearing rats and bacterial growth in tumors was significantly improved [53]. This result indicates that the targeting efficacy of *Clostridium* can be enhanced by anti-vascular agents, which enlarge regions of hypoxia in tumors.

The distinct targeting mechanisms of facultative and obligate anaerobes are complimentary. Obligate anaerobes are more selective to tumors because hypoxia is only present in tumors and not in normal tissue. This high specificity makes them preferable for treating large primary tumors. On the other hand, because facultative anaerobes rely on chemotaxis and preferential growth to target tumors, they can target metastases and regions outside tumor necrosis. When assembling the tumor therapy toolbox, these different targeting characteristics will enable personalized therapy directed to the specific characteristics of individual patient's tumors.

Controlled Therapeutic Delivery

Bacteria have the ability to manufacture and secrete proteins, which can be coupled with targeting to apply a specific and focused therapy. Using proteins with different functionality, there are three ways bacterial therapies can be tailored: controlled cytotoxicity, enzymatic drug activation, and biomolecule secretion (Figure 3). These three options, by exploiting bacterial targeting attributes, enable precise control of the time and location of therapeutic delivery in order to maximize efficacy and minimize systemic toxicity. Once the bacteria are located within the tumor environment, they can be triggered to produce compounds that will either directly or indirectly treat the disease.

Temporal control over therapeutic agents, especially for cytotoxic compounds, is necessary because gene expression during transit to the tumor site will distribute products systemically and increase toxicity in healthy tissues (Figure 3-top). Using gene promoters that are induced by non-toxic, externally applied factors such as radiation or small molecules to control expression can overcome this problem. Radiation exposure is an effective trigger of gene expression because it can easily penetrate human tissue to reach internal tumor sites. Radiation is also a commonly used cancer therapy and infrastructure already exists for its application. This strategy has been demonstrated *in vitro* with a genetically modified strain of *Clostridium* expressing TNF α under the temporal control of the radiation-induced *recA* promoter sequence [54–56]. It was shown that therapeutic radiation exposure levels result in a 44% increase in TNF α expression over non-irradiated samples [56]. This study also noted that basal gene expression under *recA* is significant, and compromises the controllability of the method. This problem can be addressed by inserting additional radio-responsive elements into the promoter sequence. One additional element decreased basal production levels by 30%

over the wild-type [55]. Coupling these promoters with expression of various therapeutic agents yields highly controllable tools for cancer treatment.

Another mechanism used to trigger gene expression is activation of promoters by small molecules. L-arabinose, which is harmless to mammalian cells and tissues, has been used to activate the P_{BAD} promoter in *E. coli* [26]. A proof-of-concept test in cell culture using the luminescent *lux* operon showed a 10^5 -fold increase over background when 0.02% L-arabinose was added to the growth medium [26]. *In vivo* mouse models showed that single intravenous and orogastric administrations of L-arabinose could activate luminescence in colonized tumors [26]. This approach may face challenges if the small molecules used for activation cannot reach the bacterial colonies because of diffusion limitations. However, in preliminary experiments this limitation was not observed, and expression within tumors was detectable within 15–30 minutes of intravenous administration [26].

A second approach for controlling therapeutic delivery is to engineer bacteria to produce enzymes that convert harmless pro-drugs into active agents (Figure 3-middle). *Salmonella* and *Clostridium* have been engineered to express cytosine deaminase (CDase) which cleaves the pro-drug 5-fluorocytosine (5-FC) to chemotherapeutic 5-fluorouracil (5-FU) [38,53,57,58]. In a pilot clinical trial utilizing 5-FC and recombinant *Salmonella* expressing CDase, two out of three patients showed tumor colonization. In these patients the average 5-FU concentration showed an intratumoral increase of 300% over blood plasma [19]. This initial trial nicely showed that a pro-drug can be activated selectively in tumor tissues using bacteria. Species of *Clostridium* have also been designed to express a series of nitroreductases for conversion of CB1954 (5-aziridinyl-2,4-dinitrobenzamide) to its 4-hydroxylamine derivative, which is 10,000 times more toxic [23]. *In vivo* models demonstrated significant tumor regression following combined prodrug and bacterial treatment. This study employed a repeat dosing structure similar to chemotherapy regimes to show that continued tumor regression could be achieved [23].

In a third strategy for therapeutic delivery bacteria could produce biologically active molecules to induce a physiological response (Figure 3-bottom). *In vitro* studies have shown that *Clostridium* can be designed to produce therapeutically relevant levels of interleukin-2 [59], a cytokine that causes T-cell-mediated neoplastic death [60]. Hypoxia-inducible factor-1 α (HIF-1 α) is a transcription factor that triggers genes that allow for cell survival in hypoxic environments [61]. Factors that target this molecule within tumors can inhibit its activity and increase the therapeutic susceptibility of the hypoxic regions. Functional anti-HIF-1 α antibody fragments have been expressed by *Clostridium*, which, when combined with hypoxic targeting, may lead to an effective cancer treatment [62]. Bacteria can also be used to deliver DNA, a process referred to as bactofection [63,64]. In this process genes are transferred from bacteria into cancer cells [65,66] that then express the mammalian therapeutic proteins. *Salmonella* containing endostatin [67] and thrombospondin-1 [41] genes have been engineered. The products of both of these genes reduce cancer growth by inhibiting angiogenesis. In mouse models, both treatment modalities decreased tumor growth and increased survival times [41, 67].

Conclusions

Cancer is difficult to treat because regions of poor vasculature present transport limitations for oxygen, nutrients, and drugs. Bacterial therapies have an advantage over passive drug molecules because they can actively target intratumoral microenvironments with preferential growth and active motile transport. The cutting edge of bacterial anticancer research is engineering strains to be more than simple vectors and transforming them into potent cancer therapies. The two issues that need to be addressed are intratumoral targeting and controllable

therapeutic delivery. Initial efforts with *Clostridium*, *Escherichia* and *Salmonella*, have demonstrated expression of temporally controlled cytotoxins, pro-drug activating enzymes, and biomolecules. The strengths of these targeting and delivery systems are complimentary. When combined, they can reach all areas of tumors or metastases and effectively kill all cancer cells. It is possible that bacterial therapies utilizing multiple genera, each expressing different therapeutic modalities, could be administered in unique combinations to individual patients to target and attack cancer in a tailored and highly effective way. Going forward, we are confident that bacterial therapies will provide a powerful tool against tumors in humans, improving efficacy of treatment and resulting in increased patient survival.

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Figure 1. Tumor targeting mechanisms of obligate and facultative anaerobic bacteria

The three modes of tumor targeting are specific chemotaxis, preferential growth, and hypoxic germination. Top) Motile facultative anaerobes direct their movements by chemotaxis, sense nutrients or chemicals (blue ellipses and purple squares) with specific chemoreceptors (complementary Y-shapes), extravasate from blood vessels (red regions), and target the tumor compartments secreting these compounds (quiescent pink region and necrotic tan region). Middle) Facultative anaerobes, *Salmonella* and *Escherichia*, are administered in active/motile form, migrate inside tumors and recognize specific regions as favorable places to proliferate. Preferential mitosis is indicated by splitting cells. Bottom) Intravenously injected spores (stars)

of obligate anaerobes, such as *Clostridium*, target avascular/necrotic regions (tan) in tumors by spore diffusion and germination in only the hypoxic environment.

Figure 2. Microenvironment Gradients Present in Tumors

Multiple microenvironments form in tumors because of concentration gradients around sparse blood vessels. Nutrients and oxygen extravasate from the blood lumen (red region), through the endothelial vessel lining (purple), and diffuse into the interstitial tissue. Close to vessels, cells are viable and proliferating. Far from vessels, hypoxia and nutrient depletion create regions of necrosis. Large inter-vessel distances also reduce the concentration of blood-borne chemotherapeutics in distal tissue.

Figure 3. Mechanisms of Controlled Therapeutic Delivery

Three mechanisms used to control therapeutic delivery include temporally controlled cytotoxin release, enzyme drug activation, and biomolecule secretion. Top) Bacteria located within a radiation field (yellow curves) express and secrete (dashed arrows) a cytotoxic compound (purple shapes), while non-irradiated bacteria do not, illustrating how irradiation can be used to spatially and temporally control delivery. Middle) Bacteria expressing (dashed arrows) an enzyme gene (dark blue shapes) are used for drug activation. The pro-drugs (light blue diamonds) enter tumors (tan) via the bloodstream (red region) and are subsequently converted to the active drug (purple triangles). Bottom) Many biologically active compounds (blue

squares), including antibodies (blue “Y” figures) and DNA (black curves) can be engineered for bacterial expression and secretion (dashed arrows).