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REVIEW

Innovative Approaches of Engineering Tumor-Targeting Bacteria with Different Therapeutic Payloads to Fight Cancer: A Smart Strategy of Disease Management

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Abstract: Conventional therapies for cancer eradication like surgery, radiotherapy, and chemotherapy, even though most widely used, still suffer from some disappointing outcomes. The limitations of these therapies during cancer recurrence and metastasis demonstrate the need for better alternatives. Some bacteria preferentially colonize and proliferate inside tumor mass; thus these bacteria can be used as ideal candidates to deliver antitumor therapeutic agents. The bacteria like *Bacillus* spp., *Clostridium* spp., *E. coli, Listeria* spp., and *Salmonella* spp. can be reprogrammed to produce, transport, and deliver anticancer agents, eg, cytotoxic agents, prodrug converting enzymes, immunomodulators, tumor stroma targeting agents, siRNA, and drug-loaded nanoformulations based on clinical requirements. In addition, these bacteria can be genetically modified to express various functional proteins and targeting ligands that can enhance the targeting approach and controlled drug-delivery. Low tumor-targeting and weak penetration power deep inside the tumor mass limits the use of anticancer drug-nanoformulations. By using anticancer drug nanoformulations and other therapeutic payloads in combination with antitumor bacteria, it makes a synergistic effect against cancer by overcoming the individual limitations. The tumor-targeting bacteria can be either used as a monotherapy or in addition with other anticancer therapies like photothermal therapy, photodynamic therapy, and magnetic field therapy to accomplish better clinical outcomes. The toxicity issues on normal tissues is the main concern regarding the use of engineered antitumor bacteria, which requires deeper research. In this article, the mechanism by which bacteria sense tumor microenvironment, role of some anticancer agents, and the recent advancement of engineering bacteria with different therapeutic payloads to combat cancers has been reviewed. In addition, future prospective and some clinical trials are also discussed.

Keywords: anticancer payload, cancer, tumor-targeting bacteria, genetic modifications, nanoparticle, targeted drug-delivery

Introduction

Cancer has been one of the main cause of deaths worldwide and poses a serious challenge and threat to human health. The current clinical therapies used for the treatment of different cancers include surgery, radiotherapy, immunotherapy, hormonal therapy, and chemotherapy. The choice can be monotherapy or combination therapy and depends on several factors like cancer origin, stage, location, and grade.[1] Even

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though these anticancer therapies can be effective, they have certain disadvantages, like: (a) they can cause pharmacological adverse effects at normal tissues; (b) they lack the ability of center-point targeting deep within tumor mass; (c) they mostly acquire drug resistance and are unable to eradicate the entire cancer cell population in the tumor.[2] Hence, there is an utmost need to develop some innovative therapeutics that should be simple, cost-effective, and could serve as a substitute to conventional treatments to fight cancer. In this regard, recent advancements in the utilization of tumor-targeting bacteria engineered with different therapeutic payloads have been found to be quite unique and effective strategies of cancer therapy.[3]

Recently, some microbes, cells, bacteria, and viruses have been found to possess unique characteristics of movement towards tumor microenvironment (TME). Thus, these candidates have been utilized as carriers of antitumor payloads including drug-loaded nanoformulations to target the cancer much more efficiently. These properties are not possessed by conventional antitumor nanoparticles (NPs) alone.

Natural cancer-targeting bacteria have the ability to selectively penetrate, colonize, and degenerate tumors.[4] These bacteria can be engineered to perform controlled delivery of specific and diverse therapeutic payloads/drug-loaded nanoformulations into TME at the desired dosage. These therapeutic payloads include cytotoxic proteins, angiogenesis modulators, immunomodulators, prodrug-converting enzymes, small interference RNAs (siRNAs), and drug-loaded nanoformulations, as shown in Figure 1.[3,5]

The toxicity issues on nearby normal tissue are a main concern for systemic injection of therapeutic agents at the tumor site. These complications have led to improve the center-point target delivery of anticancer drugs and drug nanoformulations to enhance the therapeutic potential and minimize the toxic effects. Rapid advancement in the drug-loaded nanomaterials in the past decade has been a powerful thrust for the innovation of cancer treatment. Some nanomaterials like liposomes, micelles, polymers, metal nanoparticles (NPs), etc., have been widely used as drug-loaded targeted delivery vehicles and play a significant role in cancer treatment. These nanocarriers have been loaded with different antitumor drugs, which include doxorubicin, paclitaxel, cisplatin, tamoxifen, etc.[6–8]

In comparison to normal tissues, solid tumors are more permeable to therapeutic agents including NPs due to enhanced permeability and retention effect (EPR).[9] The EPR-effect is now a well-acknowledged phenomena,

validated in different cancer models as well as in cancer patients.[10] Cancer tissues with rich blood vessels exhibit a good EPR effect and concomitantly respond to treatments, whereas tumors with reduced blood flow demonstrate poor drug delivery and treatment strategies.[11] It has been reported that nitric oxide (NO) is one of the most important factors to enhance the EPR effect through vasodilation, opening of cell junction gaps of endothelial cells, and increasing the blood flow within the hypovascular cancerous mass.

Only a few drug-loaded nanoformulations have shown remarkable success in cancer management, as many challenges still persist in the clinical application of these nanomaterials. The TME is characterized by hypoxia, acidity, immunosuppression, and high interstitial fluid pressure (IFP).[12] Therefore, the pinpoint targeted application of nanoformulations at the tumor site is still a challenge which needs to be achieved to effectively eradicate the cancer menace.

Incorporation of specific therapeutic payloads within or on the surface of a particular bacteria as a tool of tumor therapy is now considered as an innovative approach for cancer management. The TME displays a unique environment for an ideal breeding site for some obligate and facultative anaerobic bacteria.[13] Bacteria like *Bifidobacterium, Clostridium, Escherichia coli* (*E. coli*), and *Salmonella typhimurium* (*S. typhimurium*) can preferentially proliferate in immunosuppressive, eutrophic, and hypoxic environments found around tumor tissues. By the use of synthetic biological technology and genetic engineering, these engineered bacteria can achieve center-point targeted delivery of anticancer drugs, specific proteins, antibodies, enzymes, antigens, and cytokines.[14]

This article reviews the latest developments in engineering some specific tumor-targeting bacteria to enhance further their anticancer potential with immunotherapeutic agents, tumoricidal vectors and enzymes, cytotoxic agents, and drug-loaded NPs. In addition, some bacteria derived therapeutic agents like spores and membrane vesicles to carry different therapeutic payloads to deep sites of diverse tumors are also discussed. Furthermore, the prospects of the future developments and clinical trials for cancer prevention and treatment are also discussed.

Mechanisms by Which Bacteria Can Sense TME

Some bacteria love to accumulate at tumor sites as the TME provides a suitable milieu and such microorganisms
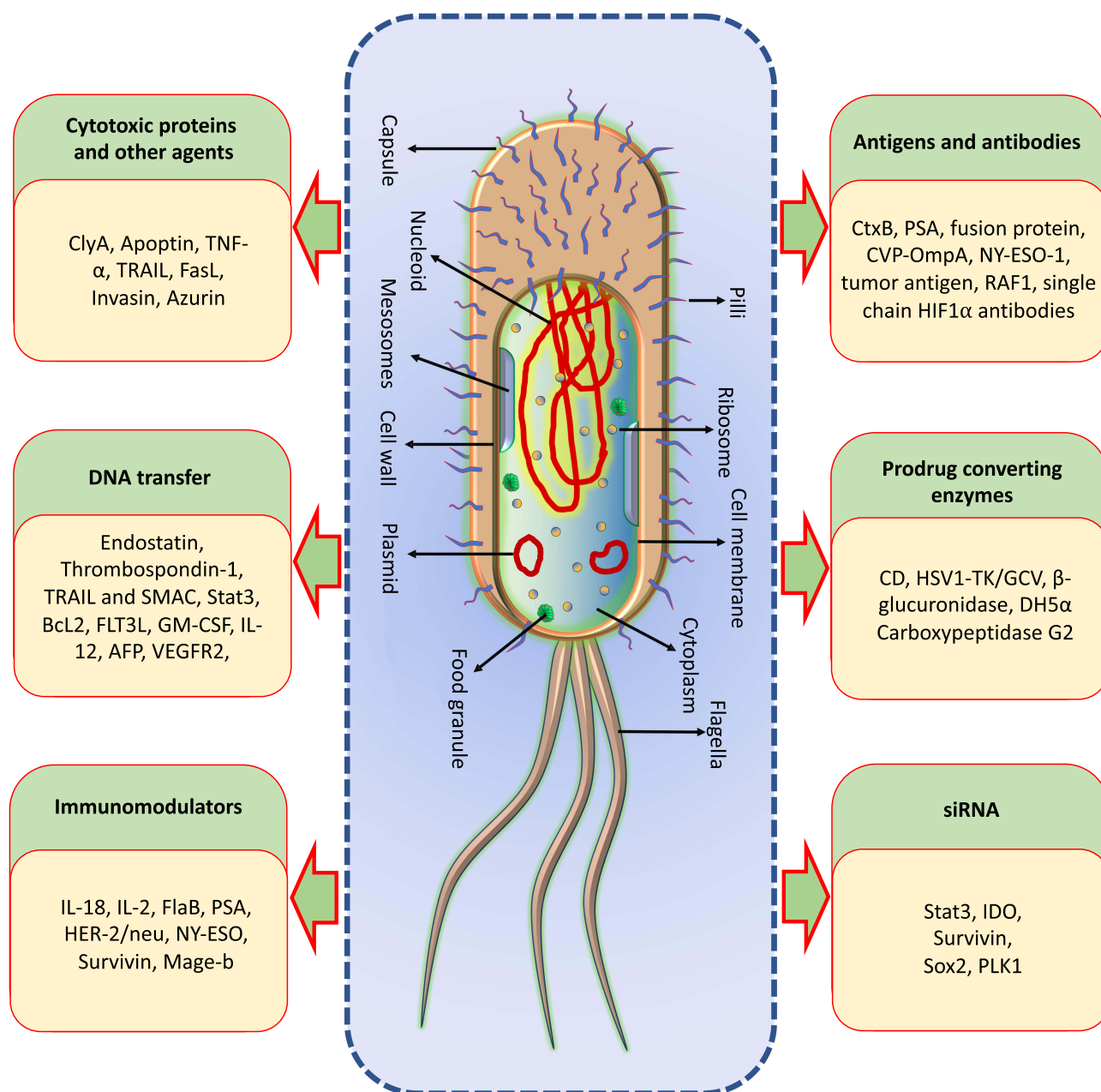
Figure 1 Diagrammatic representation of different molecules expressed by engineered-tumor targeting-bacteria, used as therapeutic agents against different cancers.

can reach this area through flagellar motion.[15] The obligate and facultative anaerobic bacteria find a suitable habitat within the TME as it is a nutrient-rich territory.[9] *S. typhimurium* and *E. coli*, as facultative anaerobes, can sense the nutrient-rich and favorable environment through their chemoreceptors and get accumulated in the periphery as well as the core of tumor region.[13] Bacteria preferably colonize in these regions as it displays an immunosuppressive environment, so is not usually cleared by neutrophils and macrophages. In contrast, the immune system quickly clears the bacteria present in the circulatory system and

other major organs. In comparison to the normal tissue, the cancerous tissue displays a chaotic vasculature and large capillary spacing that impedes the delivery of therapeutic agents. The powerful motor properties of bacteria help it to pass through the blood vessels to reach the tumor area.

Since, no oxygen is needed to survive for obligate anaerobic bacteria, they preferably migrate towards the hypoxic areas of the tumor. The flagellar motility enables some bacteria to overcome the diffusion resistance as *Bifidobacterium* and *Clostridium* have been located at hypoxic areas around the tumor. Due to the poor lymphoid

fluid drainage and blood vessel leaking, the tumor tissues possess higher IFP.[16] The increased IFP hinders the conventional therapeutic agents to reach the deeper tumor mass, thus impacts its uptake by the cancer cells. The engineered bacteria with therapeutic payloads can bypass this predicament by their flagellar motion to reach deep inside the necrotic core.[4]

Bacteria as Cancer Treatment Agents and Their Antitumor Features

Some bacteria like *Clostridium* spp., *Listeria*, and *Salmonella* have innate properties of tumor-targeting, which enables them to target, pierce, proliferate, and reduce solid tumors by different mechanisms.[3,4] *Clostridium* genus bacteria like *C. butyricum* and *C. novyi-NT* can survive in hypoxic conditions present around the tumor mass.[17] These bacteria can destroy the cancer tissue by exotoxins, which damage the cancer cell membranes and enter these cells and disrupt their essential functions.[18] These bacteria can also recruit CD8^{+} T-cells, macrophages, and granulocytes to the cancerous area and neutrophils mediate the release of TNF-related apoptosis-inducing ligand (TRAIL) (Figure 2).[19]

Listeria spp. bacteria can target the cancer tissue through tumor-infiltrating myeloid-derived suppressor cells (MDSCs), which wander to the immunosuppressive TME. A unique cell–cell spread mechanism is involved in the transport of *Listeria* from MDSCs to cancer cells.[20] *Listeria* spp. bacteria and cytotoxic T-cells in combination directly target the cancer cells and lead to shrinkage of the tumor mass.[21] These bacteria can activate NADP(+) oxidase within cancer cells and increase the intracellular Ca^{2+} level, thus triggering the production of reactive oxygen species (ROS). These biochemical changes lead to direct killing of cancer cells.[21] In addition, *Listeria* spp. can transform some infected MDSCs into immune-stimulating phenotypes that can produce interleukin-12 (IL-12), involved in natural killer (NK) and T-cell response (Figure 2).[20]

Within the TME, some metabolites produced by quiescent cancer cells act as chemo-attractants for *S. typhimurium*.[22] In the presence of tumor environment, these bacteria proliferate and trigger necrosis, apoptosis, and cell rupture, thus kill the surrounding cancer cells.[14] The cancer cells are forced to produce gap junction protein (connexin 43) by *Salmonella* spp. This protein reduces the immunosuppressive expression of indoleamine 2,3-dioxygenase (IDO) and enhances the transfer and cross-

presentation of processed tumor antigenic peptides between cancer cells and dendritic cells (DCs).[23] In addition, *S. typhimurium* flagellin reduces the frequency of regulatory T-cells (Tregs) and enhances the antitumor response of NK and CD8^{+} T-cells (Figure 2).[3]

Wild-type probiotics have been used to study bladder cancer, cervical cancer, breast cancer, liver cancer, in addition to colorectal cancer.[24] These probiotics can be directly delivered at the TME to reduce non-specific pharmacological effects on normal tissues. The tumor-targeting bacteria and probiotics have some limitations in their use as anticancer agents, as it is challenging to balance the bacterial dosage for therapeutic purpose and the measure of toxicity.[14,25] In addition, tumor-targeting and probiotics have limitations in eradicating completely the tumor mass and further probiotics lack the intrinsic therapeutic potential of tumor targeting.[17] There is still a problem of high risk infection and toxicity by using these bacteria.[26] The intratumoral injection of therapeutic bacteria at tumor sites is a good option to reduce the toxicity and infection rate, but it cannot be used during the metastatic tumor phase.[27]

Mechanism of Bacteria-Mediated Tumor Therapy

Coley used bacilli (*Streptococcus pyogenes*) for the first time in 1891 for the treatment of osteosarcoma.[28] Several mechanisms are involved in bacteria-mediated cancer suppression like the activation of immune system. The concentration of oxygen in the tumor tissue is only 7–28 mm Hg (1–4%), while it is 40–60 mm Hg (5–8%) within the normal tissue.[29] Bacteria can also recruit inflammatory cells like NK cells and granulocytes for TME, important for anti-tumor response.[30] In addition, bacteria can induce CD4^{+} T-cells in the TME to produce interferon-γ (IFN-γ) and can also activate CD8^{+} T-cells to inhibit tumor growth.[31]

The toxicity of bacteria can be minimized with the aid of genetic modifications in addition to enhanced selective targeting.[13] It involves the chromosomal deletion of purI and msbB genes of *S. typhimurium* (VNP20009) to reduce their septic shock and virulence.[32] In addition, the leu-arg-deficient genetically modified *S. typhimurium* A139 strain possesses exceptional tumor-targeting ability.[33]

The therapeutic role of bacteria can be classified into three groups as: (a) antitumor immune activation, (b) secretion of bacterial toxins, and (c) swelling and apoptosis of tumor cells by invaded bacteria. Bacteria demonstrate
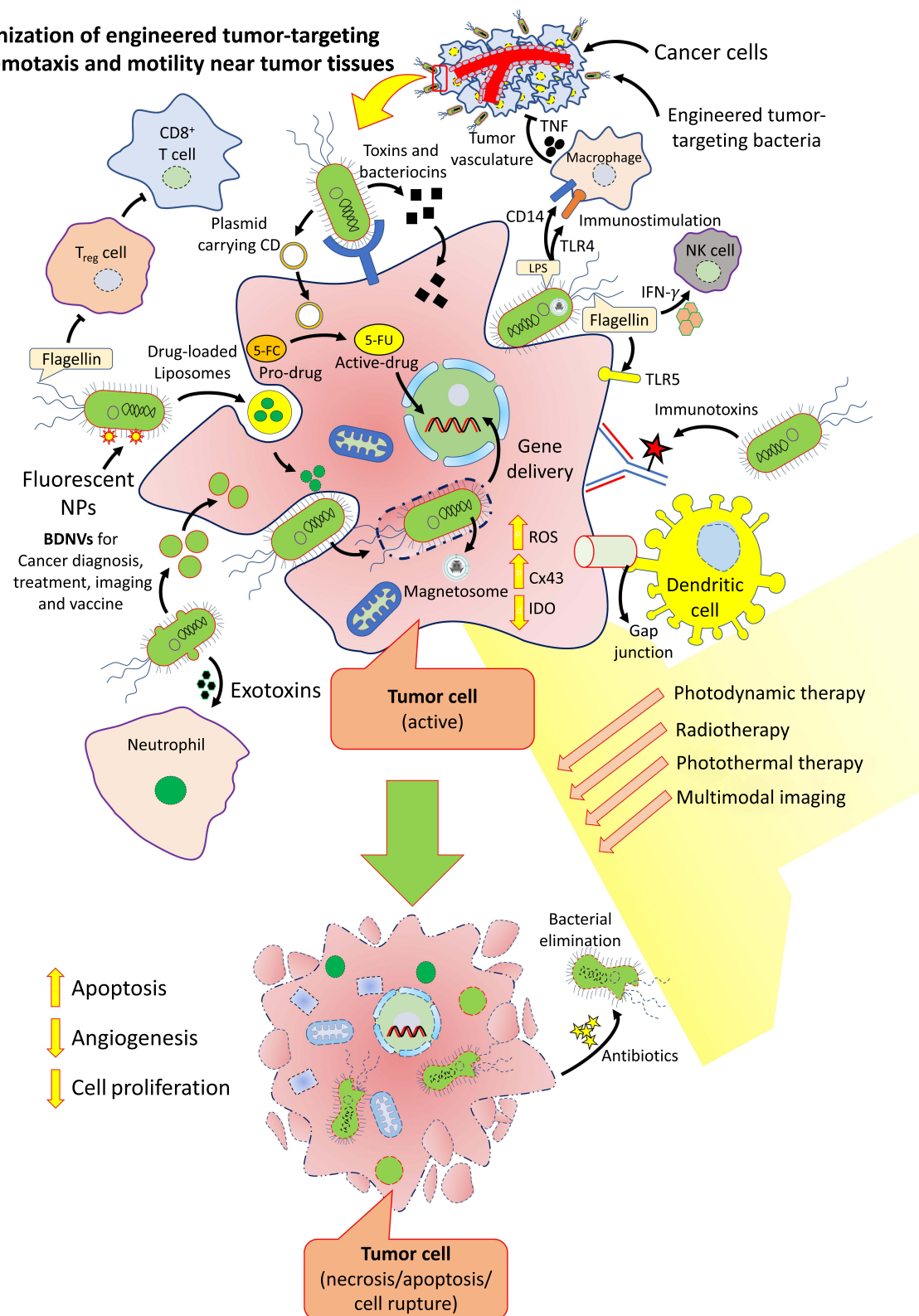
Figure 2 Diagrammatic representation of different mechanisms followed by engineered-tumor-targeting-bacteria for cancer therapy.

wonderful immune activation capability. For example, dendritic cells and macrophages get colonized in the presence of *Salmonella* and are induced to produce interleukin-1β (IL-1β). These bacteria also lead to connexin 43 (Cx43) upregulation and the gap junctions formation between tumor and the dendritic cells,[34] that leads to significant anticancer immune response. Further, the inflammatory response is also activated through pathogen-associated molecular patterns (PAMPs), which facilitates cytokine release that contributes to cancer immunotherapy.[35] For example, toll-like receptor 4 (TLR4) signal transduction is induced by lipopolysaccharides (LPS) that promotes IL-1β production from the macrophages.[36] In addition, the NK cells are stimulated by the flagellin that induces the production of IFN-γ (Figure 2).[37]

The toxins produced from bacteria can activate apoptotic pathways. For example, cytolysin A (ClyA) mediates caspase induced cell death and also forms gaps within the cell membranes.[38] ClyA, produced from *E. coli* K-12, inhibits the cancer growth. In addition, the tumor progression is correlated with nitric oxide (NO) level. The higher level of NO mediates apoptosis of cancer cells, resulting in tumor regression.[39]

Engineering of Bacteria for Tumor Management

As microscopic robots, bacteria can be reprogrammed following simple genetic rules or sophisticated synthetic bioengineering principles to produce and deliver antitumor agents based on the clinical needs. The engineering of bacteria to combat cancer is performed at different levels as virulence attenuation, enhancement of tumor targeting, targeting the tumor stroma, drug expression strategies, and the expression of cytotoxic agents. In addition, the engineering of tumor-targeting bacteria is also achieved through the biosynthesis of metal NPs and delivery of drug-loaded nanoformulations. Furthermore, the bacterial spores and bacterial membrane vesicles are also utilized as an anticancer strategy. All these strategies of antitumor approaches are briefly discussed here:

Virulence Attenuation

While using specific bacteria against a cancer, it is very important to minimize their virulence against the host immune system, keeping in view that the intrinsic antitumor activity of some bacteria are due to their virulence factors.[30,40] Therefore, the antitumor activity of a bacteria

should not be lost while attenuating them. Some highly toxic bacterial strains have been attenuated to safer strains through the deletion of major virulence genes. Deletion of purI and msbB genes in *S. typhimurium* led to the formation of VNP20009 strain, which is extensively used in cancer-bearing mice for different antitumor studies.[41] This strain has been accordingly tested in Phase I trials in human cancers, but the outcome has been disappointing.[42] The failure is expected to be due to penta-acylated lipid A, a toll-like receptor 4 (TLR4) antagonist.[43] New mutant *Salmonella* strains have been engineered by the deletion of pagL, pagP, and 1pxR genes to produce hexa-acylated lipid A with high affinity for TLR.[44]

The lipopolysaccharide (LPS)-driven septic shock has also been reduced dramatically by the deletion of msbB gene in *Salmonella* genus.[45] The integration of LPS gene within chromosome in araBAD locus resulted in production of strains with attenuated virulence and enhanced therapeutic effects.[46] The downregulation of endotoxin-associated genes led to the formation of another nontoxic *Salmonella* strain. *Salmonella* spoT and relA-mutant strains exhibited negligible toxicity as these strains are defective in ppGpp, signaling molecules involved in toxin gene expression. These strains exhibited excellent antitumor activity through the activation of inflammasome (IPAF, NLRP3), which can induce the expression of numerous proinflammatory cytokines.

The cytotoxicity of *L. monocytogenes* is achieved by the deletion of genes, involved in cell invasion and defects in phagolysosome release, achieved by HIy deletion.[47] Mutant strains of *L. monocytogenes* lacking inIA and inIB are invasion defective and the strains lacking ActA or actA PESTf-like sequences also lack intracellular diffusion ability.[48] The additional approach to attenuate virulence with enhanced tumor-specific proliferation is achieved by the introduction of specific nutrient-dependent mutations in bacteria. The examples of some attenuated strains of several tumor-targeting bacteria and their description is listed in Table 1.

Enhancement of Tumor Targeting

The approaches to enhance the bacterial tumor targeting can also improve both antitumor efficacy as well as safety aspects. Regarding this approach, the ppGpp-deficient strain SHJ2037 has been genetically engineered to exhibit cancer-specific ligands on its cell surface. An αvβ3 integrin binding with Arg-Gly-Asp peptide has

Table 1 Description of Some Genetically Modified Bacterial Strains Used for Tumor Therapy

Bacteria	Attenuated Strain	Description	References
Bifidobacterium	*B. longum* 105-A	Effective delivery of spectinomycin resistance gene	[49]
Clostridium	*C. novyi-NT*	Exhibits strong tumor-specific colonization and proteolytic features. Curing of 50% HTC116 xenograft and tumor regression as performed by COBALT technique	[50]
	C. beijerinckii NCIMB 8052	Expression of dihydropteridine gene, catalyzing the prodrug CB1955, and in vivo tumor eradication by 22-fold	[51]
	C. sporogenes NCIMB 10696	Insertion of *E. coli* Cod A gene expressing cysteine deaminase. This strain presents outstanding anticancer effects when colonized in SCCVII-tumor bearing mice followed by 5-fluorocytosine injection.	[52]
E. coli	*E. coli* (BM2710)	Insertion of inv gene derived from *Y. pseudotuberculosis* in the plasmid produces invasin which promotes the phagocytosis of the cell by mammalian cells	[53]
Salmonella typhimurium	ΔppGpp	Modified strain with the property of downregulating endotoxin genes. After injection systematically, virulent to mouse, suitable vector for targeting the delivery of antitumor molecules	[54]
	VNP20009	Possess purine dependent colonization behavior. This strain possesses a relatively better safety profile as it has been made to reduce septic shock	[55]
	A1-R	Possesses arginine and leucine dependent colonization behavior. In mouse models, inhibits the growth of different cancer types. This strain also helps to alter the cell cycle.	[56]

been fused to protein A on the outer membrane to drive its expression.[57] The resulted strains possessed enhanced cancer-specific activity and significantly augmented anti-tumor activity in mDA-MB-435 melanoma xenografts overexpressing αvβ3 integrin and mDA-MB-231 breast cancer cells. The bacteria have also been engineered to target tumor-associated genes like lymphoma-associated antigen CD20 and carcinoembryonic antigen (CEA). These strains possess reduced non-specific accumulation in the spleen and liver and effective antitumor activity.[58] The bacteria *L. monocytogenes* were coated with plasmid-loaded NPs expressing bioluminescence genes to exploit biotin-streptavidin binding. This strain, known as microrobot, could be traced by the bioluminescence imaging as it delivers the functional nucleic acid molecules within the solid tumors.[59]

A fascinating alternative to enhance the tumor selectivity is achieved by displaying synthetic adhesins (SAs) on the *E. coli* surface. These adhesins have a modular structure with stable β-domain needed for outer membrane anchoring and surface exposed antibody domains with high specificity and affinity which can be selected from large libraries.[60] Some probiotic strains have been

designed with enhanced tumor specificity and increased injection capacity of bacteria.[61]

Targeting the Tumor Stroma

The cancer growth and metastasis is equally supported by angiogenesis, and targeting this tumor neovascularization offers a favorable trend for cancer therapy. Endostatin (20 kDa C-terminal fragment from type XVIII collagen) has been found to possess inhibitory potential on tumor vessel formation with least side-effects or drug resistance.[62] The attenuated strain of *S. typhimurium* was cloned with endostatin and siRNA against transducer and activator of Stat3 and the therapeutic efficacy was investigated on HCC. It showed satisfactory reduction in cancer proliferation and metastasis and reduced the tumor vasculature as well. This strategy led to the downregulation of VEGF expression, regulatory T-cells and TGF-β expression. In addition, there was an enhancement in inflammatory cytokines including TNF-α and IFN-γ and increased CD4$^+$/CD8$^+$ T-cell population.[63]

VEGF and its receptor (VEGFR) are well known tumor angiogenesis proteins. *S. typhimurium* (SL3261) expresses the extracellular VEGFR2 domain and the oral administration of this strain led to reduced pulmonary metastasis,

neovascularization, and tumor growth. In addition, the administration of this strain led to an increased population of CD4$^+$ and CD8$^+$ T-cells near tumor regions.[64]

Endoglin (CD105) is a member of the TGF-β receptor family and its gene promoter is overexpressed in tumoral endothelial cells. Hypoxia and TGF-β1 are known to upregulate the endoglin gene promoter. Therefore, targeting the endoglin is considered as a novel strategy of cancer therapy.[65] In mouse breast cancer models, *Listeria* based vaccines have been used against CD105, Lm-LLO-CD105A, and Lm-LLO-CD105B as a treatment strategy. Such vaccines inhibited primary and metastatic tumors by the reduction of angiogenesis and elevated antitumor immune response.[66]

Drug Expression Strategies

A strict control over the production and targeting of most payloads by tumor-targeting bacteria is of utmost importance as these are toxic to both normal and tumor cells. A precise trigger for the payload expression can minimize its systemic toxicity while maximizing its therapeutic effect. By the insertion of a specific promoter sequence upstream of a drug-encoding gene, a controllable gene expression can be maintained, convening transcriptional control through external signals. The triggering for gene regulation is mainly classified into three categories as (a) internal triggering, (b) self-triggering (quorum sensing-QS), and (c) external triggering.[67] The special properties of TME like acidosis, hypoxia, and necrosis are sensed by tumor-targeting bacteria, which are utilized to improve their cancer specificity. It includes hypoxia inducible promoters (HIP-1) and pepT, activated by nitrate and fumarate reduction present in the hypoxic environment of cancerous tissue.[68] This hypoxia-inducible expression method was proposed to function during anaerobic conditions only to express essential genes like asd. Furthermore, a glucose sensor has also been engineered in *E. coli* to sense the glucose level in TME leading to its therapeutic effect.[69]

Expression of Cytotoxic Agents

The expression of cytotoxic agents can be firmly regulated to check their toxic potential on normal tissues. Bacteria like *E. coli*, *Paratyphi A*, and *S. typhimurium* produce a 34 kDa pore-forming hemolytic protein known as cytolysin A (ClyA), secreted without any post-translational modifications. Several bacterial strains have been engineered to express ClyA from a constitutive promoter.[70] In addition, ClyA is programmed to express from inducible promoters

activated by doxycycline and arabinose, and excellent tumor inhibition has been reported.

The induction of apoptosis in cancer cells is a novel alternative of tumor management. In this regard, apoptin, a virus-derived protein in chicken, has been selectively used to induce apoptosis in different human cancer cell types through the p53-independent, Bcl-2-insensitive pathway.[71] A significant cancer reduction with minimal systemic toxicity has been observed in human laryngeal cancer-bearing mice by the transformation of apoptin-encoding eukaryotic expression plasmid (pCDNA3.1) into the attenuated *S. typhimurium* strain.

Some other cytotoxic agents for the induction of apoptosis, like Fas ligands, TNF-α, and TRAIL, have limited use due to their hepatotoxicity and short half-life.[72] Some bacterial strains have been used to deliver these proteins directly within the cancerous tissues to overcome these limitations.

Yersinia express invasin on its surface which can selectively bind to β1 integrin and triggers bacterial entry into host cells. In mice, the introduction of *E. coli* strain co-expressing invasin, ovalbumin, as well as LLO has been shown to invade β1-integrin, expressing tumor cells to show strong therapeutic effects.[73] Furthermore, azurin is a low-molecular weight redox protein which initiates cancer cell apoptosis through its internalization. This protein helps to release cytochrome c from mitochondria by raising the intracellular level of p53 and Bax. The *E. coli* based azurin delivery has been reported to suppress 4T1 mouse breast cancer and B16 mouse melanoma, and this approach stimulates inflammatory response and prevents pulmonary metastasis.[74]

Different Therapeutic Payloads Delivered by Engineered Bacteria

Some specifically engineered bacteria have played a significant role in transporting different types of payloads up to extracellular TME and intracellular locations of tumor cells. Employment of some novel nanocarriers for conventional drugs and therapeutic agents helps to improve their bioavailability and pharmacodynamic and pharmacokinetic parameters. Different types of nanomaterials are used to improve the solubility of anticancer drugs, prolong circulation time, and enhance their accumulation within the TME. Native drug-loaded nanoformulations encounter diffusion limitations in the extracellular matrix and get accumulated in the periphery of the tumor rather than in the hypoxic core of the tumor.

Delivery of Anticancer-Proteins, Enzymes, and Other Agents

Non-pathogenic strains of *S. typhimurium* have been engineered under the control of prokaryotic radiation-inducible RecA promoter to secrete TRAIL protein. The TRAIL protein induces its toxicity through caspase-3 activation. On irradiation, *S. typhimurium* secreted TRAIL can lead to caspase-3-mediated apoptosis and death in 4T1 breast cancer cells in culture. In mice, the systemic injection of these engineered bacteria led to TRAIL expression by 2Gy γ-irradiation with delayed breast cancer growth.[75]

In *E. coli*, invasin genes have been cloned to express the invasin proteins.[76] These proteins are normally exploited by *Y. pseudotuberculosis* as an entry pass into the host cells during their invasion. The invasins bind with β1-integrin proteins expressed by cancerous and epithelial cells. The invasins enter the host cells through receptor-mediated endocytosis and exploit their anticancerous activity.

In the host cells, *E. coli* are armed with listeriolysin O (LLO), which forms pores in the lysosomes.[76] The expression of invasins in the cytosol results in cancer cell death. In addition, *E. coli* also helps to boost the immune system at the infection site and systematically with PAMPs expressed, recognized by Pattern Recognition Receptors (PRRs) on immune cells. The interaction of immune cells with PAMPs leads to reactive nitrogen and ROS release. This interaction also leads to the activation of T lymphocytes like CD4^{+} T-cells and CD8^{+} T-cells, which are capable of halting further proliferation of tumor cells (Figure 2).

The *E. coli* derived enzyme asparaginase (L-ASNase) has been utilized for the treatment of acute lymphocytic leukemia.[77] This enzyme catalyzes the formation of aspartate from asparagine and to some extent forms glutamate from glutamine and both the reactions are important for cancer treatment.[78] A treatment strategy was devised for acute lymphoblastic leukemia by using *Salmonella* bacteria expressing L-ASNase. The araBAD *E. coli* inducible promoter was used to design *Salmonella* cells to deliver L-ASNase to cancer cells.[79]

Delivery of Gene Therapy and Gene Silencing Agents

A promising approach to cancer therapy has been achieved by silencing specific target genes by using small interference RNAs (siRNAs). The greatest challenge to RNA interference therapy is the requirement of a specific delivery system for siRNAs to the tumor region. Mouse models have been investigated to check the activity of siRNA through bacteria-based delivery systems against indoleamine 2,3-dioxygenase (IDO),[80] Stat,[63] Sox,[81] survivin,[82] and the cell cycle-associated polo-like kinase 1 (PLK1).

Recombinant *Salmonella* has been orally administered in tumor-bearing nude mice, leads to decreased cancer growth, and displayed more sensitivity towards cis-diamine-dichloroplatinum (II) (DDP). Transforming growth factor-α (TGF-α) is a naturally occurring ligand for EGFR, which is overexpressed in tumor cells. A recombinant immunotoxin like PE38 has been constructed by conjugating TGF-α and laboratory-engineered *Pseudomonas* exotoxin A. Tumors in the mouse model as well as in vitro, PE38 exhibit a toxic effect on cancer cells which express EGFR.[83] However, dose-dependent hepatotoxicity has been reported by systemic injection of TGF-α-PE38.[84]

In one study, DppGpp *Salmonella* mutant expressing recombinant TGFα-PE38 were investigated, which showed neither attack nor proliferation within mammalian cells,[85] but exerted their anticancer effects by the expression of proinflammatory cytokines from neutrophils and macrophages, such as TNFα and IL-1β.[79] The study included the construction of a plasmid with DNA encoding TGFα-PE38, inserted into *Salmonella* cells. Breast and colon tumors with enhanced levels of EGFR expression in mouse models were employed for this study. An inducible system based on PBAD promoter from *E. coli* was used.[86] For the export of TGFα-PE38 recombinant protein from *Salmonella*, an engineered phage lysis system was employed as a bacterial membrane transport signal, fused to the proteins.[87] Both these approaches were found to be effective. It was observed that TGFα-PE38 produced from bacteria reduced cancer progression as compared to non-engineered *Salmonella* alone.[87] Increased expression of EGFR was observed by the treatment with TGFα-PE38 in cancer cells which induced the apoptosis consequently. Therefore, bacteria can be an innovative strategy for enhancing the effectiveness of immunotoxins for cancer treatment.[88]

A study was performed to investigate the cytotoxic activity of *Salmonella* strain equipped with salicylate-inducible expression apparatus, that modulates the expression of cytosine deaminase (CD).[89] 5-FU resistant *Salmonella* strains were produced for the increased production of bacterial CD. In addition, purD mutation was

developed to regulate the intracellular proliferation in the presence of adenine as well as to prevent intracellular *Salmonella* death. This approach led to the production of *Salmonella* strains CD to kill cancer cells in the presence of 5-FU.[89] As compared to other cancer-targeting bacteria, engineered *Salmonella* strains

Table 2 Some Examples of Anticancer Agents Delivered or Targeted by Different *Salmonella* Strains

Salmonella Species	Model/Type of Cell Line	Type of Cancer	Anti-Tumor Agent/Anti-Cancer Effect	References
Salmonella typhimurium	C57BL6 mice bearing an implanted prostate tumor/D2F2	Prostate tumor	Stat3-specific/Treatment of primary and metastatic cancer	[90]
Salmonella typhimurium VNP20009	Tumor-implanted mice	Melanoma	PNR/Delayed tumor growth; increased CD8(+) T-cell infiltration	[91]
Salmonella typhimurium SL7207	C57 BL/6 mice/TRAMPC1	Prostate cancer	Prostate stem cell antigen (PCSA)/Generated specific antitumor immune responses	[92]
Salmonella	BALB/c nude mice bearing A549 tumors/A549	Lung adenocarcinoma	RBM5/Apoptosis	[93]
Salmonella choleraesuis	BALB/c mice bearing 4T1 tumors	Melanoma and bladder tumor	Endostatin/Decreased intra tumoral microvessel density, reduced VEGF, CD8(+) T-cell infiltration	[94]
Salmonella typhimurium	Ectopic transplanted model of C57BL6 mice/CT26	Hepatocellular carcinoma	Stat3-shRNA	[95]
Salmonella typhimurium SC36	Mice bearing melanomas or pulmonary tumors	Melanomas or pulmonary tumor	PNR/Apoptosis	[96]
Salmonella typhimurium SC36	Mice bearing mammary carcinoma/TRAMPC1	Mammary carcinoma	PNR/Suicide gene/prodrug therapy	[97]
Salmonella typhimurium VNP20009	Mice bearing A549 tumors	Lung cancer	Sox2shRNA/Anti-angiogenesis	[98]
Salmonella typhimurium Dam (9, AroA(-)	BALB/c mice/D2F2	Breast cancer	Legumain/Suppressing tumor angiogenesis	[99]
Salmonella typhimurium - lux	C57/J mice C57 BU6 mice/CT26	Hepatocellular and colon cancer	Mouse alpha-fetoprotein (AFP) gene/Promote protective immunity	[100]
Salmonella typhimurium, MvP728 (purD/htrA)	Female BALB/c mice/CT26	Colon carcinoma and orthotopic DBT glioblastoma	Survivin/Regulated T3SS of Salmonella and NKT ligands	[101]
Salmonella typhimurium	Lewis lung carcinoma model in mice	Lung carcinoma	DNA vaccine (pcDNA3.1 -FLK1 (ECD)/Prevented recurrence and metastasis	[64]
Salmonella typhimurium	Nude mice bearing human MDAMB-231 xenografts	Tumor	PI-KI	[102]
Salmonella SL3261	Male Sprague Dawley (SD) rat model of colorectal tumor	Colorectal cancer	4-1 BBL/Enhanced T-cell immunity	[103]
Salmonella typhimurium	Mice/4T1	Mammary carcinoma	TRAIL/Reduced tumor growth	[75]

have attained a special momentum in the delivery of antitumor payloads within the TME. Table 2 describes some examples of anticancer agents delivered by different *Salmonella* strains.

Delivery of Immunomodulators

Cytokines are well-known to have antitumor potential by inducing apoptosis in tumor cells. These molecules can activate, proliferate, and differentiate immune cells via anti-angiogenesis effects on tumor vasculature. Different cytokines like IL-12, IL-18, and GM-CSF have been checked for clinical trials for tumor therapy.[104] Several cytokines have been delivered in the TME by tumor-targeting bacteria, where it augments the antitumor immune response. The primary tumor growth in mice was potentially inhibited by the intravenous administration of attenuated *S. typhimurium* strain expressing IL-18. This led to increased number of CD4$^+$ T and NK cells and massive leukocyte infiltration (especially granulocyte) at TME. This approach also led to enhanced cytokine production at TME including IFN-γ, IL-1β, TNF-α, and GM-CSF.[105]

The delivery of tumor associated antigens led by engineered bacteria can sensitize TME and overcome the self-tolerance provoked by the regulatory T-cells, thus elicit effector and memory T-cell response towards the antigen-producing cancer cells.[106] Different prostate cancer-associated antigens like prostate-specific antigen (PSA) have been worked out by bacteria-based vaccines tested on several mouse models.[107] The gene delivery of endogenous PSA has been performed by using attenuated *S. typhimurium* (SL7207), which led to alleviated immune response in murine prostate cell antigens and considerably reduced the tumor growth.[92]

Some promising cancer inhibition effects have also been observed by using a gene therapy approach by using antigens against HER-2/neu,[108] Mage-b, NY-ESO,[109] and Survivin.[110] All these findings led to deep interest in the field of immune checkpoint blockade (ICB) cancer therapy. The success of ICB therapy during clinical trials has been limited to only a few patients, some reasons include host resistance like immunosuppressive TME.[111] The bacterial tumor colonization can induce proinflammatory reactions involving enhanced expression of IFN-γ, IL-1β, and TNF-α, as well as NK and T-cell activation, thus a combination of bacterial therapies and ICB can overcome the host resistance.[112]

Delivery of Prodrug-Converting Enzyme

The conversion of prodrugs into cytotoxic agents by the expression of prodrug-converting enzymes is a smart strategy of tumor eradication. This method reduces the side-effects associated with systemic administration and improves the cancer treatment efficacy. Bacteria have been used to deliver prodrug-converting enzymes.[112] These enzymes include cytosine deaminase (CD), which converts nontoxic 5-fluorocytosine (5-FC) into a chemotherapeutic agent, 5-fluorouracil (5-FU) (Figure 2). This drug is highly toxic as it is metabolized to a product which interferes with the DNA and RNA synthesis.[113] Another prodrug-converting enzyme/prodrug combination includes the herpes simplex virus type I thymidine kinase/ganciclovir (HSV1-TK/GCV) system, widely studied for tumor therapy. The expression of cancer-specific HSV1-TK can convert nontoxic precursor ganciclovir into a toxic form, ganciclovir-3-phosphate, that kills the cancer cells. The in vivo efficacy of *Bifidobacterium infantis* strain expressing HSV1-TK and GCV was examined in a rat bladder cancer model. This led to an efficient and targeted approach inhibiting the cancer effectively via apoptosis through the enhanced expression of caspase 3.[112]

E. coli DH5α is a good example of a prodrug-converting enzyme strain which expresses β-glucuronidase that hydrolyzes glucuronide prodrug 9ACG into 9-aminocamptothecin (9AC), a topoisomerase I inhibitor which efficiently inhibits tumors.[114] Furthermore, the attenuated *S. typhimurium* (VNP20009) has been used as a vector to deliver carboxy-peptidase G2 that exhibits enhanced anticancer activity in conjunction with prodrug administration.[115]

Delivery of Drug-Loaded Liposomes

Liposomes have gained a special importance as active vehicles for the delivery of diverse therapeutic compounds. The surface modifications of conventional liposomes with different ligands have led to the formation of second generation liposomes, with higher drug loading capacity, targeted drug-delivery, and enhanced anticancer activity.[116] A novel anticancer therapeutic strategy was designed by using anticancer drug, paclitaxel (PTX) containing liposomes within *S. typhimurium*. This procedure was initiated by binding biotin molecules on the outer membrane proteins of bacteria and consequently streptavidin molecules were coated on the PTX-loaded liposomes. The motility analysis of bacteria-loaded liposomes exhibited higher average velocity as compared to free bacteria. The cytotoxicity tests were performed on breast cancer cell line (4T1) to figure out the anticancer

therapeutic efficacy of the PTX-containing liposome loaded bacteria. In addition, tumor targeting bacteria displayed robust cancer-targeting ability. These findings reveal that engineered bacteria could be an efficient alternative for anticancer therapy.[117]

Salmonella were loaded with low-temperature sensitive anticancer drug doxorubicin (DOX) loaded within liposomes targeting colon cancer cells to deliver this drug and simultaneously macrophages polarized to M1 phenotype with high intensity focused ultrasound heating (40–42°C). The studies showed that the liposomal loading was highly efficient without affecting the bacterial viability. These drug-loaded liposome-containing bacteria demonstrated efficient intracellular trafficking, excellent nuclear localization of DOX, and induced in vitro pro-inflammatory cytokine expression of colon cancer. By using murine colon tumor models, these engineered bacteria significantly enhanced the therapeutic efficacy and macrophage polarization to M1 phenotypes as compared to control samples. Further, these bacteria focused ultrasound treatments, which have the potential to improve the colon cancer therapy.[118]

Bacterial Membrane-Based Anticancer Nanoformulations

Bacterial membrane-based nanoformulations include bacteria-derived nanovesicles (BDNVs) and bacterial membrane-coated NPs. BDNVs range in size from 20–400 nm, composed of double lipid layer. BDNVs are mainly classified into four groups based on their source and structure as: outer membrane vesicles (OMVs), outer-inner membrane vesicles (OIMVs), double-layered membrane vesicles (DMBs), and cytoplasmic membrane vesicles (CMVs).[119] The BDNVs have been used against cancer, due to their cancer penetration ability, surface modification, and drug loading capacity.

Several genetically modified bacteria including *E. coli* derived 400 nm nanovesicles have been loaded with chemotherapeutic agents like DOX.[120] The feasibility of using BDNVs to transport/deliver siRNA for drug-resistant cancer treatment has also been reported.[121] Table 3 lists examples of some cargo items delivered by bacterial membrane vesicles derived from different bacteria for the strategy of cancer management.

Table 3 Efficacy of Different Therapeutic Agents Loaded in Bacterial Membrane and Targeted Against Different Cancers

Membrane Type and Source	Cargo	Cancer Type	Efficacy	References
OMV from *E. coli*	siRNA	HER2-overexpressing HCC1954 cells	Targeting of cancerous tissues through the EPR effect. Avoidance of gene leakage an protection from degradation	[122]
	ICC	CT26 and 4T1 tumors	Surface is functionalized with a calcium phosphate shell to respond to the acidic environment of the cancerous tissue. Combination of immunotherapy and photothermal therapy	[123]
	ICG	B16F1O tumor	Transdermal nanoplatform against melanoma. Combination of photodynamic therapy, photothermal, and immunotherapy	[124]
	BFGF	TC-I and B16F1O tumors	Used as a cancer vaccine. Induction of antibody production targeting tumor angiogenesis	[125]
Protoplast-derived nanovesicles from *E. coli*	Doxorubicin	Human lung carcinoma A459 cells	Bioengineered with high expression of the epidermal growth factor to target the tumor. Alleviation of systemic toxicity of the chemotherapeutic agent	[126]
DMV from *E. coli*	Doxorubicin	B16F10 tumor	Bioengineered with high expression of RGD motifs to target the tumor. Targeting of the monocytes or neutrophils that mediate transportation towards the tumor	[127]
OMV from *Salmonella*	Tegafur@F127 nanomicelles	B16F1O and 4T1 tumors	Surface is modified with RGD to preferentially accumulate in cancerous tissues. Combination of immunotherapy and chemotherapy	[128]
	Paclitaxel	Ehrlich ascites carcinoma (EAC)	Passive accumulation in tumor tissues through the EPR effect. Combination of immunotherapy and chemotherapy	[129]

In addition to gene and drug carrying potential, BDNVs also hold the capability of activating the immune response against cancer. Diverse immunostimulatory molecules loaded in OMVs have been investigated recently for vaccine and delivery system usage. The anticancer command of genetically modified *E. coli* derived OMVs exhibited excellent tumor-targeting ability due to their enhanced EPR effect.[130] Some immunomodulatory agents induce the production of anticancer agents like CXCL10 and IFN-γ, which can successfully eradicate the established tumors.

The OMVs derived from *E. coli* BL21 cells have been chemically modified with Calcium phosphate (CaP) shells. These pH-sensitive shells neutralize the acidic TME to polarize the cancer-associated macrophages and avoid the severe systemic inflammation potentially induced by CaP free OMVs. The anti-inflammatory M2 macrophage phenotypes synergized with the intrinsic immunostimulatory effect of OMVs, have eventually led to a 60% survival rate at day 80 compared with day 0 in the group applying naked OMVs.

BDNVs have also been loaded with NPs to provide additional functions like photosensitivity. Bacteria-cancer cell hybrid membrane-coated photosensitizing hollow polydopamine NPs have been synthesized recently for the approach of cancer eradication (Figure 3).[131] The anticancer cytokines were potentially produced by bacterial membranes through different immunostimulatory membrane components.

Cancer cell membrane proteins serve as excellent tumor antigens, which synergize with anticancer cytokines and induce a substantial immune response. The combination of photothermal treatment and anticancer immune therapy has been reported to eradicate melanoma. Further, the uploading of NPs within bacterial membranes adds the functionality in photothermal response and also helps to enhance the immune response to fight against cancer (Figure 3).
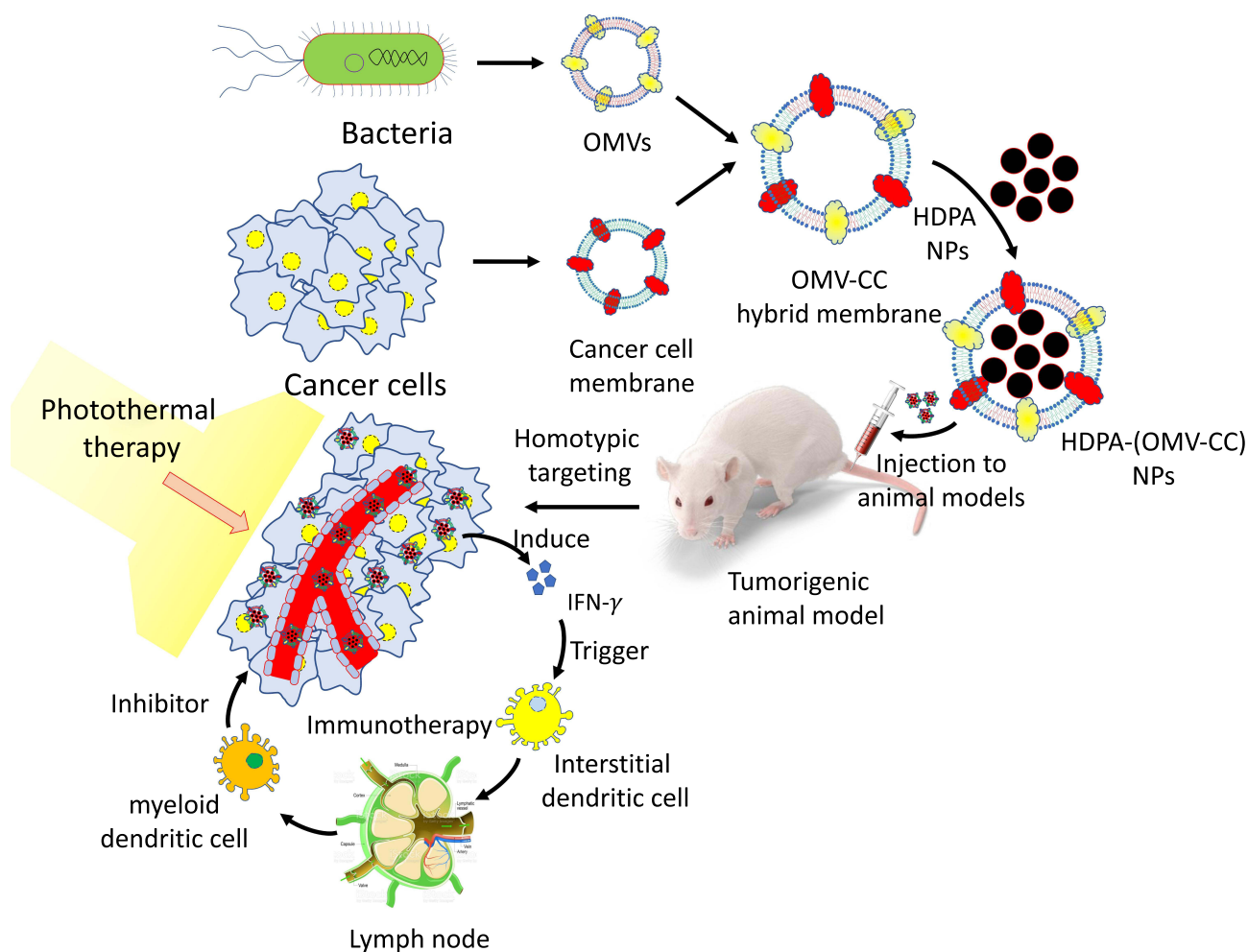

Figure 3 Diagrammatic representation of hollow polydopamine-NPs synthesis from the membranes of tumor-targeting-bacteria and cancer cells and its injection and immunotherapy/photothermal therapy in animal cancer models.

Delivery of Drug-Conjugated Nanoparticles

Bifidobacterium longum (*B. longum*) have been engineered to conjugate poly(lactic-co-glycolic acid) (PLGA) NPs (PLGA-NPs) targeting the tumor specifically to achieve precision treatment and imaging. *B. longum* selectively colonizes in hypoxic regions of the animal body, successfully targeting into solid tumors. Further, perfluorohexane (PFH) has been used to wrap the core of PLGA-NPs to improve its specificity and efficacy for cancer therapy. PFH/PLGA-NPs kills the cancer cells by the deposition of energy by affecting the acoustic environment during High Intensity Focused Ultrasound (HIFU) irradiation. This strategy has been effective in treatment and diagnosis, providing stronger imaging, a longer retention period, and much better tumor therapy.[132]

A combination of bacteriolytic therapy (COBALT) strategy was applied by using *C. novyi* devoid of its lethal toxin (*C. novyi*-NT) spores loaded with conventional chemotherapeutic drugs. It led to extensive antitumor capability against hemorrhagic cancer.[133] Bacteria-facilitated NPs delivery into the cancer cells takes the advantage of the invasive property of these microorganisms. The drug-loaded cargos are not carried inside the bacteria, rather these payloads remain attached on the microorganism surface.

S. typhimurium bacteria have been precisely engineered to transport drug-loaded nanoformulations and penetrate prostate cancer cells to deliver their antitumor cargos. Some methods established for the cargo loading and delivery include the attachment of NPs to the *Salmonella* membrane. The example includes the sucrose-conjugated AuNPs attached to the surface of *Salmonella* bacteria. The other method includes the attachment of streptavidin-conjugated fluorophores on biotinylated *Salmonella* membrane, that enhances the transport of and drug delivery.[134]

Biosynthesis and Delivery of Metal NPs

Bacteria have been significantly employed for the biosynthesis of metal NPs. The bacterial synthesis of NPs involves spontaneous and simple biochemical and biophysical processes leading to the formation of monodisperse and stable formulations. The exact mechanism of its biosynthesis at molecular level is not yet well understood.[135] The bacteria exploit different mechanisms like biosorption, solubility changes, extracellular precipitation, bioaccumulation, chelation, and metal complexation for the synthesis of metal NPs involving reducing NAD(P)H-dependent enzymes like cysteine desulfhydrase, glutathione, nitrate reductase, and sulphite reductase.[136]

Diversified bacteria growing in extreme environmental condition like archaea,[137] *Deinococcus radiodurans*,[138] and marine[139] ecosystem have been associated with metal NPs biosynthesis. Metal NPs, especially belonging to heavy and toxic group namely Au, Ag, Cd, Ni, Pd, Pt, Se, Ti and some metal oxides like CeO_2, Fe_3O_4, TiO_2, Zirconia, and ZnO along with their functional derivatives, have been reported to be synthesized by bacteria.[140]

The anticancer activity of *S. rochei* HMM13 synthesized silver NPs (AgNPs) has been checked on different tumor cell lines like breast carcinoma cells (MCF-7), hepatocellular carcinoma cells (HepG-2), prostate carcinoma cells (PC-3), colon carcinoma cells (HCT-116), intestinal carcinoma cells (CACO), lung carcinoma cells (A-549), cervical carcinoma cells (HELA), and larynx carcinoma cells (HEP-2). The percentage of all these different cancer cell lines demonstrated a dose-dependent decrease in their viability percentage by the exposure of these NPs.

The uptake of AgNPs by different tumor cells are catabolized to form amino acids and Ag ions.[141] The released Ag^+ cations interact with cellular macromolecules like DNA and proteins. These ions lead to protein modifications, DNA damage, and enhanced mitochondrial permeability of cancer cells resulting in enhanced oxidative stress. All these changes in cancer cells push them to apoptosis.[142]

Biosynthesis and Delivery of Magnetosomes

Magnetically controlled biosensors, contrast agents in MRI diagnosis, and drug delivery system popularly consist of superparamagnetic iron oxide (FeO) nanoparticles (FeONPs).[143] Magnetotactic bacteria exclusively contain magnetosomes, unique lipid bound organelles, and provide some special characteristics to these bacteria for cancer management. These magnetosomes possess narrow size distribution, regular morphology, resistance to agglomeration, and low toxicity profile, which makes them excellent for drug and gene delivery applications. The magnetosomes are nanometer-sized crystals, naturally synthesized through cytoplasmic membrane invaginations, followed by influx of iron and certain proteins, leading to magnetite crystal biomineralization.[144] These bacteria belong to the

α-Proteobacteria group and are mostly Gram-negative, having a micro-aerobic or anaerobic type of metabolism.[145] These bacteria are capable to produce naturally iron sulfide (greigite) and iron oxide (magnetite) NPs covered by a lipid bilayer.

The magnetosomes help in aligning the bacteria for external magnetic fields and optimal nutrient and oxygen conditions. The magnetosomes have been isolated from bacteria and have been useful in medical applications like peptide screening in drug development.[146] Further, these magnetosomes have been utilized for anticancer gene therapy and drug delivery.[147] These specialized bacteria have gained a distinct position as a smart drug delivery system in cancer patients.[148]

The chain alignment of magnetosomes in *Magnetospirillum gryphiswaldense* is aligned to enhance the hyperthermia outcome during cancer therapeutics.[149] In comparison to FeONPs, magnetosomes have been reported with enhanced efficacy as MRI-contrast agents.[150] As a heat sensitive system, bacterial magnetosomes have been used as a smart chemotherapeutic approach.

The magneto-aerotactic behavior of *Magnetococcus marinus* strain MC-1 has been exploited to transport up to 70 drug-loaded nanoliposomes till extremely low oxygen regions of the cancerous tissue. It has been reported that up to 55% of drug-loaded bacterial cells can penetrate the colorectal xenograft in severe combined immunodeficiency (SCID)-mice.[151] Bacterial magnetic nanoparticles (BMN) have been coated with polyethyleneimine (PEI), resulting in a size range of 45–55 nm, used to transfect DNA in mammalian cell lines.[152]

In comparison with the older methods, the bacterial magnetosomes have been complexed with anticancer antibodies (BM-Ab) to achieve greater antitumor efficacy under the magnetic therapy.[153] For the application in drug delivery and imaging protocols, these magnetic and AuNPs have been used as efficient theranostic agents.[154] Magnetotactic bacteria derived magnetosomes have been conjugated with Au nanorods and folic acid to form nanohybrids. These nanohybrids serve as effective theranostic agents for the detection and photomechanical killing of cancer cells.[155] These NPs have been applied as high contrast probes to seek out even single-cell diagnostics as well as photothermal agents for single-cell therapy (Figure 2). The application, efficacy, and theranostic mechanisms of different types of metal nanoformulations, delivered by diverse tumor targeting strains of bacteria, are summarized further in Table 4.

Bacterial Spores

The majority of anaerobic bacteria produce highly resistant spores which can survive even in an oxygen-rich environment. Once the favorable conditions like that of TME are met, these spores germinate and the bacteria thrive accordingly, targeting the nearby cancer cells. *C. novyi-NT* bacteria are genetically modified to be devoid of lethal toxins which target cancer cells without involving side-effects.[173] An intratumoral injection of *C. histolyticum* spores in mice resulted in marked lysis of cancerous tissue. A similar phenomenon has been observed by intravenous injection of *C. sporogenes* spores in mice.[156] The spores of *C. novyi-NT* are rapidly cleared by the reticuloendothelial system from circulation as observed by toxicological and pharmacological evaluation. Injection of these spores in healthy rabbits or mice even with large doses showed no clinical toxicity. However, the toxicity was related to spores dosage and tumor size in diseased mice.[174] In addition, bacterial spores have also been used as carriers of anticancer drug delivery agents, therapeutic proteins, gene therapy vectors, and cytotoxic peptides.[175]

A brief description of some important anticancer agents delivered by tumor-targeting bacteria near or within cancer cells and their concise mechanism of action has been described in various articles and is illustrated in Figure 4.

Tumor-Directed Remote Control Guidance of Bacteria

It has been reported that only a few bacteria reach TME on their own, so active research is going on in engineering other bacteria to carry or produce and deliver anticancer compounds within the tumor regions. The clinicians need to effectively navigate bacterial therapies near cancer sites, as most tumors are inaccessible by direct injection of antitumor agents. Further, the engineered bacteria should controllably and reliably release their anticancer drugs they carry or encode.[176] The incorporation of synthetic compounds within the live bacteria can allow remote control guidance of certain actions or functionality. The light has a limited ability to penetrate the cancerous tissues which hampers its approach, even though optically triggered navigation and control have enormous potential. The use of ultrasound has filled some gaps, as it has a broad range of applications in medical diagnostics and monitoring.[177]

Recently, to augment the ultrasound images of tissues, gas-filled microbubbles have been used due to their distinct

Table 4 Summary of Different Metal Based Drug-Nanoformulations Loaded in Various Tumor-Targeting Bacteria for Cancer Therapy

Bacteria	Metal NP	Type of Formulation	Application/Efficacy/ Therapeutic Mechanism	References
Synechococcus 7942	Human serum albumin nanoparticles	ICG	In situ O_2 generation enables robust immunogenic PDT against tumor growth and metastasis	[156]
Lactobacillus plantarum	AuNP	Bacterial EPS stabilized NP	Drug delivery; antibiotic	[157]
		Antibiotic-AuNP-EPS	Drug delivery against MDR	[157]
Bifidobacterium longum	PLGA	Low-boiling-point perfluorohexane (PFH)	Theranostic efficacy. Realization of high-intensity focused ultrasound therapy against cancer	[132]
Escherichia coli	Polydopamine nanoparticles	Ce6	Converts endogenic H_2O_2 into O_2 for subsequent photodynamic therapy	[158]
Magnetococcus marinus strain MC-I	–	Drug-loaded nanoliposome	Delivery of multiple drug agents	[151]
L. monocytogenes	Polystyrene nanoparticles	GFP-encoding plasmid DNA	Successful delivery of genes into the nucleus	[159]
Salmonella typhimurium YB1	PLGA	ICG	Photothermal ability to eradicate solid tumors	[160]
Microbial poly-(amino acids)	–	Poly(γ-glutamic acid) (PGGA) NP	Drug delivery; antibiotic	[161]
S. typhimurium VNP20009	PLGA	–	Remarkable nanoparticle retention and distribution in solid tumors	[162]
Escherichia coli MG1655	Magnetic Fe_3O_4 nanoparticles	–	Effective tumor colonization and Fenton-like reaction to cure cancer	[163]
B–acterial EPS	–	Gellan gum based floating bead	Drug delivery; antibiotic	[164]
	Magnetic NP	MNP-Gellan gum/Mauran nanocomplex	Drug delivery and targeting	[165]
Magnetotactic bacteria	Bacterial magnetosome	BM-PEI-siRNA	Anticancer Gene delivery	[166]
	Bacterial magnetic nanoparticle (BMP)	BMP-PEI/DNA	Gene delivery	[154]
	Bacterial magnetosome	Plasmid/Drug loaded BM	Drug/Gene delivery	[167]
	Bacterial magnetosome	Genipin (GP) and poly-I-glutamic acid (PLGA)-modified bacterial magnetosome	Anticancer drug delivery	[168]
	Bacterial magnetosome	Drug-loaded magnetosome	Anticancer delivery	[147]
	Au nanorods	BMP-Au rods-folic acid	Theranostic agents	[155]
Magnetospirillum magneticum AMB-I	Bacterial magnetosome	Protein functionalized BM	Labeling tumor markers	[169]

(Continued)

Table 4 (Continued).

Bacteria	Metal NP	Type of Formulation	Application/Efficacy/ Therapeutic Mechanism	References
Escherichia coli	Carbon nitride (C_3N_4) semiconductor nanomaterials	–	Almost 80% tumor regression superior than with E. coli alone (~20%)	[170]
Halomonas maura	–	Chitosan-Mauran EPS nanocomposite	Drug delivery; 5-FU	[171]
Shewanella oneidensis MR-1	Manganese dioxide nanoflowers	–	MnO_2 serves as tumor metabolite, lactic acid performs as an electron donor in cancer cells	[172]

and strong acoustic response. In addition, some special forms of super-powered and focused ultrasound have been used to boost the transport of drug-loaded nanobubbles by the use of acoustic pressure waves as an external energy source to push it to deeper regions of TME. This tactic has achieved some promising results in glioblastoma, as the blood–brain barrier (BBB) is a challenge to overcome for drug transport.[178]

In the recent past, ultrasound has been used to track the bacteria for therapeutic purposes in vivo. Bacteria have been genetically engineered to express the acoustic reporter gene (ARG), which encodes the compounds of gas vesicles that scatter ultrasound waves, thus generating an echo to enable the bacterial location deep inside living mice.[179] The application of magnetic fields is another source of external energy which can be remotely and safely used in the human body.

The advantage that anaerobic bacteria tend to shift to low oxygen environment, coupled with anticancer drugs and the natural homing mechanism of an externally directing magnetic field, has demonstrated enhanced penetration and accumulation for therapy in mouse tumors. The magnetotactic bacteria act like little propellers on a rotating magnetic field with tissue models on a chip, creating a flow that pushes nanomedicine out of the blood vessels and deeper in tissues.

Attaching magnetic materials to non-magnetic bacteria is an alternative to control such bacteria by the external magnetic field.[180] Tiny magnetic NPs have been attached to E. coli in addition with DOX and upon treatment with cancer cells, it has been reported that such bacteria are remotely controlled by the magnetic field to improve their tumor targeting.[181] The science of external energy source and controllable genetically engineered bacteria are a fascinating new direction in the field of cancer management. The convergence of mechanical engineering, synthetic biology, and robotics has opened up a new approach of using tiny robots to destroy different cancer types.[182]

Clinical Trials

For the management of cancer in human subjects, different bacterial strains have been selected since the use of live bacteria by Dr. Coley in 1891.[183] Among different bacterial species, *Listeria* vaccine strains have shown promising results, and some strains are tested in Phase II and Phase III clinical trials.[184] The attenuated strain of *S. typhimurium* (VNP20009) was the first strain to enter a phase I human clinical trial in 1999, tested on 24 patients with metastatic melanoma and metastatic renal carcinoma. Although different proinflammatory cytokines like IL-1β, IL-6, IL-12, and TNF-α were reported to be raised in some patients, no objective tumor regression was reported.[185] *S. typhimurium* (VNP20009) was used in another clinical trial involving metastatic melanoma patients, but no remarkable tumor response was reported.[186] To enhance the therapeutic potential, *S. typhimurium* (VNP20009) was engineered to express *E. coli* CD, that converts 5-FC to toxic 5-FU. An intratumoral injection of these bacteria was used in three patients suffering from esophageal adenocarcinoma and head and neck squamous carcinoma. Even after the six treatment cycles, no significant adverse response was observed in these patients.

Recently, some other phase I clinical trials have been reported by using *S. typhimurium* (VNP20009) and *S. typhimurium* (χ4550) expressing IL-2, as summarized in Table 5. The conclusion of these trials disclosed that the differences between human patients and preclinical animal models might be due to dissimilarities in tumor structure and growth rates that might alter bacterial TME behavior. The
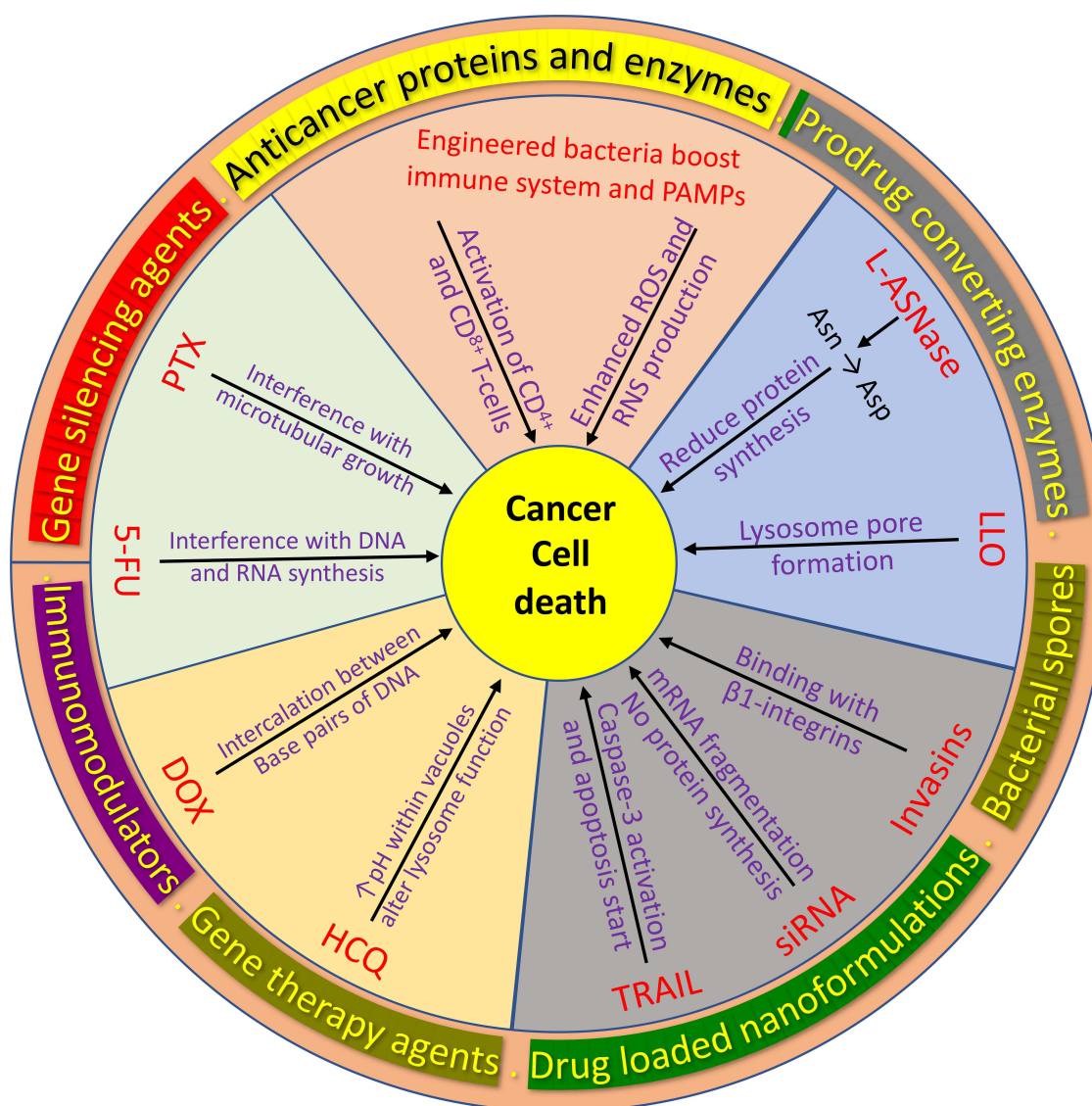
Figure 4 Some examples of anticancer agents delivered by different tumor-targeting bacteria and their brief mechanism of action.

clinical trials by *Salmonella* spp. have demonstrated that TLR4-mediated signaling is important for tumor colonization and antitumor activity, as a VNP20009 strain missing lipid A function was unsuccessful to colonize tumor sufficiently to suppress tumor growth. Although limited, these clinical trials have revealed some significant hurdles and some challenges that must be overcome for successful human application in the future. Some examples of clinical trials using several bacteria are listed in Table 5.

Future Perspective

The complete treatment of cancer is considered a challenging task as hypovascular areas provide inadequate access to drug-loaded nanoformulations. Even though some tumor-targeting bacteria have been genetically engineered to combat various cancers, several future studies are needed to address and expedite the further advancement of nanobiohybrid systems in tumor therapy.

These prospective studies need to know the shape of the nanoformulations as it is a significant parameter for nanobiohybrid systems, which impacts on bacterial transport efficiency. The loading quantity and volume of nanomaterials also affect the bacterial movement. In addition, the performance of nanobiohybrid interaction between nanomaterials and bacteria is of utmost importance to adopt varied loading strategies based on different nanomaterials to augment the performance. The attachment of NPs on the bacterial surface can affect bacterial chemoreceptors in response to TME.

Table 5 Previous and Ongoing Clinical Trials Involving Tumor-Targeting Bacteria and Cancer Bearing Human Subjects

Bacterial Strain	Cancer Type/Number of Samples	Phase	References
Clostridium novyi-NT	Colorectal cancer/2	I	https://www.clinicaltrials.gov/ct2/show/NCT00358397
	Solid tumor malignancies/5	I	https://www.clinicaltrials.gov/ct2/show/NCT01118819
	Solid tumor malignancies/24	I	https://www.clinicaltrials.gov/ct2/show/NCT01924689
Clostridium novyi-NT NCT03435952	Refractory advanced solid tumors/18-recruiting	Ib	https://clinicaltrials.gov/ct2/show/NCT03435952
Listeria monocytogenes	Metastatic pancreatic tumors/90	II	[187]
	Cervical cancer/109	II	[188]
	Cervical cancer/450- recruiting	III	https://clinicaltrials.gov/ct2/show/record/NCT02853604
S. Typhimurium VNP20009	Metastatic melanoma; metastatic renal cell carcinoma/25	I	[185]
	Melanoma/4	I	[186]
	Patients with advanced or metastatic solid tumors	I	http://www.clinicaltrials.gov/ct2/show/NCT00004216
	Unspecified adult solid tumors	I	https://www.clinicaltrials.gov/ct2/show/NCT00006254
	Neoplasm or neoplasm metastatic tumors/45	I	http://www.clinicaltrials.gov/ct2/show/NCT00004988
S. Typhimurium VNP20009 expressing TAPET-CD (cytosine deaminase)	Head and neck or esophageal adenocarcinoma/3	I	[42]
S. Typhimurium expressing human IL-2	Liver cancer/22	I	https://www.clinicaltrials.gov/ct2/show/NCT01099631
S. Typhimurium Ty21a VXM01	Pancreatic cancer/26	I	[189]

Therefore, abiotic/biological interfaces need to be carefully designed to conserve the chemotaxis and bacterial mobility.

The role of exogenous and endogenous stimuli is very important for the release of nanomaterials from the bacteria at tumor regions. It is very significant to known the spatiotemporal control of drug action at the heterogeneous environment of tumors. Furthermore, the limitations of metal toxicity in living systems need an act of balancing between the positive therapeutic effects of metal oxide NPs and their toxic side-effects.[190] Any delayed elimination or absence of dissolution/biodegradation can be followed by generation of intracellular ROS, DNA damage that triggers apoptotic cell death.[191]

The possession of bacterial immunogenicity and toxicity is very important to ensure the safety aspects. Even though a variety of bacteria are non-pathogenic, the possible toxicity may threaten immunocompromised patients with advanced stage cancer. Engineering bacteria to knock out virulence genes is of utmost importance. In addition, the complexity of the biological environment makes it necessary to develop feasible methods to control the noncatalytic therapy process to inhibit adverse catalytic reactions and prevent any damage to normal tissue. The lack of information on diverse mechanisms and side-effects of bacterial cancer therapy with development of smart

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microorganisms to treat specific cancers remains a significant challenge.

Conclusion

The therapeutic potential of different bacteria for the cancer management has been taken into significant consideration in the recent decade. Numerous bacteria possess great potential as anticancer strategies, however, this novel therapeutic approach has both advantages as well as disadvantages. The tumor-targeting bacteria possess several unique features like tumor selectivity and genetic modification capabilities. The center-point targeting of anticancer therapeutic payloads through specific bacteria is still a challenging task which can be resolved by a proper understanding about drug-nanoformulation design and its loading within bacteria, bacterial genetic setup, modifications, etc. Recent advancement in microbiology, drug-nanoformulations, and genetic engineering on the same desk have guided some anticancer bacteria to deliver different anticancer payloads at tumor sites with high precision. The bacterial anticancer therapy is still at its basic stage and more future research needs to be conducted to bypass the limitations and side-effects of this therapy by using genetic engineering and precise modifications of some antitumor agents. Despite the promising in vivo and in vitro results of anticancer bacteriotherapy, a few studies have led to clinical trials. In spite of some remarkable achievements, several critical issues like inflammation and toxicity must be resolved before the possible translation of this anticancer strategy into clinical use.

Acknowledgments

Disclosure

The author reports no conflicts of interest for this work.

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