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# *Salmonella* Typhimurium as an Anticancer Therapy: Recent Advances and Perspectives

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## Abstract

**Purpose of Review** Bacteria were first conceived as potential cancer therapeutics in the nineteenth century. Since then, a wide range of advancements has been made especially in the advent of microbial engineering, particularly in the *Salmonella* Typhimurium serovar. Recent developments include attenuated profiles of *Salmonella* for safe delivery, as well as genetic engineering for targeting to cancerous tissue and improved efficacy for antitumor effects. This review provides a summary of recent advances in the field of *Salmonella*-mediated cancer therapy and implications for further clinical testing.

**Recent Findings** A focus of recent *Salmonella*-mediated cancer therapies is genetic engineering of the bacteria for optimized tumor targeting and anticancer effects. Careful design has led to the use of attenuated S*almonella* as drug delivery vehicles and tumor-targeting therapeutics with excellent safety and therapeutic efficacy in countless murine tumor models. Moreover, *Salmonella* has the potential for use as imaging and diagnostic tools that would improve patient prognosis through early awareness.

**Summary** Here, we have detailed recent advances in the use of *Salmonella* as a therapy to combat cancer. Continued innovative and novel discovery in this field of study will yield a promising future for the use of *Salmonella*-mediated cancer therapies in cancer care.

**Keywords** Antitumor agents · Bacterial engineering · Bacterial-mediated cancer therapy · Cancer therapy

## Introduction

Advances in cancer research such as detection and effective treatments are unquestionable. Between November 2016 and October 2017, there had been 18 new cancer therapy developments, which more than doubles the previous year timeframes in 2015 and 2016 [1]. Despite this, cancer incidence is predicted to continue on a steady increase due to the growing world population and elongated average lifespan, causing an even greater need for cancer research innovation. Now more than ever, novel therapeutics and treatment strategies to combat cancer are necessary. Bacterial-mediated cancer therapies have the potential to meet this need by complementing or, in some cases, overcoming negative side effects of current cancer treatment regimens including surgery, radiation, chemotherapy, and immunotherapy [2].

### A Brief History of Bacterial-Based Cancer Therapy

The phenomenon of bacterial-mediated cancer therapy was first observed in 1868 by the German surgeon Dr. Wilhelm Busch but was later best described by Dr. William Coley, an American bone sarcoma surgeon [3]. In 1891, Dr. Coley attributed the clearance of a neck sarcoma and long-term survival of the patient to an erysipelas infection, the causative agent being *Streptococcus pyogenes* [4]. Due to toxicity, Coley heat-inactivated a bacterial mix of the *S. pyogenes* and *Serratia marcescens*, which became known as "Coley's toxins." A retrospective analysis on 1000 of Coley's cases found that nearly half were complete regression [5]. Today,

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with resistance and reduced susceptibility to common therapeutic options on the rise, bacterial-mediated cancer therapies have experienced a re-emergence in the field. The advantages of using microbes to combat cancer include the self-propagation of live bacterial agents, specificity to cancerous tissue over healthy tissue, and redirecting the host defenses to contest cancer via immunostimulation. Here, we present a summary of recent research strides that have taken place towards the use of *Salmonella* Typhimurium as an anticancer therapy. This review serves to summarize updates in the field and provide insight to future work and areas of focus.

## Key Features of *Salmonella* Typhimurium as an Anticancer Agent

### Our Ally Against Cancer

As one article cleverly coined "From spinach scare to cancer care" [6], *Salmonella* Typhimurium has not always been viewed as our friend. *S.* Typhimurium is a classic gastrointestinal pathogen found in undercooked food products such as chicken and eggs. Largely due to microbial genetic engineering, *Salmonella* can also be employed to battle cancer. Safe delivery with minimal toxic effects can be accomplished due to attenuation of *S.* Typhimurium. Moreover, *S.* Typhimurium can colonize the tumor microenvironment and elicit anticancer effects. The ways in which *S.* Typhimurium intrinsically attacks tumors selectively over normal host tissue include the following: stimulating non-specific immune responses through accumulation at the tumor site, preventing cancer cell growth through nutrient uptake, and penetrating necrotic tumor regions that are least drug-accessible [7]. Additionally, because *S.* Typhimurium is a facultative anaerobe, there is a wide variety of cancer types the bacterial species is able to infect, for example, colonization of aerobic microenvironments, such as highly vascularized tumors, and the anaerobic microenvironment of poorly vascularized tumors [3]. *S.* Typhimurium is able to survive and grow in a diverse range of pH conditions, like areas found in the tumor microenvironment [8]. Low-pH areas in the tumor microenvironment impair cytotoxic immune cell activity and cytokine secretion, consequently inhibiting host defenses [8]. The bacterial cells therefore have the capacity to exploit acidic pH areas and redirect host immune cells to the tumor site.

## Engineered *S.* Typhimurium Strains for Anticancer Effects

### VNP20009

VNP20009 was engineered by Low et al. at Yale University to target cancer. The strain was developed from the pathogenic *S.* Typhimurium 14028s [9] through chemical and UV mutagenesis. Two targeted deletions resulting in attenuation by modification of lipid A (*msbB*$^{-}$) and a dependence on purine supplementation (*purM*$^{-}$) are defining genetic characteristics of the strain. Positive preclinical results indicating antitumor activity of VNP20009 culminated in a 2001 phase 1 clinical trial towards patients with non-responsive metastatic melanoma or renal cell carcinoma. Although anticancer effects were not observed, safe delivery of VNP20009 to human patients was achieved [10]. The focus of research has since been to retain the safety profile of attenuated *Salmonella*, while eliciting anticancer effects within the tumor and/or metastatic foci.

Recently, it was discovered that VNP20009 harbors several other genetic features including 50 non-synonymous SNPs [11] and a 108-kb Suwwan deletion [11, 12], the implications of which mostly remain unknown with respect to tumor-targeting efficiency of the strain, except for the gene *cheY*, which contains a SNP rendering the strain non-chemotactic [13]. We evaluated VNP20009 *cheY*$^{+}$ in vitro and discovered a 69% restoration of chemotaxis compared to the parent strain, which we discovered at least in part to be due to the *msbB* deletion. We then compared tumor colonization and anticancer effects of VNP20009 and VNP20009 *cheY*$^{+}$ in a 4T1 mouse mammary carcinoma model and found no significant differences between tumor colonization or anticancer efficacy [14]. VNP20009 has been assessed in several murine tumor models, including melanoma, breast cancer, colon cancer, and canine spontaneous neoplasia [15, 16].

### A1/A1-R

The A1-R strain was developed at the University of California at San Diego by first mutagenizing *S.* Typhimurium 14028s with nitrosoguanidine. Then, the leucine and arginine auxotroph A1 was chosen due to selective growth in neoplastic tissues over normal tissue [17]. The strain was then further improved for tumor targeting and reduced toxicity through passaging in nude mice bearing transplanted HT-29 colon tumors resulting in the isolation of A1-R [18]. The efficacy of strain A1-R has been evaluated in several orthotopic nude mouse models of prostate [19], breast [18, 20], pancreatic [21, 22], and ovarian cancer [23], as well as sarcomas [24] and gliomas [25, 26]. Moreover, A1-R has been effective in metastatic models of cancer [27, 28]. Finally, patient-derived orthotopic xenograft (PDOX) models have been developed, for which A1-R was tested as effective [29].

### ΔppGpp

An avirulent derivative of 14028s was established that is defective in synthesis of the global regulator of gene expression, ppGpp, due to deletions of *relA* and *spoT* [30]. The resulting strain, ΔppGpp, is avirulent, presenting $LD_{50}$ values

approximately $10^5$ higher than wild-type *Salmonella* after oral or intraperitoneal inoculation [30]. In addition to tumor suppression in a CT26 mouse colon cancer model [31], ΔppGpp has been used as a vector for tumor-specific delivery of therapeutics. The engineered *Salmonella* have been successfully implemented as a theranostic agent, expressing an imaging reporter gene, *Renilla* luciferase [32, 33]. Additionally, tumoricidal agents such as cytolysin [32, 34] and Noxa [35] have been delivered by ΔppGpp.

### Other Strains

Several other strains of *S.* Typhimurium (Table 1) were constructed for the purposes of tumor targeting and eradication, including BRD509/BRD509E [36, 37], χ4550 [38], CRC2631 [7], LH340 [39–41], LVR01 [42], MvP728 [43–45], RE88 [46–48], S634/S636 [49], SA186 [50], SB824 [51], SL3261 [52, 53], SL7207 [54–57], and YB1 [58, 59].

## Intrinsic Immunostimulatory Components

The immunosuppressive environment of a growing tumor protects the tissue from immune attack [60, 61]. Some bacterial components are intrinsically immunostimulative, termed pathogen-associated molecular patterns (PAMPs). *S.* Typhimurium PAMPs include flagellin, lipopolysaccharide (LPS), and CpG-rich DNA, which can be recognized by membrane-bound toll-like receptors (TLRs) expressed by innate immune cells. In short, PAMPs are involved in the activation of innate and adaptive immune responses to differentiate foreign pathogen components from self. For example, TLR5 activation by *S.* Typhimurium flagellin has been shown to elicit potent antitumor activity in a mouse xenograft model of human breast cancer [62].

The expression of proinflammatory cytokines such as IL-1β [31] and TNF-α by immune cells has been triggered by systemic *S.* Typhimurium. TNF-α, in addition to other proinflammatory cytokines [63], has been found to play an important role in the initial phase of tumor colonization, due to increased tumor vascular disruption and hemorrhage [64]. Induction of TNF-α is a careful balancing act between immune stimulation and septicemia in *Salmonella* strain construction. Strongly attenuated bacteria such as VNP20009 have an efficacious safety profile in animals and humans, critical for use as a therapy for cancer patients [10], but may strongly reduce the favorable immunostimulatory, and therefore tumor clearance, effects. To address this, Frahm et al. constructed conditionally attenuated *Salmonella* strains by deleting genes involved in LPS synthesis, such as *rfaD* and *rfaG*, and then complementing the resulting mutants by chromosomally integrated copies of these genes under control of an arabinose-inducible promoter. This resulted in an effective balance of attenuation and therapeutic benefit, in which the conditionally attenuated *rfaD* strain delayed growth of CT26 and RenCa tumors in vivo [65].

In addition to eliciting a proinflammatory response, *S.* Typhimurium also influences the downregulation of immunosuppressive factors. Kaimala et al. found that administration of attenuated *Salmonella* led to increased accumulation and functional maturation of intratumoral myeloid cells, with decreased expression of immunosuppressive genes including arginase-1, IL-4, TGF-β, and VEGF [66]. High expression of the 2,3-dioxygenase 1 (IDO) has been found in many tumors and is associated with immune tolerance by indirectly causing T cell apoptosis via an increase in kynurenine concentration [67]. *S.* Typhimurium inhibits IDO expression in B16F10 and 4T1 tumor cells, leading to higher T cell viability and survival [67]. Overall, *S.* Typhimurium-mediated inhibition of immune evasion is a promising strategy for antitumor therapy (Fig. 1).

## The Role of Motility and Chemotaxis in Tumor Targeting of *S.* Typhimurium

Harnessing bacterial motility and chemotaxis is an appealing approach to actively direct *S.* Typhimurium towards cancerous tissue in the body and achieve distribution within the tumor. Motility has been reported as critical for in vitro tumor colonization [68]. Moreover, the importance of individual chemoreceptors for effective tumor localization in vitro has been described for SL1344 [69], where both chemotaxis and proliferation were found to be essential for bacterial accumulation of tumor spheroids [70]. VNP20009 lacking the Trg receptor localizes in vivo within regions of the tumor that are quiescent, which is a cellular, reversibly non-replicating state [71]. Using high-throughput screening of a *S.* Typhimurium gene deletion mutant library, it was presented that motility, chemotaxis, and the ethanolamine metabolic pathway confer an advantage in tumor colonization [72]. In contrast, using SL1344 mutants Δ*fliGHI* and Δ*cheY* in comparison to wild type, motility, and chemotaxis was found to be immaterial for tumor colonization in mice 24 h after administration [73]. Our group has previously shown that in the 4T1 aggressive model of mouse mammary carcinoma, VNP20009 and VNP20009 *cheY*$^+$ do not significantly differ in influencing primary tumor size and moreover have no effect on the number of pulmonary metastases or bacterial colonization of the primary tumor [14].

Overall, there is a discrepancy in the contribution of chemotaxis and motility on tumor colonization and eradication, likely due to several differences in experimental parameters including *S.* Typhimurium strain (VNP20009, VNP20009 *cheY*$^+$, SL1344), cancerous cell line (mouse mammary carcinoma 4T1, human colorectal adenocarcinoma LS174T, mouse colon carcinoma CT26), in vitro and in vivo modeling (cylindroids, microfluidic tumor in chip devices, orthotopic

**Table 1** *Salmonella* Typhimurium strains used as anticancer agents

| Name | Genotype | Description | References |
|---|---|---|---|
| VNP20009 | *msbB*$^-$, *purM*$^-$ | Chemical/UV mutagenesis; targeted deletions of *msbB*, resulting in a lipid A modification, and *purM*, resulting in purine auxotrophy. Selected for hyperinvasive trait towards B16F10 cells in vitro. | [9, 10, 14, 15, 103, 127–130] |
| A1/A1-R | *leu*$^-$/*arg*$^-$ | Chemical mutagenesis; selected for leucine and arginine auxotroph (A1). Passaged and isolated from HT-29 colon tumors in vivo (A1-R). | [17–19, 21, 24, 25, 131–139] |
| ΔppGpp/SHJ2037 | *relA*$^-$, *spoT*$^-$ | Deletions of *relA* and *spoT* resulting in a lack of ppGpp production, a global regulator. | [31, 32, 34, 35, 81, 90, 140–143] |
| BRD509/BRD509E | *aroA*$^-$, *aroD*$^-$ | Dependent on aromatic compounds for growth. | [36, 37, 144–148] |
| χ4550 | *cya*$^-$, *crp*$^-$, *asd*$^-$ | Tn mutagenesis to remove adenylate cyclase (*cya*), cyclin adenosine monophosphate receptor protein (*crp*), and aspartate semi-aldehyde dehydrogenase (*asd*). | [149, 150] |
| CRC2631 | *aroA*$^-$, *thyA*$^-$, *rfaH*$^-$ | LPS-deficient (*rfaH*) and auxotrophic for amino acids (*aroA*) and thymine (*thyA*). | [7, 151, 152] |
| LH340 | *phoP*$^-$, *phoQ*$^-$ | Lacking regulation of acid phosphatase synthesis, resulting in reduced survival in macrophages. | [39–41] |
| LVR01 | *aroC*$^-$ | Dependent on aromatic compounds for growth. | [42, 153] |
| MvP728 | *purD*$^-$, *htrA*$^-$ | Dependent on purine supplementation (*purD*) and lacking heat-shock protein induction (*htrA*). | [43, 45] |
| RE88 | *aroA*$^-$, *dam*$^-$ | Lacking DNA adenine methylase (*dam*) and dependent on aromatic compounds for growth. | 48[46–48] |
| SA186 | *znuABC*$^-$ | Deletion of the zinc transporter operon, conferring reduced virulence but maintaining stimulation of protective immunity. | [50, 154] |
| SL3261 | *aroA*$^-$ | Dependent on aromatic compounds for growth. | [53, 79, 80, 100, 155–160] |
| SL7207 | *aroA*$^-$ | Dependent on aromatic compounds for growth. | [57, 104, 161–164] |
| YB1 | *asd*$^-$, *aroA*$^-$ | Essential gene, *asd*, under control of a hypoxia-inducible promoter and dependent on aromatic compounds for growth. | [58, 59, 118, 165] |
| S634/S636 | *pagL*$^-$, *pagP*$^-$, *lpxR*$^-$, *arnT*$^-$, *eptA*$^-$, *lpxT*$^-$, *aroA*$^-$ | Aromatic compounds auxotroph and several lipid A modifications. | [49] |

syngeneic model, subcutaneous cancer cell injection), timelines ranging from hours to weeks, and mode of *Salmonella* delivery to the host (intravenous, intraperitoneal, intratumoral injection).

## Engineered *S.* Typhimurium for Cancer Therapy

### Anticancer Therapeutic Delivery

*S.* Typhimurium strains are being utilized as live delivery vehicles, made to express various anticancer therapeutics including cytokines, cytotoxic agents, regulatory molecules, tumor-associated antigens or antibodies, prodrug enzymes, and genetic material used as DNA vaccines or for RNA interference (Fig. 2). Controlled release of therapeutic agents is imperative for continued safety and efficacy. For example, *S.* Typhimurium promoters and their respective inducing molecules used for the expression of various therapeutics include the following: pBAD, inducer L-arabinose; pTet, inducer tetracycline; RecA, inducer radiation; quorum sensing, inducer bacterial density; and hypoxia-inducible promoters induced by low-oxygen concentrations [74]. Other strategies utilize the *Salmonella* type three secretion system (T3SS) allowing for efficient delivery of drugs via a molecular needle directly into the cytosol of cancerous cells. Finally, the facultative intracellular lifestyle of *Salmonella* has allowed for its use as a delivery system of a variety of cancer therapeutics, including short hairpin RNAs (shRNAs) for RNA interference that, upon entry of the eukaryotic cell, can dramatically alter cellular functions via gene silencing [16]. While examples of engineered *Salmonella* for cancer therapy are below, a detailed list is provided in Table 2.

#### Cytokines

*S.* Typhimurium has been engineered to deliver immunocompetent cytokines that can induce activation of immune cells and killing of tumor cells. Specifically, *S.* Typhimurium production of IL-2 under the control of the *nirB* promoter promoted an antitumor and pro-apoptotic intratumoral response [36]. IL-18, which stimulates NK cells and T cells to release IFN-γ, was secreted by VNP20009 under the control of the *ompC* promoter, inhibiting the growth of primary subcutaneous CT26 colon carcinoma as well as D2F2 breast carcinoma

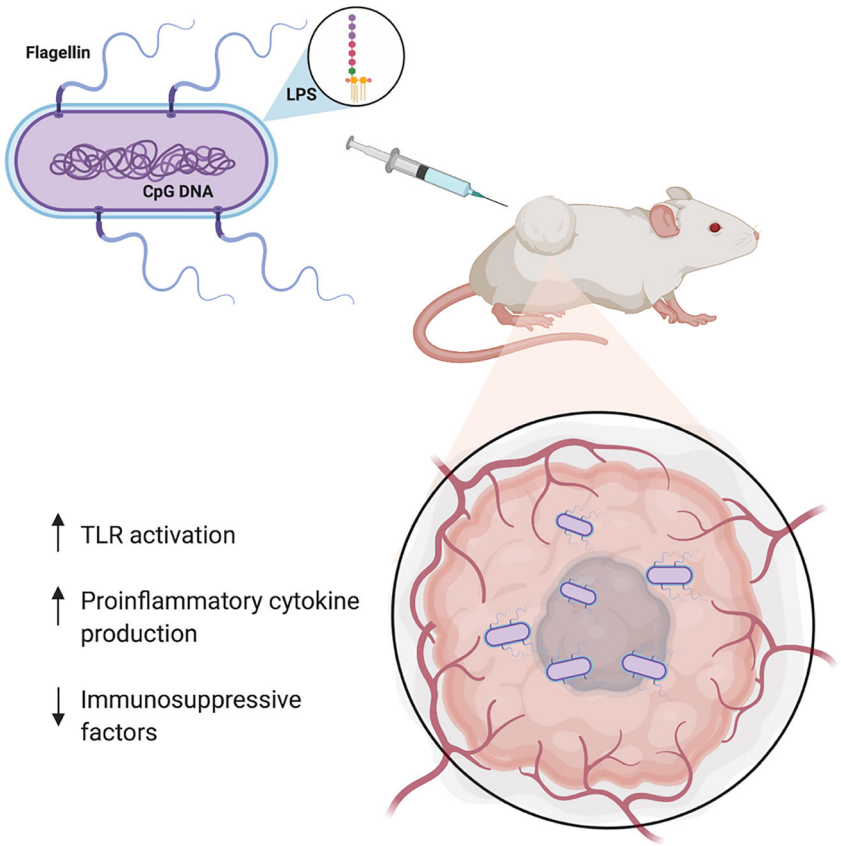


**Fig. 1** Intrinsic immunogenic components of *Salmonella* Typhimurium. Upon introduction of attenuated *Salmonella* in vivo, pathogen-associated molecular patterns (PAMPs) including flagellin, CpG-rich DNA, and lipopolysaccharide (LPS) are recognized by TLRs and elicit an immune response. Proinflammatory cytokines, such as IL-1β and TNF-α, are increasingly produced by the host, while immunosuppressive factors such as IL-4 and TGF-β exhibit reduced production

pulmonary metastases [75]. In a similar manner, Loeffler et al. have demonstrated the use of *S.* Typhimurium expressing chemokine CCL21 [76] and the cytokine LIGHT [77], resulting in antitumor activity dependent on CD4- and CD8-expressing cells. *S.* Typhimurium has also been used as a vector for cytokine gene therapy. Delivery of a eukaryotic expression plasmid producing the IL-2 cytokine prolonged survival of mice transplanted with hepatoma cell tumors [78]. Moreover, *Salmonella* has delivered IL-4 and IL-18 via eukaryotic expression vectors, mediating an IFN-γ response and increasing survival of melanoma bearing mice [79].

## Cytotoxins

Cytotoxic proteins are highly effective at mammalian cell killing and must be kept under tight control so as to not elicit adverse effects on healthy tissue [32, 34, 80]. Pore-forming cytolysins such as ClyA and HlyE delivered by *S.* Typhimurium have been shown to result in cancer cell killing and tumor clearance. Bacterial-borne HlyE under the control of a hypoxia-inducible promoter (FF+20*) was expressed only in hypoxic regions of murine mammary tumors [80]. Similarly, *S.* Typhimurium has been engineered by Min and colleagues to produce the cytotoxin ClyA under the control of the $P_{BAD}$ promoter [81]. Once the tumors have been colonized, L-arabinose can be intraperitoneally administered to activate expression of the cytotoxin and enhance tumor suppression [81]. Strain ΔppGpp expressing ClyA under the control of a *tetR*-regulated promoter was evaluated in rat advanced glioma, where the strain induced cancer cell apoptosis and lead to prolonged survival of the rats [82]. Cytotoxin delivery by *S.* Typhimurium includes secreted tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) under control of the radiation-inducible RecA promoter [83] and the hypoxia-induced *nirB* promoter, the latter causing melanoma apoptosis and reduction of tumor growth in melanoma-bearing mice [84]. Attenuated strains were engineered to express the diphtheria toxin A (DTA) chain protein in the 4T1 tumor microenvironment, resulting in significantly lower tumor volumes and 100% survival of animals through the course of the study [85]. *S.* Typhimurium has been designed to secrete chimeric *Pseudomonas* exotoxin A (ToxA) that selectively kills epidermal growth factor receptor (EGFR)-expressing tumor cells in vitro. EGFR is known to be overexpressed in neoplasias such as breast, colon, and lung prostate cancers, among others [86]. Other examples of successful tumor therapy by *Salmonella* cytotoxin delivery includes the expression of apoptin, an apoptosis-inducing small protein [87], and of FasL, the proapoptotic cytokine Fas ligand [88].

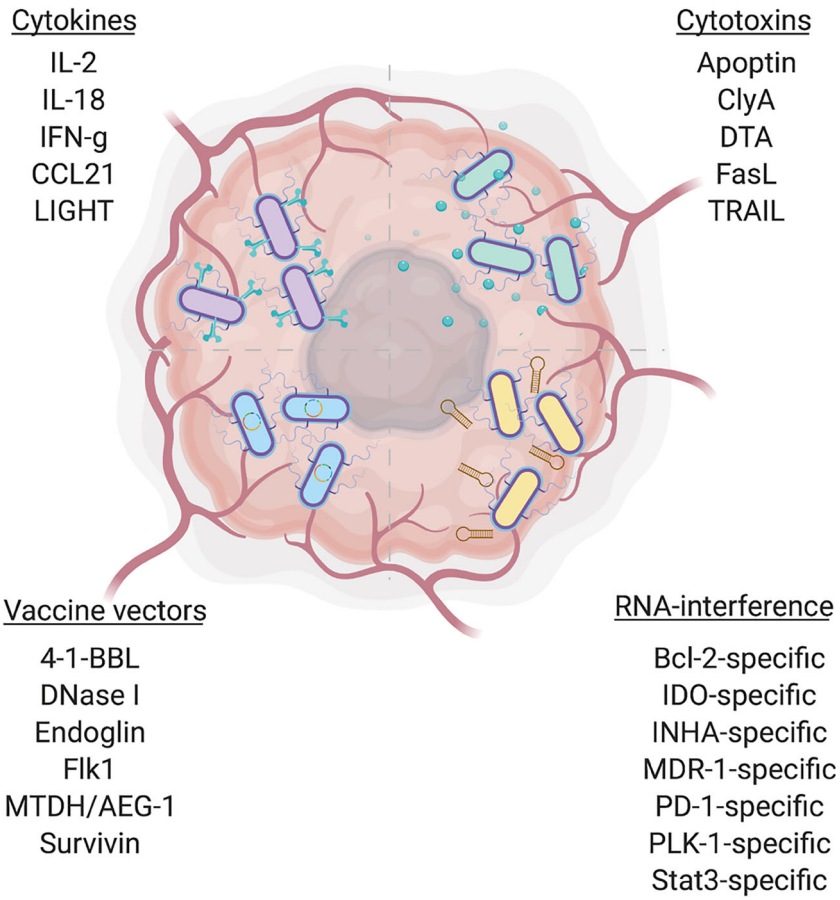


**Fig. 2** Strategies for increased therapeutic efficiency of *Salmonella* Typhimurium. After administration, *Salmonella* can directly deliver cytokines and cytotoxins to elicit anticancer effects, act as a vaccine vector prompting eukaryotic gene expression, and perform RNA interference for tumor gene silencing

### Regulatory Molecules

*S.* Typhimurium can be modified to regulate and inhibit angiogenesis as well as promote cancer cell apoptosis. Using the plasmid-bacteria balanced-lethal system [89], strain S634 delivered endostatin, an antiangiogenic agent [49]. In another study, the T3SS protein SopA was fused with endostatin for efficient tumor suppression and induction of severed necrosis in CT26 colon cancer [56]. Through controlled expression, the anticancer protein L-asparaginase demonstrated antitumor efficacy towards mice-bearing MC38, 4T1, and AsPC-1 tumors [90]. Under the hypoxia-induced *nirB* promoter, VNP20009 has been engineered to express Fas-associated protein with death domain (FADD), an adaptor protein transmitting apoptotic signals [91]. FADD-expressing VNP20009 suppressed B16F10 tumor growth and induced apoptosis of tumor cells by activating the caspase-dependent apoptotic pathway [91]. Attenuated *S.* Typhimurium has been used to deliver therapeutic cargo in the form of the mitochondrial-targeting domain (MTD) belonging to Noxa, a mediator of apoptosis induction, in the presence of mitochondrial damage. MTD fused to a novel cell-penetrating peptide (CPP) for facilitated entry was delivered to tumor cells resulting in antitumor effects towards mice-bearing CT26 tumors [35]. The entire system, including timed cell lysis and control of MTD-CPP expression, was under the control of the $P_{BAD}$ promoter activated by L-arabinose [35]. VNP20009 recently has been engineered to deliver DNase I, a nuclease that cleaves single- and double-stranded DNA, via an eukaryotic expression vector [92]. VNP20009-DNase I subcutaneously administered with the anti-inflammatory agent triptolide led to enhanced apoptosis of B16F10 cells in vitro and suppressed tumor volume in vivo [92].

### Vaccine Vectors

Bacterial-mediated vaccination is a process by which bacteria deliver tumor antigens to the host, either directly or via therapeutic plasmids, helping to prime a T cell response against the cancer-expressed targets [93]. This leads to the induction of an immune response against the tumor and effective clearance. For example, the gene MTDH/AEG-1 encoding a cell surface protein with a lung-homing domain is overexpressed in more than 40% of breast cancer patients and promotes lung metastasis [48]. Upon delivery of MTDH/AEG-1 by attenuated *S.* Typhimurium, chemosensitivity to doxorubicin was increased and breast cancer lung metastasis inhibited in vivo [48]. Using the T3SS of strain MvP728, Xu et al.

**Table 2** Anticancer therapeutic strategies using *Salmonella* Typhimurium

| Category | Strategy | Description | In vivo cancer models (s) | *Salmonella* strain | References |
|---|---|---|---|---|---|
| Cytotoxic agents | ClyA/HlyE | Pore-forming cytolysin | 4T1 mouse mammary carcinoma, CT26 colon carcinoma, rat C6 glioma | SL3261, ΔppGpp | [80–82, 166] |
| | DTA | Diphtheria toxin A | MDA-MB-231 (human) and 4 T1 (mouse) breast cancer | SL7207 | [56] |
| | TRAIL | Tumor necrosis factor-related apoptosis-inducing ligand | B16F10 melanoma, RM-1 prostate, 4T1 mammary carcinoma, human gastric cancer SGC-7901 in nude mice | VNP20009, SL7207 | [83, 84, 162] |
| | Apoptin | Apoptosis-inducing small protein | Human laryngeal Hep-2 in nude mice | LH430 | [87] |
| | FasL | Fas ligand | Murine D2F2 breast carcinoma, CT26 colon carcinoma | VNP20009 | [88] |
| Cytokines | IL-2 | Lymphocyte regulatory cytokine | B16F10 mouse melanoma | BRD509E | [36] |
| | IL-18 | IFN-γ-inducing factor, enhancing cytokine production by T and NK cells | Mouse CT26 colon carcinoma | VNP20009 | [75] |
| | CCL21 | Chemokine, controls migration of lymphocytes | Mouse D2F2 breast carcinoma, CT-26 colon carcinoma | VNP20009 | [76] |
| | LIGHT | TNF-family cytokine (also TNFSF14 or HVEM-L) | Mouse D2F2 breast carcinoma, CT-26 colon carcinoma | VNP20009 | [77] |
| Regulators | Endostatin | Antiangiogenic agent | Mouse CT26 colon carcinoma, B16F10 melanoma | SL7207, S634 | [49, 56] |
| | L-Asparaginase | Antitumor protein promoting apoptosis | Mouse 4T1 mammary carcinoma, mouse MC38 colon cancer, human AsPC1 pancreatic cancer | ΔppGpp | [90] |
| | Fas-associated protein with death domain (FADD) | Adaptor protein transmitting apoptotic signals | Mouse B16F10 melanoma | VNP20009 | [91] |
| | DNase I | Nuclease cleaving DNA | Mouse B16F10 melanoma | VNP20009 | [92] |
| Vaccine vectors | Survivin | Oncoprotein overexpressed in human cancers | CT26 colon carcinoma, A20 B cell lymphoma, murine neuroblastoma, D121 lung cancer | MvP728, SL7207, RE88 | [44, 46, 57] |
| | Endoglin (CD 105) | Co-receptor in the TGF-β receptor complex | D2F2 breast cancer, B16F10 mouse melanoma, and Renca renal carcinoma | RE88, SL2707 | [47, 54] |
| | MTDH/AEG-1 | Metadherin/astrocyte elevated gene-1, cell surface protein with a lung-homing domain | 4T1 mouse mammary carcinoma | RE88 | [48] |
| | 4-1-BBL | Ligand-enhancing T cell immunity | DMH-induced colorectal tumors in rats | SL3261 | [53] |
| | Flk1 | VEGF receptor 2, antivasculature effect | G1261 glioma, B16F10 mouse melanoma | SL7027, SB824 | [94–96] |
| RNA interference | Stat3-specific | Signal transducer and activator of transcription 3 | Mouse RM-1 prostate cancer, B16F10 melanoma, H22 hepatocarcinoma | LH430, VNP20009, MVP728 | [39, 43, 99, 167] |
| | IDO-specific | Immunosuppressive molecule | B16F10 mouse melanoma, advanced pancreatic cancer | VNP20009 | [168, 169] |
| | INHA-specific | Alpha subunit of inhibition | CT26, B16F10 | BRD509 | [101] |
| | Bcl-2-specific | B cell lymphoma 2 | B16F10 | SL3261 | [100] |
| | PD-1-specific | Checkpoint molecule | B16 | LH430 | [102] |
| | Sox2-specific | Sex-determining region Y-box 2 | Mouse xenograft A549 lung cancer | VNP20009 | [85, 170] |
| | PLK-1-specific | Polo-like kinase 1 | MDA-MB-231 (human) and 4T1 (mouse) breast cancer | SL7207 | [85] |
| | MDR-1-specific | Multidrug resistance gene | Ovarian carcinoma SKOV-3 | SL7207 | [164] |

demonstrated that survivin, an oncoprotein overexpressed in most cancers, could be delivered orally by a *Salmonella*-based vector into the cytosol of antigen-presenting cells [44]. This delivery led to therapeutic vaccination and potent antitumor activity in a CT26 mouse model [44]. Survivin has also been successfully employed for DNA-based vaccination by SL7207 and RE88 towards murine neuroblastoma [57] and murine D121 lung cancer in conjunction with chemokine CCL21 expression [46]. Other targets of *Salmonella*-based DNA vaccination include 4-1BBL, a ligand enhancing T cell immunity [53], and Flk-1, a vascular endothelial growth factor (VEGF) receptor 2 [94–96].

### RNA Interference

RNA interference (RNAi) is a mechanism of transcriptional regulation in the eukaryotic cell used for gene silencing. This mechanism can be exploited for cancer therapy, specifically to knock down mutated genes or in cancers where protein overexpression is driving tumorigenesis [97]. A current limitation in the clinical application of RNAi-based drugs is the lack of an effective delivery system [98]. *S.* Typhimurium is an intriguing delivery vehicle for RNAi therapy due to its tumor targeting and facultative intracellular nature. There are two mechanisms of bacterial-mediated RNAi delivery targeting an oncogene or tumor-expressed factor, namely delivery of plasmid-encoding shRNAs and expression of shRNAs to induce RNAi [93].

With *S.* Typhimurium expressing signal transducer and activator of transcription 3 (Stat3)-specific siRNAs against prostate tumor-bearing C57BL6 mice, tumor growth was significantly inhibited and the metastatic sites reduced [39]. Tian et al. applied *S.* Typhimurium as a vector to deliver short hairpin RNA (shRNA) targeting Stat3 in hepatocellular carcinoma, markedly delaying and reducing tumors in mice [99]. *S.* Typhimurium has delivered shRNA expressed from a plasmid to target Bcl-2 (B cell lymphoma-2). The gene was significantly silenced, delaying melanoma cell tumor growth and prolonging animal survival [100]. *S.* Typhimurium harboring an shRNA expression plasmid, that targeted the alpha subunit of inhibition (sh-INHA), was evaluated in vivo towards CT26 colon and B16F10 melanoma tumor models, where INHA expression is known to be high [101]. Results showed tumoricidal effects of *Salmonella* with and without the INHA knockdown; however, more significant and prolonged tumor growth inhibition was observed in the presence of sh-INHA activity. *Salmonella* harboring RNAi plasmid vectors are therefore an encouraging therapy strategy.

A combinational therapy was developed by Zhao et al. B16 melanoma-bearing mice received *Salmonella* delivered RNAi targeting PD-1, a checkpoint molecule involved in tumor immune escape through suppression of T cell function, as well as pimozide, a drug which has shown efficacy in some studies as a therapeutic against melanoma [102]. Results showed that combined shRNA-PD-1 and pimozide delivery significantly inhibited tumor growth and prolonged animal survival, with an increase in T cell response. The combination of a chemotherapeutic with bacterial-based immunotherapy is a promising clinical strategy in the treatment of melanoma.

## Improving Targeting Efficiency and Specificity

Limited tumor targeting in vivo has been a drawback in the use of some *Salmonella* strains for anticancer efficacy. To improve targeting, VNP20009 was engineered for inducible expression of carcinoembryonic antigen (CEA)-specific single-chain antibody fragments (scFv) on the cell surface, using the major outer membrane lipoprotein and the outer membrane protein OmpA (Lpp-OmpA) expression system [103]. The engineered strain resulted in increased accumulation of bacteria in CEA-expressing tumors vs. CEA-negative tumors [103]. Additionally, *S.* Typhimurium can be engineered to overexpress recombinant, surface expressed, single-domain antibodies to facilitate their targeting to tumor tissue [104]. Specifically, *S.* Typhimurium was constructed to express a camelid single-domain (VHH) antibody against human CD20, a well-studied tumor-associated antigen used in antibody immunotherapy as a target of non-Hodgkin and chronic lymphocytic leukemia (CLL) [105]. This engineered strain exhibited strongly reduced accumulation within spleen and liver in vivo, significantly increasing the safety profile of tumor-targeting bacteria [104]. *S.* Typhimurium ΔppGpp was constructed to display a peptide sequence termed arginine-glycine-aspartate (RGD) on the external loop of OmpA [106]. The RGD peptide is well understood as a tumor-homing peptide that binds alpha v beta 3 integrin ($\alpha v\beta 3$), which is overexpressed on cancer cells and blood vessels during cancer angiogenesis [107]. In vivo evaluation of RGD-displaying ΔppGpp introduced to nude mice bearing human breast cancer (MDA-MB-231) or human melanoma (MDA-MB-435) exhibited a 1000-fold higher targeting efficiency than control bacteria and prolonged survival of the animals [106]. This novel approach to use tumor-associated antigens for targeted delivery of *S.* Typhimurium demonstrates much promise as a future therapy.

We recently performed RNA-seq on B16-F10 melanoma tumors following *S.* Typhimurium VNP20009 intravenous administration, first confirming expression of melanoma-associated genes prior to analyzing the effect of VNP20009 on changes within the tumor transcriptional landscape. We confirmed expression of *TYRP1*, a gene encoding tyrosinase-related protein 1, within melanoma tumors where there was 1000-fold higher expression compared to spleen tissue (data unpublished). TYRP1 is located in melanocytes that produce melanin, a characteristic of B16 melanoma cells

[108]. Interestingly, the *TYRP1* gene, as well as others that fall in the category of melanocyte differentiation antigens (MDA), is a subject for vaccination therapy. The goal in these studies is the activation of cytotoxic T lymphocytes [109]. It has been described by Hara et al. that immunization against B16 melanoma can be accomplished by introduction of an antibody, mAb TA99, that recognizes gp75 (TYRP1). The stimulated immune response not only protected the animal from melanoma tumors, but also resulted in rejection of subcutaneously implanted tumors and metastases [110]. As described above, *S.* Typhimurium can be engineered to express recombinant, surface expressed, single-domain antibodies that facilitate their targeting to tumor tissue [104]. Therefore, it is possible to use this approach to more efficiently and perhaps expediently target *Salmonella* to melanoma tumors via their expression of *TYRP1*, resulting in increased and reduced colonization of the tumor and spleen, respectively.

## Using *Salmonella* for Imaging and Diagnostics

Another application of tumor-colonizing *S.* Typhimurium is the potential use in magnetic resonance and positron emission tomography (PET) for diagnostic imaging. VNP20009 expressing the reporter herpes simplex virus *thymidine kinase* (HSV1-*tk*) when delivered to mice in vivo localized within tumors and sequestered the radiolabeled nucleoside analogue 2′-fluoro-1-β-D-arabino-furanosyl-5-iodouracil (FIAU) [111, 112]. A log-log relationship was found between PET imaging and bacterial accumulation, indicating non-invasive localization of the tumor site can be achieved based on pinpointing *S.* Typhimurium. These techniques could be used to aid in tracking *S.* Typhimurium and understanding anticancer drug efficiency and requirements for duration of targeting.

In an effort to address current limitations in tomographic sensitivity, *S.* Typhimurium has been modified to release a recombinant biomarker. Specifically, VNP20009 was engineered to express and release a fluorescent reporter protein, ZsGreen, in a microfluidic-based in vitro experimental setup. The produced ZsGreen was detected using single-layer antibody dots and found to accumulate in tissue with a 2600-fold higher resolution compared to the current limit of tomographic techniques [113]. The authors recently went a step further and evaluated the fluoromarker-releasing bacterial system in tumor-bearing mice. Based on measurements gathered from viable tissue, necrotic tissue, and plasma, the system has the capability to detect tumors as small as 0.12 g [114]. The *Salmonella*-based, fluoromarker-release system has potential to identify currently undetectable microscopic tumors and facilitate early diagnostics in the future.

## Alternative Strategies Towards *S.* Typhimurium-Mediated Cancer Therapy

Exploiting tumor targeting innate to *S.* Typhimurium and the safety profile of attenuated strains, an engineering perspective has been applied to therapy options by using bacteria to deliver nano-, photo-, and thermal-therapeutics. Employing *S.* Typhimurium as a biological "mailman," to carry drug payloads via membrane attachment to intended sites in an accurate and precise way is a growing field of focus [115]. Nanoscale bacteria-enabled autonomous drug delivery system (NanoBEADS) enhanced nanoparticle retention and distribution within 3D tumor spheroids in vitro and 4T1 mouse mammary carcinoma in vivo [116]. The drug delivery system would improve therapeutic effects in cancer treatment and

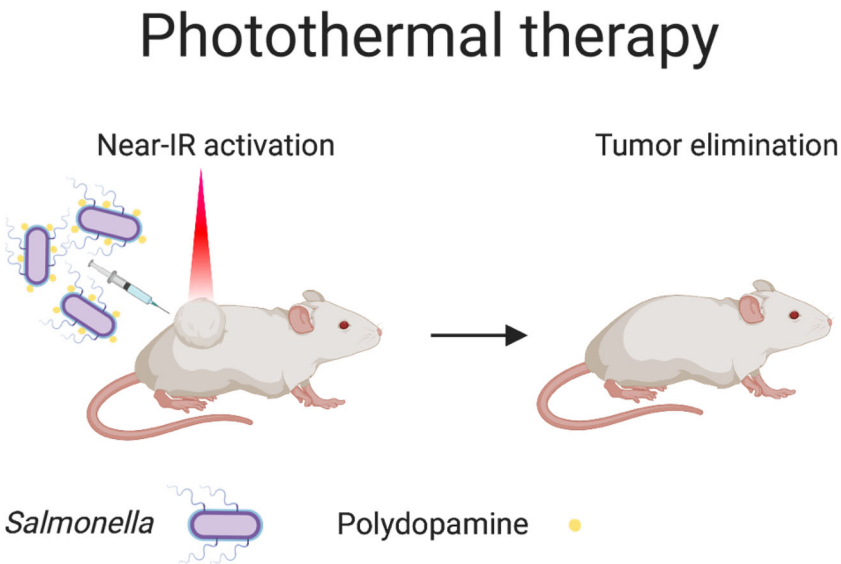


**Fig. 3** Photothermal therapy with *Salmonella*. Tumor-targeting *S.* Typhimurium are coated with polydopamine, a biocompatible photothermal agent. Following tumor colonization, near-infrared irradiation is used to activate the polydopamine, causing a light-to-heat conversion and therapeutic effects

has the potential to minimize side effects brought on by chemotherapeutics.

Attenuated strains have been developed as "thermobots" to transport membrane-attached, low-temperature sensitive liposome (LTSL), which undergo structural and chemical phase change to achieve timed doxorubicin delivery in response to high-intensity focused ultrasound (HIFU) heating [117]. The thermobots successfully triggered doxorubicin release with high nuclear localization and induced proinflammatory cytokine expression in vitro, as well as therapeutic efficacy in vivo towards CT26 colon cancer [117]. Finally, photothermal therapy, which results in the conversion of laser light to heat through absorption, has been integrated with YB1, engineered to survive only in anaerobic conditions by placing the essential gene *asd* under control of a hypoxia promoter *pepT* [118]. Nanophotosensitizers (indocyanine green-loaded nanoparticles (INPs)) activated by near-infrared laser irradiation were linked to the surface of YB1 for tumor precision therapy [119]. The YB1-INP photothermal therapy resulted in a 14-fold higher bioaccumulation within solid tumors compared to treatment with YB1 alone and eradicated solid MB49 mouse bladder carcinoma tumors in vivo [119]. Furthermore, VNP20009 has been coated with polydopamine, a biocompatible photothermal agent and heated using near-infrared irradiation (Fig. 3). In just a single dose, this therapeutic approach eliminated B16F10 tumors without relapse or metastasis [120].

*S.* Typhimurium decreases the expression of mammalian P-glycoprotein (P-gp), a multidrug resistance (MDR) transporter, in a manner dependent on the bacterial protein, SipA. Mercado-Lubo et al. constructed a gold nanoparticle system packaged with SipA for enriched delivery, followed by chemotherapeutic agents such as doxorubicin [121]. The group found suppressed tumor growth in vivo towards CT26 colon cancer with their semi-synthetic *Salmonella* nanoparticle mimic, thereby enhancing efficacy and cytotoxicity of a non-targeted chemotherapeutic [121]. Overall, the engineered biomimic was efficient in circumventing tumor MDR and exhibiting a high degree of safety [122].

## Conclusions

We have provided a review to update readers to the best of our ability on the state of *S.* Typhimurium in the bacterial-mediated cancer therapy field of study. This bacterial marvel has been tested in various stages of clinical trials [123]. VNP20009 has been evaluated in preclinical trials against canine spontaneous neoplasia, where overall survival was best in the complete responders [15]. A pilot trial (identifier: NCT00006254) was then conducted on VNP20009 towards patients with squamous cell carcinoma, where tumor colonization was observed but no tumor shrinkage [124]. Finally, VNP20009 was evaluated in a phase 1 clinical trials (identifiers: NCT00004216, NCT00004988) towards patients with metastatic melanoma, which accomplished safe delivery to the patients with minimal toxicity; however, no tumor shrinkage was observed [10, 125]. Another phase 1 clinical trial was completed in 2014, where χ4550 expressing IL-2 was orally administered to patients with unresectable hepatic metastases from a solid tumor, with results yet to be reported (identifier: NCT01099631). Despite tumor specificity and tumor-suppressive effects being well documented in preclinical testing, the translation to human oncology has fallen short. Thus, the exact mechanisms underlying *Salmonella*-mediated cancer therapy are not fully understood, highlighting the complexity of not only cancer but the interspecies relationship between *Salmonella* and residents of the tumor microenvironment.

To this end, the National Cancer Institute organized the first Microbial-Based Cancer Therapy Conference in July 2017, with the objective to share insights and stimulate conversation in the field [126]. The first NIH call specifically geared towards bacterial-mediated cancer research, named "Bugs as Drugs" was posted February 2019 (https://grants.nih.gov/grants/guide/pa-files/PAR-19-193.html). The use of *Salmonella* as a novel antitumor agent has experimentally shown much promise as a cancer therapeutic, at a time when innovation is in the greatest need.

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## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflicts of interest.

**Human and Animal Rights and Informed Consent** This article does not contain any studies with human or animal subjects performed by any of the authors.

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- • Of importance
- •• Of major importance

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