Using spore-forming bacteria to treat cancer: recent advancements in clostridial-based therapies

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Abstract: The use of bacteria in cancer therapy has emerged as a promising approach, offering unique advantages for targeted treatment and immunotherapy. This review paper explores some recent advances in the application of bacteria, particularly *Clostridium* species, in cancer therapy. For instance, the Clostridial-Directed Enzyme Prodrug Therapy (CDEPT) utilises non-pathogenic strains of *Clostridium* as carriers for targeted delivery of anti-cancer drugs to solid tumour cells. This strategy aims to minimise systemic side effects associated with traditional chemotherapy. Additionally, *Clostridium novyi* and *Clostridium sporogenes* present oncolytic properties and have shown potential for tumour regression in preclinical models. The engineering of these bacteria to produce cytokines, such as interleukin-12 (IL-12) and interleukin-2 (IL-2), further enhances their therapeutic potential by activating the immune system to target cancer cells. Clinical trials in humans have demonstrated the feasibility and safety of *C. novyi*-based therapies, and early results indicate potential efficacy in tumour regression. Overall, this review provides valuable insights into the multifaceted roles of bacteria, particularly *Clostridium* species, in cancer therapy, emphasizing their potential as targeted therapeutics and immunomodulators for improved cancer treatment outcomes.

Keywords: Spores, *Clostridium sporogenes*, *Clostridium novyi*, cancer therapy, CDEPT, hypoxia, solid tumour

Introduction

Cancer is a challenging disease that continues to pose a significant threat to human health, affecting a substantial number of individuals each year. According to projections from the American Cancer Society, an estimated 1,958,310 new cancer cases and 609,820 cancer-related deaths were anticipated in the United States for 2023 (Siegel, Miller, Wagle, & Jemal, 2023). Through the dedicated efforts of the scientific community, remarkable progress has been achieved in the realm of cancer therapeutics, leading to notable reductions in mortality rates (Siegel et al., 2023). Nonetheless, the complex nature of cancer presents a formidable challenge, as different cancer types exhibit diverse characteristics and features that hinder the development of universally applicable treatments or cures. Particularly, solid tumours pose a significant obstacle due to the formation of necrotic tissue, rendering them resistant to conventional treatment (Tharmalingham & Hoskin, 2019). Addressing this challenge entails exploring alternative approaches, such as the targeted delivery of spores of anaerobic bacteria to these tumours. This approach capitalises on the presence of hypoxic regions within solid tumours, where oxygen supply is deficient.

Bacterial endospores represent one of the most resilient life forms found on Earth, enabling bacteria to withstand extreme conditions including temperature variations, desiccation, radiation, disinfectants, and, in the case of *Clostridium*, oxygen (Setlow, 2007). These extraordinary structures feature multiple protective layers, including membranes, coat, cortex, and, in certain instances, an exosporium, all of which shield the core containing DNA (Setlow,

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2007). Such formidable defence mechanisms ensure their long-term survival, potentially spanning millions of years (Cano & Borucki, 1995; Kennedy, Reader, & Swierczynski, 1994). However, a trade-off of spore formation is the metabolic dormancy imposed on the cells. Yet, when confronted with conditions conducive to cellular viability, spores can undergo germination, reactivating metabolic processes (Setlow, 2003).

Over the years, numerous spore-forming bacteria have been identified, predominantly belonging to the taxonomic classes Bacilli and Clostridia within the Firmicutes phylum (Galperin et al., 2012). Notably, the main distinction between these classes lies in their aerobic requirements, with Clostridia typically classified as anaerobic bacteria, while Bacilli encompass both obligate and facultative aerobes (Collins et al., 1994; Ludwig, Schleifer, & Whitman, 2015). While numerous spore-forming species are typically associated with pathogenicity, it is important to acknowledge that certain species within this group are benign and exhibit intriguing applications. Notably, some of these species have demonstrated the capacity to produce solvents of commercial significance, such as *Clostridium acetobutylicum*, *Clostridium stercorarium*, and *Clostridium thermocellum* (Lamed & Zeikus, 1980; Napoli, Olivieri, Russo, Marzocchella, & Salatino, 2010; Tran et al., 2012). Additionally, *Clostridium cellulolyticum* has been investigated for its ability to efficiently degrade cellulose (Desvaux, Guedon, & Petitdemange, 2000). This review focuses on the utilisation of Clostridia in the treatment of diseases, specifically cancer (Andryukov, Karpenko, & Lyapun, 2021).

The link between spore formers and cancer goes back to at least 1947: when mice sarcomas were infected with *Clostridium histolyticum*, tumour tissue was lysed, albeit not completely (Parker, Plummer, Siebenmann, & Chapman, 1947). More recently, advances in genetic engineering allowed improvements and oncolysis can be enhanced. This is important because when the outer rim of the tumour is not fully eliminated, tumour regrowth frequently occurs (Minton et al., 1995).

This review will focus on *Clostridium sporogenes* and *Clostridium novyi*, two anaerobic spore-formers that have been extensively researched for their cancer-treatment capabilities. The combined use of these species with immunotherapy will also be explored.

Clostridium sporogenes as a versatile tool for targeted cancer therapy

C. sporogenes is rod-shaped, anaerobic, produces endospores, and can be found in a variety of environments, including soil and human/animal intestines. This species is safe, being classified as a harmless hazard group I organism by the UK Advisory Committee on Dangerous Pathogens and as a harmless biosafety level 1 organism by the American Type Culture Collection (Kubiak et al., 2015).

Extensive research has been conducted on *C. sporogenes* in the context of Clostridial-Directed Enzyme Prodrug Therapy (CDEPT). CDEPT encompasses the utilisation of non-pathogenic strains of *Clostridium* as carriers for targeted delivery of anti-cancer drugs to solid tumour cells (Kubiak & Minton, 2015; Minton et al., 1995). The development of CDEPT stemmed from the need to mitigate the undesired side effects often associated with conventional cancer treatments. Traditional chemotherapeutic agents are frequently designed to disrupt crucial cellular processes like DNA replication, mitosis, or cell proliferation, and may inadvertently impact healthy cells (Karnofsky, 1968). To overcome these challenges, the concept of a prodrug was devised, involving the delivery of a biologically inert compound to the tumour site, which is subsequently activated into a highly cytotoxic drug (Rautio et al., 2008). CDEPT employs the administration of a prodrug in conjunction with a prodrug-converting enzyme (PCE) (Kubiak & Minton, 2015; Minton et al., 1995). Ideally, the conversion of the prodrug occurs solely within the tumour microenvironment, sparing healthy tissues from harm.

One notable advantage of the CDEPT strategy lies in its utilisation of obligate anaerobes as vectors for delivering the prodrug-converting enzyme to hypoxic regions within tumours. Hypoxia, characterized by reduced oxygen levels, is a common feature observed in tumour tissues, often attributed to inadequate blood supply caused by the distance between certain tumour regions and blood vessels. This poses challenges for conventional treatment modalities such as radiotherapy and chemotherapy (Weinmann, Belka, & Plasswilm, 2004). Administration of *Clostridium* spores to cancer patients has been reported to induce tumour regression, primarily owing to their oncolytic properties, while selectively targeting hypoxic regions, as spores germinate exclusively in poorly oxygenated areas (Möse & Möse, 1964; Thiele, Arison, & Boxer, 1964).

Consequently, spores derived from *Clostridium* species have been extensively investigated as potential vehicles for delivering the prodrug-converting enzyme to hypoxic tumour tissues (Heap et al., 2014)

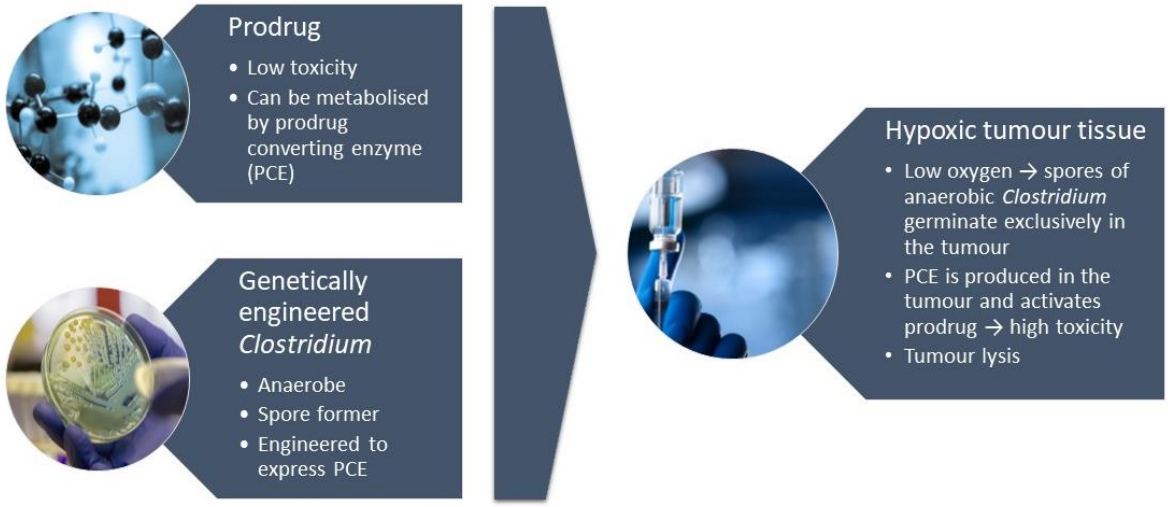

Figure 1: *Representation of the CDEPT therapy. Spores of an engineered strain of Clostridium are administered intravenously to a cancer patient. The engineered strain contains a gene encoding a prodrug-converting enzyme (PCE). Then, a prodrug is also delivered to the patient. In the tumour, the spores can germinate and express the PCE, which converts the prodrug into a toxic derivative.*

C. sporogenes has been extensively studied as the prodrug-converting enzyme delivery vector, as part of the CDEPT therapy. The successful germination of *C. sporogenes* exclusively in necrotic tumours has been demonstrated but researchers are still testing the ideal PCE-prodrug combination.

Prodrug-converting enzymes, such as nitroreductases (NTR), hold significant potential in the field. Notably, enzymes like NfsB, HsoNTR, and NmeNTR exhibit the ability to activate the prodrug CB1954 (5-(aziridin-l-yl)-2,4- dinitrobenzamide). NmeNTR, a nitroreductase from *Neisseria meningitidis*, can catalyse the reduction of the 4-nitro group found in the anti-tumour prodrug CB1954, resulting in the formation of its 4-hydroxylamine derivative. Importantly, this converted form of the drug demonstrates substantially increased toxicity compared to the prodrug itself One downside of the NmeNTR/CB1954 combination is that the toxic 4-hydroxylamine has limited ability to diffuse into neighbouring cancer cells that are not necrotic tissue and, consequently, are not colonised by the engineered *C. sporogenes*

An alternative prodrug, PR-104 (dinitrobenzamide mustard), can be converted into the PR-104A metabolite, which in turn can be further reduced to generate genotoxic drugs PR-104H (hydroxylamine) and PR-104M (amine). These metabolites exhibit the ability to induce DNA damage and interfere with cell division, thereby exerting negative effects on cellular processes In a recent study published in 2021, the NmeNTR/PR-104A prodrug converting enzyme (PCE)/prodrug combination was investigated, revealing superior activation of PR-104A by NmeNRT compared to CB1954 Moreover, the authors enhanced PCE activity by modulating the enzyme-encoding gene with an alternative promoter. Exclusive germination of *C. sporogenes* within necrotic tissue was also demonstrated, along with the inhibition of tumour growth upon intravenous administration of genetically engineered *C. sporogenes* spores alongside the prodrug PR-104. The incorporation of the tumour vascular disrupting agent 5,6-dimethylxanthenone-4-acetic acid (vadimezan), which was shown to increase tumour necrotic fraction, further augmented tumour growth inhibition and increased median survival time. Vadimezan was administered 60 minutes post-spore injection to ensure delivery of *C. sporogenes* spores to the tumour microenvironment before necrosis induction. Importantly, the ability of NmeNRT to metabolize the PET imaging agent EF5 offers

additional advantages to this therapeutic approach, enabling non-invasive imaging of therapeutic gene expression and tumour colonisation

The utilisation of β-lactamases as prodrug-converting enzymes in CDEPT therapy was proposed in 2006 This class of enzymes can cleave cephalosporin prodrugs, generating two active compounds. One notable advantage of employing β-lactamases as PCE is their natural occurrence in certain *Clostridium* species, such as *Clostridium butyricum* This inherent presence obviates the necessity for genetic modifications of bacterial strains, streamlining the implementation of the therapy.

C. sporogenes has undergone extensive evaluation within the scope of CDEPT; however, this bacterial species has also found application in other approaches to cancer treatment. Notably, *C. sporogenes* possesses the ability to produce methionine γ-lyase (MGL), an enzyme capable of catalysing the γ-elimination of L-methionine (MET). As MET plays a pivotal role in the growth of malignant tumour cells, MGL has been investigated for its potential anticancer properties. In a study conducted in 2019, the MGL gene from *C. sporogenes* was expressed in *E. coli* strain BL21. Subsequent testing of MGL, both as a standalone treatment and in combination with another compound, involved A549 human lung cancer cells. Encouragingly, when combined with doxorubicin (DOX), MGL demonstrated cytotoxicity and exhibited tumour growth inhibition (Pokrovsky et al., 2019).

Clostridium novyi: oncolytic properties and therapeutic applications

C. novyi has emerged as a compelling subject of investigation for its remarkable oncolytic properties. Upon colonisation of tumours, this species appears to produce extracellular lipases, which have been hypothesised to exert cytotoxic effects against tumour cells (Bettegowda et al., 2006).

In a study conducted in 2001, *C. novyi* ATCC 19402 was compared to 25 other anaerobic strains encompassing three distinct genera, namely Bifidobacteria, Lactobacilli, and Clostridia (Dang, Bettegowda, Huso, Kinzler, & Vogelstein, 2001). Due to its remarkable capacity to infiltrate and disperse throughout tumour tissue while sparing healthy tissue, *C. novyi* was selected by the authors for further investigation, as it demonstrated the ability to induce tumour cell death. To ensure safety, a non-toxic strain (*C. novyi*-NT) was engineered by eliminating the alpha toxin gene. To enhance the efficacy of the treatment and target both hypoxic and non-hypoxic regions within tumours, a combination bacteriolytic therapy (COBALT) approach was developed, employing spores of *C. novyi*-NT in conjunction with D10 (an agent disrupting tumour vasculature) and MMC (a DNA-damaging agent). *In vivo* experiments using mouse models with xenografts of HCT116 colorectal cancer cells demonstrated that this therapy resulted in tumour regression, albeit potentially accompanied by a phenomenon known as tumour lysis syndrome, toxicity resulting from the rapid destruction of large tumours (Dang et al., 2001). Notably, the observed toxicity appeared to be attributed to germinated cells rather than spores and was found to be proportional to both tumour size and spore dosage (Diaz et al., 2005). Nevertheless, the toxicity could be managed through the administration of the antibiotic imipenem after tumour colonisation (although this compromised the efficacy of the therapy), or through systemic hydration to counteract fluid loss during infection. Furthermore, it was observed that 90% of intravenously injected spores were cleared from circulation within 14 days (Diaz et al., 2005).

Due to its inherent oncolytic properties, *C. novyi* offers a promising avenue for treating certain tumours as a monotherapy, without the need for additional treatment combinations. A recent study conducted in 2022 explored the use of a novel non-toxic strain of *C. novyi* for the treatment of breast tumours in mice (Abedi Jafari, Abdoli, Pilehchian, Soleimani, & Hosseini, 2022). Through intratumoral injection of spores, complete remission was observed in all mice (n = 8) with tumour sizes up to 1000 mm^3. However, larger tumours demonstrated a diminished response to the treatment. The authors postulated that for smaller tumours, the bacteria primarily targeted hypoxic regions within the tumour, while the immune system response played a significant role in eliminating non-hypoxic cells. In contrast, administration of *C. novyi* alone was insufficient to entirely eradicate larger tumours (Abedi Jafari et al., 2022).

In addition to its applications in colorectal and breast cancer, *C. novyi*-NT has also been investigated as a potential treatment for glioblastoma, a form of brain cancer (V. Staedtke et al., 2022). Researchers directed their focus towards managing

host inflammatory responses, which can lead to oedema and elevated intracranial pressure. Such responses pose additional challenges by impeding the dissemination of *C. novyi*-NT throughout the tumour, hindering the lysis of tumour cells located in the outer rim. To address this issue, the authors employed neutrophil depletion achieved through the administration of the 1A8 antibody before spore delivery. Neutrophil depletion not only facilitated improved tumour clearance but also enhanced the safety of the procedure by mitigating toxicity. Encouraging results were observed in rabbit models treated with this therapy, with 70% of animals exhibiting no tumour recurrence (V. Staedtke et al., 2022).

In another study, orthotopically implanted glioblastoma rat models were utilised to assess the efficacy of *C. novyi*-NT treatment. Intravenous injection of *C. novyi*-NT spores led to tumour regression and a significant increase in survival time (Verena Staedtke et al., 2015). Although treatment-associated side effects such as brain oedema and increased intracranial pressure were observed, their impact could be controlled with the administration of steroids and antibiotics. Furthermore, combining *C. novyi*-NT treatment with a liposomal formulation of the DNA intercalating agent doxorubicin resulted in improved tumour clearance. Importantly, the authors demonstrated that spore germination occurred exclusively within tumours and not in hypoxic regions induced by stroke or myocardial infarctions (Verena Staedtke et al., 2015).

While the majority of studies involving *C. novyi*-NT have employed mouse, rat, or rabbit models with induced tumours, efforts have been made to bridge the gap between animal models and human patients by treating naturally occurring tumours in canines (Roberts et al., 2014). In a study involving 16 dogs with solid tumours, one to four cycles of intratumoral injections of *C. novyi*-NT spores were administered. Two dogs were excluded from evaluation as their tumours were surgically removed before day 21, which was set as the time point for response assessment. Among the 14 evaluated dogs on day 21, three demonstrated a complete response to treatment, three showed a partial response, five exhibited stable disease, and disease progression was observed in three dogs (Roberts et al., 2014). Moreover, a human patient with retroperitoneal leiomyosarcoma was treated using intratumoral spore injections, which resulted in a successful anti-tumour response (Roberts et al., 2014).

Promising strides have been made in exploring the safety and efficacy of *C. novyi* in early clinical trials involving human patients. In a phase I clinical trial involving 22 patients, *C. novyi*-NT was delivered through intratumoral injection, and spore germination was observed in 10 patients (Janku et al., 2021). The most common adverse effects reported were pain at the injection site, fever, and fatigue. No serious complications or deaths were reported, and any significant toxicities encountered were manageable. Notably, tumour shrinkage was reported in nine patients. Treatment with *C. novyi* stimulated a transient systemic cytokine response and enhanced systemic tumour-specific T-cell responses. Overall, the authors concluded that the treatment was both feasible and safe (Janku et al., 2021).

Finally, notable progress has been achieved in the development of imaging tools to monitor the colonisation of tumours by *C. novyi*-NT. A study utilised intratumoral injection of iron-oxide labelled *C. novyi*-NT into mice with orthotopically implanted pancreatic tumours, allowing the tracking of bacteria using magnetic resonance imaging (MRI) (Zheng et al., 2015). This *in vivo* imaging technique enables the monitoring of tumour colonisation and distribution of bacteria, facilitating the optimisation of injection procedures and enabling both intra-procedural and post-procedural monitoring. Additionally, the authors demonstrated that *C. novyi*-NT induced tumour shrinkage in a mouse model of pancreatic carcinoma (Zheng et al., 2015).

Harnessing bacteria for immunotherapy: enhancing immune response against cancer cells

Utilising bacteria for cancer treatment offers a significant advantage in addition to their oncolytic properties: they can serve as immune system modulators, directing immune cells to target cancer cells. *C. sporogenes*, for instance, has been genetically engineered to produce cytokines, thereby activating the immune system, and promoting the elimination of tumour cells.

In one study, *C. sporogenes* ATCC 3584 was engineered to secrete interleukin-12 (IL-12) (Zhang et al., 2014). The authors demonstrated that the bacteria successfully survived and secreted IL-12 within the tumour environment. This resulted in immune cell activation and destruction of tumour cells in a murine model of EMT6 mammary carcinoma. The

treatment achieved a 14.3% cure rate with no apparent toxicity (Zhang et al., 2014).

Similarly, *C. sporogenes* NCIMB 10696 was modified to produce murine interleukin-2 (IL-2) (Kubiak, Bailey, Dubois, Theys, & Lambin, 2021). The study employed an attenuated strain, *C. sporogenes*-NT, which lacked the streptolysin S operon to reduce bacterial-induced haemolysis. IL-2 plays a vital role in activating and proliferating T cells. Given that *C. sporogenes* germinates exclusively within hypoxic tumours, IL-2 production is limited to the tumour microenvironment, guiding the immune system's response against cancer. The researchers demonstrated that *C. sporogenes*-NT could successfully colonise tumours in mouse models, secrete IL-2 *in vitro*, and induce T cell proliferation (Kubiak et al., 2021). Furthermore, interleukin-2 expression was also achieved in *C. acetobutylicum* DSM792, as demonstrated in a study by Barbé et al. (2005). ELISA assays confirmed the secretion of IL-2 by *C. acetobutylicum*, and T-cell proliferation assays validated the biological activity of the cytokine (Barbé et al., 2005).

In a recent 2023 study, multifunctional porous microspheres (MPMs) were utilised to enhance the delivery and retention of *C. novyi*-NT spores within the tumour microenvironment (Bae et al., 2023). The results indicated that this delivery approach did not impair spore germination within a simulated tumour microenvironment and showcased its potential for image-guided cancer immunotherapy (Bae et al., 2023).

Conclusion

Bacterial-based cancer therapies hold immense promise, particularly due to the ability of anaerobic bacteria to specifically target the challenging hypoxic and necrotic regions of tumours that traditional treatments like chemotherapy or radiotherapy struggle to reach. In recent years, significant progress has been made, largely driven by advancements in genetic engineering techniques. Substantial improvements have been achieved not only in therapy efficacy but also in safety. Encouraging results from animal models and early-stage clinical trials underscore the potential of these approaches.

As with other therapies, the adaptation of bacterial-based treatments to specific cancer types, tumour sizes, and individual patient characteristics is likely to be crucial. *Clostridium* species have demonstrated tumour shrinkage across various cancers, including mammary/breast cancer (mouse model) (Abedi Jafari et al., 2022; Minton et al., 1995), Ehrlich carcinoma (mouse model) (Möse & Möse, 1964), sarcoma (mouse model), melanoma (hamster model) (Thiele et al., 1964), colon carcinoma (mouse model) (Dang et al., 2001; Diaz et al., 2005; Heap et al., 2014), lung carcinoma (mouse model) (Mowday et al., 2022; Pokrovsky et al., 2019), glioblastoma (mouse model) (V. Staedtke et al., 2022), glioblastoma (rat model) (Verena Staedtke et al., 2015), pancreatic cancer (mouse model) (Zheng et al., 2015), sarcoma (dog study) (Roberts et al., 2014), retroperitoneal leiomyosarcoma (human patient) (Roberts et al., 2014), and solid tumours in human patients (Janku et al., 2021). While oncolysis has been achieved with *Clostridium* injections alone (either intravenous or intratumoral), combination therapies incorporating bacteria with other agents have shown greater success in achieving complete tumour destruction. This is due to the frequent resistance of tumour rims to *Clostridium*-induced lysis, leading to tumour regrowth.

While this review focused primarily on *C. novyi* and *C. sporogenes*, other Clostridial species are being explored for their oncolytic properties. For example, *C. butyricum* has exhibited the ability to inhibit the growth of colorectal tumours in mice when combined with 5-fluorouracil (5-FU) chemotherapy, and it has also shown potential in enhancing the efficacy of immunotherapy (anti-PD-1) (Xu, Luo, Zhang, Li, & Lee, 2023).

The diverse array of bacterial strains, coupled with the possibility of combining them with other agents or treatments, offers oncologists the opportunity to tailor the most suitable therapy for their patients. Furthermore, the administration of antibiotics or other anti-infective agents can effectively eliminate the *Clostridium* species after treatment, providing an additional safety measure to prevent infections and allowing the interruption of treatment in the event of adverse effects (Mowday et al., 2022).

In conclusion, the use of bacteria, specifically *Clostridium* species, holds significant promise for cancer therapy. These bacteria offer the advantage of targeted drug delivery, oncolytic properties, and immunomodulation, contributing to improved treatment outcomes and reduced side effects. Further research and clinical trials are necessary to refine and validate these approaches, to advance bacterial-based therapies into mainstream cancer treatments. The continued exploration of bacteria in cancer

therapy opens new avenues for innovation and the potential to transform the landscape of cancer treatment in the future.

References

Abedi Jafari, F., Abdoli, A., Pilehchian, R., Soleimani, N., & Hosseini, S. M. (2022). The oncolytic activity of Clostridium novyi nontoxic spores in breast cancer. Bioimpacts, 12(5), 405-414. https://doi.org/10.34172/bi.2021.25

Andryukov, B. G., Karpenko, A. A., & Lyapun, I. N. (2021). Learning from Nature: Bacterial Spores as a Target for Current Technologies in Medicine (Review). Sovrem Tekhnologii Med, 12(3), 105-122. https://doi.org/10.17691/stm2020.12.3.13

Bae, G.-H., Ryu, Y.-H., Han, J., Kim, S. H., Park, C. G., Park, J.-H., . . . Park, W. (2023). Multifunctional porous microspheres encapsulating oncolytic bacterial spores and their potential for cancer immunotherapy. Biomaterials Science, 11(13), 4652-4663. https://doi.org/10.1039/D3BM00635B

Barbé, S., Van Mellaert, L., Theys, J., Geukens, N., Lammertyn, E., Lambin, P., & Anné, J. (2005). Secretory production of biologically active rat interleukin-2 by Clostridium acetobutylicum DSM792 as a tool for anti-tumor treatment. FEMS Microbiology Letters, 246(1), 67-73. https://doi.org/10.1016/j.femsle.2005.03.037.

Bettegowda, C., Huang, X., Lin, J., Cheong, I., Kohli, M., Szabo, S. A., . . . Zhou, S. (2006). The genome and transcriptomes of the anti-tumor agent Clostridium novyi-NT. Nature Biotechnology, 24(12), 1573-1580. https://doi.org/10.1038/nbt1256.

Cano, R., & Borucki, M. (1995). Revival and identification of bacterial spores in 25- to 40-million-year-old Dominican amber. Science, 268(5213), 1060-1064. Retrieved from http://science.sciencemag.org/content/sci/268/5213/1060.full.pdf. https://doi.org/10.1126/science.7538699

Collins, M. D., Lawson, P. A., Willems, A., Cordoba, J. J., Fernandez-Garayzabal, J., Garcia, P., . . . Farrow, J. A. E. (1994). The phylogeny of the genus Clostridium: proposal of five new genera and eleven new species combinations. International Journal of Systematic and Evolutionary Microbiology, 44(4), 812-826. Retrieved from http://ijs.microbiologyresearch.org/content/journal/ijsem/10.1099/00207713-44-4-812. https://doi.org/10.1099/00207713-44-4-812

Dang, L. H., Bettegowda, C., Huso, D. L., Kinzler, K. W., & Vogelstein, B. (2001). Combination bacteriolytic therapy for the treatment of experimental tumors. Proceedings of the National Academy of Sciences, 98(26), 15155-15160. Retrieved from https://www.pnas.org/content/pnas/98/26/15155.full.pdf. https://doi.org/10.1073/pnas.251543698

Desvaux, M., Guedon, E., & Petitdemange, H. (2000). Cellulose catabolism by Clostridium cellulolyticum growing in batch culture on defined medium. Applied and Environmental Microbiology, 66(6), 2461-2470. Retrieved from http://aem.asm.org/content/66/6/2461.abstract. https://doi.org/10.1128/aem.66.6.2461-2470.2000

Diaz, L. A., Jr., Cheong, I., Foss, C. A., Zhang, X., Peters, B. A., Agrawal, N., . . . Huso, D. L. (2005). Pharmacologic and toxicologic evaluation of C. novyi-NT spores. Toxicological Sciences, 88(2), 562-575. https://doi.org/10.1093/toxsci/kfi316.

Galperin, M. Y., Mekhedov, S. L., Puigbo, P., Smirnov, S., Wolf, Y. I., & Rigden, D. J. (2012). Genomic determinants of sporulation in Bacilli and Clostridia: towards the minimal set of sporulation-specific genes. Environmental Microbiology, 14(11), 2870-2890. https://doi.org/10.1111/j.1462-2920.2012.02841.x

Gu, Y., Patterson, A. V., Atwell, G. J., Chernikova, S. B., Brown, J. M., Thompson, L. H., & Wilson, W. R. (2009). Roles of DNA repair and reductase activity in the cytotoxicity of the hypoxia-activated

dinitrobenzamide mustard PR-104A. Molecular Cancer Therapeutics, 8(6), 1714-1723. https://doi.org/10.1158/1535-7163.MCT-08-1209.

Heap, J. T., Theys, J., Ehsaan, M., Kubiak, A. M., Dubois, L., Paesmans, K., . . . Minton, N. P. (2014). Spores of Clostridium engineered for clinical efficacy and safety cause regression and cure of tumors in vivo. Oncotarget, 5(7), 1761-1769. Retrieved from http://www.impactjournals.com/oncotarget/index.php?journal=oncotarget&page=article&op=view&path%5B%5D=1761. https://doi.org/10.18632/oncotarget.1761

Helsby, N. A., Ferry, D. M., Patterson, A. V., Pullen, S. M., & Wilson, W. R. (2004). 2-Amino metabolites are key mediators of CB 1954 and SN 23862 bystander effects in nitroreductase GDEPT. British journal of cancer, 90(5), 1084-1092. https://doi.org/10.1038/sj.bjc.6601612.

Janku, F., Zhang, H. H., Pezeshki, A., Goel, S., Murthy, R., Wang-Gillam, A., . . . Gounder, M. M. (2021). Intratumoral injection of Clostridium novyi-NT spores in patients with treatment-refractory advanced solid tumors. Clinical Cancer Research, 27(1), 96-106. https://doi.org/10.1158/1078-0432.CCR-20-2065.

Karnofsky, D. A. (1968). Mechanisms of action of anticancer drugs at a cellular level. Ca: A Cancer Journal For Clinicians, 18(4), 232-234. https://doi.org/10.3322/canjclin.18.4.232

Kennedy, M. J., Reader, S. L., & Swierczynski, L. M. (1994). Preservation records of micro-organisms: evidence of the tenacity of life. Microbiology, 140(10), 2513-2529. Retrieved from http://mic.microbiologyresearch.org/content/journal/micro/10.1099/00221287-140-10-2513. https://doi.org/10.1099/00221287-140-10-2513

Kubiak, A. M., Bailey, T. S., Dubois, L. J., Theys, J., & Lambin, P. (2021). Efficient secretion of murine IL-2 from an attenuated strain of Clostridium sporogenes, a novel delivery vehicle for cancer immunotherapy. Frontiers in microbiology, 12. Retrieved from https://www.frontiersin.org/articles/10.3389/fmicb.2021.669488. https://doi.org/10.3389/fmicb.2021.669488

Kubiak, A. M., & Minton, N. P. (2015). The potential of clostridial spores as therapeutic delivery vehicles in tumour therapy. Research in Microbiology, 166(4), 244-254. Retrieved from http://www.sciencedirect.com/science/article/pii/S0923250814002551. https://doi.org/10.1016/j.resmic.2014.12.006

Kubiak, A. M., Poehlein, A., Budd, P., Kuehne, S. A., Winzer, K., Theys, J., . . . Minton, N. P. (2015). Complete genome sequence of the nonpathogenic soil-dwelling bacterium Clostridium sporogenes strain NCIMB 10696. Genome Announcements, 3(4), e00942-00915. Retrieved from http://genomea.asm.org/content/3/4/e00942-15.abstract. https://doi.org/10.1128/genomeA.00942-15

Lamed, R., & Zeikus, J. G. (1980). Ethanol production by thermophilic bacteria: relationship between fermentation product yields of and catabolic enzyme activities in Clostridium thermocellum and Thermoanaerobium brockii. Journal of Bacteriology, 144(2), 569-578. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC294704/.

Ludwig, W., Schleifer, K., & Whitman, W. B. (2015). Bacilli class. nov. In Bergey's Manual of Systematics of Archaea and Bacteria (pp. 1-1).

Minton, N. P., Mauchline, M. L., Lemmon, M. J., Brehm, J. K., Fox, M., Michael, N. P., . . . Brown, J. M. (1995). Chemotherapeutic tumour targeting using clostridial spores. FEMS Microbiology Reviews, 17(3), 357-364. https://doi.org/10.1111/j.1574-6976.1995.tb00219.x.

Möse, J. R., & Möse, G. (1964). Oncolysis by clostridia. I. Activity of Clostridium butyricum (M-55) and other nonpathogenic clostridia against the Ehrlich carcinoma. Cancer Research, 24(2 Part 1), 212-216. Retrieved from http://cancerres.aacrjournals.org/content/24/2_Part_1/212.abstract.

Mowday, A. M., Dubois, L. J., Kubiak, A. M., Chan-Hyams, J. V. E., Guise, C. P., Ashoorzadeh, A., . . . Patterson, A. V. (2022). Use of an optimised enzyme/prodrug combination for Clostridia directed

enzyme prodrug therapy induces a significant growth delay in necrotic tumours. Cancer Gene Therapy, 29(2), 178-188. https://doi.org/10.1038/s41417-021-00296-7.

Napoli, F., Olivieri, G., Russo, M. E., Marzocchella, A., & Salatino, P. (2010). Butanol production by Clostridium acetobutylicum in a continuous packed bed reactor. Journal of Industrial Microbiology & Biotechnology, 37(6), 603-608. https://doi.org/10.1007/s10295-010-0707-8.

Parker, R. C., Plummer, H. C., Siebenmann, C. O., & Chapman, M. G. (1947). Effect of histolyticus infection and toxin on transplantable mouse tumors. Proceedings of the Society for Experimental Biology and Medicine, 66(2), 461-467. Retrieved from https://journals.sagepub.com/doi/abs/10.3181/00379727-66-16124. https://doi.org/10.3181/00379727-66-16124

Pokrovsky, V. S., Anisimova, N. Y., Davydov, D. Z., Bazhenov, S. V., Bulushova, N. V., Zavilgelsky, G. B., . . . Manukhov, I. V. (2019). Methionine gamma lyase from Clostridium sporogenes increases the anticancer efficacy of doxorubicin on A549 cancer cells in vitro and human cancer xenografts. In R. M. Hoffman (Ed.), Methionine Dependence of Cancer and Aging: Methods and Protocols (pp. 243-261). New York, NY: Springer New York.

Rautio, J., Kumpulainen, H., Heimbach, T., Oliyai, R., Oh, D., Jarvinen, T., & Savolainen, J. (2008). Prodrugs: design and clinical applications. Nat Rev Drug Discov, 7(3), 255-270. https://doi.org/10.1038/nrd2468

Roberts, N. J., Zhang, L., Janku, F., Collins, A., Bai, R. Y., Staedtke, V., . . . Zhou, S. (2014). Intratumoral injection of Clostridium novyi-NT spores induces antitumor responses. Sci Transl Med, 6(249), 249ra111. https://doi.org/10.1126/scitranslmed.3008982

Setlow, P. (2003). Spore germination. Current Opinion in Microbiology, 6(6), 550-556. Retrieved from http://www.sciencedirect.com/science/article/pii/S1369527403001371. https://doi.org/10.1016/j.mib.2003.10.001

Setlow, P. (2007). I will survive: DNA protection in bacterial spores. Trends in Microbiology, 15(4), 172-180. Retrieved from http://www.sciencedirect.com/science/article/pii/S0966842X07000261. https://doi.org/10.1016/j.tim.2007.02.004

Siegel, R. L., Miller, K. D., Wagle, N. S., & Jemal, A. (2023). Cancer statistics, 2023. Ca: A Cancer Journal For Clinicians, 73(1), 17-48. Retrieved from https://acsjournals.onlinelibrary.wiley.com/doi/abs/10.3322/caac.21763. https://doi.org/10.3322/caac.21763

Staedtke, V., Bai, R.-Y., Sun, W., Huang, J., Kibler, K. K., Tyler, B. M., . . . Riggins, G. J. (2015). Clostridium novyi -NT can cause regression of orthotopically implanted glioblastomas in rats. Oncotarget, 6(8). Retrieved from https://www.oncotarget.com/article/3627/text/.

Staedtke, V., Gray-Bethke, T., Liu, G., Liapi, E., Riggins, G. J., & Bai, R. Y. (2022). Neutrophil depletion enhanced the Clostridium novyi-NT therapy in mouse and rabbit tumor models. Neurooncol Adv, 4(1), vdab184. https://doi.org/10.1093/noajnl/vdab184

Tharmalingham, H., & Hoskin, P. (2019). Clinical trials targeting hypoxia. Br J Radiol, 92(1093), 20170966. https://doi.org/10.1259/bjr.20170966

Thiele, E. H., Arison, R. N., & Boxer, G. E. (1964). Oncolysis by clostridia. III. Effects of clostridia and chemotherapeutic agents on rodent tumors. Cancer Research, 24(2 Part 1), 222-233. Retrieved from http://cancerres.aacrjournals.org/content/24/2_Part_1/222.abstract.

Tirandaz, H., Hamedi, J., & Marashi, S.-A. (2006). Application of β-lactamase-dependent prodrugs in clostridial-directed enzyme therapy (CDEPT): A proposal. Medical Hypotheses, 67(4), 998-999. Retrieved from https://www.sciencedirect.com/science/article/pii/S0306987706003409. https://doi.org/10.1016/j.mehy.2006.05.008

Tran, H. G., Desmet, T., Saerens, K., Waegeman, H., Vandekerckhove, S., D'hooghe, M., . . . Soetaert, W. (2012). Biocatalytic production of novel glycolipids with cellodextrin phosphorylase. Bioresource Technology, 115, 84-87. Retrieved from http://www.sciencedirect.com/science/article/pii/S0960852411013733. https://doi.org/10.1016/j.biortech.2011.09.085

Weinmann, M., Belka, C., & Plasswilm, L. (2004). Tumour hypoxia: impact on biology, prognosis and treatment of solid malignant tumours. Oncology Research and Treatment, 27(1), 83-90. Retrieved from http://www.karger.com/DOI/10.1159/000075611. https://doi.org/10.1159/000075611

Xu, H., Luo, H., Zhang, J., Li, K., & Lee, M. H. (2023). Therapeutic potential of Clostridium butyricum anticancer effects in colorectal cancer. Gut Microbes, 15(1), 2186114. https://doi.org/10.1080/19490976.2023.2186114

Zhang, Y. L., Lü, R., Chang, Z. S., Zhang, W. Q., Wang, Q. B., Ding, S. Y., & Zhao, W. (2014). Clostridium sporogenes delivers interleukin-12 to hypoxic tumours, producing antitumour activity without significant toxicity. Lett Appl Microbiol, 59(6), 580-586. https://doi.org/10.1111/lam.12322

Zheng, L., Zhang, Z., Khazaie, K., Saha, S., Lewandowski, R. J., Zhang, G., & Larson, A. C. (2015). MRI-monitored intra-tumoral injection of iron-oxide labeled Clostridium novyi-NT anaerobes in pancreatic carcinoma mouse model. PLoS ONE, 9(12), e116204. https://doi.org/10.1371/journal.pone.0116204.

The study did not involve humans or animals

Conflict of Interest statement

The author declares no conflict of interest.