The potential of clostridial spores as therapeutic delivery vehicles in tumour therapy

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Received 4 September 2014; accepted 15 December 2014

Abstract

Despite substantial investment in prevention, treatment and aftercare, cancer remains a leading cause of death worldwide. More effective and accessible therapies are required. A potential solution is the use of endospore forming Clostridium species, either on their own, or as a tumour delivery vehicle for anti-cancer drugs. This is because intravenously injected spores of these obligate anaerobes can exclusively germinate in the hypoxic/necrotic regions present in solid tumours and nowhere else in the body. Research aimed at exploiting this unique phenomenon in anti-tumour strategies has been ongoing since the early part of the 20th century. Only in the last decade, however, has there been significant progress in the development and refinement of strategies based on spore-mediated tumour colonisation using a range of clostridial species. Much of this progress has been due to advances in genomics and our ability to modify strains using more sophisticated gene tools.

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Keywords: Cancer therapy; Spores; Clostridia; Hypoxia; Prodrug; Prodrug converting enzyme

1. Introduction

Cancer is a broad term for a class of diseases characterised by uncontrolled cell division, leading to abnormal growth. In a normal cell there is a finely regulated balance between all signals promoting or restraining growth. In tumour cells, however, this balance is disrupted. The great proportion of all abnormalities which occur in organisms are triggered either by inherited genetic predispositions, environmental factors or exposure to certain chemicals or harmful conditions which alter the genomic structure and processes [1,2]. According to the World Health Organisation it was reported that for 2012 there were 14 million new cancer cases occurring worldwide, which contributed to around 8.2 million deaths. It is predicted that within the next 15 years the burden of cancer will increase to 23.6 million.

To date, there is no single treatment for cancer. There are different ways to treat cancer, including surgery, radiotherapy, chemotherapy, hormone therapy, and immunotherapy. These approaches are effective in the management of many cases, but it can be difficult to control tumours in some patients, and produce a favourable outcome.

Cancer treatments currently in use suffer from number of limitations. Chemotherapy regimes typically result in unpleasant physical side effects (nausea, impotence), increased risk of developing other types of cancer and mild cognitive impairments [3]. Thus, medical and scientific research investigating new anti-cancer strategies continues.

A particularly innovative approach that is less collateral to patient’ general health and well-being is gene therapy. Although, still nascent, this new group of treatments might bring more targeted (specific) approach to treat a specific cancer type and provide better cure than the standard methods.

The concept of using proteins as anti-cancer agents has led to a multitude of strategies reliant on the use of toxins,
cytokines, immunogens or enzymes. Whilst such agents are invariably highly effective against tumour cell lines, the more exiguous problem remains their selective delivery into the tumour mass. Accordingly, much of the focus of gene therapy research has been devoted to the derivation and testing of tumour delivery vehicles, most notably antibody-based, viral, non-viral or non-biological delivery systems. Their characteristics and specificities, as well as advantages and drawbacks, have been the subject of a number of reviews [4–6]. Nevertheless, it is worth noting that these delivery strategies have created concerns over universality, safety and development costs. Additionally, the low success rate of these delivery vehicles to selectively target the microenvironment of a tumour is a key reason that many studies have been terminated after stages I or II of clinical trials.

2. Targeting tumour hypoxia

It’s estimated that more than 80% of cancers are classified as solid tumours [7]. The majority of these contain large regions of poorly oxygenated tissue, known as hypoxia. These hypoxic tumour tissues occur due to a persistent imbalance between the oxygen being delivered to the tumour through blood microvessels and that being consumed by proliferating cancer cells. In addition, some solid tumours are found to have necrotic (dead) cores, a direct effect of long-standing tissue hypoxia [8,9].

Tumour hypoxia has been demonstrated to reduce the efficacy of many standard cytotoxic drugs used in the treatment of cancer [10]. This is due to ineffective penetration of the drug into the hypoxic mass of the tumour, due to poor vascularisation. A chosen drug concentration may be inadequate for the destruction of the more distant, more hypoxic cells in the interior of the tumour.

Growing evidence suggests hypoxia acts as a prognostic for patient survival and cancer control [11]. The study of Nordsmark et al. produced evidence supporting the idea that tumour hypoxia was linked with a negative prognosis in 397 patients examined with advanced head and neck cancer following primary radiotherapy [12]. Similar findings were found for soft tissue sarcomas and cervix cancers [13,14].

Cancer survival prognosis is strongly related to the development of metastatic disease. It is estimated that around 90% of cancer-related deaths are linked to metastatic spread [15,16]. Hypoxia plays a key role in metastatic progression [11,17]. This is mainly due to chaotic angiogenesis of new blood vessels that alters the tissue microenvironment, the hypoxia-stimulated expression of pro-metastatic genes or hypoxia-triggered mutagenesis of p53 gene [11].

Patients diagnosed and surgically treated for primary tumours with severe hypoxia have a much lower chance of disease-free survival. There is a strong likelihood that metastasis has been initiated, which goes undetected at the time of surgery [13].

The challenge of hypoxia has provided opportunities for novel tumour therapies [11]. The presence of tissue with significantly reduced levels of oxygen and necrosis are unique to solid tumours. This can be exploited as a niche environment for cancer therapies. Certain novel strategies involve the use of hypoxia-activated prodrugs or development of HIF-1 inhibitors. A particularly promising option is the use of anaerobic bacteria. An oxygen-free environment creates ideal conditions for certain microbes, such as Clostridium spp., to colonise and target a necrotic/hypoxic growth. Such a therapy could potentially be used to treat relatively small tumours, preventing further development of hypoxic cancer tissue and the subsequent metastatic progression.

3. A brief historical overview on clostridial oncolysis

3.1. The use of clostridia species as a tool in cancer therapy

The genus Clostridium is one of the largest prokaryotic genera, consisting of more than 130 physiologically diverse Gram-positive bacterial species. They are obligate, rod-shaped anaerobes and, in common with the aerobic genus Bacillus, produce endospores in order to survive adversity [18].

As a grouping, Clostridium is, in the main, composed of entirely benign, non-pathogenic species that are widely distributed in the environment. Nonetheless, they are best known for the pathogenic properties of a handful of species. It is the attributes of the non-pathogenic members, however, which may ultimately be of most significance to mankind [19]. In particular, it is the ability of clostridial endospores to infiltrate, and hence selectively germinate in, the hypoxic regions of solid tumours that may have the greatest impact [20,21]. The history of the application of clostridial spores in cancer therapies has been broadly reviewed in a number of scientific publications [22–26]. Some of the most crucial findings, which laid the foundation for the most recent advances in clostridial tumour therapy, are briefly outlined in Box 1.

To avoid the use of pathogenic species, such as Clostridium histolyticum and Clostridium tetani, the use of Clostridium butyricum M-55 was investigated by Mö and Mössé in 1959. Significant oncolytic effects were observed after injecting spores into animals and later into human patients [27–29]. Thanks to these properties, the strain was named Clostridium oncolyticum and later reclassified as Clostridium sporogenes M-55 (ATCC 13732). Saccharolytic clostridia such as Clostridium beijerinckii or Clostridium acetobutylicum were also investigated and proved to be effective in tumour colonisation. However, compared to C. sporogenes the reproducibility of colonisation was inferior. Moreover, the total number of vegetative cells present in a tumour was orders of magnitude lower than C. sporogenes [30]. The initial benefits of a more aggressive coloniser have additionally been shown in a study that utilised a non-toxinogenic strain of Clostridium novyi-NT. The superior colonisation abilities of C. sporogenes and C. novyi-NT are likely a consequence of them being proteolytic species.
Box 1. The historical background of the use of Clostridium spp in cancer therapies.

The concept of using Clostridium species as tumour vectors was preceded by a number of experimental and clinical observations. As early as 1813, patients diagnosed with cancer were reported to unexpectedly defeat the illness after suffering from gangrene — infection caused by C. perfringens [82,83]. Later experiments sought to deliberately make use of clostridia in treating cancer. In 1935, Connell injected sterile filtrates of C. histolyticum containing proteolytic enzymes directly into tumour tissues as a means of causing tumour destruction [84]. Thereafter, Parker and colleagues pursued the concept that injection of C. histolyticum spores into the transplanted sarcomas of mice results could cause tumour regression. These studies resulted in noticeable lysis of tumour tissues, and represented the first time that whole Clostridium organisms were used to bring about tumour regression [85].

The most compelling evidence for the effectiveness and selectiveness of this strategy for targeting tumours was provided by a study using C. tetani. Spores were injected intravenously as opposed to directly into the tumour mass. The tumour bearing animals died (within 48 h) as a consequence of the production of lethal tetanus toxin by the actively growing vegetative cells in the colonised tumours. In contrast, healthy animals were unaffected by the injection of C. tetani spores, as they were unable to germinate and grow [86].

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3.2. Clostridia and Directed Enzyme Prodrug Therapy – CDEPT

As described in the previous section, whilst the use of the spores of unadulterated Clostridium strains showed great promise in controlling tumour growth, it was soon realised that more substantive effects were likely to accrue if combined with some other form of noxia. One particularly promising approach was to combine their use with that of prodrug-drug combinations.

An ideal cancer therapy should only target tumour cells, leaving healthy tissues unaffected. This can be achieved by using a harmless circulatory prodrug (pro-agent) that is converted to a highly toxic drug only within the tumour vicinity through the activity of a prodrug converting enzyme (PCE) that is specifically localised to the tumour. Such a strategy has been termed Directed Enzyme Prodrug Therapy (DEPT). It provides a simple practical solution to the issue of selectively exposing cancer cells to effective doses of drug that patients can tolerate [31,32].

DEPT research, to date, has focused on the discovery and formulation of effective prodrug-drug combinations and the PCEs that catalyse their conversion. One notable prodrug is CB1954 [5-(aziridin-1-yl)-2,4-dinitrobenzamide] which was found to inhibit the growth of a Walker rat carcinoma 256 cell line, yet was inactive against other, and in particular human, tumour cells [33]. It transpired that Walker 256 tumour cells produced a unique DT-diaphorase activity, not present in the other rodent and human cell lines tested, that activated CB1954 by reduction of its auxochromic nitro groups. The hydroxylamine produced is cancerostatic because it promotes the formation of interstrand C8-O6 DNA cross-links, which are ineffectively repaired by the cell [34,35]. The rat ‘DT-diaphorase’ is systematically named NQO1 (NAD(P)H:quinone oxidoreductase). Since its discovery, a number of bacterial enzymes able to activate CB1954 have also been discovered and characterised [36], including a NAD(P)H-dependent quinone reductases from Escherichia coli (NfN) and Bacillus amyloliquefaciens (YwrO). One of the most important features of DEPT strategies prodrug is the high therapeutic dose of the converted drug, achieved exclusively within the vicinity of the tumour. Each enzymatic molecule can generate large quantities of toxic derivative. In addition, neighbouring cells not exposed to the drug can display a similar response, a phenomenon called ‘bystander effect’ [37,38].

The discovery, isolation and subsequent improvements of other PCEs continue to be pursued in parallel with the application of the most suitable delivery systems. Those originally employed were based either on the fusion of the PCE to a tumour specific antibody (Antibody-Directed Prodrug Therapy, ADEPT) or on the delivery of the gene encoding the PCE using viral or non-viral vectors (Gene-Directed Enzyme Prodrug Therapy, GDEPT). The concept of using clostridia (‘Clostridial-Directed Enzyme Prodrug Therapy, CDEPT’) is a more recent addition (Fig. 1) to the DEPT arsenal [39,40]. A summary of the studies published on engineered clostridial
strains, deployment of a prodrug and their most relevant findings until 2003 are presented in Table 1. Here we review progress since this date.

4. The last decade of research on clostridia in cancer therapy

4.1. The search for better prodrugs

Although its potential as a therapeutic drug has been demonstrated in multiple preclinical studies [41–43], CB1954 prodrug has a number of significant limitations that pose questions about its use in CDEPT. Initial clinical toxicity studies revealed dose-limiting diarrhea and liver toxicity in examined cancer patients. Although the maximum safe dose was determined as 24 mg/m², some patients still suffered from mild diarrhea, severe nausea and mild reversible transaminase elevation [44]. A study on CB1954 activation reported the prodrug could be metabolised by human hepatic enzymes. The presence of 2- and 4-hydroxylamines (products of reduced CB1954) was detected in all three human liver preparations examined in this study. Significant CB1954 metabolism was observed under atmospheric levels of oxygen, but was inhibited under hyperoxic conditions [45]. This suggests a risk of clinical hepatotoxicity. Accordingly, this raises serious concerns around the safe use of CB1954 in therapeutic treatment. As a result, a lot of effort has been put into the design and development of more effective equivalents of CB1954. A promising example is the DNA cross-linking agent PR-104 [46,47]. This phosphate ester is rapidly converted in vivo to its cognate alcohol, PR-104A — bioreductive dinitrobenzamide mustard. In the study by Patterson et al., the anti-tumour effect of CDEPT was enhanced by the use of this new compound, in conjunction with increased expression of nitroreductase enzyme (NTR). New codon optimised version of an E. coli-derived nitroreductase (sNTR) was tested, both in vivo and in vitro, in combination with CB1954 and a novel PR-104. The in vivo study confirmed 20–30 fold higher activity of over-expressed E. coli-NTR with respect to its original version. In vivo data demonstrated that sNTR, CB1954 or PR-104 alone resulted in a statistically insignificant delay in tumour growth. When sNTR was combined either with a single dose of CB1954 (50 mg/kg) or with three doses of PR-104 (250 mg/kg) over a 3-week period, much greater anti-tumour activity was observed for both prodrug/enzyme treatments. A prolonged period of tumour growth delay (>13 days) was observed [46].

Although it was discovered that PR-104 could also be converted to its toxic metabolites under oxygenated conditions, the experimental evidence suggests that it offers a promising alternative to CB1954 in CDEPT approaches [48]. Through microarray analysis of human neoplastic cell lines, Patterson et al. identified a functional aldo-keto reductase, 1C3 (AKR1C3) that catalysed the reduction of PR-104A. The possibility of oxygen-based activation and the possibility of associated risks should be more extensively studied. Nevertheless, the new prodrug and AKR1C3 have also other clinical potentials. Most recently, PR-104 was applied in a phase Ib study of patients with advanced solid tumours, and is now in an ongoing phase II clinical trial, aiming to treat patients with refractory/relapsed acute leukemia (clinical trial information NCT01037556) [49]. Although, the expression of AKR1C3 could also be detected in normal tissues, its elevated level in cancerous environments supports the idea of using AKR1C3 as a marker for detecting PR-104-sensitive tumours.

More recently, a new alternative prodrug, SN 27686, was tested for activity on HCT116 cell lines, engineered to express E. coli NTR enzyme [50]. The in vitro results demonstrated that SN 27686 was 4- to 21-fold more selective than CB1954 in tissue-like cell densities. Moreover, the novel prodrug exhibited much higher bystander killing efficiency (48% over only 13% for CB1954) [50]. This study indicates that SN 27686 possesses superior properties compared to CB1954, and should be tested in CDEPT.

4.2. Towards the discovery of new prodrug converting enzymes (PCEs)

Until 2006, the most studied nitroreductase for CB1954 prodrug activation was the E. coli NfnB (or EcoNTR) enzyme. E. coli genes tend to be poorly expressed in clostridial hosts due to differences in codon usage. Thus, in addition to the use of more effective promoters, the codon optimisation of the nfnB gene to suit the clostridial host has been shown to result in improved expression and consequent increases in CB1954 conversion rates [46]. Other approaches to improve NfnB involved altering its catalytic activity against prodrugs through alterations to specific amino acid residues at the active site using site-directed mutagenesis [51,52].
Table 1
A summary of genetically modified clostridial strains employed in anti-tumour studies until 2003.

<table>
<thead>
<tr>
<th>Clostridium spp.</th>
<th>Enzyme</th>
<th>Prodrug/Drug</th>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. beijerinckii NCIMB 8052</td>
<td>Dihydropteridine reductase (nfnB)</td>
<td>CB1954</td>
<td>In vitro: 22-fold increase in tumour cell eradication; In vivo: 10/10 EMT6 tumour bearing mice were colonised with detectable nitroreductase activity;</td>
<td>Lemmon 1997</td>
</tr>
<tr>
<td></td>
<td>Cytosine deaminase (codA)</td>
<td>5-fluorouracil (5-FU) converted to 5-fluorouracil (5-FU)</td>
<td>In vivo: no anti-tumour effect</td>
<td>Fox 1996</td>
</tr>
<tr>
<td>C. acetobutylicum strains (DSM792 or N4082)</td>
<td>Mouse tumour necrosis factor alpha, mTNFz</td>
<td>None Combretastatin A-4</td>
<td>Significant levels of biologically active mTNFz were measured in both lysates and supernatants. Improved colonisation in male WAG/Rij rats bearing smaller than 1 cm³ tumours</td>
<td>Theys 1999</td>
</tr>
<tr>
<td></td>
<td>Cytosine deaminase (codA)</td>
<td>5-fluorouracil (5-FU) converted to 5-fluorouracil (5-FU)</td>
<td>In vitro: The presence of CD gene was confirmed and 5-FU conversion detected</td>
<td>Theys 2001a</td>
</tr>
<tr>
<td></td>
<td>Cytosine deaminase (codA) with vascular targeting agent</td>
<td>Combretap</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dihydropteridine reductase (nfnB)</td>
<td>CB1954 reduced to 4-hydroxylamine (4-HX) derivative of CB1954 with a kcat value of 4.3. The first study of repeated CDEPT cycles</td>
<td>Liu 2002</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C. sporogenes NCIMB 10696</td>
<td></td>
<td>Significant anti-tumour effect in SCCVII-transplanted tumours in C3H/Km mice</td>
<td>Wilson 2002</td>
</tr>
<tr>
<td></td>
<td>Cytosine deaminase (codA)</td>
<td>5-fluorocytosine (5-FC) converted to 5-fluorocytosine (5-FC)</td>
<td>Curative activity against WiDr tumours in multi-layered tissue culture model</td>
<td>Liu 2003</td>
</tr>
<tr>
<td></td>
<td>Dihydropteridine reductase (nfnB)</td>
<td>CB1954 with vascular targeting agent</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>SN24927</td>
<td>Expression of nfnB in tumour confirmed with significant anti-tumour efficacy;</td>
<td></td>
</tr>
<tr>
<td>C. novyi-NT</td>
<td>None</td>
<td>Dolastatin-10 and Combretastatin A-4</td>
<td>COBALT approach resulted in significant tumour regression and cure in 4 out of 8 HTC116 xenografts</td>
<td>Dang 2001</td>
</tr>
</tbody>
</table>

* In all cases where the gene in brackets was introduced, it was localised to an autonomous plasmid.

In an alternative approach, screening of bacterial genomes led to the isolation of nitroreductases with a greater affinity for CB1954 [39].

Out of fifteen proteins, two novel candidates (HinNTR isolated from Haemophilus influenzae and NmeNTR from Neisseria meningitides), which showed the best kinetic properties, were selected. HinNTR was found to produce only significant amounts of CB1954. Prodrug conversion translates a significantly high level (1.067 × 10³ s⁻¹) and a K_mCB1954 was found to be nearly 30-times lower (30 μM) than NfnB from E. coli. The catalytic reaction of this nitroreductase with CB1954 resulted in production of the desired 4-HX product only. Additionally, the enzyme was stable for more than 6 h at temperatures up to 50 °C, which may suggest extended activity when used in targeted anti-cancer therapy.

4.3. The first study of repeated CDEPT cycles

The gene encoding HinNTR, named NTR-H in Theys study, was codon optimised for Clostridium, cloned into an expression vector and successfully conjugated into C. sporogenes [38]. The in vivo experiments showed good, tumour-specific germination and colonisation following recombinant spore administration. Following administration of CB1954 tumour growth rates were reduced to nearly static levels in nude NMRI mice harbouring HCT116 tumours. This result clearly demonstrates that spores of C. sporogenes could deliver enough novel NTR-H enzyme to the tumour to convert significant amounts of CB1954. Prodrug conversion translates...
directly into observable anti-tumour efficacy. Additionally, recombinant *C. sporogenes* was tested in multiple consecutive treatment cycles, interspersed with antibiotic-mediated bacterial clearance. This strategy resulted in prolonged and significant anti-tumour effects when combined with CB1954 administration. The tumour only doubled in volume over the course of a 70-day treatment, whereas the HCT116 control doubled every 7 days. This study indicates that repeated cycles of CDEPT can be administered which bodes well for future clinical use. However, experiments involving immune-competent animals are required to confirm its real-world efficacy [38].

### 4.4. The integration of PCE genes into the clostridial genome

Until recently, the therapeutic genes of all the recombinant clostridial strains tested have resided on autonomous plasmids [38,55]. In the study of Heap et al., however, the therapeutic gene used was stably integrated into the genome using Allele-Coupled Exchange, or ACE [56]. This method is based on a two-step recombination process during which the plasmid vehicle and any encoded antibiotic resistance gene is eliminated from the cell. Once integrated, the therapeutic gene is stably maintained, minimising any risk both of its loss or of its dissemination by horizontal gene transfer [56].

In the most recent CDEPT study, genes encoding a novel NmeNTR enzyme and the previously studied NfnB were codon optimised and integrated into the *C. sporogenes* NCIMB 10696 genome at the pyrE locus using ACE [57]. pyrE encodes the pyrimidine biosynthesis enzyme, orotate phosphoribosyltransferase. During the recombination event, cells with inactivated pyrE were selected on growth medium supplemented with 5-fluoroorotic acid. This compound is highly toxic to cells which contain this pathway, but is harmless to cells without a functional pyrE. As a result, the integrated strains were uracil auxotrophs.

*In vitro* experiments concluded that, aside from uracil auxotrophy, all integrated NTR strains and the pyrE-negative control strain behaved identically to the parent strain. Thus, integration of the *ntr* gene had no impact on growth rate in uracil deplete media, on spore formation or germination. It follows that their potential use as tumour delivery vehicles has not been in any way compromised by these genetic modifications. An *in vivo* study demonstrated that inactivation of the pyrE gene did not affect the tumour colonising capacity of systemically administered spores. Indeed, wild-type and pyrE negative *C. sporogenes* colonised HCT116 xenografted tumours equally well. On average $10^8$–$10^9$ CFU were found per gram of tumour tissue. As expected, *C. sporogenes* vegetative cells were only present in tumours, and not in any other tissues that were examined. Tumour growth data demonstrated that NfnB- and NmeNTR-*C. sporogenes* integrants, in combination with CB1954, had anti-tumour effects. The most promising effect was observed with the strain producing NmeNTR. It was estimated that the tumour growth rate remained static over the first 25 days of observation, and in the following days, minimised to half of its initial size. After day 40, tumour regrowth was observed [57]. This could be due to multiple reasons, such as vegetative cell death, insufficient prodrug concentration or poor enzyme expression.

### 5. The overview on the use of *Clostridium* spp. In other anti-tumour strategies

In addition to CDEPT, other strategies are being explored which combine the use of clostridial spores with various different therapeutic options.

#### 5.1. Clostridium novyi-NT

In an alternative approach to CDEPT, Dang et al. (2001) proposed the use of an attenuated strain of *C. novyi-NT* in combination with conventional chemotherapeutical drugs for the treatment of cancer (termed COBALT) [58]. The experimental animals in their study suffered severe systemic toxicity, and not all tumour types were found to be equally susceptible. Subsequently, new approaches have been explored. In 2004, the same research group studied the application of microtubule-interacting chemotherapeutics alongside *C. novyi-NT* spore administration [59]. Following simultaneous treatment with bacterial spores and a novel anti-microtubule destabiliser (HTI-286), which decreases the blood flow, an enhanced haemorrhagic necrosis in all HCT116 xenografts was observed. The long-term effects of the same therapy tested on a range of different tumours were much less significant, indicating strong tumour-type specificity. In the same study, administration of two microtubule-stabilising drugs (docetaxel and MAC-321) in combination with *C. novyi-NT* resulted in gradual tumour regression. Most significantly, complete cure of some experimental animals was achieved with tumours measuring 100–250 mm$^3$, and in others, the duration of cancer remission was improved [59]. The apparent curing of 4 out of 5 HCT116-bearing animals was also recorded in other experiments on microtubule-stabilising activity alongside *C. novyi-NT* treatment [60].

The use of *C. novyi-NT* spores has also been shown to enhance the therapeutic effects when combined with radiotherapy. As oxygen deprived cells are found to be more resistant to radiation therapies, the use of anaerobic spore treatment was thought to increase the tissue sensitivity to radiation. In 2003, Betegowda et al., conducted a study on three major modes of radiation therapies: external beam, brachytherapy and radio-immunotherapy (RAIT), all combined with the *C. novyi-NT* spore treatment [61]. The overall conclusion of this work was that spores of *C. novyi-NT* demonstrated an additive effect on tumour growth inhibition. For brachytherapy specifically, only a single dose of spores resulted in 100% cure of HCT116-bearing mice and HuCC-T1 xenografts. In addition, the administration of spores allowed the use of lower doses of radiolabeled antibodies, resulting in a reduced toxic effect on healthy tissues [61].

In 2006, the use of *C. novyi-NT* to aid the release of liposomal drugs inside the tumour was explored [62]. This
species is known to lyse red blood cells and disrupt membranes. It was hypothesised that germinated spores may assist the release of liposome-encapsulated drugs at the tumour site. This would result in an enhanced therapeutic effect. In their study, Cheong and co-workers administered a single dose of liposome-encased doxorubicin to tumour-bearing mice in conjunction with *C. novyi-NT* spores. Tumour regression occurred in all treated mice and 65% survived for 90 days [62].

The first experiment utilising *C. novyi-NT* and *C. sporogenes* spores in combination with antibody treatment marked the beginning of ‘*Clostridium*-directed antibody therapy’ (CDAT). In 2007, Groot et al. decided to focus on hypoxia-inducible factor-1-alpha (HIF-1α), the activity of which is associated with resistance to radio- and chemotherapy [63]. In this study, *Clostridium* transconjugants were generated that harboured plasmid with single chain 15 kDa VHH antibody (specific towards HIF antigen). The *in vitro* experiment confirmed that antibodies isolated from clostridia retained their binding capacity and specificity towards the antigen. This study demonstrates an early stage of the CDAT approach, and it unveils new potential in combining antibody and clostridial-based therapies.

More recently, *C. novyi-NT* spore treatment has been evaluated in companion animals (dogs) with naturally occurring tumours and, in a second study, in 16 dogs and one human patient with an advanced and metastatised leiomyosarcoma (clinical trial identifier NCT01924689) [64,65]. In both studies, a severe but manageable toxicosis and abscesses in some treated canines were recorded. The authors report this was most likely a consequence of an immune response or hypersensitivity to the spores [64]. Crucially, the effectiveness of clostridial spores in colonising naturally occurring tumours was confirmed. During the phase 1 investigational study on the human patient, the intra-tumourally administered treatment resulted in a significant reduction at the tumour side and surrounding of the bone. Unfortunately an adverse effect was encountered, and the patient had to be treated for pathological fracture of the right proximal humerus [65]. Such a case demonstrates the potential challenges in *C. novyi-NT*-based therapy, and that the efficacy and safety of this treatment needs further attention.

### 5.2. *Clostridium perfringens*

Although normally associated with a food poisoning and gas gangrene, *C. perfringens* is also able to selectively colonise hypoxic tumours. Poorly vascularised hypoxic tissues of pancreatic cancers have been put through an investigation involving administration of *C. perfringens* spores [66]. In a study by Li et al., *C. perfringens* strain was genetically modified to eliminate a gene associated with oxygen tolerance — superoxide dismutase. Further, it was recombined to express Panton-Valentine Leukocidin (PVL) inflammation suppressive gene [66]. It was hypothesised that the replication, and therefore treatment efficacy of *C. perfringens*, can be enhanced in tumour-bearing, immune-competent animals when their activity is suppressed. Indeed, the treatment of mice with *C. perfringens* expressing the PVL cytotoxin resulted in reduced inflammation, enhanced tumour necrosis and prolonged survival (up to 120 days in 47% of experimental animals). However, experiments were only designed around one type of cancer and, unfortunately, in nearly half of the animals, tumours occurred, causing death. Nevertheless, the primary results are highly encouraging, and reveal a potential for combining this treatment with more standard approaches, such as chemotherapy.

In several types of cancers, such as breast, ovarian or pancreatic cancer, the expression of specific trans-membrane proteins, called claudins (such as Cldn3 and Cldn4), was found to be elevated. Their up-regulation is also correlated with advanced stages and recurrence of prostate tumours. Therefore, a novel approach of employing *C. perfringens* enterotoxin (CPE) to bind to these proteins was explored. The general mechanism of this selective gene therapy relies on CPE binding to Cldn3 and Cldn4 receptors, resulting in pore formation in epithelial cell membranes. The subsequent influx of Ca²⁺ into the cell through these pores leads to cell death. In the study by Maeda et al. (2012), Cldn3 and Cldn4 were expressed in three primary human prostate cancers and normal prostate tissues (PrEC) [67]. When CPE-mediated therapy was employed, cytotoxic effects were found to be significant in cancer cell lines, but only marginal in PrEC. Similar effects were observed in the study of Walther and co-workers, which utilised vector-based optimised CPE (optCPE) without employing bacteria as a vector [68]. Additionally, an *in vivo* experiment with this non-viral intra-tumoural gene transfer of optCPE resulted in reduced tumour growth in breast and colon cancer-bearing animals.

The targeted cytotoxicity of CPE observed in breast, colon, prostate, pancreatic and ovarian cancer studies demonstrates its positive potential [69–71]. Nevertheless, a lack of indiscriminate toxicity and long-term efficacy should to be demonstrated *in vivo* before considering *C. perfringens* therapy for clinical use. As Cldn3 and 4 are also found in some normal tissues, experiments on non-cancerous samples, as well as the feasibility of gene transfer, need to be examined in greater detail. Moreover, it is widely accepted that CPE is a major virulence factor, associated with common bacterial food poisoning caused by the *C. perfringens* type A strain, as well as other gastrointestinal diseases [72]. Although people can recover from poisoning within 24 h, for some patients the illness can be fatal. The finding in one study that 18% of screened healthy food handlers carry *C. perfringens* suggests subclinical infection is common [73]. As a result, carriage of CPE antibody could be relatively high in the general public. This may cause an undesirable immune response to be elicited in potential cancer patients treated with the CPE therapy. While non-pathogenic options exist, treatment regimes using *C. perfringens* are unlikely to be preferred. The use of any microbe in a clinical setting is controversial and, therefore, the use of a strain or its toxin that have been established as pathogenic would likely face substantial resistance.
5.3. **Clostridium acetobutylicum**

In the pursuit of a clostridia-mediated cancer treatment, saccharolytic strains of clostridia have also been investigated. Strains of *C. beijerinckii*, *C. acetobutylicum* and *Clostridium saccharoperbutylicum* were used, not only to express PCEs but also other molecules with cytotoxic activity against tumours. In the study by Theys et al. (1999), a non-pathogenic *C. acetobutylicum*, strain DSM792, was used initially to secret murine tumour necrosis factor (mTNF-α) [74]. This is a cytokine known to stimulate accumulation of macrophages, neutrophiles and T cells, and therefore indirectly triggers anti-tumour responses. However, injection of recombinant *C. acetobutylicum* producing mTNF-α did not result in a significant delay of tumour growth. Another cytokine, rat-derived interleukin-2 (IL-2), was tested and shown to have a much greater effect on tumour regression. This was studied in work of Barbe et al. in 2005 [75]. The plasmid-based rIL-2 was expressed by *C. acetobutylicum* and its biological functionality was confirmed. When spores of the therapeutic strain were intra-tumourally administered to rhabdomyoascomarc-bearing rats in combination with fractionated radiotherapy, significant tumour regression was observed. This was due to the increased activity of immune cells. The rIL-2 injected on its own, as a purified protein solution, did not have the same effect, suggesting the importance of supplying the cytokine in a clostridial vehicle [75].

Despite the efforts of employing saccharolytic clostridia, it is believed that the use of more effective colonisers, such as *C. sporogenes*, would be more advantageous in cytokine-based approaches. The verification of interleukin activity, once integrated into the bacterial chromosome, would bring further confirmation of the feasibility of these strategies.

### 6. Imaging tumour-localised clostridia and other bacteria

Evaluation of clostridial colonisation and distribution within the tumour mass would benefit from an easily accessible and non-invasive imaging methodology. In the past decade, a number of options have been explored to visualise colonisation in tumour-bearing hosts [76–78]. Although the studies conducted focused on bacteria such as *Salmonella typhimurium* and *E. coli*, their findings could potentially be adapted to clostridial-based strategies.

Genes that encode light-emitting fluorescent proteins or luciferases have been employed to detect bacteria injected into experimental animals. In the study by Yu et al., a lux-GFP fusion complex was expressed in three attenuated pathogens (*S. typhimurium, Listeria monocytogenes, Vibrio cholerae*) and *E. coli* [79]. Each bacterium were injected separately into C6 glioma-bearing, breast tumour nude mice and monitored for movements from the injection site to the tumours. It was found that all bacteria entered and replicated in primary as well as metastasised tumours. In addition, further experiments have shown that immune-competent animals were colonised equally well [79]. This real-time imaging could potentially be applied to a clostridial host, providing the in-host functionality of light-emitting proteins. As such, it has been suggested that oxygen-independent fluorescent molecules could potentially offer a solution to visual tracking of clostridial colonisers [80].

Another strategy involves the application of nuclear imaging techniques, such as positron emission tomography (PET) or single-photon emission computed tomography (SPECT). Liu et al. investigated whether endogenous bacterial enzymes, such as thymidine kinase (TK), could react with radio-labelled substrates to produce a radio-detectable reaction [81]. In this work, *C. novyi-NT* spores (amongst other bacteria) were used to colonise CT-26 colon tumour-bearing mice. After 24 h, radio labelled 1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl)-5-iodouracil (FIAU) was administered via a tail vein, and imaged at various time points using SPECT. The overall results confirmed the uptake of the [125I]FIAU probe inside the tumour. Uptake was observed as early as 16 h post-injection, peaking at 24–48 h. No uptake was detected in animals not treated with *C. novyi-NT*. Similar results were confirmed in tumour-bearing rabbits in which the uptake of probe was only recorded alongside tumour-limited bacterial germination using SPECT/CT cameras. These findings suggest that the presence of endogenous TKs in clostridial species can offer a solution to nuclear imaging techniques. More recently, the utilisation of a chemical exchange saturation transfer (CEST) MRI method for imaging intra-tumoural bacterial colonisation was demonstrated. This method relies on the chemical exchange between an exchangeable proton and its surrounding water protons, in order to transfer the selectively modified NMR information. Although the studies conducted focused on bacterial enzymes, such as thymidine kinase (TK), could react with radio-labelled substrates to produce a radio-detectable reaction [81]. At first, the endogenous CEST contrast of vegetative *C. novyi-NT* was assessed in vitro and compared to the spore profile. It was shown that spores produced no detectable CEST signal. This is likely to be a consequence of the low water content in the spore, and the thick coat surrounding, as well as a lack of metabolic activity. Further experiments monitored the CEST signal before and after systemic spore injection in colon tumour-bearing mice. The results observed confirmed visible CEST changes relating to bacterial infiltration, in contrast to images of pre-injected tumours. In addition, a preliminary assessment was made of the influence of a number of different factors and it was concluded that the profile of CEST contrast wasn’t significantly altered by stimulated changes in pH, local inflammation or necrosis. These findings illustrate that bacCEST imaging can be successfully employed in clostridial-based therapies.

### 7. Conclusions

It is now over eighty years since the concept of using clostridia to treat cancer emerged, but it is only in the last decade that meaningful progress towards this goal has been made. Much of this headway has been driven by the development of more sophisticated gene technologies with which...
the genotype of the Clostridium can be altered to display the required therapeutic attribute. The vast majority of engineered strains used to date in proof of principle studies have, however, used strains endowed with autonomous plasmids which carry antibiotic resistance genes. It follows that the engineered traits are segregationally unstable. Moreover, the use of plasmids is unacceptable from a regulatory perspective. The required modifications, therefore, need to be stably introduced into the bacterial genome. This has now been made possible by the development of Allele-Coupled Exchange (ACE) technology [56], a procedure capable of introducing DNA of any size or complexity into clostridial genomes. Indeed, ACE was used to create a CDEPT strain that was used in a mouse xenograft model of human colon carcinoma to achieve substantial tumour suppression, and in some cases cure [57]. Following these encouraging data and the creation of clinically relevant strains, it is anticipated that CDEPT will shortly enter clinical trials. In the meantime the encouraging results of the studies with C. novyi-NT spore treatment [64,65], underlines the fact that clostridia species seem destined to have a significant role to play in the future treatment of solid tumours.

Conflict of interest

None.

References


