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BACTERIA-MEDIATED ANTI-CANCER THERAPY

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A thesis submitted to the
Nanyang Technological University, Singapore,
in partial fulfillment of the requirement for the degree of
Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

6 March 2019

Date

Madhura Satish Bhawe

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

6 March 2019

Date

A handwritten signature in black ink, appearing to read 'J.H. Teoh', with a stylized flourish extending from the end.

Prof Teoh Swee Hin

Authorship Attribution

Statement

This thesis contains material from 1 paper published in the following peer-reviewed journal, in which I am listed as an author.

Chapters 4 and 5 are published as M. S. Bhavé, A. M. Hassanbhai, P. Anand, K. Q. Luo, and S. H. Teoh, Effect of Heat-Inactivated *Clostridium sporogenes* and Its Conditioned Media on 3-Dimensional Colorectal Cancer Cell Models,” *Scientific reports*, vol. 5, 2015.

The contributions of the co-authors are as follows:

- I, M. S. Bhavé, designed and performed the experiments, analysed the results, and wrote the manuscript.
- Dr A. M. Hassanbhai analysed the results, and edited the manuscript.
- Dr P. Anand designed the experiments and analyzed the data.
- A/Prof K. Q. Luo and Prof S. H. Teoh supervised the project.

6 March 2019

Date

Madhura Satish Bhavé

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Abstract

Traditional cancer treatments like chemotherapy and radiation therapy continue to have limited efficacy due to phenomena like tumor hypoxia and multi-drug resistance. Bacterial cancer therapy has the potential to overcome these problems, using anaerobic spores of bacteria such as the proteolytic *Clostridium sporogenes*. However, the use of spores or live bacteria comes with the risks of toxicity, infection and tumor recurrence. This thesis is motivated by the research question, “How can bacterial therapy be made safer while retaining the natural anti-cancer properties of bacteria?”. To address this question, heat-inactivated bacteria (IB) and conditioned medium (CM) with the secreted products of *C. sporogenes* were generated to minimize the safety and efficacy risks of live bacteria. These derivatives of *C. sporogenes*, which cannot replicate or metabolise, were tested for their anti-cancer properties for the first time. It was hypothesized that these non-viable derivatives of *C. sporogenes* can exert a direct anti-cancer effect and elicit an immune response against tumors.

The direct anti-cancer effect of IB and CM was investigated using a range of human cancer cells of various tissue origin: colorectal (HCT116), breast (MCF7), renal (A498), hepatic (HepG2), bladder (RT4) and melanoma (G361). These *in vitro* studies were conducted on 2-Dimensional (2D) monolayer and 3-Dimensional

(3D) spheroid culture models. The results showed that IB and CM have a selective anti-cancer effect on the different cell lines. Colorectal cancer cells were found to be the susceptible to IB and CM treatment in both *in vitro* models, resulting in significant decreases in cell proliferation and spheroid size.

Subsequent studies were conducted on colorectal cancer cell lines HCT116 (human) and CT26 (murine) to understand the nature of IB- and CM- mediated inhibition. The inhibition of HCT116 and CT26 cells when kept in cell culture-conditions showed that the anti-cancer efficacy of IB and CM was hypoxia-independent. This would minimize the risk of tumor recurrence associated with hypoxia-dependent spores and live anaerobic bacteria. Scanning electron microscopy imaging showed that the IB adherent to the surface of cancer cells and restricted-culture studies proved that physical contact between IB and cancer cells was crucial for inhibition. IB were found to mediate necrosis in colorectal cancer cells. On the other hand, CM was found to mediate apoptosis in the cancer cells. To understanding the mechanism of CM-mediated cancer inhibition, the active component of CM was examined. Size fractionation of CM showed that the fraction of size smaller than 3 kDa in molecular weight retained the inhibitive of CM. The findings suggested that IB and CM have intrinsically different mechanisms of cancer inhibition.

After successfully inhibiting the growth of CT26 and HCT116 colorectal cancer cells *in vitro*, the efficacy of IB and CM was tested in mouse models of colorectal tumors. Sub-cutaneous tumors of CT26 and HCT116 cells were established in BALB/cARC and athymic BALB/c-Foxn1^{nu}/ARC (nude) mice, respectively. IB and CM were administered through intra-tumoral injections over a period of 12 days, during which the tumor volume was measured. CM had limited efficacy in decreasing the tumor-burden of the mice and did not elicit an immune response. However, IB significantly inhibited the growth of the tumors and improved the survival rate

of immunocompetent CT26 tumor-bearing mice. Histological analysis showed that IB increased the necrotic area in HCT116 tumors. Immunohistochemical analysis of the tumor samples showed that IB elicited an immune response against the tumor cells. Notably, when the IB-treated mice were subsequently re-challenged with CT26 cells, the immune mediated-response prevented the establishment of a second tumor in the mice. Taken together, the results show *in vivo* efficacy of the non-viable derivatives of *C. sporogenes*.

In summary, the work of this thesis demonstrates the potential of IB and CM of *C. sporogenes* as anti-cancer agents which retain the natural anti-cancer properties of the bacteria and advantages over conventional therapy, while minimizing the safety risks.

List of Publications

Journal Publications

1. Madhura Satish Bhave, Patricia Lynn Conway, Juliana Marie Chan, Stephen Danon, Teh Bin Tean, Sven Pettersson, Swee Hin Teoh , "Inactivated Bacteria Mediate Anti-Tumor Immune Response Against Colorectal Cancer," *manuscript under review*
2. Madhura Satish Bhave, Avneet Kaur, Swee Hin Teoh, "Screening of Anti-Cancer Efficacy of Clostridial Derivatives Against Human Cancer Cell Lines," *manuscript in preparation*
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Scientific Conferences

1. Madhura Satish Bhave, "Bacterial Cancer Therapy: Anti-Tumor and Immunogenic Effects," BES 12th Scientific Meeting, 19 May 2018, Singapore. (Oral Presentation) **Gold Award**
 2. Madhura Satish Bhave, Patricia Lynn Conway, Juliana Marie Chan, and Swee Hin Teoh, "Bacteria-mediated cell death of colorectal tumors in mouse xenograft models," 4th EACR Conference, A Matter of Life and Death: From Basic Cell Death Mechanisms to Novel Cancer Treatments, 1 - 3 Feb 2018, Amsterdam, Netherlands. (Poster Presentation)
 3. Madhura Satish Bhave, Patricia Lynn Conway, Swee Hin Teoh, and Juliana Marie Chan, "In Vivo Efficacy of Heat-Inactivated *Clostridium sporogenes* For Anti-Tumor Therapy," FutureHealth 2017, 8 - 10 Nov 2017, Singapore. (Oral Presentation)
 4. Madhura Satish Bhave, "Inhibitive Effect of Non-viable Derivatives of *Clostridium sporogenes* on Colorectal Cancer Cells," 15th World Cancer Congress on Cancer Therapy, Biomarkers & Clinical Research, 5 - 7 Dec 2016, Philadelphia, USA. (Oral Presentation)
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Nomenclature

Abbreviations

2D 2-Dimensional

3D 3-Dimensional

4HBA 4-hydroxybenzoic acid

4HX 4-hydroxylamine

5-FC 5- Fluorocytosine

5-FU 5-Fluorouracil

Act Active

APC Antigen-presenting Cells

ATCC American Type Culture Collection

BCM Boiled Conditioned Media

CD Cluster of Differentiation

CD Cytosine Deaminase

CM Conditioned Media

CO₂ Carbon Dioxide

COBALT Combination Bacteriolytic Therapy

CPE Clostridium perfringens Enterotoxin

CTLA-4 Cytotoxic T-Lymphocyte-Associated Protein 4

DAB 3'-Diaminobenzidine

DAMP Danger-associated Molecular Patterns

DC Dendritic Cell

DNA Deoxyribonucleic Acid

DTT Dithiothreitol

ECM Extracellular Matrix

EDTA Ethylenediaminetetraacetic acid

ELISA Enzyme-Linked Immunosorbent Assay

EMEM Eagle's Minimum Essential Medium

EPR Enhanced Permeability and Retention

EthD-III Ethidium Homodimer-III

EthD-I Ethidium homodimer-I

FBS Fetal Bovine Serum

H&E Hematoxylin & Eosin

hLINE-1 Human Long Interspersed Nuclear Elements - 1

IB Inactivate Bacteria

IDO Indoleamine-pyrrole 2,3-dioxygenase

IFN- γ Interferon gamma

IHC Immunohistochemistry

IL Interleukin

iNOS Inducible nitric oxide synthase

IRF Interferon Regulatory Factor

IV Intravenous

LAB Lactic Acid Bacteria

LPS Lipopolysaccharide

LTA Lipoteichoic Acid

MALDI-TOF Matrix-Assisted Laser Desorption/Ionization Time-of-Flight

MAPK Mitogen-activated Protein Kinase

MDSC Myeloid-derived Suppressor Cell

MHC Major Histocompatibility Complex

miRNA MicroRNA

MWCO Molecular Weight Cut-Off

MW Molecular Weight

MYD88 Myeloid Differentiation Factor 88

NAct Non-active

NF- κ B Nuclear Factor kappa B

NK Natural Killer

NLR Nod-like Receptor

NOD/SCID Non-obese Diabetic/ Severe Combined Immunodeficient

NO Nitric Oxide

NTR Nitroreductase

NT Non-toxic

O₂ Oxygen

OD₆₀₀ Optical Density measured at 600 nm

OD Optical Density

OxPAPC 1-Palmitoyl-2-Arachidonyl-SN-Glycero-3-Phosphorylcholine

PAMP Pathogen-associated Molecular Patterns

PBS Phosphate Buffered Saline

PCLM Phase Contrast Light Microscopy

PD-1 Programmed Cell Death Protein-1

PDO Patient-derived Organoid

PDX Patient-derived Xenograft

PepG Peptidoglycan

PFA Paraformaldehyde

PICM-EF Protease Inhibitor in Conditioned Media - EDTA free

PICM Protease Inhibitor in Conditioned Media

PIM-EF Protease Inhibitor in Media - EDTA free

PI Protease Inhibitor

qPCR Quantitative Polymerase Chain Reaction

RCM Reinforced Clostridial Media

RC Restricted Co-culture

RPMI Roswell Park Memorial Institute

RT Radiation Therapy

SCFA Short Chain Fatty Acid

SCID Severe Combined Immunodeficient

SC Sub-cutaneous

SDS-PAGE Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

SD Standard Deviation

SEM Scanning Electron Microscope

TAA Tumor-associated Antigen

TGF- β Tumor Growth Factor beta

TIGIT T Cell Immunoreceptor with Ig and ITIM Domains

TIL Tumor Infiltrating Lymphocytes

TLR Toll-like Receptor

TNF- α Tumor Necrosis Factor-alpha

Treg T Regulatory

WT Wild-type

Chapter 1

Introduction

1.1 Cancer

Cancer continues to be a major healthcare burden in the 21st century, with the number of new cancer cases expected to increase to 21.7 million by 2030 [1]. The Agency for Healthcare Research and Quality (AHRQ) reported that the total costs of cancer in USA were \$87.8 billion in 2014. These numbers highlight that as the world population grows and ages, the economic burden of cancer and the demand for effective treatment methods is ever increasing.

Colorectal carcinoma, characterized by the uncontrolled growth of cells in the epithelial tissue of the large intestine, is the third most common cancer in men and second most common cancer in women around the world [2]. It is also the third highest leading type of cancer death in the United States [3]. With an estimated 97000 new colorectal cancer cases and 50000 deaths found in 2018, the urgent need for early detection and effective cancer treatments persists.

The most common modalities used in the treatment of colorectal carcinoma are

chemotherapy, radiation therapy and surgery [4]. While surgical methods like polypectomy, colectomy, proctectomy and proctocolectomy are most commonly used in the early stages of the cancer, these methods are often combined with chemotherapy or radiation therapy at the later stages of disease [4,5]. At the advanced stages of colorectal cancer, when the cancer has most often metastasized, surgery has little, or no effect and chemotherapy and radiation therapy are used instead [6].

1.2 Conventional Cancer Therapy

1.2.1 Surgery

Surgery is the first line of treatment for colorectal cancers that are detected in the early stages. In the early stages, surgery is used in the resection of tumors and polyps and the two most common methods currently used are open and laparoscopic surgery. Firstly, it is important to note that while the survival rates of surgical treatments are considerably high [5–9], the overall recurrence rates are significant too. This makes it necessary for patients to undergo surgical procedures more than once [10]. Secondly, the surgical form of treatment cannot be used in the later stages of cancer, so its effectiveness is contingent on early diagnosis of the disease. This limits the usefulness of surgery as a potent cancer treatment methodology. Furthermore, there may be several postoperative complications the result from surgeries such as internal bleeding, infections or a compromised immune system [11].

1.2.2 Radiation Therapy

Radiation therapy (RT) involves the use of ionizing radiation to curb the growth of cancer cells. It is commonly practiced in three forms [12]. The first form of RT is where it is used as a single modality treatment of tumors, although the clinical

application of this form RT is uncommon. The efficacy of RT when it used as the only form of treatment is not very high, and current research is focused on improving this efficacy. The second use of RT is as a preoperative or postoperative adjuvant therapy to resection surgery to maintain local control of the cancer. RT is most commonly used as an adjuvant treatment to surgery. Thirdly, RT can also be used to preoperatively convert an unresectable tumor to a resectable one. RT can have severe adverse effects on the health of the patients, like the risk of damage to the tissue surrounding the region of interest and the incidence of radiation-induced cancers [12, 13].

1.2.3 Chemotherapy

Chemotherapy is most commonly used in the treatment of cancer in stages II – IV. In the treatment of colorectal cancer, 5-Fluorouracil (5-FU) remains the cornerstone of chemotherapy [14]. However, the effect of 5-FU alone is known to be modest [15], which is why it is often administered in conjunction with Leucovorin [16, 17], Irinotecan and Oxaliplatin [18, 19]. These four drugs are used most commonly in the chemotherapy of colorectal cancer today and Table 1.1 compares the effectiveness of chemotherapy using these 4 drugs in treating colorectal cancer. In the table, the findings of the three most prominent chemotherapy clinical trials of advanced colorectal cancer are summarized. It can be observed that highest median survival rate is only 19.5 months, which indicates that the overall efficacy of chemotherapy drugs is still considerably low. However, low efficacy is not the only problem associated with chemotherapy. There are several other risks too. Chemotherapeutic drugs are not specific to cancer drugs and they target all rapidly dividing cells which poses the risk of damage of healthy tissues within the body [13]. Additionally, there are severe side effects associated with chemotherapy which include hand-foot skin reaction, diarrhoea, nausea, vomiting, fatigue,

sensory neuropathy, infection, neutropenic fever, hypersensitivity reaction, hypertension, gastrointestinal perforation, bleeding [18, 19].

Table 1.1: Efficacy of Chemotherapeutic Drugs with Primary End Point of Median Overall Survival Rate of Patients

Chemotherapeutic Drug	Patient Sample Size	Median Overall Survival Rate (Months)	Ref.
Fluorouracil	70	7.7	[15]
Fluorouracil + Leucovorin	142	12	
Irinotecan	226	12	[20]
Fluorouracil + Leucovorin	226	12.6	
Fluorouracil + Leucovorin + Irinotecan	231	14.8	
Fluorouracil + Leucovorin + Irinotecan	264	15	[21]
Fluorouracil + Leucovorin + Oxaliplatin	267	19.5	
Irinotecan + Oxaliplatin	264	17.4	

1.2.4 Limitations of Conventional Therapy

1.2.4.1 Tumor Microenvironment

While many advancements have been made, the effectiveness of current cancer treatments is limited due to the tumor microenvironment. The periphery of a tumor is well-connected to the vascular system with several blood vessels which support the growth of the tumor. However, the vasculature at inner regions of the tumor is poor, limiting the oxygen and nutrient supply to this part of the tumor [22]. This results in the formation of a necrotic core, composed of dead cancer cells, which is surrounded by a hypoxic region, as shown in Figure 1.1. The necrotic (anoxic) core and hypoxic region are key features of the tumor microenvironment, where oxygen levels are much lower than those in normal tissues as the oxygen and nutrient flow does not reach these parts of the tumor [23, 24]. While the oxygen levels differ among patients and within individual tumors, the median oxygen tensions in human tumors fall within the range of 1.3 - 3.9% O_2 (10 - 20 mmHg). Oxygen levels in normal tissues are much higher; 3.1 - 8.7% O_2 (24 - 66 mmHg) [25].

Figure 1.1: Schematic of tumor microenvironment

1.2.4.2 Impact on Efficacy of Conventional Therapy

Well-oxygenated tissues are normally more sensitive to ionizing radiation than hypoxic tissue. This is because oxygen molecules react with the free-radical debris of DNA formed by ionizing radiation to make the DNA damage permanent and bring about cell death. This makes the efficacy of RT heavily dependent on the presence of oxygen and thus intratumoral hypoxia greatly curbs the effectiveness of RT in treating tumors [26, 27]. Experimental evidence also strongly supports the idea that hypoxic cores in tumors play a major role in low effectiveness of RT against tumors [28, 29].

Hypoxia also limits the efficacy of chemotherapy. There are various reasons for this. Firstly, these hypoxic cancer cells are located far away from the blood vessels, preventing the delivery of drugs to cells [22, 27]. Secondly, some drugs such as melphalan [30, 31], bleomycin [32] and etoposide [33, 34] require cellular oxygen to bring about cell death and are therefore ineffective in hypoxic conditions. Finally, alkylating agents and anti-metabolite anti-cancer drugs only act against rapidly proliferating cells and as hypoxia slows down the cell-cycle, these drugs cannot effectively cause cancer cell death at the necrotic core [27, 35]. The limitations of existing cancer treatment methods have led to a pressing need to explore alternative treatment methods that are effective despite the hypoxia barrier of tumors.

1.3 Bacterial Cancer Therapy

1.3.1 History of Bacterial Cancer Therapy

The therapeutic role of bacteria against cancer can be traced back more than 200 years ago. Vautier found that gas gangrene caused by a *Clostridium perfringens* infection resulted in tumor regression in patients [36]. A German physicist, W

Busch, accidentally discovered the use of bacteria in cancer treatment in 1866 when he observed that recurring erysipelas infections cured skin cancer in one of his patient [37–39]. F Fehleisen, another German physician, discovered in 1882 that erysipelas was caused by *Streptococcus pyogenes*. He then injected cultured live bacteria into inoperable cancer patients, and observed tumor regressions in some of these patients as well [40, 41]. In 1887, P Bruns reported the cure of the cancers in 3 out of 5 patients, upon of the use of *S. pyogenes* cultures [42]. However all the aforementioned findings were either accidentally chanced upon or the result of very small scale experiments.

The first person to systematically investigate these observations for sustainable cancer treatment was the American physician William B Coley. Hypothesizing that the pathogenic bacteria were directly responsible for the regression of tumors, Coley decided to test this theory in 1891 by injecting 3 patients with live cultured Streptococcal bacteria to induce erysipelas [43]. While there was considerable regression of the tumors, 2 of the patients died due to the erysipelas infections. This highlighted the great danger of using live, wild-type bacteria which made it very difficult to control the erysipelas infection [44]. In 1893, Coley switched to a combination of heat-inactivated *S. pyogenes* and *Serratia marcescens*, which came to be known as “Coley’s toxin”, as a safer alternative. Over the next several years of his career, Coley administered these toxins to many of his patients to cure their bone and soft tissue sarcomas [45–48]. While some of his contemporaries and successors also used the toxins to cure carcinomas, lymphomas, melanomas, and myelomas [49–52], there were several others who were unable to obtain positive results. Additionally, the mechanism of this therapy was still unknown. These two factors led to widespread controversy regarding Coley’s research, with leading journals severely criticizing the toxins [53]. Four to five decades later, the first

clinical trials using bacterial therapy were carried out, but the results were only marginally beneficial [54]. Figure 1.2 provides a summary of the history of bacterial cancer therapy research.

A key milestone in the field of bacterial cancer therapy was the discovery by Malmgren and Flanigan in 1955 that anaerobic bacteria such as *Clostridium tetani* had the ability to preferentially localize and replicate in the hypoxic region within tumors [55]. Subsequently, several other bacterial strains were found to preferentially accumulate in tumors and exert an anti-tumor effect. *Salmonella*, *Clostridium* and *Bifidobacterium* are the most commonly investigated bacterial strains with respect to their anti-tumor ability.

Figure 1.2: Timeline of bacterial cancer therapy research

1.3.2 Tumor Targeting and Inhibition by Anaerobic Bacteria

While the hypoxic conditions of the tumor microenvironment are a huge obstacle for RT and chemotherapy, they are a potent tool for bacterial cancer therapy. This is because such conditions are perfect for the growth of anaerobic bacteria.

Anaerobic bacteria accumulate and proliferate in the hypoxic regions of the tumor before their natural cytotoxicity induces cancer cell death [56, 57]. There are two types of anaerobic bacteria; obligate anaerobes and facultative anaerobes. Both types of anaerobic bacteria accumulate in tumors through different mechanisms and have been found to inhibit tumor by exerting a direct cytotoxic effect.

Obligate anaerobes, like *Bifidobacterium* and *Clostridium*, can only survive in anoxic conditions and will die if they come into contact with oxygen. As such conditions are unique to the necrotic core and hypoxic region of tumor tissues, obligate anaerobes will specifically accumulate in the tumor [58–60]. This feature of obligate anaerobes allows bacterial therapy to be target-specific to tumors. *Clostridium* is a sporulating genus and the bacteria have been mainly administered as spores in experiments. Clostridial strains have been at the centre of bacterial tumor therapy since the 19th century because of the ability of their spores to selectively germinate in the hypoxic cores of tumors. *Clostridium sporogenes*, a proteolytic species, is reported to have a superior ability of tumor colonization [61, 62]. In another past study, spores of *Clostridium tetani* were injected into two sets of mice (one set with tumors and the other without) and only the mice with tumors died of tetanus infections, showing that the germination of the *C. tetani* spores only occurred in tumor-bearing mice [55]. The members of *Bifidobacterium* genus are gram-positive, non-sporulating bacteria, so they are usually administered in their whole, live form before they localize in tumors in animal models. In a study where tumor-bearing mice were injected with *Bifidobacterium bifidum*, the bacteria only accumulated in the tumor, and not in any other organ of mice [63].

In the case of *Clostridium* species, the natural anti-cancer cytotoxicity was found to be very effective, but there was a problem of toxicity. In the experiment by Parker et al., the injection of *Clostridium histolyticum* spores caused the oncolysis

of sarcoma tumors in mice, and resulted in the eventual death of the mice due to infections [64]. This saw a move towards a non-pathogenic strain of *Clostridium* known as *C. butyricum* (M-55) which was later re-classified as *C. sporogenes* [54]. A study by Dang et al. found that *Clostridium novyi* have a good tumor colonization capacity, but more than one-third of the mice used in the study died due to a lethal toxin produced by the bacteria. The group aimed to create an attenuated, non-toxic strain of bacteria. *C. novyi* spores were heated to inactivate the phage that carried the gene encoding the lethal toxin. Thus the resulting *C. novyi*-NT spores were less toxic and safer to use [56]. These spores were then administered to colon tumor-bearing mice as an adjunct form of treatment along with various chemotherapeutic drugs. This therapy, known as Combination Bacteriolytic Therapy (COBALT), had prolonged anti-tumor effects in the mice as the bacteria attacked the tumor from inside while the chemotherapeutic drugs attacked its outer surface. However, the risk of infection still persists with the use of *C. novyi*-NT spores. *Bifidobacterium* have successfully caused tumor regression in animal models. However, the non-sporulating nature of *Bifidobacterium* makes it more likely for the whole, live bacteria to cause infections in the body [65, 66]. Tables 1.2 and 1.3 summarize the findings of the studies using obligate anaerobes for the treatment of cancer.

Table 1.2: Examples of bacterial cancer therapy using obligate anaerobic bacteria in whole, live form to directly induce cancer death in tumors

Bacteria	Tumor	Model	Results	Gaps	Ref.
<i>B. infantis</i>	Meth-A sarcoma	Mouse	Tumor regression	Animals death due to infection	[65]
<i>B. adolescentis</i>	Colorectal carcinoma	Mouse	Tumor regression, prevention of tumor development	Animals death due to infection	[66]
<i>B. longum</i>	Teratocarcinoma	Mouse	Tumor regression, prolonged survival	Rim of viable tumor cells remained	[67]

Table 1.3: Examples of bacterial cancer therapy using obligate anaerobic bacteria in spore form to directly induce cancer death in tumors

Bacteria	Tumor	Model	Results	Gaps	Ref.
<i>C. butyricum</i> (M-55)	Melanoma, adenocarcinoma	Hamster	Tumor regression	Animals death due to infection	[68]
<i>C. butyricum</i> (M-55)	Glioblastoma	Human	Tumor regression	No improvement in survival rate	[69]
<i>C. butyricum</i> (M-55)	Ehrlich carcinoma	Mouse	Tumor regression	Rim of viable tumor cells remained	[70]
<i>C. butyricum</i>	Adenocarcinoma	Human	Tumor regression of large tumors only	Small tumors unaffected by treatment	[54]
<i>C. histolyticum</i>	Sarcoma	Mouse	Tumor regression	Animals death due to infection	[64]

Facultative anaerobes, like *Salmonella* and *Listeria*, can survive in both normoxic and anoxic conditions but are still capable of targeting tumors. They have a more complex mechanism of targeting tumors than obligate anaerobes. Facultative anaerobes are thought to target tumors by entrapment in tumor vasculature, entering tumors after inflammation, chemotaxis towards compounds produced by tumors, preferential growth in tumor microenvironment and protection from immune system [71]. *Salmonella* have been found to accumulate in tumors a 1000 times more than in normal tissues [72–74]. The ability of *Salmonella* to preferentially

accumulate in tumors can be attributed to their motility through chemotaxis [75]. *Salmonella* can sense and migrate towards small molecules such as serine, aspartate, ribose, thereby allowing it to identify and penetrate tumors and cancer cells [76,77]. *Salmonella* can be engineered such that they chemotaxes towards a pre-determined small molecule that can be found in a high concentration at the tumor region. A leucine and arginine auxotroph of *Salmonella*, known as *S. typhimurium* A1 was developed, and was found to direct itself towards the PC-3 prostate cancer cells which produced these two amino acids both *in vitro* and *in vivo*. A1 was found to localize in xenograft tumors in mice and cause tumor regression [78].

Wild-type *Salmonella* strains are known to be pathogenic as the presence of lipopolysaccharides (LPS) and other virulence factors can cause significant immunostimulation [74]. To reduce the pathogenicity, *S. typhimurium* was attenuated by deleting the *msbB* gene, which reduces lipid A-induced septic shock, and *purI* gene which reduces virulence due to the resulting purine auxotrophy [79]. This attenuated strain of *S. typhimurium*, VNP20009, was injected into tumor-bearing animals and was found to preferentially replicate in tumors and exert an inhibitive effect on the tumors [80]. As seen in Table 1.4, an attenuated strain *S. typhimurium* VNP20009 is currently being used in many studies as it is known to be far less toxic than its wild counterpart. However, it seems to have a less potent inhibitive effect than the wild-type strain.

Table 1.4: Examples of bacterial cancer therapy using live facultative anaerobic bacteria to directly induce cancer death in tumors

Bacteria	Tumor	Model	Results	Gaps	Ref.
<i>S. typhimurium</i> (VNP 20009)	Sarcoma	Mouse	Tumor regression	Rim of viable tumor cells remained	[72]
<i>S. typhimurium</i> (VNP 20009)	Melanoma	Mouse	Tumor regression, slow tumor growth	Rim of viable tumor cells remained	[81]
<i>S. typhimurium</i> (VNP 20009)	Melanoma	Mouse	Slow tumor growth	Large number of viable tumor cells remained	[82]

1.3.3 Gaps

Despite the many advances, bacterial cancer therapy has not gained widespread acceptance as a potential treatment method. This is because the therapy has limitations of its own, as described below.

1. Risk of Toxicity

The use of bacteria for cancer therapy poses the risk of toxicity. This has been observed in cases which used toxic, pathogenic strains of bacteria and also in some cases of attenuated bacteria. In two recent studies *C. novyi*-NT spore treatment, one involving canines and the other involving canines and a human patient with a leiomyosarcoma, the administered dose of the spores caused a

severe case of toxicosis [83, 84], showing that attenuation of *C. novyi*-NT does not successfully minimize the risk of toxicity.

2. Risk of Infection

The administration of anaerobic bacteria poses a high risk of infections after the spores have germinated or with the proliferation of the live bacteria. Several studies involving the administration of spores and live bacteria to tumor-bearing animals resulted in their death due to bacterial infections of the bacteria [55, 64–66].

3. Risk of Recurrence

Anaerobic bacteria are hypoxia-dependent and can only exert an inhibitive effect in the hypoxic core of tumors. This leaves a viable rim of peripheral tumor cells which increases the chance of tumor recurrence [67, 70, 72, 81].

4. Poor Understanding of Mechanism

Another gap in bacterial cancer therapy is that researchers have been unable to conclusively determine the real mechanism of natural cytotoxicity and immunostimulatory effect of bacteria which induces cancer cell death [56, 85, 86].

5. Failure in Complex Systems

New bacterial cancer therapy agents are commonly tested on 2D *in vitro* models, which are not physiologically relevant. While the results may be promising, the reliability of the data collected from these studies is limited and these agents often fail in more complex systems [82].

1.4 Motivation

The gaps in the field of bacterial cancer therapy need to be addressed to before bacteria can be developed as clinically relevant treatment agents. Previously, the efforts to enhance safety by attenuating bacteria have abrogated the anti-cancer efficacy of the bacteria [37, 59]. The efficacy of spores of is hypoxia-dependent and results in the risk of recurrence. This thesis is motivated by the research question, “How can bacterial therapy be made safer while retaining the natural anti-cancer properties of bacteria?”. This may be achieved by generating IB and CM from *C. sporogenes*. These non-viable derivatives of the non-toxic, non-pathogenic *C. sporogenes* will help to minimize the risk of toxicity and infection. The hypoxia-independence of bacterial derivatives and the activation of anti-tumor immunity will minimize the risk of tumor recurrence. These derivatives will be tested in 3D *in vitro* and *in vivo* cancer models to prove their efficacy in complex systems.

1.5 Aims and Hypothesis

The main aim of this thesis is to investigate the anti-cancer efficacy of the bacterial derivatives, IB and CM of *C. sporogenes*. The hypothesis and specific aims of the thesis are described below.

Hypothesis:

IB and CM can exert an anti-cancer effect against colorectal cancer *in vitro* and *in vivo*.

Specific Aim 1:

Screen efficacy of bacterial derivatives of *C. sporogenes* against

various cancer cell lines.

IB and CM were derived from *C. sporogenes* and administered to a panel of cell lines of different cancer types. The effect of the bacterial derivatives against the cancer cell lines was quantified by evaluating cell proliferation, cell viability and spheroid size.

Specific Aim 2:

Investigate the effect of IB on colorectal cancer cells *in vitro*.

IB were tested on 2D and 3D *in vitro* models of the colorectal cancer cell lines, CT26 and HCT116. The IB-cell interaction was examined and cell death analysis was conducted to understand the nature of IB-mediated inhibition of colorectal cancer cells.

Specific Aim 3:

Investigate the effect of CM on colorectal cancer cells *in vitro*.

CM was tested on 2D and 3D *in vitro* models of the colorectal cancer cell lines, CT26 and HCT116. Cell death analysis and fractionation of CM were conducted to understand the nature of CM-mediated inhibition of colorectal cancer cells.

Specific Aim 4:

Investigate the effect of IB and CM on colorectal cancer cells in immunocompetent animal models.

IB and CM were tested *in vivo* by intratumoral injection in SC tumors of colorectal cancer in immunocompetent and immunocompromised mice. Tumor volume and survival rate was evaluated and the immune response to treatment was investigated. The IB-treated mice were rechallenged with a second injection of colorectal cancer cells to test protection against tumor regrowth.

1.6 Significance of Research

Currently, the application of *C. sporogenes* in the field of bacterial cancer therapy is limited to their spore form. As previously mentioned, the use of bacterial spores poses several risks. This thesis explores the use of inactivated *C. sporogenes* (IB) and their conditioned medium (CM) as anti-cancer agents for the first time. In investigating the effect of IB and CM on colorectal cancer cells, this thesis will provide a better understanding of the underlying mechanism of the anti-cancer effect. The use of 3D spheroids in this thesis allows the evaluation of IB and CM as potential anti-cancer agents on a physiologically relevant *in vitro* platform. This will be followed by testing IB and CM *in vivo*, to establish their efficacy in a complex system, and determine their ability to elicit an anti-cancer immune response. Taken together, this thesis will help to better understand the nature and potential of *C. sporogenes* derivatives as bacterial cancer therapy agents.

1.7 Scope of Thesis

Chapter 1 introduces the background of cancer and the need for bacterial cancer therapy, and highlights the gaps in the field. Additionally, it describes the hypothesis and specific aims of the thesis.

Chapter 2 provides a literature review focused on *Clostridium sporogenes*, its current role in bacterial cancer therapy, *in vitro* and *in vivo* cancer models and the role of bacteria in cancer immunotherapy.

Chapter 3 describes the derivation of IB and CM from *C. sporogenes* and the screening of their efficacy against various human cancer cell lines. The cancer type that IB and CM were most effective against was selected for experiments described in the next three chapters.

Chapter 4 discusses the efficacy of IB against 2D and 3D *in vitro* models of colorectal cancer cells. This chapter examines the nature of IB-mediated inhibition of colorectal cancer cells *in vitro*. This chapter was partially published in Bhave *et al*, 2015, *Scientific Reports*.

Chapter 5 discusses the efficacy of CM against 2D and 3D *in vitro* models of colorectal cancer cells. This chapter investigates the key components of CM that are responsible for mediating an anti-cancer effect. This chapter was partially published in Bhave *et al*, 2015, *Scientific Reports*.

While the previous two chapters focused on the efficacy of IB and CM *in vitro*, Chapter 6 describes the *in vivo* efficacy of IB and CM against colorectal cancer models in immunocompromised and immunocompetent mice.

Chapter 7 presents conclusions of this thesis with future work recommendations.

Chapter 2

Literature Review

2.1 *Clostridium sporogenes* in Cancer Therapy

2.1.1 Clostridial Bacteria in Cancer Therapy

The use of clostridial bacteria in cancer therapy is rooted in incidental discoveries. The earliest instance can be traced back to 1813, when Vautier found that gas gangrene caused by a *C. perfringens* infection resulted in tumor regression in patients [36]. Subsequently, other clostridial species were deliberately introduced to solid tumors in animal models to induce tumor regression. Parker et al. injected *C. histolyticum* spores intratumorally in mice bearing transplanted sarcomas [64]. The anaerobic spores germinated within the hypoxic core of the tumors and caused the lysis of the tumor tissue. However, the bacterial infection resulted in the death of animals. A key milestone in the field of bacterial cancer therapy was the discovery by Malmgren and Flanigan in 1955, that anaerobic bacteria such as *C. tetani* had the ability to preferentially localize and replicate in the hypoxic region within tumors [55]. There was no germination of the spores in healthy, non-tumor-

bearing mice. This meant that the bacteria could be administered systematically and would selectively colonize solid tumors that were not accessible for direct injection. However, all the tumor-bearing mice died within 48 hours due to the tetanus toxin produced by the vegetative *C. tetani*. This highlighted the risk to toxicity associated with the use of toxic, pathogenic clostridial species.

To minimize the risk of toxicity while still retaining the ability to selectively colonize solid tumors, researchers started to use non-toxic, non-pathogenic clostridial species. Möse and Möse discovered the non-toxic, non-pathogenic, proteolytic *C. butyricum* (M-55) and tested it extensively for its oncolytic properties in mice [70]. These bacteria induced the liquefaction of solid tumors. Saccharolytic clostridia such as *C. beijerinckii* or *C. acetobutylicum* were also systemically administered as spores to tumor-bearing mice. While both bacteria were able to localize in the tumors, their colonization ability was limited [87]. This would limit the ability of the bacteria to exert an oncolytic effect [88].

A potent bacterial anti-cancer therapeutic agent would have to retain the tumor targeting ability and effective natural cytotoxicity of anaerobic bacterial spores, while enhancing the safety of the treatment. In 2001, Vogelstein's group tested 26 strains of bacteria for their ability to colonize tumors in mice. *C. novyi*, a pathogenic bacterium, was found to be the best strain for this purpose. The group developed *C. novyi*-NT, a non-toxic variant of the wild type *C. novyi* in which the gene encoding the lethal α -toxin is deleted. The tumor-bearing mice tolerated spores of *C. novyi*-NT, without side-effects, while succumbing to doses of the wild type *C. novyi* spores. The intravenous injection of *C. novyi*-NT led to germination within the tumors followed by the expansion of the necrotic regions in the tumor [56]. Thus, the engineered *C. novyi*-NT bacteria were found to be safer, while retaining the tumor-targeting and oncolytic ability of the wild-type *C. novyi*.

C. novyi-NT has since become one of the most widely researched strain of bacteria for its anti-tumor properties. Early studies by Vogelstein's group confirmed the ability of *C. novyi*-NT to colonize the hypoxic core of colorectal carcinoma tumors after systemic administration and then expand the necrotic regions of the tumor. The bacteria degraded the inner regions of the tumor, working outwards to the well-oxygenated periphery, where the anti-cancer effect of *C. novyi*-NT ceased. As result, a rim of viable tumor cells still remained [86, 89]. In 2014, the effect of *C. novyi*-NT on rat models of glioma was investigated. The intratumoral injection of the spores caused a decrease in tumor mass [83]. In another study, the systematic administration of *C. novyi*-NT spores resulted in the colonization of the syngeneic and xenograft glioblastomas in rats. Tumor regression was observed and the survival rates were prolonged in both models. However, incomplete tumor eradication in the syngeneic tumor model left a rim of viable cells and caused a regrowth of the tumor [90]. Brain edema was an observed side effect in the treated rats in both studies.

Maletzki et al. tested the effect of systematically administered *C. novyi*-NT on pancreatic tumors of 3 varying sizes (small: $< 150 \text{ mm}^3$, medium: 250 mm^3 , large: $> 450 \text{ mm}^3$). Interestingly, the study found that the efficacy of the treatment was dependent on the size of the tumor. The small tumors were unaffected by the treatment. This was attributed to the lack of hypoxic regions in tumors that were $< 150 \text{ mm}^3$ in size, which prevented the colonization of the tumors by the spores. In the large tumors ($> 450 \text{ mm}^3$), rapid necrosis was detected and tumor shrinkage occurred within a few hours. All the animals bearing large tumors died within 24 hours of treatment, possible due to the tumor lysis syndrome. The medium sized tumors (250 mm^3) responded completely to the spore injection, with rapid necrosis occurring within 48 hours and tumors being completely eradicated after 2 weeks,

showing that the medium sized tumors were optimum for this treatment [91].

The safety and efficacy of *C. novyi*-NT was tested in spontaneously occurring tumors in pet canines. A study in 2012 found that intravenously injected spores of *C. novyi*-NT could colonize natural tumors in canines. The study reported that all 6 animals experienced fever and 2 of them experienced abscess formation at the site of the primary nasal carcinoma tumor and in the spleen [84]. The abscess in the spleen shows that the bacterial spores were not fully cleared from the spleen of the canine and germinated there. This indicates that the safe and successful application of this treatment would require a greater control on the localization of the spores. In another study by Roberts et al., 16 canines with naturally occurring tumors were subjected to an intratumoral injection of *C. novyi*-NT spores. The study reported tumor destruction in all the treated animals and prolonged survival rates. Three of the animals had a complete response to the treatment, while another 3 had a partial response. The formation of tumor abscess and associated tumor pain was observed [83].

Based on the findings of the study involving canines, Roberts et al. conducted a Phase 1 human trial on a patient with advanced leiomyosarcoma. 1×10^4 *C. novyi*-NT spores were intratumorally injected, which resulted in extensive necrosis of the tumor and surrounding humerus. The patient was reported to suffer from severe pain and required surgical intervention. There was an improvement post-surgery and the patient showed no signs of infection subsequently. The patient experienced side-effects of fever, pain and an increased leukocyte count after the administration of the spores [83]. The effect of the treatment on the long-term health of the patient was not reported.

While the use of engineered *C. novyi*-NT spores as anti-cancer therapeutic agents is promising, several concerns remain. The findings of the studies by Heppner and

Möse and Maletzki et al. demonstrate that tumor size and spore dosage are both determining factors of the efficacy and safety of anaerobic spore treatment. Large tumors will respond quickly to the administration of spores, as their necrotic and hypoxic core is larger and that allows for the faster germination and proliferation of the bacteria. This will lead to the rapid expansion of the necrotic region, and the development of a large abscess, which poses the lethal risk of rupturing. As such, abscess management is a major concern in this form of treatment.

The use of anaerobic clostridial spores is based on their ability to germinate within the hypoxic cores of tumors and exert their oncolytic effect. However, this oncolytic effect ceases near the well-oxygenated, peripheral regions of the tumor. Consequently, a rim of viable tumor cells remained. The hypoxia-dependence of clostridial spores or vegetative bacteria increase the risk of tumor re-growth [59,61]. This necessitates the development of hypoxia-independent bacterial therapeutic agents. Additionally, the germination of the spores in the tumors and the subsequent proliferation of the vegetative bacteria poses the problem of abscess formation.

2.1.2 Characteristics of *Clostridium sporogenes*

Clostridium sporogenes (ATCC 13732) has been extensively studied as anti-cancer agent. Its discovery is credited to Möse and Möse, who isolated it from soil samples and classified it as *C. butyricum* (M-55) [92]. It was subsequently re-named *C. oncolyticum* before being re-classified as *Clostridium sporogenes* (ATCC 13732). It is a sporulating, Gram-positive, obligate anaerobe and has a rod-shaped structure in its vegetative form. Under conditions of high-stress, they form terminal endospores. When in culture, the bacteria occur as single bacterial cells that are 1 – 10 μm long or chains or filaments of variable length [93]. Figure

2.1 shows the morphology of *Clostridium sporogenes* DSM 795, a strain similar to *Clostridium sporogenes* (ATCC 13732), in the vegetative form and in the spore form (inset). It is known to be highly proteolytic [94, 95] and is recognized as a non-toxic, non-pathogenic surrogate of *C. botulinum*, lacking the lethal botulinum neurotoxin [92, 96–98]. The non-pathogenicity of *Clostridium sporogenes* was further proven when Möse and Möse self-injected the spores and reported no adverse reactions [99]. Apart from soil, the bacteria are also found to be a symbiont of the human gut [88, 100].

Figure 2.1: Morphology of *Clostridium sporogenes*. Reproduced with permission from [96]

2.1.3 *Clostridium sporogenes* Spores in Cancer Therapy

C. sporogenes have been used the most extensively in their spore form in the field of bacterial cancer therapy. Möse and Möse studied the use of this bacteria for therapeutic purposes as a safer alternative to pathogenic species. They intravenously self-injected the spores of *C. sporogenes* to establish their non-pathogenicity, and found the bacteria to be harmless [99, 101]. *C. sporogenes* spores are known to have a superior tumor colonization ability [58].

Möse and Möse tested the spores of *C. sporogenes* in mice bearing solid Ehrlich tumors. The hypoxic core of the solid tumors enabled the germination of the spores. Tumor lysis followed, resulting in the liquefaction of a large portion of the tumors [92]. Several studies followed where the *C. sporogenes* spores were administered systemically to tumor-bearing animals like hamsters, rats and rabbits [68, 102]. These experiments resulted in the lysis of the solid tumors too. However, all of these animals studies had similar gaps. The germination of the spores led to excessive tumor lysis, resulting in animal death. The proliferation of the vegetative *C. sporogenes* caused abscess formation. In the animals that survived, a rim of viable tumor cells remained, which caused tumor recurrence.

To address the issue of tumor recurrence due to the use of *C. sporogenes* spores, researchers combined bacterial cancer therapy with other therapeutic agents. Gericke and Engelbart administered *C. sporogenes* spores along with heavy metal compounds to tumor-bearing mice. While the treatment regime increased the extent of lysis in large solid tumors, smaller tumors remained unresponsive to the treatment [103]. This phenomenon could be explained by the absence of a large hypoxic core in smaller tumors. The normoxic conditions in these tumors are unfavourable for the germination of the spores and curb their anti-cancer efficacy. Another study involved the administration of *C. sporogenes* spores with a range of chemotherapeutic drugs, such as 5-FU, to rodents bearing solid tumors. This combination of therapeutic agents resulted in the oncolysis of large tumors. However, the smaller tumors remained unresponsive. Additionally, there was increased toxicity in the animals due to this treatment [104]. The use of *C. sporogenes* in their spore form to treat tumors had limited success. While the tumor colonization ability of the bacteria was superior and affected extensive lysis, it was ineffective in normoxic conditions and led to a risk of tumor recurrence. The proliferation of bacteria also

formed abscesses in the tumor-bearing animals. Thus, the most common application of *C. sporogenes*, as spores, has limitations which need to be addressed.

2.1.4 *Clostridium sporogenes* in Pro-Drug Cancer Therapy

Pro-drug cancer therapy is rooted in tumor-targeted gene therapy. The premise of the latter is based on the use of anaerobic bacteria as a vector to deliver anti-tumor genes to the tumor site. Pro-drug therapy involves the conversion of an inactive, non-toxic pro-drug substance into its active, toxic form by an enzyme produced by the anaerobic bacterial vector. There are several known enzyme pro-drug systems. One of them uses the Cytosine Deaminase (CD) enzyme which can convert the non-toxic pro-drug 5-fluorocytosine (5-FC) into its cytotoxic form of 5-fluorouracil (5-FU) [105]. Another enzyme pro-drug system uses nitroreductase (NTR) enzyme to convert the pro-drug CBI954 to its toxic derivative 4HX which is a DNA cross-linking agent and can induce apoptosis [106].

Various other clostridial strains had been previously utilized for pro-drug therapy. CD-recombinant *C. acetobutylicum* [107] and NTR-recombinant *C. beijerinckii* [87] were tested in animal models but had limited successes due to the low colonization ability of these strains. Other strains, like *C. novyi*-NT have better colonization abilities [56] but are difficult to transform with the anti-cancer genes. *C. sporogenes* is a preferable option as they have the advantage of their superior tumor colonization ability, which ensures that the spores target and germinate only in the hypoxic cores of tumors [108]. Additionally new techniques in gene transformation allowed the introduction of recombinant plasmids to this strain [106]. Consequently, *C. sporogenes* have been used for pro-drug therapy.

CD-recombinant *C. sporogenes* spores have been found to successfully convert 5-

FC to 5-FU, thereby inhibiting cancer cell growth *in vitro* [62] and reducing tumor burden in animal models [87, 108]. Several studies involving NTR-recombinant *C. sporogenes* have also found that the tumor-targeting spores germinate, produce the NTR enzyme and exert an anti-tumor effect in mouse models [106, 109, 110]. However, in these studies, it has been difficult to ascertain whether the natural anti-cancer effect of *C. sporogenes* or the pro-drug activation is the main reason for the observed tumor inhibition.

2.2 Bacterial Derivatives in Cancer Therapy

2.2.1 Heat-inactivated Bacteria

The earliest application of heat-treated bacteria in cancer therapy was in 1893, with William Coley using a combination of combination of heat-inactivated *S. pyogenes* and *S. marcescens*, which came to be known as “Coley’s toxin” [45]. Coley administered these toxins to cure bone and soft tissue sarcomas in his patients [45–48]. While some of his contemporaries and successors also used the toxins to regress carcinomas, lymphomas, melanomas, and myelomas [49–52], there were several others who were unable to obtain positive results. The inconsistent results and the unknown mechanism of the anti-cancer effect lowered the research interest in the use of heat-inactivated bacteria.

However, heat-inactivated bacteria hold some advantages over live bacteria or spores. Heat-treatment of vegetative bacteria kills them, making them incapable of proliferation or metabolic activity [111]. This makes them a suitable candidate of minimizing the risk of infection associated with live bacteria and spores. As proven by Coley’s work, heat-inactivated bacteria were still able to induce a anti-cancer effect. Recent studies have heat-treated various species of bacteria to utilize

them for anti-cancer treatment.

In an *in vitro* study, inactivated *Staphylococcus aureus* and *Streptococcus equisimilis* were tested on 2D models of colorectal cancer. The bacteria were heat-treated at 75 °C before being resuspended in complete growth media and administered to the 2D models of cancer cells. The inactivated bacteria caused cell viability to decrease to 82.5% of the control. When the cancer cells were co-cultured with peripheral blood lymphocytes, the cell viability decreased further [85]. This showed that the direct anti-cancer effect of the inactivated forms of these bacterial species was limited. The combined effect of immune cells was needed to elicit a significant anti-cancer effect *in vitro*. Furthermore, the study was not able to identify the mechanism for the observed anti-cancer effect. Madempudi et al. screened the anti-cancer effect of *Bacillus coagulans* Unigue IS2, heat-treated at 95 °C, against colon cancer cells *in vitro*. The heat-treated bacteria inhibited the cancer cell growth of COLO cells by 51.6%. While this indicated that the heat-treated bacteria had the potential to inhibit cancer growth, there were no further studies to investigate the mechanism of this effect [112]. Another study tested the efficacy of heat-treated *Enterococcus faecalis* and *Staphylococcus hominis* against breast cancer cells *in vitro*. These bacteria were heat-treated at 95 °C for 1 hour and then at 121 °C for 15 mins. When administered to breast cancer cells, the viability of MCF-7 cells decreased to 33.29%. This inhibitive effect was dose-dependent and was not extended to the non-malignant cell line, MCF-10A. However, the underlying mechanism for this effect was still poorly understood [113]. One of the key gaps in these studies was that efficacy of the heat-inactivated bacteria were only tested on a 2D monolayer model of cancer cells, which are not a complex *in vitro* system that are physiologically relevant. Even in the simple 2D model, the effect on cell viability was limited. This suggests that when applied to an more

complex system, such as a 3D *in vitro* model or *in vivo* tumor model, these strains of inactivated bacteria may be less effective.

Heat-inactivated forms of these bacterial species had minimal efficacy *in vivo*. In a 2017 study, *S. typhimurium*, heat-treated at 65 °C, were intratumorally administered to tumor-bearing mice along with adoptive T-cells transfer [114]. This resulted in a temporary regression of tumors before a 100% relapse in all animals. Treatment with the heat-treated *S. typhimurium* caused an influx of tumor infiltrating lymphocytes. However, the study did not investigate whether the treatment with heat-killed bacteria activated the immune memory to protect the animal from future tumor re-challenges. Conversely, a study by Kocijancic et al. showed that treatment with heat-killed *S. typhimurium* caused the regression of CT26 tumors in mice and protected the animals from future re-challenges. However, these bacteria were completely ineffective against RenCa tumors [115]. There are two main gaps in the use of heat-treated *S. typhimurium*. Firstly, there was insufficient evidence to ascertain whether tumor regression was mainly due to the heat-treated bacteria or the elicited immune response. The studies suggest that the effect of the heat-treated bacteria is observed only in the presence of the immune system. Furthermore, the mechanism of this observed effect is poorly understood.

The *in vivo* administration of heat-treated *S. aureus* and *S. equisimilis* in CT26-tumor bearing mice caused a delay in tumor growth and a 50% decrease in tumor volume [85]. These heat-treated bacteria caused a slight, non-significant decrease in tumor volume in immunocompromised mice. This study showed that there was a slight direct inhibitive effect of heat-treated bacteria, yet was not able to explain the underlying mechanism. Furthermore, the long-term effect of the heat-treated bacteria on immune memory was not tested using a tumor re-challenge.

2.2.2 Secreted Bacterial Products

Bacteria produce and secrete several proteins and metabolites when in culture. These secreted bacterial products have been found to exert an anti-cancer effect *in vitro* and *in vivo*. The bacterial cells are filtered out from the culture supernatant, giving the conditioned medium containing all the secreted bacterial products.

The secreted products of lactic acid bacteria (LAB) have been widely studied for their anti-cancer properties. Strains of LAB are classified as pro-biotics which can prevent and delay tumor growth [116]. The secreted products of five LAB (*Bifidobacterium animalis*, *B. infantis*, *B. bifidum*, *Lactobacillus acidophilus*, and *Lactobacillus paracasei*) were collected from the supernatant of fermented milk and tested for their anti-cancer effects *in vitro*. The results of this study showed that the supernatant of *B. infantis* and *L. acidophilus* inhibited the growth of MCF-7 breast cancer cells by 85% after 9 days in culture [117]. The mechanism of this inhibitive effect was poorly understood. The paper suggests that the observed effect could be due to an bacterial product released into the milk during culture, or be a component of milk transformed by the bacteria. Further analysis is needed to determine the key component responsible for the inhibition of the breast cancer cell line. The anti-cancer efficacy of the secreted products of LAB have also been tested against colon cancer cells. As LAB are commonly found in the gut microbiota, they are expected to have a greater affinity towards colon cells. A study screened the anti-proliferative effect of 54 LAB strains against colon cancer cells. Of the LAB strains tested, *Enterococcus faecium* and *Lactobacillus fermentum* had the strongest inhibitive effect on Caco-2 cells, causing a decrease of 21-29% in cell proliferation [118]. Another study found that the heat-sterilized supernatant of *B. coagulans* Unique IS2 caused growth inhibition of 22% in COLO cells. This inhibitive effect was mediated by apoptosis of the cells, through the modulation of

the ratio of pro- and anti-apoptosis proteins, Bax/Bcl2. While the paper suggests that small metabolites of *B. coagulans* are responsible for this effect, there has been no evidence to support this claim [112]. The secreted metabolites of *Enterococcus hirae* 20c, *E. faecium* 12a and L12b, have also been found to inhibit the *in vitro* proliferation of cervical and lung cancer cell lines by mediating apoptosis. When the supernatant contained the secreted bacterial products was treated with proteinase K, the anti-proliferative effect was abrogated. This suggests that the metabolites are likely to be proteins [119]. Further characterization of the metabolites would be required to improve the understanding of the mechanism of the observed anti-cancer effect.

The butanol extracts of *B. adolescentis* were tested on colon cancer cells *in vitro*. The *B. adolescentis* extracts had an inhibitive effect on Caco-2, HT-29 and SW480 cells in a dose-dependent manner. Additionally, when these extracts were applied to the murine macrophage cell line, RAW264.7, they induced macrophage activation and increased TNF- α and nitric oxide (NO) production. This showed that LAB products not only had a direct inhibitive effect on the colon cancer cells, but also had the potential to induce an immune response [120]. However, the paper did not report the characterization of the butanol extract or elucidate the mechanism for the inhibition of the colon cancer cells.

The secreted products of LAB have been used in combination with chemotherapeutic drugs. The supernatant of *Lactobacillus plantarum* was found to induce apoptotic cell death in colorectal cancer cells, HCT116 and HT-29, that are resistant to 5-FU. When the supernatant of *L. plantarum* was applied in combination with 5-FU, a synergistic anti-cancer effect was observed. Notably, the *L. plantarum* supernatant downregulated the expression of cancer stem cell markers in these chemoresistant cell lines. This showed that secreted bacterial products can

be used to overcome the problem of chemoresistance in cancer cells [121]. One of the key gaps in the use of secreted bacterial products of LAB is that the active components have not been characterized. This limits the understanding of the underlying mechanism of their anti-cancer activity. Additionally, these studies were all carried out in 2D *in vitro* cancer models. Their efficacy is yet to be tested in more complex 3D *in vitro* and *in vivo* models of cancer.

Short-chain fatty acids (SCFA) are another group of secreted bacterial proteins that have applications in cancer therapy. SCFAs such as butyrate, acetate and propionate, are synthesised by anaerobic gut microbes through the fermentation of dietary fibre and polysaccharides [122]. Butyrate has been extensively studied for its inhibitive effect on colon cancer by mediating apoptosis [123] and cell cycle arrest [124]. When exposed to physiologically relevant concentrations of butyrate, colorectal cancer cell lines HT29 and HCT116 were found to have decreased cell proliferation due to the induction of histone hyperacetylation [125]. Another study found that butyrate decreases the cell proliferation of colorectal cancer cells by modulating the expression of miRNA-dependent expression of the p21 gene [126].

Amongst the bacterial products that have been identified and characterized for their anti-cancer effect, *Clostridium perfringens* enterotoxin (CPE) has been studied extensively. CPE has been found to interact with claudin-3 and -4 receptors that are overexpressed in many types of tumors, eventually triggering cancer cell death. Studies have confirmed the anti-proliferative effect of CPE inhibits the growth of pancreatic [127], prostate [128], breast [129–131] and ovarian [132] cancer. In another study, 4-hydroxybenzoic acid (4-HBA) has been identified as the key molecule secreted by *Pseudoalteromonas haloplanktis* TAC125 which can inhibit the proliferation of lung cancer cells *in vitro* by mediating pyroptosis [133]. Before these bacterial products can be developed for systemic therapy, their long

term effectiveness and *in vivo* safety have to be established.

2.3 Cancer Models for Therapy Development

Current studies in the field of bacterial cancer therapy involve the use of both *in vitro* and *in vivo* models of cancer. Most studies use 2D monolayer cultures for cancer cells for *in vitro* experiments [113, 118, 121]. The *in vivo* studies of bacterial cancer therapy commonly involve the use of sub-cutaneous tumor models in mice [86, 115, 128]. These cancer models are discussed in this section.

2.3.1 *In Vitro* Cancer Models

The first stage of cancer therapy development involves testing the efficacy of the agent on cancer cells *in vitro*. For this purpose, a 2D monolayer model of cancer cells is used as a therapy testing platform. This model is greatly favoured due to its simplicity and low-cost. However, 2D cancer models are limited in their simulation of *in vivo* tumors. *In vivo* tumors grow in 3D spatial conformation, in which they are exposed to a heterogenous supply of oxygen and nutrients, and other mechanical and chemical stimulants. This makes the tumor microenvironment a complex entity consisting of three cellular regions. The peripheral region consists of proliferating cells that have ample access to oxygen and nutrients, the inner region of viable but quiescent cells in hypoxic conditions and a necrotic core at the centre where the diffusion of oxygen and nutrients is at the least [134]. A 2D monolayer culture consists of a single layer of cells adherent to the substrate and thus cannot simulate diffusion-limited distribution of oxygen and nutrients that is characteristic of *in vivo* tumors [135].

Therefore, 3D *in vitro* models are used as intermediate systems between 2D *in vitro* cultures and *in vivo* tumor models. These models can simulate tumor biology

better than 2D models and provide physiologically relevant data on the efficacy of a therapeutic agent [136]. Three-dimensional spheroid models are more appropriate for evaluating drug efficacy as they are less sensitive 2D monolayers. They are useful for simulating the *in vivo* microenvironment of tumors in terms of the spatial arrangement of cells, ECM deposition and inter-cellular interactions. This allows the improved assesment of drug response [137, 138]. Diffusion limitations in terms of the gradients of oxygen, nutrients and waste distribution can be observed within a spheroid. This enables the evaluation of the penetration of the drug and its corresponding efficiency at each of these gradient zones [139]. Spheroids can be generated using multiple cell types, including fibroblasts, to simulate the interactions between different cells in the tumor stroma [140]. This model can also faciliate the testing of cancer stem cell-targeting drugs by evaluating the expression of stem cell markers and maintenance of stem cells in 3D spheroid cultures [141].

One of the methods of generating 3D spheroids is by using ultra-low adhesion well-plates. The ultra-low adhesive surface of each well prevents the adherence of the cells to the substrate and promotes intercellular aggregation. Eventually, these aggregates lead to strong cell-cell interactions and form tight structures which proliferate and simulate the morphological arrangement of cells in *in vivo* tumors [142, 143]. This technique is advantageous because it is simple, cost-effective and allows the large scale generation of spheroids for high-throughput analysis [142] . This method has been used to generate spheroids of breast [141–143], pancreatic [144] and colorectal [145] cancer.

2.3.2 *In Vivo* Cancer Models

To test the efficacy of a potential cancer therapeutic, the role of the tumor microenvironment, host immune system, and angiogenesis in tumor response to

therapy has to be accounted for. It is thus necessary to use *in vivo* tumor models to assess the efficacy of therapeutic agents.

The most common *in vivo* model is the murine xenograft. The mouse strains used for this purpose commonly include classically nude (athymic), which are devoid of T lymphocytes [146], and severe combined immunodeficient (SCID) mice, which lack both B and T lymphocytes [147]. NOD/SCID mice, unlike SCID mice, are also NK cell-deficient. Subcutaneously injecting human cancer cells into an immunodeficient mouse will lead to the growth of tumor at the injection site. This model is commonly used for cancer therapy discovery as it is easy to establish, cost-effective, has tumor latency of a few weeks, and allows the visual monitoring of the tumor growth [148]. However, the use of immunocompromised mouse strains fails to account for the role of the immune system in tumor development and treatment response. Another disadvantage is that the inoculation of a homogenous cell population to form the subcutaneous tumor does not reflect the heterogeneity of real tumors [149].

Syngeneic mouse models are widely used for evaluating the role of the immune system in therapy response. These involve the use of laboratory inbred mice strains like, BALB/c or C57BL/6, which have consistent biological properties and are bio-compatible with murine cancer cell lines. This allows the establishment of subcutaneous murine tumors in immunocompetent mice [56, 85, 86, 150, 151]. However, most of the cell lines that have been successfully used in such models grow very rapidly, allowing only short dosage studies to be conducted [152].

The current trends in *ex vivo* cancer models involve the use of patient-derived xenografts (PDX) and organoids (PDO). PDXs are developed by implanting sections of human tumors in mice, where they grow and simulate the heterogeneity and molecular characteristics of clinical tumors better than cell lines or genetically

modified mouse models [?, 153]. Tumor organoids are established from patient tumor specimens grown *ex vivo* [154]. These cancer models have gained importance for personalized treatment of patients, leading the phasing out of the NCI-60 panel of 60 cancer cell lines by the US National Cancer Institute (NCI). Although PDXs and PDOs have gained importance in recent times, they have several limitations. These include a lack of dose response curve, low throughput for broad drug testing, variability of samples and high cost [154].

2.4 Bacteria in Cancer Immunotherapy

2.4.1 Immune Suppression and Evasion by Tumors

The immune system can be categorized broadly into the innate and adaptive immune system. The innate immune system is the host's first line of defence against any abnormality, such as a foreign body or cancerous growth. It consists of cells like macrophages, neutrophils and NK cells, and is not specific against immunogenic markers. On the other hand, the adaptive immune system, consisting of T and B cells, is highly specialized to target antigens. The relationship between tumors and the immune system is a complex one. The interaction of the tumor with both innate and adaptive immune cells can either stimulate or inhibit cancer growth [155].

Tumors employ various means to evade and suppress anti-tumor immunity. They recruit immune suppressor cells such as regulatory T (Treg) cells and myeloid-derived suppressor cells (MDSCs) which can suppress effector T cell expansion and function [156–159]. Tumors up-regulate the expression of immunosuppressive ligands, like PD-L1 and PD-L2, which induce T cell anergy by ligating with the PD-1 ligands present on their surface [156, 160, 161]. They also secrete cytokines like

IL-10, TGF- β , IL-6 and enzymes like IDO, arginase and iNOS which mediate immune suppression by inhibiting T cell function and disrupting dendritic cell (DC) maturation [157, 162, 163]. The DC maturation pathway has been identified as a good target for immunotherapy, because of the crucial role played by DCs in inducing an antigen-specific antitumor response. As antigen-presenting cells (APCs), DCs can capture and present tumor-associated antigens (TAAs) to stimulate innate and adaptive immune cells in the lymph node and tumor stroma [156]. When DCs are properly matured, they can present TAAs in an immunogenic manner and activate effector T cells to mediate a protective antitumor response. However, the disruption of DC maturation leads to TAAs being presented by immature DCs in a tolerizing manner and cause the deletion of effector T cells and the expansion of Treg cells [156, 164]. Tumor cells are known to manipulate DC functionality by producing factors such as VEGF [156, 165–167], TGF- β [168], IL-10 [169], IL-6 and MCS-F [170] which disrupt the functional maturation of DCs.

2.4.2 Bacterial Immunotherapeutic Agents

Immunotherapy of cancer involves the stimulation of the immune system to eradicate cancer cells. The principle of bacterial immunotherapy can be traced back to the use of Coley's toxins which aimed to induce an immune response against tumors.

Since the late 1980s, oncologists were successfully using the vaccine variant of *Mycobacterium bovis* BCG (Bacille Calmette-Guerin) as agent to prevent relapses of bladder cancer after surgically removing solid tumors [171]. Although the exact mechanism is not fully understood, it is likely that the bacteria enhance the immune response against the cancer cells by the activation of natural killer cells [172–175].

Recent advances in cancer immunotherapy involve the blocking of immunesup-

pressive checkpoints, such as PD-1, CTLA-4, and TIGIT [176–178]. Several commensal bacteria have been found to enhance the efficacy of these treatments [179, 180].

Another advancement of bacterial immunotherapy has been the result of a study conducted in 2004 which administered *C. nyovi*-NT to tumor-bearing mice [86]. After the obligate anaerobes germinated exclusively in the hypoxic core of colon carcinoma tumors and destroyed the tumor cells, the cured animals rejected a second challenge of the same tumor. This showed that the bacteria activated the immune system of the animals against the tumor cells.

Listeria monocytogenes are intracellular bacteria that can infect phagocytic and non-phagocytic cells, and elicit a significant immune response [181–183]. By engineering *L. monocytogenes* to express viral and tumoral antigens as fusion proteins, these recombinant strains elicit CD4+ and CD8+ responses in mice, which resulted in an increased antitumor effect [184–187].

While there is a precedent and a recognized potential for bacteria to be used as immunotherapeutic agents, there are a limited number of published studies in this field. The findings of this thesis explore the application of engineered bacterial derivatives for the first time and can contribute to furthering the development of bacterial immunotherapeutic agents.

Chapter 3

Screening of Bacterial Derivatives for Anti-Cancer Effect

3.1 Introduction

As discussed in Chapter 1, bacterial cancer therapy has limitations which have prevented its acceptance as a mainstream treatment modality for cancer. The administration of anaerobic bacteria as spores or live bacteria poses a high risk of infections and toxicity. Previous studies using bacterial spores to treat tumor-bearing animals resulted in their death due to bacterial infections or the toxicity of the bacteria [55,56,188]. This chapter seeks to address these gaps by developing bacterial therapeutic agents that are safer for use and effective in exerting an anti-cancer effect.

The selection of bacteria is the first crucial step in developing safe and effective bacterial therapeutic agents. *C. sporogenes*, a sporulating, Gram-positive strain of obligate anaerobic bacteria, were selected for this purpose for two reasons. Firstly,

they are non-pathogenic and non-toxic bacteria, classified the American Type Culture Collection (ATCC) as a harmless biosafety level 1 organism. These bacteria are found in soil and is also widely distributed in the intestines of humans and animals [97]. It has also been found to be non-toxic to humans when injected systemically with the number of spores ranging from 1×10^6 to 1.5×10^9 [54]. This makes it a safer candidate for use in bacterial cancer therapy, as it addresses the problem of toxicity. Secondly, *C. sporogenes* have been found to exert an oncolytic effect when administered to solid tumors [69, 70, 102, 103]. This shows that the bacteria possess natural anti-cancer properties.

The next step is addressing the problem of infections posed by the use of spores or live bacteria. This chapter aims to derive non-viable forms of *C. sporogenes* that are incapable of proliferation and metabolic activity. Heat-treatment has long been used to inactivate bacteria present in food by killing the bacterial cells [111, 189, 190]. This method of inactivating bacteria was applied to *C. sporogenes* to generate a non-viable derivative, heat-inactivated bacteria (IB), that minimizes the risk of infection. Another derivative of the bacteria is the supernatant of the bacterial culture which contains all the secreted bacterial products. The supernatant, known as conditioned medium (CM), of other clostridial strains was tested previously for their anti-cancer properties [118, 120, 191]. conditioned medium is bacteria-free and thus minimizes the risk of infection. The conditioned medium of *C. sporogenes* was tested for its anti-cancer properties for the first time in this chapter.

In this chapter, the efficacy of IB and CM as anti-cancer agents was screened against multiple human cancer cells lines. We hypothesized that IB and CM exert a differential anti-cancer effect. Renal (A498), melanoma (G361), colorectal (HCT116), hepatic (HepG2), breast (MCF-7) and urinary bladder (RT4) cancer cell lines were used. The purpose of testing IB and CM on different types of cancer

cells is to determine whether the non-viable derivatives of *C. sporogenes* have an affinity to a particular type of cancer. This will enable the targeted application of IB and CM to the cancer type that responds the best to treatment. IB and CM were first administered to 2-dimensional (2D) monolayer models of each cell line for 72 hours and the proliferation of cells was measured. A 3D *in vitro* model of each cancer cell line was then established for the screening of IB and CM effect. This is because it has been suggested in literature that cells grown in a 2D monolayer are more susceptible to drug treatment and thus not a reliable *in vitro* model [192, 193]. A 3D spheroid model is a more physiologically relevant platform for testing the effect of therapeutic agents [142]. IB and CM were tested on the 3D spheroids of each cell line over 72 hours and the viability and size of spheroids was quantified. The aim was to choose the most suitable human cancer cell line to further test IB and CM as anti-cancer agents.

3.2 Materials and Methods

3.2.1 Bacterial Culture and Derivation of Inactivated Bacteria and Conditioned Medium

The *C. sporogenes* spores (ATCC 13732), purchased from American Type Culture Collection (ATCC, USA) were inoculated in Reinforced Clostridial Media (RCM) (Oxoid, UK), in a 1:1000 ratio. An anaerobic chamber was set-up following the protocol of the GasPakTM EZ Anaerobic Container System (Becton, Dickinson and Company, USA). The bacterial culture was placed in the anaerobic chamber and incubated at 37 °C for 48 hours.

The bacterial culture was then harvested from the anaerobic chamber and heat-inactivated by placing it in an 80 °C water bath for 2 hours. After heat-inactivation,

the turbidity of the bacterial suspension was measured using UV-vis spectrophotometer (VWR, USA) to quantify the optical density (OD) of bacteria present in a 1 ml sample of the culture at 600 nm [85]. The culture was centrifuged at 2500 g for 20 minutes [118] to form the pellet of IB. After the supernatant was discarded, the IB pellet was re-suspended in complete growth media of cancer cells for use in the experiments. The optical density of the re-suspended IB were measured again as described previously. The bacteria were then enumerated by using a bacteria counter (Thomas Scientific, USA). A 1 ml suspension of IB in complete growth media contained $2.00 \pm 0.32 \times 10^7$ bacterial cells, when measured at OD₆₀₀.

For the derivation of CM, the spores were cultured in RCM using the same protocol as described above. After 48 hours, the bacterial culture was removed from the anaerobic chamber and centrifuged at 2500 g for 20 minutes to pellet the bacteria. The supernatant was collected and filtered using a 0.20 µm filter. The result is defined as CM.

3.2.2 Re-inoculation of Inactivated Bacteria and Conditioned Medium

To test the non-viability of the bacterial derivatives, IB and CM were added to RCM and complete growth media in a 1:1000 ratio. *C. sporogenes* spores were used as the positive control for re-inoculation. RCM and complete growth media were used as negative controls. Two sets of each inoculated media were prepared; one set of the inoculated media was placed in the anaerobic chamber at 37 °C, and the other was kept at cell culture conditions of 37 °C, 5% CO₂ for 48 hours. The media from each sample was collected and tested for bacterial growth by measuring the turbidity as described in Section 3.2.1.

3.2.3 Bacterial Viability Assay

IB were subjected to a bacterial viability test to confirm the effectiveness of the heat-inactivation process. The Viability/Cytotoxicity Assay kit for Bacterial Live & Dead Cells (Biotium, USA), was used to stain the IB using fluorescent nucleic acid dyes following the manufacturer's protocol. DMAO is a green dye that stains both live and dead bacteria while Ethidium Homodimer-III (EthD-III) is a red dye that only stains dead bacteria. Images were obtained using a fluorescence microscope (Olympus LX71, Japan).

3.2.4 Spore Staining of Inactivated Bacteria

Smears of IB were prepared and allowed to air-dry before heat-fixing. The slides were flooded with 2% malachite green, and heated to steam for 2 minutes. The excess was washed off with water and then counter-stained with 0.1% carbol fuchsin for 2 minutes. The slides were then rinsed with water, blot-dried and imaged using a bright-field microscope (Olympus BX51, Japan).

3.2.5 Cell Culture

The human cancer cell lines and their respective growth media are listed in Table 3.1. The selection of these cell lines was based on the commonly used cell lines of each cancer type in therapy testing. The preliminary list of cell lines was tested for 2D and 3D spheroid formation. If the cell lines were able to successfully form 3D spheroids, they were used for the experiments that followed. All the cell lines were sourced from the ATCC (USA). The complete growth media were supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, USA) and 1% Penicillin-streptomycin (Gibco, USA). The cells were cultured in a humidified atmosphere at 37 °C, 5% CO₂. The cells were seeded in 96-well tissue culture plates at a density of 5×10^3

cells/well and cultured overnight before being used as the 2D monolayer models for the experiments.

Table 3.1: Human cancer cell lines used

Cell Line	ATCC Number	Cancer Type	Growth Media
A498	HTB-44	Kidney Carcinoma	Eagle's Minimum Essential Medium (Gibco, USA)
G361	CRL-1424	Malignant Melanoma	McCoy's 5A medium (Sigma-Aldrich, USA)
HCT116	CCL-247	Colorectal Carcinoma	McCoy's 5A medium (Sigma-Aldrich, USA)
HepG2	HB-8065	Hepatocellular Carcinoma	Eagle's Minimum Essential Medium (Gibco, USA)
MCF-7	HTB-22	Breast Adenocarcinoma	Eagle's Minimum Essential Medium (Gibco, USA)
RT4	HTB-2	Urinary Bladder Transitional Cell Papilloma	McCoy's 5A medium (Sigma-Aldrich, USA)

3.2.6 3-Dimensional *In Vitro* Spheroid Model

The cancer cells were seeded at a density of 5×10^3 cells/well in round-bottomed, ultra-low adhesion 96-well plates (Corning, USA). The plates were centrifuged at 1000 g for 10 minutes using a plate centrifuge (Thermo Scientific, UK) to facilitate their collection at the bottom of the wells. The 3D spheroid culture was incubated

at 37 °C, 5% CO₂ for a period of 72 hours days to allow the development of stable spheroids with necrotic regions that simulate *in vivo* tumors [194]. After this growth period, the spheroids were used for experiments.

3.2.7 Treatment with Inactivated Bacteria and Conditioned Medium

The IB suspension in complete growth medium was diluted to a concentration of 4×10^7 bacterial cells/ml. The CM sample was prepared as 10% of CM in complete growth media (10% CM). The 2D monolayer and 3D spheroid models were incubated at 37 °C, 5% CO₂ with 200 µl of the 4×10^7 bacterial cells/ml IB, and 10% CM test sample over a 72-hour period. Complete growth media was used the untreated control in all experiments, and 10% RCM in complete growth media was used as a control to 10% CM.

3.2.8 Cell Proliferation Quantification in 2-Dimensional Monolayer Model

Cell proliferation of cancer cells in the 2D model was quantified using alamar-Blue (Invitrogen, USA). Two-dimensional monolayers of cancer cells were exposed to the IB and CM samples for 72 hours, with cell proliferation measured every 24 hours. At each time point, the test sample medium was replaced with fresh complete growth medium. AlamarBlue reagent was added to each well in a 1:10 ratio and the cells were incubated for 2 hours in darkness, at culture conditions. The fluorescence from the samples was measured at 590 nm, after excitation at 544 nm. Proliferation of cells exposed to test samples was expressed as a percentage of control. The absolute values of the proliferation of each cell line's control can be found in Appendix A.2.

3.2.9 Cell Viability Quantification in 3-Dimensional Spheroid Model

Cell viability of the cancer cells in the 3D spheroid model was quantified using CellTiter-Glo® 3D Cell Viability Assay (Promega, USA). 3D spheroids were exposed to IB and CM samples for 72 hours, with cell proliferation measured every 24 hours. At each time point, the test sample medium was replaced with fresh complete growth medium. CellTiter-Glo® reagent was added in a 1:1 ratio, the cells were incubated at room temperature for 30 minutes and luminescence was measured. Viability of cells exposed to test samples was expressed as a percentage of control. The absolute values of the viability of each cell line's control spheroids can be found in Appendix [A.2](#).

3.2.10 Evaluation of Spheroid Size

3D spheroids were exposed to IB and CM samples for 72 hours and spheroid size was quantified every 24 hours. At each time point, the spheroids were imaged using a bright-field microscope (Olympus, Japan) and the area was evaluated using the Image J software.

3.2.11 Statistical Analysis

All experiments were performed in triplicate, for statistical significance. All data were expressed as mean $\pm$ standard deviation (SD) of three separate experiments. Statistical analysis was conducted using unpaired, two-tailed Student's t-test. A p value of less than 0.05 was considered to be statistically significant. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

3.3 Results

3.3.1 Derivation and Characterization of Inactivated Bacteria

The non-toxic and non-pathogenic bacteria, *C. sporogenes*, were selected for this study to minimize the risk of toxicity associated with bacterial cancer therapy. To minimize the risk of infection posed by spore or vegetative bacteria, non-viable forms of *C. sporogenes* were derived. The first derivative was heat-inactivated *C. sporogenes* (IB), obtained by the method illustrated in Figure. 3.1. After culturing the bacteria in anaerobic conditions at 37 °C for 48 hours, they were heat-inactivated for 2 hours at 80 °C. The bacterial pellet was collected after centrifugation at 5000 g for 20 minutes and resuspended in the respective complete growth medium of each cell line. The pH of complete growth medium was 8.18 and it did not alter after IB was resuspended in it.

Figure 3.1: Schematic illustration of the process of deriving IB from the bacterial culture of *C. sporogenes*

A re-inoculation test was conducted to validate the inactivation of the bacteria. IB suspended in RCM and in complete growth media were added to RCM broth at 1:1000 ratio. Both culture broths were kept in anaerobic and in cell culture conditions for 48 hours. The turbidity of the broth was measured to determine whether

there was bacteria growth. Table 3.2 shows that results of the re-inoculation study. The bacterial growth of the positive control, of *C. sporogenes* spores, resulted in turbidity of 3.65×10^7 bacterial cells/ml in the anaerobic chamber and 4.20×10^4 bacterial cells/ml in the cell culture conditions. In comparison, IB in RCM and in complete growth medium had turbidity of 0.00 bacterial cells/ml in both anaerobic and cell culture conditions, indicating that there was no growth of the bacteria in these test samples.

Table 3.2: Turbidity of re-inoculated IB

Inoculum	Turbidity (bacterial cells/ml)	
	Anaerobic Chamber	Cell Culture Condition
IB in RCM	0.00	0.00
IB in complete growth medium	0.00	0.00
Positive control	3.65×10^7	4.20×10^4
Negative control: RCM	0.00	0.00
Negative control: Complete growth media	0.00	0.00

The heat-inactivation of the bacteria was validated by a bacterial viability assay using DMAO and Ethidium homodimer-III (EthD-III) dyes. DMAO can permeate cell membranes of both live and dead bacteria, but EthD-III can only permeate dead bacterial cells. As shown Figure 3.2 (a), live vegetative bacteria were stained green with DMAO. After heat-inactivation, the bacteria in the sample were stained red with EthD-III, further proving that the heat treatment was effective (Figure 3.2 (b)).

Spore staining of IB was conducted to characterize the bacterial derivative. There was no evidence of green-stained bacterial spores present in the sample. The staining showed that the sample consisted only of rod-shaped bacteria stained pink, indicating that heat-inactivation does not lyse the bacteria, but rather remains the rod-like morphology of *C. sporogenes*.

Figure 3.2: Characterization of IB. Fluorescence staining indicating viability of *C. sporogenes* (a) before and (b) after heat inactivation. Live bacterial cells stained green, dead bacterial cells stained red. (c) Inactivated vegetative bacterial cells stained pink with carbol fuchsin.

3.3.2 Derivation and Optimization of Conditioned Medium

The second non-viable derivative of *C. sporogenes* was obtained through the process illustrated in Figure 3.3. *C. sporogenes* was cultured in anaerobic conditions 37 °C for 48 hours and then collected at room temperature. It was then centrifuged at 5000 g for 20 mins to separate the bacteria from the media containing all the secreted products of the bacterial culture. The pellet was discarded, and the supernatant was filtered through a membrane of pore size 0.22 µm to obtain bacteria-free CM.

Figure 3.3: Schematic illustration of the process of deriving CM from the bacterial culture of *C. sporogenes*

To validate that CM was bacteria-free, a re-inoculation test of CM was conducted. CM was added to RCM broth at 1:1000 ratio. The re-inoculated RCM was kept in anaerobic and in cell culture conditions for 48 hours. The turbidity of the broth was measured to determine whether there was bacteria growth. Table 3.3 shows the results of the re-inoculation study. The bacterial growth of the positive control, *C. sporogenes* spores, resulted in turbidity 3.65×10^7 bacterial cells/ml in the anaerobic chamber and 4.20×10^4 bacterial cells/ml in the cell culture conditions. In comparison, CM in RCM and in complete growth medium had turbidity of 0.00 bacterial cells/ml, indicating that there was no growth of the bacteria in these test samples. This showed that CM was bacteria-free and its use for cancer therapy

would minimize the risk of infection.

Table 3.3: Turbidity of re-inoculated CM

	Turbidity (bacterial cells/ml)	
Inoculum	Anaerobic Chamber	Cell Culture Condition
CM	0.00	0.00
Positive control	3.65×10^7	4.20×10^4
Negative control: RCM	0.00	0.00
Negative control: Complete growth medium	0.00	0.00

The optimum concentration of CM to be used for the experiments was determined by first determining the concentration of RCM that would be safe for cells (Figure 3.4 (a)). RCM in complete growth media, at concentrations of 10%, 20% and 50%, was added to HCT116 cells for 72 hours. 20% and 50% RCM were cytotoxic to the cells, with cell proliferation dropping to 19.79% and 11.21% of control after 24 hours, respectively. After 72 hours, the cell proliferation had decreased to 15.12% and 1.67% due to 20% and 50% RCM, respectively. 10% RCM caused a slight decrease in cell proliferation to 66.06% after 72 hours. This concentration of RCM was tolerated by the cells and was taken as the maximum concentration of the bacterial broth that could be administered to the cells.

Subsequently, the optimum concentration of CM was determined by exposing HCT116 cells to 1%, 5% and 10% CM in complete growth media for 72 hours (Figure 3.4 (b)). The 1% and 5% concentrations of CM did not cause any significant decrease in cell proliferation. 10% CM inhibited cell proliferation to 61.5%, 27.00%

and 20.3% of control, after 24, 48 and 72 hours, respectively. Thus, 10% CM was used in all the experiments.

Figure 3.4: Optimization of CM concentration. Cell proliferation of HCT116 cells exposed to varying concentrations of (a) RCM and (b) CM. Data expressed as mean $\pm$ SD. t-test is a comparison with control. (***) $p < 0.001$

3.3.3 Screening of Efficacy of Inactivated Bacteria and Conditioned Medium Using 2-Dimensional Monolayer Model

The bacterial derivatives, IB and CM, were screened for their *in vitro* anti-cancer effect on multiple human cancer cells, described in Table 3.1. The inhibitive effect of heat-inactivated *C. sporogenes* (IB) was tested against each cell line cultured in 2D monolayers (Figure 3.5). IB, at a concentration of 4×10^7 bacterial cells/ml, were added to the cells for 72 hours and cell proliferation was measured every 24 hours. IB showed inhibitive activity against melanoma (G361), colorectal (HCT116), hepatic (HepG2) and breast (MCF-7) cancer cell lines in a time dependent manner. The cell proliferation decreased to 1.01%, 2.31%, 44.39% and 32.78% of control after 72 hours. G361 and HCT116 were observed to be most susceptible to IB. The renal cancer cell line A498 was unresponsive to IB, with the cell proliferation remaining similar to that of the control. The bladder cancer cell line RT4 showed a significant decrease in cell proliferation to 68.00% after 72-hour exposure to IB.

Figure 3.5: Effect of IB in 2D monolayer model. Cell proliferation of human cancer cell lines exposed to 4×10^7 bacterial cells/ml IB over 72 hours, expressed as a percentage of their respective controls. Data expressed as mean $\pm$ SD. t-test is a comparison with control. (***) $p < 0.001$. A498 = kidney carcinoma, G361 = malignant melanoma, HCT116 = colorectal carcinoma, HepG2 = hepatocellular carcinoma, MCF-7 = breast adenocarcinoma, RT4 = urinary bladder transitional cell papilloma

CM had an inhibitive effect on 2D models of five of the human cancer cell lines: G361, HCT116, HepG2, MCF-7 and RT4 (Figure 3.6 (a)). HCT116 cells were the most sensitive to CM exposure, with cell proliferation decreasing to 23.1% after 24 hours and then to 0.59% after 72 hours. G361 and HepG2 cell proliferation decreased to 5.41% and 17.9% after 72 hours, respectively. The decrease in cell proliferation of MCF-7 and RT4 was not significant for the first 24 hours. However, by the end of study after 72 hours, the cell proliferation of MCF-7 and RT4 had decreased to 24.4% and 55.3%, respectively. Exposure to CM did not have an inhibitive effect on A498 cells. The cell proliferation of these cells was not altered significantly after CM exposure for 72 hours.

Figure 3.6: Effect of CM in 2D monolayer model. Cell proliferation of human cancer cell lines exposed to (a) 10% CM and (b) 10% RCM over 72 hours, expressed as a percentage of their respective controls. Data expressed as mean $\pm$ SD. t-test is a comparison with control. (***) $p < 0.001$). A498 = kidney carcinoma, G361 = malignant melanoma, HCT116 = colorectal carcinoma, HepG2 = hepatocellular carcinoma, MCF-7 = breast adenocarcinoma, RT4 = urinary bladder transitional cell papilloma

RCM was used as a control for CM and it did not have an inhibitive effect on

any of the cell lines except G361 HCT116 (Figure 3.6 (b)). The decrease in cell proliferation of G361 due to RCM was only significant at 72 hours, yet not to the same extent as that due to CM. The decrease in cell proliferation of HCT116 due to RCM was not significant, and not to the same extent as that induced by CM.

3.3.4 Generation of 3-Dimensional Spheroids

A 3D *in vitro* platform for screening the efficacy of IB and CM was established as described previously [142]. 3D spheroids simulating *in vivo* tumors were generated through the method illustrated in Figure 3.7. 5×10^3 cells were seeded in each well. The use of ultra-low adhesion, round-bottomed well-plates and centrifugation allowed the formation of monocellular spheroids without the addition of an adhesive substance or scaffold. Due to the ultra-low adhesion coating of the well-plate, the cells were unable to adhere to the substrate. Instead, the cells formed tight cell-cell interactions with result in the development of the 3D structure of the spheroid. A key benefit of this method was that it allowed the high-throughput generation of 3D spheroids.

Figure 3.7: Schematic illustration of the process of generating 3D spheroids of the human cancer cell lines

The spheroids were cultured at 37 °C, 5% CO₂ for 6 days Figure 3.8. Upon centrifugation, the cells collected at the bottom of the well. Within the first 24 hours in culture, the cells began to form cell-cell contacts that tightly held the 3D structure together. As the time in culture increased, the 3D spheroids of HCT116, HepG2,

MCF-7 and RT4 grow larger in size. HCT116 and MCF-7 form uniform spherical spheroids, while HepG2 and RT4 are non-uniformly shaped. A498 spheroids grow more compact and smaller in size as time in culture increases. All the spheroids maintained their structural integrity over the 6 days in culture. These spheroids were cultured for 3 days at 37 °C, 5% CO₂ before being used as 3D screening platforms for the experiments.

Figure 3.8: 3D spheroid model of human cancer cell lines. Growth of spheroids with initial seeding density of 5×10^3 cells/well over 6 days. Scale bar = 200 μ m. A498 = kidney carcinoma, HCT116 = colorectal carcinoma, HepG2 = hepatocellular carcinoma, MCF-7 = breast adenocarcinoma, RT4 = urinary bladder transitional cell papilloma.

G361 cells were found to be unable to form 3D spheroids. The cells formed loose, flattened clusters at the bottom of the well as shown in Figure 3.9. These clusters disintegrated completely when the media in the wells was changed. Thus,

they were unsuitable for use in the subsequent experiments involving the test of IB and CM on 3D spheroids of the cancer cell lines. G361 cells were excluded from experiments after this point.

Figure 3.9: 3D spheroid model of G361 cell line. Initial seeding density of 5×10^3 cells/well, grown over 6 days. Scale bar = 200 μm .

3.3.5 Screening of Efficacy of Inactivated Bacteria and Conditioned Medium Using 3-Dimensional Spheroid Model

3.3.5.1 Effect of Inactivated Bacteria on 3-Dimensional Spheroid Model

To test the effect of IB on a more complex *in vitro* model, 3D spheroid models of human cancer cell lines were developed as described in Section 3.2.6. The spheroids of renal (A498), colorectal (HCT116), hepatic (HepG2), breast (MCF7) and urinary bladder (RT4) cancer cell lines were exposed to IB for 72 hours. The effect of IB was analysed by quantifying the viability of the cells in the spheroids and measuring the size of the spheroids every 24 hours.

Figure 3.10 shows the effect of IB on A498 spheroids. IB treatment did not cause a decrease in the viability of these spheroids (Figure 3.10 (a)). The viability of IB-treated spheroids remained similar to that of the control at all time points. The progression of A498 spheroids is shown in Figure 3.10 (b) and (c). The control A498 spheroids grew compact and decreased in size over the course of 72 hours in culture. In contrast, the IB-exposed spheroids were larger in size than the control spheroids over the course of 72 hours. The structure of the A498 spheroids started to get deformed and resulted in a loss of shape. Taken together, the evidence does

not suggest that IB had an inhibitive effect of A498 spheroids.

Figure 3.10: Effect of IB on A498 3D spheroid model. (a) Viability of 3D spheroids exposed to 4×10^7 bacterial cells/ml IB over 72 hours, expressed as a percentage of their respective controls. (b) Size of spheroids exposed to IB evaluated by measuring area of spheroids imaged at the 0, 24, 48 and 72-hour time points. Data expressed as mean $\pm$ SD. (c) Phase contrast imaging of spheroids exposed to IB.

HCT116 spheroid growth was inhibited by IB treatment. as shown in Figure 3.11. The viability of HCT116 spheroids significantly decreased in a time-dependent manner after exposure to IB (Figure 3.11 (a)). After 72 hours, the viability had decreased to 25.46% of control. This inhibition of HCT116 spheroids was also

reflected in the stunting of their growth (Figure 3.11 (b), (c)). While the control spheroids continued to grow larger in size, the IB-treated spheroid growth was slower and resulted in them being significantly smaller after 72 hours.

Figure 3.11: Effect of IB on HCT116 3D spheroid model. (a) Viability of 3D spheroids exposed to 4×10^7 bacterial cells/ml IB over 72 hours, expressed as a percentage of their respective controls. (b) Size of spheroids exposed to IB evaluated by measuring area of spheroids imaged at the 0, 24, 48 and 72-hour time points. Data expressed as mean $\pm$ SD. t-test is a comparison with control. (***) $p < 0.001$ (c) Phase contrast imaging of spheroids exposed to IB.

IB had an inhibitive effect on HepG2 spheroids too. The viability of IB-treated

HepG2 spheroids was significantly lower than the control spheroids as all time points (Figure 3.12 (a)). The size of IB-treated spheroids was significantly lower than that of the control spheroids (Figure 3.12 (b), (c)). However, at 72 hours, the cell viability and spheroid area was starting to increase. This suggests that the inhibitive effect of IB was no longer as strong as the first 48 hours of the experiment.

The effect of IB treatment on MCF-7 spheroids was inhibitive too. The viability of MCF-7 spheroids decreased in a significant, time-dependent manner after IB treatment. After 72 hours, viability of IB-treated spheroids decreased to 25.43% of control (Figure 3.13 (a)). However, the decrease in the spheroid size after treatment with IB (Figure 3.13 (b)) was not significant. IB treatment was also found to cause a slight deformation of the spheroid shape (Figure 3.13 (c)).

IB treatment of RT4 spheroids cause a time-dependent decrease in viability (Figure 3.14 (a)). However, the effect on spheroid size was contrary. The IB-treated spheroids were had larger sizes than the control spehroids, as shown in Figure 3.14 (b) and (c). The results of testing IB on RT4 spheroids were inconclusive in proving the inhibitive effect of IB.

Figure 3.12: Effect of IB on HepG2 3D spheroid model. (a) Viability of 3D spheroids exposed to 4×10^7 bacterial cells/ml IB over 72 hours, expressed as a percentage of their respective controls. (b) Size of spheroids exposed to IB evaluated by measuring area of spheroids imaged at the 0, 24, 48 and 72-hour time points. Data expressed as mean $\pm$ SD. t-test is a comparison with control. (***) $p < 0.001$) (c) Phase contrast imaging of spheroids exposed to IB.

Figure 3.13: Effect of IB on MCF-7 3D spheroid model. (a) Viability of 3D spheroids exposed to 4×10^7 bacterial cells/ml IB over 72 hours, expressed as a percentage of their respective controls. Data expressed as mean $\pm$ SD. t-test is a comparison with control. (***) $p < 0.001$) (b) Size of spheroids exposed to IB evaluated by measuring area of spheroids imaged at the 0, 24, 48 and 72-hour time points. (c) Phase contrast imaging of spheroids exposed to IB.

Figure 3.14: Effect of IB on RT4 3D spheroid model. (a) Viability of 3D spheroids exposed to 4×10^7 bacterial cells/ml IB over 72 hours, expressed as a percentage of their respective controls. Data expressed as mean $\pm$ SD. t-test is a comparison with control. (***) $p < 0.001$) (b) Size of spheroids exposed to IB evaluated by measuring area of spheroids imaged at the 0, 24, 48 and 72-hour time points. (c) Phase contrast imaging of spheroids exposed to IB.

3.3.5.2 Effect of Conditioned Medium on 3-Dimensional Spheroid Model

The spheroids of renal (A498), colorectal (HCT116), hepatic (HepG2), breast (MCF-7) and urinary bladder (RT4) cancer cell lines were exposed to 10% CM for 72 hours. The effect of CM was analysed by quantifying the viability of the cells

in the spheroids and measuring the size of the spheroids every 24 hours.

Figure 3.15 shows the effect of IB on A498 spheroids. CM caused a slight, non-significant decrease in the viability of the spheroids over 72 hours. the effect of RCM on viability was similar to that of that control (Figure 3.15 (a)). The change in size of A498 spheroids is shown in Figure 3.15 (b) and (c). The control A498 spheroids grew compact and decreased in size over the course of 72 hours in culture . The progression of the CM-exposed spheroids was similar to that of the control for 48 hours, after which the size of spheroids increased. RCM spheorids were larger than the control spheroids for the entire duration of the study. However, the differences in spheroid sizes of each test group were not significant.

CM-mediated inhibition was the greatest and most significant in HCT116 spheroids (Figure 3.16). There was a significant, time-dependent decrease in viability of spheroids exposed to CM. After 72 hours, the viability of CM-exposed HCT116 spheroids decreased to 27.94% of the control (Figure 3.16 (a)). There was also a significant decrease in spheroid size due to CM-treatment. CM-treated spheroids grew slower than the control and RCM-exposed spheroids, such that they were significantly smaller after 72 hours. While RCM-exposed spheroids were slightly smaller than control spheroids, the difference was not significant and not to the same extent as that of the CM-treated spheroids (Figure 3.16 (b) and (c)).

Figure 3.15: Effect of CM on A498 3D spheroid model. (a) Viability of 3D spheroids exposed to CM over 72 hours, expressed as a percentage of their respective controls. (b) Size of spheroids exposed to IB evaluated by measuring area of spheroids imaged at the 0, 24, 48 and 72-hour time points. Data expressed as mean $\pm$ SD. (c) Phase contrast imaging of spheroids exposed to IB. RCM used as a broth control. Both CM and RCM administered as 10% of complete growth medium.

Figure 3.16: Effect of CM on HCT116 3D spheroid model. (a) Viability of 3D spheroids exposed to CM over 72 hours, expressed as a percentage of their respective controls. (b) Size of spheroids exposed to IB evaluated by measuring area of spheroids imaged at the 0, 24, 48 and 72-hour time points. Data expressed as mean $\pm$ SD. t-test is a comparison with control. (***) $p < 0.001$ (c) Phase contrast imaging of spheroids exposed to IB. RCM used as a broth control. Both CM and RCM administered as 10% of complete growth medium.

The inhibitive effect of CM on viability of HepG2 spheroids was only significant after 48 hours in culture (Figure 3.17 (a)). The size of HepG2 spheroids also significantly decrease after treatment with CM (Figure 3.17 (b)). CM treatment also altered the morphology of the spheroids(Figure 3.17 (c)). RCM did not cause a decrease in spheroid viability and size.

The decrease in MCF-7 viability was non-significant at 24 hours, but was greater and significant at 48 and 72 hours (Figure 3.18 (a)). RCM-exposure did not have a significant effect on spheroid viability. Figure 3.18 (b) and (c) show the progression of MCF-7 spheroids. The control and RCM spheroids increased in size over 72 hours, with latter being larger than the former. The growth of the CM-treated spheroids was stunted and they were significantly smaller than the control spheroids by the end of the study.

Figure 3.19 (a) showed that CM significantly inhibited the viability of RT4 spheroids in a time-dependent manner. The size of RT4 spheroids exposed to CM was similar to that of the control. However, the spheroids exposed to RCM were larger in size than the control (Figure 3.19 (b) and (c)). The results of the effect of CM on RT4 spheroids were inconclusive.

Figure 3.17: Effect of CM on HepG2 3D spheroid model. (a) Viability of 3D spheroids exposed to CM over 72 hours, expressed as a percentage of their respective controls. (b) Size of spheroids exposed to IB evaluated by measuring area of spheroids imaged at the 0, 24, 48 and 72-hour time points. Data expressed as mean $\pm$ SD. t-test is a comparison with control. (***) $p < 0.001$ (c) Phase contrast imaging of spheroids exposed to IB. RCM used as a broth control. Both CM and RCM administered as 10% of complete growth medium.

Figure 3.18: Effect of CM on MCF-7 3D spheroid model. (a) Viability of 3D spheroids exposed to CM over 72 hours, expressed as a percentage of their respective controls. (b) Size of spheroids exposed to IB evaluated by measuring area of spheroids imaged at the 0, 24, 48 and 72-hour time points. Data expressed as mean $\pm$ SD. t-test is a comparison with control. (** $p < 0.01$, *** $p < 0.001$) (c) Phase contrast imaging of spheroids exposed to IB. RCM used as a broth control. Both CM and RCM administered as 10% of complete growth medium.

Figure 3.19: Effect of CM on RT4 3D spheroid model. (a) Viability of 3D spheroids exposed to CM over 72 hours, expressed as a percentage of their respective controls. t-test is a comparison with control. (***) $p < 0.001$ (b) Size of spheroids exposed to IB evaluated by measuring area of spheroids imaged at the 0, 24, 48 and 72-hour time points. Data expressed as mean $\pm$ SD. (c) Phase contrast imaging of spheroids exposed to IB. RCM used as a broth control. Both CM and RCM administered as 10% of complete growth medium.

3.4 Discussion

Heat-treatment is used to inactivate bacteria in food to prevent infections. A similar approach was adopted to inactivate *C. sporogenes* to minimize the risk of infection. Previous studies used heat-treated bacteria as controls of live bacteria or spores being tested for their effect as safer cancer therapeutic agents [85, 115, 195, 196]. In these studies, the inactivation temperatures ranged between 60 °C to 121 °C, for a time period up to 2 hours. Using the optimum inactivation temperature is crucial. If the temperature is too low, the bacteria may not be inactivated completely and may still be able to proliferate. If the temperature is too high, it may cause a denaturation of the protein content or lyse the bacteria. For the purpose of this research, *C. sporogenes* had to be inactivated while still retaining the natural anti-cancer property. Denaturation of surface molecules or lysing of the bacteria could affect the anti-cancer property. Based on existing literature about the heat-inactivation of Gram-positive bacteria [197, 198], *C. sporogenes* were heated at 80 °C to render it incapable of replication. This was confirmed by the re-inoculation test, where IB did not grow in RCM broth in either anaerobic conditions or cell culture conditions, and the viability staining assay. The re-inoculation test was carried out in cell culture conditions to confirm that the bacteria will not regrow during the experiments testing the effect of IB and CM on cancer cells stored at 37 °C, 5% CO₂. The staining of IB confirmed that heat-treatment at 80 °C does not lyse the bacteria but allows IB to retain their rod-like morphology. The second non-viable derivative of *C. sporogenes*, CM, was filtered through a 0.22 µm filter to remove all bacteria. This served to minimize the risk of infection which was validated by the re-inoculation test. A range of concentrations of CM were tested on cells and the results showed that 10% CM in complete growth media was the optimum concentration for testing against mammalian cells. Taken together,

both re-inoculation tests showed that the derivation of IB and CM generated a form of *C. sporogenes* that was a safer alternative to spores or live bacteria and would minimize the risk of infection.

The efficacy of IB and CM as anti-cancer agents was tested against various human cancer cell lines; renal (A498), melanoma (G361), colorectal (HCT116), hepatic (HepG2), breast (MCF-7) and urinary bladder (RT4) cancer. In the 3D spheroid models of MCF7 (Figure 3.13) and RT4 treated with IB (Figure 3.14), and RT4 spheroids treated with CM (Figure 3.19), there is a discrepancy between the viability and spheroid size measured. In these tests, the viability of cancer cells significantly decreased after treatment, but there was no significant effect on spheroid size. While viability is a measure of the total number of viable cells in the culture, spheroid size measurements reflect the viable and dead cell as well as the ECM of the spheroids. In the case of the MCF7 spheroids treated with IB and RT4 spheroids treated with IB and CM, the decrease in viability showed that the bacterial derivatives had a cytotoxic effect on the respective cells. However, lack of significant change in spheroid sizes could be because the dead cells remained in the spheroid or because the ECM was intact and the retained overall structure of the spheroid.

The diffusion limitation of the 3D spheroids, which are characterized by the oxygen gradient, nutrient distribution and waste accumulation, would also apply to drug or treatment agent penetration [140, 199, 200]. As the size of IB (1 - 10 μm) is considerably larger than that of the molecular components of CM, the penetration of IB to the bulk of cells in the inner regions of the spheroids would be limited. This could have reduced the inhibitive effect of IB on reducing spheroid size, as compared to that of CM.

Figure 3.20 (a) and (b) depict the effect of IB on the cell proliferation in 2D and

viability in 3D models of cancer as a function of time, respectively. Meanwhile, figure 3.20 (c) and (d) depict the effect of CM on the cell proliferation in 2D and viability in 3D models of cancer as a function of time, respectively. It is notable that the efficacy of both IB and CM against the various cell lines was not uniform. The difference in the relative efficacy of the bacterial derivatives is evident in the varying gradients of each graph line. This indicates that the anti-cancer effect of IB and CM was not based on indiscriminate cytotoxicity. The inhibitive effect is stronger against some cell lines, and limited against others. This could be due to genotype or phenotype of the cell types. This lends weight to our claim that IB and CM exert a differential anti-cancer effect against various cancer cell types. This finding was analyzed further to identify the cancer type that is most susceptible to treatment via IB and CM.

Figure 3.20: Comparison of IB and CM effect on human cancer cell lines. Effect of IB on (a) cell proliferation in 2D and (b) viability in 3D model of human cancer cells. Effect of CM on (c) cell proliferation in 2D and (d) viability on 3D model of human cancer cells. Data expressed as mean $\pm$ SD. A498 = kidney carcinoma, G361 = malignant melanoma, HCT116 = colorectal carcinoma, HepG2 = hepatocellular carcinoma, MCF-7 = breast adenocarcinoma, RT4 = urinary bladder transitional cell papilloma

One of the cell lines had to be selected for further investigation of the anti-cancer properties of the bacterial derivatives. The cell lines were ranked according to their response and suitability to IB and CM treatment (Table 3.4). Each cell line was assigned a ranking scored based on the decrease in cell proliferation (2D model) and viability (3D model) due to IB and CM. A significant decrease in spheroid size was scored 1 while a non-significant change in spheroid size was scored 0. The total of the scores was tabulated and the cell lines were ranked for suitability for

bacterial derivative treatment. As G361 cells did not form 3D spheroids, they were unsuitable for use in future experiments. As shown in Table 3.4, colorectal cancer cells, HCT116, scored the highest and were ranked first. This showed that IB and CM has the highest affinity towards these cells. It is notable that *C. sporogenes* is localized in the gut of animals and humans. Thus, its derivatives, IB and CM, are likely to be tolerated better *in vivo* as therapeutic agents targeting colorectal cancer. Therefore, the colorectal cancer cell line HCT116, was selected for further testing the anti-cancer effect of IB and CM in the forthcoming chapters.

Table 3.4: Ranking of treatment response of human cancer cell lines

Cell Line	A498	G361	HCT116	HepG2	MCF-7	RT4
IB - 2D Proliferation	1	6	5	3	4	2
IB - 3D Viability	1	NA	5	2	4	3
IB - 3D Spheroid Size	0	NA	1	1	0	0
CM - 2D Proliferation	1	5	6	4	3	2
CM - 3D Viability	1	NA	4	2	5	3
CM - 3D Spheroid Size	0	NA	1	1	1	0
Total Score	4	NA	22	13	17	10
Ranking	V	NA	I	III	II	IV

3.5 Conclusion

The non-toxic, non-pathogenic *C. sporogenes*, were selected for the generation of non-viable bacterial derivatives, IB and CM. The anti-cancer efficacy of these derivatives was screened against renal, melanoma, colorectal, hepatic, breast and urinary bladder cell lines. There are three key findings in this chapter: (1) IB and CM are incapable of bacterial proliferation and re-growth, thus minimizing the risk of infection. (2) IB and CM retain the natural anti-cancer properties of *C. sporogenes* and selectively inhibit the proliferation and viability of multiple human cancer cell lines in 2D and 3D *in vitro* models. (3) The anti-cancer effect of IB and CM is the strongest against the colorectal cancer cell line, HCT116. This cell line was chosen to be used in to further investigate the anti-cancer effect of IB and CM. The selection of *C. sporogenes* and the derivation of IB and CM minimized the risk of toxicity and infection. The next two chapters investigate the nature of inhibitive effect exerted by IB and CM on colorectal cancer cells.

Chapter 4

Effect of Inactivated Bacteria on Colorectal Cancer Cells *In Vitro*¹

4.1 Introduction

In the previous chapter, two non-viable derivatives of *Clostridium sporogenes* were developed to address the problem of infection and toxicity that is associated with the use of live bacteria and spores in bacterial cancer therapy. It was shown that heat-inactivated bacteria (IB) were incapable of re-growth. Thus as an inactive, non-viable form of bacteria, IB are a safer alternative to live bacteria or spores, with the potential to be a potent anti-cancer therapeutic agent.

Another limitation of live bacteria and spores is their hypoxia-dependence. These forms of bacteria can only exert an inhibitive effect in the hypoxic core of tumors. This leaves a rim of viable cancer cells at the periphery of tumor, which is normoxic. This increases the chance of tumor recurrence [67, 70, 72, 81]. Thus, there is a need

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for the development of a bacterial therapeutic agent whose anti-cancer efficacy is not dependent on the presence of hypoxia.

The underlying mechanism of bacterial anti-cancer activity is poorly understood [56]. It has been previously suggested that spores and live bacteria may secrete enzymes as they germinate and proliferate, respectively, which inhibit the growth of cancer cells [86]. However, there is inconclusive evidence to prove this. Understanding the nature of IB-mediated inhibition of cancer cells will be crucial for its development as a therapeutic agent.

The inhibitive effect of IB was further investigated *in vitro* using colorectal cancer cells in this chapter. In chapter 3, the colorectal cancer cells, HCT116, were shown to be the most responsive to IB and conditioned medium (CM). Additionally, since *C. sporogenes* are found in the gut of humans and animals [100], they are expected to be better tolerated *in vivo* as a therapeutic agent for colorectal cancer. In addition to the use of the human colorectal cancer cell line, HCT116, a murine cell line, CT26, was also used in this chapter to investigate the inhibitive effect of IB. The use of CT26 cells here would allow the easy progression to *in vivo* tumor models in immunocompetent animals in chapter 6. Aside from testing IB on 2D monolayer cultures, they were also tested on a physiologically relevant platform of an *in vitro* 3D spheroid model for colorectal cancer. Histological analysis of the 3D spheroids was conducted to validate the simulation of the necrotic region at the core of *in vivo* solid tumors. The 2D and 3D models were kept at normoxic, cell culture conditions and exposed to IB. The purpose of this is to investigate IB efficacy in normoxic conditions. We hypothesized that the anti-cancer effect of IB is hypoxia-independent. In this chapter, colorectal cancer cells were exposed to varying concentrations of IB to determine whether its efficacy is dose-dependent. The inhibitive effect of IB on both colorectal cancer cell lines in 2D and 3D models

was quantified by evaluating the cell proliferation, cell viability and spheroid size. The interaction of IB and cancer cells, the nature of IB-mediated cell death and the effect of IB on the extracellular matrix (ECM) of the spheroids were investigated. The aim was to achieve an improved understanding of the underlying mechanism of IB-mediated anti-cancer effect.

4.2 Materials and Methods

4.2.1 Cell Culture and *in vitro* Cancer Models

The CT26 murine colorectal cancer cells (ATCC CRL-2638) and HCT116 human colorectal cancer cells (ATCC CCL-247) were purchased from the American Type Culture Collection (ATCC, USA). CT26 cells and HCT116 cells were cultured in Roswell Park Memorial Institute (RPMI) culture medium (Hyclone, USA) and in McCoy's 5A medium (Sigma-Aldrich, USA), respectively, supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, USA) and 1% Penicillin-streptomycin (Gibco, USA) to form the complete growth media. The cells were grown in a humidified atmosphere at 37 °C, 5 % CO₂.

The 2-dimensional (2D) monolayer model was established by seeding the cells in 96-well tissue culture plates (Corning, USA) at a density of 5×10^3 cells/well. The cells were cultured overnight to allow them to adhere to the bottom of the wells in monolayers before being used for the experiments.

The 3-dimensional (3D) spheroid model was established by culturing the CT26 and HCT116 cells in round-bottomed, ultra-low adhesion 96-well plates (Corning, USA). 200 µl of the complete growth medium was added to each well and 1.5×10^3 cells were seeded in each well. The cells were spun at 1000 g for 10 minutes using a plate centrifuge (Thermo Scientific, Sorvall™ ST 16R Centrifuge, Germany)

to facilitate their collection at the bottom of the wells. The 3D spheroid culture was incubated at 37 °C, 5 % CO₂ for a period of 3 days to allow the development of stable spheroids with necrotic regions that simulate *in vivo* tumors. After this 3-day growth period, the spheroids were used for the experiments.

4.2.2 Bacterial Culture and Derivation of Inactivated Bacteria

The *C. sporogenes* spores (ATCC 13732), purchased from ATCC were inoculated in Reinforced Clostridial Media (RCM) (Oxoid, UK), at a 1:1000 ratio. An anaerobic chamber was set-up following the protocol of the GasPak™ EZ Anaerobic Container System (Becton, Dickinson and Company, USA). The bacterial culture was placed in the anaerobic chamber and incubated at 37 °C, 5 % CO₂ for 48 hours.

The bacterial culture was then harvested from the anaerobic chamber and heat-inactivated by placing it in an 80 °C water bath for 2 hours. After heat-inactivation, the optical density of the bacterial suspension was measured using UV-vis spectrophotometer (VWR, USA) quantify the amount of bacteria (OD) present in a 1 ml sample of the culture at 600 nm [85]. The culture was centrifuged at 2500 g for 20 minutes [118] to form the pellet of IB. After the supernatant was discarded, the IB pellet was re-suspended in complete growth media of cancer cells for use in the experiments. The optical density of the re-suspended IB was measured again as described previously. The bacteria were then enumerated by using a bacteria counter (Thomas Scientific, USA). A suspension of IB in complete growth media measuring 1 OD/ml (OD₆₀₀) was found to contain $2.00 \pm 0.32 \times 10^7$ bacterial cells.

4.2.3 Treatment with Inactivated Bacteria

The IB suspension in complete growth medium was serially diluted to concentrations of 1, 2, 4, 6 and 8.00×10^7 bacterial cells/ml (measured at 600 nm, as described in Section 3.2.1). The 2D monolayer and 3D spheroid models were incubated at 37 °C, 5% CO₂ with 200 µl of the IB test samples of varying concentrations, over a 72-hour period. Complete growth media was used as a control in both studies.

4.2.4 Cell Proliferation Assay

The cell proliferation of the colorectal cancer cells in the 2D and 3D models treated with IB was evaluated using the Cell Proliferation Reagent WST-1 (Roche, USA) every 24 hours. At each time point, the test samples in each well were replaced with 100 µl of fresh complete growth media. The WST-1 reagent was added to each well at a 1:10 ratio and the cells were incubated for 2 hours under culture conditions. The absorbance of the samples was measured using a microplate reader (SpectraMax M5, Molecular Devices, USA) at 430 nm with a reference wavelength of 650 nm. The absorbance value of each well is directly proportional to the number of metabolically active cells. Proliferation of cells exposed to test samples was expressed as a percentage of control. The absolute values of the proliferation of each cell line's control can be found in Appendix ??.

4.2.5 Cell Viability Assay

LIVE/DEAD® Viability/Cytotoxicity Kit for mammalian cells (Invitrogen, USA), containing Calcein AM and Ethidium homodimer –I (EthD-I) was used to determine the cell viability of the cells treated with IB in the 2D model. Every 24 hours, the test samples were removed, and the cells were washed with PBS. 100 µl

of the fluorescence dye working solution was added to each well and the microplate was incubated in darkness at room temperature for 30 minutes. A fluorescence microscope (Olympus LX71, Japan) was used to take the fluorescence images of each well. The number of viable cells per well was enumerated using the ImageJ software.

4.2.6 Cell Death Assay

HCT116 cells, grown overnight in a 6-well plate at a seeding density of 3×10^5 cells/well, were exposed to IB at a concentration of 4×10^7 bacterial cells/ml for 24 hours. The cells were then harvested and stained with Alexa Fluor 488[®] Annexin V and propidium iodide using the Dead Cell Apoptosis Kit (Invitrogen, USA), according to the manufacturer's instructions. The number of apoptotic and necrotic cells were quantified by flow cytometry.

4.2.7 Scanning Electron Microscopy

HCT116 cells were seeded on a glass slide in a petri dish at a seeding density of 5×10^4 cells/well and grown overnight in complete growth medium. The media was then replaced with 4×10^7 bacterial cells/ml IB in complete growth medium, and kept at culture conditions for 24 hours. The cells and IB on the glass slide were then fixed in 2% glutaraldehyde and then dehydrated with a series of increasing ethanol concentrations. 3D spheroids treated with 1×10^7 bacterial cells/ml of IB and control spheroids grown in complete growth media for 72 hours were fixed in 10% paraformaldehyde (PFA) and dehydrated with a series of increasing ethanol concentrations. Once the samples were completely dehydrated, they were sputter coated with platinum (current: 20 mA and sputtering duration: 60 s) and imaged with a Scanning Electron Microscope (JEOL JSM6390LA analytical SEM) (JEOL, Japan).

4.2.8 Restricted Co-culture Experiment

HCT116 cells were seeded in 24-well plates at a seeding density of 5×10^4 cells/well and cultured overnight in complete growth medium. Transwell inserts with a pore size of $0.4 \mu\text{m}$ (Corning, USA) were placed on the wells and IB at concentrations of 1, 4 and 8×10^7 bacterial cells/ml was added. IB directly added to the wells were used as control. The restricted co-culture set-up and the control set-up were incubated in culture conditions for 48 hours, after which cell proliferation was measured as described above.

4.2.9 Histology of Spheroids

3D spheroids were initially grown for 72 hours and then harvested and fixed in 10% PFA. The fixed samples were dehydrated with a series of increasing concentrations of ethanol and embedded in paraffin wax. The paraffin blocks were sectioned at $5.0 \mu\text{m}$ thickness using a rotary microtome and collected in a Polysine® slides (Thermo Scientific, USA). Subsequently, sectioned samples were stained with haematoxylin and eosin (H&E) staining and mounted with a coverslip using mounting media. The stained slides were imaged using a bright-field microscope (Olympus BX51, Japan).

4.2.10 Evaluation of Spheroid Size

The effect of IB treatment on 3D spheroid model was evaluated by monitoring the size of the spheroids at 24-hour intervals over a period of 72 hours. At each time point, the spheroids were imaged using phase contrast light microscopy (PCLM) (Olympus LX71, Japan). The ImageJ software was used to measure the diameter and calculate the area of the spheroids.

4.2.11 Immunohistochemistry of Spheroids

3D spheroid exposed to 4×10^7 bacterial cells/ml IB for 72 hours were fixed with 4% paraformaldehyde at 4 °C overnight. The fixed samples were embedded in paraffin wax. The paraffin blocks were sectioned at 5.0 μ m thickness using a rotary microtome and collected in a Polysine[®] slides (Thermo Scientific, USA). Subsequently, immunohistochemical analyses were conducted on these sections for collagen and elastin. The samples were rehydrated before blocking endogenous peroxidase activity with hydrogen peroxidase. Following antigen retrieval using sodium citrate (pH 6) at 100 °C for 5 min, immunostaining was conducted using streptavidin-biotin complex method. The slides were incubated with the primary antibody overnight at 4°C. Subsequently, biotinylated secondary antibody was added for 30 min, followed by streptavidin enzyme conjugate for 10 min. The chromogen used was 3, 3'-Diaminobenzidine (DAB) and the slides were counterstained with haematoxylin. The stained slides were imaged using a bright-field microscope (Olympus BX51, Japan).

4.2.12 Statistical Analysis

All experiments were performed in triplicates, for statistical significance. All data were expressed as mean $\pm$ standard deviation of three separate experiments. Statistical analysis was conducted using two-tailed Student's t-test. A p value of less than 0.05 was considered to be statistically significant. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

4.3 Results

4.3.1 Inactivated Bacteria Inhibit Colorectal Cancer Cells in 2-Dimensional Monolayer Model

The 2D monolayer culture of CT26 cells was exposed to varying concentrations of IB over 72 hours. Every 24 hours, the cell proliferation rate was evaluated using the WST-1 assay and expressed as a percentage of the control (Figure 4.1 (a)). At all concentrations of IB, there was a significant decrease in the cell proliferation rates. The lowest concentration of IB (1×10^7 bacterial cells/ml) decreased the proliferation to 36.9% of the control after just 24 hours. At the 48 and 72-hour time-points, the cell proliferation of the sample is reduced to 7.50% and 6.27%, respectively. The highest concentration of IB (8×10^7 bacterial cells/ml) decreased the proliferation of cells significantly to 13.11%, 2.25% and 1.08% of the control at 24, 48 and 72 hours, respectively.

Figure 4.1 (b) shows that exposure to IB caused a dose-dependent decrease in the viability of the CT26 cells. Accordingly, the live/dead staining of the CT26 cells (Figure 4.1 (c)) showed significant reduction in green live cells with increasing doses of IB exposure.

Figure 4.1: Effect of IB on CT26 cells in 2D model. (a) Cell proliferation of CT26 cells exposed to varying concentrations of IB. (b) Cell viability of 2D cell culture of CT26 cells exposed to IB. Data expressed as mean $\pm$ SD. t-test is a comparison with control. (***) $p < 0.001$ (c) Fluorescence microscopy of CT26 cells exposed to IB at varying concentrations for 24 hours. Images show cells in top left quadrant of each well.

When a similar experiment was carried out in HCT116 cells, there was a dose- and time-dependent inhibition in cell proliferation. The same experiment was carried out on the HCT116 cells using the same method. As shown in Figure 4.2 (a), 1×10^7 bacterial cells/ml of IB reduces the cell proliferation of HCT116 cells to 97.03%, 76.96% and 44.26% at 24, 48 and 72 hours respectively. Additionally, 8×10^7 bacterial cells/ml of IB reduces HCT116 cell proliferation to 40.35%, 1.49% and 0.63% at 24, 48 and 72 hours respectively. The effect of IB on the cell viability of HCT116 cells in a 2D model is shown in Figure 4.2 (b). The decrease in cell viability is also dose-dependent, with a 8×10^7 bacterial cells/ml IB reducing cell viability to 1.86% after 72 hours. This is supported by the fluorescence images taken at the 24-hour time point which showed a decrease in the number of viable cells (stained green with Calcein AM) as the concentration of IB increases (Figure 4.2 (c)).

Figure 4.2: Effect of IB on HCT116 cells in 2D model. (a) Cell proliferation of HCT116 cells exposed to varying concentrations of IB. (b) Cell viability of 2D cell culture of HCT116 cells exposed to IB. Data expressed as mean $\pm$ SD. t-test is a comparison with control. (***) $p < 0.001$) (c) Fluorescence microscopy of HCT116 cells exposed to IB at varying concentrations for 24 hours. Images show cells in top left quadrant of each well.

4.3.2 Nature of Inactivated Bacteria-Mediated Cell Death

Flow cytometry of the IB-treated cells showed that 81.67% were viable. Of the dead cells, 14.0% were found to be necrotic, while 2.33% and 2.30% were in early and late stage apoptosis (Figure 4.3). The percentage of necrotic cells in the IB-

treated sample was significantly greater than that in the control sample.

Figure 4.3: IB-mediated necrosis of colorectal cancer cells in 2D model. HCT116 cells were exposed to 4×10^7 bacterial cells/ml of IB for 24 hours and stained with Alexa Fluor 488[®] Annexin V and propidium iodide. Number of viable, apoptotic and necrotic cells were quantified by flow cytometry. Data expressed as mean $\pm$ SD. (** $p < 0.01$)

4.3.3 Characterization of Interaction between Inactivated Bacteria and Colorectal Cancer Cells

The IB-exposed cancer cells were imaged using SEM. The IB were found to adhere to the surface of the HCT116 cells, as shown by the black arrows (Figure 4.4), indicating that physical interaction between the IB and the surface of cancer cells occurs when the latter are exposed to IB.

Figure 4.4: SEM image of physical interaction between IB and HCT116 cells. HCT116 cells grown in monolayer were exposed to 4×10^7 bacterial cells/ml IB for 24 hours. Black arrows indicate IB present on cell surface.

The role of physical interaction in IB-mediated cell death was further investigated with a restricted co-culture experiment. Two co-cultures of HCT116 with IB were set-up (Figure 4.5 (a)). The first involved a direct addition of IB to the cell culture, enabling the physical interaction between IB and the cells. The second, known as a restricted co-culture, involved the separation of the IB from the HCT116 cells in the well using a transwell insert, such that the IB would not physically interact with the cells. In both co-cultures, 3 concentrations of IB were used (1, 4 and 8×10^7 bacterial cells/ml) and the cells were exposed to IB for 48 hours before cell proliferation was measured. In the restricted co-culture, the cell proliferation was 98.75%, 93.16% and 93.59% of the control, respective to each of the 3 IB concentrations in increasing order. Contrastingly, the normal co-culture resulted in a significant decrease in cell proliferation to 88.54%, 71.19% and 5.11% of the control (Figure 4.5 (b)). This shows while the direct addition of the IB to cells successfully inhibits their proliferation in a dose-dependent manner, restricting the interaction between IB and cells abrogates the anti-cancer effect of the former.

Thus, it is shown that physical interaction is crucial for IB-mediated inhibition of cancer cells.

Figure 4.5: IB-mediated inhibition of cancer cells dependent on physical interaction. (a) Schematic of direct culture set-up (IB) and restricted co-culture set-up (RC) of HCT116 cells exposed to IB. (b) Cell proliferation of HCT116 cells exposed to varying concentrations of IB for 48 hours. Data expressed as mean $\pm$ SD. (***) $p < 0.001$)

4.3.4 Inactivated Bacteria Inhibit Colorectal Cancer Cells in 3-Dimensional Spheroid Model

The development of 3D spheroid model of CT26 cells were tested at two different seeding densities (5×10^3 cells/ml and 1.5×10^3 cells/ml). Although 5×10^3 cells/ml seeding density yielded a large circular spheroid at day 0, the spherical shape collapsed as early as day 3 (Figure 4.6 (a)). On the other hand, at a lower seeding density of 1.5×10^3 cells/ml, the spheroids were smaller at day 0 with further growth to mature spherical spheroid until day 6 maintaining their spherical shape and structural integrity (Figure 4.6 (b)). The HCT116 spheroids at same density of 1.5×10^3 cells/well also maintained its shape and structural integrity until day 6 (Figure 4.6 (b)). Thus, for further experiments with 3D spheroids, an initial seeding density of 1.5×10^3 cells/ml was used.

H&E staining of CT26 spheroids after 72 hours in culture showed denser haematoxylin staining on the surface and a distinct absence of haematoxylin staining at the core region, indicating the lack of nuclei (Figure 4.7). This infers the presence of necrotic core in the spheroids which would be similar to the *in vivo* microenvironment of a tumor.

Figure 4.6: 3D spheroid model of colorectal cancer cell lines. (a) CT26 spheroids, with initial seeding density of 5×10^3 cells/well. (b) Spheroids of CT26 and HCT116 over 6 days, with an initial seeding density of 1.5×10^3 cells/well.

Figure 4.7: H&E staining of 3D spheroid after 72 hours in culture. Necrotic regions formed within spheroid stained only with eosin (white arrows).

WST-1 assay showed a continuous decrease in cell proliferation of CT26 spheroids for 72 hours (Figure 4.8 (a)). At 72 hours, the cell proliferation of CT26 spheroids exposed to 1 , 4 and 8×10^7 bacterial cells/ml IB was 50.67%, 39.57% and 23.90%

of control, respectively. These values were higher than the cell proliferation of cells in the 2D model exposed to the same concentrations of IB. The decrease in cell proliferation of 3D spheroids exposed to IB was dose-dependent and time-dependent. As anticipated, the size of control spheroids increased significantly between each time point over the entire duration of the study (Figure 4.8 (b)). However, there was a significant decrease in the area of the IB-treated CT26 spheroids. At the end of study, the spheroids exposed to 1×10^7 bacterial cells/ml IB ($3.25 \times 10^5 \mu m^2$) were 25% smaller than the control spheroids ($4.34 \times 10^5 \mu m^2$). Spheroids exposed to 4×10^7 bacterial cells/ml IB ($3.04 \times 10^5 \mu m^2$) and 8×10^7 bacterial cells/ml IB ($2.95 \times 10^5 \mu m^2$) were 30% and 32% smaller than the control spheroids, respectively. Moreover, it is interesting to note that despite the increase in size, the shape of the control spheroids was maintained throughout the study, while the shape of the CT26 spheroids exposed to IB started to get deformed after 48 hours (Figure 4.8 (c)).

The effect of IB on the 3D spheroids of HCT116 was similar. At 72 hours, the cell proliferation of HCT116 spheroids reduced to 16.24% (1×10^7 bacterial cells/ml), 9.20% (4×10^7 bacterial cells/ml) and 2.78% (8×10^7 bacterial cells/ml) of the control (Figure 4.9 (a)). The inhibition of HCT116 spheroids by IB was also observed as a decrease in spheroid size (Figure 4.9 (b)). The spheroids exposed to 1×10^7 bacterial cells/ml IB ($1.60 \times 10^5 \mu m^2$) were 44% smaller than the control spheroids ($2.85 \times 10^5 \mu m^2$). Spheroids exposed to 4×10^7 bacterial cells/ml IB ($1.42 \times 10^5 \mu m^2$) and 8×10^7 bacterial cells/ml IB ($1.14 \times 10^5 \mu m^2$) were 50% and 60% smaller than the control spheroids, respectively. While the deformation of the IB-exposed spheroids was not apparent, there was an observable stunting in the growth of the HCT116 spheroids (Figure 4.9 (c)).

Figure 4.8: Effect of IB on 3D spheroid model of CT26 cells. (a) Cell proliferation of spheroids exposed to varying concentrations of IB over 72 hours. (b) Size of spheroids exposed to IB evaluated by measuring area of spheroids imaged at the 0, 24, 48 and 72-hour time points. Data expressed as mean $\pm$ SD. t-test is a comparison with control. (***) $p < 0.001$ (c) Phase contrast imaging of 3D spheroids exposed to IB.

Figure 4.9: Effect of IB on 3D spheroid model of HCT116 cells. (a) Cell proliferation of spheroids exposed to varying concentrations of IB over 72 hours. (b) Size of spheroids exposed to IB evaluated by measuring area of spheroids imaged at the 0, 24, 48 and 72-hour time points. Data expressed as mean $\pm$ SD. t-test is a comparison with control. (***) $p < 0.001$ (c) Phase contrast imaging of 3D spheroids exposed to IB.

4.3.5 Effect of Inactivated Bacteria on Spheroid Extracellular Matrix

Figure 4.10 shows that the cells on the periphery of the control CT26 spheroids were circular in shape, packed together with tight cell contacts and thickly covered by the extracellular matrix (ECM). On the other hand, on the periphery of the spheroids exposed to IB, the cells were elongated, loosely packed with thinner ECM. The morphology of IB-exposed spheroid is deformed in contrast to the spherical morphology of control spheroid. This is in agreement with Figure 4.8 (c), where the IB-exposed CT26 spheroids appeared to have lost its morphological integrity at 72 hours.

Figure 4.10: SEM image of the surface morphology of Control and IB-exposed CT26 spheroids. Spheroids were exposed to 4×10^7 bacterial cells/ml IB for 72 hours.

The effect of IB on the ECM was further investigated by conducting immunostaining of CT26 spheroids. The sections of the spheroid exposed to sections were stained with elastin (Figure 4.11 (a)) and collagen (Figure 4.11 (b)). While prominent elastin expression was observed in both control and IB-exposed spheroids, there was no distinct collagen expression in both control and IB-exposed spheroids. Overall, there was no observable difference between the ECM of control and IB-

exposed spheroids.

Figure 4.11: Immunostaining of 3D spheroids. Histological cross-sections of 3D spheroids were immunostained for (a) elastin and (b) collagen after exposure to 4×10^7 bacterial cells/ml IB for 72 hours.

4.4 Discussion

This chapter investigated the effect of IB on 2D and 3D *in vitro* models of colorectal cancer cell lines, CT26 and HCT116. Both these cell lines were observed to behave similarly when grown in culture conditions, with similar proliferation rates over 72 hours, as demonstrated in Figure A.1. In the 2D model, the exposure to IB exerted a significant anti-proliferative effect on CT26 and HCT116 cells, in a concentration and time-dependent manner. A previous study reported that the inactivated forms of Gram-negative *S. equisimilis* and *S. aureus* bacteria induced cell death in colorectal cancer cells [85]. In this study, the incubation of 2D monolayers of colorectal cancer cells with heat-inactivated *S. equisimilis* and *S. aureus* resulted in an increase in the percentage of dead cells. After 48 hours, slightly more than 10% of the HCT116 cells exposed to heat-inactivated *S. aureus* were dead. Meanwhile, exposure to heat-inactivated *S. equisimilis* resulted in 10% dead cells. In contrast, our results showed that exposure of HCT116 to the lowest concentration of IB (1×10^7 bacterial cells/ml) lead to 53% dead cells after 48 hours (Figure 4.2 (b)). This shows that heat-inactivated *C. sporogenes* were more effective in inhibiting the growth of colorectal cancer cells than heat-inactivated *S. equisimilis* or *S. aureus*.

It has been suggested in literature that cells grown in a 2D monolayer are excessively susceptible to treatment and thus not a reliable *in vitro* model [192, 193]. The use of 3D spheroids in this study is important as it allows the evaluation of IB on a physiologically relevant platform [142, 145]. The spheroids that were grown developed necrotic regions that simulate the hypoxic, necrotic conditions of the *in vivo* tumor microenvironment [142, 201]. A 3D model is more complex as it simulates the cell-cell and cell-ECM interactions of real tumors [145]. In general,

the cells grown in a monolayer of a 2D model are more susceptible to external treatments as compared to that of a spheroid where there is a gradient of nutrient and gas exchange with the surroundings [137]. This could be a reason why the cell proliferation rate of the CT26 spheroids exposed to 1×10^7 bacterial cells/ml of IB is approximately 9 folds higher than that of its 2D model. The successful inhibition of colorectal cancer cells *in vitro* in normoxic, cell culture conditions shows that the anti-cancer effect of IB is hypoxia-independent. However, the effect of IB on the ECM was not fully understood. While SEM had shown a thinning of the ECM in 3D spheroids after IB treatment, qualifying the amount of ECM proteins through IHC staining for elastin and collagen was inconclusive. Further studies, such as ECM protein quantification assays, are required to better understand the effect of IB on ECM proteins.

The nature of IB-mediated inhibition of colorectal cancer cells was investigated. The anti-cancer effect of IB could be due to the secretion of bacterial products into the culture media or through physical contact with the cancer cells. The interaction between IB and mammalian cells was studied using SEM and IB was found to be physically adherent to HCT116 cells. Further investigation using a restricted co-culture system showed that cell proliferation was inhibited only when IB were directly added to allow the physical interaction between IB and cancer cells. In the restricted co-culture system, physical contact between IB and cancer cells was prevented by the transwell insert. As they were heat-inactivated, IB were unable to produce and secrete new products, but there was a possibility of existing bacterial molecules being released into the media. If this were the case, the restricted co-culture would contain these released bacterial molecules in the growth media. Yet, no decrease in cell proliferation was observed in the restricted co-culture set-up. This showed that physical contact between IB and cancer cells was most likely

to mediate the inhibitive effect. These observations need to be validated through further studies to confirm the role of IB-cell interaction in cancer cell inhibition.

The findings of this chapter have led to the development of the working hypothesis that the cell wall components of IB interact with receptors on the cancer cells to induce cell death, as depicted in Figure 4.12. The bacterial wall of *C. sporogenes* contains lipopeptides, lipoteichoic acids (LTA), peptidoglycans (PepG) which are macroamphiphiles and are recognized as pathogen-associated molecular patterns (PAMPs) [202]. Cancerous cells often overexpress receptors such as Toll-like receptors (TLRs) on their surface and cytosolic NOD-like receptors (NLR). NLRs are involved the caspase-1 mediated programmed cell death, pyroptosis, which is characterized by membrane disintegration and the release of inflammatory cytokines and results in the necrotic nature of dead cells [203–207]. Colorectal cancer cells overexpress TLR2 and TLR4 [208–213]. TLR2 is known to recognize and interact with PAMPs associated with Gram-positive bacteria [208, 213–217]. It has been reported that the stimulation of TLRs with bacterial ligands leads to a cross-talk between TLRs and NLRs and the activation of the latter [218]. The activation of NLR and the subsequent activation of the caspase-1 pathway after the ligation of IB PAMPs and TLR2 can lead to initiation of pyroptosis and eventual necrosis of the tumor cells. Previous studies have similarly reported that TLR2 activation by bacterial products has resulted in cell death [219, 220]. The possibility of pyroptosis occurring in IB-treated cancer cells is reflected in the necrotic dead cells found after the *in vitro* administration of IB. The involvement of the surface components of bacteria in affect cancer cell death was also supported by other studies [113, 221]. The effect of IB surface PAMPs mediating cell death can be further validated by fractionating IB into its membrane and cytosolic fractions and administering the two separately to cancer cells.

Figure 4.12: Working hypothesis of IB-mediated inhibition of colorectal cancer cells. IB exerts direct inhibitive effect on cancer cells through PAMP-TLR2 interaction, leading to pyroptosis and resulting in necrotic cell death. DAMPs = danger-associated molecular patterns; IB = inactivated bacteria; IRF = interferon regulatory factor; MAPK = mitogen-activated protein kinase; MYD88 = myeloid differentiation factor 88; NF- κ B = nuclear factor κ B; NLR = Nod-like receptor; PAMP = pathogen-associated molecular patterns; TAAs = tumor-associated antigens; TLR = Toll-like receptor. Black dashed arrows represent movement, black solid arrows activation and green double-arrows cross-talk. Green, red and yellow circles represent DAMPs, inflammatory cytokines and TAAs, respectively.

4.5 Conclusion

In this chapter, a range of IB concentrations was tested to determine dose-dependent efficacy and the nature of IB-mediated inhibition was investigated. The results showed that the anti-cancer effect of IB is hypoxia-independent. The key findings of this chapter are: (1) IB inhibits human and murine colorectal cancer, in a dose-dependent manner in 2D and 3D *in vitro* models. (2) IB mediate necrotic cell death. (3) Physical contact between IB and cancer cells is a pre-requisite for inhibition of cell growth. Subsequent chapters discuss the immunogenic properties of IB and its *in vivo* effect in mouse models of colorectal cancer.

Chapter 5

Effect of Conditioned Medium on Colorectal Cancer Cells *In Vitro*¹

5.1 Introduction

Non-viable derivatives of *C. sporogenes*, heat-inactivated bacteria (IB) and conditioned medium (CM) were developed as safer alternatives to conventional bacterial therapeutic agents. The use of these derivatives to inhibit the growth of cancer minimizes the risk of infection as compared to the use of live bacteria or spores. While chapter 4 examined the IB-mediated inhibition of colorectal cancer cells, this chapter further investigates the anti-cancer effect of CM using *in vitro* 2D monolayer and 3D spheroid models of colorectal cancer. Human (HCT116) and murine (CT26) colorectal cancer cell lines were used, to allow the easy translation to animal model studies in the subsequent chapter.

The first aim addressed in this chapter is the development of a hypoxia-independent

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bacterial therapeutic agent, which prevents the risk of tumor recurrence. Conventional bacterial cancer therapeutic agents, like live bacteria and spores, can only exert an inhibitive effect in the hypoxic core of tumors. This leaves a rim of viable cancer cells in the normoxic periphery of the tumor, thereby increasing the chance of tumor recurrence [67, 70, 72, 81]. We hypothesized that the anti-cancer efficacy of CM is hypoxia-independent. This hypothesis was tested in this chapter by administering CM to 2D and 3D models of HCT116 and CT26 cells in normoxic, cell culture conditions. The inhibitive effect of CM was evaluated by analyzing the cell proliferation, cell viability and spheroid size of the colorectal cancer cells.

The second aim of this chapter is to understand the nature of CM-mediated anti-cancer efficacy. To do this, the nature of cell death induced by CM was characterized. A key feature of using CM is that it contains all of the bacterial products of *C. sporogenes* secreted during the culture. This means that the observed anti-cancer effect could be a result of one or more secreted bacterial product. Identifying and isolating the key component of CM is important in its development as an anti-cancer therapeutic agent. The role of bacterial proteins and proteases in CM-mediated inhibition of colorectal cancer cells was investigated. Analysis of the protein content indicated that CM contained the enzyme Clostripain and its involvement in the anti-cancer effect of CM was examined. Size fractionation of CM was conducted to aid the isolation and identification of the key component of CM. Additionally, CM activity under various pH conditions was also tested. These studies serve to improve the understanding of the mechanism underlying the inhibitive effect exerted by CM.

5.2 Materials and Methods

5.2.1 Cell Culture and *in vitro* Cancer Models

The CT26 murine colorectal cancer cells (ATCC CRL-2638) and HCT116 human colorectal cancer cells (ATCC CCL-247) were purchased from the ATCC (USA). CT26 cells were cultured in the complete growth medium constituting the Roswell Park Memorial Institute (RPMI) culture medium (Hyclone, USA) and HCT116 cells were cultured in McCoy's 5A medium (Sigma-Aldrich, USA), supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, USA) and 1% Penicillin-streptomycin (Gibco, USA) to form the complete growth media for the respective cell line. The cells were cultured in flasks (Corning, USA) in complete growth medium, incubated in a humidified atmosphere at 37 °C, 5 % CO₂.

The 2-dimensional (2D) monolayer model was established by seeding the cells in 96-well tissue culture plates (Corning, USA) at a density of 5×10^3 cells/well. The cells were cultured overnight to allow them to adhere to the bottom of the wells in monolayers before being used for the experiments.

The 3-dimensional (3D) spheroid model was established by culturing the CT26 and HCT116 cells in round-bottomed, ultra-low adhesion 96-well plates (Corning, USA). 200 µl of the complete growth medium was added to each well and 1.5×10^3 cells were seeded in each well. The cells were spun at 1000 g for 10 minutes using a plate centrifuge (Thermo Scientific, Sorvall™ ST 16R Centrifuge, Germany) to facilitate their collection at the bottom of the wells. The 3D spheroid culture was incubated at 37 °C, 5 % CO₂ for a period of 3 days to allow the development of stable spheroids with necrotic regions that simulate *in vivo* tumors. After this 3-day growth period, the spheroids were used for the experiments.

5.2.2 Bacterial Culture and Derivation of Conditioned Medium

The *C. sporogenes* spores (ATCC 13732), purchased from ATCC were inoculated in Reinforced Clostridial Media (RCM) (Oxoid, UK), in a 1:1000 ratio. An anaerobic chamber was set-up following the protocol of the GasPak™ EZ Anaerobic Container System (Becton, Dickinson and Company, USA). The bacterial culture was placed in the anaerobic chamber and incubated at 37 °C, 5 % CO₂ for 48 hours.

The bacterial culture was harvested from the anaerobic chamber and centrifuged at 2500 g for 20 minutes to pellet the *C. sporogenes* bacteria. The supernatant is collected and filtered using a 0.22 µm syringe filter to obtain the bacteria-free conditioned medium (CM).

5.2.3 Treatment with Conditioned Medium

CM was diluted in the complete growth media to 10% CM and used in subsequent experiments. The 2D monolayer and 3D spheroid models were incubated at 37 °C, 5% CO₂ with 200 µl of 10% CM over a 72-hour period. Complete growth media and 10% RCM in complete growth media was used as a control in both studies.

5.2.4 Cell Proliferation Assay

The cell proliferation of the colorectal cancer cells in the 2D and 3D models treated with CM was evaluated using the Cell Proliferation Reagent WST-1 (Roche, USA) every 24 hours. At each time point, the test samples in each well were replaced with 100 µl of fresh complete growth media. The WST-1 reagent was added to each well in a 1:10 ratio and the cells were incubated for 2 hours under culture conditions. The absorbance of the samples was measured using a microplate reader (SpectraMax M5, Molecular Devices, USA) at 430 nm with a reference

wavelength of 650 nm. The absorbance value of each well is directly proportional to the number of metabolically active cells.

5.2.5 Cell Viability Assay

LIVE/DEAD[®] Viability/Cytotoxicity Kit for mammalian cells (Invitrogen, USA), containing Calcein AM and Ethidium homodimer –I (EthD-I) was used to determine the cell viability of the cells treated with CM in the 2D model. At each 24-hour time point, the test samples were removed, and the wells were washed with PBS. 100 μ l of the fluorescence dye working solution was added to each well and the microplate was incubated in darkness at room temperature for 30 minutes. A fluorescence microscope (Olympus LX71, Japan) was used to take the fluorescence images of each well. The number of viable cells well enumerated using the ImageJ software.

5.2.6 Cell Death Assay

HCT116 cells, grown overnight in a 6-well plate at a seeding density of 3×10^5 cells/well, were exposed to 10% CM in complete growth media, for 24 hours. The cells were then harvested and stained with Alexa Fluor 488[®] Annexin V and propidium iodide using the Dead Cell Apoptosis Kit (Invitrogen, USA), according to the manufacturer's instructions. The number of apoptotic and necrotic cells were quantified by flow cytometry.

5.2.7 Evaluation of Spheroid Size

The effect of CM treatment on 3D spheroid model was evaluated by monitoring the size of the spheroids at 24-hour intervals over a period of 72 hours. At each time point, the spheroids were imaged using a phase contrast light microscopy (PCLM) (Olympus LX71, Japan). The ImageJ software was used to measure the diameter

and calculate the area of the spheroids.

5.2.8 Denaturation and Inhibition of Proteins and Proteases in Conditioned Medium

Boiled Conditioned Medium (BCM) was prepared by placing the CM in a water bath at 100 °C for 30 minutes. BCM was diluted to 10% of complete growth media and tested on the 3D spheroid model of CT26 and HCT116, where cell proliferation and spheroid size were evaluated.

Proteases in CM were inhibited using a protease inhibitor cocktail, SigmaFAST™ Protease Inhibitor Tablet (Sigma, USA). For the first experiment, a 10× protease inhibitor (PI) stock was prepared by dissolving 1 tablet in 10 ml of DI H₂O. This stock was used to prepare 1× PI and 0.5× PI in complete growth media. 10% CM were added to each of these solutions. Another solution of 1× PICM was prepared by diluting the 10× PI stock in CM. 10% PICM was added to complete growth media. These solutions were tested on 2D models of HCT116 cells, and cell proliferation was evaluated.

In the second experiment, Pierce™ Protease Inhibitor Mini Tablets, EDTA Free (Life Technologies, USA) were used. 1 tablet was dissolved in 10 ml of McCoy's complete growth media (1× PIM-EF). 10% CM was added to 1× PIM. 1 tablet was dissolved in 10 ml of CM, resulting in 1× PICM-EF. 10% 1× PICM-EF was added to complete growth media. These solutions were tested on 2D models of HCT116 cells, and cell proliferation was evaluated.

5.2.9 SDS-PAGE Gel Electrophoresis and Mass Spectroscopy of Conditioned Medium

SDS-PAGE gel electrophoresis was conducted to separate the proteins in CM according to their molecular weight. Vertical 1mm thick gels comprising of a 4% stacking gel (pH 6.9) and 12% separating gel (pH 8.8) were routinely casted using the Mini-protean II system (BioRad, USA). The protein sample was mixed with 5× loading dye (BioRad, USA) in an Eppendorf tube and placed in boiling water for 5 minutes. The sample was kept on ice prior to being loaded into a SDS-PAGE gel. Electrophoresis was performed at a constant voltage of 80 V (Power Pac 200, BioRad, USA) for 120 minutes. The target protein band was excised from the gels manually with a clean spatula. This was kept in a clean centrifuge tube before being analysed. The proteins were identified using MALDI-TOF mass spectroscopy and analyzed using the Mascot program (Matrix Science, UK).

5.2.10 Treatment with Clostripain

Commercially available Clostripain from *C. histolyticum* (Sigma-Aldrich, USA) was purchased. A 40 µM stock solution of Clostripain in 1 mM CaCl₂ was prepared and an activation solution of 2.5 mM Dithiothreitol (DTT), 1 mM CaCl₂ in complete growth media was prepared. Active Clostripain was prepared by diluting the stock solution in the activation solution and incubating at 37 °C for 2 hours. Non-active Clostripain was prepared by diluting the stock solution directly in complete growth media. Active and non-active Clostripain at concentrations of 0.1, 1 and 10 µM were tested on 2D model of HCT116 over 72 hours and the cell proliferation was evaluated at every 24-hour interval.

5.2.11 Size Fractionation of Conditioned Medium

Size fractionation of CM was conducted using Amicon Ultra-15 Centrifugal Filter Units (Merck, USA). 12 ml of CM was added to ultra-filtration units with molecular weight cut-off (MWCO) of 10 kDa and centrifuged at 5000 g for 1 hour. This resulted in a filtrate of molecular weight (MW) <10 kDa and a concentrate of MW >10 kDa. The >10 kDa fraction was resuspended in 12 ml of PBS. The CM fractions were resuspended in complete growth media to give 10% <10 kDa and 10% >10 kDa solutions.

The <10 kDa fraction was further subjected to ultra-filtration with a 3 kDa MWCO unit in the same method described above and the resulting fraction were resuspended in complete growth media to give 10% <3 kDa and >3 kDa solutions. These solutions of CM fractions were tested on 2D model of HCT116 over 72 hours and the cell proliferation was evaluated at every 24-hour interval.

5.2.12 pH Adjustment of Conditioned Medium

The pH of McCoy's complete growth media, 10% CM and 10% RCM in complete growth media were measured at 25 °C. The pH of complete growth media and 10% RCM were adjusted to pH of 10% CM, which was pH 7.43, and these solutions were known as pH 7.43 Control and pH 7.43 RCM. The pH of 10% CM and 10% RCM were adjusted to pH 8.18 which was the pH of complete growth media and these solutions were known as pH 8.18 CM and pH 8.18 RCM.

Another set of solutions of complete growth media, 10% CM and 10% RCM were prepared where their pH were adjusted to pH 4.5 (denoted by acid.) and pH 9 (denoted by alk.). pH adjustment was done with NaOH (10 M) and HCl (6 M). The pH adjusted solutions were tested on 2D model of HCT116 over 72 hours and

the cell proliferation was evaluated at every 24-hour interval.

5.2.13 Statistical Analysis

All experiments were performed in triplicate, for statistical significance. All data were expressed as mean $\pm$ standard deviation of three separate experiments. Statistical analysis was conducted using two-tailed Student's t-test. A p value of less than 0.05 was considered to be statistically significant. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

5.3 Results

5.3.1 Conditioned Medium Inhibits Colorectal Cancer Cells in a 2-Dimensional Monolayer Model

The CT26 cells were cultured in a 2D monolayer and exposed to 10% CM in complete growth media over 72 hours. Every 24 hours, the cell proliferation rate was evaluated using the WST-1 assay and expressed as a percentage of the control (Figure 5.1 (a)). Cell proliferation of CT26 cells exposed to 10% CM decreased in time-dependent manner to 10.76% of the control in the first 24 hours, after which it further decreased to 4.47% (48 hours) and 2.36% (72 hours). In comparison, the cell proliferation rate of CT26 cells exposed to 10% RCM is 75.99% of the control at the first time point and it decreases to 68.08% (48 hours) and 67.85% (72 hours) over the course of study. Exposure to CM significantly inhibited the cell viability of CT26 cells in a time-dependent manner (Figure 5.1 (b)). This is validated by the fluorescence images demonstrating that CM treatment leads to decrease in CT26 cell viability (Figure 5.1 (c)). In comparison, RCM causes a slight, non-significant decrease in the proliferation and viability of CT26 cells.

Figure 5.1: Effect of CM on CT26 cells in 2D model. (a) Cell proliferation of CT26 cells exposed to CM. (b) Cell viability of 2D cell culture of CT26 cells exposed to CM. Data expressed as mean $\pm$ SD. *** $p < 0.001$, t-test is a comparison with control. ### $p < 0.001$, t-test is a comparison between RCM and CM. (c) Fluorescence microscopy of CT26 cells exposed to CM for 24 hours. Images show cells in top left quadrant of each well. RCM used as broth control. Both CM and RCM administered as 10% of complete growth medium.

The same experiment was carried out using HCT116 cells and yielded similar results. The proliferation of HCT116 cells was inhibited by 10% CM to 55.9%, 30.6% and 7.4% at 24, 48 and 72 hours respectively. On the other hand, 10% RCM slightly decreases the cell proliferation of HCT116 to 83.2% at 24 hours, 72.8% at 48 hours and 75.4% at 72 hours (Figure 5.2 (a)). The fluorescence images of the cells support the cell proliferation results (Figure 5.2 (b) and (c)). They show that the number of viable cells remaining after exposure to CM is significantly lower than those exposed to RCM.

Figure 5.2: Effect of CM on HCT116 cells in 2D model. (a) Cell proliferation of HCT116 cells exposed to CM. (b) Cell viability of 2D cell culture of HCT116 cells exposed to CM. Data expressed as mean $\pm$ SD. * $p < 0.05$, *** $p < 0.001$, t-test is a comparison with control. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, t-test is a comparison between RCM and CM. (c) Fluorescence microscopy of HCT116 cells exposed to CM for 24 hours. Images show cells in top left quadrant of each well. RCM used as broth control. Both CM and RCM administered as 10% of complete growth medium.

5.3.2 Nature of Conditioned Medium-Mediated Cell Death

The nature of cell-death induced by CM was investigated by flow cytometry. Of the CM-treated cells, 67.2% were viable. 12.0% of the cells were in early stage apoptosis and 24.67% were late stage apoptosis. The percentages of early and late stage apoptosis in CM-treated samples were both were significantly higher than those of control samples. 3.3% of the CM-treated cells were found to be necrotic (Figure 5.3).

Figure 5.3: CM-mediated necrosis of colorectal cancer cells in 2D model. HCT116 cells were exposed to 10% CM in complete growth medium for 24 hours and stained with Alexa Fluor 488[®] Annexin V and propidium iodide. Number of viable, apoptotic and necrotic cells were quantified by flow cytometry. Data expressed as mean $\pm$ SD. (** $p < 0.01$)

5.3.3 Conditioned Medium Inhibits Colorectal Cancer Cells in a 3-Dimensional Spheroid Model

The 3D spheroids were exposed to 10% CM and 10% RCM over a period of 72 hours. The cell proliferation was measured using the WST-1 assay at 24-hour intervals. In the CT26 spheroids, RCM caused a slight decrease in the cell proliferation after 24 and 48 hours. After 72 hours, the cell proliferation of RCM-exposed spheroids was similar to that of control spheroids. On the other hand, the inhibitive effect of CM is significant, with the cell proliferation of CT26 spheroids decreasing to 20.02% (Figure 5.4 (a)). CM induces a slight regression in the CT26 spheroids

from $2.73 \times 10^5 \mu m^2$ at 48 hours to $2.67 \times 10^5 \mu m^2$ at 72 hours (Figure 5.4 (b)). At the end of the study, the area of the CM exposed CT26 spheroids is 38.47% smaller than the area of the control spheroids.

Figure 5.4: Effect of CM on 3D spheroid model of CT26. (a) Cell proliferation of spheroids exposed to CM over 72 hours. (b) Size of spheroids exposed to CM evaluated by measuring area of spheroids imaged at the 0, 24, 48 and 72-hour time points. Data expressed as mean $\pm$ SD. *** $p < 0.001$, t-test is a comparison with control. ## $p < 0.01$, ### $p < 0.001$, t-test is a comparison between RCM and CM. (c) Phase contrast imaging of 3D spheroids exposed to CM. RCM used as a broth control. Both CM and RCM administered as 10% of complete growth medium.

The cell proliferation of the HCT116 spheroids exposed to CM decreased to 17.08% after 72 hours (Figure 5.5 (a)). Additionally, CM caused an overall regression of the HCT116 spheroids from $1.54 \times 10^5 \mu m^2$ at 0 hours to $1.33 \times 10^5 \mu m^2$ at 72 hours (Figure 5.5 (b)), such that the area of CM exposed HCT116 spheroids was 54.57% smaller than the control spheroids. For RCM exposed spheroids, the area, although smaller than the control, was still larger than the spheroids exposed to CM.

This inhibitive effect of CM is also reflected in the morphology of the spheroids (Figure 5.4 (c), Figure 5.5 (c)). CM causes deformation of the spheroids, stunting of the spheroid size initially and then its regression. In contrast, RCM maintained overall spheroid shape as compared to CM exposed spheroids.

Figure 5.5: Effect of CM on 3D spheroid model of HCT116. (a) Cell proliferation rate of spheroids exposed to CM over 72 hours. (b) Size of spheroids exposed to CM evaluated by measuring area of spheroids imaged at the 0, 24, 48 and 72-hour time points. Data expressed as mean $\pm$ SD. *** $p < 0.001$, t-test is a comparison with control. ## $p < 0.01$, ### $p < 0.001$, t-test is a comparison between RCM and CM. (c) Phase contrast imaging of 3D spheroids exposed to CM. RCM used as a broth control. Both CM and RCM administered as 10% of complete growth medium.

5.3.4 pH-Dependence of Conditioned Medium Activity

Complete growth medium has a pH of 8.18 and after the addition of 10% CM it has a pH of 7.43. It has been previously shown that a change in pH can alter the growth of mammalian cells. [222].

To ascertain that the decrease in cancer cell growth after CM-treatment is not due to the change in pH, the role of this pH change in CM-mediated cell death was investigated. The pH of 10% RCM and complete growth media (control) was adjusted to the pH 7.43. The pH of 10% CM and 10% RCM was adjusted to pH of the complete growth medium (pH 8.18). These solutions were then administered to HCT116 cells in an *in vitro* 2D culture model and cell proliferation of the cells was measured every 24 hours (Figure 5.6 (a)). Complete growth media adjusted to pH 7.43 (Control pH 7.43) did not alter the cell proliferation of HCT116 cells significantly as compared to the control. However, there was a significant difference in cell proliferation between the control at pH 7.43 and CM. This indicates that the change in pH in due to addition of CM is not the main cause for the observed inhibition of cancer cell growth. The proliferation of cells exposed to CM at pH 7.43 and at pH 8.18 was similar, indicating that the change in pH does not affect its activity. There was no significant difference in the cell proliferation of all three RCM samples.

Figure 5.6: Effect of pH on CM activity. (a) Cell proliferation of HCT116 cells exposed to CM, RCM and complete growth medium adjusted to pH 8.18 and pH 7.43, which were the pH values of complete growth medium and 10% CM respectively. (b) Cell proliferation of HCT116 cells exposed to CM, RCM and complete growth medium adjusted to pH 4.5 (Acid.) and pH 9 (Alk.). RCM was used as a broth control. Data expressed as mean $\pm$ SD. (NS: $p > 0.05$, * $p < 0.05$, *** $p < 0.001$)

CM activity was then evaluated at acidic and alkaline conditions of pH 4.5 and pH 9 respectively (Figure 5.6 (b)). This was to investigate the pH-dependence of CM-mediated anti-cancer effect. Acidic conditions were unfavourable for HCT116 cell growth and resulted in very low cell proliferation rates when exposed to Acid. CM, Acid. RCM and Acid. Control (complete growth medium at pH 4.5). Complete growth media and RCM that was adjusted to pH 9 (Alk. Control and Alk. RCM) resulted in cell proliferation rates that were comparable to Control. However Alk. CM resulted in cell proliferation rates that were similar to CM. This suggests that the inhibitive activity of CM is remains unchanged in alkaline conditions. The data suggests that the effectiveness of CM-mediated inhibition is not limited by pH.

5.3.5 Conditioned Medium Components Responsible for Inhibition of Colorectal Cancer Cells

In an attempt to investigate if proteins are involved in the previous observations, CM was heated at 100 °C to obtain boiled-conditioned medium (BCM). 3D spheroids were exposed to BCM in a 72-hour study. By the end of the study, it was observed that BCM has a considerably less inhibitive effect on cell proliferation. At the 72-hour time point, the cell proliferation of CT26 spheroids exposed to BCM was 61.37% of control, while those exposed to CM was 20.02% (Figure 5.7 (a)). The HCT116 spheroids exposed to BCM were observed to have a cell proliferation rate of 63.69% of control, while CM-exposed spheroids had a cell proliferation rate of 17.08% (Figure 5.7 (b)). This difference between the CM-induced and BCM-induced decrease in cell proliferation is very significant ($p < 0.001$) at all time points for both CT26 and HCT116 spheroids. The area of the CT26 (Figure 5.7 (c)) and HCT116 (Figure 5.7 (d)) spheroids exposed to BCM is consistently higher than that of the spheroids exposed to CM. The growth of the spheroids exposed to BCM is stunted, although their shape is maintained. Comparatively,

spheroids exposed to CM regress in size and also lose their shape (Figure 5.7 (e)).

Figure 5.7: Effect of protein denaturation on CM activity. BCM obtained by boiling CM contains denatured protein content. Cell proliferation rate of (a) CT26 and (b) HCT116 spheroids exposed to CM and BCM over 72 hours. Size of (c) CT26 and (d) HCT116 spheroids exposed to CM and BCM evaluated by measuring area of spheroids imaged at the 0, 24, 48 and 72-hour time points. Data expressed as mean $\pm$ SD. (***) $p < 0.001$. (e) Phase contrast imaging of 3D spheroids exposed to CM and BCM. Both CM and BCM administered as 10% of complete growth medium.

The results of the BCM experiment suggested the likelihood that the secreted bacterial proteins present in CM are likely to be involved in the inhibition of colorectal cancer cells. As *C. sporogenes* are known to be proteolytic [94, 95, 223], we investigated the involvement of secreted proteases in mediating the inhibition of colorectal cancer cells. CM was treated with a protease inhibitor cocktail (PI) at $1\times$ and $0.5\times$ concentration and then administered to HCT116 cells for a 72-hour study. The cell proliferation was measured using WST-1 assay and expressed as a percentage of control (Figure 5.8 (a)). PI $1\times$ was used as a control and it was found to exert an inhibitive effect on HCT116 cell proliferation, with was similar to the effect exerted by CM. Additionally, CM treated with $1\times$ and $0.5\times$ PI was found to have no significant difference in the decrease in cell proliferation it affected. This shows that the PI was detrimental to cell proliferation and could not be used for understanding the role of bacterial proteases in CM-mediated inhibition.

The protease inhibitor cocktail contained EDTA which is known to have cytotoxic effects on mammalian cells [224]. Thus, an EDTA-free protease inhibitor cocktail was used next to inhibit proteases in CM (PICM-EF) and test the effect on HCT116 cells (Figure 5.8 (b)). This cocktail was dissolved in complete growth media (PIM-EF) and used as a control. While PIM-EF did not have a cytotoxic effect on the cells and did not inhibit cell proliferation, PICM-EF still inhibited cell proliferation similar to CM. This shows there is inconclusive evidence regarding the contribution of secreted proteases to CM -mediated inhibition.

Figure 5.8: Effect of protease inhibition on CM activity. (a) Cell proliferation rate of HCT116 cells exposed to CM, CM in 1× PI, CM in 0.5× PI and 1× PICM over 72 hours. 1× PI and 0.5× PI were used as controls. (b) Cell proliferation rate of HCT116 cells exposed to CM and CM in PICM-EF over 72 hours. PIM-EF was used as a solvent control. Data expressed as mean ± SD. (NS: $p > 0.05$, * $p < 0.05$)

CM was subjected to gel electrophoresis to separate the protein content (Figure 5.9 (a)). A prominent protein band was found between 45-50 kDa. This was further analyzed through mass spectrometry and identified to be the enzyme Clostripain. As this enzyme was found in abundance in the sample, it was hypothesized that it was playing a part in CM-mediated inhibition. Commercially available Clostripain was obtained and administered to HCT116 cells in its active (Act) and non-active (NAct) form at concentrations of 0.1, 1 and 10 μM (Figure 5.9 (b)). While the two lower concentrations of Clostripain had no effect on the cell proliferation, regardless of activation, both the Act and NAct forms of 10 μM decreased the cell proliferation rate of HCT116 cells. There was a significant difference between the effect of Act and NAct Clostripain at this concentration. Another range of Clostripain concentrations, 1, 2, 5 and 10 μM , was tested on HCT116 cells (Figure 5.9 (c)), but only 10 μM Clostripain was found to inhibit cell proliferation. This shows that the effect of Clostripain is not dose-dependent but there is a decrease in cell proliferation at 10 μM . There is insufficient data to support its role in CM-mediated inhibition.

Figure 5.9: Role of Clostripain in CM-mediated inhibition. (a) SDS-PAGE gel electrophoresis of CM showing a protein band of Clostripain at 45 kDa. (b) Cell proliferation rate of HCT116 exposed to non-activated (NAct) and activated (Act) Clostripain at varying concentrations, between a range of 0.1 to 10 μM . (c) Cell proliferation rate of HCT116 exposed to non-activated (NAct) and activated (Act) Clostripain at varying concentrations, between a range of 1 to 10 μM . DTT activation solution (DTT Control) and PBS in complete growth medium were used as control. Data expressed as mean $\pm$ SD. (NS: $p > 0.05$, * $p < 0.05$)

To identify the key component of CM was fractionated into 3 size fractions based on their MW: < 3kDa, 3-10 kDa and >10 kDa. Each fraction was administered to a 2D monolayer culture of HCT116 and its effect was evaluated by measuring cell proliferation rate every 24 hours. The 3-10 kDa and > 10 kDa fractions did not have a considerable effect on the cell proliferation of the cells. The cell proliferation in these samples was similar to that in the control and significantly higher than the CM sample. The <3 kDa fraction caused the inhibition of the cell proliferation in a way that was similar to the effect of CM (Figure 5.10). This suggests that the active component of CM was present in the <3 kDa fraction.

Figure 5.10: Size fractionation of CM. Cell proliferation rate of HCT116 cells exposed to CM and the three CM fractions (CMF) of molecular weights of <3 kDa, 3 kDa< CMF<10 kDa and >10 kDa over 72 hours. RCM was used as a broth control. Data expressed as mean $\pm$ SD. (***) $p < 0.001$

5.4 Discussion

The supernatant of bacteria culture contains all of the secreted products, including enzymes, toxins and metabolic waste, from the bacteria. This supernatant, known as conditioned medium (CM) of various kinds of bacteria has been seen to possess anti-cancer properties [117,118,120,191,225,226]. CM of *C. sporogenes* was tested for its anti-cancer properties on colorectal cancer cells. It was administered to 2D and 3D *in vitro* models of colorectal cancer cells, diluted in the complete growth media. Initially, a range of RCM concentrations, diluted in complete media, was tested to determine the maximum concentration that can be used without adversely affecting the cell viability. Mammalian cells cannot grow in bacterial culture media and as expected, the RCM components affected cell viability to some extent. As discussed in Chapter 3, 10% RCM in cell culture media was found to be the most ideal and thus used to compare against 10% CM in cell culture media for all the subsequent experiments.

In the 2D model, the exposure to CM was found to also have a strong inhibitive effect on the cells. CT26 cells were found to be more sensitive to CM in the 2D model. In a study by Arimochi et al. 10% CM of *C. perfringens* was administered to colorectal cancer cells, which resulted in a 60% decrease in cell proliferation after 4 days. Comparatively, 10% CM of *C. sporogenes* caused a 97% decreased in cell proliferation of CT26 cells after just 3 days. The successful inhibition of colorectal cancer cells *in vitro* in normoxic, cell culture conditions shows that the anti-cancer effect of CM is hypoxia-independent. This shows that our clostridial derivative has a the potential to be developed as an anticancer agent that can minimize the risk of tumor recurrence. The effect of CM on CT26 and HCT116 spheroids was highly inhibitive while the exposure to RCM was shown to have had a less inhibitive effect

on the cells and spheroids. Therefore, the main factor in affecting anti-proliferation of the cells was likely to be a bacterial product secreted into the culture medium.

When compared to the efficacy of IB against 2D colorectal cancer cells *in vitro*, CM was observed to have a significantly stronger inhibitive effect at every time point against CT26 (Figure 5.11 (a), (b)) and HCT116 (Figure 5.11 (c), (d)) cells.

Figure 5.11: Comparison between effect of IB and CM on 2D model of colorectal cancer. (a) Cell proliferation and (b) cell viability of CT26 cells exposed to IB and CM over 72 hours. (c) Cell proliferation and (d) cell viability of HCT116 cells exposed to IB and CM over 72 hours. Data expressed as mean $\pm$ SD. * $p < 0.05$, *** $p < 0.001$, t-test is a comparison between IB and CM. Cells exposed to 1×10^7 bacterial cells/ml IB and 10% CM in complete growth medium.

In the 3D model, CM had a significantly stronger effect against the proliferation

of CT26 spheroids at all time points than IB did (Figure 5.12 (a)). In the case of the HCT116 spheroids, the efficacy of CM was significantly higher than IB in inhibiting cell proliferation at 24 hours (Figure 5.12 (c)). However, the effect was similar at 48 and 72 hours. CM and IB had a similar effect on spheroid size (Figure 5.12 (b) and (d)). Taken together, this analysis shows that CM has a stronger anti-cancer effect *in vitro* than IB.

Figure 5.12: Comparison between effect of IB and CM on 2D model of colorectal cancer. (a) Cell proliferation and (b) spheroid size of CT26 spheroids exposed to IB and CM over 72 hours. (c) Cell proliferation and (d) spheroid size of HCT116 spheroids exposed to IB and CM over 72 hours. Data expressed as mean $\pm$ SD. * p < 0.05, *** p < 0.001, t-test is a comparison between IB and CM. Spheroids exposed to 1×10^7 bacterial cells/ml IB and 10% CM in complete growth medium.

pH conditions impact the growth of mammalian cell cultures [222]. The addition

of CM caused a change in the pH levels of the *in vitro* culture. This possibility of pH change decreasing the proliferation of colorectal cancer cells was investigated. However, the results of this study confirmed that the change in pH was not the main cause for the decrease in colorectal cancer cell proliferation. Furthermore, CM-mediated anti-cancer activity was shown to be independent of the pH of the cell culture.

This then lead to investigating the key component of CM responsible for mediating the observed anti-cancer effect. To analyze the role of proteins in CM, the effect of CM was compared with that of BCM. BCM's inhibitive effect was diminished to levels similar to that of RCM, suggesting that the denaturation of the protein content of CM abrogates its anti-cancer activity. In particular, this could be due to the secreted proteases of *C. sporogenes*, that degrade the tumor tissue [36,94]. To test this, a protease inhibitor cocktail was used to negate the activity of the proteases present in CM. When the protease inhibitor-CM solution was tested on colorectal cancer, it was found to have a similar effect to CM. This shows that it is difficult to attribute the inhibitive effect of CM to the proteases it contains. Another outcome of preparing BCM could be the denaturation of other CM components, such as carbohydrates, lipoproteins and glycoproteins. Further research into the role of these molecular groups in CM, using specific inhibition, is needed to verify this possibility.

Protein analysis showed an abundance of the enzyme Clostripain, and its effect on colorectal cancer cells was tested. However, the evidence did not suggest that Clostripain has an inhibitive effect on cancer cells. To confirm that Clostripain was identified correctly as a component of CM, commercially obtained Clostripain can be used as a positive control in the SDS-PAGE electrophoresis of CM in future experiments.

Additionally, CM was found to mediate apoptotic cell death, as shown in Figure 5.3. This result warrants further investigation into the mechanism of CM-mediated cell death. Caspase assays which quantify apoptosis through caspase activity in the cell samples, and mitochondria membrane potential assays can be used to validate the apoptotic nature of CM-mediated inhibition. Future work should also test the effect of CM on cell cycle for any alterations and the cytostatic effects of CM should also be investigated using EdU assay. These experiments will provide a better understanding of the nature of the cytotoxic effect mediated by CM.

To identify the active component of CM, it was fractionated based on the molecular weights of its component. The fraction containing molecules of molecular weight lower than 3 kDa retained the anti-cancer effect of CM while the larger fractions (3 – 10 kDa and >10 kDa) did not have an inhibitive effect on colorectal cancer cells. This explains why Clostripain (MW = 45 kDa) did not inhibit the growth of cancer cells. The active component of CM can thus be concluded to be of a molecular weight lower than 3 kDa. It is possible that CM activity is the result of more than one molecule working together. Further research is required to identify and isolate key components of CM from the <3 kDa fraction which could be used as an anti-cancer therapeutic agent.

5.5 Conclusion

The *in vitro* efficacy of CM against colorectal cancer cells was examined in greater depth. The results showed that the anti-cancer effect of CM is hypoxia-independent. The nature of CM-mediated cell death and the key component responsible for this was investigated. This chapter has the following key findings: (1) CM inhibits human and murine colorectal cancer in 2D and 3D *in vitro* models.

(2) CM mediates apoptotic cell death. (3) The active component of CM is likely to be of a molecular weight lower than 3 kDa. *In vivo* testing of CM is required to validate its efficacy as a therapeutic agent to mediate cancer cell inhibition. The next chapter discusses this in detail, highlighting the *in vivo* efficacy of CM in mouse models of colorectal cancer.

Chapter 6

Effect of Inactivated Bacteria and Conditioned Medium on Colorectal Cancer *In Vivo*

6.1 Introduction

We successfully validated the *in vitro* inhibitive effect of heat-inactivated *C. sporogenes* (IB) and its conditioned medium (CM) on 2D monolayer and 3D spheroid cultures of HCT116 and CT26 colorectal cancer cells in previous chapters. However, *in vitro* systems cannot simulate several aspects of human physiology, thus making it necessary to validate the efficacy of therapeutic agents using *in vivo* systems [227]. To determine the effectiveness of our bacterial derivatives in complex biological systems, IB and CM were tested in two sub-cutaneous (SC) colorectal tumor models in mice. We hypothesized that IB and CM will exert an anti-tumor effect in mouse models of colorectal cancer.

Subcutaneous models of colorectal cancer have been used in previous studies for test new therapeutic agents [86, 128]. SC tumor models in immunocompromised or nude mice are commonly used for cancer therapy discovery as they are easy to establish, cost-effective, have tumor latency of a few weeks, and allow the visual monitoring of the tumor growth [148]. While they are useful to investigate a direct anti-cancer effect of therapeutics, they do not account for the role played by the immune system in tumor progression and treatment. Tumors employ various means to evade and suppress anti-tumor immunity. However, bacterial therapy has the means of evoking an immune response against the tumors, as reported by several publications over the years [86, 171, 173, 179]. Yet, it remains to be seen whether the non-viable derivatives of *C. sporogenes* can elicit an anti-cancer immune response. This motivates the use of SC tumor models in immunocompetent mice to test the immunotherapeutic potential of IB and CM.

This chapter explores how IB and CM address the gaps of bacterial cancer therapy; efficacy in complex systems and risk of tumor recurrence. Testing the bacterial derivatives *in vivo* validated their efficacy in complex biological systems, which will be necessary for developing them into therapeutic agents in the future. The SC tumors in both mouse models consisted of the hypoxic tumor microenvironment that is known to limit therapy efficacy. The presence of hypoxia reduces the efficacy of radiation therapy [26] and chemotherapy [31]. Contrastingly, the presence of hypoxia allows anaerobic bacterial spores and vegetative bacteria to exert an anti-tumor effect. Yet, the anti-tumor effect ceases in the normoxic areas of the tumor periphery, leaving a viable rim of tumor cells [70, 72, 81]. This poses the risk of tumor recurrence. It is therefore necessary to develop a form of bacterial therapy that is neither hindered by hypoxia nor contingent on it. Testing IB and CM against *in vivo* tumors helped to evaluate their hypoxia-independence. Stim-

ulation of the immune system against tumors is another strategy for preventing tumor recurrence [86]. The SC tumor model in immunocompetent mice provided the platform for testing immune stimulation ability of IB and CM. Section 6.2 details the experimental approach followed in this chapter, while sections 6.4 and 6.5 describe and discuss the results of testing IB and CM *in vivo*, respectively.

6.2 Experimental Approach

Firstly, a human xenograft tumor model of HCT116 colorectal cancer cells was established in BALB/c-Foxn1^{nu}/ARC (nude) mice. Nude mice are athymic and therefore deficient in T cells. This allows the establishment of a solid, sub-cutaneous tumor from a cell culture of HCT116 cells [228,229]. These tumors remain localized, do not metastasize and retain their original histology, karyotype and tumorigenicity [230]. They are known to have a hypoxic core characterized by low oxygen levels and a central region of necrosis [23,24]. This model was thus used to validate the direct inhibitive effect of IB and CM in the absence of a robust immune system *in vivo*. Secondly, a syngeneic tumor model of murine CT26 colorectal cancer cells was established in immunocompetent BALB/cARC mice. CT26 cells are known to form highly immunogenic SC tumors [231]. This model is able to simulate the interaction between the immune system and the tumor tissue and was therefore used to validate the ability of IB and CM to elicit an anti-tumor immune response.

Figure 6.1 details the workflow for these studies. Both tumor models were subjected to regular intratumoral doses of IB and CM, and their effect was evaluated by monitoring the volume of the tumors and survival rate of the animals. At the end of the study, tumor samples were excised and subjected to histological and immunohistochemical analysis to determine the immune response to treatment. The ability of the IB in developing the immune memory of the host against tumor cells

was evaluated through a tumor re-challenge experiment.

Figure 6.1: Workflow for *in vivo* experiments. (a) IB and (b) CM tested in SC tumor models in mice.

6.3 Materials and Methods

6.3.1 Cell Culture

The CT26 murine colorectal cancer cells (ATCC CRL-2638) and HCT116 human colorectal cancer cells (ATCC CCL-247) were purchased from the ATCC (USA). CT26 cells and HCT116 cells were cultured in Roswell Park Memorial Institute (RPMI) culture medium (Hyclone, USA) and were in McCoy's 5A medium (Sigma-Aldrich, USA), respectively, supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, USA) and 1% Penicillin-streptomycin (Gibco, USA) to form the complete growth media. The cells were grown in a humidified atmosphere at 37 °C, 5 % CO₂.

6.3.2 Bacterial Culture and Derivation of Inactivated Bacteria and Conditioned Media

The *C. sporogenes* spores (ATCC 13732), purchased from ATCC were inoculated in Reinforced Clostridial Media (RCM) (Oxoid, UK), in a 1:1000 ratio. An anaerobic chamber was set-up following the protocol of the GasPak™ EZ Anaerobic Container System (Becton, Dickinson and Company, USA). The bacterial culture was placed in the anaerobic chamber and incubated at 37 °C, 5 % CO₂ for 48 hours. IB and CM were prepared as described previously in Section [3.2.1](#).

6.3.3 Immunodeficient Mouse Tumor Model and Treatment

Female BALB/c-Foxn1^{nu}/ARC athymic (nude) mice, 6-8 weeks of age, were obtained from Animal Resources Centre, Australia. The study was approved by the University of New South Wales, Sydney Australia Animal Care and Ethics Committee (ACEC 17/69B). The immunodeficient mice were inoculated with HCT116

cells by the sub-cutaneous (SC) injection of 4×10^6 cells in PBS, on their right flank. Tumors were established after 10-12 days when, at which time the animals were treated with either IB or CM. the average size of tumors when treatment was initiated was $\approx 100 \text{ mm}^3$. Treatment was administered in 4 doses, every 3 days through intratumoral injection of IB (1, 4, 8×10^6 bacterial cells/ 50 μl of PBS) or CM (5%, 10%, 15% in 50 μl of PBS). As controls, one tumor-bearing group and one healthy group received intratumoral injections of PBS, while another tumor-bearing group received 15% RCM injections. The sample size was $n = 5$. Tumor growth was assessed by measuring the size of the minor (width) and major (length) axes of the tumor every 3 days. Tumor volume was calculated using the formula: tumor volume = width² $\times$ length $\times$ 0.5 [86]. The animals were euthanized at day 12 post-treatment or when they became moribund due to a $>20\%$ decrease in weight or extensive tumor growth ($>1000 \text{ mm}^3$). Tumors were collected from the animals of all groups for further analysis.

6.3.4 Immunocompetent Mouse Tumor Model and Treatment

Female BALB/cARC mice, 6-8 weeks of age, were obtained from Animal Resources Centre, Australia. The study was approved by the University of New South Wales, Sydney Australia Animal Care and Ethics Committee (ACEC 17/69B). The immunocompetent mice were inoculated with CT26 cells by the sub-cutaneous (SC) injection of 4×10^6 cells in PBS, on their right flank. Tumors were established after 10-12 days when, at which time the animals were treated with either IB or CM. the average size of tumors when treatment was initiated was $\approx 100 \text{ mm}^3$. Treatment was administered in 4 doses, every 3 days through intratumoral injection of IB (1, 4, 8×10^6 bacterial cells/ 50 μl of PBS) or CM (5%, 10%, 15% in 50 μl of PBS). As controls, one tumor-bearing group and one healthy group received

intratumoral injections of PBS, while another tumor-bearing group received 15% RCM injections. The sample size was $n = 5$. Tumor growth was assessed by measuring the size of the minor (width) and major (length) axes of the tumor every 3 days. Tumor volume was calculated using the formula: tumor volume = width² $\times$ length $\times$ 0.5 [86]. The animals were euthanized at day 12 post-treatment or when they became moribund due to a >20% decrease in weight or extensive tumor growth (>1000 mm³). Tumors were collected from the animals of all groups for further analysis.

6.3.5 Tumor Re-challenge

IB-treated immunocompetent BALB/cARC mice ($n=5$) were re-challenged with the SC injection of 4×10^6 CT26 cells in PBS on their left flank. Naïve BALB/cARC mice were SC injected with CT26 cells as controls. The growth of the tumors was measured over 10 days, as described above.

6.3.6 Histology of Tumors

The tumor samples harvested from the BALB/cARC and nude mice were fixed in fixed in 10% paraformaldehyde (PFA). The fixed samples were dehydrated with a series of increasing concentrations of ethanol and embedded in paraffin wax. The paraffin blocks were sectioned at 5.0 μ m thickness from different parts of the tumor tissue ($n=3$). Subsequently, sectioned samples were stained with haematoxylin and eosin (H&E) and and mounted with a coverslip using mounting media. The stained slides were imaged using a bright-field microscope (Olympus BX51, Japan). The mean estimated area of necrosis was calculated using the ImageJ software.

6.3.7 Immunohistochemistry of Tumors

The tumor samples harvested from the BALB/cARC and nude mice were fixed in fixed in 10% paraformaldehyde (PFA). The fixed samples were dehydrated with a series of increasing concentrations of ethanol and embedded in paraffin wax. The paraffin blocks were sectioned at 5.0 μm thickness. Subsequently, immunohistochemical analyses were conducted on these sections for positive cytoplasmic staining of CD3 to indicate the presence of T cells. The samples were rehydrated before blocking endogenous peroxidase activity with hydrogen peroxidase. Following antigen retrieval using sodium citrate (pH 6) at 100 °C for 5 min, immunostaining was conducted using streptavidin-biotin complex method. The slides were incubated with the primary antibody, Anti-CD3 antibody (Abcam, USA) overnight at 4 °C. Subsequently, biotinylated secondary antibody (Abcam, USA) was added for 30 min, following streptavidin enzyme conjugate for 10 min. The chromogen used was 3, 3'-Diaminobenzidine (DAB) and the slides were counterstained with haematoxylin. The stained slides were imaged using a bright-field microscope (Olympus BX51, Japan). T cells were quantified as the average number cells with positive cytoplasmic staining across 3 tumor sections of each model, using the ImageJ software.

6.3.8 Statistical Analysis

The *in vivo* studies had a sample size of $n = 5$ for statistical significance. Tumor volume data were expressed as mean $\pm$ standard deviation. Statistical analysis was conducted using two-tailed Student's t-test. A p value of less than 0.05 was considered to be statistically significant. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

6.4 Effect of Inactivated Bacteria on Colorectal Cancer *In Vivo*

6.4.1 Results

6.4.1.1 Effect of Inactivated Bacteria on Colorectal Tumor Xenograft in Immunocompromised Mouse Model

The *in vivo* efficacy of IB was tested in a HCT116 tumor-bearing immunodeficient mouse model using athymic nude mice. IB were intra-tumorally injected every three days, for a total of four doses. Three concentrations of IB, $1, 4, 8 \times 10^6$ bacterial cells/ 50 μ l of PBS, were tested. The tumor size was quantified by measuring the tumor volume.

The response of HCT116 tumors to IB treatment was such that the volumes of IB-treated tumors were comparable, or slightly higher than the control tumors. Within 24 hours of the first dose, inflammation was observed at the site of the tumor. None of the tumors showed signs of ulceration or necrosis. At the end of the study, the volume of the 1×10^6 IB treated tumors was 172.7% of the initial tumor volume, while that of the control tumors was 227.7%. The 4 and 8×10^6 IB treated tumors had higher average tumor volumes than the control groups (Figure 6.2). All the groups in the HCT116 tumor model had a 100% survival rate.

Figure 6.2: Effect of IB on tumor burden in immunocompromised mouse. Tumor volume of HCT116 tumors in nude mice. Mice were treated intratumoral injections of 1, 4 and 8×10^6 IB in PBS every 3 days. Control group was treated with PBS. Data expressed as mean $\pm$ SD. (NS: $p > 0.05$)

As seen in Figure 6.3, the histological evaluation of the HCT116 tumors showed increased necrosis after treatment with IB. The estimated area of necrosis in the tumors, denoted by N, were seen to increase in area, proportional to the dosage of IB. This indicated that the direct anti-tumor effect of IB increases the extent of necrosis in HCT116 tumors.

Figure 6.3: (a) Hematoxylin and eosin staining of HCT116 tumors treated with varying concentrations of IB. Tumors were harvested at the end of 12 days showing the extent of necrosis. Viable regions of the tumor stained purple (T). IB treatment caused extensive area of necrosis, stained pink (N). Scale bar = 200 μ m. (b) Estimated area of necrosis as a % of tumor area. Data expressed as mean $\pm$ SD. (n=3)

6.4.1.2 Inactivated Bacteria Inhibit Growth of Syngeneic Colorectal Tumor in Immunocompetent Mouse Model

IB treatment reduced tumor burden in immunocompetent mice by inhibiting the growth of CT26 tumors. Figure 6.4 (a) shows CT26 tumor volumes as percentages of the initial tumor volume at the start of the study in BALB/cARC. In these immunocompetent mice, inflammation developed at the site of the tumor within 24 hours of IB administration. Some of the tumors became ulcerous during the study. At the end of the study, it was found that IB treatment inhibited tumor growth at all concentrations. The final volume of the control tumors was 610.8% of the initial volume. In contrast, the final tumor volume for the 1×10^6 IB group was 267.2% of the initial volume and that of the 4 and 8×10^6 IB group being 345.3% and 344.3% respectively. This shows that IB treatment significantly inhibited the tumor growth of CT26 tumors since the volume of the control tumors was more than double that of the IB treated mice. Figure 6.4 (b) shows the progression of IB-treated CT26 tumors in comparison to the control mice, highlighting that IB-treatment resulted in a much smaller tumor size as compared to the control.

The histological evaluation of the CT26 tumors showed increased necrosis after treatment with IB (Figure 6.5). The necrotic regions in the tumor, denoted by N, grew more extensive as the concentration of IB increased. Treatment with 8×10^6 IB resulted in a very large portion of tumor tissue being necrotic, with a small area of viable tumor tissue remaining, denoted by T. This shows that the direct anti-tumor effect of IB is amplified in the presence of an immune system.

Figure 6.4: Effect of IB on tumor burden in immunocompetent mouse. (a) Tumor volumes of CT26 tumors in BALB/cARC mice. Mice were treated with intratumoral injections of 1, 4 and 8×10^6 IB in PBS every 3 days. Control group was treated with PBS. Data expressed as mean $\pm$ SD. (** $p < 0.01$) (b) Tumor progression of control and IB-treated CT26 tumors over 12 days.

Figure 6.5: (a) Hematoxylin and eosin staining of CT26 tumors treated with varying concentrations of IB. Tumors were harvested at the end of 12 days showing the extent of necrosis. Viable regions of the tumor stained purple (T). IB treatment caused extensive area of necrosis, stained pink (N). Scale bar = 200 μ m. (b) Estimated area of necrosis as a % of tumor area. Data expressed as mean $\pm$ SD. (n=3)

IB treatment improved the survival rates of CT26-bearing mice (Figure 6.6). In

the control group, 60% of CT26-bearing BALB/cARC mice died/were culled due to due to extensive tumor growth, while 40% survived the entire study. The 1×10^6 IB group had a survival rate of 80%. Notably, 100% of the mice treated with 4 and 8×10^6 IB survived the entire study, showing that IB treatment improved the survival rates in CT26 tumor-bearing mice.

Figure 6.6: Survival curve of CT26 tumor-bearing mice after treatment with 1, 4 and 8×10^6 IB. (n=5)

6.4.1.3 Inactivated Bacteria Elicit Anti-Tumor Immune Response in Immunocompetent Mouse Model

The difference in the results of the nude mouse model and BALB/cARC model suggested the involvement of the immune system in the effectiveness of IB treatment. This was confirmed when the treatment of CT26 tumors with IB elicited an immune response in the BALB/cARC mice.

Swelling and redness was observed at the tumor site after doses, indicating that IB treatment elicited an immune response. To further analyze this response, the tumor samples were examined by immunohistochemical (IHC) staining for the

presence of CD3+ T-cells. The brown-colored, positive cytoplasmic staining of cells indicated the CD3+ T-cells that had infiltrated the tumor in the during the study (Figure 6.7 (a)). The number of T-cells in the IB-treated tumors were expressed relative to those in the control tumor (Figure 6.7 (b)). It can be seen that IB treatment almost doubled the number of T-cells infiltrating the tumors, with a 1.95-fold increase after treatment with 1×10^6 IB dose.

Figure 6.7: Effect of IB on immune response in CT26-bearing immunocompetent mice. (a) Immunohistochemistry showing CD3+ T cells (brown stained) in CT26 tumors. (b) Mean (n=3) CD3 positivity per 100 tumor cells as determined by IHC, expressed relative to control. Data expressed as mean $\pm$ SD. Scale bar = 400 μ m.

Next, the effect of the IB-mediated treatment on the immunologic memory of the BALB/cARC mice was evaluated. In the first part of the study, CT26 tumors were established on the right flank of the mice. In this re-challenge experiment, IB-

treated mice and naïve BALB/cARC mice (control) were sub-cutaneously injected with 4×10^6 CT26 cells on the left flank and tumor growth was monitored for 10 days. While the CT26 tumors were established in 100% of the control mice, a tumor-like growth was found to be growing in only 20% of the IB-treated group. Furthermore, while the average volume of the naïve control tumors was 72.44 mm^3 , the volume of the growth in the IB-treated group was only 9.45 mm^3 which was about 1/8th of that of the control animals.

Histological analysis of the tumor-like growth in the IB-treated group showed no evidence of tumor mass or neoplastic cells. It consisted of a central area of necrosis surrounded by fibrosis (Figure 6.8 (a)). The intense inflammation in the tumor-like growth was qualified by IHC staining for CD3+ T-cells showed that there were 2.9 times more T-cells in the IB-treated group than the naïve control group (Figure 6.8 (b) and (c)). This shows that IB treatment pre-emptively rejected the establishment of new CT26 tumors, providing complete immune protection against tumor re-growth.

Figure 6.8: Tumor re-challenge of IB-treated immunocompetent mice with CT26 cells. (a) Hematoxylin and eosin staining of re-challenge tumor established in naïve control and IB-treated mice. Viable regions of the tumor stained purple (T), necrotic region stained pink (N). Scale bar = 200 µm (b) Immunohistochemistry showing CD3+ T-cells (brown stained) in re-challenge tumors. Scale bar = 400 µm. (c) CD3 positivity per 100 tumor cells as determined by IHC, expressed relative to naïve control. Data expressed as mean ± SD.

6.4.2 Discussion

Our previous research showed that the administration of IB inhibits the growth of CT26 (murine) and HCT116 (human) colorectal cancer cells *in vitro* in 2D and 3D models, demonstrating the direct anti-cancer effect of this derivative [194]. This section of the chapter investigated the *in vivo* effect of IB in two mouse models. The first model involved the sub-cutaneous establishment of the HCT116 tumor in nude mice, while the second model used immunocompetent BALB/cARC mice sub-cutaneously injected with the syngeneic CT26 cell line. Both models involved a treatment regime of intratumoral injections of IB.

In the HCT116 tumor model, the IB treatment resulted in an increase of the extent of necrosis in the tumors as compared to the control, however, the tumor volumes were similar to that of the control. This showed that the overall tumor burden in immunocompromised mice did not reduce after IB treatment. Conversely, the CT26 tumor model showed an increase in the extent of necrosis as well as a decrease in the tumor volume, indicating a decrease in tumor burden. These results show that the direct anti-cancer effect of IB is compounded by an immune response leading to a greater inhibition of tumor growth *in vivo*. Additionally, IB treatment improved the survival rate of the tumor-bearing mice.

However, in both models, the inhibition of tumor volume or increase in necrosis was not proportional to the increase in IB dose. A possible explanation for this could be the concentrations of IB used, 1, 4 and 8×10^6 bacteria. At a microbial level, a $4\times$ or $8\times$ increase in concentration is not sufficient to observe a significant difference in their effect on the host [232].

Notably, IB treatment of mice stimulated the immune system to exert an anti-tumor effect in immunocompetent mouse model. CT26 tumors are known to be

highly immunogenic with the up-regulated expression of MHC class I molecules, which results in levels of intratumoral immune cells [233], something that was confirmed by our IHC analysis of the control tumors. Despite the presence of tumor infiltrating lymphocytes (TILs), these tumors are able to evade an anti-tumor immune response. They do this by producing immune suppressive factors [234,235], up-regulating the expression of immunosuppressive ligands [156,160,161], secreting cytokines like IL-10, TGF β , IL-6 and enzymes like IDO, arginase and iNOS which mediate immune suppression by inhibiting T cell function and disrupting dendritic cell (DC) maturation [162,163,236]. Tumors also recruit immune suppressor cells such as regulatory T (Treg) cells and myeloid-derived suppressor cells (MDSCs) which can suppress effector T cell expansion and function [156,158,159,236]. This suppression of the immune system allows the tumor to continue to thrive.

IB treatment was able to stimulate the immune system to act against the tumor. Figure 6.9 illustrates the proposed mechanism for this. As discussed in Chapter 4, IB induce pyroptosis in tumor cells, which leads to their necrotic cell death and the release of DAMPs, TAAs and inflammatory cytokines. These molecules can cause the activation of T-cell to target and kill the remaining tumor cells. IB treatment has the potential to induce DC maturation which can elicit a cytotoxic T-cells response against the tumor cells. PAMPs on the IB surface can interact with the TLR2 present on DCs and can induce DC maturation. This PAMP-mediated DC maturation has been previously found to strongly stimulate T-cells expansion and secretion of perforins, IFN- γ and TNF β , which are cytotoxic to tumor cells [164,237–240]. This proposed action of IB-mediated immune response is supported by other studies which have reported that inactivated bacteria and bacterial cell wall extracts can similarly elicit an anti-tumor immune response [85,115,195,214,240–242]. Consequently, it is proposed that the direct

immunogenic effects of IB contributes to the anti-tumor immune response observed. This suggests that the direct anti-tumor action of IB may have a minor role while the immune response elicited by IB plays a major role in affecting tumor inhibition. This would explain the apparent dose-independence of IB mediated inhibition *in vivo*. The immune response generated by this range of IB doses, up to 8-fold increase in IB concentration, is likely to be similar. Thus, the decrease in overall tumor volume is seen to not vary much between IB doses (Figure 6.4).

Figure 6.9: Working hypothesis of IB-mediated anti-tumor immune response. DAMPs, TAAs and inflammatory cytokines released from necrotic tumor cells and PAMPs from IB trigger DC maturation. Activated DC present TAAs to T-cells, and trigger their activation along with DAMPs released from the necrotic tumor cells. This elicits a specific anti-tumor immune response of the activated T-cells against the other viable tumor cells. DAMPs = danger-associated molecular patterns; DC = dendritic cell; IB = inactivated bacteria; PAMP = pathogen-associated molecular patterns; TAAs = tumor-associated antigens. Black dashed arrows represent movement, black solid arrows activation and red capped line inhibition. Green, red and yellow circles represent DAMPs, inflammatory cytokines and TAAs, respectively.

The results of this study also show that IB can prime the immune memory to

reject future occurrences of the same tumor, which is supported by the findings of a previous study by Agrawal et. al which used *C. novyi*-NT spores [86]. It is notable that the use of IB is a safer alternative due to the inactive state of *C. sporogenes* bacteria.

The effectiveness of IB in mediating tumor growth inhibition in these *in vivo* tumors showed that it is not limited by the presence of hypoxia. The effectiveness of IB was hypoxia-independent and it was able to stimulate the immune system against the tumor. Thus, it would reduce the risk of tumor recurrence.

6.5 Effect of Conditioned Medium on Colorectal Cancer *In Vivo*

6.5.1 Results

6.5.1.1 Conditioned Medium Limits Growth of Colorectal Tumor Xenograft in Immunocompromised Mouse Model

HCT116 tumors had a minimal response to intratumoral injections of CM. As seen in Figure 6.10, volumes of tumors treated with 5% and 15% CM were lower than control for the first 9 days of the study. However, between day 9 and 12, the volume of 5% CM-treated tumors increased from 115.6% to 275.2% of initial tumor volume and the 15% CM-treated tumors had an increase in volume from 145.3% to 219.9%. At the end of the study, the control tumor volume was 227.7% of initial tumor volume. Conversely, 10% CM-treated had a higher tumor volume than the control till day 9 for the study, after which tumor growth was inhibited, resulting in the final volume (162.3% of initial tumor volume) being lower than the control's. The difference in the final tumor volumes was not significant. This shows

that CM exerted an inhibitive effect on HCT116 tumors only for short durations of the study. The tumor volume of 15% RCM groups were comparable to the control group. All the groups in the HCT116 tumor model had a 100% survival rate.

Figure 6.10: Effect of CM on tumor burden in immunocompromised mouse. Tumor volume of HCT116 tumors in nude mice. Mice were treated with intratumoral injections of 5, 10, 15% CM in PBS every 3 days. Control group was treated with PBS. 15% RCM in PBS was used as a control for the CM. Data expressed as mean $\pm$ SD.

The histological evaluation of the HCT116 tumors showed that the extent of necrosis increased slightly after treatment with CM in a dose dependant manner (Figure 6.11). This shows that the CM-treatment induces cell death *in vivo* which is reflected in the increase in necrotic area. However, the overall tumor growth is only inhibited for a short period of time during the study, and tumor outgrowth occurs towards the end of the study. In an immunocompromised mouse model, the *in vivo* efficacy of CM is limited and unable to reduce the overall tumor burden.

Figure 6.11: (a) Hematoxylin and eosin staining of HCT116 tumors treated with varying concentrations of CM. Tumors were harvested at the end of 12 days showing the extent of necrosis. Viable regions of the tumor stained purple (T). CM treatment increased the extent of necrosis, stained pink (N). Scale bar = 200 μ m. (b) Estimated area of necrosis as a % of tumor area. Data expressed as mean $\pm$ SD. (n=3)

6.5.1.2 Conditioned Medium Limits Growth of Syngeneic Colorectal Tumor in Immunocompetent Mouse Model

CM had an inhibitive effect on CT26 tumors for the first half of the study, where tumor volumes were inhibited by at least 68% in CM-treated groups on day 6. The volume of 15% CM-treated tumors was significantly lesser than that of the control tumors. However, after day 6, tumor growth increased at a rate comparable to that of the control group. This resulted in 5% CM-treated tumors having a volume of 596.7% of initial tumor volume at the end of the study, which was very close to the control volume of 610.8% of initial tumor volume. Groups treated with 10 and 15% CM had final volumes 764.5% and 721.6% of initial tumor volume (Figure 6.12 (a)). This shows that the inhibitive effect of CM on CT26 tumors is no longer observable in the second half of the study. The difference between the mean tumor volume of the control group and that of the 15% CM group is not significant due to the high standard deviation in the latter group. Figure 6.12 (b) shows the tumor progression of the CT26 tumors treated with 5, 10 and 15% CM. The histological evaluation of the CT26 tumors showed that the necrotic area increased slightly after treatment with CM (Figure 6.13).

Figure 6.12: Effect of CM on tumor burden in immunocompetent mouse. (a) Tumor volumes of CT26 tumors in BALB/cARC mice. Mice were treated with intratumoral injections of 5, 10, 15% CM in PBS every 3 days. 15% RCM in PBS was used as a broth control. The control group was treated with PBS. Data expressed as mean $\pm$ SD. (* $p < 0.05$) (b) Tumor progression of control and CM-treated CT26 tumors over 12 days.

Figure 6.13: (a) Hematoxylin and eosin staining of CT26 tumors treated with varying concentrations of CM. Tumors were harvested at the end of 12 days showing the extent of necrosis. Viable regions of the tumor stained purple (T). CM treatment increased the extent of necrosis, stained pink (N). Scale bar = 200 μ m. (b) Estimated area of necrosis as a % of tumor area. Data expressed as mean $\pm$ SD. (n=3)

CM treatment also improved the survival rates of the animals bearing CT26

tumors. While the control group had 40% survival, CM treatment improved the survival rates of CT26-bearing mice (Figure 6.14). In the control group, 60% of CT26-bearing BALB/cARC mice died/were culled due to extensive tumor growth, while 40% survived the entire study. 15% CM group had 60% survival while 5 and 10% CM groups both had 80% survival.

Figure 6.14: Survival curve of CT26 tumor-bearing mice after treatment with 5, 10, 15% CM in PBS. (n=5)

Unlike IB-treatment, the treatment of CT26 tumors with CM did not elicit an immune response in the BALB/cARC mice. There was no inflammation observed at the tumor site after doses. The tumor samples examined by immunohistochemical staining for the presence of CD3+ T-cells did not show a significant increase in the number of T cells (with brown-colored, positive cytoplasmic staining for CD3) (Figure 6.15 (a)). The number of T-cells in the CM-treated tumors, expressed relative to those in the control tumor (Figure 6.15 (b)), are similar to that in the control.

Figure 6.15: Effect of CM on immune response in CT26-bearing immunocompetent mice. (a) Immunohistochemistry showing CD3+ T cells (brown stained) in CT26 tumors. (b) Mean (n=3) CD3 positivity per 100 tumor cells as determined by IHC, expressed relative to control. Data expressed as mean $\pm$ SD. Scale bar = 400 μ m.

6.5.2 Discussion

The aim of this section was to evaluate the efficacy of CM *in vivo* against colorectal tumors using immunocompromised and immunocompetent mouse models, to assess its efficacy in complex biological systems and ability to reduce the risk of tumor recurrence. The tumor volumes of CT26 and HCT116 tumors exposed to CM were not significantly different from those of their respective controls. CM treatment caused a decrease in the tumor volumes in the early part of the study in both tumor models, but tumor outgrowth was observed towards the end of the study. The average tumor volume in the mice treated with 10% and 15% CM at day 12 was greater than that of the control. However, the difference between the data sets was not significant. The extent of necrosis in the tumor core increased only slightly for all concentrations of CM. The effect of CM *in vivo* also differed from that of IB in its ability to stimulate the immune system. The number of CD3+ T-cells infiltrating CM-treated tumors did not differ significantly from the control tumors. Additionally, the effect of CM on tumor volume in the immunocompromised mice was not significantly different from that in the immunocompetent mice. This indicated that the immune system was not stimulated by CM to exert an anti-tumor effect.

Figure 6.16 shows the end-point comparison between the effect of IB and CM against *in vivo* subcutaneous colorectal tumor models. Against HCT116 tumors in nude mice (Figure 6.16(a)), the end-point tumor volume of mice treated with 4×10^6 IB was significantly higher than that of the 10% CM group. As the standard deviation in the data sets were considerable, no significant difference was observed between the other groups. In immunodeficient mice bearing CT26 tumors, CM treated groups had higher end-point tumor volumes than the control group as well as all the IB treated groups (Figure 6.16(b)). This shows that CM had lower

efficacy *in vivo* than IB. This is the opposite of what was observed *in vitro*, where CM mediated significantly stronger inhibition of 2D and 3D models of colorectal cancer than IB (Figure 5.11, Figure 5.12).

Figure 6.16: Comparison between *in vivo* effect of IB and CM on subcutaneous colorectal cancer models. Tumor volumes at end-point (Day 12) of (a) HCT116 and (b) CT26 tumors treated with IB and CM. Data expressed as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, t-test is a comparison between IB and CM.

These results are in line with the trend where therapeutic effects observed *in vitro*

are not always translated *in vivo*. To understand the lack of success of CM *in vivo*, the various aspects of CM treatment have to be re-examined. The route of delivery of therapeutic agents is crucial for effectiveness of the treatment. Intravenous injections of CM would have likely led to loss of the majority of key therapeutic components. Thus, intratumoral injections are the preferred method for administering secreted bacterial products such as CPE [127,129,243]. While these studies reported a significant reduction in tumor burden after intratumoral administration of CPE, CM failed to demonstrate similar effectiveness *in vivo*.

One of the reasons for the low efficacy of CM could be that the concentrations selected (5, 10, 15% of complete growth media) were too low to observe an anti-cancer effect *in vivo*. Furthermore, Section 5.3.5 showed that the key component of CM is likely to be less than 3 kDa in size. This could have resulted in the active component of CM being cleared away from the host body too quickly. The enhanced permeability and retention (EPR) effect that enables the targeting of macromolecular therapeutic agents to tumors does not apply to small molecules [244]. Low-molecular weight agents, such as small molecule drugs and toxins, rapidly diffuse into the blood stream before being cleared by the renal system [245,246]. This lends weight to the speculation that the small molecular weight of the key component of CM, less than 3 kDa, results in the poor retention of CM in the SC tumors. Consequently, an inhibitive effect of CM against colorectal cancer models *in vivo* is not observed.

The *in vitro* efficacy of CM belies the strong anti-cancer effect demonstrated *in vitro*. This suggests that the complexity of *in vivo* tumors is not sufficiently encompassed by *in vitro* cancer models such as the 2D monolayer and 3D spheroid model. Furthermore, the use of homogenous cancer cell cultures to establish *in vivo* tumors does not recapitulate the heterogeneity of clinical tumors [247]. The use of

cell lines to test the efficacy of therapeutic agents like CM is reductive because of their limitations; change in genotype and phenotype, and lack of tumor microenvironmental factors [248]. These factors could have resulted in the discrepancy between the *in vitro* and *in vivo* results of CM against colorectal cancer.

6.6 Conclusion

There are three key findings of this chapter: (1) IB have a dual anti-cancer effect of directly inhibiting tumor growth and mediating an anti-tumor immune response. (2) IB treatment develops host immunity to reject future re-challenges of the tumor. (3) CM has a limited efficacy *in vivo*. The effect of CM can be investigated further by testing a larger range of doses. The potential of IB as an immunotherapeutic agent can be maximized with further research that evaluates the effects over a longer period of time and analyzes the changes in the tumor-immune milieu over the course of time. Additionally, the safety and efficacy of the treatment can be further validated by conducting toxicity assays and testing a larger range of doses.

Chapter 7

Concluding Remarks and Future Outlook

7.1 Concluding Remarks

Bacterial cancer therapy is able to overcome the limitations of conventional forms of cancer therapy. However, there are several gaps of bacterial cancer therapy that need to be addressed before it can be developed as a clinically relevant treatment option. This thesis aimed to address these gaps by generating the bacterial derivatives, inactivated bacteria (IB) and conditioned medium (CM), of *Clostridium sporogenes* and testing them for their anti-cancer efficacy.

The first gap was the risk of toxicity associated with several bacterial strains that have been used for cancer therapy previously. The selection of the non-toxic, non-pathogenic *C. sporogenes* contributed to the minimization of this risk. Previous studies with *C. sporogenes* had proved their anti-cancer properties, but the application of this bacteria in its spore form posed the risk of infection. Thus, two

non-viable derivatives, IB and CM, were generated. IB and CM are incapable of bacterial proliferation and re-growth, thus minimizing the risk of infection. The anti-cancer efficacy of these derivatives was screened against renal, melanoma, colorectal, hepatic, breast and urinary bladder cell lines. Chapter 3 showed that IB and CM retain the natural anti-cancer properties of *C. sporogenes* and differentially inhibit the proliferation and viability of multiple human cancer cell lines in 2D and 3D *in vitro* models, with the strongest inhibition of the colorectal cancer cell line, HCT116. However, as the screening panel only included one cell line per cancer type, it would be premature to dismiss renal cancer, melanoma or urinary bladder cancer, as unsuitable candidates for treatment with IB and CM. There is a need to test multiple cell lines of these cancer types to determine the diseases that can be targeted and treated by IB and CM. As IB and CM demonstrate a higher efficacy against colorectal cancer cells, the subsequent chapters used these cells for further experiments.

To investigate the anti-cancer properties of IB in greater depth, it was tested on 2D and 3D *in vitro* models of human and murine colorectal cancer cells lines (Chapter 4). To gain a better understanding of the underlying mechanism of IB-mediated inhibition of cancer, a range of IB concentrations was tested. When tested *in vitro* in cell culture conditions, IB were found to inhibit the growth of human and murine colorectal cancer cells in a dose-dependent manner. This shows that the anti-efficacy of IB is hypoxia-independent. This would help to minimize the risk of recurrence associated with the use of hypoxia-dependent anaerobic spores. Studying the interaction between IB and the cancer cells revealed that physical contact is critical for an anti-cancer effect. Additionally, IB mediated necrotic cell death. Taken together, these results suggested that the underlying mechanism is likely to be the activation of pyroptosis through the ligation of the PAMPs on

the IB with the TLR2 receptors present on the cancer cell surface. This working hypothesis was proposed based on the findings of this chapter. There is a need to test this hypothesis to gain a better understanding of the mechanism of the anti-cancer effect of IB.

The *in vitro* efficacy of CM against colorectal cancer cells was discussed in Chapter 5. Testing the efficacy of CM *in vitro* in cell culture conditions showed that the anti-cancer effect of CM is hypoxia-independent. This suggests that CM would be able to minimize the risk of recurrence associated with hypoxia-dependent bacterial agents. The underlying mechanism and key component of CM-mediated cell death were investigated. CM mediates apoptotic cell death, indicating its mechanism was different from that of IB-mediated inhibition of cancer cells. Further studies showed that the active component of CM is likely to be of a molecular weight lower than 3 kDa. The identification and isolation of the key component of CM-mediation inhibition is a critical step which will allow the development of a highly purified and specific therapeutic agent. Future work should be focused on this to gain a better understanding of the mechanism of CM-mediated anti-cancer effect. Once the key component has been identified and isolated, an appropriate delivery system can be developed to ensure viable administration of the therapeutic.

All the *in vitro* studies involved 3D models of cancer, to test the efficacy of IB and CM in complex systems. To further validate their clinical relevance, both derivatives were also tested in animal models of colorectal cancer. The sub-cutaneous tumor models in BALB/cARC and nude mice modelled the hypoxic conditions at the core of real tumors. IB and CM were intratumorally administered to test their efficacy. Immunocompetent mice were used to test the immune stimulation ability of IB and CM. IB were found to exert a dual anti-cancer effect of directly inhibiting tumor growth and mediating an anti-tumor immune response. It reduced the

tumor burden and improved survival rate in tumor-bearing mice. Additionally, IB treatment also developed host immunity to reject future re-challenges of the tumor. This suggests that IB minimizes the risk of recurrence. CM was found to have a limited efficacy *in vivo*. While it improved the survival rate, it did not directly decrease the tumor volume or elicit an anti-tumor immune response.

The contribution of this thesis to the field of bacterial cancer therapy can be summarized through 3 key findings. Firstly, it established that the non-viable derivatives of *C. sporogenes*, IB and CM retain the anti-cancer effect previously demonstrated in its spore or vegetative form. Secondly, IB and CM have an advantage over previously used spore or live forms of bacteria, as they are non-viable and can minimize the risk of infection, and they are hypoxia-independent and can limit the risk of tumor recurrence. Thirdly, IB have the potential to not only elicit a potent anti-tumor immune response against the existing tumor but also prime the immune memory to reject future occurrences of the same tumor. This thesis has shown the potential of non-viable derivatives of *C. sporogenes* as anti-cancer agents and that there is much to look forward to in the development of IB and CM as effective therapeutic agents.

7.2 Future Outlook

This thesis has established the anti-cancer effect of the non-viable derivatives of *C. sporogenes*, IB and CM, against colorectal cancer models. In addition to these results, more work can be done to improve the understanding of bacteria-mediated inhibition of cancer and develop IB and CM as therapeutic agents.

7.2.1 Mechanism of Inactivated Bacteria-Mediated Anti-Cancer Activity

The results discussed in Chapter 4 showed that physical contact between IB and cancer cells was critical for anti-cancer activity through IB-mediated necrosis of the cells. The proposed explanation for this was the activation of the pyroptosis pathway through the ligation of PAMPs on IB with TLR2 receptors on the cancer cells. To test this hypothesis, IB can be fractionated into membrane fraction and intracellular fraction, using an extraction method previously described [249]. After fractionation, each fraction of IB can be administered to cancer cell cultures. The effect of the two fractions on the cancer cells can be evaluated as using the WST-1 assay to quantify the effect on cell proliferation. This will confirm whether the key anti-cancer component of IB is present in its outer membrane. Going further, the two most abundant groups of surface molecules found on *C. sporogenes*, proteoglycans [250] and cell wall proteins [251], can be isolated and tested on cancer cell cultures. The role TLR2 in IB-mediated tumor inhibition can be investigated by using OxPAPC, a TLR2 inhibitor, to block the corresponding pathway. Commercially available TLR2 antagonists, such as Sparstolonin B, can be used as positive controls. The effect on IB on TLR2-blocked cell cultures can be evaluated using cell proliferation assay, WST-1.

7.2.2 Mechanism of Conditioned Medium-Mediated Anti-Cancer Activity

This thesis has shown that the key component in CM is likely to be of a molecular weight of < 3 kDa. The identification of this component will be vital for the development of CM as a therapeutic agent. The proteins in this molecular weight

fraction of CM can be identified using MALDI-TOF mass spectroscopy and analyzed using the Mascot program. The isolated components can then be tested separately by administering them to cancer cells in culture and evaluating their effect on cell viability and cell proliferation. The type of cell death that cancer cells undergo when exposed to CM is another starting point for understanding the mechanism of CM-induced inhibition of cancer cells. Chapter 5 showed that CM mediates apoptosis of the cancer cells. Apoptosis induction can be examined via caspase activity (caspase 3/7) across different time points. Cyclins are key regulatory components, directing the cell cycle machinery through the different phases. The total levels and/or phosphorylation status of individual cyclins can be easily and rapidly measured using specific antibodies by immunoblotting. The Cyclin D1 ELISA kit can be used, allowing for a more quantitative assessment of expression, where the absorbance reading of the sample is proportional to the quantity of cyclin D1 protein. Together with the apoptosis data, the cell cycle analysis will allow the effect of CM on cancer cell cultures to be better understood.

7.2.3 Safety and Efficacy of Inactivated Bacteria *In Vivo*

The preliminary *in vivo* study discussed in Section 6.4 showed that IB exert an anti-cancer effect in mouse models of colorectal cancer. The sample size for the study can be decided upon by conducting power calculation. The safety and efficacy of IB *in vivo* can be further investigated. The maximum tolerated dose (MTD) of IB can be determined in two phases of dose range finding studies. For the single dose phase, 4 dose levels of IB can be administered ($0, 50, 500, 5000 \times 10^6$ IB/kg in PBS through intravenous (IV) injection) in healthy mice and monitored for 7 days. Based on the results of the single dose phase, the dose levels of the repeated dose phase can be decided, and the doses can be administered in 4 levels daily for 5 days. For both phases, the following parameters and end points can be evaluated in this

study: mortality, clinical signs, body weights, food consumptions, haematology and serum biochemistry parameters, post mortem evaluation included gross examination for all the animals at the terminal necropsy and histopathological examination of major organs in the control and high dose groups [252]. Biodistribution of IB can be evaluated in naïve and HCT116-tumor bearing female athymic mice, using previously established protocols [253]. The mice can be ^{125}I -labeled IB supplemented with unlabelled IB such that the total number of IB injected is $15,000 \times 10^6/\text{kg}$. Mice in groups of three or four can be euthanized by CO_2 inhalation at 1 h, 1 day, 3 days, 7 days, and 14 days post-injection, and their blood (0.1 ml), brain, heart, lungs, liver, spleen, kidneys, muscle, bone, small and large intestines, and tumor will be removed, weighed, and counted in an automated gamma counter.

Based on the results of the dose range finding studies, 3 doses of IB can be intratumorally administered to HCT-116 tumor-bearing female nude mice, with a vehicle control of PBS. The objective of this is to evaluate the dose-dependent effect of IB on tumor volume. The tumor volume will be evaluated daily and weight will be measured before each dose and at termination. At the endpoint, tumors and major organs will be harvested. The tumors will be processed for histological analysis, which will include haematoxylin & eosin staining, gram staining. TUNEL staining will be conducted to evaluate the apoptosis of tumor cell. Quantification of circulating DNA (such as hLINE-1) is commonly used to evaluate xenograft tumor burden in animal models [254, 255]. hLINE-1 will be quantified by qPCR after blood samples collection, DNA purification from plasma. Decreasing levels of hLINE-1 correspond to the decreased tumor burden and increasing survivability.

7.2.4 Immune Response to Inactivated Bacteria

The immune response to IB needs to be studied further in order to develop it as an anti-cancer therapeutic agent. The *in vivo* experiment with BALB/cARC mice bearing CT26 tumors conducted previously can be repeated, with IB treatment. At the endpoint, tumors and major organs can be harvested. Lymphocytes and splenocytes from the lymph nodes and spleens and leukocytes from the bone marrow and peripheral blood of IB-treated, tumor-bearing and healthy control mice can be isolated for future use. The tumors can be processed for histological analysis, which will include haematoxylin & eosin staining, gram staining. TUNEL staining can be conducted to evaluate the apoptosis of tumor cell. IHC staining of immune markers can determine the nature of immune cell infiltration.

Another tumor re-challenge study can be conducted in two experiments. In the first experiment, IB-treated mice from the tumor regression study detailed above, can be re-challenged with a SC injection CT26 cells on the opposite flank of the mice. Naïve Balb/c mice can be inoculated for the first time with CT26 cells and used as a control for this experiment. The growth of the tumors can be monitored for 10 days, with tumor sizes being measured every 3 days. Splenocytes collected in the first study can be cultured for 3 days before the supernatant of the culture is collected for cytokine analysis. An ELISA kit can be used to analyse the cytokine levels, giving us a profile of the cytokine regulation that occurs due to IB treatment. The leukocytes isolated from the first study can be counted by flow cytometry to give an indication of leukocyte levels after IB treatment.

For the second experiment, CD4+ and CD8+ cells can be purified from the lymphocytes collected in the previous studies, and adoptively transferred to naïve Balb/c mice. These mice can then be challenged with a sub-cutaneous injection of

CT26 cells. Establishment of tumors can be monitored through tumor measurements over 10 days.

7.2.5 Effect of Inactivated Bacteria and Conditioned Medium on Other Cancerous and Non-Cancerous Cell Lines

The results described in Chapter 3 showed that the renal cancer cell line, A498, did not respond well to treatment. This was the reason it was not used for further experiments. However, one cell line cannot be entirely representative of the entire cancer type. Therefore, it is important to screen the efficacy against multiple cell lines of a cancer type. Future studies can be conducted using 3 to 4 cell lines per cancer type to provide a more wholistic understanding of the efficacy of IB and CM against different types of cancer. Additionally, other prevalent cancer types, such as prostate cancer, can also be used to test the effect of IB and CM.

Furthermore, examining the effect of IB and CM on non-cancerous cells originating from the native tissue of the cancer type (kidney, colon, breast, liver, skin) will allow us to understand the targeted specificity of these as therapeutic agents.

Chapter 3 showed that the breast (MCF-7) and liver (HepG2) cell lines respond well to treatment with IB and CM. Further studies on these cell lines will enable a better understanding of the therapeutic potential of IB and CM. The *in vitro* and *in vivo* experiments can be conducted as mentioned previously, using the MCF-7 and HepG2 cell lines. To validate the efficacy of IB and CM using a more clinically relevant platform, it is recommended that patient-derived xenografts are additionally used for these studies.

Appendix A

Appendix

A.1 Correlation of CT26 and HCT116 Growth

Figure A.1: Cell proliferation of CT26 and HCT116. Both cell lines were seeded at a density of 5000 cells/well and grown in a humidified atmosphere at 37 °C, 5 % CO₂. Cell proliferation was measured every 24 hours.

A.2 *In Vitro* Growth of Cell Lines

Figure A.2: Growth of A498 cells. (a) Cell proliferation of 2D culture of A498 cells. Fluorescence measured at 590 nm. (b) Viability of 3D spheroid culture of A498 cells. Cells seeded at 5000 cells/well and grown for 72 hours. Cell proliferation and spheroid viability were measured every 24 hours.

Figure A.3: Growth of G361 cells. Cell proliferation of 2D culture. Fluorescence measured at 590 nm. Cells seeded at 5000 cells/well and grown for 72 hours. Cell proliferation was measured every 24 hours.

Figure A.4: Growth of HCT116 cells. (a) Cell proliferation of 2D culture. Fluorescence measured at 590 nm. (b) Viability of 3D spheroid culture. Cells seeded at 5000 cells/well and grown for 72 hours. Cell proliferation and spheroid viability were measured every 24 hours.

Figure A.5: Growth of HepG2 cells. (a) Cell proliferation of 2D culture. Fluorescence measured at 590 nm. (b) Viability of 3D spheroid culture. Cells seeded at 5000 cells/well and grown for 72 hours. Cell proliferation and spheroid viability were measured every 24 hours.

Figure A.6: Growth of MCF7 cells. (a) Cell proliferation of 2D culture. Fluorescence measured at 590 nm. (b) Viability of 3D spheroid culture. Cells seeded at 5000 cells/well and grown for 72 hours. Cell proliferation and spheroid viability were measured every 24 hours.

Figure A.7: Growth of RT4 cells. (a) Cell proliferation of 2D culture. Fluorescence measured at 590 nm. (b) Viability of 3D spheroid culture. Cells seeded at 5000 cells/well and grown for 72 hours. Cell proliferation and spheroid viability were measured every 24 hours.

Figure A.8: Growth of CT26 cells. Cell proliferation of (a) 2D and (b) 3D spheroid culture. Absorbance measured at 430 nm, with reference at 650 nm. Cells seeded at 5000 cells/well (2D culture) and 1500 cells/well (3D spheroids) and grown for 72 hours. Cell proliferation was measured every 24 hours.

Figure A.9: Growth of HCT116 cells. Cell proliferation of (a) 2D and (b) 3D spheroid culture. Absorbance measured at 430 nm, with reference at 650 nm. Cells seeded at 5000 cells/well (2D culture) and 1500 cells/well (3D spheroids) and grown for 72 hours. Cell proliferation was measured every 24 hours.

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