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pH-Dependent Release and Folate Receptor Alpha (FRα) Targeting To Allow Exclusive Targeting of Lipid-Nanoparticles (LNP)

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Abstract

Introduction: Colorectal cancer rates continue to rise in Canada, placing an increasing economic burden on the healthcare system and profoundly impacting patients' quality of life. Here, we propose a novel method for delivering anti-colorectal cancer treatment that uses a Eudragit-coated folate-LNP conjugation to allow pH-sensitive release and specificity for cancerous colon cells.

Methods: GFP-tagged IL-10 mRNA will serve as a biomarker to evaluate folate receptor alpha (FRα) binding, transfection, and release of therapeutic contents by the novel LNP formulation in LS174 human cells, measured by flow cytometry. To confirm exclusive delivery, tissue samples from the small and large intestines of $Apc^{Min/+}$ mice treated with the formulation will be analyzed using ELISA following oral gavage. MTT assay of healthy, non-cancerous CCD 841 CoN will confirm consistent cell viability across non-targeted colonic tissue.

Expected Results: The folate-LNP formulation is expected to bind FRα on the LS174 cells efficiently and release the encapsulated IL-10 mRNA after endosomal escape, evidenced by significantly higher fluorescence in treated cells than the negative controls. In the mouse model, the Eudragit coating is anticipated to dissolve exclusively in the colonic tissue of the mouse models, with minimal dissolution in the small intestine, allowing targeted binding to cancerous colonic enterocytes.

Discussion: Our proposed folate-LNP formulation is designed to achieve targeted delivery to colonic tissue by leveraging pH-sensitive release and FRα upregulation in colorectal cancer cells. Efficient binding and successful translation of IL-10 mRNA in the LS174 cells will demonstrate the formulation's specificity and therapeutic potential. Exclusive localization to cancerous colonic tissue in the mouse model will confirm the ability to bypass non-target tissues.

Conclusion: This approach represents the first known proposal for a Eudragit-coated folate-LNP molecule targeting the colon. If successful, this approach could offer a more effective, targeted treatment for colorectal cancer, minimizing systemic side effects and improving patient outcomes. Future research and clinical validation are required to confirm the safety and efficacy of this approach, as well as to determine the patients who will benefit most.

Keywords: colorectal cancer; lipid nanoparticles; cancer therapy; exclusive organ targeting

Introduction

Colon cancer is one of the most prevalent forms of cancer worldwide and is characterized by abnormal cell growth in the colon or rectum. In Canada, colon cancer is the second leading cause of cancer-related deaths among men and the third leading cause among women [1]. Despite advances in screening and treatment, the disease remains a significant cause of morbidity and mortality, particularly when diagnosed in later stages. Conventional therapies, such as surgery, chemotherapy, and radiation, can be effective but often lead to significant side effects and are not always successful in preventing recurrence or metastasis [2]. In particular, the non-specific nature of these traditional cancer treatments result in both healthy and cancerous cells being impacted. For example, chemotherapy often eliminates beneficial gut bacteria in addition to cancerous cells, leading to chronic inflammation and gastrointestinal damage [3]. With high-risk and intensive surgeries, patients often face

higher risks of infection, intestinal leakage, and longer recovery times [3]. As such, this uncertainty has spurred interest in innovative therapeutic strategies that can more specifically target the underlying mechanisms of cancer development.

One promising approach involves the use of lipid nanoparticles (LNPs), which encapsulate therapeutic molecules, such as nucleic acids for therapy. This delivery system is useful as LNPs can protect therapeutic agents from degradation in the body and improve their cellular uptake into specific tissues or organs. However, they do not naturally protect against gastric juices due to their acidity, so an outer coating is often necessary. The lipid shell consists of four lipids: ionizable lipids, helper lipids, cholesterol, and polyethylene glycol (PEG) lipids [4, 5]. LNPs work by mimicking the environments of cell membranes, which helps them fuse with cell surfaces or enter cells via endocytosis [4]. Once taken up by the cell, LNPs are typically trapped in an endosome. To facilitate endosomal escape, ionizable lipids become positively charged in the acidic environment of the endosome, which helps disrupt the endosomal membrane and release the therapeutic cargo into the cell's cytoplasm [4, 5]. Once in the cytoplasm, the released therapeutic molecule (e.g., mRNA) can reach its target within the cell, such as the ribosome, where it can be translated into proteins.

Currently, LNPs modified with surface charge-altering SORT molecules have proven successful in driving the targeted delivery of mRNA to the liver, lung, and spleen [6]. Recent reviews [5, 7] report that LNPs have demonstrated to be effective vehicles for therapeutic delivery. To further enhance the targeting properties of the LNPs, Hao et. al [8] recommend modifying the LNP surface with tumour-specific ligands, such as folate.

We propose expanding on the premise of this nanotechnology to achieve targeted delivery to the colon. By targeting cancer cells more precisely, modified LNPs offer the potential to reduce systemic toxicity, enhance therapeutic efficacy, and pave the way for more personalized cancer treatments. However, intravenous administration of medication, as used in the SORT approach, is often less preferred by patients than oral delivery. In a study conducted by Eek et. al [9], it was reported that patients saw oral administration as providing greater autonomy and convenience, and eliminated issues associated with intravenous therapy, such as pain and difficulty starting an IV line. Current anti-colon cancer treatments offered in tablet form include Capecitabine [10] and Trifluridine and Tipiracil [11], albeit neither is colorectal cancer specific [12, 13]. In leveraging the convenience of oral formulations with the specificity provided by modified LNPs, the approach to colon cancer therapy could be transformed, enabling more efficient drug delivery to the tumor site, while preserving patients' quality of life and sense of normalcy.

When targeting colon cancer, several key factors must be considered for effective therapeutic delivery using LNPs to the colon. Current literature demonstrates that like many other cancers, colon cancer cells have abnormal expression of folate receptors, including folate receptor alpha (FRα) [14]. Shia et al. [15] found that FRα was positively higher in carcinomas when compared to healthy tissue. These findings suggest that FRα could serve as a biomarker for distinguishing cancerous from non-cancerous colon tissue and may also be a suitable target for therapies aimed at FRα-positive cancer cells [8].

In this study, we propose an oral Eudragit-coated folate-LNP formulation designed for the exclusive delivery of therapeutic contents to cancerous colon tissue. Cellular modelling with LS174 human cells followed by flow cytometry will validate uptake, while oral gavage tube administration to $Apc^{Min/+}$ mice and an ELISA of tissue from the colon and small intestine will confirm exclusivity. This study seeks to answer whether a folate-LNP formulation with Eudragit coating can effectively target cancerous colon tissue, providing exclusive delivery and minimizing systemic side effects.

Methods

Folate-LNP Formulation

This study utilizes IL-10, an interleukin that functions to maintain immune balance [16], mRNA tagged with GFP as a marker for transfection and monitoring the survival of our formulation under various conditions. This tagging will occur through *in situ* hybridization and is a non-invasive approach to monitoring. Since the mRNA will be translated by cytosolic ribosomes, no nucleic entry or DNA alteration is necessary, unlike other approaches such as the Cre-*LoxP* system that requires genetic deletions [17]. This protocol aims to confirm highly specific uptake and cargo unloading of our formulation such that it can deliver future anti-cancer medications, rather than the ability to conduct genetic modifications, so a tagged-mRNA-based approach is sufficient. In addition, the use of IL-10 as a carrier for GFP is unlikely to cause cell death that would inhibit our monitoring. It is an anti-inflammatory cytokine whose deficiency under stressful cellular conditions, such as infections, is more associated with immunopathology [18].

Folate is added to the LNP formulation for improved specificity, as it promotes LNP binding to cancerous cells abnormally expressing upregulated FRα [14]. We will first prepare our base LNP following the Onpattro formulation with 4A3-SC8, DSPC, cholesterol, DMG-PEG, and folate-PEG at a molar ratio of 50/10/38.5/1.0/0.5, and IL-10 mRNA [19]. The LNP will be prepared using an ethanol dilution method, where one part of ethanol immersed with the lipids is mixed with three parts of aqueous buffer containing the mRNA. The LNP can be mixed by three different methods based on the available equipment and required scale of the study. These methods include pipette, vortex, and microfluidic mixing. Pipette mixing is suitable for small batches, involving manual rapid pipetting. Vortex mixing, appropriate for medium-scale batches, uses a vortex mixer for vigorous agitation. In contrast, microfluidic mixing is

ideal for medium to large-sized batches and uses a custom-built microfluidic device with polydimethylsiloxane (PDMS) channels that are integrated with syringe pumps. Microfluidic mixing can also use commercial systems like the NanoAssemblr (Precision Nanosystems). Our protocol follows the vortex mixing method [20].

Eudragit Coating

After the successful formulation of the LNPs, an outer coating of Eudragit S100, an anionic copolymer soluble at pH >7, will be applied to enable pH-sensitive release to the colon [21]. This coating will protect the folate-conjugated LNPs in the gastric environment and ensure LNP release in the colon (pH = 7.4) [21]. A mixture of acetone and isopropanol in a 40:60 ratio is commonly used as the organic solvent to prepare Eudragit S100 coatings. The coating process involves spray-coating or dip-coating techniques to ensure a uniform layer over the LNP. Parameters, such as spray rate and drying conditions, will be considered carefully to ensure coating integrity and to prevent aggregation. To enhance the specificity and mechanical strength of the Eudragit S100 coat, polymers such as talc or hydroxypropyl methylcellulose (HPMC) can be combined. The solvent will be evaporated through air or oven drying, leaving the coated LNP behind [21].

Cellular Modelling

We propose the LS174 human cell line for modelling delivery to the colon [22]. These cells were extracted from a patient with colorectal adenocarcinoma and are frequently used for therapeutic research on colon cancer. Cellular modelling will be conducted with the naked LNP to ensure successful transfection into colonic epithelial cells after Eudragit dissolution. Untreated cells will be used as negative controls to confirm successful binding and endosomal escape. The LS174 cells will also be compared against treated and non-treated cells from the non-cancerous human colon cell line, CCD 841 CoN [23] to verify selective targeting to cancer cells, while preserving healthy tissue. Cells will be cultured in DMEM media for 48 hours at 37 ˚C to allow optimal growth while preventing cell overcrowding. The LNP formulation will be resuspended in PBS by vortexing to avoid aggregation. Afterwards, varying concentrations of the LNP molecules will be cultured alongside the human cells to determine the optimal concentration for transfection efficiency. These will be cultured at 37 °C for 4-6 hours, after which the media will be replaced, and no LNPs will be added beyond this point to avoid LNP toxicity. Cells will be cultured overnight at 37˚C to allow translation of the GFP-mRNA [23].

After harvesting, the cells will be washed with PBS to remove contaminants. The cells will then be resuspended. GFP activity will be measured through flow cytometry to confirm the successful transfection of the formulated LNP and translation of the mRNA.

Cellular Viability

An MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay will be used to assess cell viability of healthy, non-cancerous cells following treatment exposure by measuring mitochondrial activity [24]. The living cells convert MTT into formazan crystals, and the number of crystals produced correlates with the number of viable cells. This assay is commonly used to evaluate the cytotoxic effects of drugs on cell lines. In this study, the MTT assay will be used to confirm the LNP formulation does not reduce cell viability in the non-cancerous CCD 841 CoN cells. Cells exposed to the previously determined optimal concentration of the treatment will be compared against untreated CCD 841 CoN cells.

Confirmation of Gastric Stability

Before modelling the administration of our formulation in mouse models, the stability of the coated folate-LNP in a gastric environment will be simulated. A 0.01M hydrochloric acid solution (pH 2) will mimic the 1-3 pH environment of the stomach [25]. Consistent with a normal dietary transit time of 15 minutes to 4 hours [25], multiple preparations of the optimal concentration of the formulation, as determined in the previous step, will be submerged in the HCl solution and monitored hourly for six hours to account for slower gastric emptying rates in some individuals. At each interval, a subset of the solutions will be brought to a pH of approximately 6.5 with 0.1M NaOH to mimic the colonic environment [26]. Centrifugation will separate the released LNPs from the neutralized liquid. After discarding the supernatant, the pelleted LNPs will be washed three times and resuspended in PBS. The solution will be applied to cultured LS174 cells for four to six hours at 37 °C. The media will then be replaced with non-LNP-containing PBS and the cells will be cultured overnight at 37 °C. The following day, successful uptake of the LNP and translation of the IL-10 mRNA will be quantified through GFP expression, measured by flow cytometry.

Animal Modelling

We propose using the colon cancer mouse model $Apc^{Min/+}$ to demonstrate exclusive delivery of our formulation to the colon. This model possesses a germline mutation in the Apc gene, predisposing the mice to the spontaneous formation of intestinal and colonic adenomas, mirroring human familial adenomatous polyposis (FAP) and sporadic colorectal cancer found in humans [27]. The $Apc^{Min/+}$ mice will be maintained under standard laboratory conditions, with a controlled diet to minimize external influences on the gastrointestinal tract. $Apc^{Min/+}$ mice treated with saline only will serve as negative controls. The coated particles will be suspended in saline according to the previously determined optimal concentration and delivered to the mice through an oral gavage tube inserted into the stomach to ensure accurate dosing. After 24 hours, the mice will be euthanized. The intestines will be harvested from the

mice and separated into their large and small constituents. They will be homogenized in an ice-cold homogenization buffer to release the expressed proteins. An Enzyme-Linked Immunosorbent Assay (ELISA) will be conducted on each sample to determine the level of GFP expression in each component and confirm exclusive delivery to the colon.

Anticipated Results

We expect our formulation will efficiently bind FRα on the LS174 cells and release the encapsulated IL-10 mRNA following endosomal escape. We anticipate this expectation to remain true after simulated gastric exposure. Treated LS174 cells are expected to demonstrate significantly higher GFP fluorescence when compared to untreated controls, validating receptor-specific binding and intracellular delivery. In addition, healthy colonic cells and untreated controls are anticipated to demonstrate no fluorescence as receptor binding through FRα is not expected to occur, thereby prohibiting GFP-IL-10 translation. We expect the MTT assay to demonstrate consistent high cell viability between treated and untreated healthy CCD 841 CoN cells, confirming the treatment's selective and safe nature. In the mouse model, we expect the colonic tissue of treated mice to demonstrate significantly higher fluorescence when compared to the negative controls, while small intestinal tissue from these mice is expected to exhibit low, insignificant levels of fluorescence, consistent with successful bypass of the small intestine.

Discussion

In this protocol, we have proposed a novel method of developing a folate-LNP molecule that facilitates exclusive delivery of therapeutic contents to the colon. We expect our proposed model to leverage the Eudragit pH-sensitive release and FRα upregulation in colorectal cancers to bypass the small intestine and achieve targeted entry into colonic tissue. In the LS174 model, significantly increased fluorescence of treated cells when compared to untreated and healthy controls would confirm successful FRα-mediated binding and validate endosomal escape, confirming that contents such as IL-10 mRNA can be successfully delivered to these cells. In our animal model, significant increases in fluorescence of large intestinal tissue when compared to small bowel tissue will confirm the exclusive delivery of our formulation to the colon, supporting the effectiveness of pH-sensitive release and FRα targeting. Consistent high cell viability across healthy, non-cancerous controls after exposure will support the targeted nature of the treatment. These results will reinforce the potential of this approach for targeting cancerous colon tissue while minimizing off-target effects in the small intestine. The statistical significance of these measurements will be assessed using one-way ANOVA with Tukey post-hoc tests, assuming a parametric data distribution.

Our approach represents the first known proposal for a Eudragit-coated folate-LNP molecule designed for targeting colon cancer, representing a novel area of study that has the potential to open new treatment avenues. The current treatment approach for colorectal cancer is often surgery (polypectomy, local excision, resection of the colon with anastomosis or colostomy, radiofrequency ablation, or cryosurgery) followed by chemotherapy, radiation, immunotherapy, or other targeted therapies to halt the metastasis and survival of remaining cancer cells [28]. These therapies can have systemic effects, such as causing sexual health issues in men and women, anemia, and hair loss in the case of non-specific chemotherapy [29]. By targeting colon cancer cells exclusively, our approach could minimize or eradicate some of these effects, leading to a more comfortable treatment experience and potentially increased therapeutic efficacy.

Colorectal cancer was projected to be the third most common cause of cancer in Canadian males and females in 2024, and this diagnosis was expected to be the second and third most common cause of cancer deaths in these individuals, respectively [30]. These increasing rates place a substantial economic burden on the Canadian healthcare system, accounting for a projected $3.5 billion in direct health system costs [31]. Colorectal cancer also has a profound impact on a patient's quality of life due to physical, emotional, and psychological challenges that spread to family members and caregivers. Our proposed formulation, if successful, could address these challenges by minimizing the avenues required for patients to achieve remission.

Our strategy holds great promise in colon cancer research, but limitations in our proposed methodology must be addressed before widespread implementation. First, we suggest $Apc^{Min/+}$ mice as an animal model for our study. However, this and other colon cancer mouse models have not demonstrated an upregulation of FRα, lending the need to further study this expression before modelling our approach. Second, we suggest surgical separation of the small and large intestines in the animal models to confirm exclusive delivery. This task is intensive and must be conducted by a skilled individual who may be unavailable to a research group. Third, while our suggested separation would help confirm the validity of our approach, further testing is essential to address areas of pH overlap between these organs that may allow premature dissolution of the Eudragit. Finally, there are unique differences between patients that may impact the effectiveness of this treatment.

To address these challenges, representative clinical trials must be developed to identify patients most likely to benefit from this treatment to avoid unnecessarily increasing the economic burden on patients, caregivers, and families. In addition, further research on FRα expression and surgical optimization in colon cancer models must be conducted to confirm the validity and feasibility of our approach.

Conclusions

In conclusion, our proposed folate-LNP formulation is a promising strategy for targeted colon cancer treatment,

potentially reducing systemic side effects and improving treatment efficacy. Our intention with this publication is for our suggested formulation to serve as a foundation for future research into folate-LNP-based therapies, particularly in achieving exclusive colonic targeting. Further research and clinical validation of our approach are required to optimize this formulation and confirm its safety and efficacy in clinical settings.

List of Abbreviations
ELISA: enzyme-linked immunosorbent assay
FAP: familial adenomatous polyposis
FRα: folate receptor alpha
HPMC: hydroxypropyl methylcellulose
LNP: lipid nanoparticle
PDMS: polydimethylsiloxane
PEG: polyethylene glycol
SORT: selective organ targeting

Conflicts of Interest
The authors declare that they have no conflict of interest.

Ethics Approval and/or Participant Consent
This study did not require ethics approval or participant consent as it presents a protocol that has not been performed on human or animal participants.

Authors' Contributions
MAC: developed the core study design, expected results, and discussion, and provided final approval of the version to be published.
DR: contributed to study design, planning, and methods development, and provided final approval of the version to be published.
TL: contributed to study design and planning and provided final approval of the version to be published.

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