Title:

IDENTIFICATION OF STRATEGIES AIMED AT INHIBITING ERK5 NUCLEAR TRANSLOCATION FOR TARGETED CANCER THERAPY

Background and Aim: Cancer presents persistent challenges in modern medicine, demanding innovative therapeutic strategies. While progress has been made, existing approaches face limitations. This study aims to unravel the intricate role of Extracellular Signal-Regulated Kinase 5 (ERK5) nuclear translocation in cancer, offering potential avenues for disrupting this process and revolutionizing targeted therapy.

Methods: Cancer cell lines will be cultured and ERK5 expression validated using advanced techniques. Genetic manipulation and pharmacological inhibitors will modulate ERK5 levels, and a screening assay will identify inhibitors disrupting nuclear translocation. Optimal inhibitor concentrations will be determined through assays, molecular modeling, and in vivo xenograft models. Combination therapies will explore synergies. Data will be rigorously analyzed, employing statistical and bioinformatics tools.

Expected results: The expected results encompass a comprehensive characterization of ERK5 inhibition effects on cancer cell behavior upon disrupting nuclear translocation. The study anticipates the identification of potential inhibitors targeting ERK5 nuclear translocation, presenting a promising avenue for innovative targeted therapy approaches. Furthermore, the research foresees the potential for enhanced treatment efficacy through synergistic interactions between ERK5 inhibition and established treatments, leading to improved treatment outcomes. By gaining insights into resistance mechanisms, the study aims to inform the development of combination therapies to mitigate treatment resistance. The findings are expected to enable patient stratification and personalized treatment strategies based on distinct ERK5 characteristics, potentially revolutionizing the approach to cancer care. Moreover, the research is poised to contribute to the advancement of targeted therapies, diversifying the available options for cancer treatment. Ultimately, the successful validation of ERK5 inhibitors holds the potential to translate laboratory discoveries into tangible clinical applications, opening avenues for future clinical trials and significantly impacting the field of cancer therapy.

Keywords: *Cancer, ERK5, Nuclear translocation, Targeted therapy, Inhibitors, Synergies, Personalized treatment, Clinical trials*

1. Introduction

Cancer continues to be a formidable challenge in the realm of modern medicine, prompting relentless efforts to identify innovative and effective therapeutic strategies. While significant progress has been made in understanding the intricate molecular underpinnings of various cancers, current therapeutic approaches still face formidable challenges that limit their efficacy and long-term success (1, 2). The traditional cancer treatment triumvirate of surgery, chemotherapy, and radiation therapy, while pivotal in cancer management, often confronts limitations that impact both patient outcomes and quality of life. Radiation therapy, while capable of targeting specific tumor regions, can inadvertently damage surrounding healthy tissues, resulting in complications that impede treatment optimization and patient recovery. Immunotherapy, a promising innovation that harnesses the immune system's power to recognize and attack cancer cells, has shown remarkable potential. However, its success is contingent on overcoming the immunosuppressive tumor microenvironment and the development of durable responses in a subset of patients (3, 4). Moreover, targeted therapies that exploit specific molecular vulnerabilities within cancer cells have demonstrated remarkable successes. However, the clinical utility of these agents is often hindered by the development of resistance mechanisms (5).

Among the numerous molecular players implicated in cancer progression, the Extracellular Signal-Regulated Kinase 5 (ERK5) pathway has emerged as a critical factor in tumorigenesis. Central to its role is ERK5's ability to translocate into the nucleus, where it exerts its pro-tumorigenic effects by modulating gene expression and signaling cascades. Therefore, the quest to unravel strategies that hinder ERK5 nuclear translocation presents a promising avenue for targeted cancer therapy. ERK5, a crucial member of the mitogen-activated protein kinase (MAPK) family, has emerged as a focal point in cancer research due to its intricate engagement in fundamental cellular processes. Its multifunctional role spans cell proliferation, survival, differentiation, and even migration, implicating ERK5 in the orchestration of pivotal physiological responses. As such, it holds a dual significance – contributing to normal cellular functions while also being a potent driver of malignancy (6-11).

One of the most compelling aspects of ERK5's involvement in cancer lies in its ability to translocate into the cell nucleus. This nuclear translocation serves as a molecular switch that activates a cascade of downstream events, including the modulation of gene expression and the initiation of oncogenic programs. By exerting its influence within the nucleus, ERK5 empowers cancer cells to acquire enhanced proliferative capacity, evade apoptosis, and bolster their invasive potential, ultimately promoting tumor growth and metastasis (12). However, ERK5's nuclear localization serves as more than a facilitator of aggressive cancer behavior; it also acts as a safeguard against the efficacy of current therapeutic approaches. The aberrant activation of ERK5 can lead to the development of resistance to various treatment modalities, rendering established therapies less effective over time. This phenomenon underscores the urgent necessity to uncover novel strategies that specifically disrupt ERK5's nuclear translocation, thereby dismantling the foundation of its oncogenic influence (13-15).

The driving force behind this research proposal is the compelling necessity to confront the pressing challenges that persist within current cancer treatment paradigms. This study is dedicated to unraveling the intricate molecular mechanisms that underlie ERK5's nuclear

translocation, a phenomenon pivotal in driving oncogenic programs and fostering resistance. The primary objective is to uncover innovative strategies capable of disrupting this process, thereby opening avenues for a paradigm shift in targeted cancer therapy. By achieving this, the research carries the profound potential to spark a transformative evolution in the treatment landscape, offering a promising route towards novel therapeutic approaches that not only enhance the effectiveness of treatments but also curtail the emergence of resistance, ultimately culminating in an elevated standard of care and improved well-being for individuals engaged in the battle against cancer.

2. Methodology

The research methodology for this study has been primarily formulated drawing upon two key sources: a 2018 study conducted at the University of Florence under the leadership of Tusa (16), and a subsequent study in 2022 led by Tubita et al. (17) at the same institution in Italy.

2.1. Cancer Cell Line Culture and ERK5 Expression Evaluation

A diverse range of cancer cell lines, encompassing various malignancies, will be cultivated and maintained, with meticulous attention given to ensuring optimal culture conditions and maintaining cellular purity. The comprehensive validation of ERK5 expression and its intricate subcellular localization will be meticulously pursued, employing an array of cutting-edge techniques, including advanced immunofluorescence microscopy methodologies, high-resolution Western blotting analyses, state-of-the-art confocal imaging modalities, innovative single-cell RNA sequencing protocols, and robust quantitative reverse transcription polymerase chain reaction (RT-PCR) assays.

2.2. Genetic Manipulation and Pharmacological Inhibitors

Genetic manipulation techniques, encompassing the precise utilization of siRNA and CRISPR/Cas9 technologies, will be implemented to enact controlled modulation of ERK5 expression levels. The consequential impact of these modifications on nuclear translocation will be systematically assessed, employing state-of-the-art imaging methods. Furthermore, the exploration extends to employing pharmacological inhibitors specifically targeting ERK5 activation, permitting a detailed elucidation of the intricate signaling pathways dictating its nuclear transport. "XMD8-92," is used as a common pharmacological inhibitor known to selectively inhibit the activity of ERK5 by binding to its ATP-binding pocket. Concomitantly, real-time imaging techniques will be harnessed to monitor and capture alterations in ERK5 subcellular localization, with subsequent quantitative analyses unveiling the nuanced spatial and temporal dynamics underlying ERK5's movement within the cellular milieu.

2.3. Screening Assay

An extensive collection of diverse compounds, encompassing FDA-approved drugs as well as naturally occurring compounds, will be assembled and organized into a screening library. Subsequently, a highly robust and tailored screening assay will be developed, harnessing the power of cutting-edge technology such as high-content imaging and fluorescence-based assays. This sophisticated screening platform will be employed with precision to systematically identify compounds within the library that possess the capacity to disrupt ERK5 nuclear translocation, ultimately unveiling potential candidates with the ability to intricately modulate this pivotal cellular process.

2.4. Determination of Optimal Concentrations of ERK5 Nuclear Translocation Inhibitors

Lead compounds, identified through the initial screening based on their potency and specificity, will be carefully prioritized. Subsequent steps will encompass the execution of dose-response experiments, enabling the precise determination of optimal concentrations that effectively inhibit ERK5 nuclear translocation. In-depth confirmation of inhibitor activity will be undertaken through the strategic deployment of orthogonal assays, including co-immunoprecipitation, fluorescence resonance energy transfer (FRET), and proximity ligation assays. These assays, each selected for their capacity to offer unique insights, will collectively contribute to the robust validation of the identified candidate inhibitors, providing a multifaceted understanding of their efficacy and establishing a solid foundation for their potential utilization in targeted cancer therapy.

2.5. ERK5 Modeling by PyMol

The ERK5 protein will be modeled using PyMol, enabling the prediction of potential binding sites where inhibitors might interact. To gain a deeper understanding of these interactions, molecular docking simulations will be executed to systematically assess the binding affinities and intricate dynamics governing the interactions between the ERK5 protein and the candidate inhibitors. The veracity of these computational predictions will be further substantiated through the judicious application of cutting-edge structural biology techniques, including X-ray crystallography or cryo-electron microscopy.

2.6. Xenograft Models

Human cancer cells will be employed to develop xenograft models, facilitating an in-depth evaluation of the efficacy of ERK5 nuclear translocation inhibitors within an in vivo context. Throughout this process, attention will be dedicated to the monitoring of crucial parameters, including tumor growth dynamics, metastatic progression, and the overall therapeutic response. These assessments will be underpinned by the application of non-invasive imaging techniques, exemplified by methodologies such as bioluminescence imaging or positron emission tomography, thereby enabling the visualization and quantification of the effects exerted by the ERK5 inhibitors on the multifaceted facets of cancer progression within living organisms.

2.7. Combination Therapies:

ERK5 nuclear translocation inhibitors will be combined with conventional chemotherapy or targeted agents, with the overarching aim of unveiling potential synergies. This exploration will span both in vitro and in vivo domains, encompassing the careful assessment of combination treatments. In-depth analyses will be undertaken, scrutinizing critical parameters including tumor regression kinetics, shifts in apoptotic markers, and the emergence of potential side effects. Through this inquiry, the interplay between the ERK5 nuclear translocation inhibitors and established therapeutic agents will be elucidated, offering insights into the potential enhancement of treatment outcomes and the potential mitigation of unwanted effects.

2.8. Data Analysis:

The data will be subjected to rigorous scrutiny and interpretation, facilitated by the adept application of appropriate statistical methodologies. These encompass widely recognized techniques, including t-tests, analysis of variance (ANOVA), and comprehensive regression analysis.

2.9. Bioinformatics Analysis:

Transcriptomic and proteomic data originating from cells subjected to ERK5 inhibitor treatment will be processed and analyzed through the adept utilization of advanced bioinformatics tools. This process will entail the systematic extraction of insights from intricate datasets, leading to the identification of crucially altered signaling pathways, downstream molecular targets, and the potential emergence of mechanisms contributing to resistance phenomena. By employing computational methodologies and analytical algorithms, this bioinformatics inquiry will distill intricate patterns and relationships embedded within the data, unveiling a deeper understanding of the intricate interplay between ERK5 inhibition and cellular responses.

2.10. Clinical Studies:

The validation of the significance of ERK5 nuclear translocation and the efficacy of identified inhibitors will be undertaken using patient-derived samples, encompassing tumor tissues and blood samples. These clinical specimens will be diligently analyzed to discern the presence and implications of ERK5 nuclear translocation, bolstering our understanding of its relevance in human cancer. Moreover, a detailed analysis will be conducted to establish correlations between ERK5 localization patterns and the clinical outcomes of patients, thereby unraveling potential associations with treatment responses and disease progression.

2.11. Ethical Approvals

Necessary ethical approvals for animal experiments and patient sample collection will be diligently obtained, ensuring full compliance with all relevant regulations and guidelines. This process will involve obtaining approvals from institutional review boards and regulatory bodies, addressing animal welfare considerations for experiments and ensuring informed consent and confidentiality protocols for patient sample collection.

3. Expected results

The findings of this study will reveal:

1. **Characterization of ERK5 Inhibition Effects**:
   The investigation is anticipated to reveal the precise impact of inhibiting ERK5 nuclear translocation on cancer cell behavior.
2. **Identification of Novel Inhibitors**:
   The study is likely to unveil a repertoire of novel compounds or agents that effectively hinder ERK5 nuclear translocation. These inhibitors could serve as promising candidates for targeted cancer therapy, offering potential alternatives or synergistic options to existing treatments.
3. **Enhanced Treatment Efficacy**:
   The development of strategies to inhibit ERK5 nuclear translocation could potentially enhance the efficacy of existing cancer treatments. Combining ERK5 inhibition with conventional therapies might lead to synergistic effects, reducing resistance and improving overall treatment outcomes.
4. **Insights into Resistance Mechanisms**:
   By exploring the consequences of ERK5 inhibition, the research might shed light on potential resistance mechanisms that cancer cells might employ in response to these interventions. Such insights could inform the design of combination therapies to counteract resistance emergence.
5. **Patient Stratification and Personalized Therapy**:

The study's findings could potentially contribute to the stratification of patients based on their ERK5-related characteristics. This could enable a more personalized approach to cancer therapy, tailoring treatments to individual patients for improved outcomes.

6. **Advancement of Targeted Therapies**:
The research has the potential to advance the field of targeted cancer therapy by uncovering a new avenue for intervention. ERK5 nuclear translocation inhibition could emerge as a viable therapeutic strategy, contributing to the diversification and enhancement of treatment options.

7. **Translational Impact**:
Successful identification and validation of ERK5 inhibitors could pave the way for preclinical and clinical trials, facilitating the translation of laboratory findings into tangible clinical applications.

4. Brief Bibliography

The Mitogen-Activated Protein Kinase (MAPK) pathway, which includes ERK5, has garnered considerable attention due to its pivotal role in regulating critical cellular processes like proliferation, survival, and differentiation. The elucidation of ERK5's involvement in these processes has prompted researchers to explore its potential as a therapeutic target for cancer treatment. In a 2018 University of Florence study titled "Targeting the ERK5 Pathway to Suppress Leukemia Stem Cells," researchers found that MEK5/ERK5 inhibition significantly reduced key abilities of Chronic Myeloid Leukemia (CML) cells in vitro. MEK5/ERK5 inhibition suggested its potential as an innovative therapeutic approach to suppress cancer progenitor/stem cells linked to CML (16). A review study by Tubita et al. (2020) from the University of Florence, Italy, investigated ERK5's trafficking between the cytoplasm and nucleus. The study discussed the potential utilization of these mechanisms for designing novel strategies in cancer treatment (17). In a 2020 study involving the University of Siena, Italy, a notable discovery emerged indicating that post-translational modification of ERK5's C-terminal tail, which holds significance in cancer cells due to observed hyperphosphorylation of ERK5. This finding suggests a potential link between this modification and cancer-related signaling, opening avenues for understanding cancer pathogenesis and devising targeted therapeutic approaches (18). A study involving the University of Siena, Italy, in 2022 revealed that the inhibition of ERK5 effectively overcomes breast cancer resistance to anti-HER2 therapy, primarily by targeting the G1/S cell cycle transition. These findings collectively underscore the therapeutic promise of ERK5 inhibitors in enhancing the clinical efficacy of targeted HER2 therapies, potentially advancing the treatment outcomes for patients (19). A 2022 study led by Tubita et al. at the University of Florence, Italy, explored the impact of ERK5 inhibition on melanoma cells. The researchers discovered that inhibiting ERK5 elicited cellular senescence in melanoma, mediated by the cyclin-dependent kinase inhibitor p21. Notably, ERK5 knockdown or inhibition using XMD8-92 in melanoma xenografts induced cellular senescence in vivo. These findings underscore the potential of small-molecule compounds targeting ERK5 as a promising avenue for pro-senescence drugs, presenting a novel approach for melanoma treatment, offering a potential strategy for therapeutic intervention (20).

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Title:

Anticancer Effects of Extracellular Vesicles Derived from Human Intestinal Bacteria on Colon Cancer Cells

Background and Aim: Colon cancer poses a significant health challenge in Europe, prompting considerable research interest in the human gut microbiota, especially gut bacteria, and their potential impact on cancer development and treatments. Bacterial extracellular vesicles (BEVs) from the gut have shown promising anticancer properties, although the findings are currently inconclusive. The aim of this research proposal is to investigate the potential anticancer effects of EVs derived from human gut bacteria on colon cancer cells in vitro. The study will comprehensively explore the cytotoxicity of bacterial EVs against colon cancer cells and evaluate their impact on apoptotic pathways. Furthermore, in vivo experiments using animal models will assess the antitumor effects of these EVs.

Methods: Methods include isolating human gut bacteria from fresh fecal samples using culture techniques, followed by the identification of bacterial strains through molecular methods such as PCR, 16S rRNA gene sequencing, or MALDI-TOF mass spectrometry. After isolating the bacterial EVs from cell-free supernatants, their characterization will be performed using techniques such as nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM), or dynamic light scattering (DLS) to determine size and concentration. In vitro cytotoxicity assays using MTT, bioluminescence imaging, and CCK-8 assays will determine the impact of EVs on colon cancer cells. Apoptosis evaluation will be conducted by assessing the expression levels of relevant apoptotic markers using Real-Time PCR, flow cytometry, and western blotting. Animal experiments will involve inducing colon tumors in rats and injecting EVs into the tumor site, monitoring tumor progression through the IVIS Lumina imaging system and ultrasound imaging.

Expected results: Expected results include the demonstration of human gut bacterial EVs' cytotoxicity against colon cancer cells in vitro, confirming their antitumor effects in vivo. The investigation will reveal insights into the underlying mechanisms involved in EV-induced apoptosis in colon cancer cells. The study's findings will advance our understanding of the therapeutic potential of gut bacteria-derived EVs and contribute to novel strategies for colon cancer treatment.

1. Introduction

Colon cancer is a major public health challenge in Europe, accounting for a significant number of cancer cases and mortality rates. The rising incidence of colon cancer in the region has become a cause for concern, necessitating urgent attention and research efforts. As one of the most prevalent cancers, colon cancer poses substantial challenges to healthcare systems, demanding comprehensive strategies for prevention, early detection, and effective treatment. The impact of colon cancer goes beyond the burden on healthcare resources; it also has profound social and economic implications. The disease affects individuals across various age groups, with potential impacts on their quality of life and productivity. Additionally, colon cancer places emotional and financial strain on patients and their families, further highlighting the urgency to address this growing health issue.Each year, thousands of new cases are reported, making it a major public health challenge (1,2).

In recent years, the emerging field of cancer research has increasingly focused on the human gut microbiota, with a particular emphasis on gut bacteria, including *Bacteroides fragilis*, *Escherichia coli*, *Enterococcus faecalis*, *Lactobacillus spp*., *Clostridium spp*., *Bifidobacterium spp., Streptococcus spp., Peptostreptococcus spp., Fusobacterium spp*., and *Prevotella spp* (3, 4). Understanding the interplay between gut bacteria and cancer holds promise for advancing our knowledge of cancer etiology and developing novel therapeutic approaches.

Extracellular vesicles (EVs) are fascinating nanoscale structures that play crucial roles in intercellular communication and information transfer. Released by various cell types, including bacteria, EVs are enveloped by a lipid bilayer membrane and act as messengers, shuttling bioactive cargo between cells and tissues. These bioactive molecules encapsulated within EVs include proteins, lipids, nucleic acids (such as DNA, mRNA, and microRNAs), and metabolites, making them essential carriers of information and signaling molecules. EVs are involved in diverse physiological processes, regulating cellular functions, and maintaining tissue homeostasis. They can either transmit signals to nearby cells (paracrine signaling) or travel over long distances to influence remote cells (endocrine signaling). In addition to their roles in normal physiological processes, EVs have emerged as key players in various pathological conditions, including cancer, infectious diseases, and neurodegenerative disorders. In cancer, tumor-derived EVs are known to promote tumor growth, angiogenesis, and metastasis by manipulating the tumor microenvironment and modulating immune responses. On the other hand, bacterial EVs, also known as bacterial extracellular vesicles (BEVs), have drawn attention for their potential contributions to therapeutic applications against cancer.

The investigation of EVs, including BEVs, has opened new avenues in the fields of cell biology, immunology, and cancer research. In the context of the gut microbiota, extracellular vesicles derived from intestinal bacteria have emerged as intriguing mediators of host-microbe interactions and are believed to play a pivotal role in modulating human health and disease (5, 6).

Numerous studies have shown that EVs derived from intestinal bacteria possess promising anticancer properties, particularly in targeting digestive system cancers. In vitro investigations have revealed the pro-apoptotic effects of EVs derived from Lactobacillus rhamnosus GG on hepatic cancer (HepG2) cells (7). Moreover, research has

demonstrated the anticancer effects of EVs derived from the gut bacterium *Akkermansia muciniphila* on prostate cancer (8). Additionally, recent reports have highlighted the cytotoxic effects of EVs derived from *Staphylococcus aureus* on breast cancer MCF-7 cells (9). In another study, it has been observed that extracellular vesicles derived from the human gut bacterium *Lacticaseibacillus paracasei* PC-H1 (LpEVs) possess the ability to enter colon cancer cells. Notably, LpEVs have been found to inhibit the proliferation, migration, and invasion of colon cancer cells, while inducing apoptosis through the PDK1/AKT/Bcl-2 signaling pathway (10). Furthermore, a very recent investigation unveiled that EVs derived from *E. coli* induce oxidative stress and mitophagy via mTOR pathways in colon cancer (HT-29) cells (11). These findings hold significant implications for the bioactivity of extracellular vesicles and their potential role in colon cancer progression. Despite these promising findings, it is essential to acknowledge that certain aspects of the research remain contentious, and the number of studies in this area is significantly limited, necessitating further exploration and clarification. Continued investigations in this field may shed more light on the therapeutic potential of intestinal bacteria-derived EVs in colon cancer treatment and management.

This research endeavors to investigate the potential anticancer properties of EVs derived from human gut bacteria, with a specific emphasis on their impact on colon cancers in vitro. Through a comprehensive exploration of the anticancer effects exhibited by these EVs against colon cancer cells, this study aims to elucidate the underlying mechanisms that may play a role in colon cancer prevention. The findings from this investigation hold promise for advancing our understanding of the interplay between gut bacteria-derived EVs and cancer cells, contributing to the development of novel therapeutic strategies targeting gastrointestinal malignancies.

2. Methodology

2.1. Cell line and culture

The human embryonic kidney cell line (Hek293), human colorectal adenocarcinoma cell line (HT29) will be procured from the National Cell Bank. The cell line will be cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin.

2.2. Human gut bacteria isolation

Fresh fecal samples will be collected from human volunteers to obtain a diverse and representative population of gut bacteria. The isolation process will involve culture techniques, which will allow the bacteria to grow and multiply in a controlled environment. Once the gut bacteria are successfully isolated, their identification will be carried out using advanced molecular methods. Polymerase Chain Reaction (PCR) will be employed to amplify specific regions of the bacterial DNA, allowing us to detect and analyze the presence of target bacterial genes. Furthermore, 16S rRNA gene sequencing, a powerful tool in microbiome research, will be utilized to characterize the gut bacterial community at a deeper level. By sequencing the conserved 16S rRNA gene regions, we can differentiate between various bacterial species based on their unique DNA sequences. In addition to PCR and 16S rRNA gene sequencing, Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) will be employed for bacterial identification. MALDI-TOF MS is a rapid and accurate method that relies on the detection of characteristic protein profiles unique to different bacterial species. This technique can

efficiently identify a wide range of bacteria within a short time, making it an excellent complement to the other molecular methods.

2.2. Human gut bacterial extracellular vesicles (BEVs) isolation

Following the isolation of human gut bacteria from fresh fecal samples using culture techniques, the next step in this research involves the extraction of bacterial extracellular vesicles (BEVs). To achieve this, a cell-free supernatant is obtained by subjecting the bacterial cultures to centrifugation. During centrifugation, the bacterial cells are separated from the supernatant, which contains the released extracellular vesicles. The supernatant is then carefully collected and subjected to additional filtration steps to remove any remaining cellular debris or contaminants. This filtration process ensures that the extracted BEVs are free from unwanted particles that may interfere with subsequent experiments. The isolated BEVs are then separated and purified from the supernatant using ultracentrifugation, a high-speed centrifugation technique. This step allows for the efficient isolation of BEVs based on their size and density, separating them from other components present in the supernatant. To further ensure the quality and purity of the extracted BEVs, a washing step is performed.

Throughout the entire isolation process, strict aseptic techniques are followed to prevent any potential contamination and maintain the integrity of the BEVs. The successful isolation of high-quality BEVs is crucial for this study, as it will enable us to investigate their potential anticancer effects on colon cancer cells.

2.3. Bacterial extracellular vesicles characterization

After successful isolation of BEVs, the next step in our research involves their comprehensive characterization. One of the primary techniques used for BEV characterization is nanoparticle tracking analysis (NTA). NTA allows us to measure the size and concentration of individual BEVs in a liquid suspension. By tracking the Brownian motion of the vesicles, NTA provides us with information about their size distribution and concentration, enabling us to determine the average size and quantity of BEVs present in the sample. Transmission electron microscopy (TEM) is another powerful tool used for BEV characterization. TEM allows for high-resolution imaging of individual BEVs, providing detailed information about their morphology, size, and membrane structure. Dynamic light scattering (DLS) is also commonly used to characterize BEVs. DLS measures the fluctuations in the intensity of scattered light from particles in solution, allowing us to determine the size distribution of the BEVs based on the Brownian motion of the vesicles. Once the BEVs are characterized, they are preserved under appropriate conditions to maintain their integrity and bioactivity for subsequent experiments or analysis. The comprehensive characterization of BEVs using NTA, TEM, and DLS will provide valuable insights into their physical properties and composition, contributing to a deeper understanding of their potential role in cancer therapy.

2.4. Cytotoxicity assay

In the upcoming research phase, we will conduct in vitro cytotoxicity assays to assess the effects of BEVs on colon cancer cells and healthy cells. To evaluate the cytotoxicity against colon cancer cells, we will employ three widely used and complementary assays: the MTT assay, a bioluminescence imaging system (BLI), and the Cell Counting Kit-8 (CCK-8) assay. The MTT assay is a colorimetric method that measures cell viability based on the ability of viable cells to convert the yellow tetrazolium salt (MTT) into purple formazan crystals. In conjunction with the MTT assay, we will utilize a bioluminescence imaging

system (BLI) to gain real-time insights into the cytotoxic effects of BEVs. By monitoring the changes in bioluminescence signal over time, we can dynamically assess the effects of BEVs on the metabolic activity and viability of colon cancer cells. Furthermore, to ensure safety and evaluate potential side effects of BEVs on healthy cells, we will perform the MTT and CCK-8 assays on the Hek293 cell line. By combining the MTT assay, BLI, and CCK-8 assay, we aim to obtain comprehensive data on the cytotoxicity of BEVs against colon cancer cells while considering their effects on healthy cells. These assays will enable us to determine the selectivity and specificity of BEVs in targeting cancer cells, providing valuable information for potential therapeutic applications.

2.5. Apoptosis evaluation

To comprehensively investigate the apoptotic effects of BEVs on colon cancer cells, we will assess and confirm induced apoptosis through multiple approaches. Firstly, we will employ Real-Time PCR to analyze the expression levels of key apoptotic regulators, including BAX, Bcl-2, P53, caspase-3, -8, and -9. By examining the expression levels of these pro-apoptotic and anti-apoptotic genes, we can gain insights into the molecular mechanisms underlying the apoptotic response induced by BEVs in colon cancer cells.

Flow cytometry will also play a crucial role in assessing apoptosis. Using fluorescently labeled annexin V and propidium iodide staining, we will determine the percentage of apoptotic cells. Furthermore, we will utilize western blotting to detect and quantify specific apoptotic markers in colon cancer cells. By probing the protein lysates with antibodies against BAX, Bcl-2, P53, caspase-3, -8, and -9, we can assess the changes in protein expression levels and activation status.

2.6. Animal experiments

To investigate the in vivo effects of BEVs on colon tumors, a rat model of colon cancer will be established using dimethylhydrazine (DMH) induction. Rats will be injected with DMH to induce the development of colon tumors, mimicking the pathogenesis of human colon cancer. Once the tumors have developed and reached a suitable size, the BEVs will be administered directly to the tumor site. The administration of BEVs will be carefully controlled and performed using precise techniques to ensure accurate delivery and reproducibility of the treatment. Tumor growth progression will be closely monitored using advanced imaging techniques, including the IVIS Lumina imaging system and ultrasound imaging. Ultrasound imaging will complement the IVIS Lumina system by providing additional information on tumor size, location, and morphology. After completion of the in vivo experiments, tumor masses will be collected from the rats for ex vivo study. The tumors will be dissected and weighed to quantitatively assess the impact of BEV treatment on tumor growth. Additionally, histological analysis of the tumor tissues will be performed to evaluate changes in tumor cell morphology, cell proliferation, and apoptotic rates. This comprehensive analysis will provide valuable insights into the effects of BEVs on tumor biology and validate their potential as therapeutic agents for colon cancer.

2.7. Data analysis

The data will be analyzed using a two-tailed Student's t-test and one-way analysis of variance (ANOVA).

3. Expexpected results

In this study, we explore human gut BVEs anticancer effects against colon cancer in vitro and in vivo. The findings of this study will reveal:

1. The in vitro cytotoxicity assays will demonstrate the ability of human gut BEVs to induce cytotoxic effects on colon cancer cells, potentially leading to reduced cell viability and increased apoptosis.
2. In the in vivo experiments using a rat model of colon cancer, the administration of human gut BEVs to colon tumors is expected to show antitumor effects, leading to reduced tumor growth and enhanced apoptosis.
3. The investigation of apoptotic pathways in colon cancer cells treated with human gut BEVs will reveal alterations in the expression levels of apoptotic regulators, supporting the hypothesis of BEV-induced apoptosis.
4. The assessment of human gut BEVs on healthy non-cancerous cells in vitro will determine their specificity in targeting cancer cells while minimizing potential side effects on healthy tissues.
5. The comprehensive characterization of human gut BEVs using NTA, TEM, and DLS will provide valuable insights into their physical properties and composition, contributing to a deeper understanding of their potential role in cancer therapy.
6. The study's findings will advance the knowledge of the molecular mechanisms underlying human gut BEV-induced apoptosis in colon cancer cells, shedding light on their therapeutic potential.
7. The in vivo experiments utilizing advanced imaging techniques will provide visual evidence of the effects of human gut BEVs on colon tumor growth, morphology, and apoptosis in a living organism.
8. The validation of human gut BEVs' potential therapeutic applications in colon cancer treatment may lead to the development of novel and targeted strategies for managing this prevalent and challenging disease.
9. The research's outcomes will contribute to the broader understanding of the role of gut bacteria-derived EVs in cancer research and their implications for colon cancer progression and management.

4. Brief bibliography

The study of bacterial extracellular vesicles (BEVs) has garnered significant attention in the last decade. While extracellular vesicles have been studied for many years, the focus on BEVs and their importance in cancer research has emerged more recently (12,13). Evidence of BEVs in Gram-positive bacteria was first reported in 1990 when they were observed on the surfaces of Bacillus cereus and B. subtilis using transmission electron microscopy. More recent studies have found that Staphylococcus aureus and B. anthracis produce extracellular vesicles, termed membrane vesicles (MVs), during in vitro culture (14-16). In 2019, J. Yang et al. reported on Microbe-derived extracellular vesicles as a smart drug delivery system (17). In 2021, J Park found that Gut microbe-derived EVs in colorectal cancer (CRC) had a distinct microbial composition compared to controls, suggesting that profiling of microbe-derived EVs may serve as a novel biomarker for detecting and predicting CRC prognosis (18). In 2022, Shi et al. conducted in vitro and in vivo studies demonstrating that extracellular vesicles from Lacticaseibacillus paracasei PC-H1 inhibited the growth of colorectal cancer cells in vitro and induced apoptosis through the PDK1/AKT/Bcl-2 signaling pathway (10). More recently, in 2023, Marzoog et

al. reported that bacterial extracellular vesicles induced oxidative stress and mitophagy through mTOR pathways in colon cancer (HT-29) cells (11).

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Objectives:

1. To investigate the cytotoxicity of human gut bacterial extracellular vesicles (BEVs) against colon cancer cells in vitro.
2. To evaluate the potential antitumor effects of human gut BEVs on colon tumors in vivo using a rat model of colon cancer.
3. To elucidate the effects of human gut BEVs on apoptotic pathways in colon cancer cells in vitro.
4. To assess and determine the possible side effects of human gut BEVs on healthy non-cancerous cells in vitro.
5. To comprehensively characterize the physical properties and composition of human gut BEVs using techniques such as nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM), and dynamic light scattering (DLS).
6. To explore the molecular mechanisms underlying the apoptotic response induced by human gut BEVs in colon cancer cells, focusing on the expression levels of key apoptotic regulators, including BAX, Bcl-2, P53, caspase-3, -8, and -9.
7. To determine the selectivity and specificity of human gut BEVs in targeting colon cancer cells compared to healthy cells, providing valuable information for potential therapeutic applications.
8. To investigate the effects of human gut BEVs on colon tumor growth, morphology, and apoptosis in an in vivo rat model of colon cancer using advanced imaging techniques, such as the IVIS Lumina imaging system and ultrasound imaging.
9. To validate the potential therapeutic applications of human gut BEVs in colon cancer treatment and management, based on the findings from the in vitro and in vivo experiments.
10. To contribute to the understanding of the therapeutic potential of gut bacteria-derived EVs in cancer research, particularly in the context of colon cancer progression.