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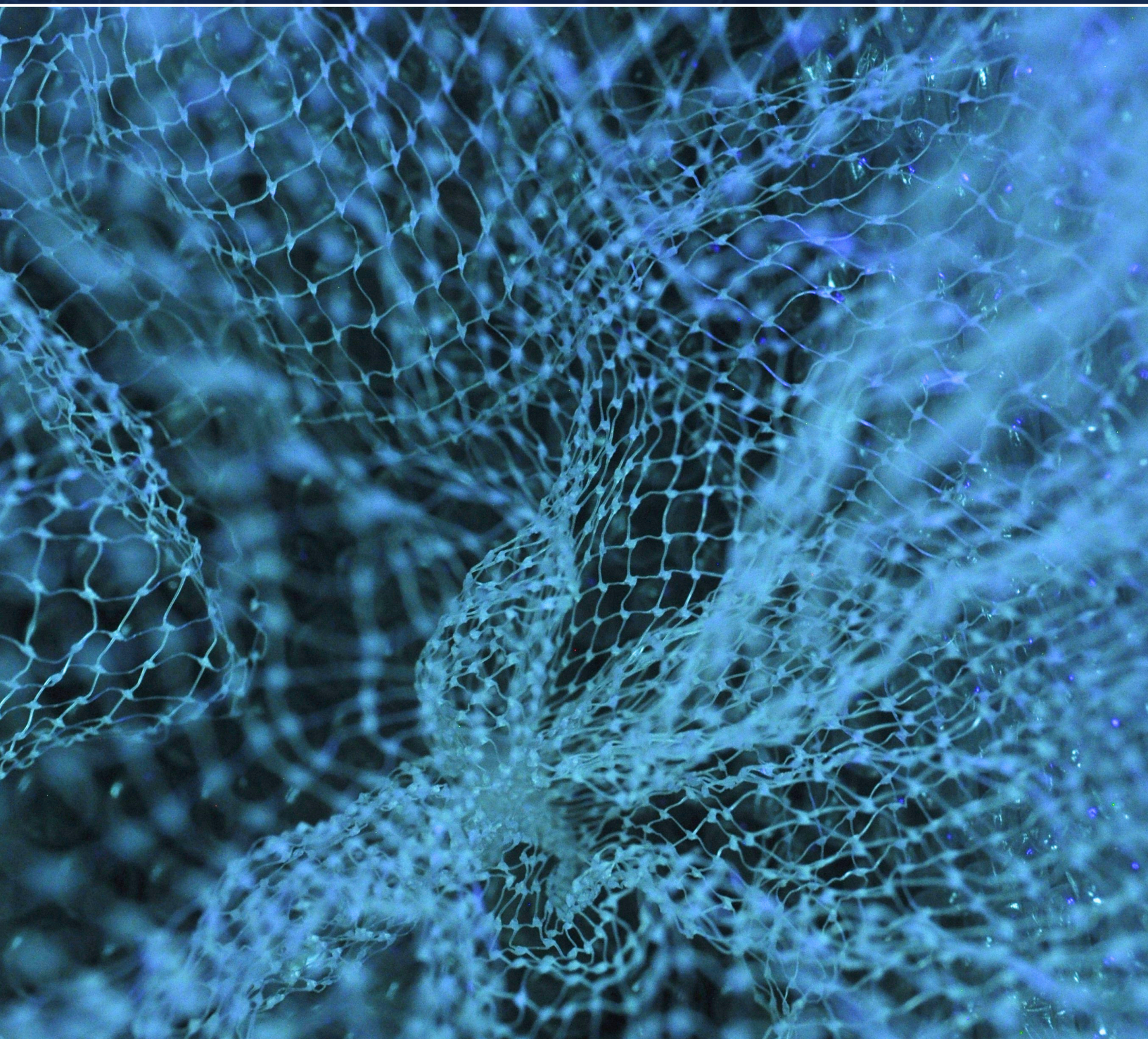

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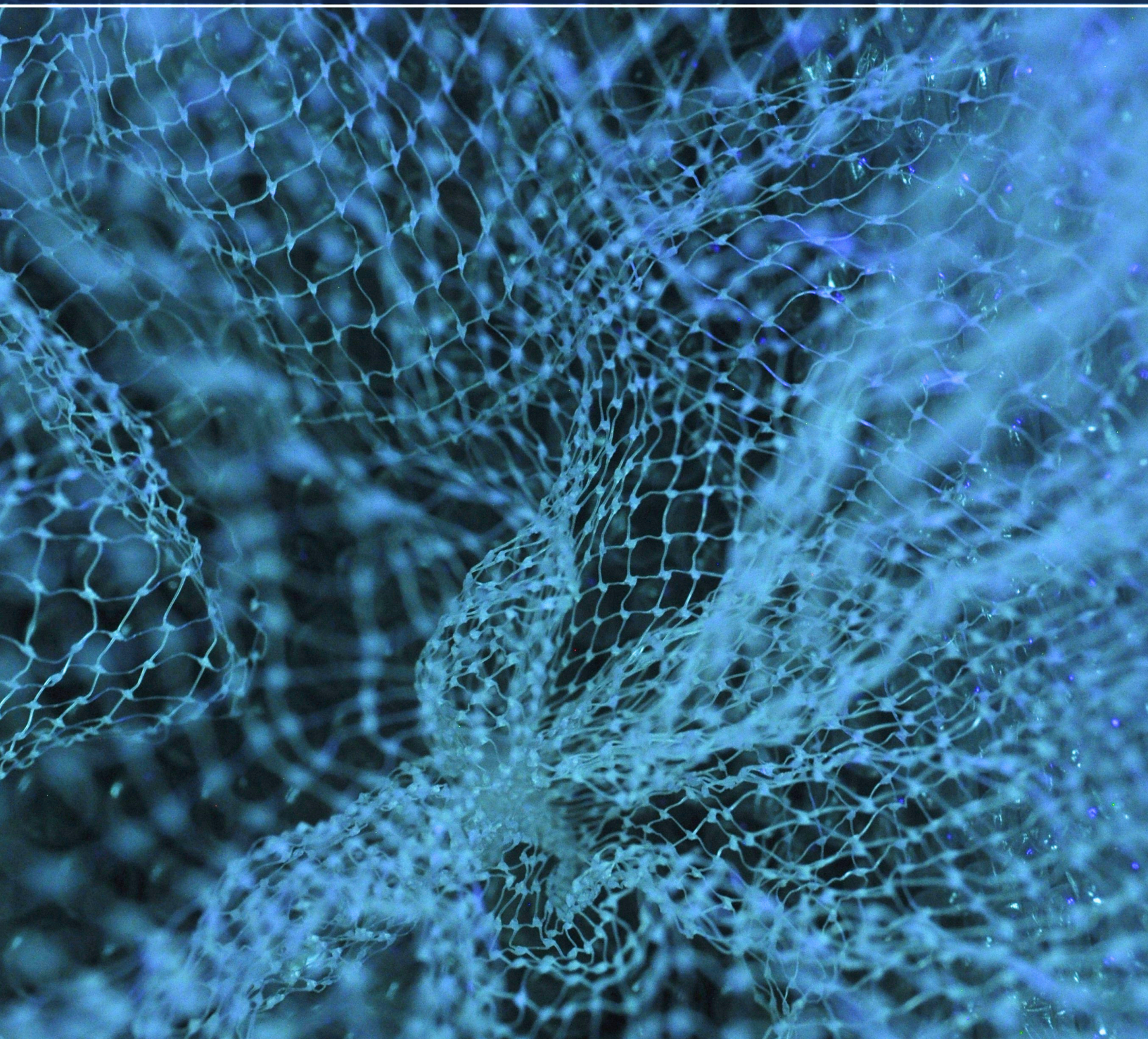

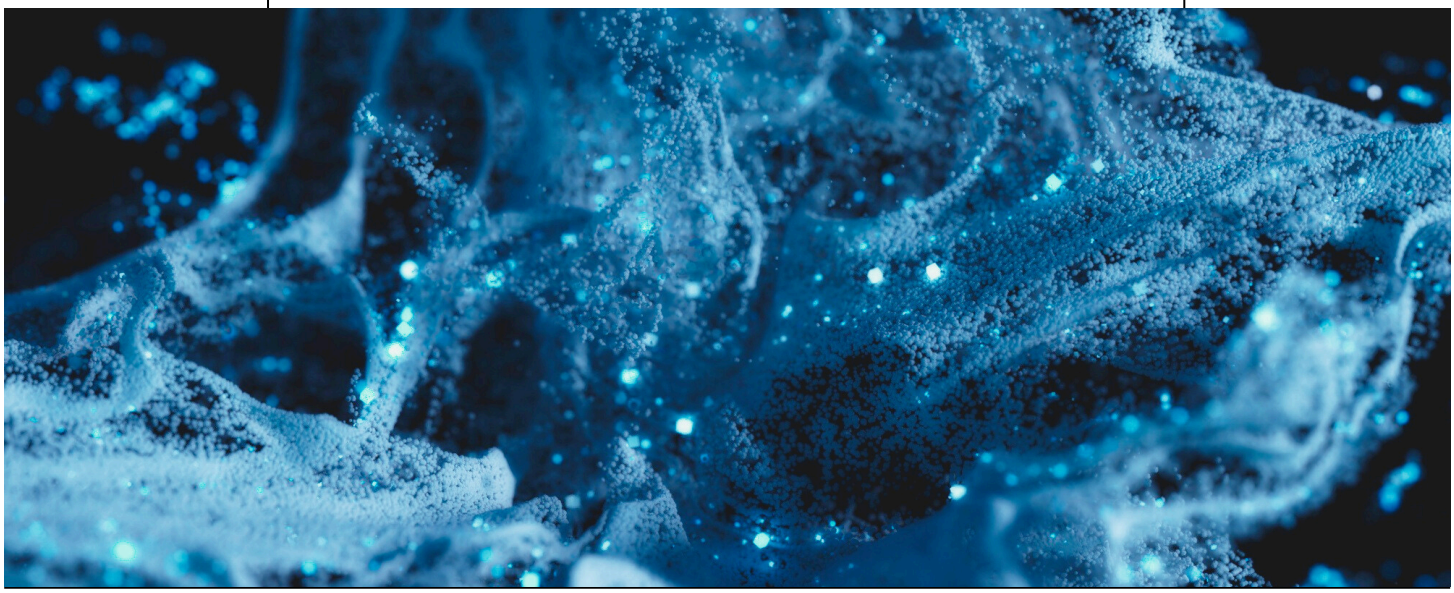

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Investigating E. coli Strain HT115-mediated RNA Interference in the Treatment of Gastrointestinal Nematode Infection by Ascaris lumbricoides

Adam Al-Khalili[1], Tabassum Howlader[1], Annie Xiang[1]

[1] Faculty of Medicine, University of Ottawa, Ottawa, Ontario, Canada

Abstract

Nematodes are parasitic worms in the body that can lead to a variety of health problems. One subset of these nematodes are called Ascaris lumbricoides, which proliferate within the intestine. This is the most common parasitic worm affecting humans, infecting hundreds of millions globally. A common method of nematode infection is through contact with soil contaminated with their eggs. This experiment attempts to disrupt the VENOM gene of A. lumbricoides to prevent their function in human hosts and lead to expulsion of the worm. This experiment begins with editing a gut bacterium to deliver RNA interference molecules to target the VENOM gene and lead to parasite expulsion. We propose two delivery methods of secretion: the first is to use the outer membrane vesicles to package and deliver the RNA inference molecules and the second is to directly secrete the RNAi using a bacterial Type 3 secretion system. We propose that in both methods after the release of the RNAi, it will be absorbed by the worm and downregulate the VENOM gene leading to the worm's death. These findings will be able to provide valuable insight into improving current therapies and potentially give insight on how we can prevent recurrence of nematode infection.

Résumé

Les nématodes sont des vers parasites présents dans le corps qui peuvent entraîner divers problèmes de santé. Un sous-groupe de ces nématodes s'appelle Ascaris lumbricoides, qui prolifèrent dans l'intestin. Il s'agit du ver parasite le plus courant chez l'humain, infectant des centaines de millions de personnes mondialement. Un mode courant d'infection par les nématodes est le contact avec un sol contaminé par leurs œufs. Cette expérience tente de perturber le gène VENOM du A. lumbricoides, afin d'empêcher son fonctionnement chez l'hôte humain et de provoquer l'expulsion du ver. L'expérience commence par la modification d'une bactérie intestinale pour qu'elle délivre des molécules d'interférence ARN ciblant le gène VENOM, entraînant ainsi l'expulsion du parasite. Nous proposons deux méthodes de livraison pour la sécrétion: la première consiste à utiliser des vésicules de la membrane externe pour emballer et transporter les molécules d'interférence ARN et la seconde consiste à sécréter directement l'ARNi à l'aide d'un système de sécrétion bactérien de type 3. Nous supposons que dans les deux méthodes, après la livraison de l'ARNi, celui-ci sera absorbé par le ver et régulera négativement le gène VENOM, menant à la mort du ver. Ces résultats pourraient offrir des informations précieuses pour améliorer les thérapies actuelles et potentiellement fournir des pistes sur la manière de prévenir la récurrence des infections par les nématodes.

Introduction

Background

Gastrointestinal (GI) nematode infection refers to the colonization and proliferation of parasitic nematodes in the GI tract, resulting in a range of pathological conditions that can cause significant morbidity and mortality in both humans and animals. Nematodes, also known as roundworms, are part of the Nematoda phylum (1) and are among the most abundant organisms on Earth (2). Soil-transmitted helminths (STHs) are the most prevalent form of nematode infection in humans, specifically by A. lumbricoides, T. trichiura, and hookworms, affecting billions worldwide (3). STH infections are transmitted through direct contact with feces-contaminated soil where development of infectious worm eggs and larvae occur. Once in the body, the parasitic worms undergo maturation to reach the adult stage, eventually residing in the small intestine, the large intestine, and occasionally, the cecum. Most GI nematodes cause chronic intestinal inflammation, leading to a reduced absorption of nutrients. Other GI nematodes causing hookworm infection and trichuriasis induce iron-deficiency anemia by blood feeding and hemorrhage (3).

Most common symptoms include diarrhea, vomiting, nausea, and loss of appetite. However, maturation in the small intestine is often asymptomatic or associated with mild symptoms, thus leading to many GI nematode infections going unnoticed. The intensity of symptoms experienced by an afflicted individual is characterized by the number of adult worms residing in the intestine (3).

Diagnosis and Current Therapies
Shortly after World War II, there was an explosive increase of STH infections in Japan. In order to conduct mass-examination, a novel stool examination method called “cellophane thick smear technique” (4) was invented by Kato and Miura in 1954, which still remains the most widely used technique for the diagnosis of gastrointestinal STH infections (5). The cellophane thick smear technique, also known as the Kato-Katz technique, is employed to quantify the number of helminth eggs per gram of feces, in order to determine the intensity of the infection (6).

The current therapy for GI nematode infection is via anthelmintic pills. The mechanism of action for anthelmintics is variable, but primarily focuses on inducing paralysis within the nematode to eventually expel it (7). However, these drugs only provide short-term relief, and the frequent use of anthelmintics has led to increased rates of resistance (8) prompting research in novel therapies.

Ascaris lumbricoides
In this study, we are investigating Ascaris lumbricoides, a parasitic nematode that is responsible for most GI nematode infections in humans. A. lumbricoides affects over 25% of the population globally, inducing intestinal blockage and hemorrhage when present in high concentrations (9). A. lumbricoides infection occurs in the jejunum of the small intestine, following ingestion of embryonated eggs (10). The exact feeding mechanism of A. lumbricoides is unknown, but is hypothesized to primarily involve the competition for nutrients via absorption through the cuticle within the GI tract, contributing to its proliferation and reproduction (11).

Rationale & Objectives

Rationale
One of the genes in A. lumbricoides that is vital to its function is the VENOM gene, with this gene being upregulated upon infection from this nematode (12). By engineering a bacterium that can silence this vital VENOM gene, it will hinder the function of the nematode, ultimately leading to its death. The use of RNA interference (RNAi) has been explored with potential feasibility regarding nematodes (13). RNAi is a process that can be used as a silencing complex to inhibit certain gene expression (14). This process will be used to silence the VENOM gene leading to the death and expulsion of the worm. This study will also aim to conclude the best secretion method for the RNAi through testing various methods to determine which results in the greatest efficiency of the worm killing. Current studies have explored RNAi via bacterial feeding by nematodes, however since most nematodes infecting humans do not feed on bacteria, different methods of RNAi must be explored for effective treatments to be uncovered.

Objectives
The objective of this study is to investigate a novel therapy for the treatment and prevention of recurrence of gastrointestinal nematode infection by Ascaris lumbricoides. Due to the increasing rates of resistance to current anthelmintic therapies, it is crucial to determine alternative methods to combating these parasites. In this study, we investigate the administration of genetically engineered Escherichia coli strain HT115 bacteria to mice, suppressing the vital VENOM gene in A. lumbricoides via secretion of RNAi molecules. We hypothesize that this novel therapy could provide a long-term solution to the problems presented with current anthelmintic therapies, as the RNAi is able to replicate within E. coli HT115 as it proliferates as opposed to current short-term drug treatments.

Methods
RNAi Design
We propose that RNA interference can be used to silence the VENOM gene in A. lumbricoides in order to hinder its parasitic function. Recent research has shown upregulation in expression of the VENOM gene, also known as Venom allergen 3, in adult humans infected by A. lumbricoides. Although the exact function of the gene is unknown, it has been found to be homologous to the SCP/TAPS protein, which is hypothesized to be involved in the evasion of host immune defense mechanisms (12). Thus, given that this gene is essential to the nematode’s ability to survive, we expect to see a decrease in the number of A. lumbricoides eggs per gram of feces following treatment with RNAi.

The target gene sequence to be used for VENOM will be the one identified by Mohd-Shaharuddin et al with a corresponding accession number of ERG80545. In vitro dsRNA will be synthesized by inserting a segment of the target gene sequence into a plasmid vector designed for bidirectional transcription by bacteriophage mediated promoter T7 RNA polymerase enzymes (15, 16). This vector will then be inserted into the Escherichia coli strain HT115, given previous success of bacterial mediated RNAi delivery using this bacterium and its RNAse deficiency to prevent degradation of the inserted RNA. Success in related model organism A. suum shows that dsRNA on its own can cause RNA interference (17), however A. lumbricoides nematodes have been found to have working siRNA pathways, which subjects the engineered dsRNA to cleavage by the host nematode’s Dicer enzyme complex into small interfering RNA (siRNA) fragments (18).

Method of Delivery
The outer membrane vesicles (OMVs) and E. coli strain HT115 will be delivered to the small intestine based on a traditional oral delivery system. An enteric coating on the pill protects the contents from the acidic pH of the stomach (~2), and from enzymatic degradation (19, 20). The coating allows for release of the drug in a location-specific manner, preventing delivery of the drug in the stomach and only permitting release via dissolution of the coating in the small intestine. This mechanism is engineered by using polymers that are only soluble at intestinal pH (~6), and insoluble at stomach pH. Synthetic

polymers such as cellulose acetate phthalate, methacrylic acid copolymers, and hydroxypropyl methylcellulose phthalate, are typically used (20).

Secretion Method #1: RNA packaged in Outer Membrane Vesicles

E. coli strain HT115 naturally produces outer membrane vesicles (OMVs), which can be used to deliver products such as RNA or proteins out of the bacterial cell (21). After engineering the bacteria with a vector with the dsRNA, the OMVs formed can be isolated and purified in vitro. RT-PCR could then be used to confirm expression of the implemented RNA. The isolated OMVs will be tagged with a small lipid to aid fusion to the lipid rich cuticle of the nematode. After encapsulation, the OMVs will be delivered to the intestine where they are hypothesized to fuse with the cuticle and empty their contents into the nematode (22).

Secretion Method #2: Direct RNAi Secretion

Another method of RNAi delivery to the target sequence will test in direct RNA secretion using the bacterial Type 3 secretion system (T3SS). This secretion system is typical of gram-negative pathogenic bacteria as a method of injecting virulence factors directly into the host environment (23). The E. coli strain HT115 chosen for this study is not pathogenic, as to avoid potential disruptions to the gut microbiome upon bacteria delivery. However, to optimize the secretory abilities of the bacteria, we propose engineering it with a gene for the T3SS. Upon delivery to the intestine where the A. lumbricoides nematodes reside, the RNA designed to interfere with the target VENOM gene in the nematode will be secreted via T3SS into the intestinal lumen. A. lumbricoides feeds by absorbing surrounding nutrients in its environment, therefore we hypothesize that the secreted RNA will potentially be absorbed through the worms' cuticle. Once in the organism, it will have the ability to cleave the target mRNA for the VENOM gene to silence it, interfering with the nematode's survival ability and eventually leading to its expulsion.

Experimental Design

To conduct the experiment, there will be different treatment conditions for mouse groups to determine the success of the treatment. The first experiment will test whether RNAi packaged in outer membrane vesicles is able to kill the A. lumbricoides worm. The treatment groups for this experiment will include three different groups of mice, with three mice per group. The first group will be a negative control group where WT mice receive E. coli strain HT115 bacteria that has not been modified with vesicles that secrete RNAi. The second group will consist of mice that are only infected with the A. lumbricoides worm, but without any bacterial modifications. The final group of mice will both be infected by the A. lumbricoides worm as well as be treated with the bacteria that contains the RNAi packaged in the outer membrane vesicles. These groups will all be analyzed using their fecal samples via Kato-Katz thick smear technique.

This will determine the number of A. lumbricoides eggs per gram of feces which will allow us to conclude the effectiveness of treatment for the nematode infection.

The second experiment will be testing whether the efficiency of delivery is improved through direct secretion of RNAi to kill the A. lumbricoides worm. Here, there will similarly be three different experiment groups, each group containing three mice. The first group represents a negative control group where WT mice receive an unmodified version of the E. coli strain HT115 bacteria. The second group consists of mice being infected with the worm, and an unmodified version of the bacteria. This will allow for visualization of the nematode infection at a baseline. Lastly, the third group of mice will be infected with A. lumbricoides worm as well as the engineered version of the E. coli strain HT115 bacteria to facilitate the RNAi secretion via T3SS. In all three treatment groups, their fecal samples will be analyzed via Kato-Katz thick smear technique to determine the number of A. lumbricoides eggs per gram of feces, and if this was a more efficient delivery method. We expect a decrease in the number of A. lumbricoides eggs per gram of feces following treatment with RNAi.

Conclusion

The purpose of this study was to explore a potential novel therapy for A. lumbricoides nematode infection. The results from this study will serve as an indicator for the potential of RNA interference as an effective method of silencing genes in parasitic worms. Should the results be negative, the methodology could be modified to test other strains of bacteria, nematode species, target genes and methods of RNAi delivery to optimize the protocol. Given the success of RNAi in other disease treatments.

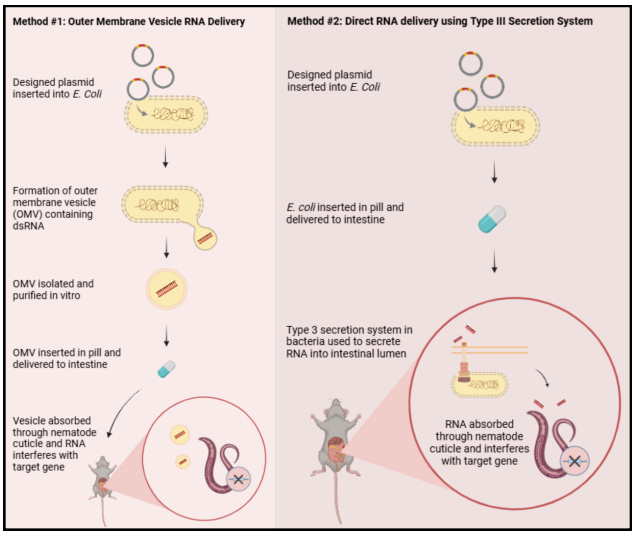

Figure 1. Graphical abstract outlining experimental delivery methods of RNA. 2 methods are illustrated, outer membrane vesicle RNA delivery (left) and direct RNA delivery using Type II secretion system (right).

Competing interests

The authors declare that they have no competing interests.

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How Medicinal is Dried Angelica archangelica Relative to the Fresh Plant?

Monica Dimitrova,[1] Sharon Barden,[2] Paul Mayer [2]

[1] Faculty of Sciences, University of Ottawa, Ottawa, Ontario, Canada
[2] Faculty of Chemistry and Biomecular Sciences, University of Ottawa, Ottawa, Ontario, Canada

Abstract
The chemical composition of natural products is responsible for their medicinal properties. Angelica archangelica is known for its antimicrobial properties, which stem from terpenes that make up the bulk of the extract of the fresh plant.The dried root can be purchased from commercial sources and is sold for the above-mentioned medicinal characteristics.Though thorough identifications of the fresh plant's composition have been made, there has been little in the way of characterization of the chemical components of the dried root. In this study, we use both organic solvents and supercritical CO_2 to extract the dried root and compare the results to the essential oil (made from the fresh plant). The results show that the dried roots used in this study were devoid of the terpenes linked to the plant's medicinal properties, and rather contain osthole, a coumarin derivative found in relatively small abundance with respect to the plant's terpenes.

Résumé
La composition chimique des produits naturels est responsable de leurs propriétés médicinales. Angelica archangelica est bien connue pour ses propriétés antimicrobiennes, attribuées aux terpènes qui constituent la majeure partie de l'extrait de la plante fraîche. La racine séchée peut être achetée auprès de sources commerciales et est vendue pour ses propriétés médicinales évoqués précédemment. Malgré l'étude approfondie de la composition de la plante fraîche, peu d'analyses ont été effectuées concernant ses composants chimiques de la racine séchée. Dans cette étude, nous utilisons à la fois des solvants organiques et du CO2 supercritique pour pouvoir extraire les composés de la racine séchée, et nous comparons les résultats à ceux de l'huile essentielle (obtenue à partir de la plante fraîche). Les résultats démontre que les racines séchées utilisée dans cette étude étaient dépourvus des terpènes associés aux protéines médicinales de la plante, et contenaient à la place de l'ostal, un dérivé de la coumarine présent en relativement faible quantité en comparaison aux terpènes de la plantes

Introduction

Background
Angelica archangelica (Figure 1), commonly known as angelica root, is a medicinal plant that grows natively in Syria but is cultivated and widely distributed in various regions of Europe and western Asia [1]. [BS1] For many generations, the plant garnered a reputation for healing diseases in folklore medicine. Its popularity led to numerous studies examining the active molecules in the plant to explain the factors that could describe these observations. In vitro experiments observed bacterial growth inhibition, supporting the claim that Angelica archangelica possesses antimicrobial properties. One study identified the active molecules of a-pinene and limonene, the main contributors to the plant's chemical composition, to have considerable activity against Escherichia coli and Staphylococcus aureus [2]. These studies used and analyzed the plant freshly harvested; however, consumers purchase and receive the raw material in the form of dried, cut roots, whose components have not been explored in detail[BS2] . We considered whether the dried consumer product exhibits the same health benefits as fresh plant material[BS3] . Herein, we describe both organic solvent and supercritical CO_2 (sc-CO_2) extractions of the dried Angelica archangelica roots. Gas chromatography-mass spectrometry (GC-MS) analysis is conducted to quantify and identify the molecules in the raw dried plant material. The results are then compared to the plant's essential oil to address the legitimacy of taking dried Angelica archangelica roots as herbal supplements for medicinal purposes[BS4] . Nykänen and co-workers previously performed sc-CO_2 extraction on dried Angelica archangelica root [3] and found a predominance of terpenes, notably a-pinene, a- and b- phellandrene, d-3-carene.There was also a large abundance of 7-methoxy-8-(3-methyl-2-butenyl)-2H-1-benzopyran-2-one (osthole) identified, which was not previously observed in dried root extracts. In some cases, osthole was also found to be a major component [4]. Osthole, a coumarin derivative, is found in many medicinal plants and has been linked to the treatment of bone-related diseases [5]. The essential oil was found to be largely a-pinene, b- phellandrene and d-3-carene [6].

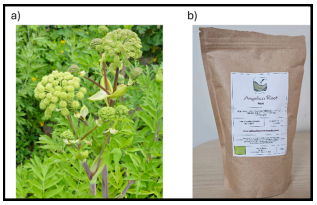

Figure 1. a) *Angelica archangelica* and b) the purchased dried root.

Materials and Methods

Dried Angelica archangelica root was purchased from Amazon Inc., and the essential oil, produced from the fresh plant, was purchased from Aromatics International. The essential oil (1 □L) was diluted with 1 mL ethyl acetate, and 1 □L of this mixture was then injected into the GC-MS. The dried root was extracted with three different solvents to compare their chemical composition: chloroform, ethyl acetate, and hexanes. In each case, 5 grams of ground plant material was mixed with 15 mL of the solvent and the extraction was allowed to proceed for seven hours. Samples were then filtered, and 1 mL of each extract was diluted with 12 mL of ethyl acetate prior to injection on the GC-MS. For sc-CO2 extraction, the dried root was ground into a fine powder and 25 g was placed in the sample holder of the SFT-250 SFE System from Supercritical Fluid Technologies Inc. CO2 was pressurized to 300 bar at a temperature of 40ºC and passed through the sample and the extract was collected in an external vial. 1 mg of extract was diluted with 1 mL of ethyl acetate. 1 □L of extract was then injected into the GC-MS. The instrument was an Agilent 6890 GC with MSD. The column was a 30m Agilent 19091J-433 HP-5 column, with an internal diameter of 250 mm, stationary phase thickness of 0.25 mm, He carrier gas with a flow rate of 30 cm/s. The oven temperature was programmed from 150ºC to 310ºC at 15ºC/min. Chemical identification was carried out by comparing the mass spectra of each chromatographic peak with the NIST database (see Supporting Information). The hydrosol results were quantitated using a 4-ethylguaiacol internal standard. The calibration curve is found in Figure S2.

Results and Discussion

The chromatograms obtained for the three organic solvent extracts of the dried root are shown in Figure 2a. The three are essentially indistinguishable and they all agree that the major constituent in the dried root is osthole at 24% (structure shown). Several unidentified components do not appear in the NIST database. The sc-CO_2 extract is slightly different in that angelicin and osthole were observed along with at least one fatty acid, Figure 2b.However, both extraction methods agree that osthole is the major component, as was previously reported by Paroul and co-workers [4], but different from the observations of Nykänen et al [3], who found mostly terpenes[BS1] .

These results for the dried root contrast with those for the essential oil (Figure 3). The essential oil is characterized by a series of terpenes, as identified by Wedge et al.[6]. These terpenes are absent in the extracts of the dried root, presumably due to their volatility and thus loss during the drying process[BS2] [BS3] [BS4] .

Conclusion

Combining the two sets of results with the literature data suggests that the drying process (temperature and duration) could ultimately effect both the chemical composition of the dried root and its reported medicinal properties. Given the complete lack of information on the commercial drying process reported for the purchased roots, it cannot be assumed that the purchased product will necessarily have the desired medicinal characteristics of the fresh plant, therefore appearing to be a case of “buyer beware”.

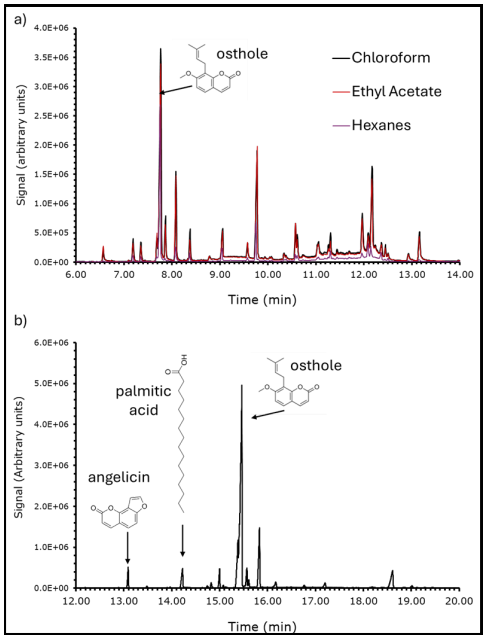

Figure 2. The GC-MS results for a) organic solvent extracts of the dried root of Angelica archangelica and b) the sc-CO_2 extract.

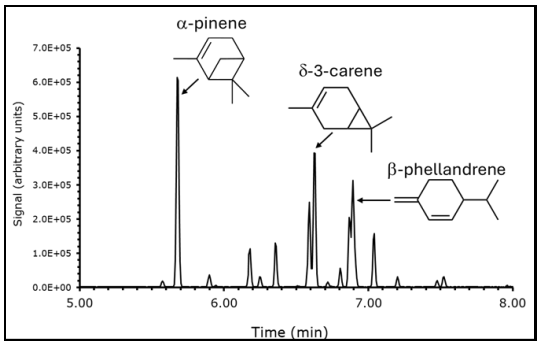

Figure 3. The GC-MS of the essential oil of Angelica archangelica.

Competing interests
The authors declare that they have no competing interests.

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Don't Get a Toothache! New Insight into the Chemical Composition of Miswak, the Natural Toothbrush

Batoul Al Hussein[1], Sharon Barden[2], Paul Mayer[2]

1 Faculty of Sciences, University of Ottawa, Ottawa, Ontario, Canada
2 Faculty of Chemistry and Biomecular Sciences, University of Ottawa, Ottawa, Ontario, Canada

Abstract

Salvadora Persica, best known as the toothbrush tree, holds a distinctive place in middle eastern folkloric traditions, particularly for its roots, which have served as a natural toothbrush for many centuries. Recent studies demonstrated its antimicrobial properties, attributing this efficacy to the presence of benzyl isothiocyanate, BITC. This study aimed to quantify the BITC molecule and identify additional components present in these roots with two novel extraction methods. A hydrosol of the root was produced with microwave hydrodistillation and a supercritical-CO_2 (s-CO_2) extract was prepared from the roots and both were analyzed using gas chromatography-mass spectrometry (GC-MS). The hydrosol was found to contain BITC, benzylcyanide, and 5-(isothiocyanatomethyl)-1,2,3-trimethoxybenzene, while the s-CO_2 extract lacked the trimethoxy compound, but exhibited the presence of preservatives and fatty acids.

Résumé

Salvadora persica, connue surtout sous le nom d'arbre à brosse à dents, occupe une place particulière dans les traditions folkloriques du Moyen-Orient, notamment pour ses racines qui servent depuis des siècles de brosse à dents naturelle. De nombreuses études récentes sur cette plante ont démontré ses propriétés antimicrobiennes, attribuant leur efficacité à la présence de benzyl isothiocyanate (BITC). Cette étude avait pour but de quantifier la molécule BITC et d'identifier d'autres composants présents dans ces racines grâce à deux méthodes d'extraction innovantes. Un hydrosol de la racine a été produit par hydrodistillation assistée par micro-ondes, et un extrait au CO2 supercritique (s-CO2) a été préparé à partir des racines ; les deux extraits ont été analysés par chromatographie en phase gazeuse couplée à la spectrométrie de masse (GC-MS). L'hydrosol contenait du BITC, du benzylcyanure et du 5-(isothiocyanatométhyl)-1,2,3-triméthoxybenzène, tandis que l'extrait s-CO2 ne contenait pas le composé triméthoxy, mais présentait des agents conservateurs et des acides gras.

Introduction

Salvadora Persica, best known as the toothbrush tree, holds a distinctive place in middle eastern folkloric traditions, particularly for its roots, which have served as a natural toothbrush for many centuries. Recent studies demonstrated its antimicrobial properties, attributing this efficacy to the presence of benzyl isothiocyanate, BITC. This study aimed to quantify the BITC molecule and identify additional components present in these roots with two novel extraction methods. A hydrosol of the root was produced with microwave hydrodistillation and a supercritical-CO_2 (s-CO_2) extract was prepared from the roots and both were analyzed using gas chromatography-mass spectrometry (GC-MS). The hydrosol was found to contain BITC, benzylcyanide, and 5-(isothiocyanatom-ethyl)-1,2,3-trimethoxybenzene, while the s-CO_2 extract lacked the trimethoxy compound, but exhibited the presence of preservatives and fatty acids.

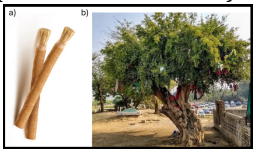

Figure 1. Image of a) miswak natural toothbrushes and b) Salvadora persica L. tree.

The effectiveness of miswak in dental hygiene stems largely from the compound benzyl isothiocyanate, BITC (Figure 2), which is a major component of the biologically available chemicals in the twig [2-5].BITC has been found to have antimicrobial and anticancer properties [2, 4-10]. Upon plant tissue damage, BITC might penetrate through the outer bacterial membrane and possibly interfere with the bacterial redox systems, thus hampering the ability of the bacterium to maintain its membrane potential [5]. The mechanical action of chewing facilitates the penetration of the freshly released BITC into deeper tissues, where it reacts with the bacterial redox systems and disrupts the bacterium's ability to maintain its membrane potential [5].

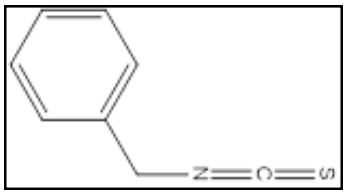

Figure 2. Benzyl isothiocyanate (BITC)

The previous work in the literature focused on organic solvent extracts of the miswak material [11]. The aim of our study was to explore two previously unused extraction processes: water-based microwave extracts (hydrosols) and supercritical carbon dioxide extraction of the miswak toothbrush to determine if any novel, previously

unassigned, molecules can be identified that are related to the oral hygiene environment.

Methods

Miswak was purchased from a commercial source, chopped, and ground into a pulp (Figure 3).

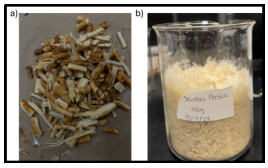

Figure 3. Chopped (a) and ground (b) miswak

Microwave Exraction

A hydrosol extract was generated from the ground miswak by the following procedure (refer to Figure 4). A collector beaker was placed in the middle of a glass jar and ground miswak was placed around it in the bottom of the jar. An ice cone was suspended on the top of the jar. Upon microwave heating, volatiles evaporate from the ground material, condense on the ice cone and the resulting hydrosol dripped into the collector beaker. A total of six 9-minute heating cycles were used, each with a new ice puck. All extracts were pooled into a common pot and 10 mL of hydrosol was added to each of six vials. An internal standard, 4-ethylguaiacol, was then added to each vial.

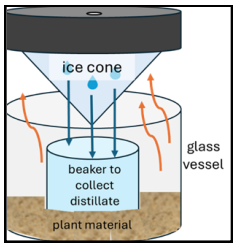

Figure 4. Extraction vessel from the microwave hydrosol generator.

Solid Phase Extraction (SPE)

Six 3 mL C-18 solid phase extraction (SPE) cartridges were conditioned with 2 mL of methanol followed by 2 mL of deionized (DI) water to prepare them for sample loading. Each 10 mL of extract was passed over a cartridge and organics were eluted with 1 mL of ACN:MeOH (1:1). 1 mL of extract was then injected into the gas chromatography-mass spectrometer. The instrument was an Agilent 6890 GC with MSD. The column was a 30m Agilent 19091J-433 HP-5 column, with an internal diameter of 250 mm, stationary phase thickness of 0.25 mm, He carrier gas with a flow rate of 30 cm/s. The oven temperature was programmed from 40ºC to 250ºC in 15 min. Chemical identification was carried out by comparing the mass spectrum of each chromatographic peak with the NIST database (see Supporting Information, Figure S1). The hydrosol results were quantitated by internal standards. The calibration curve is found in Figure S2. Supercritical CO2 extract The ground miswak (25 g) was placed in the sample holder of the SFT-250 SFE System from Supercritical Fluid Technologies Inc.CO_2 was pressurized to 300 bar at a temperature of 40ºC and passed through the sample and the extract collected in the external vial. The column temperature ramp was altered from the previous program to be 40ºC to 320ºC at 20ºC/min and a final hold time of 5 min, to access larger molecular weight analytes.

Results and Discussion

The gas chromatogram of the hydrosol is shown in Figure 5a. Three major components are observed in the hydrosol, the previously observed BITC and precursor benzylcyanide, and a third novel compound, 5-(isothiocyanatomethyl)-1,2,3-trimethoxybenzene (TMBITC). In a previous study, 3-methoxybenzylisothiocyanate was observed and thought to be due to the reaction of an alcohol solvent with BITC at increased temperatures [3], but a study by Abdel-Kader et al. explored this and no methoxy derivatives were observed [11]. Thus, TMBITC is a novel compound found in our hydrosol. It has previously been identified in peppergrass (Lepidium coronopus (L.) Al-Shehbaz) [12], and Lepidium densiflorum [13], and is an inhibitor of calcineurin-dependent gene expression in yeast and recombinant human calcineurin [14]. Figure 5a also shows the results of quantitation, with concentrations of benzylthioisocyanate being approximately 9.2 mg/L of hydrosol. The gas chromatogram of the hydrosol is shown in Figure 5a. Three major components are observed in the hydrosol, the previously observed BITC and precursor benzylcyanide, and a third novel compound, 5-(isothiocyanatomethyl)-1,2,3-trimethoxybenzene (TMBITC). In a previous study, 3-methoxybenzylisothiocyanate was observed and thought to be due to the reaction of an alcohol solvent with BITC at increased temperatures [3], but a study by Abdel-Kader et al. explored this and no methoxy derivatives were observed [11]. Thus, TMBITC is a novel compound found in our hydrosol. It has previously been identified in peppergrass (Lepidium coronopus (L.) Al-Shehbaz) [12], and Lepidium densiflorum [13], and is an inhibitor of calcineurin-dependent gene expression in yeast and recombinant human calcineurin [14]. Figure 5a also shows the results of quantitation, with concentrations of benzylthioisocyanate being approximately 9.2 mg/L of hydrosol.

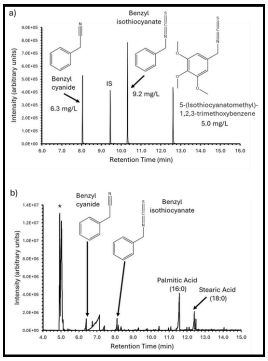

Figure 5. Gas chromatogram of a) the miswak hydrosol. IS = the internal standard 4-ethylguaiacol and b) the miswak s-CO_2 extract.
The asterisk denotes the two peaks due to propanoic acid and glycerol. The fronting on the peak at 7 min is due to water present in the extract.

Conclusion

As expected, benzyl isothiocyanate (BITC) was the main active component found in the miswak extracts. BITC's natural origin underscores the importance of exploring plant-derived compounds in modern medicine [10]. Interestingly, the hydrosol was found to be a very clean extract, uncontaminated with fatty acids or the preservative/humectant additives. This suggests that miswak hydrosol could be explored further for its antimicrobial properties and potential for a topical antibiotic. It would also be important to determine if BITC could contribute to antimicrobial resistance [2, 10]. We also identified 5-(isothiocyanatomethyl)-1,2,3-trimethoxybenzene in the extract. The next steps for this molecule could include researching its fundamental biochemical and microbiological mechanisms by which it affects bacteria. This comprehensive approach could pave the way for the development of new, plant-based antimicrobial therapies that address the growing issue of antibiotic resistance.

Competing interests
The authors declare that they have no competing interests.

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Sociodemographic factors associated with access to preventative care for children: secondary analysis of the 2022 National Survey of Children's Health data in the United States

Varna Prapakaran[1]

[1] Faculty of Health Sciences, University of Ottawa, Ottawa, Ontario, Canada

Abstract

Since the beginning of the 21st century, there has been an ongoing decline in children's physicals, delayed immunizations, and well visits. This study investigates sociodemographic factors, including poverty levels (PDL), employment status, adequacy of insurance, and race, that affect the accessibility of preventative care check-ups. The research from this study has been derived from the 2022 National Survey of Children's Health survey that sampled 53,621 participants from diverse socioeconomic and racial backgrounds. This study uses Binary regression analysis and Pearson's chi-squared tests to examine and test the association between key factors and access to care. For example, participants in the lowest poverty level (0-99%) experienced poorer health outcomes (27.2%) compared to those in 400%+ income level (12.4%). Furthermore, when compared to the unemployed or unpaid class of participants, the participants who work full-time are associated with better health outcomes (OR=1.219, 95% CI = 1.100, 1.352). Racial minorities, including Hispanic (OR=0.763), Black (OR=0.801), and Asian (OR=0.553) participants, showed lower odds of positive outcomes compared to participants of White descent. This research seeks to encourage the creation of targeted intervention strategies focusing on poverty reduction, stable income, and equitable, universal access to preventative measures for children across diverse populations.

Keywords: preventative care, children, sociodemographic factors, United States

Résumé

Depuis le début du 21e siècle, il y a un déclin continu des examens physiques, des vaccinations retardées et des visites médicales des enfants. Cette étude examine les facteurs sociodémographiques, incluant les niveaux de pauvreté dépendant (NPD), le statut d'emploi, l'adéquation de l'assurance et la race, qui influencent l'accessibilité aux soins préventifs. Les données de cette recherche proviennent de l'enquête du National Survey of Children's Health (NSCH) de 2022, qui a interrogé 53 621 participants issus de milieux socioéconomiques et raciaux variés. Cette étude utilise une analyse de régression binaire et les tests du chi carré de Pearson pour examiner et tester l'association entre les facteurs clés et l'accès aux soins. Par exemple, les participants appartenant au niveau de pauvreté le plus bas (0-99%) ont présenté des résultats de santé moins favorables (27,2%) comparativement à ceux ayant un revenu de 400% ou plus (12,4%). De plus, en comparaison avec les participants sans emplois ou non rémunérés, les participants travaillant à temps plein sont associés à de meilleurs résultats de santé (OR=1.219, 95% CI = 1.100, 1.352). Les minorités raciales, notamment les participants hispaniques (OR = 0,763), noirs (OR = 0,801) et asiatiques (OR = 0,553), ont présenté des probabilités moindres d'avoir des résultats positifs par rapport aux participants d'origine blanche. Cette recherche vise à encourager la création de stratégies d'intervention ciblées sur la réduction de la pauvreté, la stabilité des revenus et un accès équitable et universel aux mesures préventives pour les enfants de diverses populations

Mots-clés: soins préventifs, enfants, facteurs sociodémographiques, États-Unis.

Introduction

The accessibility of preventative care is crucial for the health and well-being of children since it promotes positive long-term health outcomes through the use of immunizations, screenings, and visits. These strategies are essential for early detection and ensuring children are meeting their developmental milestones.

From 1995 to 2004, prenatal visits by family physicians decreased by 50% and reduced the possibility of intervention strategies to limit the progression of new or current diseases [1]. When looking at race/ethnic background factors against access to care study between 1996-2000 [2], children of Asian, Hispanic, and Black descent experience significant deficits in accessing medical care compared with whites primarily due to their dissatisfaction with the quality of medical care. Furthermore, a study conducted on KiGGS Wave 2 study reports that families of higher income status have greater access to preventative check-ups than those from lower-income backgrounds [3]. The main goal of my research is to examine the sociodemographic factors that hinder access to preventative care for children in the United States, using secondary data from the 2022 National Survey of Children's Health. The study aims to pinpoint the disparities in accessing essential health services like immunizations, screenings, and regular check-ups. Through this study, these factors can be thoroughly understood and help foster targeted interventions to reduce health inequities and ensure every child, regardless of their social identity, has access to preventive care services, which is fundamental to promoting the health and wellbeing of children throughout the course of their life.

Methods

The variables selected for this study (Poverty-Dependent Level, Insurance Adequacy, No Referral Problem, Employment Status, Housing Instability, and Race) were chosen due to their established relevance in promoting access to preventative care for children [See Figure 1]. Poverty Dependent Level (PDL) is a percentage that relates to a participant's family economic status on a scale ranging from 0 to 400%+. This variable is a key determinant of healthcare access to pay for the necessary fees associated with accessing care. Insurance Adequacy (InsuranceAdeq) reflects the coverage and comprehensiveness of health insurance of the individual, which is a main factor in ensuring children receive necessary preventative care. The access to specialized preventative care (NoRefProblem) may be delayed by diagnostic tests not being completed by a specialist and communicated with the patient's family physician within a reasonable timeframe. The employment status variable (EmployStat) plays a major role in providing families with health insurance coverage through their income bracket or benefits, which most individuals rely on to cover the cost of healthcare. Housing Instability (HousingInstab) is linked to a family's ability to maintain regular healthcare routines aside from the mental toll it has on the participants.

Finally, Race is examined due to its strong, well-known association with health inequities causing barriers in accessing appropriate care. This study employs two analyses, Chi2 test and Binomial Regression Analysis to study the association between the socio demographic variables and response variables. First, the dataset was filtered to include the relevant variables and removed all missing values that may skew the results of the study. First, a chi-square test of independence was used to examine associations between preventative care and the categorical sociodemographic factor variables. This method was chosen because both the independent variables (e.g., race, PDL, InsuranceAdeq, EmployStat, etc.) and the dependent variable (access to care: yes/no) are categorical and assess whether the observed percentage distributions differ across groups. After the chi-squared test was used, the binomial logistic regression analysis was utilized to model the likelihood of children receiving preventative care based on multiple sociodemographic predictors. Logistic regression was appropriate because the outcome variable was binary (yes/no), and the estimation of odds ratios was used to quantify the effect of each sociodemographic factor on the likelihood of receiving care.

Results

[See Figure 2] Of the data reported, 1846 participants (27.2%) who had a 0-99% score on the federal poverty level (PDL) reported difficulties in accessing care ("No") compared to 2828 people (12.4%) in the 400%+ PDL group. Regarding insurance adequacy, 6072 participants with adequate insurance (16.8%) were in the "No" group compared to 2282 people (15.2%) without adequate insurance. Employment status also showed variation between the results, with 695 individuals (24.7%) belonging to the unemployed or unpaid category being present in the "No" group whereas 7745 (16.5%) of full-time workers and 500 (23.0%) part-time workers reported having no access to care for their children. In terms of housing instability, 1462 participants (20.7%) of those with housing instability were in the "No" group, compared to 7644 people (16.9%) without such experience. Lastly, racial differences were observed and showed that 861 (26.4%) of Asian participants stated having no access to care when compared to 5261% (15.3%) of White participants. With respect to all the variables, the chi-squared test shows that there is a statistically significant association between PDL levels and preventative care ($p<0.001$). [See Figure 3] After extrapolating the data, it is seen that participants with in the 400%+ PDL (odds = 2.169, 95% CI = 1.996, 2.356) had increased odds of being in the "Yes" group compared to those in the 0-99%, 100-199% (odds = 1.159, 95% CI = 1.065, 1.263), and 200-399% range. When examining the variable, employment status (EmployStat), it is seen that there is an association with participants with full-time employment who had higher odds (OR = 1.219, 95% CI: 1.100, 1.352) compared to the unemployed or unpaid group. Finally, the race variable concluded that Hispanics (OR = 0.801, 95% CI: 0.724, 0.886) and Black participants (OR = 0.763, 95% CI: 0.710, 0.821) had lower odds. The lowest odd ratio is present in Asian participants (OR = 0.553, 95% CI: 0.501,

0.611), while multiracial participants showed no difference (OR = 0.962, 95% CI: 0.876, 1.057) in the data.

Significance

The essence of the study emphasizes the association of sociodemographic characteristics that play a role in accessing care services. Also, the takeaways closely reflect from similar research. When comparing higher income levels, Lower PDLs (0-99%) were correlated to worsen health conditions. This further proves that poverty increases risks for chronic illnesses and mental health issues due to limited access to care and heightened stress [4]. Additionally, the Employment factor is very significant as full-time workers show better health outcomes (OR=1.219) compared to the unemployed group. According to an article on Working Conditions [5], stable employment effectively improves access to healthcare and ultimately, reduces psychosocial stress. Lastly, the odds of racial backgrounds of Hispanic, Black, and Asian descent participants having access to preventative care compared to participants of White descent are lower due to racial disparities. These results are consistent with well-established systemic obstacles that lead to worse health outcomes in marginalized areas, including discrimination and limited access to healthcare [6]. Overall, it is essential to go to preventative care appointments and ensure that they are accessible as these appointments address arising health issues (e.g. obesity, diabetes, etc.), manage existing health conditions, and aim to promote good health. Additionally, these appointments are crucial for children to remain healthy throughout the rest of their lives as proper education in conjunction with preventative measures keeps them healthy as they get older. On the other hand, by analyzing and studying the factors that presume to have a relationship with access to preventative care, policymakers, and medical professionals can effectively spearhead initiatives to combat these issues to allow all children, regardless of their socio-demographic identity, to have equal access to care.

Limitations

A limitation present in the study that affects the quality of the results is the underrepresentation of racial and ethnic minorities and participants who come from lower socioeconomic statuses. This limitation can skew the trends seen throughout the study, therefore creating an inaccuracy in the results interpreted. As seen, the dataset predominantly sampled participants of Caucasian descent and participants within sufficient household income (FPL of 300% or more), and lower response rates of individuals from a lower socioeconomic class or ethnic backgrounds.

Further research should strive to select and sample more diverse populations to incorporate in this study to provide valuable insights into diverse populations within the United States.

Conclusion

In conclusion, this analysis summarizes that children of low-income families and racial minorities, including but not limited to Asian, Black, and Hispanic, experience worsened health outcomes and delays in health services. These findings emphasize the need for focused interventions on poverty reduction, stable employment, and equitable healthcare access to ensure children receive necessary healthcare services, regardless of their socioeconomic status or racial background.

Competing interests

The authors declare that they have no competing interests.

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Traditional Chinese Medicine as an Alternative Treatment for Adults with Hypercholesterolemia: A Systematic Review

Tasfia Hussain [1]

[1] Faculty of Health Sciences, University of Ottawa, Ottawa, Ontario, Canada

Abstract

Understanding whether Traditional Chinese Medicine (TCM) offers effective treatments for hypercholesterolemia could potentially open doors to finding new lipid-lowering drugs with fewer adverse effects. This systematic review attempts to compile and analyze the available research relating TCM to hypercholesterolemia. Using keywords from the research question, 658 records were identified on the MEDLINE (Ovid) database. Ovid database was used to search for articles on TCM and hypercholesterolemia from 2005 to 2025. After assigning limits and screening articles based on the set criteria, the five remaining articles are read in full and narrative synthesis is done. The five chosen articles are appraised using the Jadad score, and all are included in the review to give critical insights into different Chinese herbs. Five studies from 2190 human, 62 rat, and 48 rabbit subjects in clinical settings were analyzed, and only Palmiwon, Lingzhi, Xuezhikang, and Daming capsules showed positive effects on hypercholesterolemia. This systematic review finds that Palmiwon, Xuezhikang, Xiaozhiling and Daming have positive effects on treating hypercholesterolemia. However, there is not enough evidence to support their clinical use compared to HMG-CoA reductase inhibitors (statins). This is likely due to the limited use of TCM in Western medicine, resulting in a lack of peer-reviewed research. TCM such as Palmiwon, Xuezhikang, Xiaozhiling and Daming may have the potential to be used in the clinical management of hypercholesterolemia in comparison with standard pharmacological treatment. However, further research is needed before establishing it as an alternative treatment.

Résumé

Comprendre si la médecine chinoise constitue un traitement efficace contre l'hyperlipidémie pourrait ouvrir la voie à la découverte de nouveaux hypolipidémiants avec moins d'effets indésirables. Cette revue systématique vise à rassembler les recherches disponibles sur la médecine chinoise et l'hyperlipidémie. À l'aide de mots-clés issus de la question de recherche, 604 enregistrements ont été identifiés dans la base de données MEDLINE (Ovid). Après application de limites et sélection des articles selon des critères établis, les six articles restants ont été lus intégralement et une synthèse narrative a été réalisée. Les six articles sélectionnés ont été évalués à l'aide du score de Jadad, et tous ont été inclus dans la revue pour fournir des perspectives critiques sur différentes herbes chinoises. Des études portant sur 2676 sujets humains et rats en contexte clinique ont été analysées, et seuls Palmiwon, Lingzhi, Xuezhikang et les capsules Daming ont montré des effets positifs sur l'hypercholestérolémie. Cependant, ces résultats restent non concluants en raison de la petite taille des échantillons et de biais dans la génération de la séquence d'allocation aléatoire, la dissimulation de l'allocation, l'aveuglement des participants, les données de résultats incomplètes et le rapport sélectif des résultats. Cette revue systématique révèle que certaines médecines chinoises ont des effets positifs dans le traitement de l'hypercholestérolémie. Toutefois, il n'existe pas suffisamment de preuves pour soutenir leur utilisation clinique comparée aux inhibiteurs de la HMG-CoA réductase (statines). Cela est probablement dû à l'utilisation limitée de la médecine chinoise en médecine occidentale, entraînant un manque de recherches évaluées par des pairs. Certaines médecines chinoises ont le potentiel de gérer l'hypercholestérolémie en comparaison avec les traitements pharmacologiques standards. Cependant, des recherches supplémentaires sont nécessaires avant d'établir leur usage clinique.

Introduction

Hypercholesterolemia is a growing concern as it is a risk factor for many serious and chronic conditions, such as cardiovascular diseases and diabetes [1]. Hyperlipidemia is the presence of excess lipids (fats) in the blood. Hypercholesterolemia is a type of hyperlipidemia that is defined as increased cholesterol levels in the blood. There are two types of cholesterol: HDL (high-density lipoprotein) and LDL (low-density lipoprotein) [2]. Lipoprotein is a molecule that transports lipids; LDL

transport lipids from the liver to peripheral tissues while HDL brings lipids back to the liver. Hence, LDL is considered “bad cholesterol”, as excessive cholesterol in the periphery tissues may lead to plaque formation [8]. Hypercholesterolemia is associated with a total increase in blood cholesterol, lower levels of HDL, or increased levels of LDL [2]. In 2019, all risk-related deaths and disability-adjusted life-years due to high LDL-C increased by 46.7% and 41.5%, respectively, since 1990 [4]. This means the future population is at an increasing risk of developing high blood cholesterol-related diseases. Therefore, it is pertinent to delve into available research on all kinds of lipid-lowering remedies to find a suitable cure, particularly ones that would accompany the least adverse effects. Hypercholesterolemia occurs as a consequence of modifiable risk factors such as the consumption of a high-fat diet and a sedentary lifestyle [2]. Cholesterols are naturally occurring lipids in the body that are necessary for metabolic processes [8]. However, an excess accumulation of cholesterol in the blood may eventually result in the development of atherosclerosis, characterized by plaque formation in the blood vessels, which cause interruptions in blood flow [3].

Hypercholesterolemia may also cause other complications such as hypertension, coronary heart disease (CHD) and diabetes mellitus [1]. These conditions further lower the quality of life and require extensive clinical management such as pharmacotherapy, surgical intervention, etc. Fortunately, hypercholesterolemia can be managed through lifestyle changes as well as with lipid-lowering drugs such as 3-hydroxy-3-methylglutarylcoenzyme A (HMG-CoA) reductase inhibitors, also known as statins [2]. Due to its efficacy, statins are usually administered to treat hypercholesterolemia as the first line of standard treatment [2]. Other standard pharmaceutical drugs that may be administered include fibrates, nicotinic acids, bile acid sequestrants, hormones, etc. [2].

In contrast, Traditional Chinese Medicine (TCM), a 3000-year-old holistic medicine practice based on the belief that opposing forces reside within the body, uses medicinal herbs, acupuncture, diet, massage, and therapeutic exercise to treat and prevent diseases by restoring the body's balance of opposing forces. Chinese medicinal herbs include extracts from any part of a plant in whole or powdered form, etc. TCM may offer affordable and safe alternatives to pharmaceuticals [2]. This systematic review attempts to seek whether TCM can be considered as an alternative to standard pharmaceutical drugs. The TCM methods that will be evaluated are among those that have been studied as potential remedies for lowering cholesterol. The research question that will be studied in this systematic review is, “Can TCM reduce hypercholesterolemia in adults with hypercholesterolemia compared to standard pharmacological interventions?” Scientific literature addressing this topic will be summarized in this review to determine whether TCM has any effects on treating hypercholesterolemia in the adult population.

Methods

Inclusion Criteria
To answer the research question regarding whether TCM has any effect on hypercholesterolemia compared to standard pharmaceutical interventions, studies that mentioned using only TCM, as opposed to combining treatments, as the intervention for addressing high cholesterol levels were included. The studies were limited to full-text availability and the English language. Relevant articles published in the last 20 years (2005 to 2025) were included. Due to the limited availability of studies on the topic, both randomized controlled trials (RCTs) and review papers were initially included.

Quality Appraisal Strategy
The quality of the five selected studies was determined using the Jadad score [5]. The Jadad score is a widely recognized appraisal tool that takes into account study-specific methods including RCT standards, double-blinding, and explains dropouts; all of which are indicators of a study's reliability and validity [5]. Using this score, we can understand whether the study's findings were valid [5].

Data Collection and Analysis
After conducting the literature search and retrieving relevant articles, a narrative synthesis was provided for the findings from the included articles. The following data was extracted: the population's characteristics, types of intervention, comparison groups, and outcomes of the treatments. Then, the quality of the articles was assessed using the Jadad score to determine the reliability and validity of the studies, as well as reviewing their strengths and limitations. The results were summarized in a table.

Results

Critical Appraisal
Results The first study assigned subjects randomly into groups, but there was no mention of the randomization method. It did not mention blinding, withdrawals or dropouts. The second study did not indicate the randomization method, blinding or dropouts. The third paper is another systematic review. It intended to use randomized studies and assess their type of randomization, but it did not end up satisfying the criteria set, so it was not included. However, points are given for consideration. The fourth article randomly assigned groups, but the randomization method was not described. It did not mention blinding, withdrawals or dropouts. The fifth study was randomized using the assignment of random numbers. Double-blinding, withdrawals or dropouts were not noted. Overall, 50% of the studies scored below 3. As the number of studies that meet the criteria are few, all are included to provide a comprehensive overview of the topic.

Discussion

Key Findings, Strengths and Limitations

We compiled the findings of studies done by Go et al., Tao et al., Liu et al., Li et al., and Jing et al. on various Chinese herbs to determine their efficacy in treating hypercholesterolemia. These findings reveal that TCM, such as Palmiwon, Xuezhikang, Xiaozhiling and Daming, may have positive effects on cholesterol levels compared to standard pharmaceutical medications like statins, while TCM like Xinkeshu do not. Go et al. [3] concluded that Palmiwon can be a potential treatment for addressing hepatic lipid accumulation and lipid-related diseases. They found that in a menopausal rat model, the use of Palmiwon reduced total cholesterol and LDL levels compared to simvastatin. As the herb is already available for treating diabetes, hypertension, urinary problems, and kidney disorders, Palmiwon shows as promising alternative to the standard estrogen therapy. Tao et al. [6] found that the Xinkeshu tablet did not significantly affect the levels of total cholesterol, LDL or HDL compared to atorvastatin. Given this finding, Xinkeshu, which is currently used to treat angina pectoris and arrhythmia, may not be an effective treatment for lowering cholesterol in the blood. Liu et al. [2] found that some TCM may offer possible treatment for hypercholesterolemia. Amongst the findings, red yeast rice extract (also known as Xuezhikang) has been shown to induce a significant difference in total cholesterol and LDL levels compared to inositol nicotinate and marine triglycerides, but not atorvastatin. Compared to pravastatin, Daming capsules lowered total cholesterol levels while 9 Xiaozhiling significantly improved the levels of LDL and HDL. Given Xuezhikang's effect on cholesterol levels in comparison with standard treatments, it could be further implored as a treatment for hypercholesterolemia. Li et al. [7] found Xuezhikang powder to reduce hypercoagulation and tissue factor expression, which explains the improvement of cardiac outcomes following its administration. Although the authors did not observe a significant reduction in total cholesterol or LDL following the administration of Xuezhikang compared to lovastation, their finding that the herb can ameliorate the plasma hypercoagulable state provides a base for its use in treating thrombosis that is caused by atherosclerosis. Jing et al. [1] concluded that Daming capsules could be a viable option for treating hypercholesterolemia. The study demonstrated that Daming capsules significantly decreased ($p < 0.05$) total cholesterol and LDL levels compared to pravastatin. In summary, using TCM, such as Palmiwon, Xuezhikang, Xiaozhiling and Daming, has shown to improve cholesterol levels, which make them viable options to treat hypercholesterolemia.

This review looked at many controlled clinical trials and other systematic reviews, which are considered the strongest in the hierarchy of scientific evidence. However, due to the small sample size of individual studies as well as the extent to which they were studied (e.g. in mice), the mentioned TCM cannot be established as alternative treatments until thorough research on the specific herbs (Palmiwon, Xuezhikang, Xiaozhiling and Daming) are conducted with larger sample sizes and human subjects.

Implications for future work

For further research, conducting well-designed RCTs with long-term outcomes, larger sample sizes and comparisons with standard treatments, such as statins, will help achieve homogeneity between studies to establish the efficacy and safety of TCM as an alternative treatment option for adults with 215 220 225 230 hypercholesterolemia. Addressing methodological limitations, underlying mechanisms of action, and exploring combination therapies is also encouraged to better utilize TCM's potential in managing hypercholesterolemia. Furthermore, population variability can be determined by studies that asses the effects of sex, age and ethnicity on treatment efficacy.

Conclusion

This systematic study reveals that TCM such as Palmiwon, Xuezhikang, Xiaozhiling and Daming may present alternative treatment options to manage hypercholesterolemia compared to conventional pharmaceutical drugs. However, further research is warranted to establish their replacement with the standard treatment.

Competing interests

The authors declare that they have no competing interests.

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Therapeutic Approaches to Restore Dopamine Homeostasis and Alleviate Motor Symptoms in Parkinson's Disease – a Narrative Review

Jordan J. Yin,[1] Josh A. Zeldin[1], Huy K. Nguyen[1]

[1] Faculty of Medicine, University of Ottawa, Ottawa, Ontario, Canada

Abstract

Parkinson's disease (PD) is the second most common neurodegenerative disorder, characterized by progressive dopaminergic neuronal loss in the substantia nigra pars compacta, leading to motor impairments such as bradykinesia, tremors, and rigidity. Current therapeutic strategies aim to restore dopamine homeostasis and alleviate motor symptoms through pharmacological, surgical, and non-pharmacological interventions; however, there are no current treatments that treat PD as a whole. This narrative review highlights three primary pharmacological approaches: (1) dopamine replacement with levodopa, the gold standard therapy, which is effective but associated with long-term complications such as motor fluctuations and dyskinesias; (2) dopamine receptor agonists, which offer an alternative to levodopa but exhibit increased non-motor side effects; and (3) inhibitors of dopamine degradation enzymes, including monoamine oxidase B (MAO-B) and catechol-O-methyltransferase (COMT) inhibitors, which prolong dopamine availability and reduce motor fluctuations. Emerging strategies focus on multifunctional compounds targeting both MAO-B and COMT, offering neuroprotection and improved dopaminergic stability. Despite advancements in PD management, an urgent need remains for novel therapeutics that provide sustained symptom relief with minimal side effects.

Résumé

La maladie de Parkinson (MP) est la deuxième maladie neurodégénérative la plus fréquente. Elle se caractérise par une perte progressive de neurones dopaminergiques dans la substantia nigra pars compacta, entraînant des troubles moteurs tels que bradykinésie, tremblements et rigidité. Les stratégies thérapeutiques actuelles visent à rétablir l'homéostasie de la dopamine et à atténuer les symptômes moteurs par des interventions pharmacologiques, chirurgicales et non pharmacologiques. Cette revue narrative met en lumière trois approches pharmacologiques principales : (1) la substitution de la dopamine par la lévodopa, le traitement de référence, qui est efficace mais associé à des complications à long terme telles que les fluctuations motrices et les dyskinésies ; (2) les agonistes des récepteurs de la dopamine, qui offrent une alternative à la lévodopa mais présentent davantage d'effets secondaires non moteurs ; et (3) les inhibiteurs des enzymes de dégradation de la dopamine, y compris les inhibiteurs de la monoamine oxydase B (MAO-B) et de la catéchol-O-méthyltransférase (COMT), qui prolongent la disponibilité de la dopamine et réduisent les fluctuations motrices. Les stratégies émergentes se concentrent sur des composés multifonctionnels ciblant à la fois la MAO-B et la COMT, offrant une neuroprotection et une meilleure stabilité dopaminergique. Malgré les progrès réalisés dans la prise en charge de la MP, il reste un besoin urgent de nouvelles thérapeutiques qui soulagent durablement les symptômes avec un minimum d'effets secondaires.

1. Introduction

Parkinson's disease (PD) is classified as the second most common neurodegenerative disorder, with over 1 million diagnoses every year(1). PD typically begins between the ages of 60 and 70 years old, with males being affected more often. In terms of clinical symptoms, PD is mainly defined by motor disturbances, such as bradykinesia and rigidity; however, there are also other functions of the nervous system that are affected(2–4). With physical disabilities only getting worse as one ages, PD patients have limited independence, often having their families caring for them. Moreover, patients with PD often develop anxiety and depression, affecting the quality of life for the entire family(2, 3, 5). As such, this disease not only puts a socioemotional burden on the individuals and families experiencing it, but also an economic burden on healthcare facilities and treatment groups. Ongoing neurodegenerative research aims to identify and understand the underlying hallmarks and mechanisms affecting PD, and to integrate this information into the development of an effective therapy. Currently, there are eight hallmarks that characterize the pathological mechanisms and processes underlying PD. Amongst these, some include pathological protein aggregation, synaptic and neuronal network dysfunction, altered energy metabolism, and neuronal cell death(6). By targeting these hallmarks, certain symptoms of PD can be diminished, thereby yielding more effective and safer treatments. Current PD treatments can be characterized into pharmacological treatments, surgical treatments, and non-pharmacological treatments(7).

1.1. The role of substantia nigra and dopamine.
The substantia nigra (SN) is a component of the midbrain, and plays a critical role in motor control, reward processing, and cognitive functions. The SN can be differentiated into two main parts: the pars compacta (SNpc) and the pars reticulata (SNpr). SNpc is heavily populated with dopaminergic neurons that produce dopamine, a neurotransmitter essential for modulating motor activity and reward-related behaviors(8–10). Dopamine production in these specialized neurons involves the enzyme tyrosine hydroxylase, which is responsible for the conversion of tyrosine to L-DOPA, which will then be decarboxylated into dopamine. To note, dopamine is synthesized and released from the dendrites within the SNpc, rather than the synaptic terminals(11, 12). In PD, the core manifestations of motor system degeneration are primarily from the progressive loss of dopaminergic neurons in the SNpc(8). When SNpc undergo oxidative stress, mitochondria dysfunction, and calcium dysregulation, it results in a manifestation in neurodegenerative diseases(9).

1.2. Molecular mechanisms and risk factors in Parkinson's disease.
As the second most common age-related and idiopathic neurological disorder, PD, serves as a complex neurodegenerative disorder marked mainly by the progressive loss of dopaminergic neurons in the SNpc. Due to this progressive loss, a reduced level of dopamine and impaired motor function manifests in patients(13, 14). Moreover, another hallmark of PD is the presence of Lewy bodies. These are intracellular aggregates that are composed of misfolded alpha-synuclein (α-syn), which affect synaptic function and axonal transport. Additionally, hyperphosphorylated tau protein contributes to neuronal instability as these are also protein that are found in the neuron during PD development(13, 15). Both α-syn and tau are known to behave in a prion-like manner, meaning that the aggregation formed is able to leave its cell of origin and induce further aggregation in neighboring cells (via templated conformational change) (16). Moreover, PD may result from both genetic and environmental factors. Familial and age-related PD is linked to mutations in genes such as synuclein alpha (SNCA), leucine-rich repeat kinase 2 (LRRK2), protein deglycase DJ-1 (Parkinson disease protein 7), PTEN-induced putative kinase 1 (PINK1), and Parkin (PRKN)(17). Through studies, mutations in proteins such as PINK1, PRKN, and DJ-1 impair mitochondrial quality control through disturbances of mitophagy, thus leading to mitochondrial dysfunction. With this, reduced ATP production and increased generation of reactive oxygen species (ROS) will occur, damaging lipids, proteins, and DNA(8, 13, 14, 18). Moreover, mutations in SNCA and LRRK2 genes play a critical role on maintaining dopaminergic neurons(19, 20). This specifically hinders tyrosine hydroxylase expression, resulting in decreased dopamine production(20). In parallel, mutations in LRRK2, such as R1441C and G2019S, disrupt dopamine metabolism and neurotransmission. Dopamine release is impaired due to a malfunction of the D2 receptor, further contributing to PD pathology(21, 22).

Environmental factors that are implicated in PD include exposure to certain pesticides and herbicides, traumatic brain injuries (TBI), neurotoxins, and the use of beta-blockers(17). TBI significantly contributes to parkinsonism as it impacts dopamine production and induces neurodegenerative changes in the brain. TBI causes neuroinflammation, ROS production, mitochondrial dysfunction, and α-syn production, which damages the dopaminergic neurons in the SNpc(23). Studies have shown that TBI reduces the expression of tyrosine hydroxylase, an enzyme that is required for dopamine production, leading to decreased dopamine levels and impairments in the nigrostriatal pathway. Moreover, this disruption not only affects the motor system defects in PD, but also exacerbates non-motor symptoms, such as cognitive decline and anxiety(23–25).

1.3. The need to treat PD-associated motor symptoms.
In brief, PD is a neurodegenerative disorder driven by dopamine dysfunction due to the loss of dopaminergic neurons in the SNpc. Overall, disruptions in dopaminergic signaling, in PD patients, result in motor deficits such as bradykinesia, tremors, and rigidity, further highlighting the need for proper treatment. Our review will explore current pharmacological approaches to combat motor symptoms and has identified 3 key strategies: dopamine replacement through levodopa, dopamine agonists, and inhibition of enzymes breaking down dopamine (MAO-B and COMT).

2. Levodopa: The Flawed Gold Standard

2.1. Significance of levodopa.
PD is characterized by a deficiency in dopamine, a neurotransmitter essential for multiple elements of tic communication, due to the death of dopaminergic neurons of the SNpc(26–28). This loss of dopamine can lead to motor symptoms such as tremors and stiffness(26). Therefore, a viable therapeutic strategy to treat this condition is through dopamine supplementation(27, 28). However, dopamine cannot pass through the blood-brain barrier (BBB) due to its positive charge, creating a barrier in treatment(28, 29). This signifies that there is a need for a biological molecule that compensates for the lack of dopamine, while passing through the BBB.

2.2. Levodopa as a dopamine replacement therapy.
Levodopa (L-DOPA), discovered by Torquato Torquati in the Vicia Faba bean in the early 1910s and harnessed as a therapeutic in the 1960s, was identified as a biological molecule that can pass through the BBB and replenish dopamine levels(27, 28, 30). It is able to reach the brain by passing through the sodium-dependent L-neutral amino acid carrier system at the BBB, and it is converted to dopamine by aromatic amino acid decarboxylase (AAAD) (27, 29).

2.3. Early trials and clinical validation.
Many studies have explored the efficacy of levodopa since its first human trial in 1961(30). Perhaps the most pivotal paper to the clinical validation of levodopa was the work of Cotzias et al(30, 31). Prior trials showed mixed results

for the efficacy of levodopa, as an insufficient dose was unobtainable due to dose-limiting gastrointestinal side effects(27, 30, 31). However, Cotzias et al. achieved a high dose through initiating with a very low dose, allowing patients to get tolerant to the drug, before escalating to a dose as high as 16g per day. Eight of the 16 patients showed dramatic levels of improvement with regards to stiffness, tremors, and other motor symptoms, while 2 showed mild improvements(30, 31). Thus, levodopa is a widely used therapeutic for managing motor symptoms(26, 30).

2.4. Combination with carbidopa.

Furthermore, L-DOPA is often paired with carbidopa to further avoid gastrointestinal side effects(28). A major cause of side effects in L-DOPA therapy is that peripheral AAAD converts L-DOPA to dopamine at the gastrointestinal tract and rest of the body(32–35). There, L-DOPA is unable to ameliorate the effects of the death of dopaminergic neurons, but instead dopamine acts on the area postrema of the brain. Pairing L-DOPA with the AAAD inhibitor carbidopa makes the therapy more tolerable for patients. However, carbidopa does not impede L-DOPA conversion to dopamine in the brain as carbidopa cannot pass the BBB.

2.5. Limitations of L-DOPA therapy.

Despite the management of gastrointestinal side effects, L-DOPA therapy has substantial motor side effects(35). It has been proposed that levodopa is initially an effective therapeutic, with the increased dopamine being stored and utilized by neurons in the brain. As PD progresses, neuronal death results in a decreased ability for neurons to store excess dopamine, resulting in extracellular concentrations of dopamine to reach abnormal levels, leading to dyskinesia. As well, because of the low half-life of L-DOPA, dopamine levels can fluctuate dramatically, resulting in "OFF" periods caused by low dopamine concentrations.

2.6. Long-term impact of L-DOPA usage.

Further work has characterized the impact of L-DOPA in long-term usage of this drug. A subsequent trial by Cotzias et al. explored chronic dopamine usage and found various motor side effects. In 28 patients who received levodopa, 14 patients experienced dyskinesias such as grimacing, gnawing, myoclonus, and other dyskinesias in addition to the gastrointestinal side effects identified(30, 36, 37). A few patients experienced dose failure, required redosing, and one patient succumbed to aspiration pneumonia despite continued use of levodopa. For instance, Hely et al. found that 95% of participants experienced dyskinesia after 15 years of usage, with 12% describing their dyskinesia as severe(37, 38). Ninety-six percent of patients have experienced instances of dose-failure(38). The study concluded that L-DOPA ultimately does not slow the progression of PD. Other studies exploring motor side effects of L-DOPA includes work done by Luquin et al., who found 84% of patients experienced dyskinesia (incidence of "OFF" periods was not recorded)(37, 39). Holloway et al. found that within 4 years of starting L-DOPA therapy, 62.7% of patients experienced dyskinesias (6.9% of patients characterized their dyskinesia as disabling) and 54.0% experienced wearing OFF periods (37, 40). However, despite these side effects, the incidence of discontinuation of levodopa therapy is low according to the study done by Gray et al., with only 7% of patients discontinuing levodopa after 7 years of use. This is possibly due to alternatives having worse side effects(34). In short, the gold standard dopamine replacement therapy levodopa can dramatically improve the symptoms of PD in the short-term, but its long-term potential is limited due to the emergence of dyskinesia. Other pharmacological agents have been tried, either as a monotherapy, or as a combination therapy with levodopa. We will explore some alternatives and additives to levodopa.

3. Dopamine agonists

3.1. Introduction to dopamine agonist therapy.

An alternative to dopamine replacement therapy in PD is to employ dopamine agonists, molecules that can mimic dopamine and stimulate dopamine receptors in the brain(41). The first dopamine agonist, apomorphine, was discovered in 1869, and researchers in the early 1950s were able to effectively reverse symptoms of PD through intravenous administration. The first effective oral dopamine agonist, bromocriptine, was used in 1974 by

Calne et al. They found that patients, who were on levodopa or other anticholinergic drugs, had substantial improvement in reduction of tremors and rigidity(42, 43). Current dopamine agonists used include cabergoline, pramipexole, ropinirole, and lisuride and others, either as a monotherapy or in combination with other therapies(26, 43, 44).

3.2. Dopamine agonists vs levodopa.

Many studies have explored the efficacy of dopamine agonists in reducing PD motor symptoms as well as comparing their efficacy to other options. For instance, Holloway et al. compared the efficacy of the dopamine agonist pramipexole to levodopa(40). They found that pramipexole had lower rates of motor side effects, with 52% of participants on pramipexole having motor-related side effects compared to 74% of those taking levodopa. However, pramipexole had a higher incidence of non-motor side effects than levodopa For instance, pramipexole treatment had a greater incidence of edema (42.4% vs 14.7%), and hallucinations (14.6% vs 8.0%) compared to levodopa. Similar results are found across the literature, with systematic reviews highlighting that although dopamine agonists result in lowered rates of motor side effects, they have higher rates of non-motor side effects, such as hallucinations, edema, and somnolence(44–46). Furthermore, there is evidence to suggest that the relief from the L-DOPA-associated side effects is outweighed by the non-motor side effects since patients on dopamine agonists have much higher dropout rates due to these side effects(44, 45).

In short, despite the reduced occurrence of motor side effects on dopamine agonist treatment, the non-motor side effects provide a significant caveat to the utility of dopamine agonists over levodopa.

4. Inhibiting dopamine degradation: MAO-B and COMT inhibitors as adjuvants for dopaminergic medication

4.1. Pharmacological challenges with levodopa.
Levodopa is the gold standard for managing motor symptoms in PD due to its ability to restore depleted dopamine levels. However, its efficacy is hindered over time by complications such as motor fluctuations and dyskinesias(47), including "OFF" periods, where dopamine levels are insufficient, and "ON" periods, marked by excessive dopamine that can lead to involuntary movements. These fluctuations arise from levodopa's short half-life and irregular absorption(48). Additionally, peripheral metabolism of levodopa by enzymes such as monoamine oxidase B (MAO-B) and catechol-O-methyltransferase (COMT) reduces its bioavailability, requiring higher doses and leading to more side effects(49, 50).

4.2 MAO-B inhibitors
4.2.1 Prolonging dopamine availability by preventing the breakdown role of MAO-B in metabolism and the "cheese effect". MAO-B is an enzyme responsible for breaking down dopamine in glial cells, particularly in the brain. In PD, increased MAO-B activity not only accelerates dopamine catabolism but also exacerbates oxidative stress by producing hydrogen peroxide, a by-product that contributes to dopaminergic neuronal death(51, 52). Historically, non-selective MAO inhibitors posed significant safety concerns due to the "cheese effect" - a hypertensive crisis triggered by consuming tyramine-rich foods such as cheese. Tyramine competes with dopamine for transport and storage in neurons, causing a dangerous increase in blood pressure. However, selective MAO-B inhibitors avoid this effect by sparing MAO-A, which metabolizes tyramine(51, 52).

4.2.2. Current advancements in MAO-B inhibitors.
Selective MAO-B inhibitors such as selegiline and rasagiline are particularly effective at reducing OFF-time and improving motor symptoms(47, 50). Recent advancements have introduced reversible inhibitors like safinamide, which not only inhibits MAO-B but also modulate glutamate release. This dual mechanism addresses non-motor symptoms such as pain, improving overall quality of life. Safinamide has demonstrated sustained efficacy in increasing ON-time without inducing dyskinesias, a notable improvement over earlier therapies(53). Emerging compounds, such as phenethylamide derivatives and 6-benzyloxyphthalides, show promise with their high potency (e.g., IC50 = 11 nM for phenethylamides) and neuroprotective effects, including reducing oxidative stress and α-syn aggregation in preclinical models. C14, a benzofuran derivative with IC50 = 0.037 μM was identified in 2023 for MAO-B inhibition. C14 demonstrated superior pharmacokinetics (bioavailability >80%) and BBB permeability compared to safinamide(52). 6-benzyloxyphthalides, particularly compounds 8f and 14a were developed in 2022, and showed IC50 values of 1.33 nM and 0.02 nM, respectively for inhibiting MAO-B(51). These advancements represent a significant step toward safer and more effective MAO-B inhibitors(51, 52).

4.3. COMT Inhibitors
4.3.1. Extending levodopa's therapeutic window.
COMT is an enzyme that methylates both levodopa and dopamine, reducing their availability in the brain. This process leads to the accumulation of 3-O-methyldopa (3-OMD), a metabolite associated with reduced therapeutic efficacy and increased motor complications. Long-term levodopa therapy exacerbates this issue, as elevated 3-OMD impairs dopamine uptake and promotes oxidative stress(50, 54).

4.3.2. Current COMT inhibitors.
Entacapone and tolcapone are the primary COMT inhibitors used in clinical practice. Tolcapone was well-tolerated initially but adverse effects (e.g., diarrhea, nausea) increased with selegiline addition, but no cardiovascular issues were observed(55, 56). Entacapone, a peripheral COMT inhibitor, enhances levodopa's bioavailability by reducing its peripheral metabolism, thereby prolonging ON-time and minimizing OFF-time. Clinical studies have shown that entacapone increases ON-time by an average of 1.7 hours while reducing OFF-time by 1.5 hours(57). It also allows for lower daily levodopa doses, potentially delaying the onset of levodopa-induced dyskinesias. Entacapone is well-tolerated, with mild gastrointestinal side effects, and lacks the hepatotoxicity risks associated with tolcapone(58). Preclinical studies using a Japanese encephalitis virus (JEV)-induced rat model of Parkinson's disease demonstrate that combining entacapone with levodopa and dopa decarboxylase inhibitor (DDCI) significantly improves motor function, reducing pole test descent time from 13.2 seconds (JEV group) to 7.7 seconds, comparable to healthy controls at 7.1 seconds. This combination also increased striatal dopamine levels from 53.4 to 102.3 ng/mg protein, outperforming standalone monotherapies(59).

4.4. Comparing MAO-B and COMT inhibitors: complementary but distinct roles.
While both MAO-B and COMT inhibitors serve to enhance levodopa therapy, their mechanisms and clinical applications differ. MAO-B inhibitors primarily act in the brain, preventing dopamine breakdown and reducing oxidative stress. They are particularly useful for patients in the early stages of or those experiencing mild motor fluctuations(47, 50). COMT inhibitors act peripherally, improving levodopa's bioavailability and prolonging its effects. They are more suitable for managing advanced PD with significant motor fluctuations(57, 60). Combining these inhibitors with levodopa offers a multitarget approach to stabilizing dopamine levels, minimizing OFF-time, and improving motor outcomes. However, careful equilibration is required to balance their distinct side effect profiles and maximize their complementary benefits.

4.5. Future directions: toward multifunctional therapies.
MAO-B inhibitors provided better patient-rated quality of

similar in efficacy to MAO-B inhibitors but associated with more side effects (e.g., dyskinesia, impulse control issues). COMT inhibitors showed less efficacy in quality-of-life improvement(61). Emerging multifunctional compounds that target both MAO-B and COMT, such as chalcones, offer a promising avenue for comprehensive PD management(62). These agents not only inhibit dopamine degradation but also provide neuroprotective effects by neutralizing ROS and disrupting toxic α-syn fibrils. Preclinical studies suggest that such dual-targeting strategies could enhance both motor and non-motor symptom control, representing a significant advancement in PD therapy(63, 64).

5. Conclusion

Many strategies have been proposed to combat motor symptoms by restoring reduced dopamine levels. The gold standard approach is to use levodopa, a dopamine precursor. While in the short-term it restores dopamine levels and alleviates motor symptoms, as PD progresses and neuronal cells struggle with maintaining dopamine. increased MAO-B activity not only accelerates dopamine catabolism but also exacerbates oxidative stress by producing hydrogen peroxide, a by-product that contributes to dopaminergic neuronal death(51, 52). Historically, non-selective MAO inhibitors posed significant safety concerns due to the "cheese effect" - a hypertensive crisis triggered by consuming tyramine-rich foods such as cheese. Tyramine competes with dopamine for transport and storage in neurons, causing a dangerous increase in blood pressure. However, selective MAO-B inhibitors avoid this effect by sparing MAO-A, which metabolizes tyramine(51, 52).

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5. Conclusion

Many strategies have been proposed to combat motor symptoms by restoring reduced dopamine levels. The gold standard approach is to use levodopa, a dopamine precursor. While in the short-term it restores dopamine levels and alleviates motor symptoms, as PD progresses and neuronal cells struggle with maintaining dopamine. homeostasis, significant motor side effects such as dyskinesia and non-responsiveness, colloquially called “OFF” periods, emerge. Thus, other ways to treat PD have been proposed. Namely, dopamine agonists have been found to reduce the frequency of motor side effects, but they have increased incidence of non-motor side effects that result in much higher withdrawal rates. MAO-B and COMT inhibitors serve as critical adjuvants to levodopa therapy, addressing its limitations by stabilizing dopamine levels and reducing motor fluctuations. MAO-B inhibitors, such as safinamide, offer additional neuroprotective benefits but may cause side effects like insomnia or headache. COMT inhibitors like entacapone effectively extend levodopa's therapeutic window but can lead to mild gastrointestinal issues and, in rare cases, hepatotoxicity with tolcapone. Despite these limitations, these complementary mechanisms improve ON-time, minimize OFF-time, and delay dyskinesias, offering a more stable motor symptom relief in PD patients. However, there is still a need for therapeutics that provide motor symptom relief with reduced side effects.

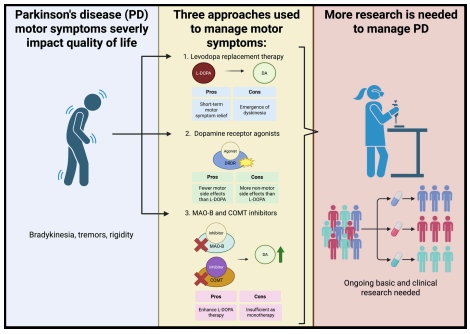

Figure 1. Graphical abstract highlighting ways to treat motor symptoms in Parkinson’s disease (PD). Dopamine deficiency due to the death of dopamine-producing cells in the striatal nigra results in motor symptoms. There are several therapeutic strategies to provide motor symptom relief, namely levodopa, dopamine agonists, and inhibitors of dopamine breakdown enzymes (COMT and MAO-B).

ABBREVIATIONS

PD	Parkinson’s Disease	**SNCA**	Synuclein Alpha
SN	Substantia Nigra	**LRRK2**	Leucine-Rich Repeat Kinase 2
SNpc	Substantia Nigra pars compacta	**PINK1**	PTEN-Induced Putative Kinase 1
SNpr	Substantia Nigra pars reticulata	**PRKN**	Parkin
α-syn	Alpha-Synuclein	**ATP**	Adenosine Triphosphate
TBI	Traumatic Brain Injury	**MAO-B**	Monoamine Oxidase B
BBB	Blood-Brain Barrier	**COMT**	Catechol-O-Methyltransferase
L-DOPA	Levodopa	**3-OMD**	3-O-Methyldopa
AAAD	Aromatic Amino Acid Decarboxylase	**IC50**	Half Maximal Inhibitory Concentration
ROS	Reactive Oxygen Species		

Competing interests
The authors declare that they have no competing interests.

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Commentary

Neurobiological Delusions: Regional Cerebral Blood Flow insights into Cotard's Syndrome and Schizophrenia

Nur Zeynep Camci[1]

[1] Faculty of Health Sciences, University of Ottawa, Ottawa, Canada

Abstract

Cotard's syndrome is a clinically rare condition that is characterized by nihilistic or immortality delusions, which are often accompanied by thoughts of suicide, depression, and anxiety. These symptoms vary among individuals but are generally distinguished as a neuropsychiatric disorder. Cotard's syndrome remains under-explored, especially in the context of psychiatric conditions such as schizophrenia. The cause of this condition was further investigated in a recent case study by Nomura et al. (2024), where a 52-year-old male patient diagnosed with schizophrenia and mild intellectual disability was examined. The patient suffered from several lumbar fractures due to an attempted suicide, and after admission to the hospital, the patient was diagnosed with Cotard's syndrome. This study aimed to assess the longitudinal changes of regional cerebral blood flow (rCBF) on a single-photon emission computed tomography (SPECT) scan with respect to a schizophrenic patient displaying Cotard's syndrome. The symptoms of Cotard's syndrome appear to dissipate through the administration of lurasidone, an atypical antipsychotic medication used in psychiatric treatment, which is observed through SPECT and changes in rCBF. The observed correlation between the development of Cotard's syndrome and an increased rCBF provides a strong basis on the nature of the condition, and enhanced understanding of neuropsychiatric conditions.

Résumé

Le syndrome de Cotard est une affection rare, qui se caractérise par des délires nihilistes ou d'immortalité, souvent accompagnés de pensées suicidaires de dépression et d'anxiété. Ces symptômes diffèrent selon les individus mais sont souvent considérés comme des manifestations d'un trouble neuropsychiatrique. Le syndrome de Cotard reste insuffisamment étudié, en notamment dans le contexte de pathologies psychiatriques comme la schizophrénie. La cause de ce syndrome a été étudiée de façon plus approfondie dans une étude de cas récente dirigée par Nomura et al. (2024), au sujet d'un patient âgé de 52 ans atteint de schizophrénie et d'une légère déficience intellectuelle. Ce patient a subi beaucoup de fractures lombaires a la suite d'une tentative de suicide, et après son admission à l'hôpital, il a été diagnostiqué avec le syndrome de Cotard. Cette étude à évaluer les modifications longitudinales du flux sanguin cérébral régional par tomographie monophotonique (SPECT) chez un patient atteint de schizophrénie présentant un syndrome de Cotard. Les symptômes du syndrome semblent diminuer avec l'administration de la lurasidone, un antipsychotique atypique prescrit en psychiatrie, comme en témoignent les résultats du SPECT et les variations du rCBF. Le lien identifié entre le développement du syndrome de Cotard et une augmentation du rCBF donne un fondement solide pour mieux comprendre la nature de cette affection ainsi que les troubles neuropsychiatriques en général.

Introduction

Nihilistic delusions range from individuals who feel as though they are dead, have lost a part of their body, their soul, or simply do not exist. This is commonly attributed to patients diagnosed with Cotard's syndrome. A recent case report published by Nomura et al. (2024) describes a 52-year-old schizophrenic patient who is hospitalized after a suicide attempt, where he was then diagnosed with Cotard's syndrome [1]. The patient is further investigated to identify the correlation between schizophrenia and Cotard's syndrome. Although both conditions may be observed together, the causes, diagnosis, and treatment, are divergent. To distinctly analyze Cotard's syndro, the authors used a single-photon emission computed tomography (SPECT) scan to observe the longitudinal changes in rCBF between conditions with and without the syndrome. As a part of the treatment plan, Lurasidone, an atypical antipsychotic medication, was used and observed in initial and follow-up imaging. The results suggested that Cotard's syndrome is associated with an increased rCBF in the prefrontal cortex and decreased rCBF in the right occipital and parietal lobes, highlighting the importance of cerebral blood flow (CBF) studies in comprehending complex neuropsychiatric conditions. Longitudinal changes in Cotard's syndrome and schizophrenia can be

investigated through the correlations between psychiatric symptoms and rCBF patterns using SPECT imaging. SPECT imaging with 99mTc-ethyl cysteinate dimer was conducted at varied time points to measure rCBF and plot brain activity. This approach using SPECT imaging provides an outlook on brain activity during differing clinical states presented in symptoms of those with the syndrome. Longitudinal monitoring of cerebral perfusion can be achieved to map how shifts in rCBF may correlate to the presence and remission of symptoms. The importance of cerebral blood flow has been recognized in previous schizophrenia research, which supports the use of perfusion imaging- adding that it is a non-invasive and functional method to capture relationships between behavior and the brain [5]. Other methods used to conduct this case study included baseline imaging and neurovascular coupling. The following baseline imaging was then used to analyze the progression and potential remission of symptoms. An analysis of rCBF patterns was used to establish fluctuations pertaining to the patient with psychiatric symptoms and nihilistic delusions. This procedure supports the idea that SPECT can track neurobiological changes that correspond to psychiatric disorders.

Neurovascular coupling is the process by which increased neuronal activity is correlated with the local regulation of cerebral blood flow- securing an adequate supply of oxygen to exceed demands for brain function [2]. Neurovascular coupling provided an overview of the relationship between changes in rCBF and neurological disturbances. Altered CBF has effects on certain brain regions along with their associated functions. Hypoperfusion in the frontal lobe is linked to impaired function and self-awareness, which may be a cause of delusions [3]. Alternatively, hypoperfusion in the parietal lobe is linked to disrupted spatial and body awareness, which may specifically contribute to the nihilistic thoughts accompanied by Cotard's syndrome. It is important to note that neurovascular coupling may vary in areas with related neuronal populations, which may be due to differences in functional processes linking neural activity through metabolic changes in local CBF [3]. These observations are now a routine part of practice within the imaging community- given that detailed studies on coupling have been exclusive to cortical areas, which reinforces the need to study non-cortical areas [3]. These variations may influence results for the study on Cotard's syndrome, as it may instigate a contrast in how neuronal activity corresponds to alterations in rCBF [3]. In addition, SPECT imaging could vary in accuracy of reflecting neuronal dysfunction. This limitation is important to consider in terms of future implications of therapeutic application, research advancements, and diagnostic dependability [4]. Lurasidone was used as a pharmacotherapy treatment after initial imaging was conducted, and its effectiveness was then observed in clinical assessments and follow-up imaging. The key findings of this study display an improvement in symptoms after the lurasidone treatment, specifically with relation to increased circulation in the frontal and parietal regions- particularly in the left hemisphere.

The initial imaging displayed hypoperfusion, which was improved through the administration of lurasidone. Despite the observed improvements in the psychiatric symptoms of Cotard's syndrome, some neurobiological abnormalities and symptoms linger during remission. Current studies provide evidence that altered CBF in schizophrenia, more specifically the frontal and parietal lobes, are consistent features of the condition [5]. This supports the notion that hypoperfusion in the brain regions is correlated to symptoms of delusions and cognitive disorders [5]. Schizophrenia studies using SPECT and other imaging tests have revealed both cerebral hyperperfusion and hypoperfusion, which have also demonstrated strong associations between positive symptoms of schizophrenia to different rCBF values [6]. Such findings help interpret the distinguished inconsistencies in patterns of perfusion in untreated schizophrenia patients [6]. Acknowledging these associations provides a strong basis for how CBF in schizophrenia further reinforces the key role of CBF aberrations on the psychopathology of schizophrenia and conditions similar to Cotard's syndrome [5].

This study's approach to schizophrenia and Cotard's syndrome provides a different perspective and insight into the functions and mechanisms that occur in the brain and how they contribute to psychiatric conditions. Some psychiatric implications may suggest the use of interventions that focus on recovery through CBF improvements in the affected regions. This includes potentially using SPECT imaging to diagnose and derive a prognosis for schizophrenia or related disorders. A longitudinal perspective is not commonly reported for Cotard's syndrome, which is why this study provides new discoveries and benefits of using these advanced and personalized procedures. The positive results attributed to lurasidone establish the benefit of these medical interventions. By addressing the challenges in the accuracy of the procedure, a more reliable and clarified understanding of neuronal activity and CBF can be derived. Overall, the gap between psychiatry and neurobiology can be bridged with SPECT imaging and may open more opportunities for research on neurovascular mechanisms in complex psychiatric disorders.
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Competing interests

The authors declare that they have no competing interests.

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Targeted genomic profiling identifies a Thai-specific variant in SCN5A contributing to Brugada syndrome

Marwan Bakr [1]

[1] Faculty of Science, University of Ottawa, Ottawa, Canada

Abstract

Brugada syndrome, a disorder that can lead to sudden cardiac arrest, is a leading cause of death among young men in Thailand. However, the genetic factors underlying its high prevalence in Southeast Asia remain inadequately studied due to limited representation in genomic studies. A recent study by Walsh et al. identified a Thai-specific noncoding variant in the *SCN5A* locus and demonstrated its causal role in Brugada syndrome. This study highlights the importance of population-specific genomic research and advances our previously limited understanding of noncoding regulatory variants in cardiac disease.

Keywords: Brugada syndrome, *SCN5A*, electrophysiology, noncoding variants

Résumé

Le syndrome de Brugada, est un trouble qui peut contribuer à un arrêt cardiaque soudain, est une cause majeure de décès chez les jeunes hommes thailandais. Cependant, les facteurs génétiques à l'origine de sa forte prévalence en Asie du Sud- Est ne sont pas suffisamment étudiés, due à une faible représentation dans les études génomiques. Une étude qui a été récemment menée par Walsh et. al a trouvé une variante non codante spécifique à la population thaïlandaise dans la région du gène *SCN5A* et a montré son rôle causal dans le syndrome de Brugada. Cette étude met en évidence l'importance de la recherche génomique ciblée selon les populations et nous aide à mieux comprendre le rôle des variantes régulatrices non codantes dans les pathologies cardiaques.

Mots clés: Le Syndrome de Brugada, *SCN5A*, électrophysiologiques, variantes non codantes

In the 1980s, the Centers for Disease Control and Prevention (CDC) reported an alarming increase in sudden deaths among young, healthy, Southeast Asian men in the United States, with most victims dying during sleep (1). This phenomenon, often without preceding symptoms, was termed 'sudden unexplained death syndrome' and had long been known in Asia, where it was called *Lai Tai* in Thailand, *Bangungut* in the Philippines, and *Pokkuri* in Japan (2). Epidemiological studies soon highlighted the high prevalence of sudden unexplained death syndrome in Thailand, identifying it as the leading natural cause of death in young Thai men (3, 4). In these cases, the absence of pathological findings on autopsy—such as neurological injury, pulmonary failure, or other major abnormalities that could explain the cause of death—implicated the heart as the likely source (5). The lack of structural heart defects further pointed to a disturbance in cardiac electrical conduction rather than a morphological abnormality, later supported by the discovery of a common electrocardiographic pattern (ST-segment elevation) (6). This electrical irregularity could be reliably reproduced in suspected patients by administering sodium channel-blocking medications, confirming the role of impaired cardiac sodium current in the disease's pathogenesis (7). The syndrome was clinically described by cardiologists Josep and Pedro Brugada in 1992 and is now termed Brugada syndrome (BrS) (6).

The discovery of genetic contributions to BrS has since provided insight into its pathogenesis. The major genetic contributor to BrS, implicated in approximately 20% of cases, is a group of variants in the *SCN5A* locus (8, 9). *SCN5A* encodes the alpha subunit of the sodium channel $Na_V1.5$, which is essential for propagating electrical impulses through the heart muscle. $Na_V1.5$ channels mediate the influx of sodium ions, facilitating the rapid depolarization of cardiac muscle cells, and thus allowing for rhythmic contractions of the heart (Fig. 1A) (10). Pathogenic variants in the *SCN5A* locus reduce the function of $Na_V1.5$ channels, leading to electrical conduction delays which can cause abnormal heart rhythms and sudden cardiac death (10). BrS is most prevalent in Southeast Asia, particularly in Thailand, yet many of the large-scale genomic studies to date have focused primarily on patients of European ancestry (11, 12). This has limited the understanding of the genetic basis of BrS in Southeast Asian populations. Consequently, genetic testing protocols commonly used in European populations are often less effective for Thai patients, as they may miss variants specific to this population (11, 12). A study by Roddy Walsh and colleagues, recently published in

Circulation, performed genome sequencing in a Thai population to identify BrS-associated variants enriched in Thailand (13). One variant in the *SCN5A* gene was identified as significantly enriched in BrS patients and prioritized by the authors. This variant, termed regulatory element 5 (RE5), was not located in *SCN5A*'s coding region, but in a previously characterized intronic regulatory region affecting *SCN5A* expression. Notably, this variant was found to be restricted to Southeast Asian populations, indicating its likely contribution to the high prevalence of BrS in the region.

The authors next sought to characterize the functional impact of the RE5 variant by introducing it into a human induced pluripotent stem cell-derived cardiomyocyte cell line. Using RNA sequencing, the authors found reduced *SCN5A* expression in variant-carrying cells compared to controls (data publicly available through the Gene Expression Omnibus under accession number GSE264359). Importantly, they identified the likely cause of this reduced expression to be a disruption of a binding site for the cardiac transcription factor (TF) family Mef2. The effect of the variant on cardiac electrophysiological function was also examined, specifically by measuring the cardiac sodium current modulated by *SCN5A*-encoded $Na_V1.5$ channels. This revealed a 30% reduction in the density of this current in variant-carrying cells, confirming the functional effects of the variant.

The results of this study represent a critical step in addressing the low yield of genetic testing for BrS in Thailand, where the disease burden is highest. By incorporating the *SCN5A* RE5 variant into Thai genetic testing protocols, clinicians could improve diagnosis and management of the disease. Notably, a substantial number of disease-associated genetic variants are located in noncoding regions, which makes it challenging to determine their causal mechanisms. This study, however, represents one of the first examples of a rare regulatory variant in a cardiac disease gene proven to be pathogenic. This marks a significant advancement, not only in understanding the genetic architecture of BrS, but also in providing a framework for studying the pathogenic mechanism of variants in noncoding regulatory regions. This research also highlights the importance of expanding genetic studies to include populations beyond those typically represented in genomic research. As of 2019, approximately 71.8% of large genomic study participants were recruited from just three countries—the United States, United Kingdom, and Iceland—leaving large segments of the global population underrepresented (14). By focusing on a Thai cohort, Walsh and colleagues demonstrate the significant insights that can arise from studying diverse populations and the necessity of inclusive genomic research for effective, population-specific medical interventions.

It is also important to note the complexity of the genetic architecture of BrS in Thailand and globally, as the disease has been associated with other genomic loci beyond *SCN5A* (Fig. 1B) (15-17). The variant identified in this study is present in approximately 4% of BrS patients in Thailand, indicating the importance of future research building upon the present study's framework to characterize other genetic contributors to Thailand's high BrS prevalence. Many cardiac TF loci have been implicated in genome-wide association studies, indicating the key role of transcriptional regulation in BrS pathogenesis (15). This includes *HEY2* and *TBX20*, known to be expressed in the outer layer of the heart's ventricular muscle (epimyocardium), as well as *TBX5*, *IRX3* and *IRX5*, known to be expressed in the inner layer (endomyocardium), where *SCN5A* is also strongly expressed. Along with the enrichment of *SCN5A* expression in the endomyocardium, the variation in expression pattern of BrS-associated TFs across the ventricular well suggests that the pathogenic mechanism of mutations in these TFs may involve disruption in their maintenance of *SCN5A*'s endomyocardial-enriched expression pattern. Specifically, mutations in endomyocardial-enriched TFs may result in decrease of endomyocardial *SCN5A* expression, and mutations in epimyocardial-enriched TFs may result in aberrant increase of *SCN5A* expression in the epimyocardium. Indeed, the mouse ortholog of IRX5 has been shown to regulate several cardiac properties that vary across the ventricular wall, including repolarization and contractility (18, 19). Notably, *TBX5*, *IRX3* and *IRX5* have all been shown to regulate the expression of cardiac ion channels, including promotion of *SCN5A* expression (20-22). Therefore, further research into variants in these genes and others is important to elucidate their contribution to BrS, especially in the understudied Southeast Asian population.

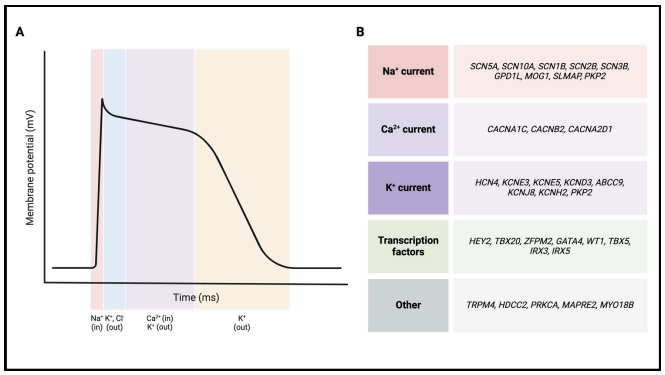

Figure 1. Genetic contributors to Brugada syndrome (confirmed and suspected). (A) Schematic of a ventricular cardiomyocyte action potential, highlighting the major ion currents and their timing. (B) Genes associated with Brugada syndrome, grouped by functional category. These genes have been identified through rare variant analyses, genome-wide association studies, and functional validation in human and model systems (15-17)

Competing interests

The authors declare that they have no competing interests.

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Flow Cytometry and Pharmacology Reveal Breast Cancer Senescence Mechanisms

Sofea Prabaharan[1]

1 Faculty of Science, University of Ottawa, Ottawa, Ontario, Canada

Abstract

Senescence was considered an irreversible cell cycle arrest triggered by stress, such as chemotherapy, which halts the division of cancer cells. However, some cancer cells can escape senescence, resuming proliferation and forming tumours, presenting challenges in cancer treatment. In a recent study, Tóth et al. investigated senescence dynamics in breast cancer cell lines MCF-7 and MDA-MB-231 using novel flow cytometry-based methods to track senescence escape in real time. They explore the effectiveness of azithromycin, a senolytic drug, combined with sitagliptin, a DPP4/CD26 inhibitor, to elucidate senescence escape mechanisms. Their findings reveal treatment- and cell-line-specific responses, suggesting potential therapeutic strategies for targeting senescent cancer cells

Résumé

Dans une étude récente intitulée Investigation in vitro de la sénescence induite par traitement et de l'échappement à la sénescence dans des cellules de cancer du sein via une méthode innovante de cytométrie en flux, Tóth et ses collaborateurs ont analysé la sénescence dans deux lignées cellulaires de cancer du sein (MCF-7 et MDA-MB-231), soumises à trois traitements inducteurs de sénescence différents : bromodésoxyuridine (BrdU), gemcitabine (GEM) et palbociclib (PALBO) (2). Les auteurs proposent également une stratégie pharmacologique ciblant les cellules sénescentes, en explorant le rôle de la protéine DPP4/CD26, surexprimée à la surface des cellules sénescentes, ainsi que l'efficacité de l'azithromycine testée pour la première fois comme agent sénolytique dans le contexte du cancer du sein. De plus, les chercheurs ont mis en place une méthode innovante de cytométrie en flux permettant de détecter en temps réel les cellules échappant à la sénescence, grâce à la combinaison de marqueurs de prolifération et de sénescence. Cette approche surmonte les limites des méthodes classiques, qui nécessitent 2 à 3 semaines d'incubation et ne permettent pas de détecter précocement les mécanismes d'échappement ou les phénotypes moins agressifs.

Senescence was previously believed to be a stable and irreversible state of cell cycle arrest in response to cellular stress, naturally induced by the body to prevent damaged cells from proliferating (1). Therapy-induced senescence (TIS), triggered by chemotherapy, leverages this mechanism to suppress tumour cells. Senescent cancer cells often experience increased glycolysis and mitochondrial dysfunction, contributing to their survival despite being damaged (2, 3). These adaptations also promote cell-cycle reentry, allowing cancer cells to regain their ability to proliferate and form tumours (4, 5). While inhibiting proliferation via TIS is a favourable outcome in cancer treatment, senescence-escaped cells pose significant risks to patients, as they can contribute to therapy resistance and lead to recurrent tumours (6). To mitigate recurrence and improve patient recovery, it is critical to uncover the mechanisms underlying senescence and senescence escape and to develop high-confidence tests for detecting and eliminating senescent cells.

In a recent study titled In Vitro Investigation of Therapy-Induced Senescence and Senescence Escape in Breast Cancer Cells Using Novel Flow Cytometry-Based Methods, Tóth et al. investigate senescence in two breast cancer cell lines (MCF-7 and MDA-MB-231) using three different senescent-inducing treatments (BrdU, GEM, and PALBO) and suggest a pharmacological strategy to target senescent cancer cells (7). This research examines the role of DPP4/CD26, a protein highly expressed on the surface of senescent cells, and the effectiveness of azithromycin, tested as a senescent-targeting drug for the first time in the context of breast cancer. Furthermore, the researchers employ an innovative flow cytometry method to detect senescent-escaping cells in real time, utilizing both proliferation and senescence markers. This approach addresses the limitations of previous detection methods, which required 2-3 weeks of sample incubation, making it difficult to detect less aggressive phenotypes and characterize early senescence escape mechanisms. To first study the senescence mechanism between treatments, the researchers stained for Ki-67, a

protein in the nucleus of proliferating cells, and γH2AX, a histone variant phosphorylated in the presence of double-stranded DNA breaks (8).

Together, these biomarkers confirm the induction of senescence and characterize its mechanism. Bromodeoxyuridine (BrdU) is a synthetic thymidine analogue that is incorporated into nascent DNA (9, 10). Palbociclib (PALBO) is a kinase-inhibitor medication that hinders cell proliferation (11, 12). Across both cell lines, BrdU- and PALBO-treated cells are stained mostly negative for γH2AX, indicating that they do not induce senescence via DNA damage but possibly through the upregulation of senescence-associated genes. Additional transcriptomic analyses should focus on identifying these genes, particularly those linked to metabolic remodelling and cell-cycle regulation. Functional analyses could then elucidate the role of these genes in senescence escape pathways and investigate their potential as therapeutic targets, aiming to induce or stabilize senescence in cancer cells by mimicking the necessary upregulation. GEM is a deoxycytidine analogue; this chemotherapy drug integrates into replicating DNA and prevents further synthesis, leading to DNA damage that kills proliferating cells (13, 14). In both cell lines, GEM induced senescence via DNA damage. When measuring senescence-associated secretory phenotypes (SASPs) IL-6 and IL-8, the responses of MCF-7 and MDA-MB-231 cells varied. The expression of these proteins increased in BrdU- and GEM-treated MCF-7 cells; conversely, the PALBO-treated MCF-7 cells did not have the same effect. In contrast, across all treatment types, MDA-MB-231 cells exhibited IL-6 and IL-8 expression. Thus, the use of differing treatments across two cell lines demonstrated both treatment- and cell-line-specific senescent phenotypes, further highlighting the challenge of identifying senescent cells.

Senescence escape was tracked by measuring Ki-67 fluctuations using flow cytometry. Senescent cells in BrdU- and GEM-treated cultures underwent a more stable form of senescence induction, as escaped cells were only evident after five to seven days. For PALBO, senescence escape began after the removal of the drug, suggesting a more temporary senescent state. However, the mechanism by which the cells were escaping senescence remained unclear. To address this, the authors focused on the potential role of the DPP4/CD26 protein in senescence escape. They tested this by treating the senescent cells with sitagliptin, a medication that inhibits the activity of DPP4, normally used to treat type 2 diabetes (15). In MCF-7 cells, sitagliptin treatment decreased senescence escape, whereasin GEM-treated MDA-MB-231 cells, senescence escape unexpectedly increased. These observations indicate that the enzymatic activity of DPP4 regulates senescence escape in a treatment- and cellline-specific manner. Research is underway to investigate DPP4 as a potential therapeutic target (16, 17).

Senolytic drugs offer an alternative approach to targeting senescent cancer cells by selectively inducing apoptosis, facilitating their removal while minimizing harm to normal cells. One such drug is azithromycin (AZI), a common antibiotic that has demonstrated up to 97% efficiency in killing and eliminating senescent cells via autophagy inhibition in low doses (18). In this study, its effectiveness on senescent breast cancer cells was tested for the first time. Tóth et al. reveal that while the viability of both cell lines decreased, MCF-7 exhibited greater resistance to AZI and fewer senescence-escaped cells following BrdU treatment. The varying responses to AZI led investigators to deduce that MCF-7 cells rely on autophagy-independent survival mechanisms, whereas MDA-MB-231 cells are autophagy-dependent. A combinatorial approach, treating with both AZI and sitagliptin, improved AZI's effectiveness on MCF-7 cells, further reducing the number of senescence-escaped cells. The potential restoration of proliferation and migration capabilities emphasizes the importance of determining the functionality of senescence-escaped cells. The senescent cells from each breast cancer line were labeled with CFSE. This dyebinds to intracellular proteins, to reveal proliferating cells as the intensity of their fluorescence diluted post-cell division. An increase in Ki-67 expression also confirmed senescence escape. Senescence-associated ß-galactosidase (SA-β-gal) activity was analyzed using fluorescence-based detection and fluorescence-activated cell sorting (FACS), identifying cells with low activity as senescence-escaped. By measuring these markers simultaneously through flow cytometry, Tóth et al. present an effective approach to quantify and characterize senescence-escaping dynamics in breast cancer cells in real-time, shortly after senescence escape. Their results showed that both MCF-7 and MDA-MB-231 senescent cells could re-enter the cell cycle, regaining proliferation abilities.

Previous studies indicate that senescent-escaped cells surviving senolytic treatment can re-enter the cell cycle with increased proliferation and tumour formation (6). However, the authors note that combined treatment with sitagliptin decreased senescence escape in AZI-resistant MCF-7 cells. This highlights antibiotic resistance as a potential challenge for AZI in cancer therapy, prompting further investigation into the effectiveness of this combinatorial approach in vivo. Furthermore, as phenotypes and responses vary between cell lines and treatment types, exploring these factors in more detail offers additional insight into senescence and escape mechanisms, potentially extending beyond breast cancer. The novel rapid isolation method ensures observed proliferating cells originated from a once senescent state, as opposed to ambiguous ancestry, thus enhancing the accuracy of results. This enables the potential application of multi-omic approaches to comprehensively explore mechanisms driving senescence and escape.

Although inducing senescence is an effective strategy to decelerate uncontrolled tumour proliferation, senescence escape dynamics create a challenge for navigating the complexities of cancer recurrence. This study explores a combinatorial pharmaceutical approach while characterizing senescent and escaped cell phenotypes in MCF-7 and MDA-MB-231 breast cancer cell lines. Their findings provide valuable insights for future research on

senescence, aiming to ultimately improve patient outcomes and mitigate the adverse effects of senescent cells in cancer therapy.

Competing interests

The authors declare that they have no competing interests.

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