QËNDRESA HOTI

AVERMECTIN B1A EXHIBITS ANTI-PROLIFERATIVE AND ANTI-CANCER PROPERTY IN HCT-116 CELLS BY PROMOTING TUBULIN POLYMERIZATION

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We certify that we have read the thesis submitted by Qëndresa Hoti titled **"Avermectin B1a Exhibits Anti-proliferative and Anti-cancer Property in HCT-116 cells by Promoting Tubulin Polymerization"** and that in our combined opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Philosophy in Medical Biochemistry.

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Declaration

I hereby declare that all information, documents, analysis and results in this thesis have been collected and presented according to the academic rules and ethical guidelines of Institute of Graduate Studies, Near East University. I also declare that as required by these rules and conduct, I have fully cited and referenced information and data that are not original to this study.

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Qëndresa Hoti

Abstract

Avermectin B1a Exhibits Anti-Proliferative and Anti-Cancer Property in HCT-116 Cells by Promoting Tubulin Polymerization

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The avermectins are a group of macrocyclic lactones that are produced by *Streptomyces avermitilis*, a soil microorganism. They are commonly used as pesticides to treat pests and parasitic worms. Abamectin, one of the avermectins, known as avermectin B1, contains at least 80% avermectin B1a and no more than 20% avermectin B1b. Some members of the avermectin family, such as ivermectin, have shown promising anti-proliferative effects on cancer cells. Colon cancer is a significant cause of death worldwide, thus, novel approaches and therapeutic molecules are needed for efficient therapy. This study aimed to explore the potential anti-cancer properties of avermectin B1a using the HCT-116 colon cancer cell line. The MTT assay was used to determine the IC_{50} by exposing cells to increasing doses of avermectin B1a for 24, 48, and 72 hours. Flow cytometry was used to evaluate apoptosis following a 24-hour incubation of cells with the IC_{50} concentration of avermectin B1a. Using a wound healing assay, the migration capacity of HCT-116 cells in the absence or presence of avermectin B1a was also evaluated. Subsequently, tubulin polymerization in the presence of avermectin B1a was evaluated at 340 nm for one hour. Avermectin B1a inhibited the growth of HCT-116 cells with an IC_{50} value of 30 μM. Avermectin B1a was found to promote tubulin polymerization at a concentration of 30 μM. In addition, avermectin B1a also caused HCT-116 cells to undergo apoptosis and significantly reduced their capacity to migrate. These findings demonstrate that avermectin B1a exhibits significant anti-cancer activity and promotes tubulin polymerization, indicating that avermectin B1a can be used as a promising microtubule-targeting agent for the development of future anti-cancer medications.

***Keywords*:** Avermectin B1a, tubulin polymerization, HCT-116 cell line, apoptosis, cell migration

Özet

Avermektin B1a HCT-116 Hücrelerinde Tübülin Polimerizasyonunu Arttırarak Anti-Proliferatif ve Anti-Kanser Özellik Göstermektedir

Hoti, Qëndresa

Doktora, Tıbbi Biyokimya Anabilim Dalı

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Avermektinler, bir toprak mikroorganizması olan *Streptomyces avermitilis* tarafından üretilen bir makrosiklik lakton grubudur. Genellikle haşereleri ve parazit solucanları tedavi etmek için böcek ilacı olarak kullanılırlar. Avermektinlerden biri olan ve Avermektin B1 olarak bilinen abamektin, en az %80 avermektin B1a ve en fazla %20 avermektin B1b içerir. İvermektin gibi avermektin ailesinin bazı üyeleri, kanser hücreleri üzerinde umut verici anti-proliferatif etkiler göstermiştir. Kolon kanseri dünya çapında önemli bir ölüm nedenidir, bu nedenle etkili tedavi için yeni yaklaşımlara ve terapötik moleküllere ihtiyaç vardır. Bu çalışma, HCT-116 kolon kanseri hücre hattını kullanarak avermektin B1a'nın potansiyel anti-kanser özelliklerini araştırmayı amaçlamıştır. MTT yöntemi IC_{50}'yi belirlemek için, hücreleri 24, 48 ve 72 saat boyunca artan dozlarda avermektin B1a'ya maruz bırakarak uygulandı. Avermektin B1a'nın IC_{50} konsantrasyonu ile hücrelerin 24 saatlik inkübasyonunu takiben apoptozu değerlendirmek için akış sitometrisi kullanıldı. Yara iyileşme yöntemi kullanılarak, avermektin B1a'nın yokluğunda veya varlığında HCT-116 hücrelerinin migrasyon kapasitesi de değerlendirildi. Ardından, avermektin B1a varlığında tübülin polimerizasyonu bir saat boyunca 340 nm'de takip edildi. Avermektin B1a'nın anti-proliferatif aktivitesi 30 µM'lık bir IC_{50} değerine sahipti. Avermektin B1a'nın 30 µM'lik bir konsantrasyonda tübülin polimerizasyonunu desteklediği bulundu. Ek olarak avermektin B1a, HCT-116 hücrelerinin apoptoza girmesine neden oldu ve göç kapasitelerini önemli ölçüde azalttı. Bu bulgular, avermektin B1a'nın önemli anti-kanser aktivitesi sergilediğini ve tübülin polimerizasyonunu desteklediğini göstermektedir, bu da avermektin B1a'nın gelecekteki anti-kanser ilaçlarının geliştirilmesi için umut verici bir mikrotübül hedefleme ajanı olarak kullanılabileceğini göstermektedir.

***Anahtar Kelimeler*:** Avermektin B1a, tübülin polimerizasyonu, HCT-116 hücre hattı, apoptoz, hücre migrasyonu

Table of Contents

List of Figures

List of Abbreviations

MTT:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
CaCCs:	Ca2+-activated Cl channels
CSCs:	Cancer stem cells
CC:	Colon cancer
CRC:	Colorectal cancer
dTDP:	Deoxythymidine diphosphate
DMSO:	Dimethylsulphoxide
DMEM:	Dulbecco's Modified Eagle Medium
FBS:	Fetal Bovine Serum
GSH-Px:	Glutathione Peroxidase
GST:	Glutathione-S- transferase
IL:	Interleukin
MLs:	Macrocyclic Lactones
MAPK:	Mitogen-activated Protein Kinase
MDR:	Multidrug Resistance
PS:	Phosphatidylserine
PTMs:	Post-translational Modifications
PI:	Propidium Iodide
ROS:	Reactive Oxygen Species
SOD:	Superoxide Dismutase
TNF-a:	Tumor Necrosis Factor-alpha

CHAPTER I

Introduction

According to data from the United Nations Food and Agriculture Organization, pesticides play a crucial role in preventing biological disasters and crop loss in agriculture, saving more than 30% of global crop production annually. Pesticides are also heavily used, with over 2.4 million tons applied annually worldwide (Yu et al., 2017).

Avermectins are a drug class with multiple therapeutic targets. They are commonly used as pesticides to treat pests and parasitic worms due to their anthelmintic and insecticidal properties (Pitterna et al., 2009). Avermectins are derived from a 16-member lactone ring and are naturally produced by *Streptomyces avermitilis*, a soil actinomycete (Batiha et al., 2020).

Eight distinct avermectins, consisting of four pairs of avermectin homologs (A1a, A2a, B1a, B2a, A1b, A2b, B1b, and B2b), are derived from *S. avermitilis* fermentation. Abamectin, moxidectin, milbemycin oxime, doramectin, selamectin, ivermectin, and eprinomectin are all members of the avermectin family. Among them, abamectin and ivermectin are the most extensively used. Notably, abamectin is the only one approved for both agricultural production and medicinal purposes (Bai & Ogbourne, 2016a).

Abamectin, also known as avermectin B1, is a combination of 80 percent avermectin B1a and 20 percent avermectin B1b. It is frequently used in insecticides (Campbell, 2012). Abamectin is an analog of ivermectin, differing by the presence of an unsaturated double bond at the C22-C23 position, whereas ivermectin has a saturated single bond at the same position (Molinari et al., 2010).

Tumors present a significant threat to human health with their high morbidity and mortality rates, making tumor treatment one of the most challenging endeavors in medicine today. Pathological investigations have revealed that the most critical biological characteristic of a tumor is the uncontrolled proliferation of tumor cells. Consequently, new targets such as the protein tubulin have gained popularity for the

development of anti-tumor therapies, given their pivotal role in cell proliferation (Borisy et al., 2016; la Sala et al., 2019). Tubulin is a small protein molecule found in mitochondria and cell nuclei. It collaborates with microtubule-binding proteins to create microtubules. There are eight distinct varieties of tubulin, each serving a specific purpose (Zhu et al., 2021). To form protofilaments, α- and β- tubulin monomers heterodimerize and align head-to-tail. Microtubules usually consist of 12 or 13 parallel-oriented protofilaments, with tubulin rapidly assembling at one end and gradually assembling or even disassembling at the other (Pellegrini & Budman, 2009).

Tubulin polymerization is a dynamic process influenced by the concentration of tubulin dimers and subunits (Nogales, 2003). The concentration of tubulin dimers in the solution is regulated by the critical concentration. When the dimer concentration exceeds the critical concentration, microtubule polymerization occurs, leading to growth. Conversely, when the dimer concentration falls below the critical concentration, the microtubule undergoes disassembly (Alberts et al., 2002).

Given the rapid proliferation of tumor cells, the predictable rate of tubulin polymerization and depolymerization, and the critical role of tubulins in cell division and mitosis, the research and application of tubulin inhibitors offer crucial concepts and strategies for tumor treatment (Manzoor et al., 2018). Tubulin was discovered over 50 years ago, and since then, tubulin inhibitors have been approved for use in various therapeutic anti-tumor treatments. Among them, direct microtubule system inhibitors stand out as some of the most popular and effective chemotherapy drugs for treating tumors (Aryapour et al., 2017).

Recent research on ivermectin, a member of the avermectin compound family, has revealed a direct interaction with nematode and human tubulin even at micromolar doses (Ashraf et al., 2015). Furthermore, ivermectin inhibited the proliferation of HeLa cells, and this effect was reversible. It is possible that ivermectin achieved this by stabilizing mammalian microtubules, similar to the impact of taxol, a positive control known for stabilizing tubulin polymerization and causing mitotic arrest (Ashraf & Prichard, 2016). Therefore, our primary focus in this work was the ivermectin analog, abamectin.

Purpose of the Study

The purpose of this study is to investigate how avermectin B1a affects colon cancer cell lines and its potential involvement in tubulin polymerization. There is currently no existing literature examining or reporting on the impact of avermectin B1a on colon cancer cells. As a result we conducted experiments, such as the MTT assay, apoptosis analysis, cell migration study and tubulin polymerization assay to evaluate the anti-cancer properties of avermectin B1a. The findings indicate that avermectin B1a has an influence on tubulin polymerization and displays promise in inhibiting the growth of colon cancer cells by inducing apoptosis and reducing cell migration.

Significance of the Study

This study focuses on four key aspects:

1. Determining the half-maximal inhibitory concentration (IC_{50}) of avermectin B1a that inhibits the growth of HCT-116 colon cancer cell lines.
2. Investigating the anti-cancer effects of avermectin B1a on HCT-116 colon cancer cell lines through apoptosis assays.
3. Monitoring tubulin polymerization in the presence of avermectin B1a using a spectrophotometer.
4. Assessing cell migration using a wound healing assay.

Limitations

The primary limitation of this research is the use of a single cancer cell line for experimentation.

CHAPTER II

Literature Review

History of Avermectin Discovery

In the 1970s, researchers at the Kitasato Institute in Japan discovered a previously unknown bacterium, and scientists at Merck in the USA found that this bacterium produced a novel chemical substance effective against a wide range of arthropods and especially the roundworm parasites (Campbell, 2012; Campbell et al., 1984). This bacterium, *Streptomyces avermitilis*, an actinomycete isolated from the soil, produce avermectins naturally as a byproduct of fermentation (Yoon et al., 2004a). These discoveries attracted a lot of attention due to their scientific implications, therapeutic importance, economic, and commercial success. The end result was a crop of scientific discoveries, valuable improvements in human and veterinary health, and corporate initiatives aimed at uncovering and creating new products within the same chemical class—the macrocyclic lactones (Campbell, 2012).

Abamectin (avermectin B1) and ivermectin were the first macrocyclic lactones to be utilized as veterinary insecticides and established as pesticides for agriculture. The other avermectin compounds, such as doramectin, selamectin, milbemycin oxime, moxidectin, and eprinomectin are subsequent macrocyclic lactone insecticides. Each of these later compounds has its own characteristics that distinguish them from one another and determine their therapeutic and commercial importance (Campbell, 2012).

The remarkable discovery of two major natural product molecules, avermectin and artemisinin, and their purification, earned the 2015 Nobel Prize in Physiology and Medicine. Avermectin's commercial use garnered significant attention. The fact that avermectin is a natural product is encouraging and extremely significant, supporting the widely acknowledged reality that a sizable fraction of medications in current clinical practice are derived from natural resources (Efferth et al., 2015).

Avermectins Compound Family

Avermectins are a class of medications that can be used to treat a variety of conditions. Due to their anthelmintic and insecticidal properties, they are commonly used as

pesticides to control pests and parasitic worms (Pitterna et al., 2009). Eight distinct structures were identified and divided into four major groups (A1a, A2a, B1a, and B2a) and four minor groups (A1b, A2b, B1b, and B2b) (Campbell, 2012). Ivermectin, abamectin, doramectin, eprinomectin, moxidectin, and selamectin are examples of avermectins (Campbell, 2012). Their structures are similar to antibacterial macrolides and antifungal macrocyclic polygenes, but their mechanisms of action are different (Batiha et al., 2020). Avermectin is a class of macrocyclic lactones (MLs) that possess acaricidal, nematocidal, and insecticidal activities. Since the 1980s, avermectin has gained significant therapeutic value in both humans and animals (Ashour, 2019).

Biosynthesis of Avermectins

A group of sequenced genes encodes all the necessary enzymes in this pathway, forming the basis for avermectin production. The biosynthesis of avermectin involves four stages. The initial phase is the biogenesis of starting units. In the second phase, the first aglycons are created using polyketide synthases, requiring four distinct proteins. The third stage involves post-polyketide modification, which may include oxidative cyclization, reduction, and/or methylation to produce avermectin aglycons. The final stage in avermectin production is the glycosylation of avermectin aglycons using the active sugar deoxythymidine diphosphate (dTDP)-L-oleandrose (Yoon et al., 2004; Batiha et al., 2020).

Ivermectin

Ivermectin is Abamectin’s cishydrogenated form. Ivermectin is a macrolide antiparasitic drug that is a member of avermectin composed of 20% 22, 23-dihydro avermectin-B1b, and 80% 22, 23-dihydro avermectin-B1a. It was authorized for use in humans by the U. S. Food and Drug Administration (FDA) in 1987 and has been established as the most successful drug among other members of the avermectin family compounds which has been approved for human use (Prichard & Geary, 2019). Ivermectin is a broad-spectrum antiparasitic medication with a unique mechanism of action (King et al., 2020).

Moreover its known efficacy in treating parasites recent studies have highlighted the anti-cancer properties of ivermectin. These findings indicate that it holds potential, for addressing a range of malignant conditions (Juarez et al., 2018).

Abamectin (Avermectin B1)

Abamectin, which is also referred as avermectin B1, belongs to a group of compounds known as the avermectin family. The specific structure of abamectin is consisting 20% of B1b ($C_{47}H_{70}O_{14}$) and the remaining 80% being avermectin B1a ($C_{48}H_{72}O_{14}$) (Nasr et al., 2016). It is a pesticide derived from the soil bacteria *Streptomyces avermitilis*, and it is among the most widely used pesticides for controlling pests in various agronomic, vegetable, and fruit crops. Abamectin degrades quickly under direct sunlight, leading to a short half-life and low usage rate (Yu et al., 2017). Numerous studies have been done to demonstrate how safe abamectin is for usage in agriculture, both for people and the environment (Bai & Ogbourne, 2016a). Additionally, studies have demonstrated that abamectin is easily degraded by soil microorganisms (Lasota & Dybas, 1990). Residues of abamectin on or in crops are extremely low, often less than 0.025 ppm. This means that harvesting or consuming treated crops exposes people to very little of the chemical. Abamectin does not persist or accumulate in the environment. Its bioavailability to non-target organisms is limited due to its instability, poor water solubility, strong soil binding, and its prevention from leaching into groundwater or entering the aquatic system (Lasota & Dybas, 1990).

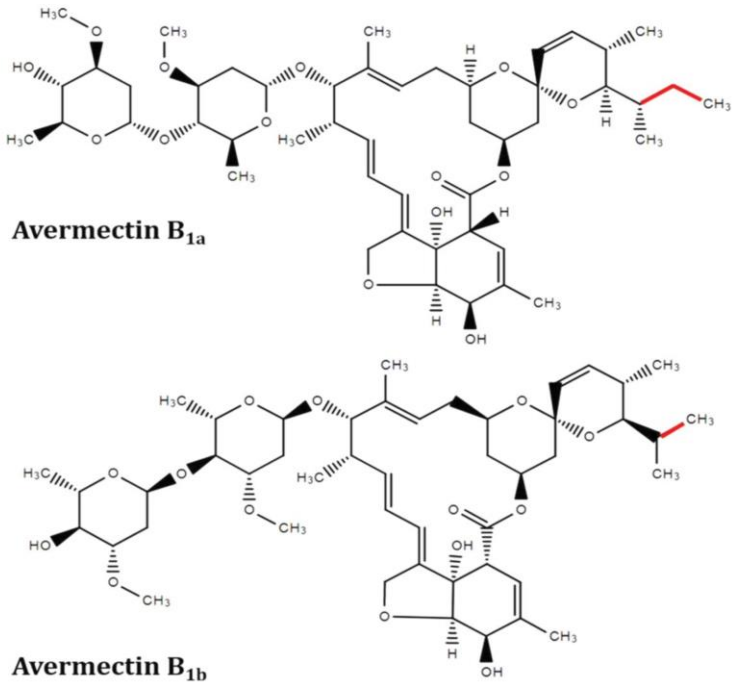
Abamectin is often used alongside bait to attract insects and is employed in the control and regulation of pests such as *Panonychus citri, Plutella xylostella, Cnaphalocrocis medinalis,* and other damaging pests of vegetables and fruits (Nasr et al., 2016). In 1985, Merck launched abamectin for use in cattle in Australia when the Australian regulatory body had not yet authorized ivermectin. Later, it quickly spread to other regions, partly due to its lower production costs. As mentioned earlier, it also gained widespread use as an agricultural pesticide (Campbell, 2012).

Consequently abamectin is composed of a blend of avermectins B1a and B1b. The difference, between B1a and B1b lies in the fact that B1a has an ethyl group connected to a location of disaccharide attachment on one of the ring structures whereas B1b has a

methyl group connected to a location of disaccharide attachment, on one of the ring structures (Teralı et al., 2018) as shown in Figure 1.

FIGURE 1

Abamectin Structure (Teralı et al., 2018).

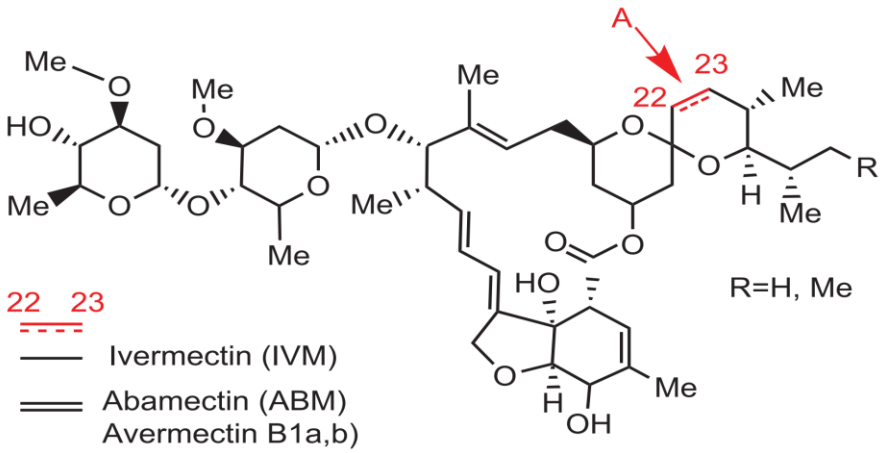


Among the compounds within the avermectin family, the most extensively used ones are abamectin and ivermectin. Abamectin, in particular, is considered for use in both agricultural production and pharmaceutical applications (Bai & Ogbourne, 2016a).

Abamectin and ivermectin have a similar structure. The main distinction lies in the unsaturated double bond, at the C22-C23 position in abamectin. In contrast ivermectin has a saturated bond, at that position (Figure 2) (Molinari et al., 2010).

FIGURE 2

Abamectin and Ivermectin Structure (Asatryan et al., 2014).



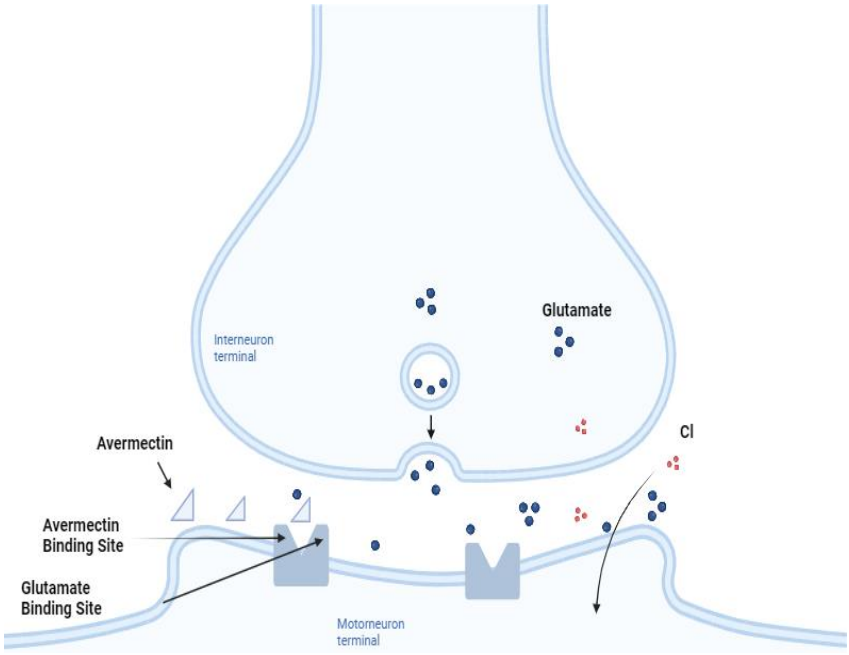
Mechanism of Action of Avermectins

Avermectin primarily affects organisms' neurological system, where it is known to enhance the function of glutamate-gated chloride ion channels and the gamma-aminobutyric acid (GABA) receptor (Zhang et al., 2016). This allows more chloride ions to enter the cells, leading to hyperpolarization and paralysis of the neuromuscular systems in invertebrates. Consequently, since neurons sensitive to GABA are confined to the mammalian central nervous system, it is generally assumed that the toxicity of avermectin is low in mammals (Figure 3) (Subbanna et al., 2020).

The primary mode of action is by disrupting the GABA receptors in the nerve cells of insects. This disruption leads to the inhibition of both nerve-to-nerve and nerve-to-muscle connections, ultimately resulting in the paralysis or death of the insect (Molinari et al., 2010).

FIGURE 3

Avermectin Mechanism of Action. (Created by BioRender).



Avermectins Family Compounds, with Emphasis on Avermectin B1a: Pharmacological Effects and Significance

Safety and Uses of Avermectin B1a in Humans

Avermectins are generally considered safe, but in extreme cases of overdose, they can lead to unconsciousness, hypotension, acidosis, and, in some instances, prove fatal. In humans, avermectin B1a is used to treat onchocerciasis, which is caused by the filarial nematode *Onchocerca volvulus.* Nevertheless, the potential adverse effects on humans are not yet well understood (Aminiahidashti et al., 2014).

Ivermectin, the analog of avermectin B1a, has been widely used for the treatment of various diseases in humans since 1985 (Geary, 2005). It is also employed to prevent the prevalence and spread of many diseases, and when combined with different drugs, its effectiveness has been demonstrated. Currently, ivermectin is used to treat a range of infections, including *leishmaniasis* (Santos et al., 2009), *strongyloidiasis*, *scabies*,

pediculosis, gnathostomiasis, myiasis, and *onchocerciasis* (Batiha et al., 2020). Ivermectin can also be used to reduce the frequency and transmission of various infectious disorders caused by helminthic parasites transmitted through soil. These disorders include *enterobiasis, strongyloidiasis, ascariasis, trichuriasis*, hookworm infections, and infections caused by *Trichuris* and *Ascaris.* These diseases have been identified as leading causes of mortality in underdeveloped children with poor nutrition (Moncayo et al., 2008).

While avermectin B1a is generally considered to have low toxicity, a report by Chung *et al.* mentioned that the majority of poisoning cases involving avermectin B1a resulted from suicide attempts (Chung et al., 1999). In 50% of instance 2, a toxic dosage of 30 mg/kg of mouse body weight was detected. According to a study, the fatal dosage of avermectin B1a is reported to be 10 mg/kg of body weight (Aminiahidashti et al., 2014). Additionally, avermectin B1a exhibits a variety of interaction effects, but it is only toxic when ingested orally. Intoxication symptoms may include mydriasis, vomiting, tremors, seizures, partial ptosis, disorientation, and coma (Soyuncu *et al*., 2007). Symptoms of mild intoxication, such as weakness, nausea, vomiting, and diarrhea have been reported (Chung et al., 1999). In contrast, severe poisoning is characterized by hypotension, unconsciousness, and respiratory failure (Karunatilake et al., 2012). Another study has considered the impact of chronic exposure to this pesticide on male infertility and semen quality (Celik-Ozenci et al., 2012). In conclusion, there is currently no established treatment for pesticide poisoning, and our understanding of its toxicity to humans remains limited.

Avermectin B1a's Antibacterial Effects

According to Campbell, Avermectins seem to have so far been unable to exhibit strong antibacterial or antifungal activity (Campbell, 2012). Avermectins are primarily used as insecticides, nematocides, antihelminthics, and arachnicides. Recent research indicates that avermectin is effective against certain bacterial species, although it does not exhibit broad-spectrum antibacterial activity (Lim et al., 2013). Ivermectin, selamectin, doramectin, and moxidectin are avermectins that have been studied for their potential antibacterial properties, and these avermectins have been shown to be effective *in vitro*

against *Mycobacterium ulcerans*, and *Mycobacterium tuberculosis* with minimum inhibitory concentration values of 1 to 8 mg/L and 4 to 8 mg/L, respectively (Lim et al., 2013; Kirienko et al., 2019).

Recent research indicates that ivermectin has antistreptococcal activity, at doses of 6.25 and 12.5 g/ml against two isolates of *Staphylococcus aureus*, and has an antibacterial effect (Ashraf et al., 2018).

Avermectin B1a's Antiviral Effect

Avermectins have been shown to have antiviral effects in several investigations, thus further focused research was done to gain a deeper understanding. According to research that evaluated 3000 bioactive substances in various cell lines containing Chikungunya virus replicons, the Chikungunya virus that belongs to the alphavirus genus, it was discovered that ivermectin and avermectin B1a had an antiviral impact against these alphaviruses by preventing viral RNA and protein accumulation and affecting virus replication (Varghese et al., 2016).

In one study, 2080 chosen compounds were screened for antiviral properties against three culturable caliciviruses: feline calicivirus, murine norovirus, and porcine sapovirus. Avermectin B1a was found to have wide antiviral activity against all three caliciviruses (Ohba et al., 2016).

Furthermore, a recent study demonstrated that avermectin B1a has antiviral activity against the Japanese encephalitis virus, a significant cause of acute encephalopathy primarily transmitted by mosquitoes and prevalent in Asian and Pacific regions. Despite the absence of authorized medicines for Japanese encephalitis virus infections, avermectin B1a's potent antiviral action was confirmed *in vitro* (Ruan et al., 2021).

Ivermectin is one of the avermectin family members that has shown broad-spectrum antiviral potential against human and animal viruses. Ivermectin's efficiency against parvoviruses in a freshwater crayfish (Cherax quadricarinatus) model was established in the first report on the drug's *in vivo* effectiveness against viruses (Sharun et al., 2020). Moreover, ivermectin, an analog of avermectin B1a, has been reported to have potential antiviral effects by inhibiting the replication of viral proteins through transport blocking.

This mechanism has also been considered for the SARS-CoV-2 virus (Caly et al., 2020). It has shown effects, against various RNA viruses, such as the Zika virus, Venezuelan equine encephalitis virus, influenza A virus, porcine reproductive and respiratory syndrome virus, West Nile virus, Newcastle disease virus, and human immunodeficiency virus (Sharun et al., 2020).

Avermectins as Potential Drug for Treatment of Alcoholism

Avermectins were investigated for their potential role in anti-alcohol therapy, and it was observed that ivermectin lowers alcohol consumption in numerous models that replicate various forms of human alcohol consumption, such as binge drinking, social drinking, and alcohol-induced behavior, at doses between 1.25 and 10 mg/kg (Yardley et al., 2014; Batiha et al., 2020).

Additionally, from the study of the mechanism of avermectins as an anti-alcohol drug, they discover that avermectin B1a improved P2X4R knockout mice potentiation more than ivermectin did at greater dosages and that it acts as a direct agonist in P2X4R stimulatory effects as it increases P2X4R activity in the absence of ATP (Batiha et al., 2020).

Avermectins' Anti-Inflammatory Properties

Avermectin B1a has the ability to treat parasitic infections while also reducing the inflammatory symptoms caused by the parasitic infection. Avermectin B1a has been shown to upregulate IL-10 and downregulate pro-inflammatory cytokines including tumor necrosis factor-alpha (TNF-a) and interleukin (IL)-1b *in vitro*. Additionally, it has been shown to inhibit the nuclear transcription factor kappa-B (NF-κB) and mitogen-activated protein kinase (MAPK) activation pathway (Ci et al., 2009).

The anti-inflammatory effects of avermectin B1a were investigated in acute lung injury, characterized by diffuse inflammatory parenchymal processes, often occurring in the context of multisystem organ failure. *In vivo* studies demonstrated that avermectin B1a effectively reduced lung tissue damage. This protective effect in Japanese encephalitis virus-susceptible mice was attributed to its anti-inflammatory action, with contributions from NF-κB and MAPK inhibition (Zhang et al., 2011).

The Important Role of Avermectin B1a as an Anti-tumor Agent

It has been elucidated that avermectin B1a effectively inhibits the multi-drug resistance of P388 and Hep-2 tumor cells. Hence it has been suggested that avermectin B1a can be used as anti-tumor chemotherapy to eliminate multidrug resistance of tumor cells and to enhance its effectiveness, also it is possible to use avermectin B1a in combination with an anti-tumor agent (Korystov et al., 2004). Other results show that avermectin B1a affects the proliferation of P388 lymph leukemia and murine ascites Ehrlich carcinoma, and 755 carcinoma tumor cells and solid Ehrlich, demonstrating clearly that avermectin B1a is capable of showing antitumor activity in mice (Molinari et al., 2010). In contrast, the same research group previously reported the opposite results (Molinari et al., 2010).

Cancer stem cells (CSCs), a tiny subpopulation of tumor cells with the capacity to self-renew, differentiate, and produce secondary or tertiary tumors, are highly associated with cancer recurrence (Gupta et al., 2009). CSCs are resistant to conventional radiation and chemotherapy (Maccalli & de Maria, 2015). Although most cancer cells are destroyed by current treatments, which effectively reduce tumor mass, CSCs remain and continue producing new tumors that are frequently much more aggressive. Intense research is being done on substances that can destroy CSCs in a targeted manner and alter the microenvironments that sustain these cells (Chen et al., 2013). Although several cell surface indicators and multiple aberrant signaling pathways have been discovered as possible CSC therapeutic targets, no clinically licensed medication that specifically targets CSCs is yet available (Janikova & Skarda, 2012).

As a result of recent high throughput screening of 16,000 chemicals, encompassing collections of natural extracts and commercial resources, among those compounds the avermectin B1a and the salinomycin, etoposide, and nigericin were the only ones to display notable CSC specificity. Moreover, avermectin B1a was discovered to be a selective inhibitor of immortalized human breast epithelial cells ($HMLE^{shEcad}$) that induced an epithelial-mesenchymal transition. Avermectin B1a demonstrated moderate to remarkable selectivity (IC_{50} for $HMLE^{shEcad}$ cells was 10-fold lower than $HMLEsh^{Control}$ cells). Therefore, there is a critical need to find novel, specific chemicals to supplement this restricted collection of anti-CSC agents (Gupta et al., 2009).

Recent studies have reported promising results of ivermectin having anti-cancer effects. The first study reported the use of ivermectin as a possible revert of multidrug resistance (MDR) (Didier & Loor, 1996), and more reports have been published that emphasize the potential of ivermectin in cancer treatment (Kobayashi et al., 2018). The advantages of ivermectin with regards to cancer treatment also include the effective suppression of tumor proliferation and inhibition of tumor metastasis, the promotion of the death of cancer cells at non-toxic doses to normal cells, excellent efficacy against drug-resistant cancer cells, its combination with chemotherapy drugs has a significant desirable effect on cancer (Tang et al., 2021).

Ivermectin has several patterns for the death of tumor cells, however, apoptosis is the primary way of cell death in ivermectin (Nagata, 2018).

Another finding suggests that avermectins have an anti-cancer effect, it has been proven that the natural substance avermectin B1 and four of its structural counterparts inhibit the TMEM16A-encoded CaCCs (Zhang et al., 2020), the TMEM16A which belongs to the transmembrane protein/anoctamin family (TMEM16A-K or ANO1-10) and has been identified as the molecular identifier of endogenous Ca^{2+}-activated Cl channels (CaCCs) (Guo et al., 2021). In numerous forms of human tumors, including head and neck squamous cell carcinomas, esophageal squamous cell cancer, gastrointestinal stromal tumors, colorectal cancer, and esophageal cancer, it has been shown that TMEM16A is up-regulated (Crottès & Jan, 2019).

In addition to inhibiting TMEM16A-encoded CaCCs, the avermectins also prevent the proliferation and migration of LA795 cancer cells *in vitro* and the development of xenograft tumors *in vivo*. The findings show that avermectins have anti-cancer benefits (Zhang et al., 2020).

Cytotoxicity Induced by Avermectin B1a

Several pesticides cause cytotoxicity by inducing autophagy. Avermectin B1a has been reported to have adverse effects on the cells of mammals, and these effects include oxidative stress, apoptosis, and double-strand breaks of DNA (Zhang et al., 2016). The safety of avermectin B1a has been studied recently due to the various reported toxicities to non-target organisms. However, it is noteworthy to know that little is still known about the underlying mechanisms that induce toxicity with avermectin B1a. In a study

by Liang et al., 2019, the cytotoxicity of avermectin B1a in mouse macrophages and its mechanisms were investigated. The study reported oxidative stress, characterized by increased reactive oxygen species (ROS) indicator intensity. Also, they reported DNA damage even at relatively low dosage administration (Liang et al., 2019).

Treatment using avermectin B1a activated ATM/ATR and MAPK signaling pathways, which was demonstrated by an increase in ATR, JNK, and ATM. The inhibition of signaling pathways for ATR/ATM and JNK indicated a partially improved cell viability, which implies the overproduction of ROS induced by avermectin B1a and DNA damage may lead to cytotoxicity in the ATR/ATM and JNK signaling pathways (Liang et al., 2020).

Another study evaluated the cytotoxic effect of avermectin B1a on a non-target organ, the food ingestion primary organ, investigating human gastric cell lines the MGC803, it observed a significant effect of avermectin B1a on those cells. Avermectin B1a influences the accumulation of intracellular ROS and suppresses the expression of PI3K and AKT proteins, enhancing the apoptosis of MGC803 cells. Moreover, it was observed that abamectin induces apoptosis by affecting the mitochondrial apoptotic pathway by upregulating the Bax and Bcl-2 in MGC803 (Zhu et al., 2019).

On the other hand, a study emphasizes that abamectin reduces the activity of antioxidant enzymes such as glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and glutathione-S- transferase (GST), in male rat hepatocytes, moreover damage in the membrane of hepatocytes were observed due to increase the leakage of liver function enzymes (El-Shenawy, 2010). Moreover, it was observed that avermectin B1a has relatively efficient inhibitory activity on the mammalian butyrylcholinesterase enzyme at micromolar level with (IC_{50} = 10.6 µM) (Teralı et al., 2018).

Avermectin B1a is thought to be relatively harmless to humans and other mammals. On the other hand, its longevity in the environment raises possible ecological concerns in various ecosystems. According to several studies, avermectin B1a degrades slowly due to its lipophilicity and water insolubility (Hong et al., 2020). Reports show that 80–98% of abamectin is eliminated in feces (Novelli et al., 2016).

Recent research investigates the lethal toxicity of avermectin B1a on fish species *Schizothorax prenanti;* in the liver, avermectin B1a induced oxidative stress, DNA damage, and apoptosis in this freshwater commercial fish. After a caspase cascade, the toxicity's underlying mechanism is likely connected to ROS production via mitochondrial and death receptor pathways. Hepatic damage is enhanced when antioxidants were inhibited (Hong et al., 2020).

Tubulin Protein

Tubulin is a small globular protein molecule, with eight distinct forms identified, each characterized by unique physiological functions and labeled as α, β, δ, ε, ζ, η, θ, and ι-tubulin. The most well-understood types of tubulin are α-tubulin and β-tubulin. α-tubulin is comprised of 295-519 amino acids, while β-tubulin is composed of 104-495 amino acids, and has a molecular weight of around 55 kDa (Zhu et al., 2021). Heterodimers are formed by the combination of α-tubulin and β-tubulin, and protofilaments are created as multiple heterodimers are bound end-to-end. These protofilaments are responsible for the assembly and structure of microtubules, which are elongated, hollow cylinders made up of tubulin dimers (Kaur et al., 2014; Zhu et al., 2021).

Microtubules

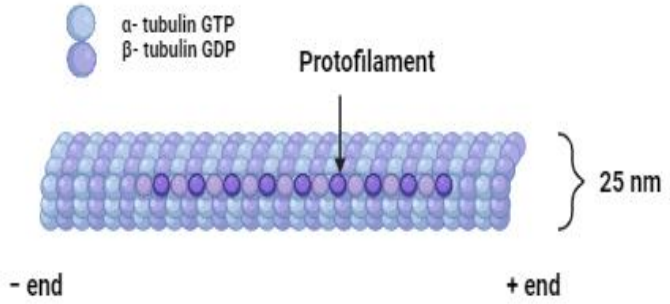
Microtubules are one of the significant parts of a cytoskeleton. They are found in eukaryotic cells and are involved in mitosis, intercellular transport, cell motility, and the overall maintenance of cell shapes. They are the essential elements of the mitotic spindle apparatus, which separates the chromosomes. A microtubule is a tubulin dimer cage involved in the chromosome’s formation of the mitotic spindle during cell division; over the years they have been placed as important targets in anticancer therapy (Sana et al., 2019). If the cell's division machinery is compromised, defective microtubules will result in irregular cell shape and eventually cell death.

Microtubules are hollow and cylindrical structures formed by thirteen laterally associated protofilaments of α/β-tubulin heterodimers that interacting head-to-tail (Figure 4) (Lopes & Maiato, 2020). Tubulin isotypes; α- and β-tubulin proteins are

responsible for the encoding of several genes that diverge in the C-terminal tail concerning the composition of amino acids (Janke, 2014). Tubulin isotypes; α- and β, sometimes undergo post-translational modifications (PTMs) as the isotopes heterodimers undergo polymerization into microtubules. This has been associated with regulating fundamental cellular processes' properties and underlying functions (Janke & Magiera, 2020).

FIGURE 4

Microtubule Structure. (Created by BioRender).



Microtubules play a pivotal role in the mitotic apparatus, responsible for the separation of chromosomes during both metaphase and anaphase of mitosis. Specifically, during metaphase, the plus ends of microtubules undergo elongation and contraction. This dynamic behavior enables them to probe and connect with kinetochores, a process often referred to as 'search and capture' (Kline-Smith & Walczak, 2004). Once chromosomal translocation to the equatorial region is achieved, highly dynamic microtubules become stabilized, facilitating the subsequent separation of chromosomes during anaphase. As microtubules shorten, they exert force on the chromosomes, propelling them towards the poles of the cell (Battaje & Panda, 2017).

Microtubules are versatile cellular structures that play vital roles in multiple cellular processes. During cytokinesis, microtubules are instrumental in midbody development, contributing to the successful completion of cell division. Furthermore, microtubules have the ability to assemble into specialized structures with specific functions. Axonemes, for instance, are highly stable, nine-fold symmetric microtubule structures.

They serve as the structural foundation for essential cellular components such as centrioles, cilia, and flagella. These structures are crucial for various cellular functions, including cell signaling and motility. Microtubules also play a pivotal role in cell differentiation, influencing changes in cell morphology and participating in intracellular rearrangements. In blood platelets, microtubules form the marginal bands, which help maintain the discoid shape of these cells. In neuronal cells, microtubules organize into complex arrays that are indispensable for neuronal polarization, intracellular cargo trafficking, neuronal migration, and differentiation (Prassanawar & Panda, 2019).

Microtubules exhibit remarkable versatility in forming intricate structures with distinct morphological and functional characteristics, tailored to the specific needs of various tissues. Their behavior varies depending on cell types and functions, contributing significantly to cellular dynamics (Huang et al., 2022). During interphase, microtubules, originating from centrosomes, create an extensive network that spans the inner surface of the cell. This network plays a critical role in facilitating intracellular movement, preserving cell polarity, and maintaining cell shape, making it indispensable for cellular homeostasis (Akhmanova & Kapitein, 2022). However, during cell division, microtubules undergo a profound transformation. They become highly dynamic and are involved in the formation of an active mitotic spindle. This reorganization of microtubules from their comparatively stable interphase state is crucial for successful cell division, highlighting the adaptability and functional diversity of microtubules in different cellular contexts (Lacroix & Dumont, 2022).

Microtubules are dynamic polymers composed of tubulin, a soluble heterodimer that is found in the cytoplasm (Steinmetz & Prota, 2018). The dynamic nature of microtubules is crucial for various cellular processes, and their disruption could halt cell division at the spindle checkpoint. Consequently, substances aiming to interfere with microtubule dynamics have the potential to serve as effective treatments for cancer, fungal infections, and parasitic diseases (Battaje & Panda, 2017).

Tubulin Polymerization

The polymerization of tubulin is a process that involves the addition or removal of monomers, depending on the concentration of tubulin dimers comprising α and β

subunits. This concentration of tubulin dimers is regulated within the solution relative to the critical concentration. The critical concentration represents the point at which neither disassembly nor assembly occurs at the microtubule end. When the dimer concentration exceeds the critical point concentration, polymerization takes place within the microtubule, leading to its growth. Conversely, if the concentration falls below the critical concentration, the microtubule will undergo disassembly (Alberts et al., 2002).

The Dynamic Assemble of Microtubules

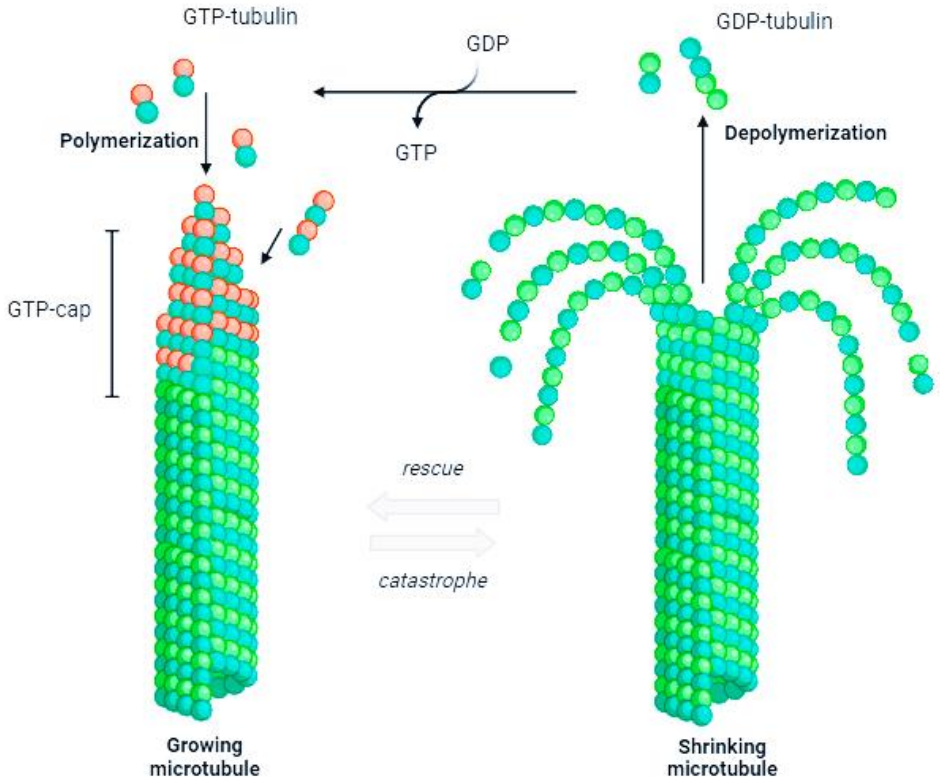
The microtubules are made up of α/β-tubulin heterodimers organized head-to-tail to produce linear 13 protofilaments sheets that connect longitudinally within a 24-nm microtubule. The sheets are pressed together to form a cylindrical tube. In eukaryotic cells, microtubules are in a high-dynamic equilibrium, with one end of the microtubule constantly polymerizing while the other depolymerizes (Battaje & Panda, 2017).

The energetic process of GTP hydrolysis controls the dynamic instability of microtubules. Tubulin-bound GTP is hydrolyzed into tubulin-GDP and inorganic phosphate (Pi) when tubulin is added to the end of the microtubule (Zhu et al., 2021). As further noted by Zhu et al., the microtubule core, consisting of GDP and microtubules, forms once Pi is separated from the microtubules. GTP or GDP-Pi tubulin-binding proteins remain stable for depolymerization at the ends of microtubules, but the release of tubulin-GDP and inorganic phosphate Pi simultaneously causes the tubulin molecules to change conformation, resulting in microtubule polymer formation (Ilan, 2019; Zhu et al., 2021).

The generation of the microtubule polymer results in the formation of an unstable polymer that shortens or damages the microtubules (Battaje & Panda, 2017). Different microtubule-binding proteins have an optimal structure to regulate the dynamic instability of the microtubules because GTP hydrolysis causes changes in the conformation of the microtubule ends (Liu et al., 2021). When GTP is hydrolyzed at the ends, the GTP cap is lost, revealing the GDP-tubulin structure. GDP-tubulin units have a weaker interaction with one another, curving away from the tube and eventually disassembling (Figure 5) (Zhu et al., 2021).

FIGURE 5

Visualizing the Structure and Kinetics of Microtubule Assembly and Disassembly in Cells. (Created by BioRender).



The biological role of dynamic instability has been postulated to involve the recycling of microtubules that have missed their objectives (Erickson & O'Brien, 2003). Although tubulin concentration affects the polymerization reaction, depolymerization tends to happen independently (Housman et al., 2016). Catastrophe is the transition from a lengthening to a shortened state, whereas rescue is the transition from a shortened state to a lengthening one (Walker et al., 1988). The catastrophe has been attributed to the 'aging' of microtubule ends. The ends of microtubules become shaped as they grow, and this modification is independent of GTP hydrolysis. While the proteins that alter microtubule end shapes may contribute to the catastrophe or the shortening (Coombes et al., 2013).

The Interaction of Avermectins with Tubulin

The direct interaction between ivermectin and nematode and human tubulin has been recently reported even at micromolar concentrations. Ivermectin inhibited the growth of HeLa cells and the effect was reversible suggesting that ivermectin stabilized mammalian microtubules. It prevented depolymerization by increasing polymerization, mimicking the effect of taxol, the positive control that stabilizes tubulin polymerization, leading to mitotic arrest (Ashraf & Prichard, 2016). Another finding in *Haemonchus contortus* indicated that ivermectin at micromolar affinity can bind to α and β-tubulin isotype 1, and that competing for the same binding site on tubulin with taxol (Ashraf et al., 2015).

Previous research demonstrated that abamectin significantly reduced the expression of P-glycoprotein, cytoskeletal proteins β-tubulin, and β-actin (Sun et al., 2010). The β-tubulin III is a microtubule protein marker that is primarily produced in neuronal cells and found in cancerous cells resistant to various anti-cancer medications (Banerjee, 2002). These results suggest that this macrocyclic lactone may suppress the expression of P-glycoprotein and cytoskeletal proteins, thus preventing neurite formation (Sun et al., 2010), which is linked to the expression of the cytoskeleton (Bakos et al., 2013). The cytoskeleton provides the framework that defines, supports, and maintains the structure of neurons, and any change in cell morphology may signal changes within the cytoskeleton (Kevenaar & Hoogenraad, 2015).

Microtubules are widely recognized as essential for regulating neurite formation in differentiating cells (Roychowdhury & Rasenick, 2008). The levels of β-actin and β-tubulin III in developing N2a cells were significantly reduced after abamectin administration, indicating that this may be a major factor in the toxicity of avermectins. It is conceivable that the cytoskeleton is the part of the cell most susceptible to damage by avermectins, as the quantities of cytoskeleton proteins, such as β-tubulin III, are reduced in vinblastine-resistant human colon cancer HCT-8R cells after exposure to abamectin. However it is still not entirely clear how variations in β-tubulin levels impact the transmission of signals from avermectin receptors (Sun et al., 2010).

Cancer

Cancer is one of the major causes of death worldwide (Cao et al., 2021). It is characterized by increased cell proliferation, often associated with the down-regulation of pro-apoptotic pathways (Pistritto et al., 2016). In terms of its prevalence, progression, and treatment, cancer remains a significant global health concern (Gupta et al., 2018).

Recent research reveals that in 2021, approximately 19.3 million new cancer cases were diagnosed worldwide (Sung et al., 2021). According to the analysis, in which basal cell carcinoma and nonmelanoma skin cancer will not be included in the estimated 28.4 million new instances of cancer by 2040. Many medical experts' ultimate goal is to find and generate new cancer chemotherapy drugs (Fedorov et al., 2019). In addition, other techniques, either alone or in combination, are required to treat cancer, including surgery, chemotherapy, and radiation therapy (Kumar et al., 2017). Several new target-oriented drugs are being studied, and some have already demonstrated good activity/efficacy, primarily when used in combination with chemotherapy (Labianca et al., 2010).

Potential targets for anticancer therapies encompass a range of factors. These include cancer stem cells, known for their self-renewal abilities within tumorigenic cell populations (Yang et al., 2020); kinases, such as tyrosine kinases and cyclin-dependent kinases (Bharate et al., 2013); monoclonal antibodies (Khiavi et al., 2019); vascular targeting drugs (Ghosh et al., 2020); and the microtubule protein tubulin (Vindya et al., 2015).

Given the multifaceted role of numerous proteins in cancer cell progression, differentiation, and dissemination, any substance capable of interacting with essential autophagy, cell cycle, apoptosis, and necrosis-related proteins and inducing significant structural and functional changes can be considered a potential cancer treatment (Seeger-Nukpezah et al., 2015). Proteins represent a crucial class of biomacromolecules with distinct structures, dynamics, and functions (Zhang et al., 2022). For instance, tubulin, which has recently garnered significant attention, stands out as a promising therapeutic target for cancer (Čermák et al., 2020; Liu et al., 2020). Finding cancer therapies that are both safe and efficient while minimizing toxicity has proven

challenging (Hari et al., 2022). There are several main types of anti-cancer medications, including alkylating and intercalating substances (DNA-targeting therapies), topoisomerase inhibitors, hormone therapies, antimetabolites, signal transduction inhibitors, and mitotic spindle inhibitors. Among these categories, compounds that inhibit microtubule growth are particularly important for cancer treatment (Ebenezer et al., 2022). Research has demonstrated that tubulin-mediated pathways are among the most significant mechanisms promoting cancer cell growth, proliferation, and metastasis (Čermák et al., 2020; Liu et al., 2020). Therefore, in this study, we focused on targeting tubulin in a colon cancer cell line.

Colon Cancer, Epidemiology, and Pathology

Colon cancer (CC) is one of the most common cancers worldwide (Assessment, 2009). It arises from the transformation of the normal colonic epithelium into an adenomatous polyps. Genetic changes play a significant role in its development, and its incidence has been steadily increasing in recent years. The alarming rise in the prevalence of CC can be attributed to recent industrialization and changing lifestyles in urban areas globally (Banerjee et al., 2017). Studies suggest that approximately 30% of colorectal cancer (CRC) cases have a genetic origin (Sopik et al., 2015). In about 75% of CRC cases, the cause remains unclear, while the remaining minority can be attributed to familial occurrences or inflammatory bowel disease. Over 33% of familial cases have a genetic basis (Triantafillidis et al., 2009).

The inability of colonic cells to undergo programmed cell death in response to a lesion is a crucial indicator of their tendency to develop into cancer. Additionally, an imbalance between cell renewal and cell death, with a preference for proliferation, is a known mechanism underlying the development of colorectal cancer. Organ size and colonic crypt structure are maintained through a balance between new and old cells. Tumor growth is influenced by both the rate of apoptosis and the rate of proliferation (Huerta et al., 2006).

Colonic polyps on the mucosa, give rise to colon cancer. One of the most prevalent histologic type is hyperplastic polyps. They have an increased number of glandular cells with decreasing cytoplasmic mucus but no nuclear hyperchromatism, stratification, or

atypia. The other typical histologic type is called adenomatous nuclei, which are typically palisade-shaped, hyperchromatic, larger, and cigar-shaped. Adenomas can be tubular or villous in nature. Villous adenomas have digitiform villi grouped in a frond, whereas tubular adenomas histologically consist of branching tubules. Both components are present in tubulovillous adenomas (Cappell, 2005). The treatment strategy for colorectal cancer is determined by the disease's stage. Colorectal cancer (CRC) can be categorized into five stages (0, I, II, III, and IV) based on the degree of local invasion depth, the development of metastases, and the involvement of lymph nodes. Stage IV is the most advanced stage and is associated with poorest prognosis. Therefore, surgery is recommended for the removal of tumors at stage 0. Patients with stage II and upper invasive cancer that has crossed the basement membrane may require more involved treatments, such as surgery, chemotherapy, or radiation therapy (Gurba et al., 2022). Furthermore, treatment trials have shown that the effectiveness of certain treatments varies depend on patients age and the stage of the patients' cancer. Younger patients tend to have better outcomes compared to older ones. However, it is important to note that standard-of-care treatments for older patients produce long-term results that are comparable to those seen in the younger population (Mishra et al., 2013).

Surgery serves as the primary treatment modality for the majority of colorectal cancer cases (Akgül et al., 2014). While chemotherapy is considered the most effective option for colon cancer, its highly adverse side effects significantly diminish the patient's quality of life, thus limiting its potential for substantial therapeutic benefit (Ji et al., 2018). Consequently, extensive research is underway to explore new target-oriented drugs, some of which have already demonstrated promising efficacy, especially when employed in conjunction with chemotherapy (Labianca et al., 2010). The most formidable challenge in achieving a successful drug-targeting strategy lies in preventing drug absorption and/or degradation in the upper gastrointestinal tract before reaching the colon (Banerjee et al., 2017).

CHAPTER III

Methodology

Materials

Chemicals

- Avermectin B1a (BIA-A1010, BioAustralis, Australia).
- Paclitaxel (TXD01, Cytoskeleton, Denver, CO, USA).
- Colchicine (C9754, Sigma Aldrich, USA).
- Dimethylsulphoxide (DMSO, Carlo Erba, France).
- Dulbecco's Modified Eagle Medium (DMEM-LPXA Capricorn, Germany).
- Fetal Bovine Serum (FBS) (04-127-1A, Biological Industries, Israel).
- Penicillin/Streptomycin (03-031-1B, Biological Industries, Israel).
- L-Glutamine (200 mM), (GLN-B Capricorn, Germany).
- Trypsin/EDTA (03-052-1B, Biological Industries, Israel).
- Dulbecco's PBS (PBS-1A, Capricorn, Germany).
- Trypan blue, 0.4% (15250-061, Thermo Fisher Scientific, USA).

Reagent Kits

- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT (M6494, Thermo Fisher Scientific, USA)
- Annexin V-FITC/PI Apoptosis Kit (E-CK-A211, Elabscience, USA).
- Tubulin polymerization HTS Assay Kit (BK004P, Cytoskeleton, Denver, CO, USA).

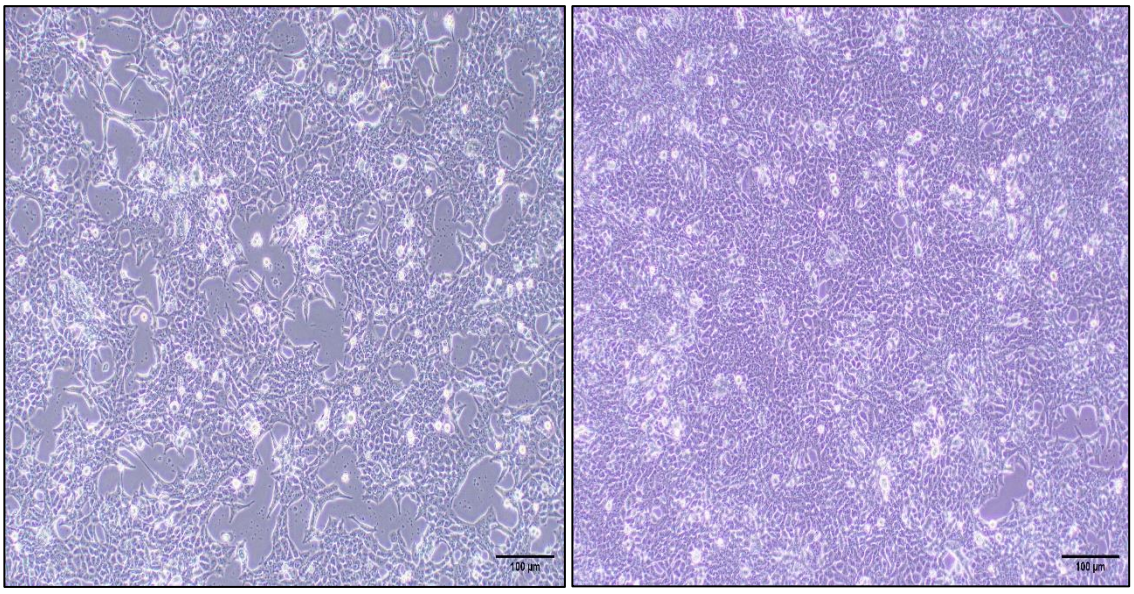
Cell Culture Conditions

In this study, the human colon cancer HCT-116 cell line shown in Figure 6, obtained from the American Cell Culture Collection (CCL-247, ATCC), were utilized. The cells were cultured and maintained in DMEM medium, supplemented with 10% FBS, 1% of 200 mM L-glutamine, and 1% penicillin/streptomycin. They were incubated at 37°C in a humidified air atmosphere containing 5% CO_2. Upon reaching a high level of confluence, routine subculturing was performed. Furthermore, a portion of the cells was cryopreserved using a cell freezing medium and stored at -80°C. Cell passages,

proliferation, and tracking were regularly monitored using an inverted microscope (Olympus IX53, Tokyo, Japan). Before the cell viability test, washing was conducted by adding 2 mL of 1x PBS to 25 cm^2 flasks and 4 ml to 75 cm^2 flasks. After the washing process, the PBS was removed. Trypsin-EDTA was added to the flasks, and the cells were incubated for 5 minutes in the cell culture incubator. Once the cells were detached, the trypsin enzyme was neutralized by adding 4 mL of full DMEM medium to each flask. The cell suspension was collected and transferred to a sterile 15 mL falcon tube. The falcon tube was centrifuged at 1200 rpm for 5 minutes. After centrifugation, the supernatant was removed without damaging the pellet. The remaining pellet was suspended in a fresh growth medium, and cell counting was performed.

FIGURE 6

Images of HCT-116 cells under an inverted microscope are presented, showcasing the cells within a standard culture medium.



Cell Counting

The Neubauer chamber and coverslip were thoroughly cleaned with ethanol, dried, and then the coverslip was placed. Next, 10 µL of collected cells were added to each side of the chamber with a 1:1 dilution ratio of 0.4% trypan blue dye. Cell counting was performed using an inverted microscope (Olympus IX53, Tokyo, Japan) within 8

squares, and the average count was calculated. The following formula was used for cell counting:

$NC \times 10^4 \times DF$ = cells /mL

NC: average number of cells in total large squares;

DF: dilution factor

Cell Viability and MTT Assay

The HCT-116 cells, at a concentration of $5x10^3$ cells/mL in the growth medium, were transferred into three 96-well plates and incubated at 37°C in a 5% CO_2 incubator for 24 hours. After 24 hours of incubation, the cells, observed under inverted microscopy were found to be attached and had achieved more than 80% confluency. Subsequently, the medium was then removed, and the cells were treated with different concentrations of the tested compound. Each application, consisting of 3 µL of the compound in 100 µL of DMEM medium, was repeated four times. Following the application, the plates were incubated at 37°C in a 5% CO_2 incubator.

Avermectin B1a was dissolved in DMSO, and the cells were exposed to varying concentrations of avermectin B1a (2.5, 5, 10, 15, 20, and 30 µM) in the cell culture. Additionally, the first blank comprised the medium without cells,the second blank contained the medium with cells, and the nontreated control cells were treated with DMSO instead of compound of interest. This procedure was applied to three 96-well plates during 24, 48, and 72 hours of incubation. The amount of DMSO in the assay medium was kept constant at a concentration of 0.1%.

The MTT assay, a colorimetric technique widely used for evaluating cell viability and proliferation by measuring metabolic activity, was employed. This assay relies on the ability of NADPH-dependent cellular oxidoreductase enzymes to convert the yellow tetrazolium salt into a purple-colored insoluble formazan crystal. The capacity of death cells to convert MTT into formazan is lost, rendering color formation an indicator of viable cells. The formazan product is deposited as an insoluble precipitate close to the cell surface, inside cells, and in the culture medium. Before absorbance readings were

taken, the appropriate solvent was used to dissolve the formazan (Kuete et al., 2017; Riss et al., 2016).

The MTT assay was utilized to assess the cytotoxicity of avermectin B1a on the HCT-116 cell line after 24 hours of incubation. After the completion of the incubation period, 10 µL of MTT working solution (5 mg/mL in PBS) was added to each well using a multichannel pipette to induce formazan crystal formation. The plate were covered with aluminum foil and incubated for 3 hours at 37°C in a 5% CO_2 incubator. Subsequently, 100 µL of DMSO was added to each well to ensure the dissolution of the formazan salts, and the plates were incubated for 15 min at room temperature. Absorbance was determined at 570 nm using a UV–visible spectrophotometer multi-plate reader (Versa Max, Molecular Device, Sunnyvale, CA). The entire assay was conducted in a dark. The assay was performed at time intervals of 24, 48, and 72 hours across the three different plates. The percentage of cell viability was determined using the following formula:

$$\text{Viable cells \%} = \frac{Absorbances\ treated - Absorbance\ blank}{Absorbance\ untreated - Absorbance\ blank} \bullet 100$$

Apoptosis and Flow Cytometry Analysis

To confirm the expected induction of cell apoptosis by our compound of interest, avermectin B1a, the Annexin V-FITC/PI Apoptosis Kit (E-CK-A211, Elabscience, USA) was employed. The appearance of phosphatidylserine (PS) residues is an early indicator of apoptosis, which are usually concealed within the plasma membrane, on the cell surface. This phenomenon can be utilized for apoptosis identification and qualification since PS translocate from the cytoplasmic face of the plasma membrane to the cell surface during apoptosis. Annexin V, with its robust, Ca^{2+}-dependent affinity for PS, was utilized as a probe for apoptosis identification (Lee et al., 2013).

Sixteen 25 cm^2 flasks were used to seed HCT-116 cells at a concentration of $5x10^5$ cells/mL, distributed among four different experimental groups: Avermectin B1a, DMSO, untreated control unstained (AN-/PI-), and untreated control stained (An+/PI+). These cell populations were incubated for 24 hours at 37 °C in 5% CO_2 environment. On the following day, the medium was removed, and cells in each group were treated with 6 mL of medium with four replicates for each: 30 µM of Avermectin B1a, 30 µM of

DMSO, and two different blank groups. Subsequently, the cells were incubated for another 24 hours at 37°C in a 5% CO_2 incubator.

Apoptosis measurements were conducted at medium speed, with a minimum count of 20,000 cells in the selected area. Readings were obtained in channel FL1-H for Annexin V (FITC) analysis and in channel FL3-H for PI (PerCp/Cy5.5) analysis. The analysis was carried out in triplicate for each cell group.

Initially, debris was excluded from the cell population by setting a gate in the FSC-H/SSC-H graph. Subsequently, a FL1-A/FL3-A density graph containing the cells within the gate was created, and a quadrant was added to the graph. FCS files obtained from the FACSCalibur flow cytometer device were then imported into FCS Express Cytometry 7 software for analysis.

The percentages of cells distributed in the quadrants were then subsequently exported to GraphPad software, where statistical analysis was performed to determine any statistically significant differences between the groups in terms of apoptotic and early-late apoptotic cell populations (%). Based on this data, relevant graphs were generated to visualize and interpret the results.

Performing the apoptosis assay:

The medium was discarded, and washing step was carried out by adding 2 mL of PBS to each flask. Following this, the PBS was removed, and trypsin-EDTA was added into the flasks. The cells were then incubated for 5 minutes in the incubator. After incubation, the cells were detached, and the trypsin enzyme was neutralized by adding 2 mL of DMEM full medium to each flask. The cell suspension from each flask was collected and transferred to separate sterile 15 ml falcon tubes. These tubes were then centrifuged at 1200 rpm for 5 minutes and, after the centrifugation, the supernatant was carefully discarded without disturbing the cell pellet. The remaining pellet was gently suspended with 2 mL of PBS, and cell counting was performed for each tube. HCT-116 cells at a concentration of 5×10^5 cells/mL were divided into tubes, and 2 mL of PBS was added to each tube to suspend the cells. The tubes were then centrifuged at 1250 rpm for 5 minutes. Following centrifugation, the supernatant was removed, and the cells were

gently resuspended in 500 µL of 1X annexin V binding buffer, which had been prepared by diluting the 10× annexin V binding buffer with distilled water.

Subsequently, the cell suspension of each group was separately transferred into FACS tubes and placed in the dark. To each tube, 5 µL of annexin V-FITC and 5 µL of propidium iodide were added, except for the untreated group (AN-/PI-). The cells were gently vortexed and then incubated at room temperature for 15 minutes in the dark. Following incubation, the cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) with excitation at 488 nm and emission at 530 nm.

Tubulin Polymerization Assay

The Tubulin polymerization HTS Assay Kit (BK004P, Cytoskeleton, Denver, CO, USA) was employed. The tubulin protein utilized in the assay had been purified from porcine brain and processed a high purity level of 97%. All components of the kit were reconstituted in accordance with the provided instructions and were appropriately stored at the required temperature to ensure their stability and functionality.

The reconstitution of kit components:

The General Tubulin Buffer was reconstituted by adding 10 mL of distilled water, and the resulting solution was stored at 4°C. The GTP Stock tube was reconstituted by adding 100 µL of ice-cold distilled water, resulting in a 100 mM stock solution. This stock solution was then divided into 10 x 10 µL aliquots and stored at -70 °C for long-term storage. However, during use, it was kept on ice at -20°C to maintain its stability. The paclitaxel stock tube was reconstituted by adding 100 µL of DMSO, leading to the creation of a 2 mM stock solution. Similar to the GTP stock, this solution was also stored at -70 °C for long-term storage and placed on ice at -20°C while being used to ensure its integrity and functionality during the experiment.

The spectrophotometry instrument settings:

The UV-visible spectrophotometer multi-plate reader (Versa Max, Molecular Device, Sunnyvale, CA) was powered on and set to an absorbance wavelength of 340 nm in kinetic mode. The settings were adjusted to enable 120 cycles of readings, with one

reading being captured every 30 seconds, all at a constant temperature of 37°C. Plate shaking was configured to occur once at the initiation of the reaction, lasting for 5 seconds. The 96-well plate was positioned in the spectrophotometer to be warmed at 37°C for 30 minutes before commencing the assay.

The preparation of assay reagents:

Paclitaxel was used as an enhancer control and colchicine was used as an inhibitor control for polymerization assay. The preparation of assay reagents for different controls and avermectin B1a is as follows:

1. Paclitaxel:
    - A dilution of 5 µL from the 2 mM paclitaxel stock solution with 95 µL of room-temperature general tubulin buffer was mixed to achieve a concentration of 10 µM.
    - This solution was kept at room temperature and utilized within 1 hour.
2. Colchicine:
    - 8 µL from the 1.25 mM colchicine stock solution, which was dissolved in DMSO, was diluted in 92 µL of general tubulin buffer to obtain a final concentration of 10 µM.
3. Standard Assay Control:
    - 8 µL DMSO was diluted with 92 µL of room-temperature general tubulin buffer to achieve a final concentration of 10 µM.
4. Avermectin B1a:
    - From the 30 mM stock solution of avermectin B1a, 6.25 µL was diluted in 43.75 µL of DMSO.
    - In addition, 8 µL of the resulting solution was further diluted with 92 µL of room-temperature general tubulin buffer, creating a 10x strength in G-PEM and resulting in a final concentration of 30 µM.
5. G-PEM buffer:1 mL of G-PEM Buffer with 5% glycerol was prepared by adding 80 mM 906.7 µL of general tubulin buffer, which consisted of 80 mM PIPES pH 6.9, 2 mM $MgCl_2$, and 0.5 mM EGTA. To attain a final concentration of 1 mM GTP, 10 µL of GTP stock (100 mM) was added. Subsequently, the mixture was supplemented with 83.3 µL of tubulin glycerol buffer (5% glycerol).

After the combination of these components, the G-PEM buffer with 5% glycerol was prepared. Subsequently, it was then kept on ice and used within 1 hour to maintain its stability.

6. Tubulin: The final vial prepared prior to the addition of components to the buffer was the 4 mg tubulin vial. To reconstitute the tubulin, 1 mL of ice-cold G-PEM buffer with 5% glycerol was added into the vial, yielding a final protein concentration of 4 mg/mL. The reconstituted tubulin was promptly placed on ice and used for the experiment.

Tubulin Polymerization Assay:

For the blank wells, 10 µL of general tubulin buffer was added in the wells of the pre-warmed plate. Similarly, for the standard assay control 10 µL of general tubulin buffer was added to the corresponding wells to facilitate standard assay control polymerization. 10 µL of each of the prepared assay reagents (paclitaxel, colchicine, avermectin B1a, and DMSO) were pipetted into their respective wells. These reagents were assigned to represent the enhancer control polymerization, inhibitor control polymerization, the tested compound, and the control, respectively. The final concentration of each paclitaxel, colchicine, and DMSO was maintained at 10 µM. The plate was then incubated for 2 minutes at 37°C. Following the incubation period, 100 µL of tubulin at a concentration of 4 mg/mL in G-PEM with 5% glycerol was pipetted into the necessary wells. The blank well was contained G-PEM with 5% glycerol, devoid of tubulin. Immediately after tubulin addition, the plate was promptly inserted into the UV-visible spectrophotometer multiplate reader (VersaMax, Molecular Devices, Sunnyvale, CA) set at 37°C. The kinetic settings were configured to initiate the recording of the reaction at 340 nm absorbance.

To ensure the reproducibility and accuracy of the results, the assay for all compounds was conducted in duplicate.

Wound Healing Assay

HCT-116 cells were seeded in 6-well plates at a density of $6x10^4$ cells/mL in DMEM medium, supplemented with 10% FBS, 1% of 200 mM L-glutamine, and 1% penicillin/streptomycin. Subsequently, the plates were incubated at 37°C in a 5% CO_2

incubator until the cells reached over 80% confluence. The cell culture medium was carefully withdrawn from the wells, scratch were introduced onto the cell monolayer using 10 μL pipette tips. Following the scratching procedure, detached cells were washed with 1 mL of PBS, and the initiation of each scratch was designated as the zero time point for the experiment. The wells were treated in quadruplicates with the following conditions:

Experimental group was treated with 30 μM avermectin B1a, negative control was treated with DMSO, and the blank cells were treated with DMEM. After treatment, the cells were incubated for 24 hours at 37°C in a 5% CO_2 incubator.

Following the 24-hour incubation, the medium was withdrawn, and the cells were washed with PBS. Photographs of the identical scratched areas were captured using an inverted microscope (Olympus IX53, Tokyo, Japan) equipped with a 10X objective. ImageJ software was employed for measuring the scratch area, wound coverage and wound width standard deviation. These measurements played a crucial role in quantifying the effects of the treatments on cell migration and wound healing. The wound closure percentage was calculated using the following formula:

$$\text{Wound Closure \%} = \left(\frac{Wound\ area\ at\ 0h - Wound\ area\ at\ 24h}{Wound\ area\ at\ 0h}\right) \times 100$$

Statistical Analysis

The statistical analysis of the experimental data was conducted using GraphPad Prism version 9 software. To evaluate significant differences in cell viability and assess the apoptotic rate, the One-Way ANOVA Kruskal-Wallis test was applied. For the wound healing assay, the Mann-Whitney test was utilized Statistical significance was considered at p-values less than 0.05.

CHAPTER IV

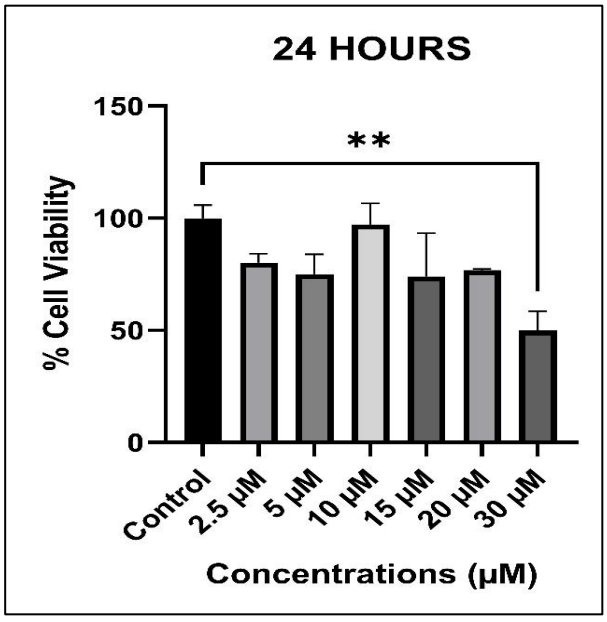
Findings and Discussion

The anti-proliferative effect of Avermectin B1a on the HCT-116 cell viability

The anti-proliferative effect of avermectin B1a on HCT-116 cell viability was determined in a dose and time-dependent manner. The effect of avermectin B1a on HCT-116 cell viability is illustrated in Figure 7. Based on the data derived from the MTT assay, the treatment with 30 µM avermectin B1a for 24 hours resulted in a notable inhibition of HCT-116 cell viability, amounting to 50.1% ($p<0.01$). This concentration was deemed as the IC_{50} value for the study.

FIGURE 7

*The impact of different concentrations of avermectin B1a on the percentage of HCT-116 cell viability after a 24-hour incubation is depicted, with comparison to the control group. Values are mean ± standard error (SE) of three replications (p < 0.01**).*



Apoptosis Analysis with Flow Cytometry

The Annexin V-FITC/propidium iodide (PI) assay was used to determine the effect of avermectin B1a on cell apoptosis. According to the data obtained from the cell viability

assay, 30 μM avermectin B1a was applied to HCT-116 cells. Table 1 displays the groups that were applied. Treated cells were incubated for 24 hours at 37°C in a 5% CO_2 incubator.

Table 1

Treatment groups used for apoptosis assay

Group	Preparation
Control 1	HCT-116 cells with 7 ml DMEM, untreated unstained (AN-/PI-).
Control 2	HCT-116 cells with 7 ml DMEM, untreated stained (AN+/PI+).
DMSO	7 μM DMSO mixed with 7 ml DMEM, added to the cells.
Avermectin B1a	7 μM avermectin B1a dissolved in 7 ml DMEM, for final concentration 30 μM, added to the cells.

After the incubation, annexin V-FITC and PI were applied to each group for staining. By this technique, cells were categorized into distinct groups as living cell population (annexin V-/PI-), early apoptotic cell population (annexin V+/PI-), late apoptotic cell population (annexin +/PI+), and necrotic cell population (annexin V+/PI+). The apoptotic cell population was considered to be composed of both the early and the late apoptotic cells. Figure 8 displays an example of the apoptosis graphs obtained from each application and control group as a result of apoptosis assay.

The total percentage of apoptotic, necrotic, and viable cells was determined for each group. The average percentage of apoptotic, necrotic and viable cells were calculated. Using the percentage cell population data distributed in the quadrants in the apoptosis graphs, the statistical analysis was used to evaluate whether there was a significant difference in total apoptotic and early-late apoptotic cell populations in the cells treated with avermectin B1a compared to the control group (Figure 8).

As a result of the analysis it was found that 19.13% of cells were apoptotic, 2.36% were necrotic, and 78.51% were viable in the DMSO-treated group. However, no statistically significant differences were observed between the control group and the DMSO-treated group in terms of cell populations of interest ($p>0.05$). However, it is found that 39.83% cells were apoptotic, 4.5% of them were necrotic and 57.99% of the cells were viable in the cell population treated with 30 µM avermectin B1a (Figure 9). It was demonstrated that there was a statistically significant difference between control group and avermectin B1a treated group in terms of apoptotic and necrotic cell ratios.

FIGURE 8

Dot plots of apoptosis graphs obtained by flow cytometry. The cells in the upper left quadrant (Q1) were considered as necrotic, those in upper right quadrant (Q2) were considered as late apoptotic cells. Cells in lower left quadrant (Q3) were considered viable, and the cells in the lower right quadrant (Q4) were considered early apoptotic.

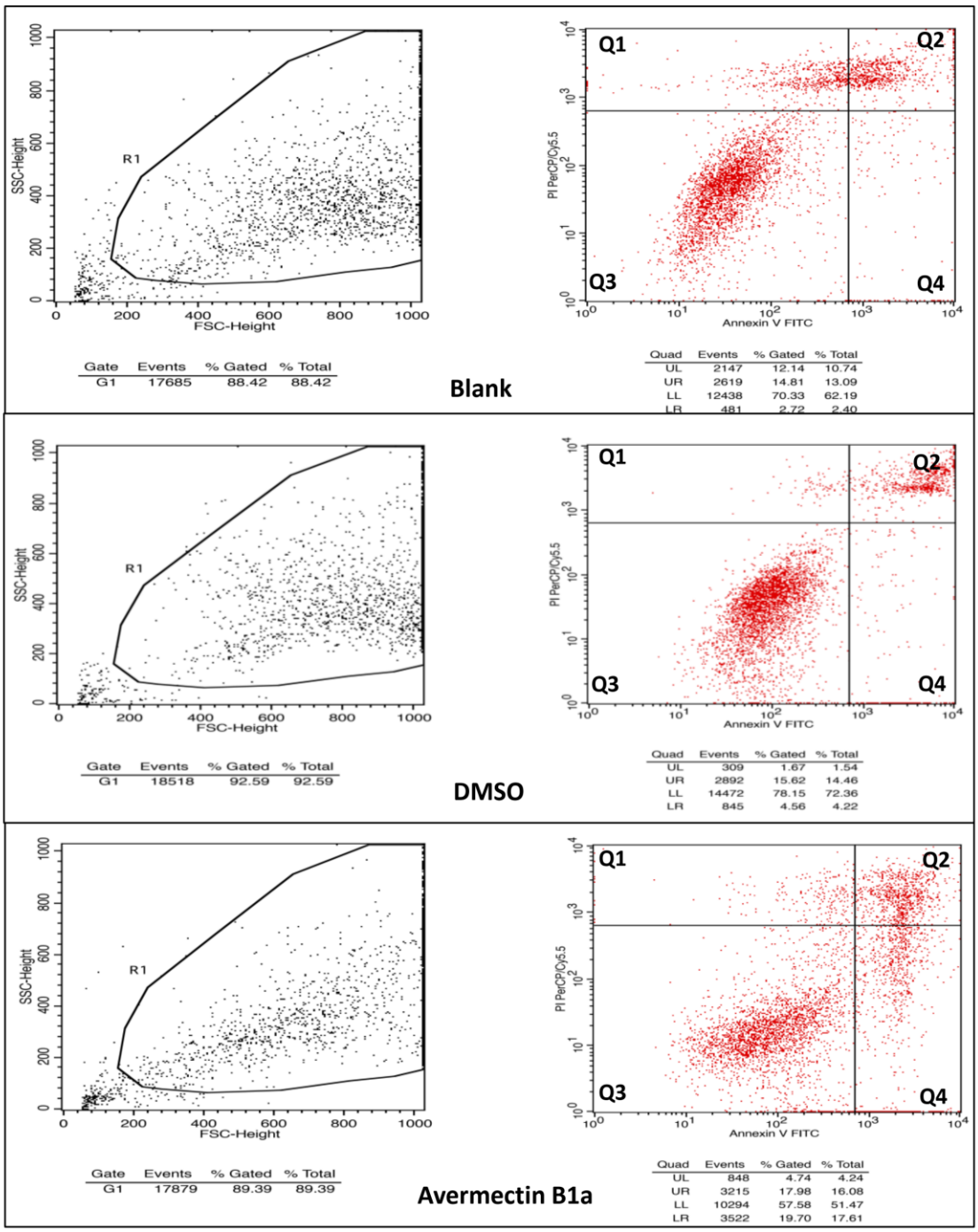
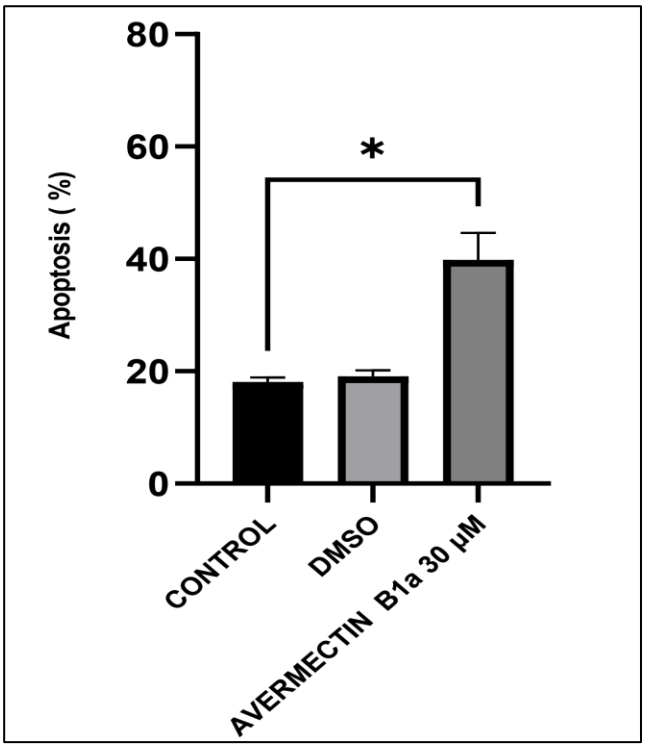


FIGURE 9

Bar graph comparing the percentage of apoptotic cells in the total cell population in the cell groups treated with avermectin B1a treatment compared to the control and DMSO group. ($p \leq 0.0225$). $p < 0.05$* was considered statistically significant compared to the control.*



Tubulin Polymerization Assay Results

The interactions between tubulins and other proteins affect microtubule dynamics. This was prompted by a previous study showing that ivermectin, a molecule analogous to avermectin B1a, stabilized mammalian microtubules by promoting polymerization, making it a recognized tubulin polymerization stabilizer (Ashraf & Prichard, 2016). Here we investigated the possibility that avermectin B1a could influence microtubule dynamics. To assess the development of polymerized microtubules over time in the *in vitro* tubulin polymerization experiment, we utilized optical density, which is a standard method for monitoring this process (Gaskin, 2011).

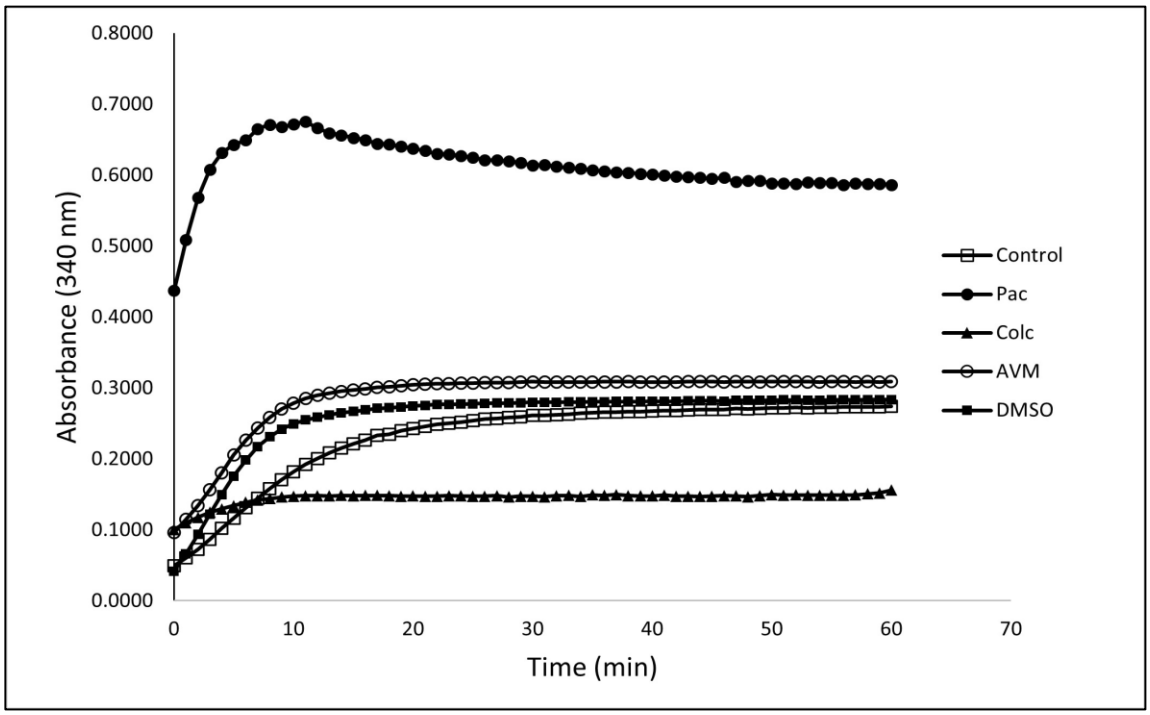
To investigate the effect of avermectin B1a on tubulin polymerization, we used tubulin with a purity of over 97%. The tubulin samples were treated with both avermectin B1a

and control compounds and then incubated for 1 hour at 37°C. During this incubation period, the proportion of polymerized microtubule components was monitored by measuring the absorbance at 340 nm at regular intervals of 30 seconds for one hour, which elevated as the polymerization occurs. We used absorbance increase to assess the stabilizing activity since the experiment was being kinetically monitored. The findings demonstrate that all of the study's groups displayed varying degrees of stimulating activity. This allowed us to track the changes in tubulin polymerization triggered by avermectin B1a and compare them with the control compounds.

We observed at a concentration of 30 µM, avermectin B1a exhibited a significant impact on microtubule polymerization, increasing the polymerization of mammalian tubulin (Figure 10). This alteration in tubulin polymerization dynamics could potentially lead to cell death. Additionally, paclitaxel, a well-known microtubule stabilizer, was used as a positive control to enhance protofilament assembly. Similar to paclitaxel, avermectin B1a also demonstrated the ability to stabilize tubulin assembly and potentially stimulate protofilament assembly. This suggests that avermectin B1a has the potential to function as a microtubule stabilizer. Among the tested substances, paclitaxel exhibited the highest stimulating activity in this assay, while colchicine showed the lowest level of activity.

FIGURE 10

Effects of avermectin B1a on tubulin polymerization. Tubulin polymerization was monitored in the presence of 30 µM of avermectin B1a, 10 µM of paclitaxel (as a positive polymerization control), 10 µM of colchicine (as a negative polymerization control), and DMSO. The observed increase in absorbance indicated the progressive polymerization of tubulin, which was promoted by avermectin B1a. The polymerization was measured at 340nm in kinetic mode.



Wound Healing Assay Results

The interference with cell migration through the targeting of microtubules has emerged as a promising approach in cancer research. By disrupting microtubule dynamics, these tubulin-targeting compounds can impair the formation of cellular structures essential for cell movement and invasion. In particular, the trailing region of migrating cells, where highly dynamic microtubules are concentrated, plays a crucial role in driving cell motility and invasion into surrounding tissues (Ganguly et al., 2012). The ability of avermectin B1a to reduce cell migration by modulating microtubule dynamics in this region holds significant potential for the development of novel anti-cancer therapies.

$6x10^4$ cells were seeded into 6 well plates and incubated. At the end of the incubation period, scratches were generated and photographed. The cells were subsequently treated with 30 µM avermectin B1a and incubated. DMSO treated and non-treated cells were used as controls. The scratched areas were re-photographed and considered as a 24 hour time point. Photographs of wound areas on 0 and 24 hour time points are represented in Figure 11. The experiments were performed quadruplicate and ImageJ software was used to calculate the migration of cells to wound areas. 432 pixels were counted in 1mm in ImageJ software to quantify the pictures. The scratched HCT-116 cell monolayer provided an experimental model mimicking wound healing, where cells migrate to close the gap created by the scratch. The treated cells were compared with the control groups created for them in terms of wound closure and GraphPad Prism version 9 software was used to create the migration assay graph. The data obtained as a result of the statistical analysis are shown in Figure 12. As shown in Figure 12, after 24 hours of incubation, the cell migration of HCT-116 cells was inhibited in non-treated, DMSO treated and 30 µM of avermectin B1a treated group by 1.92 %, 9.59 % and 84.39 % respectively ($p \leq 0.05$ *). As a result of the analysis, the considerable reduction in cell migration observed following treatment with 30 µM of avermectin B1a. This finding highlights the compound's ability to inhibit the migration capacity of cancer cells.

The results from this study not only underscore the potential of avermectin B1a as an anti-cancer agent but also suggest a possible role for microtubule stabilization in controlling cell motility. By stabilizing microtubules in the trailing region, avermectin B1a appears to limit the dynamic remodeling required for efficient cell movement.

Moreover, the discovery of compounds that specifically target microtubule dynamics in migrating cells could have broader implications beyond cancer treatment. Cell migration is a fundamental process in various physiological and pathological contexts, such as tissue repair, embryonic development, and immune response (Gagliardi et al., 2015). Therefore, elucidating the role of avermectin B1a in inhibiting the cell migration could offer valuable insights into the broader biology of microtubules and their influence on cellular behavior.

FIGURE 11

Representative images of scratches in HCT-116 cells, illustrating the migration capacity following treatment with avermectin B1a, controls treated with DMSO, and non-treated cells.

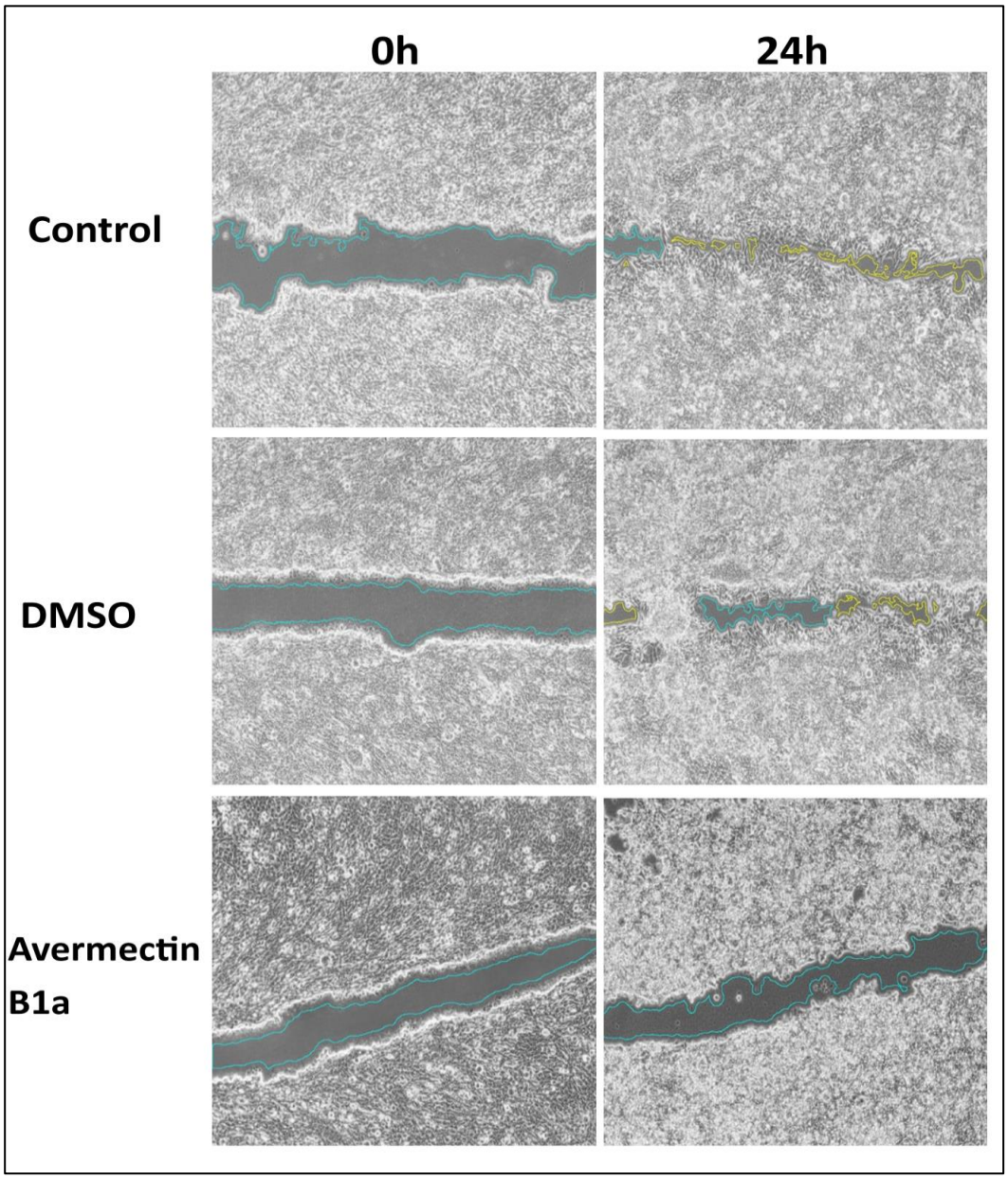
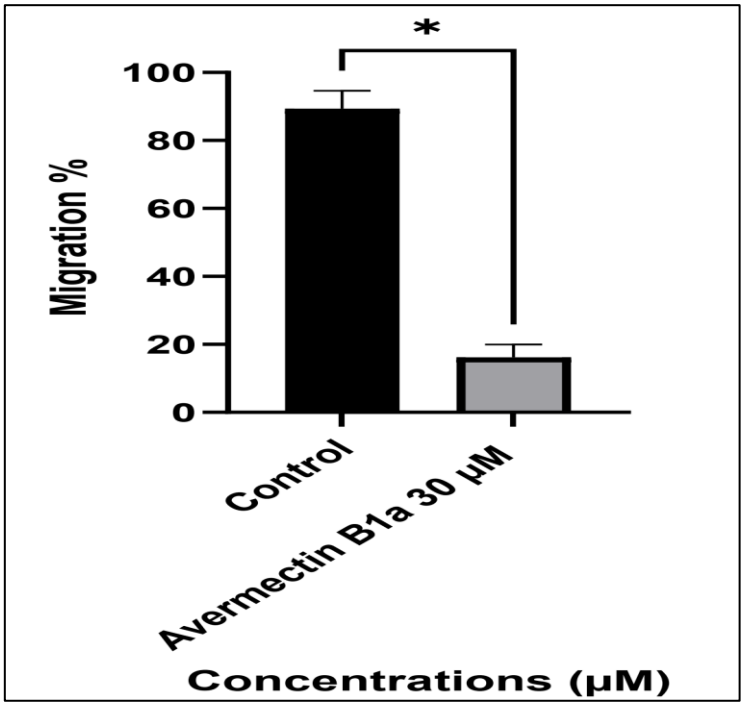


Figure 12

The percentage of cell migration inhibition following a 24-hour treatment period is shown in the summary bar graph. The values represent the means ± standard error (SE) of four replications ($p \leq 0.05$).*



CHAPTER V

Discussion

Pesticides are chemicals that can be biological or chemical compounds, and they are used in agriculture and veterinary medicine to protect crops from insects and other animals that could damage them, as well as to stop the transmission of diseases that insects carry (Taha & Mohammed, 2021).

This study aimed to explore a compound featuring a macrocyclic lactone structure with the potential to be developed into an anti-cancer drug derived from natural small molecules. Avermectin B1a, a frequently employed chemical within the avermectin family, has demonstrated efficacy in both agricultural and medicinal applications (Bai & Ogbourne, 2016b). Prior studies have indicated that compounds from the avermectin family exhibit notable anti-cancer properties and are more adept at inhibiting the development of tumor cells (Batiha et al., 2020; Dominguez-Gomez et al., 2018; Dou et al., 2016).

Microtubules, composed of dynamic subunits (α-tubulin and β-tubulin), play a vital role in cellular functions such as movement, cell shape maintenance, intracellular trafficking, and mitosis through their continuous process of polymerization and depolymerization (Chen et al., 2020). Due to the well-established and clinically validated status of tubulin as a target, and the widespread use of tubulin-targeted chemotherapeutic drugs in clinical settings, microtubules have emerged as an appealing target for the development of highly effective anti-cancer medications (Chen et al., 2017). Natural products that interact with microtubules play a significant role as sources of microtubule-binding agents in cancer therapy. The identification of potent inhibitors targeting tubulin and their structures constitute essential aspects of drug development (Chen et al., 2020). Hence, in this research, we aimed to explore the anti-cancer effect of avermectin B1a.

In this study, we assessed the impact of avermectin B1a on cell proliferation and observed that it effectively inhibited the growth of HCT-116 colon cancer cells, with an IC_{50} value in the micromolar range, which was 30 μM. From our finding indicate that

avermectin B1a holds promise as a potential agent for anti-proliferative therapy in cancer.

The destabilization of microtubule polymerization either by promotion or inhibition of polymerization may result in cell apoptosis and mitosis blockage (Zdioruk et al., 2020). In addition, apoptosis has been widely acknowledged as a unique pathological mechanism in colon cancer, and agents that stimulate apoptosis have been utilized in the treatment of colon cancer (Zhou et al., 2017). According to our findings, apoptosis induction was shown to have happened. The percentage of cells that induced apoptosis was determined to be 39.83%, 24 hours after treatment with 30 µM avermectin B1a, compared to 18.07% in the control group (Figure 9). Furthermore, the apoptotic rate generated by avermectin B1a was found to be much higher than the rate caused by the DMSO, which was 19.13%, as indicated by the ($p \leq 0.0225$*) notation. Our findings are consistent with those of a prior investigation that found ivermectin causes apoptosis in HeLa cells through the intrinsic pathway, which is mediated by mitochondria. Ivermectin also enhanced the expression of the apoptosis protein Bax while suppressing the expression of the apoptosis protein Bcl-2. Additionally, it cleaved PARP, which ultimately caused apoptosis, and activated the downstream caspase-3/-9 that induces apoptosis (Zhang et al., 2019). Ivermectin's ability to cause colorectal cancer cells to undergo apoptosis is further supported by a different study that found that the drug's effects on apoptosis were dose-dependent. Additionally, the same study revealed that the ivermectin increased the activity of caspase-3/-9 (Zhou et al., 2021).

According to a different study, ivermectin caused esophageal squamous cell carcinoma cells to undergo apoptosis, which was linked to the mitochondrial pathway, furthermore, ivermectin-induced apoptosis is activated by ROS produced from mitochondrial dysfunction via the NF-κB signaling pathway (Xu et al., 2021).

Microtubule dynamics are impacted by the interactions between tubulins and other proteins. This was brought by a prior study that showed ivermectin, that is similar to avermectin B1a, was recognized as tubulin polymerization stabilizer because it promoted polymerization and stabilized mammalian microtubules (Ashraf & Prichard, 2016). In this study, we looked into the potential impact of avermectin B1a on

microtubule dynamics. Moreover, we observed at a concentration of 30 µM, avermectin B1a exhibited a significant impact on microtubule polymerization, increasing the polymerization of mammalian tubulin. This alteration in tubulin polymerization dynamics could potentially lead to cell death. Additionally, similar to paclitaxel, avermectin B1a also demonstrated the ability to stabilize tubulin assembly and potentially stimulate protofilament assembly. This suggests that avermectin B1a has the potential to function as a microtubule stabilizer.

Studies demonstrating the active role of microtubules in cancer cell migration are limited. Chemicals like taxol that stabilize microtubules generally prevent cells from migrating, although different types of cells respond differently to drugs that disturb microtubules, such colchicine and nocodazole. In the near term, the cellular response to drugs that disrupt microtubules is to promote cell migration in certain cells while decreasing it in others. Unless the microtubules are dynamically regulated or released from the microtubules organising centres, the microtubules that radiate from the microtubules organising centres to the cellular peripheries should theoretically offer great resistance to changes in cell shape and to migration (Du et al., 2020).

Cell migration plays a critical role in the mortality and morbidity of individuals with colon cancer, as it drives cell invasion and the progression of metastasis (Luan et al., 2021). From the wound healing assay, our findings demonstrate that after 24 hours of incubation, the cell migration of HCT-116 cells in the control group was 98.08%, and 90.41% for the DMSO, whereas it was reduced to 15.61% following treatment with 30 µM of avermectin B1a ($p \leq 0.05$ *). The results from this study not only underscore the potential of avermectin B1a as an anti-cancer agent but also suggest a possible role for microtubule stabilization in controlling cell motility. By stabilizing microtubules in the trailing region, avermectin B1a appears to limit the dynamic remodeling required for efficient cell movement. This finding opens up new avenues for further investigation into the molecular mechanisms by which avermectin B1a exerts its anti-migratory effects.

CHAPTER VI

Conclusion and Recommendation

Conclusion

In this study, we investigated the anti-cancer activity of avermectin B1a, a naturally occurring compound belonging to the avermectin family, on the human colon cancer cell line HCT-116. The cell viability assay revealed that avermectin B1a exhibited the highest anti-proliferative activity against the HCT-116 cancer line, with IC_{50} value of 30 μM. We evaluated the effectiveness of avermectin B1a on colon cancer cells as a potential candidate for microtubule-targeting agent in cancer therapy.

The investigations into the anti-cancer mechanism suggested that avermectin B1a could potentially promote the protofilament assembly of tubulin, leading to an increase in the tubulin polymerization rate by mimicking the effect of paclitaxel, as observed in the tubulin polymerization assay. Additionally, avermectin B1a demonstrated the ability to reduce the cancer cell migration rate and induce apoptosis in HCT-116 cells. Based on these findings, this naturally derived compound shows promise as a potential microtubule-stabilizer lead structure for future anti-cancer drug discovery.

In conclusion, avermectin B1a, which interacts with tubulin, displays anti-proliferative and anti-migration properties and triggers apoptosis in colon cancer cells. This study provides compelling evidence of the potential of avermectin B1a as an anti-cancer agent due to its ability to suppress cell migration through microtubule stabilization. The findings contribute significantly to our understanding of how microtubule-targeting compounds can influence cancer cell behavior and offer promising prospects for developing new microtubule-stabilizing agents for cancer therapy and beyond. Further research in this direction may lead to the discovery of innovative strategies to combat cancer and other diseases that involve aberrant cell migration.

Recommendation

In addition to the findings presented in this study, it is highly recommended that future research explores the anti-cancer activity of avermectin B1a using different cancer cell lines, such as brain and breast cancer cell lines. Investigating its effects on a broader

range of cancer types would provide valuable insights into its potential as a versatile and effective therapeutic agent.

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Appendices

Appendix A

Curriculum Vitae

1. PERSONAL INFORMATION

NAME, SURNAME:	Qëndresa Hoti
DATE of BIRTH and PLACE:	31.05.1991, Hun/Libya
Nationality:	Kosovar
ELEPHONE: +38349346383 E-MAIL: qendresahoti1@gmail.com	

2. EDUCATION

YEAR	GRADE	UNIVERSITY	DEPARTMENT
2018-now	Postgraduate	Near East University	Medical Biochemistry
2015-2017	Masters	Near East University	Medical Biochemistry
2009-2013	Undergraduate	University of Sirte	Medical Technology of Laboratory

3. EXPERIENCE

PERIOD	TITLE	INSTITUTION
Currently	Assistant lecturer	Alma Mater Europaea Campus College "Rezonanca"

4. LANGUAGES

LANGUAGE	READING	SPEAKING	WRITING

Albanian	Excellent	Excellent	Excellent
English	Excellent	Excellent	Excellent
Arabic	Excellent	Excellent	Excellent
Turkish	Good	Good	Good

5. CONFERENCES AND SYMPOSIUM

Poster Presentation (PB1258/T)

"Altered expression levels of TAS1R1, and TAS1R3 genes among SARS-CoV-2 variants"

American Society of Human Genetics Annual Meeting in Los Angeles, California, October 2022.

Poster Presentation (P-08.2-59)

"Avermectin B1a Exhibits Antiproliferative and Anticancer Activity in HCT-116 Cells by Enhancing Tubulin Polymerization"

47th- FEBS, The Federation of European Biochemical Societies, Tours, France, July 2023.

6. PUBLICATIONS

Peer-reviewed articles

Teralı, K., Dalmizrak, O., Hoti, Q., & Ozer, N. (2018). Evaluation of the inhibitory effect of abamectin on mammalian butyrylcholinesterase: Enzyme kinetic and molecular docking studies. Journal of Environmental Science and Health, Part B, 53(11), 713-718.

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Hoti Q, Rustem DG, Dalmizrak O. Avermectin B1a Shows Potential Anti-Proliferative and Anticancer Effects in HCT-116 Cells via Enhancing the Stability of Microtubules. *Current Issues in Molecular Biology*. 2023; 45(8):6272-6282. https://doi.org/10.3390/cimb45080395

Book Chapters

Tuncel, G., Hoti, Q., Mocan, G., & Ergoren, M. C. (2022). A review of the Mediterranean diet and nutritional genomics in relation to cancer in women. Journal of Preventive Medicine and Hygiene, 63(2 Suppl 3), E81.

Appendix B

Turnitin Similarity Report

Turnitin Orijinallik Raporu

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Qendresa Hoti, Duygu Gencalp Rustem, Ozlem Dalmizrak. "Avermectin B1a Shows Potential Anti-Proliferative and Anticancer Effects in HCT-116 Cells via Enhancing the Stability of Microtubules", Current Issues in Molecular Biology, 2023

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Gaber El-Saber Batiha, Ali Alqahtani, Omotayo B. Ilesanmi, Abdullah A. Saati et al. "Avermectin Derivatives, Pharmacokinetics, Therapeutic and Toxic Dosages, Mechanism of Action, and Their Biological Effects", Pharmaceuticals

1% match (Ying-Jian Sun, Ding-Xin Long, Wei Li, Wei-Yuan Hou, Yi-Jun Wu, Jian-Zhong Shen. "Effects of avermectins on neurite outgrowth in differentiating mouse neuroblastoma N2a cells", Toxicology Letters, 2010)
Ying-Jian Sun, Ding-Xin Long, Wei Li, Wei-Yuan Hou, Yi-Jun Wu, Jian-Zhong Shen. "Effects of avermectins on neurite outgrowth in differentiating mouse neuroblastoma N2a cells", Toxicology Letters, 2010

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Xuan Zhang, Gaohua Zhang, Wenjing Zhai, Zhijun Zhao, Sheng Wang, Jianfeng Yi. "Inhibition of TMEM16A Ca2+-activated Cl- channels by avermectins is essential for their anticancer effects", Pharmacological Research, 2020

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