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The effect of combination treatment of vascular endothelial growth factor receptor 3 inhibitor, MAZ-51, and zingerone on melanoma cell proliferation

By

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Submitted in partial fulfilment of the requirements for the degree

MSc in Human Physiology

This dissertation is presented in publication format

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December 2024

Declaration of originality

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Dedication

To my grandparents

Mosima Mirriam Mabitsela

1929 - 2000

and

Joseph Nakedi Mabitsela

1928 - 2013

Acknowledgements

I would like to extend my sincerest gratitude to my supervisors Dr Yvette Hlophe and Professor Trevor Nyakudya for their unwavering guidance and support throughout this entire journey. I am grateful for all the opportunities you have given me to learn and grow. Your feedback, both positive and constructive, has been invaluable to my development as a researcher.

To Professor June Serem: Your support and guidance throughout my master's journey is truly appreciated.

Dr Nontobeko Gumede and Dr Mlindeli Gamede: Your mentorship, guidance, motivation and support carried me through this past year. I appreciate everything that you have done for me and will forever be grateful for the words of motivation and wisdom that you have imparted onto me.

To my lab mates Sandra Tatchum, Carolyn Nadasen, Aminat Adigun and Charlise Basson: Thank you for walking this journey with me.

To my friends Kgatelopele Cheune, Khanyisa Maswanganyi, Amogelang Sedibe and Karabo Molomo: This journey has been nothing short of amazing. I cherish your contribution to my life and will forever be grateful for your presence in my life.

My siblings Kago Letsoalo and Mosima Letsoalo: You have been with me since the beginning and continue to support, guide and love me. Thank you for everything.

To my mommy Mpule Mautla: You are my favourite person and my strength. Your love, support and sacrifices are the reason why I am here today. You are everything and more to me. Ke go rata ka pelo ea ka eohle.

Lastly, I would like to thank myself for believing in me, for all the hard work, never giving up and always showing up.

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Research Outputs

Publications

1. **K Letsoalo**, E Nortje, S Patrick, T Nyakudya and Y Hlophe. Decoding the synergistic potential of MAZ-51 and zingerone as therapy for melanoma treatment in alignment with the sustainable development goals. Cell Biochemistry and Function. 2024;42:e3950. doi:10.1002/cbf.3950.

Submitted manuscript

1. **K Letsoalo**, C Basson, T Nyakudya, Y Hlophe. Exploring phytochemical adjuvant therapy in melanoma treatment: The effects of MAZ-51 and zingerone on melanoma cell proliferation. Cancer Medicine Journal. Manuscript ID: CAM4-2024-12-6838

Oral presentations

1. **K Letsoalo**, S Patrick, T Nyakudya and Y Hlophe. Decoding the synergistic potential of MAZ-51 and zingerone as therapy for melanoma treatment in alignment with the sustainable development goals. Cancer Research Elective, University of Pretoria, 8th November 2023
2. **K Letsoalo**, S Patrick, T Nyakudya and Y Hlophe. Unravelling the synergistic potential of a chemotherapeutic, MAZ-51, and zingerone in alignment with the sustainable development goals in cancer treatment. 50th Annual Conference of the Physiological Society of Southern Africa, University of Limpopo, 25-28th August 2024.

Poster presentations

1. **K Letsoalo**, T Nyakudya and Y Hlophe. The *in-vitro* effect of combination treatment of vascular endothelial growth factor receptor 3 inhibitor, MAZ-51, and zingerone on melanoma cell proliferation. Health Science Faculty Day, University of Pretoria, 28-29th August 2024.

Abstract

Melanoma is an aggressive form of skin cancer that is characterised by the carcinogenic transformation of melanocytes. Globally approximately 132 000 new melanoma cases are recorded annually and South Africa has recorded an incidence of 2.7 per 100 000 individuals. Various treatment strategies such as chemotherapy, targeted therapy and surgical excision are currently employed to inhibit melanoma growth, survival and progression however, the use of current treatments often results in unintended side-effects and drug resistance. Therefore, to combat this negative effect alternative treatment strategies such as medicinal plants and their bioactive phytochemicals have been widely studied and accepted as an alternative treatment, suggesting that the combined use of phytochemicals and synthetic drugs may inhibit cancer growth and proliferation with limited toxicity observed to noncancerous cells. Therefore, this study aims to investigate the individual and combined effects of (3-(4-Dimethylamino-naphthelen-1-ylmethylene)-1, 3-hydroindol-2-one) (MAZ-51) and a derivative of ginger, zingerone, on melanoma cell proliferation and survival in the melanoma (B16-F10) and human keratinocyte (HaCaT) cell lines. The cytotoxic effects of MAZ-51 (0.002-0.005 mg/mL) and zingerone (0.5-2 mg/mL) at 24, 48 and 72 hours were investigated using crystal violet assay. Additionally, crystal violet analysis was used to investigate the effects of MAZ-51 and zingerone co-treated with vascular endothelial growth factor (VEGF) on cell numbers. The morphological changes induced by the compounds were investigated using polarization optical transmitted differential contrast (PlasDIC) and hematoxylin and eosin (H&E) staining. The effects of the compounds on cell cycle progression were determined using flow cytometry. The results indicate that MAZ-51 and zingerone significantly inhibited cell growth at 48 and 72 hours ($p<0.05$). Morphological changes included the formation of apoptotic bodies, cellular protrusions, cell swelling and cell rounding suggesting cell death. In addition, MAZ-51 and zingerone resulted in a cell cycle block. Our findings demonstrate that MAZ-51 and zingerone exhibit significant antiproliferative effects on melanoma cells, with zingerone showing potential in reducing melanoma cell viability.

Keywords: Melanoma, chemotherapeutics, phytochemicals, zingerone and MAZ-51

List of abbreviations

Abbreviation	Explanation
AJCC	American Joint Committee on Cancer
Akt	Protein Kinase B
APs	Antineoplastics
BCL-2	B-cell lymphoma 2
bFGF	Fibroblast growth factor
BSA	Bovine serum albumin
CCM	Complete culture medium
CP	Cyclophosphamide
CDDP	Cisplatin
Cdk4	Cyclin dependent kinase 4
CDKN2A	Cyclin dependent kinase inhibitor 2A
CNS	Central nervous system
CPDs	Cyclobutane pyrimidine dimers
CXCL12	C-X-C chemokine ligand 12
CXCR4	C-X-C chemokine receptor 4
DAG	Diacylglycerol
DITC	Dacarbazine
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EMT	Epithelial mesenchymal transition
Erk	Extracellular signal-related kinase
ETS	E26 transformation specific
FA	Focal adhesion
FAK	Focal adhesion kinase
FAO	Food and Agricultural Organization

FAT	Focal adhesion targeting
FDA	Food and drug administration
GDP	Guanine diphosphate
GPCRs	G-protein coupled receptors
Grb-2	Growth factor receptor binding protein 2
GSK3β	Glycogen synthase kinase-3 beta
GTP	Guanine triphosphate
H&E	Hematoxylin and eosin
IC_{25}	Inhibitory concentration twenty-five
IC_{50}	Half maximal inhibitory concentration
IF	Ifosfamide
IL-1	Interleukin 1
IP3	Inositol (1,4,5)- trisphosphate
LEC	Lymphatic endothelial cell
LVD	Lymphatic vessel density
LYVE-1	Lymphatic vessel endothelial hyaluronan receptor-1
MAPK	Mitogen activated protein kinase
MC1R	Melanocortin 1 receptor
mg/mL	Milligrams per millilitre
MMPs	Matrix metalloproteinases
ng/mL	Nanogram per millilitre
NOC	Nocodazole
OECD	Organisation for Economic Co-operation
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PI	Propidium iodide
PIGF	Placental growth factor
PI3K	Phosphoinositide 3-kinase
PFS	Progression free survival
PlasDIC	Polarization optical transmitted differential contrast

PLC-β	Phospholipase-c-beta
ROS	Reactive oxygen species
SGDs	Sustainable development goals
SH2	Src homology 2
Shc	Src homology containing protein
SFKs	Src family kinases
SLN	Sentinel lymph node
TβR	Transforming growth factor beta receptor
TGF-β1	Transforming growth factor beta 1
TME	Tumour microenvironment
TMZ	Temozolomide
TNM	Tumour thickness, Nodal involvement and Metastasis
UV	Ultraviolet radiation
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WHO	World Health Organization
v/v	Volume per volume
v/w	Volume per weight
4-HC	4-hydroxycoumarin
5-FU	5-fluorouacil

1. Chapter 1

1.1 Introduction

Cancer is characterised by the unregulated growth of mutated cells.[1] The 2022 data from the World Health Organisation recorded 20 million new cases and 9.7 million cancer-related deaths with lung, breast and colorectal cancers being the prominent forms of malignancy.[2] The skin is the largest organ protecting the body from environmental factors thus, skin cells are highly susceptible to malignant transformations.[3] Skin cancer is characterised by the unregulated proliferation of mutated skin cells and is subcategorised into two groups namely: melanoma and nonmelanoma skin cancer.[1] According to the National Cancer Registry's 2022 statistics, melanoma is the 4th and 5th most widespread form of cancer in South African men and women respectively.[4] Globally approximately 132 000 new melanoma cases are recorded annually and South Africa has recorded an incidence of 2.7 per 100 000 individuals.[5] Melanoma is a highly metastatic type of skin cancer, contributing to 90% of skin cancer-related deaths and is characterised by the carcinogenic transformation of melanocytes.[1] Melanoma is sub-categorised into four groups namely: lentigo, nodular, superficial spreading and acral lentiginous. This is further classified into stage 0-IV depending on tumour depth, ulceration and metastatic potential.[5-6]

Risk factors such as exposure to ultraviolet radiation, genetic predisposition, the use of cosmetics and the presence of common or atypical mole increases the predisposition of developing melanoma.[1] Physiological conditions are characterised by the homeostatic balance of growth and inhibitory factors. However, as a result of the accumulation of mutations, cancerous cells become insensitive to these inhibitory factors.[7] In addition, the tumour microenvironment is infiltrated with various regulatory factors that activate angiogenic and lymphangiogenic signalling pathways thus, promoting tumour survival and metastasis.[8]The sprouting of lymphangiogenic vessels and metastasis to the sentinel lymph nodes is a prominent melanoma prognostic factor.[1] Vascular endothelial growth factor receptor 3 (VEGFR-3) and its ligands (VEGF-C and D) are key markers of melanoma progression and have been shown to be hyperactivated in various malignancies including melanoma, colorectal and lung adenocarcinoma.[8-9] Therefore, targeting this signalling pathway provides a potential therapeutic strategy.

The compound (3-(4-dimethylaminonaphthelen-1-ylmethylene)-1,3-dihydroindol-2-one) MAZ-51 is an indolinone based molecule that inhibits VEGF-C induced phosphorylation of VEGFR-3, thus inactivating the downstream signalling pathways that promote lymphangiogenesis and inhibiting the metastatic potential of melanoma.[1,10] Current treatment options such as dacarbazine and temozolomide have been extensively studied and resulted in a positive patient outcome however, their use induces toxicity to noncancerous cells thus, resulting in the development of unintended side effects.[1,11] As a result, the use of alternative treatments such as medicinal plants and their phytochemical constituents has become widely accepted as a treatment modality. Ginger (*Zingiber officinale*) is a well-known flavouring agent that has gained significant attention as a medicinal plant due its pharmacological activities in various diseases such as arthritis, cramps, rheumatism, sprains, muscular aches, hypertension and dementia. The biological activities of ginger are primarily attributed to its phytochemicals, including 6-gingerol, 6-shogal and zingerone.[12]

Zingerone is a bioactive phytochemical compound that possess pharmacological properties namely: anti-inflammatory, anticancer, antidiabetic and antispasmodic properties.[13-14] The chemotherapeutic properties of zingerone have been observed in malignancies where it inhibited cell cycle progression in neuroblastoma and exhibited antiangiogenic and antiproliferative properties in rat colon, ovarian and colorectal cancer.[1,15-16] To our knowledge, the chemotherapeutic properties of zingerone have not been observed in melanoma however, a derivative of zingerone, acetyl-zingerone, has been shown to improve ultraviolet (UV) radiation induced DNA mutations in melanocytes by the downregulation of reactive oxygen species (ROS).[17-18] The initial stages of melanoma are characterised by melanin-induced over-expression of ROS thus, fostering the carcinogenic transformation of melanocytes.[19] Zingerone possess antioxidant properties by scavenging ROS.[1] In addition, a study by Kim *et al* observed the ability of zingerone to inhibit transforming growth factor beta-1 (TGF-β1) induced epithelial mesenchymal transition (EMT) therefore, inhibiting metastasis.[20] Thus, considering the abovementioned attributes of zingerone, we hypothesise that zingerone is a potential chemotherapeutic for melanoma.

Chapter 2 is a published paper that provides an overview of melanoma detailing its epidemiology, aetiology, current treatments and their side-effects and the different signalling pathways involved in melanoma progression. In addition, the paper delves

into the long-term environmental and economic effects associated with the use of cytotoxic drugs and highlights the significance of phytochemicals as adjuvant therapy to combat the undesirable effects of current treatment.

Chapter 2: Decoding the synergistic potential of MAZ-51 and zingerone as therapy for melanoma treatment in alignment with the sustainable development goals

This review article has been published in Cell Biochemistry and Function Journal

Letsoalo K, Nortje E, Patrick S, Nyakudya T, Hlophe Y. Decoding the synergistic potential of MAZ-51 and zingerone as therapy for melanoma treatment in alignment with sustainable development goals. Cell Biochemistry and Function. 2024;42:e3950. doi:10.1002/cbf.3950

Received: 25 September 2023 | Revised: 28 December 2023 | Accepted: 29 January 2024

DOI: 10.1002/cbf.3950

REVIEW ARTICLE

CELL BIOCHEMISTRY & FUNCTION WILEY

Decoding the synergistic potential of MAZ-51 and zingerone as therapy for melanoma treatment in alignment with sustainable development goals

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Funding information
National Research Foundation, Grant/Award Number: N1F580A1F685; University of Pretoria Postgraduate Funding Scheme

Abstract

Melanoma, an invasive class of skin cancer, originates from mutations in melanocytes, the pigment-producing cells. Globally, approximately 132,000 new cases are reported each year, and in South Africa, the incidence stands at 2.7 per 100,000 people, signifying a worrisome surge in melanoma rates. Therefore, there is a need to explore treatment modalities that will target melanoma's signalling pathways. Melanoma metastasis is aided by ligand activity of transforming growth factor-beta 1 (TGF-β1), vascular endothelial growth factor-C (VEGF-C) and C-X-C chemokine ligand 12 (CXCL12) which bind to their receptors and promote tumour cell survival, lymphangiogenesis and chemotaxis. (3-(4-dimethylaminonaphthelen-1-ylmethylene)-1,3-dihydroindol-2-one) MAZ-51 is an indolinone-based molecule that inhibits VEGF-C induced phosphorylation of vascular endothelial growth factor receptor 3 (VEGFR-3). Despite the successful use of conventional cancer therapies, patients endure adverse side effects and cancer drug resistance. Moreover, conventional therapies are toxic to the environment and caregivers. The use of medicinal plants and their phytochemical constituents in cancer treatment strategies has become more widespread because of the rise in drug resistance and the development of unfavourable side effects. Zingerone, a phytochemical derived from ginger exhibits various pharmacological properties positioning it as a promising candidate for cancer treatment. This review provides an overview of melanoma biology and the intracellular signalling pathways promoting cell survival, proliferation and adhesion. There is a need to align health and environmental objectives within sustainable development goals 3 (good health and well-being), 13 (climate action) and 15 (life on land) to promote early detection of skin cancer, enhance sun-safe practices, mitigation of environmental factors and advancing the preservation of biodiversity, including medicinal plants. Thus, this review discusses the impact of cytostatic cancer drugs on patients and the environment and examines the potential use of phytochemicals as adjuvant therapy.

KEYWORDS
chemotherapeutics, MAZ-51, melanoma, phytochemicals, sustainable development goals, zingerone

1 | INTRODUCTION

Cancer is a heterogeneous disorder characterised by unchecked cell growth and proliferation, with transformed cells invading surrounding tissue and mobilising to distant sites.[1] The World Health Organization (WHO) 2020 statistics recorded cancer as the second leading cause of death globally, with approximately 10 million deaths reported.[2] South Africa reported a total of 107,467 new cases and 57,373 cancer-related deaths in 2018.[2] Breast, lung, colon and rectum, prostate, and nonmelanoma skin and stomach cancers are the most widespread types of cancers.[2]

Skin cancer manifests as the uncontrolled growth of mutated skin cells,[3] with over 1.5 million cases estimated worldwide in 2020.[4] South Africa ranks second in skin cancer incidence, with 20,000 new cases and 700 fatalities recorded annually.[5] The condition is categorised into two types: melanoma and nonmelanoma skin cancer, with melanoma constituting merely 1% of cases but contributing to as much as 90% of skin cancer-related deaths.[6] The next section of the review delves into a detailed discussion of metastatic melanoma providing its biology.

Significance statement

- Cytostatic drugs, despite being effective treatment agents, induce nontarget effects harming noncancerous cells.
- This review highlights the use of phytochemicals as adjuvant therapy in cancer treatment to combat the nontarget effects of cytostatic drugs.
- Additionally, phytochemicals as adjuvants decrease the environmental burden incurred by cytostatic drugs, promoting environmental sustainability.

2 | MELANOMA

2.1 | Epidemiology

Malignant melanoma is a neoplasm characterised by the malignant transformation of melanocytes.[7] Albeit contributing to 1% of skin cancer cases, melanoma contributes to 90% of skin cancer-related deaths.[6] Global estimates recorded an average of 132,000 new cases annually, while South Africa recorded an incidence of 2.7 per 100,000 individuals.[5] Melanoma incidence is increasing globally with an alarming element being the discrepancy in age and gender. While males exhibit a 1.5-fold greater incidence, younger females aged 20–45 report higher incidence rates. However, a sharp incline in incidence rate is recorded in males greater than 50 years.[8,9] Younger females possess a greater predisposition to melanoma as they are more likely to be exposed to ultraviolet (UV) radiation, directly from the sun or from artificial sources.[10] Additionally, the difference in metabolic activity of males and females in response to androgens and oestrogens influences melanoma development.[10]

The occurrence of malignant melanoma is dependent on the synergistic interplay between risk factors such as common or atypical nevi, the use of cosmetics, chronic sun exposure (particularly during childhood) and geographical zone.[7,11] Incidence rates are particularly elevated in Australia, New Zealand, Europe and Northern America while lower rates are observed in Africa.[7] This disparity is concordant with ethnicity, lifestyle as well as genetic composition. Populations with fair skin are frequently diagnosed with cutaneous melanoma whereas, individuals from Asia and Africa develop acral and mucosal melanomas at lower incidence rates.[7]

2.2 | Melanoma etiology

2.2.1 | The role of UV radiation exposure

UV radiation is the most prominent carcinogenic factor inducing melanoma and nonmelanoma skin cancer. The UV spectrum consists of UV-A (315–400 nm), UV-B (280–315 nm) and UV-C (100–280 nm).[12] UV-C despite having the shortest wavelength, does not penetrate the ozone layer and therefore, has no observed effect on melanoma genesis.[12] Approximately 5% of UV-B and 95% of UV-A rays reach the earth's surface however, UV-B is the most potent form as it directly induces DNA mutations whereas, UV-A penetrates deeper layers of the skin (dermal stratum papillare) and results in indirect damage resulting in oxidative stress.[3,12,13] In instances where UV-induced mutations affect genes dictating signalling pathways such as cell cycle, proliferation, apoptosis or DNA repair then malignancy may be induced.[13]

Consequent to UV-radiation-induced DNA damage, skin keratinocytes produce melanocyte stimulating hormone (MSH) which in turn binds to melanocortin 1 receptor (MC1R) inducing the production of melanin and thus preventing UV-induced DNA damage.[14]

Melanin contains pro-oxidant and antioxidant properties; its transformation from an antioxidant to a pro-oxidant due to UV-radiation induces carcinogenesis.[15] Its pro-oxidant properties increase intracellular oxygen radical concentrations promoting DNA mutations of melanocytes. These mutations prompt the overactivation of various signalling pathways resulting in uncontrolled cell proliferation.[15]

2.2.2 | The role of cosmetics

Cosmetics such as facial makeup are frequently applied to the skin and thus their constituents are exposed to and absorbed by the skin for extended periods of time.[11] Facial products are expected to consist of ingredients that are safe for consumer use and comply with regulatory standards. However, potential carcinogens such as parabens, ethoxylated compounds, formaldehyde donors, urea and silica have been detected in facial products.[11] The carcinogens are categorised into two groups mainly genotoxic and nongenotoxic compounds. Genotoxic compounds induce direct DNA damage following application or proceeding xenobiotic metabolism.[11] Conversely, nongenotoxic compounds result in indirect DNA damage by inducing the formation of reactive oxygen species (ROS), immunosuppression and inducing inflammation.[11]

2.2.3 | The role of hereditary factors

Melanoma is typically a consequence of somatic mutations however, a shared family history increases disease risk because of inherited mutations and common sun exposure habits.[16]

Germline mutations in cyclin dependent kinase inhibitor 2 A (CDKN2A) and to a lesser extent cyclin dependent kinase 4 (CDK4), are genetic impairments affiliated with familial melanoma. Inheritance of mutated CDKN2A allele increases an individual's risk of developing melanoma. Seventy percent of cutaneous melanoma cases reported a mutation in the CDKN2A gene.[16] This gene is situated on chromosome 9p21 and encodes $p16^{INK4A}$ and $p14^{ARF}$, tumour suppressor proteins. Under normoxic conditions, these proteins promote cell-cycle arrest however, mutations inhibit their cancer-suppression activity promoting uncontrolled cell proliferation.[16,17] In addition, $p16^{INK4A}$ negatively regulates cell cycle progression by suppressing the activity of CDK4 inhibiting Gap 1-synthesis checkpoint progression whereas, $p14^{ARF}$ positively regulates p53, a tumour suppressor protein, by inhibiting the phosphorylation of murine double minute 2-a p53 regulator- thus preventing cell cycle progression.[17] Therefore, impairment of CDKN2A by deletions, promoter silencing or mutations results in uncontrolled cell growth and proliferation.[17]

Cdk 4, located on chromosome 12q3, binds to, and is regulated by p16. Mutations in its binding domain inhibit it from associating with p16 therefore, promoting cell cycle progression.[17]

3 | MELANOMA DIAGNOSIS AND STAGING

Melanoma classification is outlined by the American Joint Committee on Cancer (AJCC) based on the tumour thickness, nodal involvement and metastasis (TNM) system.[18,19] Tumour thickness, within the TNM framework, is characterised by the primary tumour's thickness and ulceration. Conversely, the Breslow measurement considers the depth of invasion of the neoplasm.[6,19] Nodal involvement signifies whether the tumour has progressed to proximal lymph nodes. In the TNM system, the 'M' assesses dissemination to distal lymph nodes and organs, with the brain, lungs and liver being the preferred metastatic sites.[6,19]

Melanoma staging may further be classified into stages 0–IV.[5] Stage 0, also known as in situ, denotes cancer cells that are confined to the epidermis lacking infiltration into the dermis, lymph nodes or distant organs. Stage I is characterised by a tumour measuring approximately 1 mm in thickness with or without ulceration.[5] Stage II is characterised by a tumour of 4 mm thickness without metastatic evidence.[5,20] Stage III is characterised by a tumour size ≥4 mm, coupled with dissemination to proximal lymph nodes.[5,20] Stage IV arises when melanoma cells metastasize to distant organs such as the brain, lungs and liver, in addition to distal lymph nodes.[5,20]

4 | MELANOMA SUBTYPES

Melanoma is subcategorised into four subtypes: acral lentiginous, superficial spreading, lentigo malignant and nodular melanoma.[5,21] Understanding the characteristics of each subtype is crucial for early detection, accurate diagnosis and tailored treatment strategies.

4.1 | Superficial spreading melanoma

Superficial spreading melanoma is the most prevalent form[22] accounting for half to three-quarters of diagnosed malignancies.[21] Superficial spreading melanoma stems from pre-existing moles or nevi and is restricted to the epidermis, for a period before the vertical growth phase takes place. Nevi are ubiquitously positioned around the body with increased density on the back and legs in females, and the back and trunk in men.[5,21]

4.2 | Nodular melanoma

Nodular melanoma is the most invasive class of melanoma, contributing to around 15%–20% of all South African cases, and is renowned for its ability to rapidly penetrate the skin.[5] It is characterised by the emergence of moles that exhibit dark blue–black, blue–red and occasionally colourless hues, typically found on the neck, head and trunk.[19,21]

4.3 | Lentigo melanoma

Lentigo melanoma is a less prevalent subtype of melanoma responsible for 5%–15% of cases, with lesions located on areas of the body susceptible to photodamage including the face, ears, arms or upper torso.[5,19] This form of melanoma is less invasive with an extended radial growth phase. It is further characterised by blue,

black, tan or brown lesions that appear to be flat with irregular borders.[21]

4.4 | Acral lentiginous melanoma

Acral lentiginous melanoma is an infrequent form of melanoma, but frequently diagnosed in individuals with African ancestry.[21] It presents as tan to brown–black patches with uneven borders and is customarily located on the underside of nail plates, soles of the feet and the palms.[5,19,21]

Through a comprehensive understanding of these melanoma subtypes, healthcare professionals can enhance their diagnostic accuracy and treatment strategies, ultimately leading to improved outcomes for patients. In the subsequent sections of this review, we will assess the emerging therapeutic approaches, shedding light on the intricacies of melanoma management.

5 | CURRENT MELANOMA TREATMENT OPTIONS

Cancer is characterised as heterogenous, tissue-specific and evolutionary. Consequently, several treatment modalities have been developed and are currently devised to eradicate tumour cells and mitigate the risk of recurrence.[1]

5.1 | Surgical excision

The primary treatment approach for early-stage melanoma involves surgical excision of both the neoplastic cells and the neighbouring noncancerous tissues. This approach is supported by a 92% overall survival rate. Furthermore, subsequent steps often involve sentinel lymph node (SLN) biopsy and the excision of surrounding lymph nodes.[7,14,23]

Surgical excision during the initial stages of pathogenesis results in improved prognosis. However, with a 10% chance of a 5-year survival rate, metastatic cells demonstrate resistance to surgical intervention. Consequently, advanced stages of melanoma necessitate additional treatment plans, such as chemotherapy.[7,23]

5.2 | Chemotherapy

Over the recent decades, chemotherapy has been the conventional therapeutic approach. The first drug approved by the Food and Drug Administration (FDA) for metastatic melanoma was dacarbazine (DITC), receiving approval in 1975.[23] DITC is an alkylating agent that induces DNA damage by inserting alkyl groups in guanine bases resulting in cell death.[24] Despite being regarded as one of the most potent chemotherapeutic drugs, DITC is minimally effective, yielding a median survival ranging from 5 to 11 months, coupled with a 1-year survival rate of 27%.[23] The side effects associated with DITC include nausea, vomiting, leukopenia and anaemia.[25] Temozolomide (TMZ), an analogue of DITC, presents similar pharmacological activities to DITC however, unlike DITC, TMZ can cross the blood-brain barrier targeting central nervous system (CNS) metastasis.[26] Retrospective studies by Agarwala and colleagues and Paul and colleagues consisting of 122 patients presenting with intracranial disease demonstrated an overall response rate of 7% with a 77% decrease in CNS metastasis.[27,28]

Combination therapy targeting multiple cell cycle components is a common therapeutic strategy to combat tumour resistance and to reduce adverse side effects associated with monotherapy.[26] Polytherapy of DITC with other cytostatic drugs such as vinblastine, vindesine, cisplatin, carboplatin and taxane has been investigated however, no significant overall survival rates have been recorded when comparing DITC monotherapy to DITC polytherapy.[7]

With the advancement of research, the underlying molecular mechanisms dictating melanoma initiation, growth and progression have been explored and detailed derailing treatment modalities from cytotoxic drugs to more specific treatment strategies such as targeted therapy.[24] A phase III clinical trial comparing vemurafenib, a BRAF inhibitor, to DITC in patients with V600E- mutant metastatic melanoma observed a response rate of 48% in vemurafenib and 5% for DITC.[29] Additionally, vemurafenib-treated patients exhibited a progression-free survival (PFS) of 5.3 months compared to a PFS of 1.6 months in DITC.[29] A median PFS of 4.8 months was observed in trametinib-treated patients compared with 1.5 months for the chemotherapy group. An overall survival rate of 81% for trametinib and 67% for chemotherapy was recorded at 6 months.[29]

5.3 | Targeted therapy

The mitogen-activated protein kinase (MAPK) pathway is a signalling cascade that modulates cell survival, differentiation and proliferation. The pathway constitutes of RAS, RAF, MEK and ERK intermediary kinase proteins transducing outside-in signalling. Approximately 90% of melanomas display irregularities within the MAPK pathway. The most prevalent genetic anomalies linked to disrupted MAPK signalling in melanoma involve mutations in the BRAF and NRAS gene.[14] Currently, no specific targeted therapy for NRAS mutations have been identified however, the impact of BRAF inhibitors, specifically vemurafenib and dabrafenib, on disease outcomes has been investigated. The findings demonstrate improved survival rates and increased tolerance however, patients eventually develop resistance to the treatments, resulting in the resurgence of the MAPK pathway.[7,23] To counteract treatment resistance and relapse, combination therapy with MEK inhibitors, cobimentinib and trametinib, were explored. Preclinical studies documented increased apoptosis and a decrease in treatment resistance. Nevertheless, significant side effects have been recorded with the use of MAPK pathway inhibitors.[7]

5.4 | Challenges associated with conventional cancer therapies

Conventional cytotoxic therapies have shown favourable patient outcomes. However, it is important to note that cytostatic drugs do not distinguish between cancerous and noncancerous cells thus, resulting in undesired side effects associated with their use.[1] Table 1 shows drugs used for cancer treatment and their side effects.

Chemotherapy has been proven to eliminate cancer cells however, its cytotoxic activity is observed in noncancerous cells resulting in adverse side effects.[1] The most prevalent side effects recorded are vomiting, nausea, fatigue, malaise, diarrhoea, headaches, rashes, pain, infections, mucositis, alopecia and loss of appetite.[39]

Chemotherapeutic drug utilisation results in immunosuppression, as cytotoxic agents target dividing hematopoietic cells, resulting in neutropenia and cytopenia. This increases the susceptibility to infections, including those caused by oncogenic viruses.[39] Additionally, cytotoxic drugs induce both epigenetic and genetic damage. Consequently, their use not only adversely affects healthy rapidly dividing cells, but also increases an individual's vulnerability to secondary malignancies and other diseases.[39] Cytotoxic drugs can lead to neurological side effects, including memory loss, cognitive dysfunction, vision impairment, seizures, dementia and cerebral infarctions.[39] These effects impact a significant proportion of patients, ranging from 4% to 75% following the completion of treatment.[39] Moreover, treatment protocols include the use of combination and adjuvant chemotherapy; long-term use of the latter culminates in chronic fatigue, sexual dysfunction, musculoskeletal abnormalities and skin changes.[39] Although combination chemotherapy targets multiple pathways, it is associated with the development of multidrug resistance. Therefore, it is imperative to explore alternative forms of treatment.[39,40]

The development of melanoma is an intricate process involving various intracellular signalling pathways. Knowledge in the weak points of these signalling pathways allows researchers to enhance treatment strategies moving from current conventional therapeutics to more effective and efficient treatment strategies that inhibit cancer cell growth without inducing nontarget effects. The following section reviews signalling pathways involved in melanoma, describing the aberrations resulting in malignancy.

6 | ROLE OF THE TUMOUR MICROENVIRONMENT IN CANCER PROGRESSION

The tumour microenvironment consists of tumour cells and stroma namely cancer-associated fibroblasts, tumour-associated macrophages, tumour endothelial cells, leukocytes and pericytes that mould an environment that supports tumour growth and progression.[41,42]

Tumour cells secrete regulatory factors such as cytokines, growth factors and chemokines that recruit stromal, immune cells and enzymes that remodel the extracellular matrix (ECM) to construct a favourable environment that promotes tumour growth, proliferation and metastasis.[42]

Cytokines have been observed to promote melanoma cell growth, proliferation and survival.[43] In vitro studies have recorded that several melanoma cell lines secrete cytokines and growth factors that function in an autocrine and/or paracrine manner to mediate growth, invasion and angiogenesis.[43] Additionally, cytokines operate as adhesion molecules and have been shown to possess antiapoptotic properties.[43] Dysregulation of melanoma cytokine levels affect malignancy by altering the sensitivity to therapeutics and mitigating disease progression.[44] Interleukin (IL)-1, fibroblast growth factor (bFGF), transforming growth factor-beta (TGF-β) and platelet-derived growth factor (PDGF) stimulate neovascularisation required for melanoma cell growth; these factors are elevated in melanoma

TABLE 1 List of food and drug administration approved synthetic cancer drugs and their associated side effects.

Drug name	Mode of action	Side effect	References
Dacarbazine	Inhibits DNA and RNA synthesis. BRAF inhibitor.	Fatigue, loss of appetite, nausea, vomiting, kidney and liver malfunction	Milijašević et al.[30]
Paclitaxel	Induces a metaphase/anaphase halt and cell death	Alopecia, nausea and vomiting, mucositis, neutropenia, leukopenia, anaemia	Kampan et al.[31]
Cisplatin	Promotes DNA damage and induces apoptosis	Nephrotoxic, cardiotoxic, and hepatoxic	Dasari et al.[32]
Vemurafenib	Inhibits activation of the MAPK, inhibiting proliferation	Photosensitivity, maculopapular eruptions and hyperkeratosis	Trunzer et al.[33]
Temozolomide	Deposits methyl group on DNA guanine bases	Nausea and vomiting	Wesolowski et al.[34]
Ipilimumab	Inhibits cytotoxic T-lymphocyte antigen-4	Dermatological, gastrointestinal/hepatic, endocrine and pulmonary system abnormalities	Darnell et al.[35] and Youssef et al.[36]
Nivolumab	Inhibits programmed cell death-1 receptor and its ligand	Colitis, hepatitis, skin toxicities, hypophysis and thyroid dysfunction	Koppolu et al.[37] and Spain et al.[38]

patients.[44] Elevated concentrations of IL-10 have been detected in stage II and IV melanoma patients. IL-10 downregulates antitumour responses and functions as a tumour growth factor.[44] The cytokine, IL-6, is a pleiotropic cytokine secreted by multiple cells such as lymphocytes, monocytes, macrophages, keratinocytes, tumour and endothelial cells.[45] In in vitro studies, elevated IL-6 concentrations inhibit melanocyte growth however, IL-6 promotes the growth and proliferation of cells isolated from metastatic melanoma.[45] Melanoma patients with elevated IL-6 concentrations present with a poor response to treatment, have a lower survival rate and are resistant to IL-2 therapy.[44] Therefore, it is evident that cytokines play a significant role in promoting melanoma cell growth, proliferation and survival.

7 | GROWTH FACTORS THAT ACTIVATE INTRACELLULAR SIGNALLING PATHWAYS

7.1 | Transforming growth factor-beta signalling in melanoma

TGF-β is a pleiotropic cytokine forming part of a family that consists of more than 30 members in mammals and manages several cellular processes including apoptosis, angiogenesis, wound healing, embryonic development, immune surveillance and tumour biology.[46,47]

TGF-β's three isoforms (TGF-β 1,2 and 3), are synthesized as inactive complexes and transported to the extracellular matrix (ECM) where activation takes place through proteolysis.[47,48] TGF-β mediates signal transduction through nonsmad (noncanonical) and smad (canonical) pathways. Active TGF- β 1 binds to its receptors (TβR II and TβR I) with serine/threonine kinases. TβR II binds to and phosphorylates TβR I transducing intracellular signalling through the activity of smad proteins, Figure 1.[41,47]

Signalling may conversely be conveyed through nonsmad pathways such as protein extracellular signal-regulated kinase (Erk) 1/2, kinase B (Akt), p38 MAPK and phosphoinositide 3-kinase (PI3K) prompting cancer cell progression, invasion and metastasis, Figure 1.[48,49]

TGF-β1 functions dichotomously; it acts as a tumour suppressor in melanocytes and premalignant cells by modulating cyclin-dependent kinase inhibitors, p21 and p15, and c-myc, an oncogene that endorses cell proliferation.[47] However, the accrual of genetic and epigenetic modifications procures TGF-β 1 insensitive to its inhibitory effects.[50]

Paracrine secretion induces cancer cell growth and invasion through the modification of the tumour microenvironment (TME) resulting in the activation of stromal fibroblasts and their conversion to myofibroblasts.[49] Overexpressed TGF-β 1 prompts ECM stiffening through increased production of collagen I and III and fibronectin, proteins involved in ECM adhesion, augmenting communication between the ECM and fibroblast.[49] Additionally, fibroblast-induced signalling is conveyed through integrins initiating downstream signalling cascades, prompting malignancy.[51]

TGF-β 1 advances tumour angiogenesis by upregulating the secretion of vascular endothelial growth factors (VEGFs) and interleukin 8.[47,52] A study performed by Kyung and colleagues observed TGF-β 1 induced expression of vascular endothelial growth factor-C (VEGF-C) by activating the smad pathway in a gastric cancer cell line.[53] Furthermore, TGF-β 1 induces epithelial cell growth

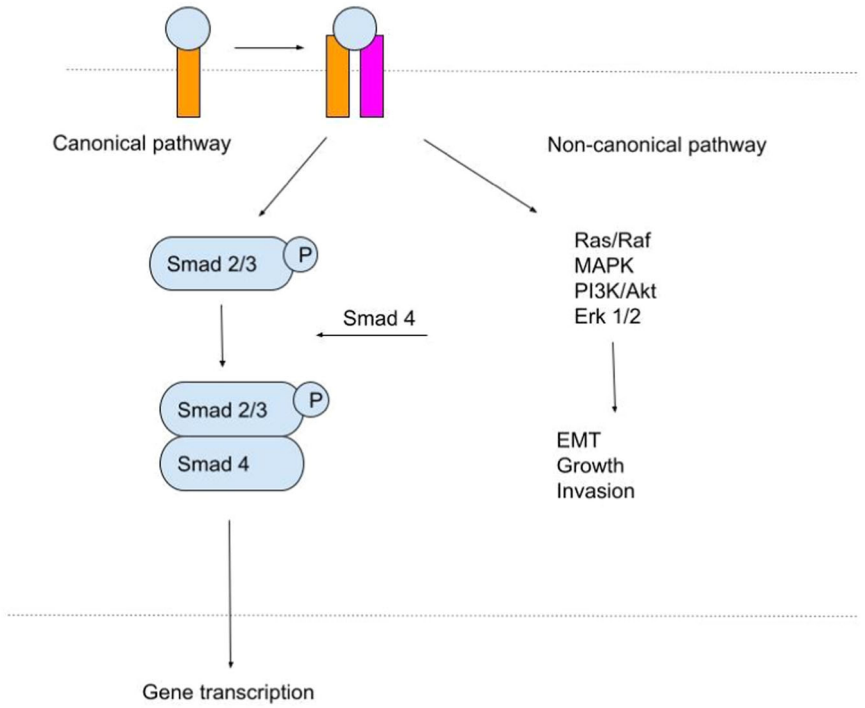

FIGURE 1 Transforming growth factor-beta signalling pathway. TGF-β1 binds to TβR II (receptor II) which recruits and phosphorylates TβR I (receptor I) resulting in the activation of the canonical and noncanonical signalling pathways. Active canonical pathway results in the recruitment and phosphorylation of smad 2/3 and smad 4 proteins which migrate to the nucleus and induce gene transcription. Noncanonical signalling includes Ras/Raf, MAPK, PI3K/Akt and Erk1/2 signalling pathways which promote cell growth, proliferation and survival.[36–39] (Image was designed by K. Letsoalo in Microsoft Word 2016). EMT, epithelial mesenchymal transition; MAPK, mitogen activated protein kinase; PI3K, phosphoinositide 3-kinases.

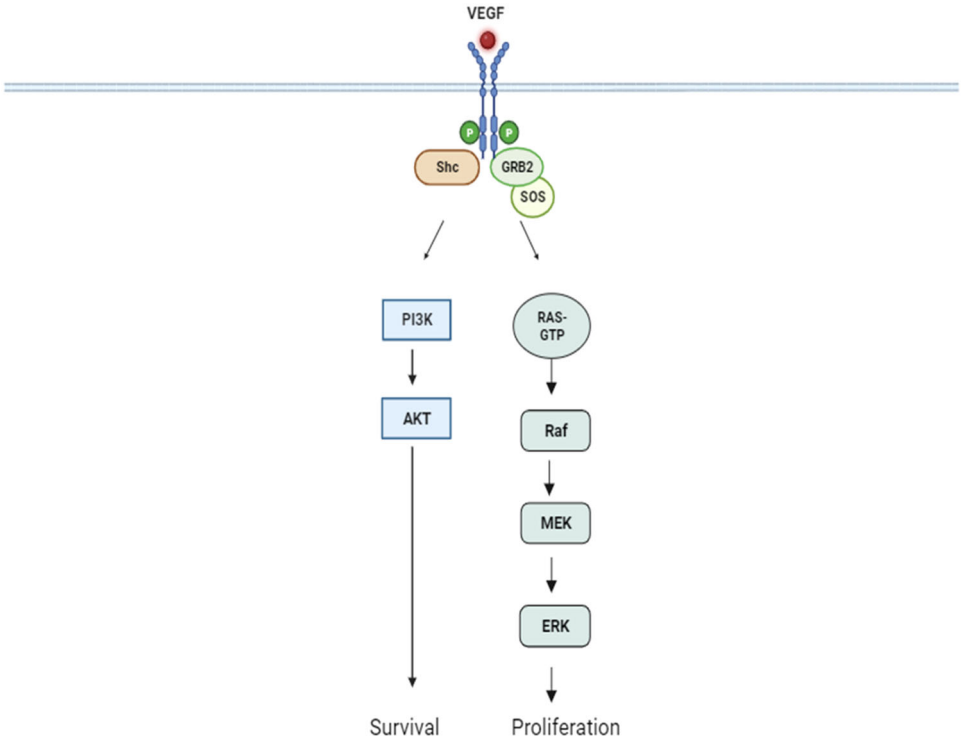

FIGURE 2 VEGFC signalling pathway. VEGF-C binds to its receptors resulting in receptor homo or heterodimerization. Receptor tyrosine residues are phosphorylated creating docking sites for adapter proteins. Ligand binding transduces signalling through the MAPK and PI3K/Akt signalling cascades which promote tumour cell proliferation and survival.[58] (Image was designed by K. Letsoalo with BioRender.com). GRB2, growth factor receptor binding protein 2; Shc, src homology containing protein; SOS, son of sevenless protein; PI3K, phosphoinositide 3-kinases; VEGF, vascular endothelial growth factor.

prompting the development of peritumoral neo-vasculature thus, prompting tumour growth and metastasis.[52]

7.2 | Vascular endothelial growth factors and their receptors in melanoma signalling

The differentiation of endothelial cells during the embryonic period, postnatal vasculature development and sprouting of vessels during pathology is reliant on VEGFs and their corresponding receptors (VEGFRs).[54] VEGFs are structurally related angiogenic and lymphangiogenic factors that are constituents of the PDGF subgroup of the growth factor cystine knot group.[55] In mammals the VEGF family consists of VEGF-A, B, C and D, placental growth factor (PIGF) as well as the homologs that are expressed in Orf viruses including VEGF-E and VEGF-F expressed in snake venom.[55–57] Alternative splicing and proteolytic processing yield various isoforms of the proteins.[55,56]

VEGFs induce signalling through their receptor tyrosine kinases, VEGFR 1,2 and 3 with ligand binding yielding receptor homo or heterodimerisation, conformational changes and auto or transphosphorylation of tyrosine residues, ultimately activating signalling cascades that promote cell proliferation, survival and migration.[55,58]

The primary mechanism of mortality in cancer patients is the dissemination of tumours to secondary locations through the vasculature.[59] Tumour dissemination to secondary locations utilises three mechanisms mainly: direct spread (invasion of surrounding tissues and organs), hematogenous (metastasis to secondary locations using the bloodstream) and lymphatic metastasis (metastasis to secondary locations and lymph nodes utilising the lymphatic vasculature).[60] VEGF-C binding to VEGFR-3 promotes lymphangiogenesis, providing a metastatic route for melanoma cells.

7.2.1 | VEGFR-3/VEGF-C signalling pathways

VEGF-C is a lymphangiogenic growth factor signalling through VEGFR-2 and 3.[56] VEGF-C and its receptors are predominantly expressed by endothelial cells however, their expression is observed in tumour cells thus paracrine/autocrine signalling between tumour cells, vasculature and nonendothelial cells activates the VEGFR-3/VEGF-C axis inducing biological responses including tumour growth, proliferation and migration.[56]

Ligand binding yields receptor dimerization and autophosphorylation of tyrosine residues Y1230/Y1231, recruiting adapter proteins: growth factor receptor binding protein 2 (grb-2) and src homology containing protein (Shc).[61] Active receptors activate the MAPK and PI3K/Akt pathway (Figure 2).[61]

7.2.2 | Role of VEGF-C/VEGFR-3 signalling pathway in promoting lymphangiogenesis

Tumour growth and metastasis to regional lymph nodes is the initial step in melanoma dissemination and serves as a significant indicator of disease prognosis.[62] In melanoma lymphangiogenesis is the emergence of lymphatic vessels from pre-existing vessels and is induced by VEGF-C binding to its receptor VEGFR-3 expressed by endothelial cells, inducing lymphatic endothelial cell (LEC) proliferation and the development of peritumoral and intratumoral vessels thus, promoting metastasis to lymph nodes.[63]

In the past, limited knowledge and research of lymphatic molecular markers impeded research that would allow researchers to distinguish blood vessels from lymphatic vasculature in the TME and thus elucidate lymphatic metastasis.[59,62] However, the emergence of VEGF-C and D, their receptors and lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1), a lymphatic specific marker, has contributed to the significance of the lymphatic vasculature in promoting metastasis in various malignancies including melanoma.[59,62]

A study performed by Liu and colleagues established the contribution of VEGF-C and D in promoting lymphangiogenesis and lymph node metastasis.[64] The continuous overexpression of these factors by tumour cells and stroma promotes lymph vessel growth cultivating metastasis.[59] In a melanoma clinical study, VEGF-C overexpression correlated with SLN spread.[65] Additionally, a melanoma animal model observed a positive relation between overexpressed VEGF-C and lymphatic vessel quantity and peritumoral vessel diameter.[59] However, a soluble VEGFR-3 inhibitor minimised tumour induced lymphangiogenesis and metastasis.[59] Several tumours express elevated concentrations of growth factor C and/or D with growth factor expression paralleling lymphatic vessel density (LVD), lymphatic metastasis and disease outcome.[66] From these studies it is evident that the VEGF-C/VEGFR-3 signalling pathway is significant in fostering a metastatic niche by promoting lymphatic metastasis.

Tumours procure a premetastatic niche at the SLN before metastasis taking place.[67] The phenomenon was initially observed and exemplified by Michael Detmar and his colleagues in a skin-cancer animal model.[59] SLN metastasis is coupled with increased distant metastatic prevalence as well as hematogenous spread justifying that hematogenous and lymphatic spread act synergistically to promote distant metastasis.[59] Primary tumour cells secrete VEGF-C/A which are transported via the lymphatic vessels to SLN where they procure a premetastatic niche. Arrival of the tumours at the SLN results in elevated concentrations of VEGF-C/A which journey to distant lymph nodes resulting in the increased capacity of the lymph vessel network.[66,67] SLN lymphangiogenesis is correlated with increased metastasis at distant lymph nodes however, the absence of lymph node metastasis impedes distant organ metastasis thus, substantiating the contribution of the lymph nodes and vessel in tumour dissemination.[66] Additionally, chemokines secreted by LEC employ the lymphatic vasculature to generate a gradient inducing directional migration of tumour cells from the primary location to secondary sites promoting lymph node and distant metastasis.[66]

8 | CONTRIBUTION OF CHEMOKINE SIGNALLING IN LYMPHATIC METASTASIS

Chemokines are cytokines consisting of a wide-range family of small (8–15 kDa) proteins characterised by their chemotactic properties and similar composition, facilitating heparin binding and the regulation of the host's defence system.[68] Chemokines are characterised by their amino acid sequence and positioning of the cysteine residues within the protein.[69] Variation in the N-terminal cysteine organisation gives rise to four subgroups/families mainly: CC, CXC, CX_3C and XC with C denoting the N-terminal domain and X denoting an amino acid.[69,70] Initially chemokines were named according to their function however, in the year 2000 a systemic nomenclature system was described that includes subfamily designation (CC, CXC, CX_3C and XC) followed by L (representing the ligand) and a number representing the gene it was initially isolated from.[70]

C-X-C chemokine receptor 4 (CXCR4) and its corresponding ligand C-X-C chemokine ligand 12 (CXCL12) are the most widespread chemokine receptor/chemokine pair in various malignancies including melanoma.[5,71] Under normoxic conditions, chemokine signalling is responsible for foetal development, tissue repair and the homing of hematopoietic cells and leukocytes to specific sites and tissues. However, tumourigenesis is characterised by the directional migration of receptor-expressing tumour cells to ligand-expressing metastatic sites.[66]

CXCR4 upregulation is a key metastatic factor in various malignancies including human melanoma. CXCL12 binds to CXCR4 and activates a plethora of signalling cascades that promote melanoma growth, adhesion, angiogenesis and migration.[72] Kim and colleagues observed the role of chemokine receptors in melanoma and colorectal cancer liver metastasis.[73] Microarray analysis classified CXCR4 as the most prominent receptor in both cell lines; 89% of melanoma patients and 97% of colorectal cancer patients expressed CXCR4. Additionally, in vitro treatment with CXCL12 increased cell migration in the cell lines.[73] These findings correlate with other cancer research outputs classifying CXCR4 expression as a prognostic factor. In addition, inhibition of CXCR4 signalling pathway abrogates cell migration in metastatic melanoma.[74,75] Therefore, the CXCR4/CXCL12 signalling pathway is prominent in melanoma metastasis and thus, targeting this pathway provides a therapeutic approach to prevent migration.

Lymph nodes express elevated concentrations of CXCL12 driving a gradient that facilitates the attraction of CXCR4-expressing malignant cells, therefore, promoting directional migration toward the lymph nodes. Moreover, tumour-associated lymphatic vasculature and not normal lymphatics express CXCL12, justifying the role of the lymphatic endothelium in metastatic spread.[66] Evidently, the CXCR4/CXCL12 gradient induces distant metastasis mainly to CXCL12-expressing tissue such as the lungs, liver and bone-melanoma

metastatic sites.[66] A study performed by Kim and colleagues observed the proximity of $CXCR4^+$ melanoma cells with CXCL12-producing lymphatic vessels in metastatic lymph nodes and lung tissue with $CXCR4^+/CD133^+$ cells presenting a greater metastatic activity than $CXCR4/CD133^-$ cells.[76] Moreover, inhibition of CXCR4 abolished melanoma growth and metastasis thus, justifying the role of the CXCR4/CXCL12 axis in promoting lymph node and distant metastasis by adopting the lymphatic vasculature.[76]

8.1 | CXCR4/CXCL12 signalling in melanoma

Signalling through CXCR4/CXCL12 prompts downstream signalling cascades resulting in multiple responses such as chemotaxis, cell survival, proliferation and gene transcription.[77,78] Ligand binding initiates G-protein activation through the exchange of guanine diphosphate (GDP) for guanine triphosphate (GTP) resulting in the dissociation into GTP-bound and α and βγ subunits.[78] Dissociated βγ subunits initiate the activation of phospholipase-c-β (PLC-β) and PI3K. Phospholipase-c-β cleaves phosphatidylinositol (4,5)–bisphosphate into secondary messengers mainly: inositol (1,4,5)–triphosphate (IP3) and diacylglycerol (DAG). Inositol (1,4,5)–triphosphate prompts intracellular Ca^{2+} release through binding to its endoplasmic reticulum receptors, Figure 3.[79]

Gα and Gβγ subunits drive PI3K activation resulting in the phosphorylation of focal adhesion proteins such as focal adhesion kinase (FAK), cytoskeletal protein paxillin and proline-rich kinase-2 and thus facilitate cell migration by reorganisation of the actin cytoskeleton.[68] Active PI3K promotes the rapid production of phosphatidylinositol (3,4,5)–triphosphate prompting Akt pathway activation.[80] Moreover, the active Akt pathway induces the activation of Bcl-2 associated agonist of cell death (BAD), an antagonist of B-cell lymphoma 2 (BCL-2), thus contributing to cell survival.[68] Additionally, CXCR4 signalling through Akt diminishes the activity of glycogen synthase kinase-3 beta (GSK3β)[80] and initiates the stabilization of β-catenin which translocates to the nucleus and prompts gene transcription and proliferation, Figure 3.[68]

9 | INTRACELLULAR ADHESION PROTEINS AND INTEGRINS ACTIVATED BY CHEMOKINES BINDING THEIR SPECIFIC RECEPTORS

9.1 | Integrins

Integrins are large, complex and heterodimeric glycoproteins that bridge signal communication between the internal and external environments.[81] These proteins are composed of alpha and beta subunits forming noncovalent heterodimers. In mammals, there are 18 alpha and 18 beta subunits, giving rise to 24 distinct integrin heterodimers.[5] Integrins primarily bind to various components of the ECM such as vitronectin, fibronectin, laminin or collagen. This binding provides essential anchorage for cell adhesion and invasion processes.[81] Integrin binding to the ECM induces their clustering in the membrane plane and enables them to recruit and activate several signalling and adapter protein such as Src family kinases (SFKs), FAK and scaffolding molecules such as p130CRK-associated substrate to assemble focal adhesions (FA).[82] Moreover, they integrate the ECM into the actin cytoskeleton by recruiting proteins such as paxillin, talin, α -actinin, vinculin and tensin. Thus, FA protein recruitment and activity is regulated by integrin activity and directs cell adhesion and migration.[81] Integrins serve as meditators of bidirectional signalling where intracellular signalling transforms extracellular process (inside-out signalling); conversely, extracellular ligand binding activates intracellular signalling cascades (outside-in signalling) by activating the Ras/Rho signalling pathways.[83]

9.2 | Focal adhesion kinase background

FAK is a 125kDa[84] nonreceptor tyrosine kinase whose activity is regulated by integrin signalling, GPCRs, cytokines and growth factors.[85] FAK mediates multiple cellular processes including proliferation, survival, adhesion and migration.[5] Furthermore, FAK advances tumour stemness, epithelial mesenchymal transition, chemotherapeutic resistance tumour angiogenesis and fibrosis in the stroma. FAK is a ubiquitously expressed protein with three domains: an N-terminal domain, a central kinase domain and a C-terminal focal adhesion targeting (FAT) domain.[85] The C-terminal domain associates with FA-associated proteins such as talin and paxillin. Subsequent to growth factor or integrin signalling, FAK is autophosphorylated at Y397 resulting in the formation of a Src binding site which further phosphorylates other tyrosines on FAK therefore, yielding additional binding sites for Src homology 2 (SH2) domain-containing proteins.[84,85] Moreover, an active FAK/Src complex activates downstream proteins including paxillin.[84]

9.2.1 | Focal adhesion kinase in the development of melanoma

The FAK promoter region contains a p53 binding site where wild-type p53 inhibits FAK transcription.[86] However, mutant p53 which is observed during malignancy is incapable of binding to the promoter region and thus, displays no inhibitory effects on FAK promoter activity, therefore, promoting continuous FAK transcription and overexpression.[86]

Anoikis is a form of programmed cell death initiated because of obstructions between the cell and the ECM and the loss of FAK activity.[87,88] Overexpression of FAK encourages resistance to anoikis despite the detached state of cells from the ECM. Furthermore, increased activity of the FAK/Src complex stimulates the induction of PI3K/Akt and MEK/Erk 1/2 signal transduction, promoting cell survival in the detached conformation.[87] TGF-β stimulates FAK and Akt expression utilising smad3 and p38 MAPK respectively, conferring anoikis resistance and promoting tumour survival.[89]

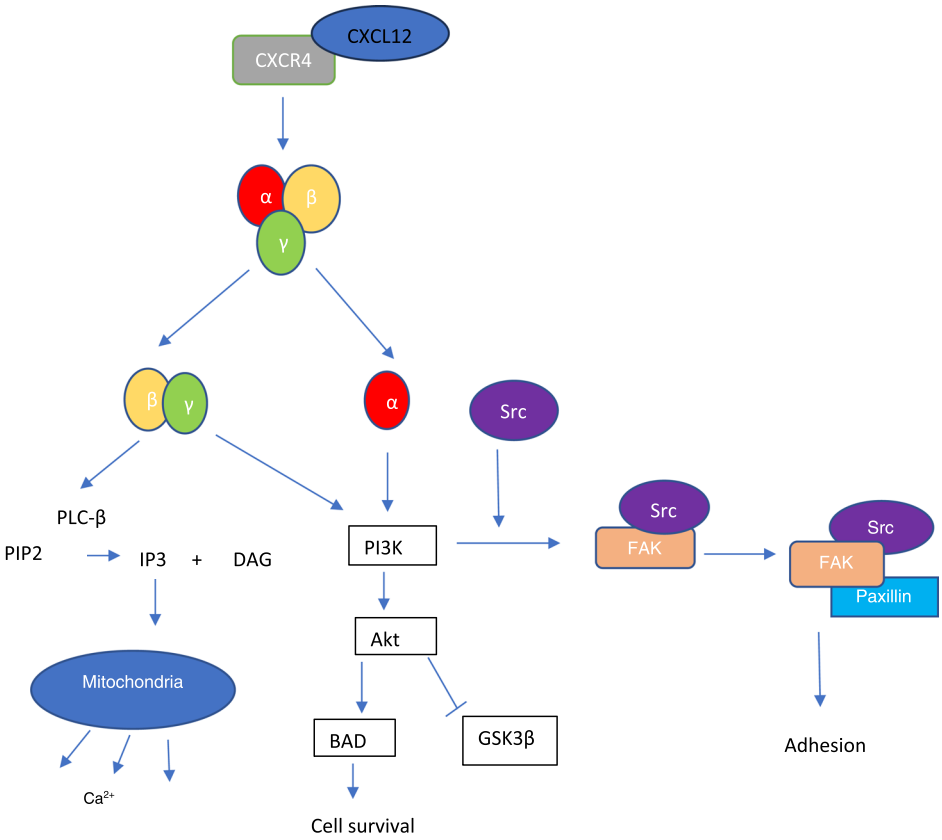

FIGURE 3 CXCR4/CXCL12 signalling pathway. Active CXCR4/CXCL12 results in the exchange of GDP for GTP resulting in the dissociation of Gα and Gβγ subunits. Gα and Gβγ activate PI3K driving adhesion through the activity of FAK and paxillin. PI3K additionally inhibits GSK3β and activates BAD protein promoting tumour cell survival. Gβγ subunits induce the production of IP3 and DAG with IP3 promoting mitochondrial calcium secretion.[67,74,76] (Image was designed by K. Letsoalo in Microsoft Word 2016). BAD, Bcl-2 associated agonist of cell death; CXCL12, CXC chemokine ligand 12; CXCR4, CXC chemokine receptor 4; DAG, diacylglycerol; FAK, focal adhesion kinase; GSK3β, glycogen synthase kinase-3 beta; IP3, inositol (1,4,5)–triphosphate; PI3K, Phosphoinositide 3-kinases; PIP2, phosphatidylinositol (4,5)–bisphosphate; PLC-β, phospholipase-c-β.

Additionally, FAK overexpression inhibits the induction of caspase-3-mediated apoptosis whereas FAK inhibition induces apoptosis.[87]

FAK overexpression and phosphorylation is associated with cell-cycle progression through modulation of cell-cycle proteins, this further reiterates the role of FAK in tumour cell survival.[87] Overexpressed FAK facilitates cyclin D1 expression and the inhibition of p21 therefore, prompting cell-cycle progression through the G1 phase. Overexpressed FAK is delineated to regulate the E26 transformation specific (ETS) binding site located within the cyclin D1 promoter which further modulates the transcription of cyclin D1.[87] FAK further promotes cell-cycle progression by inhibition of p53 tumour suppressor mediated apoptosis.[87] Therefore, FAK regulates cancer cell proliferation by modulating cell-cycle molecules or promoting turnover of tumour suppressor proteins.

9.3 | Paxillin background

Paxillin is the principle constituent of FAs which plays a critical role in signal transduction following the interaction between the ECM and integrins.[90,91] Paxillin is a scaffolding protein and recruits kinases and phosphatases, co-factors, structural and oncoproteins whose activity is required for intracellular signalling.[91] Activation of the above-mentioned proteins reorganises the actin cytoskeleton and induces assembly/disassembly of FAs that are essential for cell adhesion, metastasis and migration.[91] Paxillin positively regulates cell adhesion as it is recruited to nascent FAs at the anterior of the cells inducing the assembly of adhesion complexes; conversely, it is attributed to FA disassembly at the rear end of the cell during cell migration.[91] Paxillin activity is not only localised to FAs, but also to the cytoplasm and nucleus with its activity exerted on gene transcription. Therefore, it bridges signal transduction from the plasma membrane and cytoskeleton to the nucleus.[91] Despite its interaction with protein complexes and enzymes, paxillin does not present any enzymatic activity itself. Instead, paxillin serves as a docking site for other proteins, facilitating the assembly of multiprotein complexes.[91]

Paxillin activity and localisation is tightly regulated by its phosphorylation in response to diverse stimuli. Tyr31 and Tyr118 are the well-known phosphorylation sites however, paxillin may also be phosphorylated at various serine and tyrosine residues.[91] In

response to integrin association with the ECM, paxillin is phosphorylated prompting the recruitment of proteins such as talin, vinculin, tensin and FAK that are required for FA assembly.[91] Paxillin phosphorylation at the tyrosine residue provides a scaffold for the recruitment of FAK and Src. Furthermore, Src-induced phosphorylation at Tyr88 and Tyr118 as well as phosphorylation at Tyr118 and Tyr31 by FAK at the N-terminus initiates paxillin interaction with downstream effectors inducing p130Crk-associated substrate (p130cas) extracellular signal transduction into cellular responses mediated by MAPK.[91]

9.3.1 | The role of paxillin in cancer development

Paxillin acquires gain of function mutations which are associated with tumour progression in several malignancies including melanoma.[92] Paxillin functions in conjunction with other adhesion proteins to regulate cell migration and adhesion thus, enhancing metastasis.[91,92] To evaluate the role of paxillin in melanoma metastasis Velasco-Velazquez and colleagues transfected B16-F10 melanoma cells with paxillin-siRNA.[93] Transfection significantly inhibited the metastatic potential of the B16-F10 cells.[93] Furthermore, increased levels of phospho-paxillin have been observed in melanoma cells compared to melanocytes.[93] To further determine the role of paxillin in melanoma metastasis, B16-F10 cells were treated with 4-hydroxycoumarin (4-HC). 4-HC reduced α and β isoforms of paxillin mRNA levels.[93] Additionally, 4-HC mitigated paxillin signalling pathways resulting in decreased phosphorylation of FAK and GTP-bound Rac-1.[93] Therefore, it is evident that paxillin is a significant contributing factor of melanoma metastasis.

10 | POTENTIAL SYNTHETIC COMPOUND TO INHIBIT MELANOMA CELL PROLIFERATION

10.1 | MAZ-51(3-(4-dimethylaminonaphthelen-1-ylmethylene)-1,3-dihydroindol-2-one)

MAZ-51 (3-(4-dimethylaminonaphthelen-1-ylmethylene)-1,3-dihydroindol-2-one) is an indolinone-based molecule synthesised to inhibit VEGFR-3 phosphorylation in endothelial cells.[94,95] Indolinones contain distinct amino acid components at position three that form part of adenosine triphosphate-competitive inhibitors of receptor kinases and have been proven to bind to specific receptor tyrosine kinases such as fibroblast growth factor, epidermal growth factor, PDGF and VEGF.[90,94] Indolinone derivatives exhibit anti-proliferative properties and induce apoptosis in cancer and endothelial cell lines. MAZ-51 competitively binds to VEGFR-3 inhibiting VEGF-C induced phosphorylation of VEGFR-3 therefore, inactivating signalling cascades that promote cell survival and proliferation in endothelial and cancer cell lines.[90] To our knowledge, limited studies have observed the effect of MAZ-51 on melanoma cell proliferation. However, melanoma is a VEGFR-3-expressing tumour and has been proven to utilise the VEGFR-3/VEGF-C signalling pathway to promote lymphatic metastasis thus, we hypothesize that VEGFR-3 expressed by melanoma cells will be sensitive to MAZ-51 treatment.[90,96] A study conducted by Lee and colleagues observed the effect of MAZ-51 on VEGFR-3 inhibition in B16-F10 melanoma cell line.[95] MAZ-51 treated cells exhibited a decrease in intratumoural lymphatic vessels in the lungs, decrease in tumour size as well as a decrease in the expression of VEGF-C, VEGFR-3 and Prox-1.[95] Therefore, from the available literature, it is evident that MAZ-51 is a potential therapeutic strategy for melanoma by targeting the lymphangiogenic pathway.

11 | THE ROLE OF PHYTOCHEMICALS AS POTENTIAL CHEMOTHERAPEUTIC AGENTS

11.1 | Implications of using phytochemicals/plants for cancer treatment

Harmonious to the limited efficacy of conventional cancer treatments, the use of medicinal plants and their bioactive compounds as anticancer agents has been accepted as a form of medical intervention.[97] The use of medicinal plants for primary healthcare is a long-standing practice with approximately 80% of populations in developing countries being reliant on phytomedicine for their primary health care.[98,99] Their accessibility, low costs and reduced side effects contribute to their increased consumption in comparison to synthetic drugs.[98] The health-promoting effects of phytochemicals are attributed to their biological properties such as antioxidants, anti-inflammatory, antimicrobial and anticancer activities.[1] With the increase in efficacious experimental findings, phytochemicals are postulated to possess significant anticancer capabilities.[99]

Carcinogenesis is dependent on the aberrant activation of signalling pathways that promote cell growth, proliferation and inhibit the sensitivity of pathways to regulatory molecules that maintain a homeostatic balance.[100] The proposed mechanism of action utilised by phytochemicals is their ability to regulate signal transduction pathways by increasing proapoptotic proteins and decreasing antiapoptotic proteins, increasing expression of regulatory proteins thus, promoting cell cycle arrest, decreasing the sensitivity of cells to mitogens and mitigating invasion and metastasis by inhibiting epithelial mesenchymal transition (EMT).[99,101] Several signalling pathways such as the MAPK/Erk and PI3K/Akt are dysregulated during pathogenesis promoting cancer cell growth and metastasis.[101] Targeting these pathways provides a solid foundation to prevent and treat malignancies. Table 2 provides a list of phytochemicals that have been investigated for melanoma and provides their mode of action in combating malignancy.

Both in vitro and in vivo studies advocate for phytochemicals as alternative treatment modalities for cancer. However, clinical studies supporting the sole use of phytochemicals are limited due to challenges such as their low bioavailability and the high doses

required for effective treatment outcomes.[111,112] The combined application of medicinal plants with existing cytotoxic drugs has garnered significant attention and widespread acceptance. This approach is favoured for its potential to decrease the chemotherapeutic drug dose, reduce toxicity, enhance drug bioavailability, lower resistance development and capitalise on the synergistic effects of phytochemicals and synthetic drugs to jointly inhibit tumour cell proliferation.[113]

One such phytochemical that has been identified as a potential anticancer agent is zingerone.

11.2 | Zingerone

Zingerone (4-(4-hydroxy-3-methoxyphenyl)-2 butanone) is a non-toxic bioactive phytochemical compound that has pharmacological activities.[114] It is a ketone that is a 4-phenylbutan-2-one. It belongs to a group of compounds called methoxyphenols where a methyl group is attached to a benzene ring. Zingerone contains pharmacological properties including antidiabetic, anti-inflammatory, antidiarrheic, antilipolytic, antispasmodic and anticancer properties.[114,115]

Zingerone's chemoprotective properties have been observed in in vitro and in-vivo studies where it induces cell cycle arrest in neuroblastoma cells, prevents angiogenesis and its antiproliferative effects investigated in in rat colon cancer, ovarian cancer and colorectal cancer.[116,117] Zingerone possesses several pharmacological properties that render it a suitable chemopreventative and chemotherapeutic agent. The following section details the pharmacological properties of zingerone in cancer management.

11.2.1 | Mechanism of action of zingerone against melanoma development

Zingerone's chemotherapeutic properties have been observed in multiple malignancies however,[116–118] to our knowledge zingerone's ability to induce melanoma cell death has not been studied. Nevertheless, acetyl zingerone—a derivative of zingerone—has been shown to ameliorate DNA mutations in melanocytes following sun exposure.[119–121] Cyclobutane pyrimidine dimers (CPDs) are UV-radiation-induced photoproducts resulting in DNA lesions and mutations and are strongly correlated with melanoma and non-melanoma skin cancer.[119,121] Additionally, CPDs are correlated to photoaging and the production of immunomodulatory cytokine tumour necrosis factor alpha which has been shown to promote melanoma signalling pathways.[119] Studies by Chaudhuria and colleagues and Srivastava and colleagues observed that acetyl zingerone is effective in inhibiting the formation of CPDs in

TABLE 2 List of phytochemicals utilised for melanoma treatment.

Phytochemical name	Source	Mode of action	References
Fisetin	Apples, onions, grapes, cucumbers, and strawberries	Promotes mesenchymal to epithelial transition and targets the NFkB and MAPK signalling	Chandra Pal et al.[21,102]
Indole-3-carbinol	Broccoli and brussel sprouts	Stabilises PTEN. Induces G1 cell cycle arrest and apoptosis.	Aronchik et al.[103]
Epigallocatechin gallate	Green tea	Inhibits expression of PD-L1 and PD-L2. Induces cell cycle arrest and apoptosis.	Chandra Pal et al.[21] and Ravindran Menon et al.[104]
Eugenol	Cloves, bay leaf and cinnamon leaf	Induces S-phase cell cycle arrest and apoptosis. Inhibits E2F1.	Ghosh et al.[105]
Resveratrol	Grapes, mulberries peanuts, eucalyptus, and cranberries	G1/S cell-cycle arrest. Upregulates p53.	Chandra Pal et al.[21] and Pourhanifeh et al.[106]
Capsaicin	Chilli peppers	Activates caspase 3,8 and 9. Downregulates Bcl-2.	Chandra Pal et al.[21]
Apigenin	Parsley, celery, artichokes	Downregulates ERK 1/2 and PI3K/Akt signalling	Chandra Pal et al.[21]
Genistein	Soybeans	Inhibits angiogenesis, proliferation, and metastasis and promotes apoptosis. Upregulates p53, p21 and checkpoint kinase 2.	Chandra Pal et al.[21]
Curcumin	Turmeric	Targets Akt, NFkB and AP-1	Chandra Pal et al.[21]
Silymarin	*Silybum marianum* L. Gaertin	Induces apoptosis and cell cycle arrest. Downregulates Bcl-2 and upregulates Bax.	Chandra Pal et al.[21] and Vaid et al.[107]
Procyanidin	Cocoa, berries, apples grapes	Targets 67 kDa Laminin receptor signalling	Bae et al.[108]
Luteolin	Broccoli, raw brussel sprouts, carrots, peppers and parsley	Inhibits expression of MMP-2 and 9. Targets PI3K/Akt pathway.	Rocchetti et al.[109] and Yao et al.[110]

melanocytes following UV exposure.[119,121] Acetyl zingerone inhibits the formation of CPDs by upregulating the expression of nucleotide excision repair pathway, decreasing reactive oxygen species (ROS) and neutralizing free radicals and scavenging peroxynitrite.[119,121]

According to the literature, zingerone promotes cancer cell death by inhibiting the disintegration of the ECM, inhibiting angiogenesis, promoting cell cycle arrest and apoptosis.[108,122,123] However, in melanoma cells, zingerone targets oxidative stress by inhibiting the formation of ROS. In melanocytes, melanin suppresses the formation of ROS. However, during malignancy melanogenesis is a source of oxidative stress. Overexpressed ROS induces a melanocyte homeostatic imbalance thus compromising their viability and fostering their malignant transformation.[124] Taking into consideration the antioxidant activity of zingerone and acetyl zingerone and the role of ROS in promoting melanoma, we hypothesize that zingerone is a suitable therapeutic strategy to target oxidative stress and thus, inhibit melanoma genesis.

Current cancer treatment approaches utilise cytostatic cancer drugs to halt cancer cell growth and proliferation. However, these drugs are inefficiently metabolised and are excreted into environmental water systems through bodily waste. This raises concerns about their impact on the environment, with drug residues detected in water bodies and soil. Striking a balance between effective cancer treatment and minimising environmental harm is a pressing challenge, urging the development of more eco-friendly treatment strategies. The next section of the review provides a detailed discussion of the impact of cytostatic cancer drugs on the environment.

12 | THE EFFECTS OF CYTOSTATIC CANCER DRUGS ON THE ENVIRONMENT

Cancer is the second leading cause of death with its global burden on the rise, 9.6 million cancer-related deaths were recorded in 2018[125] with an estimation of 29.5 million cases will be recorded in 2040.[126] The increase in cancer incidence is positively correlated with an increase in the prescription of chemotherapeutic drugs and the presence of antineoplastics (APs), anticancer drugs, in the aquatic environment.[125]

Anticancer drugs are inefficiently metabolised by the human body and are excreted either as the parental compound or its metabolite via urine and faeces, some traces are found in sweat and vomit.[126] Cancer treatment frequently takes place at the hospital with wastewater derived from hospitals and pharmaceutical factories being noteworthy contributors of anticancer drugs in the aquatic environment.[126]

Elevated concentrations of AP agents,[125] reaching levels on the order of micrograms per liter (μg/L), have been identified in in hospital effluents.[127] Despite this, there are limited environmental regulations overseeing their safety thresholds and wastewater treatment options for these compounds.[125] The presence of APs in untreated wastewater may result in acute and chronic effects in vulnerable aquatic species such as zebrafish and crustaceans.[127]

Chronic exposure to APs alters the genetic composition and cell-cycle of aquatic flora and fauna with researchers deeming them as pseudo-resistant pollutants.[128] Individual APs are present at low concentrations with limited aquatic effects however, their combined effect results in additive and synergistic effects.[129] The combination of cyclophosphamide (CP), ifosfamide (IF) and their metabolites in alga *Synechococcus leopoliensis* brought about increased growth inhibition in comparison to the individual drugs.[116] A study performed by Elersek and colleagues indicates that a single high dose of APs is comparable to the combination of multiple APs at low concentrations.[128] Additionally, bioaccumulation and biomagnification of APs may further increase their concentration in the aquatic environment.[128]

The presence of APs in the environment is a well-known phenomenon however, information about their biological effects on organisms is scarce.[126,127] Anticancer agents are cytotoxic and cytostatic drugs that target cancerous and noncancerous cells thus, eukaryotic organisms are prone to the side effects.[127] With the global incidence of cancer on the rise, several AP agents have been detected in the aquatic environment with the five most widely studied being: CP, IF, methotrexate, tamoxifen and 5-fluorouracil (5-FU).[125]

Tamoxifen, an oestrogen receptor antagonist, is used individually or in combination with other drugs to treat hormone receptor-positive breast cancer.[125,127] A study performed by Pagano and colleagues observed the effects of tamoxifen on fertilization, embryogenesis and mitotic effects in the sea urchin.[127] Researchers observed the effects of tamoxifen on developmental toxicity resulting in early embryonic death. CP is an alkylating agent utilised for the treatment of various malignancies including ovarian and breast cancer, retinoblastoma, multiple myeloma and mycosis fungoides to name a few.[130] Li and colleagues observed the effects of CP at concentrations of 0.5–50 μg/L on zebrafish.[130] Results indicated that exposure to CP induced malformation, histopathological alterations in the retina and liver and decreased swimming mobility of the zebrafish.[130] Additionally, CP hampered the transcription of genes required for the MAPK signalling pathway.[130] Novak and colleagues studied the individual effects of CP and IF as well as in combination with 5-FU and cisplatin (CDDP) in zebrafish liver cell lines.[131] Individually, CP and IF induced minimal cytotoxicity however, in combination with 5-FU and CDDP, the compounds induced DNA strand breaks.[131]

Chemotherapeutics, although required for cancer treatment enter the wastewater system and endanger the aquatic life with their combinations acting additively and/or synergistically.[129] The intersection of cancer treatment and ecological preservation underscores a call for sustainable progress, aligning with multiple UN Sustainable Development Goals (SDGs).

13 | CONTRIBUTION OF CHEMOTHERAPEUTICS TO THE SDGs

In cancer treatment, residual chemotherapeutic agents infiltrating aquatic systems impact SDGs 14 and 15 (life below water and on land). Nanotechnology offers promise, aligning predominantly with SDGs 3 (good health) and 9 (innovation). Engineered nanoparticles,

targeting drug delivery, reinforce SDG 6 (clean water). Addressing contamination pathways resonates with SDGs 6 and 12 (responsible consumption). Enhanced wastewater management and innovative treatment resonate with SDGs 6 and 11 (sustainable cities). The synthesis of medical exigency and ecological imperatives advances SDG 3 while nurturing the planet (SDG 13).

13.1 | Sustainable development goal 3—Good health and well-being

Anticancer drugs are administered both within hospitals and to outpatients as a result, both hospital and domestic wastewater are a source of AP agents, entering and contaminating the aquatic ecosystem.[126] Residential sewage treatment plants are inadequately equipped to process AP waste and thus, serve as a contamination route for surface and groundwater.[125] Moreover, several developed countries utilise septic tank systems which are ineffective in the removal of pharmaceuticals suggesting an additional route of contamination for the soil and groundwater.[125]

Although concentrations of APs are nonsignificant in drinking water, vulnerable populations such as foetuses, children and breastfeeding individuals are at risk.[132] Chemotherapeutics target rapidly multiplying cells sensitising foetuses, babies and children to their adverse side effects due to the rapid growth experienced by these groups. However, there is a knowledge gap about the risk associated with exposure through drinking water, skin and oral exposure.[132]

Chemotherapeutics are genotoxic resulting in the occurrence of malignancy when they interface proto-oncogenes or tumour suppressor genes.[133] Furthermore, exposure to genotoxic agents induces sequences of events resulting in the occurrence of adverse side effects. The beneficial effects of chemotherapeutics outweigh the detrimental risks in patients however, healthcare workers and caregivers are exposed to the drugs and are subjected to the side effects without any beneficial activity of the drug.[133] Baniasodi and colleagues studied the urinary concentrations of CP and IF in healthcare workers involved in the preparation and administration of chemotherapeutic drugs.[134] Results observed the presence of CP in five preshift and nine postshift samples. IF was detected in one preshift and four postshift samples whereas, the drugs were not detected in the control group. Additionally, the healthcare workers reported a headache as the most frequent adverse effect.[134] A study performed by Elshaer recorded the effects of APs on exposed nurses. Thirty one percent of exposed nurses presented with infertility and 36.36% recorded oral ulcers.[135] Additionally, white blood cell counts were reduced whereas, there was an increase in creatine levels.[135] Exposure to APs is associated with a twofold increase in spontaneous abortions, congenital malformations, infertility and the development of acute myelogenous leukaemia and myelodysplastic syndrome.[133] The use of chemotherapeutics albeit being beneficial for the initial malignancy results in adverse side effects and the development of secondary malignancies. Moreover, exposure of APs to healthcare workers is associated with the development of several disorders. APs not only affect the health and well-being of patients but also of their caregivers thus, calling for research for alternative treatment options.

13.2 | Sustainable development goal 8—Decent work and economic growth

Limited studies have been conducted to characterise the effects of APs on the aquatic ecosystem.[126] It is postulated that APs are mutagenic and genotoxic affecting various trophic levels of aquatic life resulting in a decrease in the quality and population of the aquatic ecosystem.[131]

Countless people worldwide are dependent on healthy aquatic ecosystems as sources of food and employment. Additionally, aquatic ecosystems sustain economic growth, modulate the climate and aid in the prosperity of coastal communities.[136] Therefore, it is of utmost importance to sustain and manage this natural resource. The Organisation for Economic Co-operation and Development (OECD) has reported that oceans contribute approximately US$1.5 trillion annually to economic growth.[136] According to the Food and Agricultural Organization (FAO), around 58.5 million individuals worldwide are employed in primary fish production, with women accounting for 21% of this workforce.[136] The department of agriculture, fisheries and forestry states that the fisheries sector has a net worth of approximately R8 billion annually with 28,000 people employed in the commercial sector in South Africa.[137] Even though the South African fisheries industry contributes less than 1% of the total gross domestic product (GDP) and only 5% of the Western Cape's provincial GDP, this sector is a significant contributor to food security, employment and environmental impact.[137]

Given the fisheries sector's role in ensuring food security and providing employment opportunities, contamination of aquatic ecosystems and oceans can profoundly impact the economic growth and development of countries, particularly developing countries. Many communities in these regions depend on commercial and recreational fishing for both sustenance and job security, making them especially vulnerable to the negative consequences of ecosystem contamination.[136] Additionally, the decrease in aquatic populations and increase in genetic aberrations affects research and development as several aquatic organisms such as the zebrafish are involved in experimental scientific research.[131] Thus, pharmaceutical contamination of aquatic ecosystems is a cause for concern affecting various sectors that affect economic growth and development.

13.3 | Sustainable development goal 9—Target 9.5, indicator 9.5.1: Research and development spending

Cancer cells are heterogenous exhibiting frequent mutations and insensitivity toward traditional treatment options.[138] Considering the challenges associated with current chemotherapy, the use of plant-based anticancer agents is on the rise. Although the use of

phytochemicals is gaining attention with several researchers advocating for their employment, they present with low bioavailability, limited genotoxic profiling and variable immune response.[138]

One of the outcomes stemming from the combined use of chemotherapeutics and phytochemicals is the potential for herb-drug interactions. This poses a particular concern for patients undergoing treatment for chronic conditions, as they may be at a higher risk for such interactions.[139] The most prevalent mechanism behind herb-drug interactions involves the obstruction and/or induction of drug-metabolising enzymes and transport proteins by herbs, resulting in the reduced efficacy of the chemotherapeutic drug.[139] Accurate analysis of phytochemical-drug interaction and increased bioavailability to the target tissue using advanced imaging tools pave a pathway for successful anticancer drug intervention.[138]

Current knowledge of phytochemical-drug interaction rests on in vitro, in vivo and in silico models with minimal clinical trials studies. Therefore, research of phytochemical interaction in human studies and the development of advanced imaging tools will enhance the understanding of mechanisms of action and predict clinical effects.[140]

14 | CONCLUSION

This paper reviewed melanoma, detailing its biology, and signalling pathways. Additionally, it explored the current treatment strategies providing their mechanism of action and their associated side effects and explored the potential use of phytochemicals as adjuvant therapy.

Medicinal plants and their derivatives are gaining traction as potential therapeutic agents due to their reduced toxicity, affordability and capacity to alleviate side-effects often seen with the use of synthetic drugs. Research is ongoing to understand their effectiveness and mechanisms of action. These natural compounds, phytochemicals, are preferred over their synthetic counterparts because of their lower toxic profiles, affordability and ability to minimise side effects. Furthermore, they have a positive environmental impact, contrasting with synthetic drugs that can have unintended consequences, jeopardizing patients, caregivers and our ecosystem. The nontarget effects of synthetic drugs can be detrimental to both human health and the environment.

Although phytochemicals have shown promising results in laboratory settings, their limited bioavailability and stability have hindered their advancement in clinical trials. Contemporary cancer treatments often combine various methods, including surgery, radiation and chemotherapy. However, the concurrent use of multiple synthetic drugs can result in increased resistance to these drugs and unintended harm to healthy cells. Recent research has highlighted the potential benefits of pairing phytochemicals with traditional cancer treatments, suggesting that this combination can reduce the need for synthetic drugs, decrease toxicity and combat drug resistance. This combined approach could lead to more effective and targeted treatments for cancer, ultimately resulting in the destruction of cancer cells.

Incorporating environmental contaminants and SDGs 3 (good health and well-being), 9 (industry, innovation, and infrastructure) and 8 (decent work and economic growth), the findings of this review underscore the importance of sustainable and environmentally friendly treatment options. Using medicinal plants and phytochemicals aligns with these SDGs by promoting health, fostering innovation in treatment modalities and ensuring that the means of production do not harm the environment or the workforce. Further research will be crucial to unlock the full potential of phytochemicals in clinical practice and to address challenges related to their bioavailability and stability. This paves the way for a new era of cancer treatment that is not only more effective but also environmentally conscious.

ACKNOWLEDGEMENTS

The research supporting this review was funded by the National Research Foundation (NRF) of South Africa (N1F580A1F685) and the University of Pretoria's Postgraduate funding scheme.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

No additional data is available.

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How to cite this article: Letsoalo K, Nortje E, Patrick S, Nyakudya T, Hlophe Y. Decoding the synergistic potential of MAZ-51 and zingerone as therapy for melanoma treatment in alignment with sustainable development goals. *Cell Biochem Funct*. 2024;42:e3950. doi:10.1002/cbf.3950

Chapter 3

3.1 Purpose of the study

Skin cancer is a continuously evolving malignancy with South Africa recording the second highest incidence rate and approximately 700 mortalities recorded annually.[5] Therefore, there is a need to develop novel treatment modalities. Current treatment strategies utilise monotherapy to eradicate cancerous cells.[11] However, chemotherapeutics are non-selective and target rapidly dividing cancerous and noncancerous cells resulting in the development of unintended side-effects such as immunosuppression, fatigue, malaise, diarrhoea, infections and alopecia.[11,21] A common side-effect observed in cancer patients is drug resistance. To circumvent this, the concurrent use of multiple treatment strategies such as surgery and chemotherapy and a combination of multiple chemotherapeutics has become the cornerstone of cancer treatment.[21] Despite the improved treatment response of combination therapy of cytotoxic drugs, their side-effects remain a prominent concern.[1,21] Therefore, there is a need to develop safe and affordable alternative treatment strategies that effectively eradicate cancerous cells without inducing cytotoxic effects on noncancerous cells.

The use of medicinal plants and their phytochemicals has received significant consideration as potential chemotherapeutics due to their accessibility, low costs and reduced toxicity.[22] Various studies have reported the efficacy of phytochemicals against cancer progression however, there are few clinical studies that have investigated their individual use due to their reduced bioavailability and high doses required to elicit a significant decrease in viability.[23-24] As such, combined treatment of phytochemicals and current chemotherapeutics is widely accepted as a potential therapeutic as phytochemicals are hypothesised to target multiple signalling pathways, reduce the dose of the cytotoxic drug, limit toxicity and drug resistance and enhance drug availability. [1,25]

Aim: To investigate the individual and combined effects of MAZ-51 and zingerone on melanoma cell proliferation in the B16-F10 melanoma and HaCaT human keratinocyte cell lines.

Objectives:

1. To determine the cytotoxic effects of MAZ-51 and zingerone in the B16-F10 and HaCaT cells using crystal violet.
2. To investigate the morphological changes induced by MAZ-51 and zingerone in the B16-F10 and HaCaTs using polarization optical transmitted light differential contrast (PlasDIC) and hematoxylin and eosin (H&E) staining on the B16-F10 and HaCaT cells.
3. To determine the effects of MAZ-51 and zingerone on cell cycle progression in the B16-F10 and HaCaT cells using flow cytometry.
4. To investigate the effects of MAZ-51 and zingerone on cell numbers in B16-F10 and HaCaT cells co-treated with vascular endothelial growth factor (VEGF) using the crystal violet assay.

Chapter 4: Exploring phytochemical adjuvant therapy in melanoma treatment: The effects of MAZ-51 and zingerone on melanoma cell proliferation

Data from this study has been submitted to Cancer Medicine Journal

Letsoalo K, Basson C, Nyakudya T, Hlophe Y. Exploring phytochemical adjuvant therapy in melanoma treatment: The effects of MAZ-51 and zingerone on melanoma cell proliferation. Cancer Medicine Journal (under review). Manuscript ID: CAM4-2024-12-6838

Abstract

Current melanoma treatment results in adverse effects. Recent research supports using phytochemicals as adjuvant therapy to reduce the reliance on synthetic drugs and combat drug resistance. This study aims to investigate the *in-vitro* effect of (3-(4-Dimethylamino-naphthelen-1-ylmethylene)-1, 3-hydroindol-2-one) (MAZ-51) and zingerone, a ginger derivative, on melanoma cell proliferation in B16-F10 melanoma and HaCaT human keratinocyte cell lines. The individual effects of MAZ-51 (0.002-0.005 mg/mL) and zingerone (0.5-2 mg/mL) at 24, 48 and 72 hours, as well as the combined effects (at IC_{50} at 48 and 72 hours), were assessed to determine cell numbers using a crystal violet assay, which was also utilised to investigate the effects of vascular endothelial growth factor (VEGF) co-treated medium on cell numbers. Morphological changes were examined using hematoxylin and eosin (H&E) staining and polarization optical density inferential contrast (PlasDIC) and cell cycle progression by means of flow cytometry. MAZ-51 and zingerone significantly reduced cell viability at 48 and 72 hours ($p<0.05$). Cells treated with both MAZ-51 and zingerone, in combination with VEGF co-treatment, exhibited a decrease in cell numbers. PlasDIC imaging and H&E staining revealed characteristics of cell death. Flow cytometry analysis showed that zingerone and MAZ-51 induced a mitotic block. Our findings demonstrate that MAZ-51 and zingerone exhibit significant antiproliferative effects on melanoma cells, with zingerone showing potential in reducing melanoma cell viability.

Keywords: Melanoma, phytochemicals, zingerone, MAZ-51 and chemotherapeutics

1. Introduction

Cancer initiation and progression is a multifaceted process that transforms normal cells into malignant cells.[26] According to the International Agency for Research on Cancer (IARC), more than 18.1 million new cases and 9.5 million cancer-related deaths were recorded in 2020.[27] In addition, the IARC estimates an additional 29.5 million new cases and 16.4 million cancer-related deaths will be recorded by 2040.[27] South Africa recorded approximately 108 168 new cases and 56 802 cancer-related deaths in 2020. Prostate, lung, colon-rectum cancer and Kaposi sarcoma are the most prevalent types of cancers among South African males whereas, breast, cervical-uterine, colorectum and lung cancers are prominent in South African females.[27] Melanoma, an aggressive form of skin cancer, derives from the neoplastic transformation of melanocytes and is classified into four subgroups namely: superficial spreading, nodular, lentigo and acral lentiginous melanoma.[1] Despite accounting for less than 1% of skin cancer cases, melanoma is responsible for 90% of skin cancer-related deaths due to its high metastatic potential.[1]

In contrast to normal cells, which respond to exogenic stimuli to regulate growth and metastasis, tumour cells through the accumulation of genetic mutations become insensitive to these anti-mitogenic signals, allowing them to evade the apoptotic and repair mechanisms.[28] To establish a metastatic niche, tumour cells along with tumour-associated macrophages (TAMs) and stromal cells secrete various regulatory factors such as growth factors, cytokines and chemokines that promote a favourable environment for melanoma growth by stimulating the proliferation of lymphatic endothelial cells (LECs) prompting lymphangiogenesis.[1,8] Lymphangiogenesis, the sprouting of lymphatic vasculature from pre-existing vessels, is primarily driven by vascular endothelial growth factor receptor 3 (VEGFR-3) and its ligands (VEGF-C and D).[8-9] Activation of VEGFR-3 signalling pathway stimulates LEC proliferation and lymphatic vessel remodelling, creating a metastatic route to lymph nodes and secondary locations. The elevated expression of VEGFR-3/VEGF-C axis is a key prognostic factor in multiple cancers including, melanoma, colorectal, lung adenocarcinoma and breast cancer.[1] Therefore, targeting this signalling pathway is a potential therapeutic for melanoma growth and progression.

Inhibiting the VEGFR-3 pathway by disrupting the binding of VEGF-C/D to their receptor may combat lymphangiogenic metastasis and proliferation.[8] (3-(4-dimethylaminonaphthelen-1-ylmethylene)-1,3-dihydroindol-2-one) MAZ-51, an indolinone-based synthetic molecule, prevents receptor tyrosine kinase (RTK) activity of VEGFR-3.[1,29] By hindering VEGF-C induced activation of VEGFR-3, MAZ-51 impedes receptor phosphorylation and activation of downstream signalling pathways and thus, inhibits lymphangiogenesis and proliferation.[1]

A significant challenge in modern cancer treatment strategies is the development of unintended side-effects due to traditional cancer drugs, which are cytotoxic to both cancerous and noncancerous cells, leading to common side-effects such as alopecia, neutropenia, neurological disorders, vomiting, nausea and increased susceptibility to secondary infections.[11,21] Additionally, combination therapy often involves using multiple chemotherapeutic agents to target different signaling pathways. However, this approach can lead to multidrug resistance, potentially promoting tumour growth and proliferation.[1] Additionally, metabolites of anti-cancer drugs are excreted in wastewater systems posing an environmental burden.[1]

Given the challenges associated with the use of pharmacological agents in the management of cancer, there is a need to align effective cancer treatment with environmental sustainability by exploring alternative treatment strategies. In light of the accumulating evidence against the use of synthetic drugs due to their unintended side-effects, high costs, limited specificity and toxicity, medicinal plants and their phytochemicals have gained significant attention as alternative potential chemotherapeutic agents.[1,30] Phytochemicals possess antimicrobial, anticancer and anti-inflammatory properties making them promising alternative therapeutics.[31] Their low cost, increased accessibility and limited side-effects enhance their suitability compared to synthetic drugs.[22] The antiproliferative properties of phytochemicals are attributed to their ability to modulate signalling pathways, promote cell cycle arrest and apoptosis, inactivate carcinogens, inhibit proliferation and regulate the immune system.[31-32] Ginger (*Zingiber officinale*) is a medicinal plant that possesses pharmacological properties and elicits its therapeutic effects as result of it phytochemicals namely: 6-gingerol, 6-shogal and zingerone.[12]

Zingerone (4-(4-hydroxy3-methoxyphenyl)-2-butanone), a bioactive phytochemical compound derived from ginger, exhibits pharmacological properties such as anti-inflammatory, antidiabetic, antidiarrhoeic, anticancer and antispasmodic properties.[1] Although limited studies have investigated the effect of zingerone on melanoma cells, its chemotherapeutic properties have been observed in malignancies such as prostate cancer, neuroblastoma, ovarian and colorectal cancer where it was shown to exhibit antiproliferative properties by inducing cell cycle arrest and inhibiting angiogenesis.[1]

Although phytochemicals show promising results as chemotherapeutic agents, their clinical use is limited due to low bioavailability and the high dose required to achieve significant beneficial effects.[1,23-24] However, combining phytochemicals with conventional cytotoxic drugs has gained significant attention. This approach has the potential to reduce the synthetic drug doses, improve drug bioavailability, limit drug resistance and maximise on the synergistic effects of phytochemicals with synthetic drugs to inhibit cancer growth and proliferation.[1,25] Therefore, this study aimed to investigate the individual and combined effects of MAZ-51 and zingerone on melanoma cell proliferation using a cell survival assay, microscopy and flow cytometry to identify cell cycle changes.

2. Methodology

2.1 Ethics approval

Ethics approval for this study was granted by the University of Pretoria's Faculty of Health Science Research Ethics Committee (Ethics committee number: 192/2022).

2.2 Cell lines: Melanoma and keratinocyte cell lines

B16-F10 melanoma, a highly metastatic cell line, expresses the receptors CXCR4 and VEGFR-3 and was purchased from the American Type Culture Collection (ATCC® CRL-6475™). It is a murine cell line produced as the 10th serial passage subclone of the B16 parent tumour line in the C57BL/6J mice strain.[17] The HaCaT cell line is a non-tumourigenic monoclonal human keratinocyte cell line that was derived from an adult human skin. The cell line exhibits a partial to fully differentiated phenotype. The cell line was purchased from CELLONEX, South Africa and served as a control cell line.[18] The B16-F10 and HaCaT cells were utilised from passage 2-12 and passage 45-55 respectively.

2.3 Sample preparation

MAZ-51 and zingerone were dissolved in dimethyl sulfoxide (DMSO) with the final concentration less than 0.1% (w/v). Serial dilutions were made by adding ddH_2O to the compounds. Control samples were exposed to a vehicle control of 0.01% DMSO (v/v). An additional control sample at 0.6% DMSO (v/v) and a medium control sample were included following the calculation of the maximal inhibitory concentrations (IC_{50}) to account for the increased DMSO concentration for zingerone at IC_{50} values. However, nonsignificant differences were recorded between the controls therefore 0.01% DMSO was used as the standard control. Cells were exposed to MAZ-51 (0.002- 0.005 mg/mL), zingerone (0.5-2 mg/mL) and nocodazole (NOC) at 0.004 mg/mL for 24, 48 and 72 hours. Nocodazole served as a positive control as it is known to result in a G2/M mitotic phase block.[19] Following the determination of the IC_{50} values, subsequent experimental assays were performed at 48 and 72 hours due to nonsignificant data observed at 24 hours. Cells were treated with IC_{50} values of MAZ-51, zingerone and a combination of MAZ-51 and zingerone.

2.4 Cell culture

The cells were grown in T25 or T75cm^2 culture flasks and were maintained in Dulbecco's modified Eagles's medium (DMEM) (Whitehead Scientific, Brackenfell, Cape Town, South Africa), supplemented with 10% heat-inactivated foetal calf serum (FCS) and 1% penicillin/streptomycin to form complete culture medium (CCM). Cells were then stored in a humidified Esco Life Sciences incubator at 37ºC and 5% CO_2. Cell growth was monitored daily with CCM changes taking place every 2-3 days.

After reaching 70-90% confluency, the B16-F10 and HaCaT cells were sub-cultured by removing the medium, gently washed twice with 3 mL of 1 × phosphate buffered saline (PBS) and a dissociation agent namely TrypLE™ Express phenol red and 5% trypsin was utilised to aid B16-F10 and HaCaT cell detachment respectively. This was followed by incubation for 3-5 minutes. Thereafter, 5 mL of CCM was added to the cell suspension to inactivate the TrypLE™ Express phenol red and 5% trypsin. The cell suspension was transferred to a 15 mL tube and centrifuged for 5 minutes at 1429 × *g*. Following centrifugation, the supernatant was discarded followed by resuspension with 1 mL of CCM.

2.5 Spectrophotometry

2.5.1 Determination of cell viability: Crystal violet

B16-F10 and HaCaT cells were seeded at a volume of 0.09 mL and concentrations of 5×10^4 cells/mL (5×10^3 cells/well) and 10×10^4 cells/mL (9×10^3 cells/well) respectively in 96-well plates and allowed to attach overnight. Cells were treated with the compounds: zingerone (0.5-2 mg/mL), MAZ-51 (0.002-0.005 mg/mL) and NOC, a positive control, at 0.004 mg/mL followed by incubation for 24, 48 and 72 hours. Following incubation, termination was aided by the addition of 200 µL of 1% glutaraldehyde dissolved in ddH_2O to the 96 well plates. The plates were incubated for 30 minutes followed by the removal of glutaraldehyde. Crystal violet,100 µL of 0.1 %, solution was dissolved in ddH_2O and added to stain the plates for 30 minutes at room temperature (RT) followed by rinsing of the plates under running water. The plate was left to dry overnight followed by the addition of 100 µL of 10% acetic acid dissolved in ddH_2O to solubilise the crystal violet dye. The absorbance was read at 630 nm using a spectrophotometer Epoch Microplate Reader (Agilent Bio-Tek). The results were reported as a percentage of cell viability against the vehicle control group.

2.6 Determination of cell morphology

2.6.1 Polarization optical differential inferential contrast and Hematoxylin and Eosin staining

The cells were seeded on heat-sterilized coverslips in a 24-well plate at a volume of 0.45 mL and concentrations of 10×10^4 cells/mL (4.5×10^4 cells/well) and 20×10^4 cells/mL (9×10^4 cells/well) for the B16-F10 and HaCaT cells respectively and were left to attach overnight. Following overnight incubation, cells were exposed to the different compounds: NOC, MAZ-51, zingerone and a combination of MAZ-51 and zingerone at the calculated B16-F10 IC_{50} values for 48 and 72 hours. For PlasDIC, images were taken using the Zeiss inverted microscope (Axiovert CFL40 Zeiss; Oberkochen, Germany). Following PlasDIC, the CCM was removed and the cells were fixed with Bouin's fixative for an hour. Bouin's fixative was removed and 70% ethanol was added to the coverslips for 20 minutes at RT. Coverslips were then rinsed for two minutes with tap water followed by the addition of Mayer's haematoxylin for 20 minutes and rinsed under tap water for two minutes. Coverslips were treated with 70% ethanol followed by the addition of 1% eosin dissolved in ddH_2O for five minutes. The eosin was removed and the coverslips were dehydrated twice with increasing concentrations of ethanol at 70%, 96% and 100% followed by the addition of xylene. Coverslips were

mounted on microscope slides with resin and were left to dry. Zeiss Axiovert MRc microscope (Zeiss, Oberkochen, Germany) were used to acquire images at 100 × magnification with immersion oil.

2.7 Flow cytometry:

2.7.1 Cell cycle analysis

B16-F10 and HaCaT cells were seeded in a 24-well plate at a volume of 0.45 mL and concentrations of 10 ×10^4 cells/mL (4.5 ×10^4 cells/well) and 20 × 10^4 cells/mL (9 × 10^4 cells/well) respectively and allowed to attach overnight. Cells were treated with NOC, zingerone, MAZ-51 and a combination of zingerone and MAZ-51 at the B16-F10 IC_{50} values for 48 and 72 hours, followed by trypsinisation and resuspension in 1 mL of CCM. The cells were centrifuged for five minutes at 1429 × g followed by resuspension of the pellet with 200 µL ice-cold PBS. The cells were centrifuged for five minutes and the supernatant was discarded. Cells were treated with ice-cold 70% methanol that was added in a drop-wise manner and stored at -20ºC before analysis took place. Prior to analysis, cells were pelleted by centrifugating them for five minutes at 1429 × *g*. The supernatant was removed followed by washing the cells in 0.5% bovine serum albumin (BSA)/PBS solution. Cells were resuspended in 1 mL of PBS that contains propidium iodide (PI) (40 µg/mL), 100 µg/mL RNase A and 0.1% triton-X and incubated at 37ºC, 5% CO_2 for 45 minutes. FC500 flow cytometer (Beckman Coulter, Johannesburg, South Africa.), was used for cell analysis. Distribution of the cell cycle was calculated using Kaluza C software (version 1.2.1) by allocating deoxyribonucleic acid (DNA) content per cell to sub-G_1, G_1, S and G_2/M phases.

2.8 The effect of a growth factor on cell numbers

2.8.1 Vascular endothelial growth factor

B16-F10 and HaCaT cells were seeded at a volume of 0.09 mL and concentrations of 5 x 10^4 cells/mL (5 × 10^3 cells/well) and 10 × 10^4 cells/mL (9 × 10^3 cells/well) respectively in a 96-well plate. Cells were exposed to MAZ-51, zingerone and a combination of MAZ-51 and zingerone at the B16-F10 IC_{50}. Cells were further exposed to either 20 ng/mL or 60 ng/mL of vascular endothelial growth factor (VEGF) (Sigma-Aldrich, Kempton Park, South Africa) for the stipulated time period of 48 and 72 hours. Additional control samples were included, either exposed to VEGF or not exposed to VEGF at 20 ng/mL or 60 ng/mL. Crystal violet analysis was then carried out in the same manner as explained in section 2.5.1.

2.9 Statistical analysis

Each experiment was performed with three technical and three biological repeats for all cell lines and was tested for normality using the Shapiro-Wilks test. Data obtained from quantitative experiments are represented by the mean ± standard error of mean (SEM) and were statistically analysed using the one-way analysis of variance (ANOVA). The Bonferroni test was used as a *post-hoc* test and $p \leq 0.05$ was considered statistically significant. GraphPad Prism version 9.5.1 was utilised for statistical analysis.

3. Results

3.1 Crystal violet staining of B16-F10 and HaCaT cells: Zingerone and MAZ-51 induced a decrease in cell viability

The effects of the compounds MAZ-51 and zingerone at concentration ranges of 0.002-0.005 mg/mL and 0.5-2 mg/mL respectively were evaluated on the B16-F10 melanoma and HaCaT human keratinocyte cell lines for viability for 24, 48 and 72 hours.

Zingerone-treated B16-F10 cells significantly decreased cell numbers from concentrations of 1.5-2 mg/mL and 0.5-2 mg/mL for 48 and 72 hours respectively ($p<0.05$; Figure 1A) whereas, HaCaT cells displayed a significant decrease in cell numbers at concentrations of 1-2 mg/mL at 24, 48 and 72 hours ($p<0.05$; Figure 1C). A significant decrease in cell numbers was also observed in MAZ-51 treated B16-F10 cells from 0.004 and 0.005 mg/mL at 72 hours ($p<0.05$; Figure 1B). However, a nonsignificant decrease was observed in MAZ-51 treated HaCaT cells at 24, 48 and 72 hours (Figure 1D).

The positive control, NOC induced a significant decrease in cell numbers in the B16-F10 cells at 48 and 72 hours ($p<0.01$; Figure 1A) whereas, a significant decrease at 24, 48 and 72 hours was observed in the HaCaT cells ($p<0.05$; Figure 1C&D).

The IC_{50} determined by linear regression for zingerone was 27.9, 2.199 and 1.219 mg/mL at 24, 48 and 72 hours respectively. The IC_{50} for MAZ-51 was 0.05428, 0.03162 and 0.01204 mg/mL at 24, 48 and 72 hours respectively (Table 1). Due to the non-statistical decrease in cell numbers observed at 24 hours, subsequent experimental assays were conducted at 48 and 72 hours. The IC_{50} values resulted in lower

percentage cell numbers in the B16-F10 cell lines in comparison to the HaCaT cells at 48 hours (Figure 2). However, at 72 hours treatment with zingerone at IC_{50} and a combination of MAZ-51 and zingerone was more potent in the control cell lines compared to the B16-F10 cell lines (Figure 2)

Due to the significant reduction in cell numbers observed in zingerone-treated HaCaT cells at IC_{50} values, the effects of zingerone at IC_{25} values (1.0995 mg/mL for 48 hours and 0.60695 mg/mL for 72 hours) were further investigated. This study also examined the combined treatment of MAZ-51 at IC_{50} and zingerone at IC_{25} concentrations in the B16-F10 and HaCaT cells. In comparison to the IC_{50} values, zingerone at IC_{25} values resulted in higher percentage cell numbers at both cell lines at 48 and 72 hours ($p<0.05$; Table 2). In addition, combination treatment also resulted in an increase in the percentage cell numbers ($p<0.05$; Table 2).

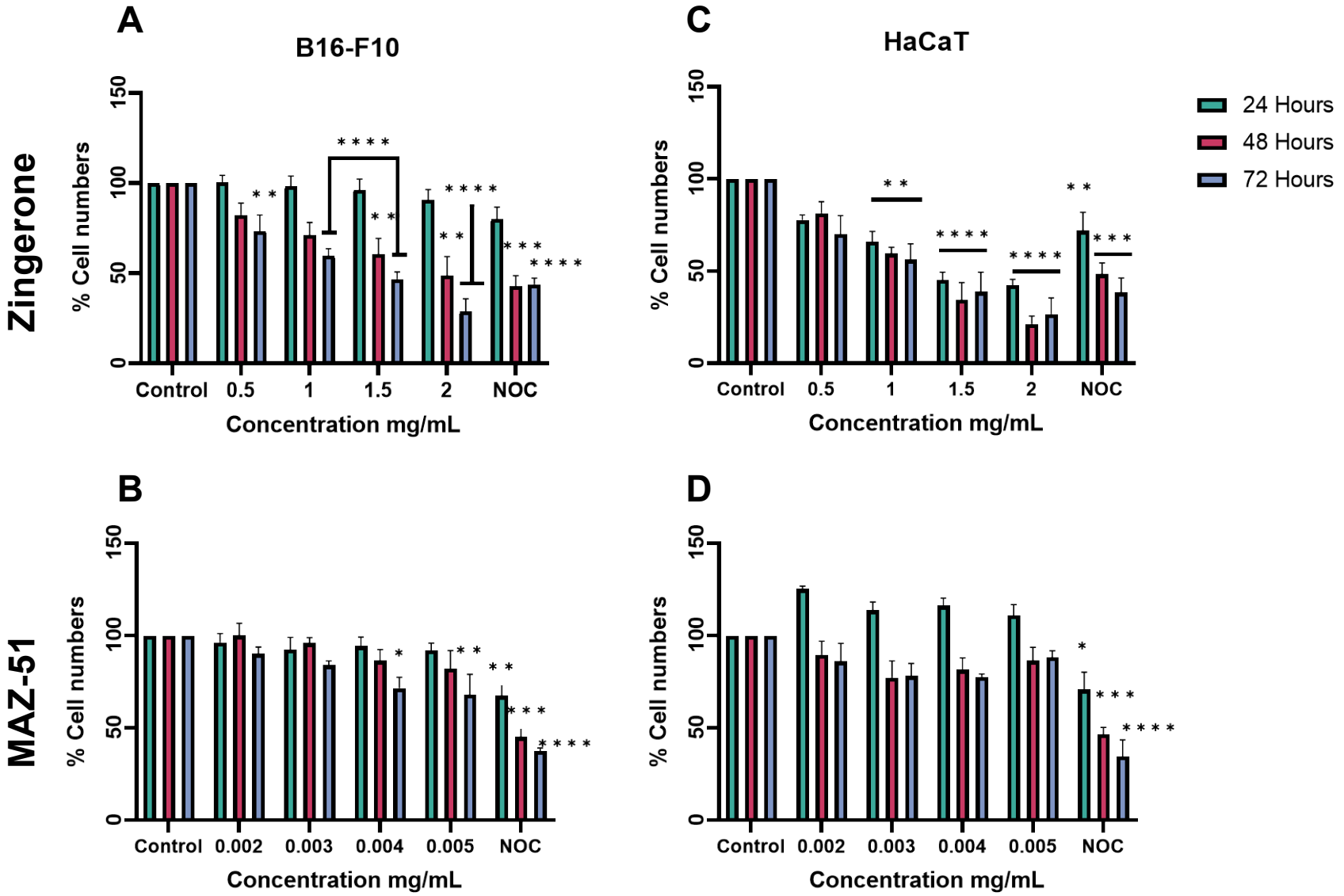

Figure 1: The effect of zingerone and MAZ-51 on the cell number of the B16-F10 (A&B) and HaCaT (C&D) cells at 24, 48 and 72 hours. * p<0.5, ** p<0.01, *** p<0.001, **** p<0.0001 indicates significance in comparison to the control.

Table 1:Table depicting the maximal inhibitory concentrations (mg/mL) for the B16-F10 and HaCaT cell lines at 24, 48 and 72 hours

	B16-F10			**HaCaTs**		
	24h	**48h**	**72h**	**24h**	**48h**	**72h**
MAZ-51	0.05428	0.03162	0.01204	1.131E+51	1.557E+46	0.0176
Zingerone	27.9	2.199	1.219	1.548	1.074	1.027

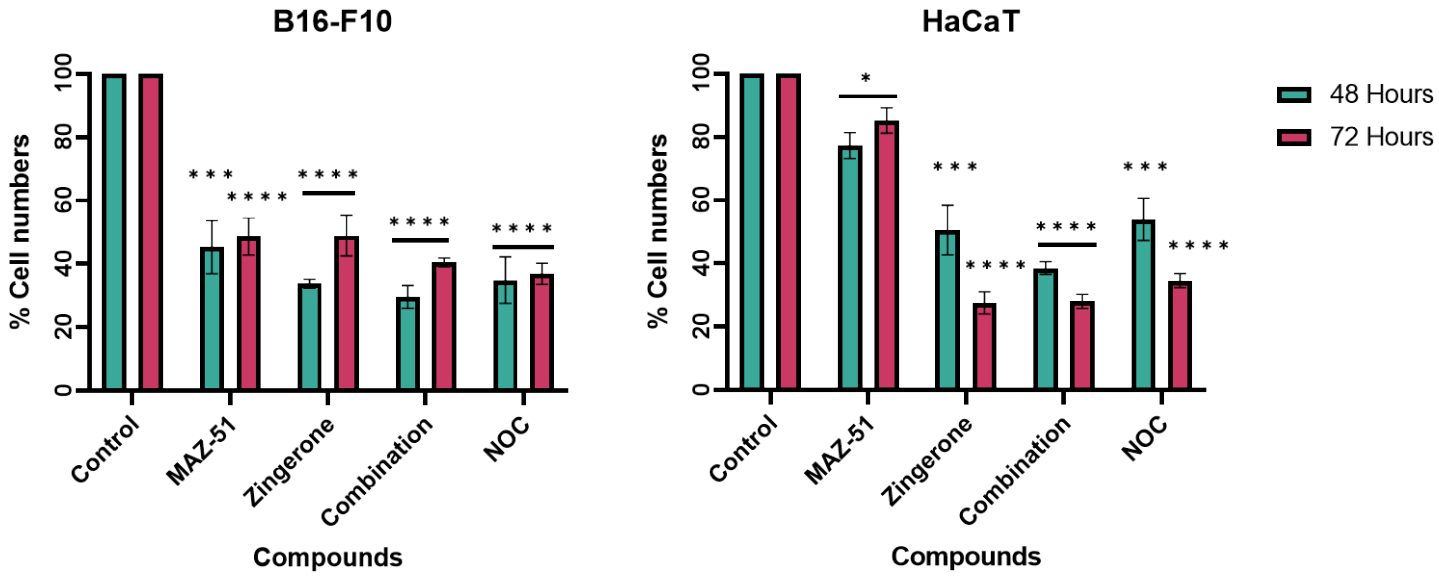

Figure 2:The effect of the half maximal inhibitory concentrations of MAZ-51 and zingerone on the B16-F10 and HaCaT cells at 48 and 72 hours. * p<0.5, ** p<0.01, *** p<0.001, **** p<0.0001 indicates significance in comparison to the control

Table 2: Table comparing the effects of zingerone at IC_{50}, IC_{25} and combination treatment on percentage cell numbers on the B16-F10 and HaCaT cell lines at 48 and 72 hours. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 indicates significance in comparison to the control.

		Treatment Compounds			
		Zingerone		MAZ-51 + Zingerone	
Timelines	**Cell lines**	Zn-IC_{50}	Zn-IC_{25}	MAZ-51 IC_{50} + Zn IC_{50}	MAZ-51 IC_{50} + Zn IC_{25}
48 hours	B16-F10	34% ****	47% ****	30% ****	46% ****
	HaCaTs	50% ***	51% **	39% ****	52% **
72 hours	B16-F10	49% ****	53% ****	41% ****	60% ***
	HaCaTs	28% ****	61% **	28% ****	61% **

3.2 The effect of zingerone and MAZ-51 on cell morphology

3.2.1 Polarization optical differential inferential contrast imaging of B16-F10 and HaCaT cells treated with zingerone and MAZ-51

In PlasDIC micrographs, B16-F10 melanoma cells treated with zingerone at IC_{50} values at 48 and 72 hours exhibited apoptotic bodies and protrusions (Figure 3 and 4). Samples treated with MAZ-51 exhibited a decrease in density and cell rounding (Figure 3 and 4). Combination treatment at IC_{50} with MAZ-51 and zingerone resulted in apoptotic bodies and protrusions (Figure 3 and 4). NOC-treated samples revealed cell debris, cell swelling and a decrease in density (Figure 3 and 4).

HaCaT cells treated with zingerone at IC_{50} at 48 and 72 hours showed protrusions whereas, exposure to MAZ-51 led to the development of cell rounding and cell swelling (Figure 3 and 4). Combination treatment at IC_{50} resulted in protrusions. NOC treated cells revealed cell swelling and debris (Figure 3 and 4).

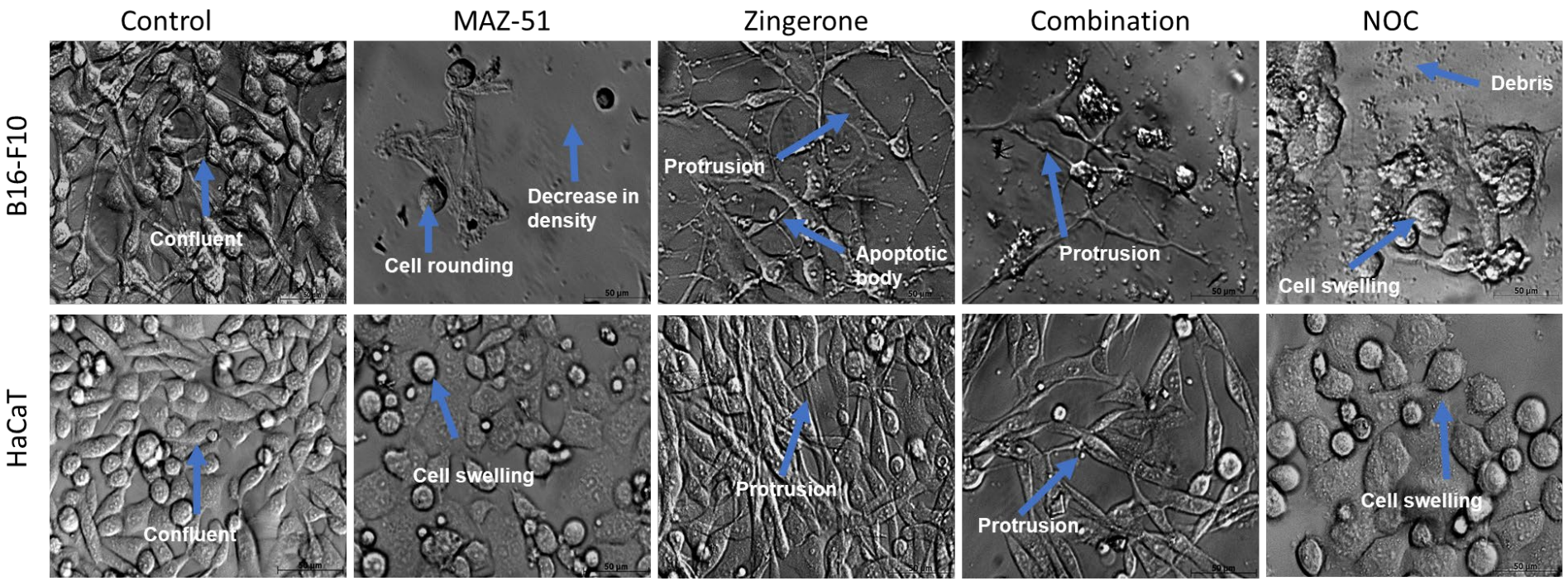

Figure 3: Polarization optical differential inferential contrast imaging of B16-F10 and HaCaT cells at 48 hours. Images were taken at 40× magnification. Scale bar- 50 µm

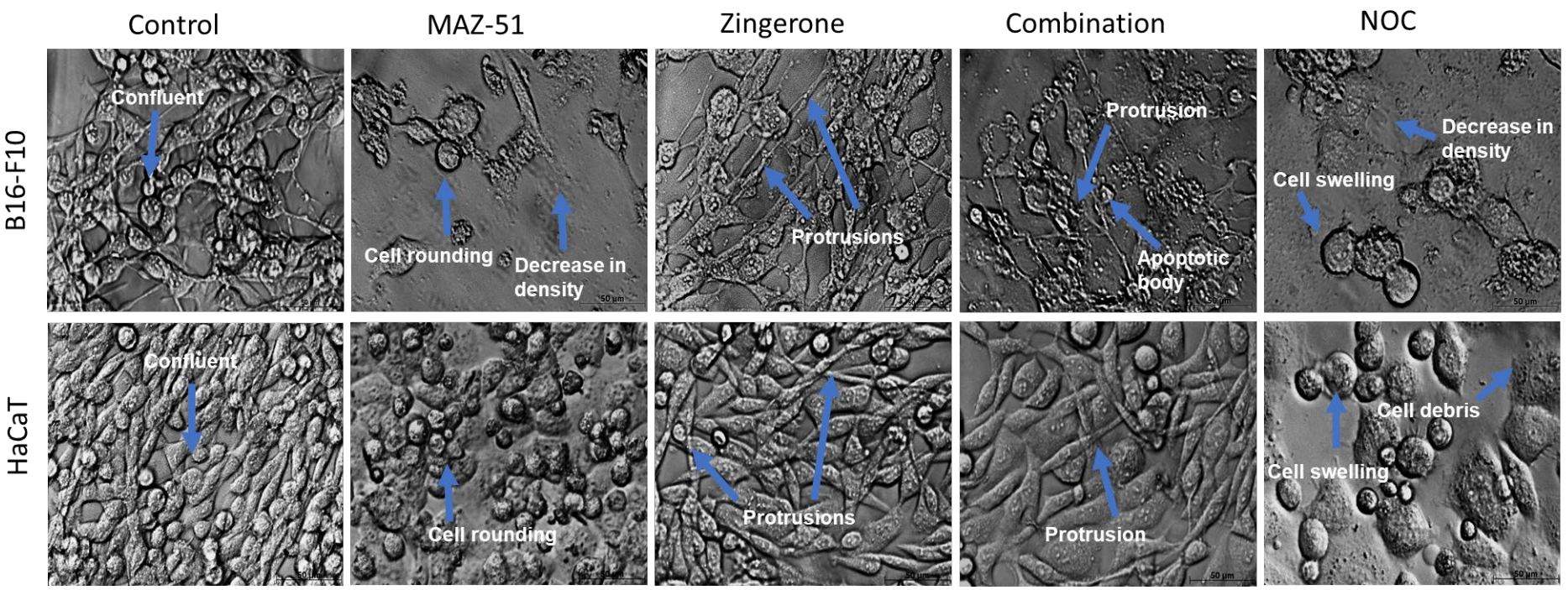

Figure 4: Polarization optical differential inferential contrast imaging of B16-F10 and HaCaT cells at 72 hours. Images were taken at 40× magnification. Scale bar- 50 µm

3.2.2 Hematoxylin and eosin staining of B16-F10 and HaCaT cells treated with zingerone and MAZ-51

H&E-stained control B16-F10 melanoma cells exhibited confluent cells (Figure 5 and 6). Treatment with zingerone revealed protrusions and apoptotic bodies (Figure 5 and 6). MAZ-51-treated cells exhibited pyknosis, cell swelling and a decrease in density (Figure 5 and 6). Combination treatment showed a decrease in density, protrusions and cell swelling (Figure 5 and 6). NOC-treated samples resulted in cell swelling, membrane blebbing and karyorrhexis (Figure 5 and 6).

H&E-stained HaCaT control cells at 48 and 72 hours exhibit confluent cells (Figure 5 and 6). Cells treated with zingerone and a combination of zingerone and MAZ-51 exhibited protrusions. MAZ-51 treated cells resulted in karyorrhexis (Figure 5 and 6). NOC-treated cells revealed a decrease in density, cell swelling, membrane blebbing and karyorrhexis (Figure 5 and 6)

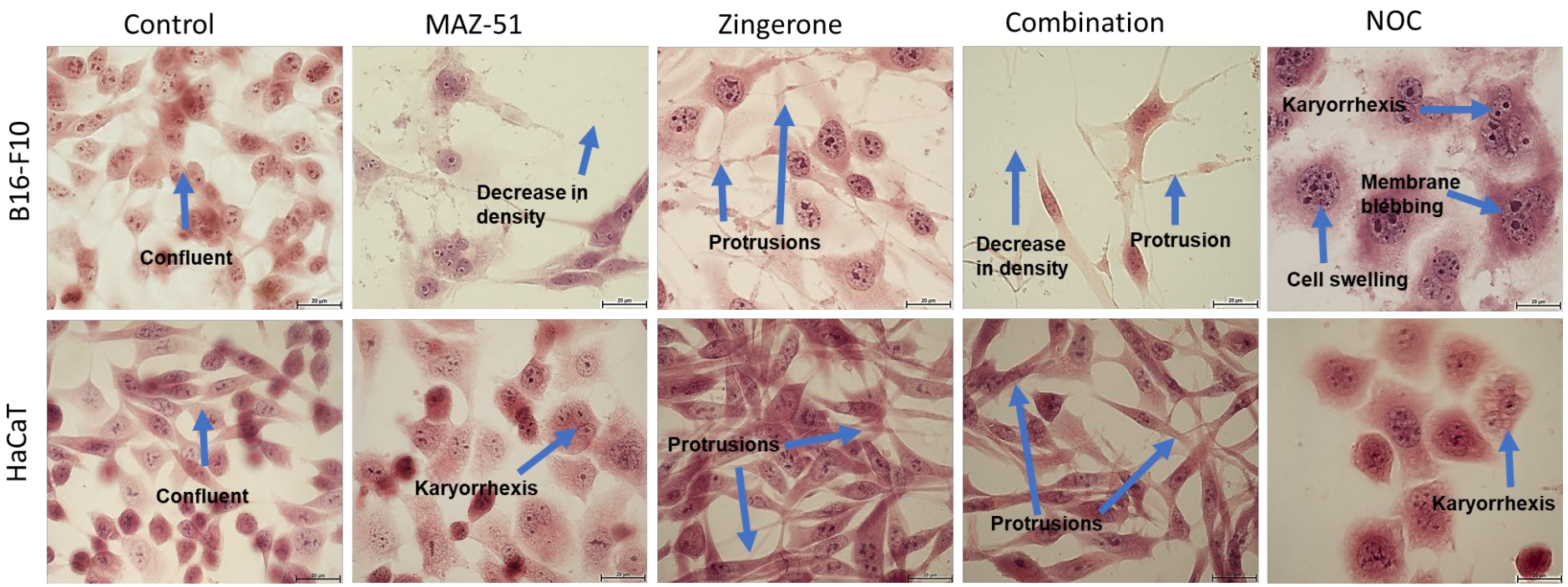

Figure 5: Hematoxylin and eosin staining of B16-F10 and HaCaT cells at 48 hours. Images were taken at 100× magnification. Scale bar- 20 µm.

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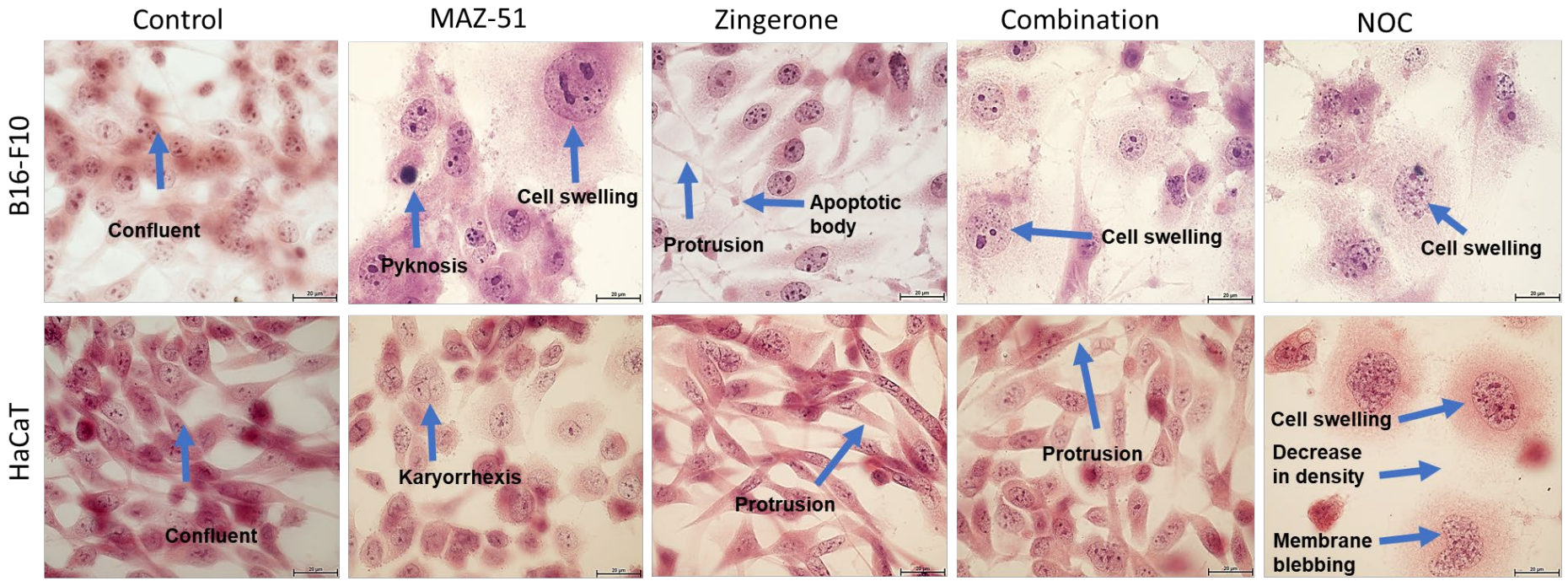

Figure 6: Hematoxylin and eosin staining of B16-F10 and HaCaT cells at 72 hours. Images were taken at 100× magnification. Scale bar- 20 µm

3.3 Cell cycle analysis of B16-F10 and HaCaT cells

3.3.1 MAZ-51 and zingerone resulted in a cell cycle arrest

Cell cycle analysis showed that there was a significant increase in the number of cells in the sub-G_1 phase in the B16-F10 cells treated with MAZ-51, zingerone, combination of zingerone and MAZ-51 at IC_{50} and NOC at 48 hours. This was followed by a decrease in the number of cells in the G_1 phase ($p<0.05$; Figure 7). At 48 hours, HaCaT cells exhibited a significant increase ($p<0.001$) in the number of zingerone and combination treated cells in the sub-G_1 phase followed by a decrease in the number of cells in the G_1 phase ($p<0.05$; Figure 7). NOC-treated cells exhibited a significant increase ($p<0.001$) in the number of cells in the G_2/M phase in the B16-F10 cells at 48 hours and HaCaT cells at 48 and 72 hours, indicative of a G_2/M cell cycle block.

MAZ-51, zingerone and combination treatment at IC_{50} had no significant effect on the B16-F10 cells at 72 hours. HaCaT cells treated with zingerone and combination treatment at IC_{50} resulted in a significant increase ($p<0.01$) in cell numbers in the sub-G_1 phase at 72 hours. This was followed by a significant decrease ($p<0.001$) in cell numbers in the G_1 phase (Figure 7). MAZ-51 treated HaCaT cells resulted in a significant decrease ($p<0.001$) in cell numbers in the G_1 phase at 72 hours.

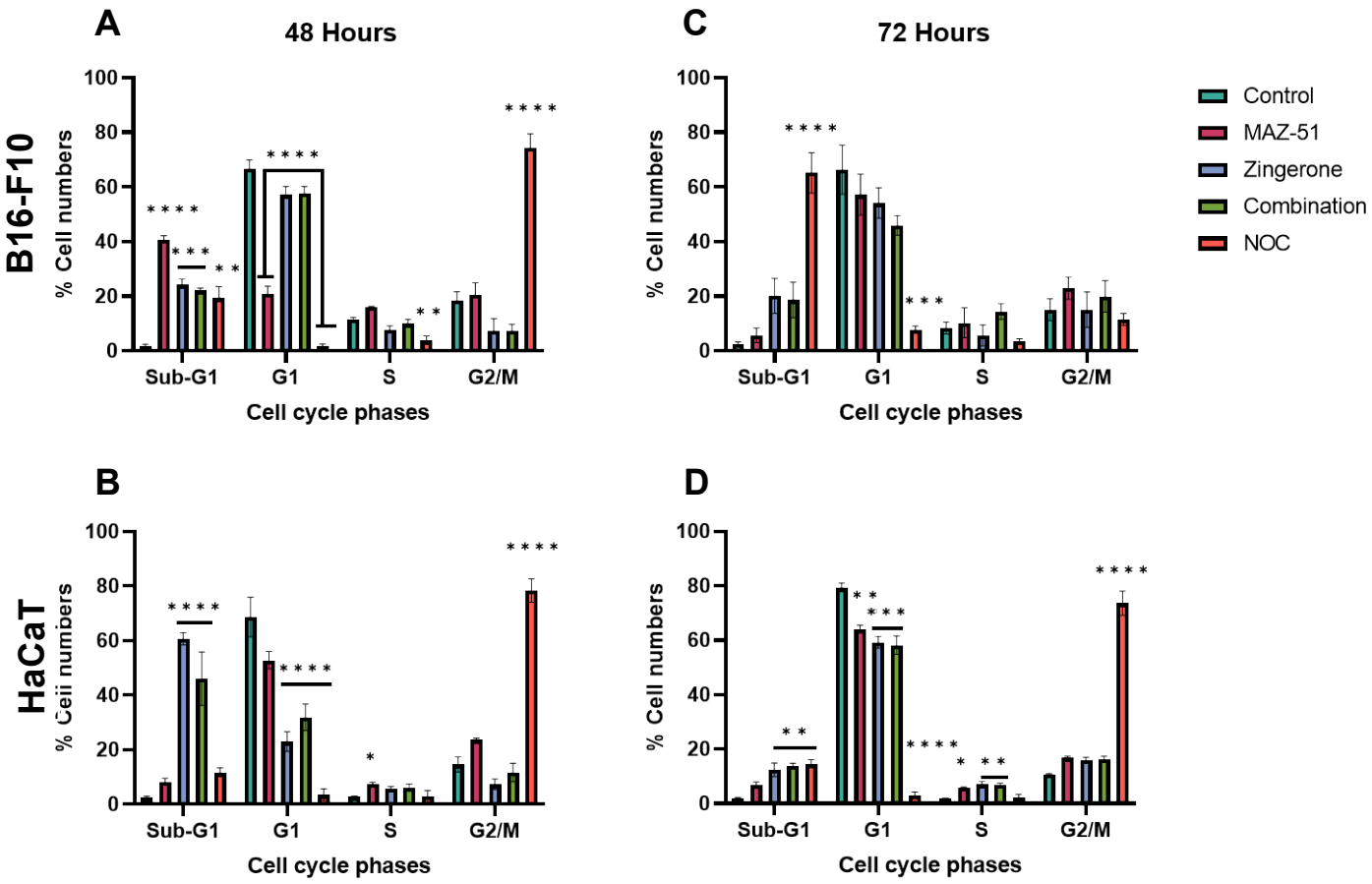

Figure 7: The effect of the half maximal inhibitory concentrations of MAZ-51 and zingerone on the B16-F10 and HaCaT cells on cell cycle progression at 48 and 72 hours. * p< 0.05, ** p<0.01, *** p<0.001, **** p<0.0001 indicates significance in comparison to the control

3.4 The effects of vascular endothelial growth factor on the B16-F10 and HaCaT cells

B16-F10 cells treated with MAZ-51 and VEGF at 20 and 60 ng/mL (MAZ-51/VEGF+) resulted in an increase in the percentage number of cells at 48 and 72 hours in comparison to cells that were only treated with MAZ-51 (MAZ-51/VEGF-) ($p<0.05$; Table 3&4). However, MAZ-51/VEGF+ resulted in a decrease in cell numbers in the HaCaT cells at 48 and 72 hours (Table 3&4). Zingerone/VEGF+ and combination treatment/VEGF+ treated B16-F10 cells resulted in a decrease in percentage cell numbers compared to the VEGF- cells at 48 and 72 hours ($p<0.0001$; Table 3&4). HaCaT cells exhibited a decrease in percentage cell numbers in zingerone/VEGF+ and combination/VEGF+ cells at 48 hours ($p<0.0001$; Table 3&4) however, an

increase in cell numbers was observed at 72 hours in comparison to the VEGF- cells ($p<0.001$; Table 3&4).

Table 3: Table depicting the effect of vascular endothelial growth factor at 20 ng/mL on percentage cell numbers in the B16-F10 and HaCaT cells at 48 and 72 hours. * p< 0.05, ** p<0.01, *** p<0.001, **** p<0.0001 indicates significance in comparison to the control.

		Treatment Compound					
		MAZ-51		Zingerone		MAZ-51 + Zingerone	
Timelines	**Cell lines**	VEGF -	VEGF +	VEGF -	VEGF +	VEGF -	VEGF +
48 hours	B16-F10	45% ***	69% *	34% ****	19% ****	30% ****	18% ****
	HaCats	77% *	70% **	51% ***	30% ****	39% ****	31% ****
72 hours	B16-F10	48% ****	64% **	49% ****	35% ****	41% ****	35% ****
	HaCaTs	85% *	76%	28% ****	43% ***	28% ****	35% ****

Table 4: Table depicting the effect of vascular endothelial growth factor at 60 ng/mL on percentage cell numbers in the B16-F10 and HaCaT cells at 48 and 72 hours. * p< 0.05, ** p<0.01, *** p<0.001, **** p<0.0001 indicates significance in comparison to the control.

		Treatment Compounds					
		MAZ-51		Zingerone		MAZ-51 + Zingerone	
Timelines	**Cell lines**	VEGF -	VEGF +	VEGF -	VEGF +	VEGF -	VEGF +
48 hours	B16-F10	45% ***	66% ***	34% ****	19% ****	30% ****	18% ****
	HaCaTs	77% *	68% *	51% ***	38% ****	39% ****	43% ****
72 hours	B16-F10	48% ****	65% **	49% ****	39% ****	41% ****	39% ****
	HaCaTs	85% *	80%	28% ****	45% ****	28% ****	37% ****

4. Discussion

Skin cancer continues to evolve, as such, there is a need to develop novel treatment strategies. Phytochemicals have acquired substantial attention as adjuvant therapy to inhibit cancer growth without eliciting cytotoxic effects on noncancerous cells.[1] Therefore, this study investigated the individual and combined effects of MAZ-51 and zingerone on melanoma cell proliferation. The study findings indicate that MAZ-51 and zingerone are cytotoxic to the B16-F10 melanoma cells. In addition, the compounds induced morphological changes indicating cell death and resulted in a cell-cycle arrest.

Consistent with results obtained by Scholtz and Mabeta who recorded a significant decrease in the cell viability of endothelial and B16-F10 melanoma cells treated with MAZ-51,[33] this study observed a significant decrease in cell numbers in B16-F10 cells at 72 hours at concentrations of 0.004 and 0.005 mg/mL. MAZ-51 had no significant effect on the HaCaT cells at 24, 48 and 72 hours thus, demonstrating differential toxicity in the cell lines. This was further confirmed by the experimental IC_{50} data where MAZ-51 reduced cell numbers to 45% ($p<0.001$) and 49% ($p<0.0001$) at 48 and 72 hours respectively in the B16-F10 cells. In the HaCaT cells, MAZ-51 reduced cell numbers to 77% ($p<0.05$) and 85% ($p<0.05$) at 48 and 72 hours respectively. This data indicates that MAZ-51 is cytotoxic however, it is more potent in the B16-F10 cells compared to the HaCaT cells, suggesting its specificity to transformed cancerous cells.

Current literature supports the use of phytochemicals as potential cancer treatments, suggesting that they will result in therapeutic effects, with limited cytotoxicity to noncancerous cells compared to current synthetic drugs.[34-35] However, this study observed that zingerone was more cytotoxic to the B16-F10 and HaCaT cells compared to treatment with MAZ-51. The literature also states that phytochemicals are required at higher concentrations to elicit therapeutic effects in cancer cells.[23-24] Findings from this study indicate that the increased concentrations of zingerone decreased the B16-F10 cell numbers. An enhanced cytotoxic effect was observed in the noncancerous HaCaT cell lines at IC_{50} at 48 and 72 hours, with enhanced toxicity observed at 72 hours. To combat this, the zingerone IC_{25} was determined and

increased the HaCaT cell number, indicating a potential therapeutic concentration. In addition, zingerone at 48 hours was more cytotoxic to the B16-F10 compared to at 72 hours. This demonstrates that treatment at 48 hours yielded optimal cytotoxic effects in the B16-F10 cells with reduced toxicity exerted on the HaCaT cells. In order to elucidate the additional characteristics of the compounds of interest, morphological studies were done on the B16-F10 and HaCaT cells treated with MAZ-51, zingerone combination of MAZ-51 and zingerone at IC_{50} at 48 and 72 hours.

Morphological observations using PlasDIC and H&E staining showed the formation of cellular protrusions and apoptotic bodies in zingerone-treated cells. This observation is in agreement with findings by Chu *et al.* who observed cytoskeleton elongations and dendrite-like structures in zingerone-treated B16-F10 cells.[36] Consistent with results recorded by Park *et al.* in glioma cells,[37] MAZ-51 treated B16-F10 and HaCaT cells exhibited cell rounding, karyorrhexis, pyknosis and cell swelling which is characteristic of apoptosis and necrosis. The morphology data indicated that MAZ-51 and zingerone are cytotoxic and induce structural changes that indicate cell death. Since the study observed structural changes that are characteristic of both apoptosis and necrosis, the type of cell death induced by the compounds needs to be further investigated. To further examine the effects of MAZ-51 and zingerone on melanoma growth and proliferation, flow cytometry analysis was used to determine the effects of the compounds on cell cycle progression.

Flow cytometry analysis exhibited a significant increase in the number of cells in the sub-G_1 phase of B16-F10 cells treated with MAZ-51 at 48 hours, and a non-significant effect was observed at 72 hours. MAZ-51 at 72 hours displayed non-significant effects on cell cycle progression in the B16-F10 cells. HaCaT cells treated with MAZ-51 at 48 and 72 hours exhibited a non-significant increase in cells in the sub-G_1 phase followed by a significant decrease in cell numbers in the G_1 phase at 72 hours. This indicates that MAZ-51 inhibits HaCaT cell proliferation without inducing cell death. These findings are supported by results obtained by Park *et al* who observed the effects of MAZ-51 on the rat C6 and human U251MG glioma cells where the cytotoxic effects of MAZ-51 where non-significant on the control cell lines.[37] This indicates that MAZ-51 possesses antiproliferative properties however, its cytotoxic properties target transformed cells. In addition, as MAZ-51 induced significant changes at 48 hours in the B16-F10 with limited toxicity observed in the HaCaTs at 48 hours, this indicates

that treatment at this timeline yields maximal results which is confirmed by the cell viability data.

Zingerone-treated B16-F10 cells resulted in a significant increase in the percentage of cells in the sub-G_1 phase at 48 hours with a less potent increase observed at 72 hours. Similar results were observed in zingerone-treated HaCaT cells. Choi *et al* observed an increase in the number of cells in prometaphase and a decrease in cells in metaphase in zingerone-treated neuroblastoma cells, indicating mitotic arrest.[15] This data indicates that zingerone is cytotoxic to the B16-F10 and HaCaT cells. The quantitative flow cytometry data is supported by the qualitative data, suggesting that zingerone is toxic to the cells and induces apoptosis.

Angiogenic and lymphangiogenic factors such as VEGFs and their corresponding receptors are dysregulated during carcinogenesis and thus, are critical targets for cancer therapy.[37] MAZ-51 inhibits the VEGF-C-induced phosphorylation of VEGFR-3, inhibiting proliferation.[10] This study observed an increase in cell numbers in MAZ-51/VEGF+ cells compared to MAZ-51/VEGF- in the B16-F10 cells. Even though this study utilised non-specific VEGF, the findings provide a potential mechanism of action utilised by the treating compounds to inhibit cell proliferation and survival. Given that MAZ-51 specifically targets VEGFR-3, the results suggest that the additional VEGF activates alternative signalling pathways such as VEGFR-2[10] that are activated in melanoma progression thus explaining the increase in cell numbers observed in MAZ-51/VEGF+ cells compared to MAZ-51/VEGF- cells. Conversely, zingerone-treated B16-F10 cells supplemented with VEGF caused a decrease in cell numbers compared to VEGF- cells. This is supported by results by Kung *et al.* who recorded a decrease in VEGF and VEGFR expression in endothelial cells treated with zingerone nanoparticles.[38] This data suggests that zingerone is cytotoxic in the presence of VEGF indicating that it diminishes proliferation and survival by targeting VEGFs.

Literature recommends the use of phytochemicals as alternative treatment for cancer due to their potential therapeutic value. Literature has highlighted that the individual use of phytochemicals often exhibits limited bioavailability and requires high concentrations to induce cytotoxic effects. When combined with pharmacological agents, phytochemicals demonstrate enhanced anti-inflammatory and antioxidant properties.[1] This study investigated the combined effects of MAZ-51 and zingerone on melanoma cell proliferation. The findings revealed that combined treatment

significantly decreased cell numbers, induced morphological changes such as cellular protrusions and cell rounding and caused a mitotic block in the B16-F10 melanoma cells.

However, the combination treatment of MAZ-51 and zingerone was also cytotoxic to the HaCaT cells, contradicting the anticipated protective effects typically associated with the use of phytochemicals when used alongside pharmacological agents. Furthermore, the combination index (CI) exceeded one, indicating an antagonistic interaction between MAZ-51 and zingerone.[39] To address this, the study evaluated the combination treatment of zingerone at IC_{25} and MAZ-51 at IC_{50}. This approach demonstrated reduced toxicity to both B16-F10 melanoma and HaCaT noncancerous cells at 48 and 72 hours compared to treatment at IC_{50} for both agents. This indicates that zingerone at reduced concentrations is less toxic to both cancerous and noncancerous cells. Although the reduced toxicity to the noncancerous cells is a desirable effect, the CI remains above one and indicates antagonism between zingerone and MAZ-51. This antagonistic effect may be attributed to phytochemical-drug interaction that alter drug metabolising enzymes or transport proteins, modification of the chemotherapeutic by the phytochemical and incompatibility of the two drugs resulting in the modification of the physical properties of the drugs such as drug solubility and stability.[40] Although previous studies have reported additive or synergistic effects of phytochemicals with pharmacological agents, supporting their role as adjuvant therapies, the findings of this study emphasise the importance of carefully evaluating drug interactions, delivery mechanisms and compound compatibility.[41-42] Future studies investigating the anticancer effects of MAZ-51 and zingerone should consider an individual compound approach to optimise therapeutic outcomes.

5. Conclusion

In conclusion, the findings of this study demonstrate that MAZ-51 and zingerone effectively inhibit melanoma cell proliferation and survival, with zingerone showing promise as a potential therapeutic agent for melanoma treatment. While cytotoxic effects were observed at zingerone IC_{50} in both B16-F10 and HaCaT cells, treatment at IC_{25} significantly inhibited B16-F10 cell proliferation while exhibiting reduced toxicity in HaCaT cells. The results underscore the potential of zingerone as an alternative

treatment option. However, due to the observed antagonistic interaction between MAZ-51 and zingerone both at IC_{50} and IC_{25}, future studies should investigate the individual effects of MAZ-51 at IC_{50} and zingerone at IC_{25}. Additionally, further studies should investigate the effects of zingerone on TGF-β1-induced expression of VEGF-C/D, focal adhesion kinase and paxillin as these proteins are implicated in melanoma growth and metastasis.

6. Author contribution

Kganya Letsoalo: Experimental and data analysis (lead); conceptualization (equal); writing – original draft (lead); writing – review and editing (equal). **Charlise Basson:** Writing – review and editing (equal). **Trevor Nyakudya:** Conceptualization (equal); writing – review and editing (equal). **Yvette Hlophe:** Conceptualization (equal); writing – review and editing (equal).

7. Funding statement

The research supporting this review was funded by National Research Foundation (NRF) of South Africa (N1F580 A1F685) and the University of Pretoria's Postgraduate funding scheme.

8. Conflict of interest

The authors declare no conflict of interest

9. Data availability

The data supporting this study is available upon request

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Chapter 5: General discussion and conclusion

5.1 Discussion

Cancer is an aggressive malignancy that is continuously transforming and skin cancer and melanoma incidence rates continue to increase, which necessitates the development of various treatment strategies.[1,11] Despite the advancement in cancer research and improved treatment strategies, cancer incidence remains high with current treatment resulting in cytotoxic effects on noncancerous cells.[21] To address these unintended effects associated with cytotoxic drugs, this study investigated the individual and combined efficacy of MAZ-51, a VEGFR-3 inhibitor, and zingerone, a bioactive compound, on melanoma cell proliferation.

This study employed several methods to investigate the individual and combined effects of MAZ-51 and zingerone on melanoma cell proliferation and survival. Cytotoxicity was assessed using crystal violet analysis at 24, 48 and 72 hours. Treatment at 48 and 72 hours significantly inhibited melanoma cell proliferation and subsequent assays were performed at these time points. Structural changes were examined using PlasDIC imaging and H&E staining, while the effects of the compounds on cell cycle progression were analysed using flow cytometry. Since VEGFs are dysregulated during malignancy, this study also investigated the effects of MAZ-51 and zingerone on melanoma cell proliferation in the presence of VEGF.

The experimental IC_{50} data revealed that both MAZ-51 and zingerone significantly reduced cell numbers at 48 and 72 hours. MAZ-51 and zingerone induced structural changes indicative of cell death. This was confirmed by the increase in cell numbers in the sub-G_1 phase of zingerone-treated B16-F10 (48 hours) and HaCaT cells (48 and 72 hours), suggesting that zingerone results in apoptosis. Similar to zingerone, MAZ-51 increased cell numbers in the sub-G_1 phase of B16-F10 cells at 48 hours and no significant effect observed at 72 hours. In addition, MAZ-51 had no significant effect on the HaCaT cell cycle progression at 48 hours. However, treatment with MAZ-51 resulted in an increase in cell numbers in the G_1 phase, indicating that it induces a mitotic block in the HaCaT cells. VEGF co-treatment experiments revealed differential effects of the two compounds. MAZ-51-treated B16-F10 cells (MAZ-51/VEGF+) showed increased proliferation in the presence of VEGF, confirming its specificity for the VEGFR-3 rather than VEGF. In contrast, zingerone demonstrated an inhibitory

effect on B16-F10 cell proliferation when co-treated with VEGF at 48 hours, suggesting that it targets VEGFs directly. These findings highlight the potential of MAZ-51 and zingerone as complementary agents in melanoma therapy, with distinct mechanisms of action that could be further optimised for targeted treatment strategies.

This study observed that treatment with MAZ-51 effectively induced cytotoxicity in the B16-F10 melanoma cells, with minimal cytotoxicity effects in the HaCaTs compared to zingerone and their combination treatment. While these results challenge our initial hypothesis that phytochemicals are expected to be less toxic than synthetic drugs, it is important to note that current treatment strategies yield minimal long-term therapeutic effects. Conventional therapies such as dacarbazine offer limited long-term benefits with a one-year survival rate of 27%.[43] Even polytherapy combining multiple cytotoxic drugs results in nonsignificant survival rates.[44]

In addition to therapeutic efficacy, the environmental impact of current cancer drugs remains a growing concern.[1] Metabolites of cytotoxic agents are frequently detected in wastewater. Since these drugs are genotoxic, they alter the metabolic activity of aquatic organisms therefore, reducing their viability.[1] These disruptions in turn affects the ecological and socioeconomic effects, thus compromising Sustainable Development Goal 8 - *Decent Work and Economic Growth* as many communities worldwide rely on the aquatic ecosystems for their livelihoods and to generate income.[1] Therefore, there is a need to develop alternative treatment and complementary strategies. Despite the toxicity observed in zingerone-treated cells at IC_{50}, treatment at IC_{25} increased cell numbers in the HaCaT cells, suggesting potential therapeutic value for this compound. Although this study did not observe synergistic activity between MAZ-51 and zingerone, existing literature have recorded synergism in phytochemical-chemotherapeutic treatments.[41-42] These findings support the ongoing investigation of phytochemicals as adjuvant therapies, which could enhance efficacy while potentially reducing the side-effects associated with conventional cancer therapeutic strategies.

5.2 Limitations and future studies

In this study, the human skin keratinocyte HaCaT cells were used as the control noncancerous cell line. Considering that melanoma is caused by mutations in the

melanin producing melanocytes,[1] future studies should investigate the effect of the compounds on human melanocyte cell lines.

This study investigated the effects of generic VEGF on melanoma cell numbers. However, melanoma progression is characterised by the specific expression of VEGF-C which specifically targets VEGFR-2 and 3.[10] The current results provide a potential mechanism of action to inhibit proliferation. To elucidate the effects of the compounds on melanoma lymphangiogenesis, future studies should investigate the effects of MAZ-51 and zingerone on the expression of VEGF-C and its associated receptors, VEGFR-2 and 3.

This study also observed a combination index (CI) that is greater than one for both MAZ-51 IC_{50} – zingerone IC_{50} and MAZ-51 IC_{50} – zingerone IC_{25} combination treatment. A CI>1 indicates that the compounds are antagonistic. Therefore, future studies should investigate MAZ-51 and zingerone individually. Since zingerone IC_{25} resulted in reduced toxicity in the HaCaT cells in comparison to zingerone IC_{50}, future studies should investigate the effects of zingerone at IC_{25}.

Melanoma relies on the interplay of different signalling pathways such as the VEGFR-3/VEGF-C and CXCR4/CXCL12 pathways that promote growth, adhesion and metastasis.[10] The abovementioned pathways are also activated by transforming growth factor beta-1 (TGF-β1) which also directly influences the expression of adhesion proteins (focal adhesion kinase and paxillin).[10] Therefore, future studies should investigate the effects of MAZ-51 and zingerone on the expression of TGF-β1, focal adhesion kinase, paxillin as well as the ability to inhibit the activation of the CXCR4/CXCL12 and VEGFR-3/VEGF-C signalling pathways.

5.3 Conclusion

In conclusion, this study provided novel insight on MAZ-51 and zingerone highlighting their antiproliferative effects on melanoma cells and revealed the potential of phytochemicals in cancer therapy. Despite the toxicity observed at zingerone IC_{50} values, treatment with zingerone IC_{25} was cytotoxic to the B16-F10 cells with a decrease in toxicity observed in the HaCaT cells. Further research is warranted to optimise dosage regimens, explore synergistic combinations, and minimise environmental and ecological impacts, thereby contributing to more sustainable and effective cancer treatment strategies.

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Appendix

Section A: Supplementary data

This data represents results for the RAW 264.7 macrophage cell line. This cell line was initially used as the control cell line for the study. Upon the inclusion of an appropriate human skin keratinocyte (HaCaT) control cell line, subsequent experimental assays were performed on the B16-F10 and HaCaT cell lines.

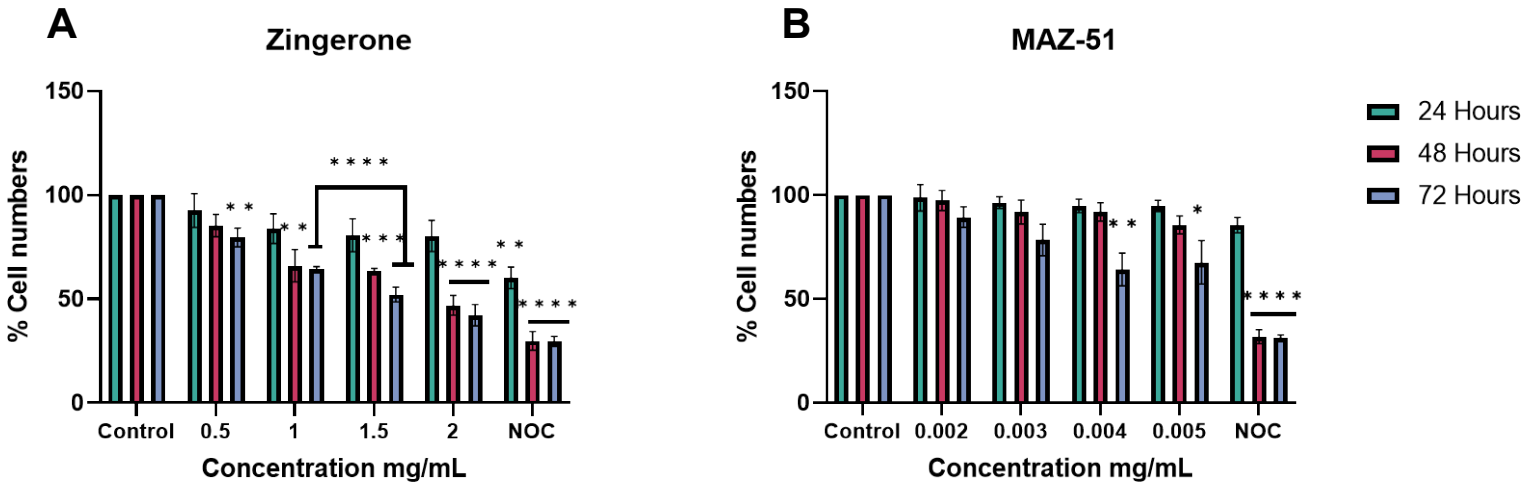

Figure 8: The effect of zingerone and MAZ-51 on the cell numbers of the RAW 264.7 cells at 24, 48 and 72 hours. * p<0.5, ** p<0.01, *** p<0.001, **** p<0.0001 indicates significance in comparison to the control.

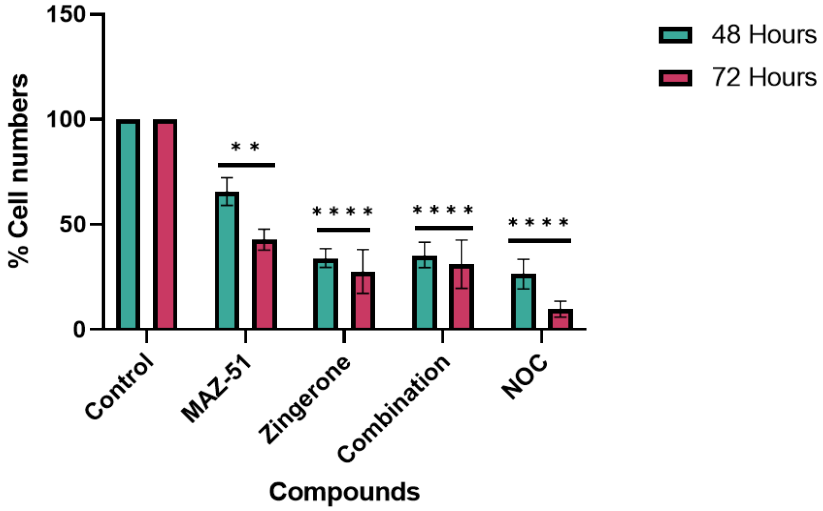

Figure 9 : The effect of the half maximal inhibitory concentrations of MAZ-51 and zingerone on the RAW 264.7 cells at 48 and 72 hours. * p<0.5, ** p<0.01, *** p<0.001, **** p<0.0001 indicates significance in comparison to the control.

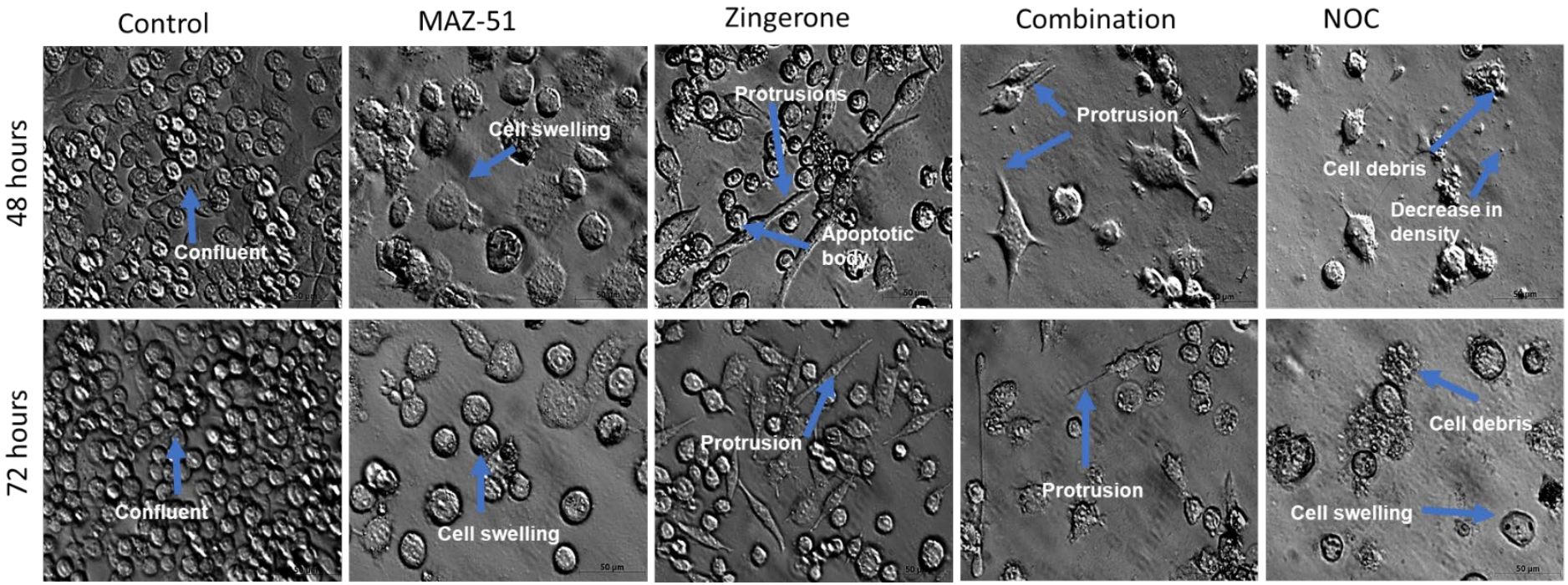

Figure 10: Polarization optical differential inferential contrast imaging of the RAW 264.7 cells at 48 and 72 hours. Images were taken at 40× magnification. Scale bar- 50 µm

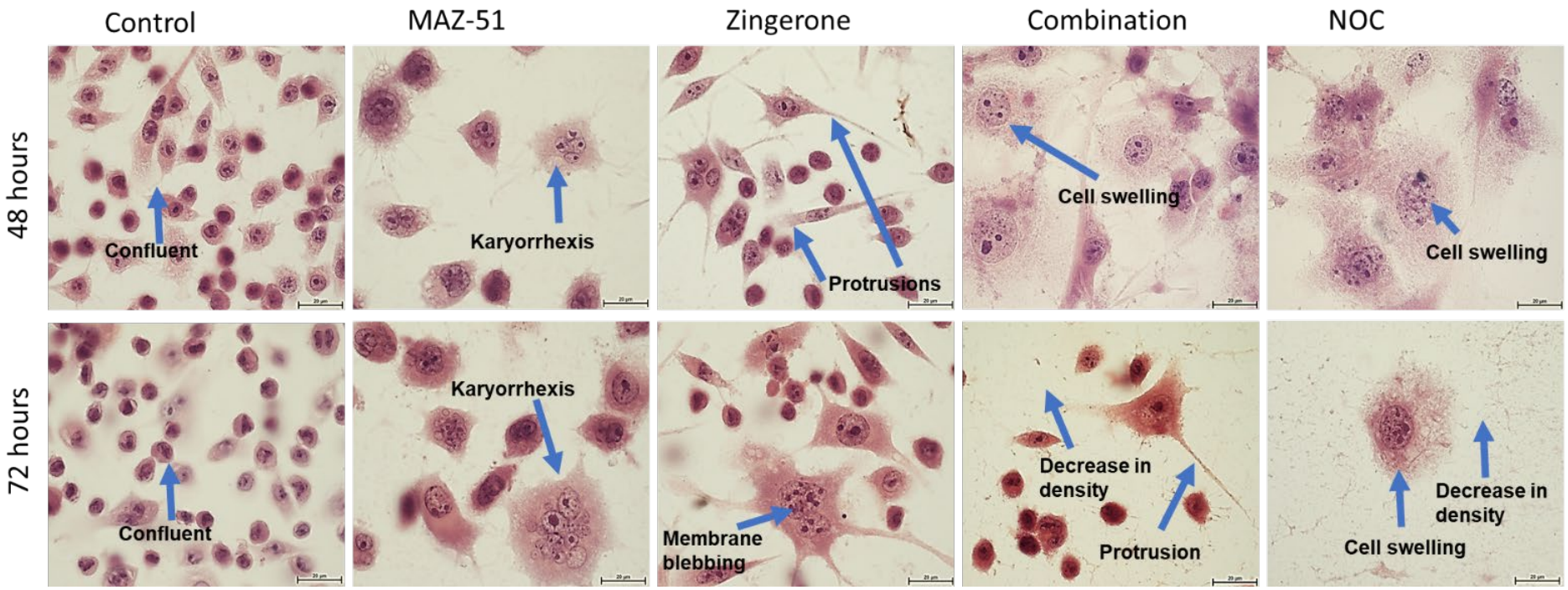

Figure 11: Hematoxylin and eosin staining of the RAW 264.7 cells at 48 and 72 hours. Images were taken at 100× magnification. Scale bar- 20 µm.

Section B: Ethical clearance

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 18 March 2022 and Expires 18 March 2027
- IORG #: IORG0001762 OMB No. 0990-0279 Approved for use through [illegible] 2025 and Expires: 07/20/2028.

Faculty of Health Sciences **Research Ethics Committee**

19 July 2024

Approval Certificate
Annual Renewal

Dear Ms RK Letsoalo,

Ethics Reference No.: 192/2022 – Line 3
Title: The effect of combination treatment of vascular endothelial growth factor receptor 3 inhibitor, MAZ-51, and zingerone on melanoma cell proliferation

The **Annual Renewal** as supported by documents received between 2024-06-18 and 2024-07-17 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2024-07-17 as resolved by its quorate meeting.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2025-07-19.
- The Research Ethics Committee (REC) must monitor your research continuously. To this end, you must submit as may be applicable for your kind of research:
  a) annual reports;
  b) reports requested *ad hoc* by the REC;
  c) all visitation and audit reports by a regulatory body (e.g. the HPCSA, FDA, SAHPRA) within 10 days of receiving one;
  d) all routine monitoring reports compiled by the Clinical Research Associate or Site Manager within 10 days of receiving one.
- The REC may select your research study for an audit or a site visitation by the REC.
- The REC may require that you make amendments and take corrective actions.
- The REC may suspend or withdraw approval.
- Please remember to use your protocol number (192/2022) on any documents or correspondence with the Research Ethics Committee regarding your research.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

On behalf of the FHS REC, Professor Theresa (TM) Rossouw
***Chairperson**: Faculty of Health Sciences Research Ethics Committee*

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

Research Ethics Committee
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Gezina 0031, South Africa
Tel +27 (0)12 356 3084
Email: deepeka.behari@up.ac.za
www.up.ac.za

Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

Section C: Letter of clearance from the biostatistician

Date: 19 / 04 / 2022

LETTER OF CLEARANCE FROM THE BIOSTATISTICIAN

This letter is to confirm that, Remmotile Kganya Letsoalo from UP **discussed with me the study titled:** The effect of combination treatment of vascular endothelial growth factor receptor 3 inhibitor, MAZ-51, and zingerone on melanoma cell proliferation

I hereby confirm that I am aware of the project and also undertake to assist, if possible, with the Statistical analysis of the data generated from the project.

The analytical tool(s) that will be used is(are): Data summary will report, but may not be restricted to, mean, standard deviation and if feasible 95% confidence interval (CI) by level of factors and in combination of factors. Cell viability, morphology, apoptosis, cell cycle and protein quantification data will be analysed in *three/two factor analyses of variance* with Stata Release 17, or later, statistical software.

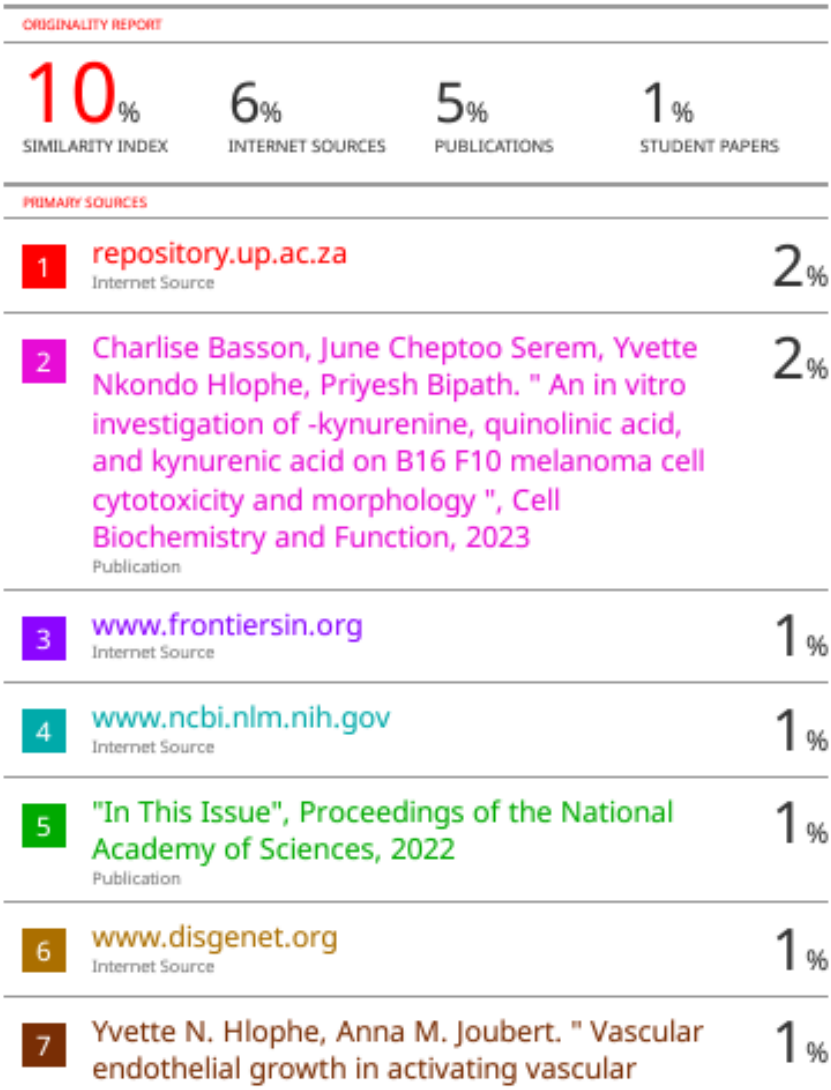
The experiments will be conducted as two factor study designs where treatment has three levels (MAZ-51; Zingerone; MAZ-51 & Zingerone) and two cell lines (B16-10; RAW264.7) for morphology, apoptosis, cell cycle and protein quantification. Cell viability will have an additional factor time at three levels (24h; 48h; 72h). For morphology, apoptosis, cell cycle and protein quantification there will be six observations per treatment-cell combination, while for cell viability three observations will be made for each treatment-cell-time combination. These two designs will respectively have 30 and 40 degrees of freedom (df) for residual mean squares, and hence meets the requirement of at least 30 df. Testing will be done at the 0.05 level of significance and the family-wise error will be maintained for the pairwise comparisons of the three levels for time.

Signature 

PJ Becker (Tel: 012-319-2203**)**

Research Office, Faculty of Health Sciences

Section D: Turnitin report



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Section E: Proof of manuscript submission

09-Dec-2024

Dear Dr. Letsoalo:

Your manuscript entitled "Exploring phytochemical adjuvant therapy in melanoma treatment: The effects of MAZ-51 and zingerone on melanoma cell proliferation" by Letsoalo, Kganya; Basson, Charlise; Nyakudya, Trevor; Hlophe, Yvette, has been successfully submitted online and is presently being given full consideration for publication in Cancer Medicine.

The submitting author is the contact author for this paper, as such all further correspondence will be sent to Dr. Kganya Letsoalo.

Co-authors: Please contact the Editorial Office as soon as possible if you disagree with being listed as a co-author for this manuscript.

Your manuscript ID is CAM4-2024-12-6838.

Please mention the above manuscript ID in all future correspondence or when calling the office for questions.

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