A STUDY OF HOW TWO POTENTIAL ANTICANCER AGENTS AFFECT THE MAJOR CANCER HALLMARKS: APOPTOSIS AND METASTASIS

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To my amazing daughters Madawi, Sultana and my unborn baby boy, my greatest motivators, thank you for hanging in there with me

To my wonderful husband Dr. Saleh Alsaif, may the next chapter of our adventures involve less video calls

To my best friends Dr.Nadin Almosnid & Dr.Fatimah Hani, you made every day less bitter

To my beloved parents Najla Aldawood and Dr.Abdulaziz Alsaif,

I did it

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Lastly, to our great leader, who chose to invest in developing the minds of the youth, King Abdullah Bin Abdulaziz Al-Saudi, May God grant you mercy.

Abstract

Cancer is characterized by continuous growth of cells that divide uncontrollably, escaping the standard eliminating mechanism known as apoptosis, which is a form of programmed cell death or suicide. Although abundant efforts have been dedicated to finding an effective cure for cancer, treatment remains a difficult challenge. Many of the current cancer therapies, including chemotherapy, radiation, surgery, immunosuppression, cause many side effects for patients. For that reason, many researchers focus on natural products as they have been used for thousands of years to prevent many chronic diseases, including cancer. Cancer cells are distinguished from healthy cells by certain hallmarks, where each one of those hallmarks requires distinctive capabilities. These hallmarks were defined by Hanahan and Weinberg in 2000, and they include: sustaining proliferating signaling, evading growth suppressors, resisting cell death “apoptosis”, enabling replicative immortality, angiogenesis, activating invasion and metastasis, reprogramming of energy metabolism and evading immune destruction. In this study, we focused on the ability of natural products, either aurones or an oligostilbene, to affect two of the primary cancer hallmarks, inducing apoptosis and inhibiting cancer cell metastasis. Our findings suggest the ability of four aurone derivatives (A3, A5, A10 and A14) based upon five-membered heteroaromatic rings as well as *cis*- and *trans*- gnetin H, trimeric resveratrol oligostilbenes, to exhibit the most anticancer activity; growth inhibition was associated with induction of apoptosis as well as repression of cell motility at low concentrations. Together, these findings suggest that aurone derivatives and both *cis*- and *trans*- gnetin H could be potential leads for new anti-cancer agents.

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CHAPTER 1

INTRODUCTION

Cancer is defined as group of diseases characterized by uncontrolled growth and spread of abnormal cells. Cancer remains the most diagnosed disease and results in one of the highest mortality rates globally. According to the American Cancer Society (ACS) there will be approximately 1,762,450 new diagnosed cancer cases with 606,880 estimated deaths in 2019, which corresponds to 1,700 deaths per day (Siegel et al., 2019).

The World Health Organization (WHO) have emphasized four main steps in order to control cancer globally. These four steps include:(1) cancer prevention, which provides the most cancer cost-effective and long term cancer control, (2) early detection of the precancerous lesions, which will lead to more controllable and effective treatment, (3) diagnosis and treatment, as a first step of cancer management, ranging from surgery, radiotherapy, chemotherapy, hormone therapy and targeted therapy,(4) palliative care which is mostly practiced among patients with advanced stages of cancer; this practice is recommended to control pain using inexpensive oral pain medication depending on the patient's needs (Center et al., 2011).

Cancer cells are robust enough to resist any cellular stress caused by the addition of certain compounds; cells achieve this by altering their cellular biology, enabling them to survive and proliferate. Despite the various choices in cancer treatment, most of them fail due to their adverse reaction or drug resistance, accounting for about 90% of chemotherapy failure and deaths due to invasion and metastasis of cancer (Mansoori et al., 2017). A possible alternative to overcome the drug resistance problem might be by

the use of natural products, where many studies have shown their potential to act on multiple potential targets by increasing the intracellular concentration of the chemotherapeutic drug leading to increased drug absorption or by inducing the programmed cell death or apoptosis (Yuan et al., 2017).

Historically, in drug discovery, natural products from plants have been a rich source of compounds, making up most of the pharmacologically compounds used to treat cancer. Up until 2014, 136 drugs were employed as anticancer agents and 83% of those drugs were either exclusively from natural products or else derived from natural products (Newman & Cragg, 2016). In the process of drug discovery from plants, initially, a plant with promising properties is identified by a botanist, ethnobotanist, or plant ecologist, which is then followed by a biological screen of the plant extract to identify its different therapeutic activities. Once this is confirmed, the extract goes through an isolation process to identify its active compound. Then the compound goes through many biological studies to identify its mode of action and its possible molecular targets (Balunas & Kinghorn, 2005). Natural products continue to be important as pharmaceuticals, both for their diverse biological properties, and the ability to use their chemical structure as a template to produce other chemical derivatives that may yield more potent yet safer drugs (Priyadarshani et al., 2016).

For many years, Traditional Chinese Medicine (TCM) has played a crucial role in cancer prevention and treatment where a large number of herbal medicines have demonstrated an intense inhibitory activity against carcinogenesis (Jiao et al., 2018). TCM mainly focuses on ways to promote and maintain the flow of qi, which is also known as the "life

energy". TCM also focuses on balancing the opposites in order to achieve maximum harmony and the healthy flow of qi. In 1981, Chinese practitioners began to interact with western physicians allowing the merger of both TCM and western medicine, permitting old ideas to commingle through new perspectives (崔月犁, 1981).

A Chinese herbal mixture known as Anti-tumor B (ATB) or Zing-Shing-Ping has shown anticancer properties *in vivo* caused by reducing the N-butyl-(4-hydroxybutyl) nitrosamide BBN by 90.7 %(Fan, 1993). ATB anticancer activity corresponded to the modulation of gene expression affecting multiple signaling pathways, such as the MAPK pathway and the NFkB pathway, allowing the induction of apoptosis by increasing the executioner caspase-3 while decreasing Bcl-2 production (Guan et al., 2012). Prunella vulgaris (PV) and Rosmarinic Acid (RA) are both polyphenolic phytochemical compounds. PV and RA were both tested for their chemo-preventative properties, showing their ability to eliminate reactive oxygen species (ROS) production by inhibiting IL-6 cytokine release in HaCat keratinocytes (Vostálová et al., 2010). PV induced gene expression and the release of cytokines through activation of both the NFkB and MAPK pathways (Han et al., 2009), while RA inhibited ROS by inhibiting TNF-α and NFkB activation while enhancing TNF-α leading to the induction of apoptosis in the human leukemia U937 cell line (Moon et al., 2010).

Taxol is one of the most well-known plant-derived anticancer drugs isolated in 1971 from the bark of *Taxus breviolia* Nutt (Western yew). The FDA approved taxol in 1992 for treating ovarian cancer, where it possesses its cytotoxic effect by disrupting the microtubule assembly by binding to the polymerized microtubules and altering their conformation (Wani et al., 1971). Moreover, Taxol binds to the microtubules binding

proteins (MAPs) that usually rearrange both α and ß tubulin in the microtubules. This binding to the MAPs will further form a complex that helps the compound stabilize the microtubules from continuing division (Xiao et al., 2012). Overall, this biding to the microtubule leads to arrest of the mitotic spindle formation and thus inhibits cancer cell growth (Prota et al., 2013).

Camptothecin (CPT), a compound isolated from the *Campototheca acuminate* tree found in China (Wall et al., 1966) acts by trapping the topoisomerase I-DNA complexes; by blocking the rejoining of cleavage/re-ligation reaction of the topo-I leading to inhibition of DNA replication. However, due to its low efficiency, low water solubility and high toxicity, this compound was discontinued in the 1970s. The CPT mechanism of action continued to be an area of interest to many researchers to produce CPT analogs that overcame the problems of the original CPT compound, such as FL118, where the improved antitumor activity mainly depended on the free hydroxyl group in its lactone ring and steric configuration (Wani et al., 1986; Zhao et al., 2014).

One of the problems that natural products possess is that they are prone to oxidation and degradation. Besides their chemical stability, most natural products are water-insoluble and have poor bioavailability, requiring high concentrations to enhance bioactivity. The poor solubility of the natural products and the lack of *in vivo* bioavailability have led to limitations in using these compounds in clinical trials; prompting the development of synthetic compounds possessing a similar structure to the original natural compound without compromising the mode of action. To overcome biodegradation, Coimbra et al., 2011 prepared a liposomal formulation to incorporate lipophilic compounds into a liposome bilayer, increasing intravenous efficiency by 70%, allowing compounds to be

encapsulated without being degraded. To overcome the limitation of the plant availability to produce CPT, researchers have invested in structure-activity relationship studies (SAR) to produce a semi-synthetic or synthetic derivatives that are more stable than the original CPT such as the FDA approved compounds irinotecan (9-[(dimethylamino)methyl]-10-hydroxy-camptothecin), and topotecan (7-ethyl-10-[4-(1-piperi-dino)-1-piperidino] carbonyloxycamptothecin) (Martino et al., 2017).

It is important to remember that the goal of cancer research is to understand the biology of cancer cells and what major biochemical pathways are disrupted to successfully develop anticancer agents. Nevertheless, with the growing complexity of cancer treatments, we need to re-consider the appropriate approach to understand what identifies cancer cells from normal cells and once cancer has developed how it does progresses. For that reason (Hanahan & Weinberg, 2000) have pointed out six significant cancer hallmarks that alternates the cell physiology into a malignant state. These six hallmarks include: 1) sustaining self-proliferation, 2) evading growth suppressors, 3) activating invasion and metastasis, 4) enabling replicative immortality, 5) inducing angiogenesis and 6) resisting cell death.

Since cancer is a scenario of decreased apoptosis, targeting programmed cell death via this route is a critical therapeutic approach to cancer therapy. Apoptosis is a form of programmed cell death that facilitates the elimination of useless and unwanted old cell components; this process maintains the normal homeostasis of the cells. Apoptosis induction occurs through two major pathways: the extrinsic pathway, also known as the death receptor pathway or the intrinsic pathway, also known as the mitochondrial pathway (Matthews et al., 2012). The extrinsic pathway depends on ligand-receptor

interactions for the cell death effector activity (Elmore, 2007). The main characterized ligands are FasL/Fas, TNF-α/TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5, also known as TRAIL. The most studied pathways are FasL/Fas, TNF-α/TNFR1 and TRAIL (Yaacoubet al., 2016). Each one of those ligands must bind to a specific receptor; FasL binds the Fas receptor then binds Fas- associated protein with death domain (FADD) adaptor protein, TNF-α binds TNF then binds to the Tumor necrosis factor receptor type1- associated death domain (TRADD) adaptor protein, while TRAIL binds to two receptors TRAIL-R1 and TRAIL-R2 that also recruits the FADD adaptor protein. The recruitment of FADD protein will dimerize procaspase-8 forming the Death Inducing Signaling Complex (DISC), leading to the activation of pro-caspase-8 into caspase-8 to stimulate the executioner caspases (McIlwain et al., 2015).

The intrinsic pathway requires destruction of the mitochondrial membrane caused by intracellular stress directly activating caspase-3 or by cleaving Bid. Bid is a Bcl-2 homology 3 domain (BH3 only protein) that binds to the mitochondrial membrane and releasing cytochrome c into the cytoplasm. Cytochrome c forms what is known as the "death wheels" or the apoptosome with the apoptotic protease activating factor-1 (APAF-1) as well as with the inactive form of the pro-caspase-9 leading to the release of caspase-9, which results in activation of the executioner caspases 3, 6 and 7. The B-cell lymphoma-2 (Bcl-2) family can partially regulate intrinsic pathways by either activating the pro-apoptotic proteins Bid, Bad, Bim, Bax, Bak, Bok, Puma and Noxa, or by inhibiting the anti-apoptotic proteins Bcl-2 XL and Mcl-1. Once the pro-apoptotic proteins are activated, this will lead to mitochondrial outer membrane permeabilization (MOMP). Most of the chemotherapeutic and targeted therapies kill tumor cells using this

route, this facilitates the release of cytochrome c that binds to the apaf-1 along with procaspase-9 leading to its activation into caspase-9, which in turn activates the executioner caspases (Loreto et al., 2014). As mentioned earlier, Taxol is known to inhibit microtubule formation; it was also believed to induce apoptosis in different cancer cell lines through the phosphorylation of Bcl-2, which is accompanied by the loss of its anti-apoptotic function through the activation of R-Ras/Bcl-2 associated Raf-1 kinase (Blagosklonny et al., 1996). It was also observed that Taxol induces apoptosis in a cell-type-specific manner in both MCF-7 breast cancer cells as well as the SKOV3 ovarian cancer cell line. Apoptosis was induced independently of caspases-3 and 9 (Ofir et al., 2002) Taxol induced apoptosis in the human leukemia HL-60 cell line was caspase-9 dependent (Ibrado et al., 1997).

Once cancer progresses to advanced stages, it can cause invasion within the same organ or metastasize to distal organs. Cancer metastasis requires multiple cellular processes, which progress through invasion, intravasation, dissemination, extravasation and finally colonization (Salvador, Llorente, & Gomis, 2019). This lengthy process involves the degradation of the extracellular matrix (ECM) followed by the escaping of the primary cancer cell into the circulating blood system and migrating to a distant organ allowing the vessels to penetrate and further nourish the new colonizing tumor (Su et al., 2015). Bioactive compounds found in natural products have the ability to inhibit cancer metastasis, such as in berries, including proanthocyanidins, anthocyanins, and ellagitannins (Kennedy et al., 2001). A flavonoid from lowbush blueberries (Vaccinium angustifolium) inhibits prostate cell metastasis by downregulating the Matrix

Metalloproteinase (MMP) activity that is important for the degradation of the ECM while increasing the Tissue Inhibitor Metalloproteinase (TIMP) (Matchett et al., 2006).

As plants continue to be an essential source of novel cancer therapies, the Tennessee Center for Botanical Medicine Research (TCBMR) at Middle Tennessee State University (MTSU) has developed several testing protocols that utilize different human cancer cell lines and mouse animal models to test the bioactivity of potential anticancer compounds. This was facilitated by the collaboration with the Biology and Chemistry departments at MTSU as well as with many institutions in China such as the Guangxi Botanical Garden of Medicinal Plants (GBGMP), and the Institute of Medicinal Plant Development (IMPLAD), where both of these institutes provided the TCBMR with compounds to be tested for their potential activities in the hopes of producing new anticancer agents.

In this study, we evaluated the anticancer properties of 14 semi-synthetized aurone derivatives for their potential anticancer properties and further evaluated the mechanism of two novel compounds, *cis*- and *trans*-gnetin H, trimeric oligostilbene derivatives of resveratrol isolated from *Paeonia suffraticosa* to induce apoptosis and inhibit cancer cell migration.

Aurones, the "golden flavonoids" were first discovered in 1943 from the *Coreopsis grandiflora* plant. They belong to the family of flavonoids, which are secondary metabolites that are distributed among different parts of the plants and possess diverse biological activities. Aurones are the most desirable form of the flavonoids due to their simplicity, high stability and drug-likeness scores (Alsayari et al., 2019). Due to their limited presence in nature, yet high therapeutic potential, many researchers have been interested in developing aurone analogs using synthetic or semisynthetic techniques that

possess high activity, with a specified mode of action against cancer, such as telomerase inhibitors (Ballinari et al., 2004), adenosine receptor inhibitiors (Gao, Z. et al., 2002) and CDK1 inhibitors (Schoepfer et al., 2002). As shown by many studies synthetic analogues may be more potent than the parent aurone (Priyadarshani et al., 2016).

Resveratrol, a naturally occurring polyphenolic stilbene that occurs extensively in many of the TCM compounds, can be found in grape skin and other plants. The anti-cancer activity of resveratrol was first reported by (Jang et al., 1997a); since then, many researchers have investigated resveratrol's other biological activities, such as its ability to act as an inflammatory and anti-tumorigenesis agent (Baur & Sinclair, 2006). It has been observed that resveratrol can inhibits NF-kB through different mechanisms, either by blocking the upstream activator of PKCδ and activating the inhibitors of SIRT1, or by directly inhibiting major proteins within the same pathway such as the inhibitory apoptosis proteins IAPs (XIAP, cIAP1, and cIAP2), the anti-apoptotic family proteins, survivin, and caspases (Tang et al., 2014; Yeung et al., 2004). In particular, the oligostillebenes *cis*-/*trans*-gnetin H are trimers of resveratrol; extracted from the seeds of *Paeonia Suffruticosa.* Structurally, *cis*- and *trans*- gnetin H differ from each other in the olefinic moiety between two carbon atom C-7`/C-8` (Gao et al., 2015a). Both compounds have been shown to exert anti-proliferative and anti-metastatic activities against different cancer cell lines, lung, breast and bone and induces apoptosis by directly affecting the mitochondrial pathway (Gao et al., 2015a, Gao and He, 2017).

CHAPTER 2

EVALUATION OF FOURTEEN AURONE DERIVATIVES AS POTENTIAL ANTI-CANCER AGENTS

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Abstract

Aurones are a sub-set of the flavone family that possess a number of biological activities, including anti-cancer, anti-inflammatory, anti-microbial, anti-parasitic and anti-viral. In this study, a series of aurone derivatives with simple unsubstituted coumaranone (benzofuranone) fragments and a range of alkylidene fragments have been prepared and tested for anti-proliferation activity against human cancer cell lines A549 (lung), BT20 (breast) and MCF7 (breast), and anti-metastasis activity against A549. Interestingly, several of these compounds displayed significant levels of activity and high levels of selectivity for the inhibition of the growth of cancerous cell lines versus the corresponding normal cell lines. This growth inhibition was found to be associated with the induction of apoptosis in cancer cells. Moreover, several aurone derivatives showed remarkable inhibition on the motility of lung cancer cells A549. Analysis of the structure-activity relationship revealed that the aurone derivatives based upon five-membered heteroaromatic rings exhibited the most significant anti-cancer activity. Thus, aurone derivatives devoid of the unusual oxygenation found in the coumaranone fragment are potential leads for new anti-cancer agents.

Keywords

Aurones, benzofuranone, anti-cancer, selectivity, apoptosis, cell motility

1. Introduction

In the United States, cancer is the second leading cause of deaths exceeding the deaths caused by heart diseases, and it is expected to increase in the next two decades by 70% (Siegel et al., 2015). According to the American Cancer Association, it is estimated that in 2016 there were 1,685,210 new cancer cases and a total of 595,690 deaths, with prostate cancer (21%) and breast cancer (29%) being the most occurring in male and female, respectively (Siegel et al., 2016).

Aurones are an interesting, though small, sub-set of the flavone family of natural products. Structurally, they can be viewed as being composed of two fragments: benzofuranone and an aromatic ring linked via an exocyclic alkene (Fig. 1A). They are generally isolated in small quantities from plants that produce yellow flowers and are responsible for this coloration (Nakayama, 2002). In recent years, they have been found to display a number of interesting biological activities, including anti-cancer, anti-inflammatory, anti-microbial, anti-parasitic and anti-viral (Boumendjel, 2003). Particularly, much of the attention has been focused on their application as anti-cancer agents. A number of aurone analogs have been synthesized and evaluated for their anti-cancer activity (Fig. 1B). Boumendjel et al.(Boumendjel, 2003; Václavíková et al., 2006) and Go et al.(Sim et al., 2011; Sim et al., 2008) have noted several 4, 6-dimethoxy substituted aurones, such as **1** and **2**, displayed significant levels of inhibition of ABCB1 (P-glycoprotein) or ABCG2 and thus could potentially act as inhibitors of multidrug resistance (MDR) in cancer treatment. In both cases, these reports have noted little intrinsic cytotoxicity. Zhou has reported a series of 5-hydroxy substituted aurones (such as **3**) that exhibited low μM levels of growth

inhibition against breast and lung cancer cell lines (Cheng et al., 2010). Further, Lawrence prepared a series of aurones (such as **4**) which were mimics of combretastatin A-4 that similarly displayed both anti-cancer and anti-tubulin activities (Lawrence et al., 2003) . Some 5-ureido-substituted aurones (**5**) were reported by Tsou and co-workers as specifically being inhibitors of the ATP-binding site of mTOR and thus have potential as anti-cancer agents that act by interfering with angiogenesis(Tsou et al., 2010; Zhang et al., 2010). In addition, Pal and co-workers have studied aurones (such as **6**) as SIRT inhibitors that could moderately inhibit the growth of cancer cells at 10 μM (Manjulatha et al., 2012). Interestingly, this last report was the first one to note significant anti-cancer activity of aurones that are unsubstituted on the benzofuranone section of the molecule. Recently, Chen *et al.* synthesized two aurones, (Z)-2-(4-methylbenzylidene) benzofuran-3(2H)-one and (Z)-2-(4-chlorobenzylidene) benzofuran-3(2H)-one, that exhibited cytotoxicity against two breast cancer cell lines MDA-MB-231 (IC_{50} value of 10.2 μM) and MCF-7 (IC_{50} value of 6.4 μM) (Chen, Qi, & Qiu, 2014). Elhadi et al. synthesized another two aurone derivatives, Z-2'--(dichlorobenzylidene)-5,7-dichlorobenzofuran-3(2H)-one and Z-4'-(methylbenzylidene)-5,7-dichlorobenzofuran-3(2H)-one, that showed growth inhibition against human chronic myelogenous leukemia cells K562 with IC_{50} values of 23 μM and 20 μM, respectively (Elhadi et al., 2015). The growth inhibition effects of these aurones were found to be associated with cell cycle arrest in the G0/G1 phase and an increase of cellular apoptotic death in cancer cells.

Although a number of different routes for the synthesis of aurones have been developed over the years, the most common and most general remains the condensation of a benzofuranone with an aldehyde under a wide variety of conditions, including acidic, basic,

or neutral (Fig. 1A). One attractive feature of the use of neutral conditions is that a diverse range of aldehydes are commercially available and many benzofuranones are either commercially available or readily prepared in 2-4 steps. In addition, the only by-product of this condensation is water, which can render product purification more straightforward. In light of our recent report of a facile and mild method for synthesizing aurone derivatives using neutral reaction conditions(Hawkins & Handy, 2013), we have explored the anti-proliferation and anti-metastasis activity of these aurone derivatives against three cancer cell lines and herein report the results of this assessment.

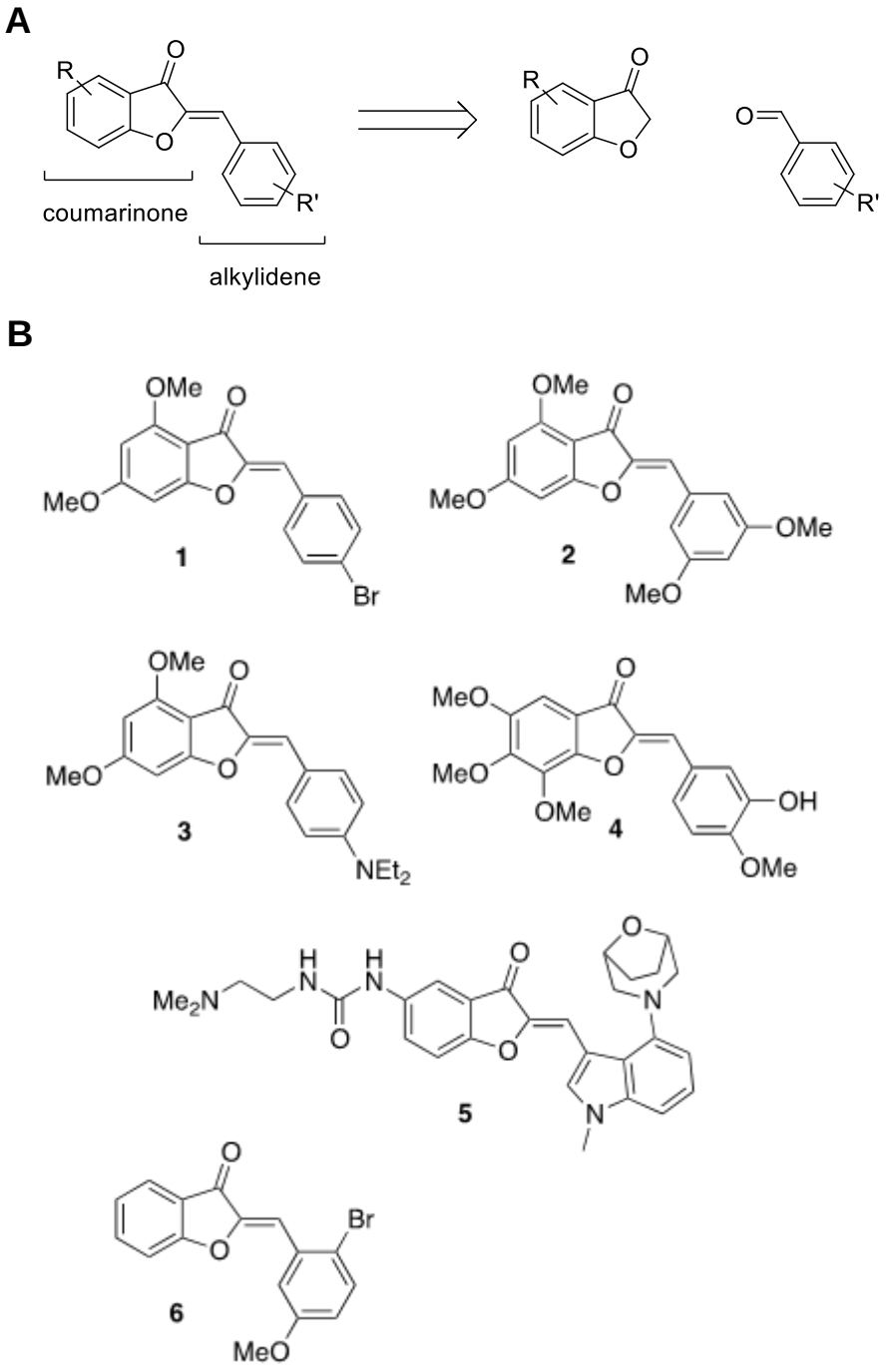


Figure 1. (A) General aurone structure (left) and general synthesis of aurones (right). (B) Representative anti-cancer aurones.

2. Material and methods

2.1. Cell culture and cytotoxicity assay

The cytotoxicity of the aurones against cancer cells was determined in cultured cancer cell lines, human lung carcinoma cell line A549, as well as human breast carcinoma cell lines BT20 and MCF-7. Two typical subtypes of breast cancer cell lines, triple negative BT20 cells (ER^-PR^- $HER2^-$) and Luminal-A phenotype MCF-7 cells ($ER^+PR^+HER2^-$), were used. A549 and BT20 cells were maintained in RPMI-1640 (Sigma) supplemented with 10% FBS (Gibco) and 100 U/mL penicillin/streptomycin. MCF-7 cells were maintained in EMEM (Sigma) supplemented with 0.01 mg/mL human recombinant insulin (Sigma), 10% FBS (Gibco), and 100 U/mL penicillin/streptomycin. To test the selectivity of the aurones against normal cells, two normal cell lines, Human Peripheral Lung Epithelial Cells (HPL1A) and Human Mammary Epithelial Cells (HMEC), were used as a control for the lung cancer cells and breast cancer cells, respectively. HPL1A cells were maintained in DMEM/F-12K (Sigma) supplemented with 10% FBS (Gibco) and 100 U/mL penicillin/streptomycin. HMEC cells were maintained in McCoy's 5A (ATCC, USA) supplemented with 10% FBS (Gibco) and 100 U/mL penicillin/streptomycin.

In vitro cytotoxicity was measured by a fluorescence dye staining method. Cells were harvested by trypsinization, diluted in medium to a density of 4~5 X 10^4 cells/mL, and seeded in a 96-well tissue culture plate. Aurones were dissolved in pure DMSO (Sigma-Aldrich) at 10 mM as a stock solution, and diluted to 6.25 to 100 μM using cell culture medium in the following assays, which resulted in a final DMSO concentration of 1% or

less. Cells were treated with individual aurones the day following seeding. Cells were further incubated for 48 h and then Alamar Blue dye (Invitrogen) was used to assess the viability of cells according to manufacturer's protocol. The fluorescence intensity was read on a SpectraMax M2e microplate reader (Molecular Devices Inc.) at Ex 560 nm and Em 590 nm.

2.2. IC_{50} determination

IC_{50} tests were performed in cancer cell lines A549, BT20, and MCF7. After cells were treated with six serial dilutions (100, 32, 10, 3.2, 1, and 0.32 μM) of aurones for 48 h, cells viability was determined with Alamar Blue dye as described above. The data was fitted to a Four-Parameter Logistic model. IC_{50} values were calculated using non-linear regression analysis (percent inhibition versus log concentration) with GraphPad Prism 6.0 software (GraphPad Software Inc).

2.3. Apoptosis assay

A549 cells were seeded in a 96-well plate and incubated overnight. Then cells were treated with aurones at different concentrations (100, 50, 25 μM) for 20 h. Next, cells were stained with a mixture of three dyes (Hoechst, FTIC-Annexin V, and PI) for 15 min. The Excitation⁄Emission (nm) for Hoechst, FTIC-Annexin V, and PI are 350⁄461, 494⁄518, and 535⁄617, respectively. Cells were imaged using an Arrayscan VTI high-content screening (HCS) reader (Thermo Scientific) with appropriate filters and the data was analyzed using the vHCS Scan software.

2.4. Cell motility assay

First, blue fluorescent microspheres (Life Technologies, Eugene, OP, USA) were used to coat a 96-well plate. In brief, the microspheres were washed twice with PBS and centrifuged for 1 min at 13,000×g, then 75 μL of microspheres were added to each well and incubated for 1 h at 37˚C. The plate was washed for five times with 200 μL of PBS and examined using the microscope to confirm that an even monolayer of microspheres had formed. Subsequently, the A549-GFP cells were harvested, suspended in serum-free medium, and seeded in each well at an intensity of 500 cells/well in the coated plate. After incubation at 37˚ C for 1 h, the cells were treated with aurones at different concentrations (50, 25, 12.5, 6.25 μM) in 20% FBS-containing medium for 18 h. Cells that were treated with serum-free medium served as negative control, while cells treated with 20% FBS-containing medium served as positive control. Cells were imaged using an Arrayscan VTI HCS reader (Thermo Scientific). The data was analyzed by vHCS Scan software.

2.5. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0 software (GraphPad Software Inc). All data were reported as mean ± SD and were analyzed with unpaired *t*-test. Differences are considered statistically significant if $p < 0.05$.

3. Results and discussion

3.1. Aurones exhibited mild to modest anti-proliferation activity against human cancer cells.

A total of fourteen aurones were synthesized for this study (Fig. 2, Table 1). The structures were selected to cover a range of substitution patterns (o, m, and p) and electronic variations (electron-rich and deficient) on the benzaldehydes as well as a few heteroaromatics. None of the synthesized compounds surpass the limits set by Lipinski's Rules or Veber's Rules. The synthesis and characterization of the aurones used in this study have all been reported previously (Hawkins & Handy, 2013) except A14, (Z)-2-((1H-imidazol-2-yl) methylene) benzofuran-3(2H)-one, which was prepared as a new addition using a related procedure as described below. All of the compounds displayed high levels of purity (>95%) on the basis of their spectral data and were fully soluble in DMSO.

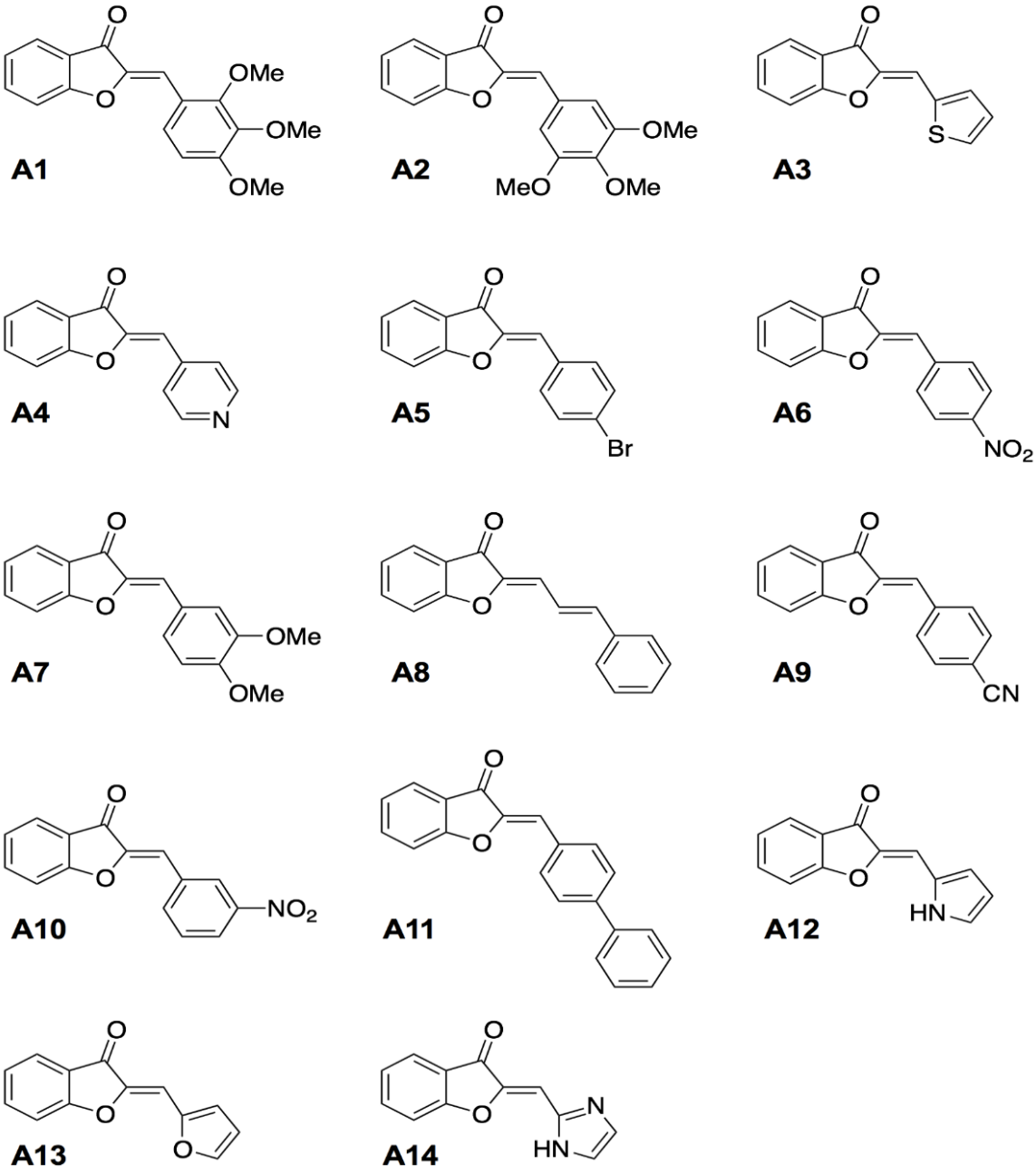


Figure 2. Chemical structures of the fourteen synthesized aurones.

Code	Chemical Name	Solubility	Purity	Lipinski's Rules2				Veber's Rules	
				H Bond Donors	H Bond Donors	M.W	LOG P	Rotatable Bonds	Polar surface Area
A1	2,3,4-Trimethoxybenzaldehyde	Fully soluble	>95%	0	5	312.321	2.1892	4	53.99
A2	3,4,5-Trimethoxybenzaldehyde	Fully soluble	>95%	0	5	312.321	2.1892	4	53.99
A3	Thiophene-2-carboxyldehyde	Fully soluble	>95%	0	2	228.265	2.5501	1	26.3
A4	Pyridine-4-carboxaldehyde	Fully soluble	>95%	0	3	223.231	1.2313	1	38.66
A5	4-Bromobenzaldehyde	Fully soluble	>95%	0	2	301.139	3.3973	1	26.3
A6	4-Nitrobenzaldehyde	Fully soluble	>95%	0	3	267.24	2.995	2	78.11
A7	3,4-Dimethoxybenaldehyde	Fully soluble	>95%	0	4	282.295	2.3156	3	44.76
A8	trans-Cinnamaldehyde	Fully soluble	>95%	0	2	248.281	3.0838	2	26.3
A9	4-Cyanobenzaldehyde	Fully soluble	>95%	0	3	247.253	2.6017	2	50.09
A10	3-Nitrobenaldehyde	Fully soluble	>95%	0	3	267.24	2.995	2	78.11
A11	4-Phenylbenzaldehyde	Fully soluble	>95%	0	2	298.341	4.2436	2	26.3
A12	Pyrrole-2-carboxaldehyde	Fully soluble	>95%	1	3	211.22	1.112	1	38.33
A13	2-Furyl	Fully soluble	>95%	0	3	212.204	1.1841	1	35.53
A14	2-((1H(imidazol-2-yl) methylene) benzofuran-3(2H)-one	Fully soluble	>95%	1	4	212.208	0.5194	1	50.69

Table 1. Chemical names, solubility, purity of the fourteen aurone derivatives and structural analysis based on Lipinski's Rule and Verber's Rule.

To synthesize aurone A14, benzofuranone (1.00 mmol) and imidazole-2-carboxaldehyde (1.00 mmol) were combined in a dry vial. 3.5 g of neutral alumina was then added followed by 5 mL of dichloromethane. The reaction mixture was stirred for 12 h at 25 °C. The reaction mixture was then filtered and the dichloromethane layer collected and concentrated to dryness *in vacuo* to afford the desired aurone. The crude reaction mixture was then purified by trituration with ether to yield 24.0 mg (11 %) of aurone A14 as a brown solid (Decomp. = 92-94 °C). IR (neat, thin film): 2950, 2820, 1700, 1610, 1500, 1310, 1090, 750; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 9.77 (s, 1H), 7.83 (dt, J = 9.0, 1.4 Hz, 1H), 7.68 (dq, J = 9.0, 1.4 Hz, 1H), 7.38-7.21 (m, 4H), 7.06 (s, 1H). ^{13}C NMR ($CDCl_3$, 75 MHz): 183.34, 181.22, 165.47, 165.17, 147.72, 146.48, 141.18, 137.60, 137.08, 124.92, 124.82, 124.11, 123.31, 121.79, 113.01, 112.68, 112.41, 102.14.

We first confirmed the ability of the fourteen synthesized aurones to inhibit the growth of different human carcinoma cell lines by employing the Alamar blue assay. Because it is important in cancer chemotherapy to selectively kill cancer cells without affecting normal cell growth, we also evaluated aurones for their cytotoxicity towards noncancerous cells. In the initial screening, some interesting aurones were identified. Six aurones (A3, A5, A10, A12, A13 and A14) exhibited greater than 50% inhibition of the growth of human lung cancer cells A549 at 100 μM concentration, with five of them exhibiting little or no inhibition of the growth of normal lung cells HPL1A (Fig. 3A). In particular, A3, A5, A10 and A14 showed greater than 90% inhibitory activity against lung cancer cells and did not inhibit normal lung cells. Ten aurones also showed growth inhibitory effects against two breast cancer cell lines BT20 and/or MCF7 with greater than

50% growth inhibition at 100 μM, with five of them exhibiting much lower (less than 50%) inhibition of the growth of normal breast cells HMEC (Fig. 3B). In particular, A3 and A10 showed >90% inhibitory activity against breast cancer cells and were significantly less toxic to normal cells. In general, this study showed that good selectivity for cancerous versus normal cell lines can be obtained for aurones. This is particularly the case for lung cancer, in which only A13 displayed high toxicity towards both the cancerous and normal cell lines. The four aurones (A3, A5, A10, and A14) that displayed significant inhibitory activity against both lung cancer and breast cancer cells, while showing little inhibitory activity against normal control cells, were the most promising for additional studies, and their IC_{50} values were determined (Table 2).

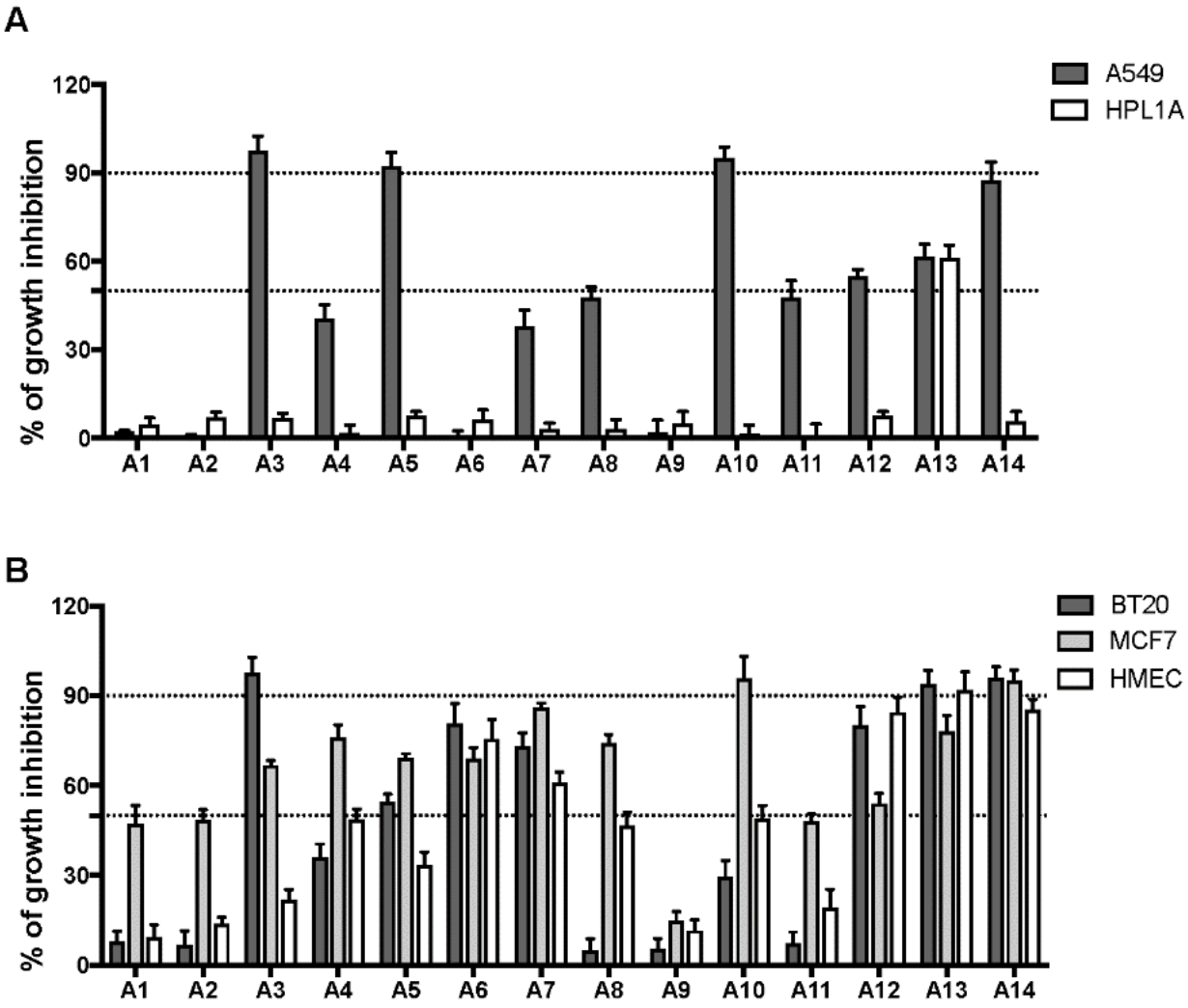


Figure 3. Anti-proliferation activity of the fourteen aurones against human cells.

	Cancer cells			Normal cells	
	A549	**BT20**	**MCF7**	**HPL1A**	**HMEC**
A3	22.5	41.3	>100	>100	>100
A5	40.8	>100	79.2	>100	>100
A10	40.6	>100	13.7	>100	>100
A14	57.9	28.1	24	>100	47.9

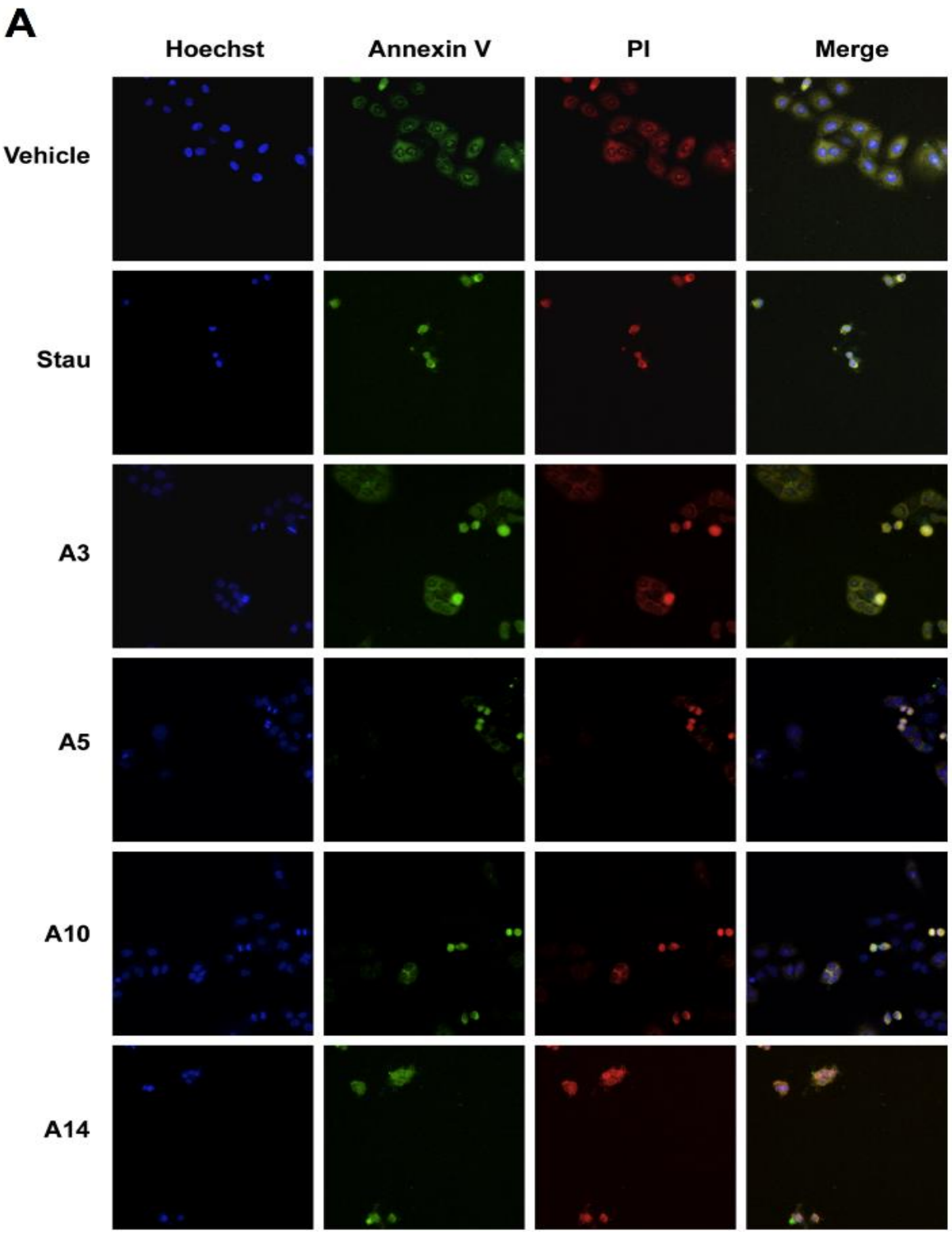
Table 2. IC_{50} values of the effective aurone derivatives (μM)

Beyond the interesting observation that oxygenation of the benzofuranone portion of the aurone structure is not essential for anti-cancer activity, our cytotoxicity studies showed that the aurone derivatives containing five-membered ring heteroaromatics (A3, A12, A13 and A14) were generally the most active, and A3 and A14 were also the most selective. Considering how little is known about the potential of aurones as anti-cancer agents, this may be an interesting area for future study. Although it is too early to draw any specific conclusions, one hypothesis that warrants investigation is that the presence of the heteroatom in the five-membered ring and the oxygen in the benzofuranone serve as some form of metal chelator or perhaps stabilize or interrupt some key hydrogen-bonding net-work.

3.2. Aurones induced apoptosis in human lung cancer cells A549.

Apoptosis is a type of programmed cell death and is controlled by certain enzymes, such as caspases that remove cellular organelles and eventually lead to cell death by two different pathways, the intrinsic or extrinsic pathway (Lockshin & Zakeri, 2004). The four aurones (A3, A5, A10 and A14) that showed a cytotoxic effect were tested to see if their cytotoxicity was due to apoptotic cell death. Apoptotic cells could be identified by cell shrinkage that reduced their size and resulted in a more dense color. In addition, Propidium Iodine (PI) was used as a vital dye to differentiate live and dead cells by staining the cell wall, and FTIC-Annexin V dye was used as a specific indication for the disruption of membrane integrity accompanying the last stage of apoptotic cell death. We accessed the ability of the aurones to induce apoptosis using high-content analysis (HCA) as these distinguishable characteristics, reduced cell size and increased Annexin V/PI straning,

could be easily seen under light microscopy and numerically confirmed by HCA. An apoptosis assay was performed on A549 with 20-h treatment with the four aurones (A3, A5, A10 and A14) at 25, 50, and 100 µM concentrations. As shown in (Fig. 4A), aurones A3, A5, and A14 showed apoptotic induction at all tested concentrations (25, 50 and 100 µM) (*$p \leq 0.05$, **$p \leq 0.01$ or ***$p \leq 0.001$), while aurone A10 induced apoptosis at higher concentrations (50 and 100 µM) (***$p \leq 0.001$) (Fig. 4B). These data suggest that the growth inhibition of A549 cancer cells by aurones was associated with induction of apoptosis.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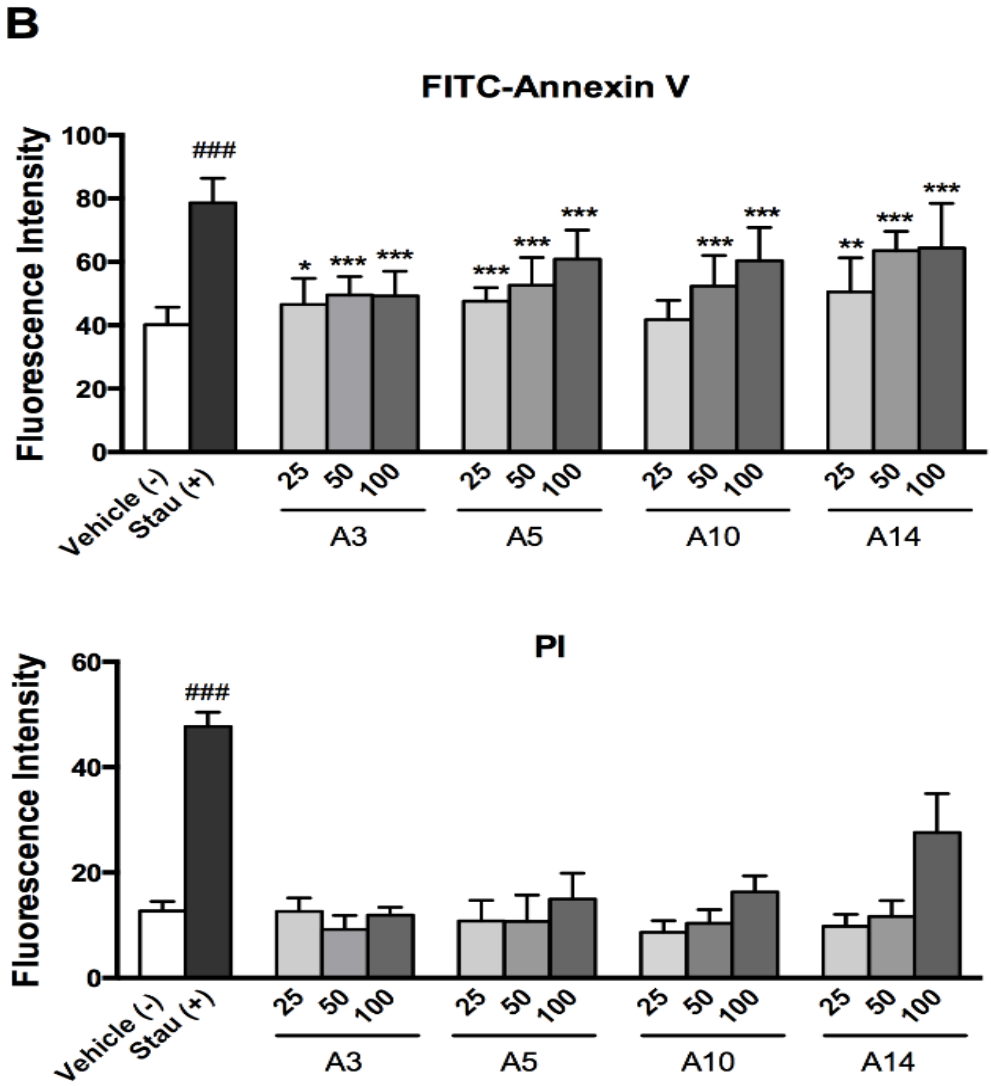

Figure 4. Induction of apoptosis in A549 cells by aurones A3, A5, A10, and A14.

Cells were treated with the test compound at different concentrations for 20 h, then stained using a mixture of Hoechst, FITC-Annexin V, and PI. Cells treated with 1 μM Staurosporine served as a positive control, and cells treated with vehicle (PBS) only served as a negative control. (A) Representative fluorescent cell images were observed using a HCS reader. (B) Quantitation of fluorescent intensity obtained from (A). *$p \leq 0.05$, **$p \leq 0.01$, or ***$p \leq 0.001$.

3.3. Aurones suppressed the motility of human lung cancer cells A549-GFP.

One of the major features of cancer cells is their ability to migrate, leading to recurrent episodes of cancer in different parts of the body and increased mortality. This has led to increased research efforts to discover anti-metastatic drugs. Metastasis of cancer cells is a multistep process involving not only morphological cell changes, but also the detachment of a tumor cell by degrading the extra cellular matrix (ECM) whereupon the tumor cells travel through the blood vessels to give rise to new tumor cells at a new site (Martin et al., 2013). We determined the ability of the four aurones (A3, A5, A10 and A14) to inhibit the motility of A549 cells after 18-h treatment by employing a fluorescence-conjugated human lung cancer cell line A549-GFP. Before the motility assay, A549-GFP cells were tested for viability after treatment with the respective aurone at different concentrations in the presence of 20% FBS-containing medium for 18 h. Aurones at concentrations of 6.5, 12.5, 25, or 50 μM showed greater than 80% viability to A549-GFP cells (data not shown) and these concentrations were selected for the motility assay. After treatment, the average track area generated by each cell, which is proportional to the cells movement, was measured. Cells treated with 20% FBS-containing medium served as a positive control and showed 167.6% longer track areas than the cells treated with vehicle only ($^{\#\#\#}p \leq 0.001$). As shown in Fig. 5, aurones A3, A5, A10, and A14 significantly inhibited the motility of A549-GFP cells by showing smaller track areas at concentrations of 6.5-50 μM ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$, or $^{***}p \leq 0.001$). Aurones A3, A5, A10, and A14 showed a decrease in A549-GFP motility by 31.7%, 62.4%, 53.6% and 51.1%, respectively, at the concentration of 6.25 μM after 18-h treatment.

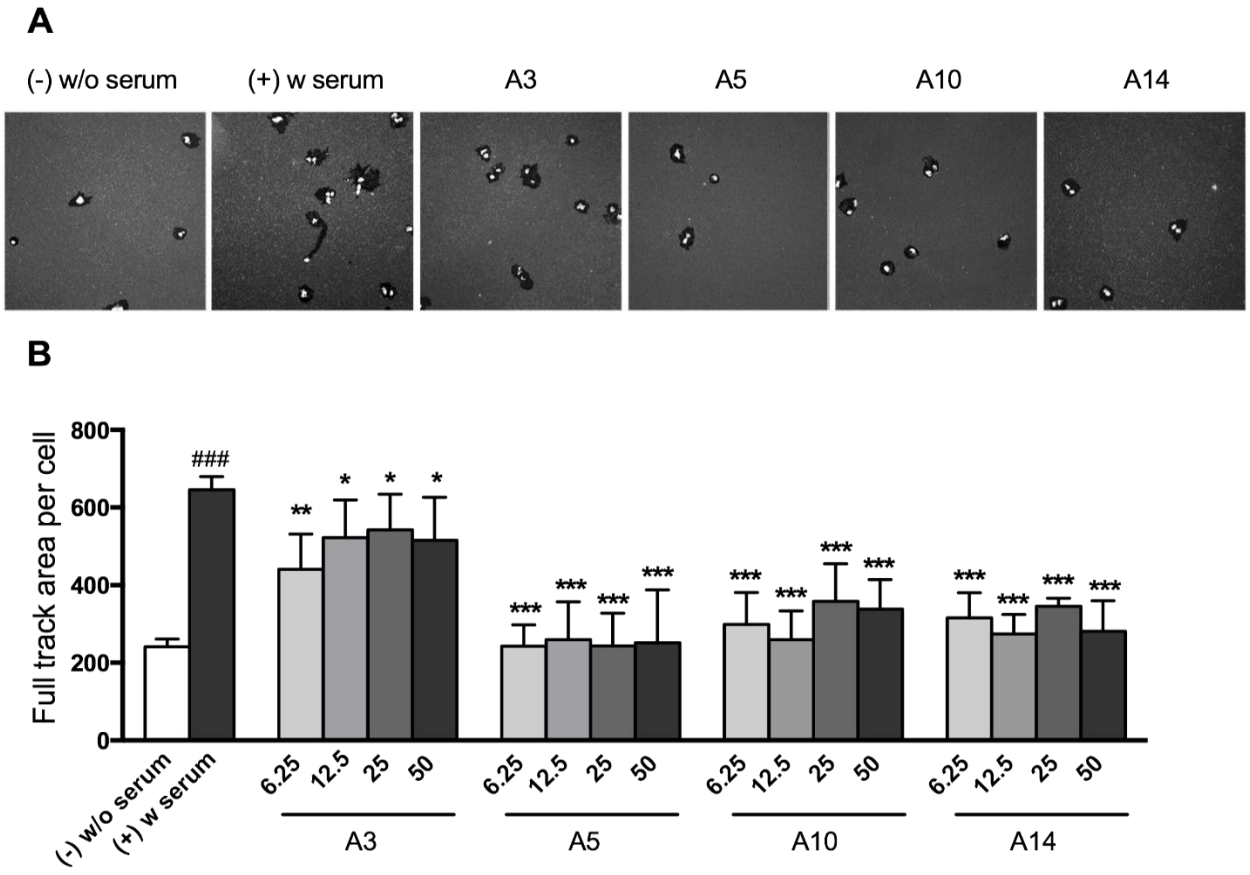

Figure 5. Inhibition of cell motility of A549 cells by aurones A3, A5, A10, and A14.

Cells were treated with the test compound at different concentrations and 20% serum-containing medium for 18 h, Cells treated with 20% serum-containing medium served as a positive control, and cells treated with non-serum-containing medium were served as a negative control. The tracks of the cells were defined and the track area per cell was measured using a HCS reader. *$p \leq 0.05$, **$p \leq 0.01$ or ***$p \leq 0.001$.

4. Conclusion

Due to their high availability, simple synthesis, and generally low toxicity, aurones could be attractive candidates for safer cancer drugs. Besides their reported potential of inhibiting multidrug resistance, cell growth, and angiogenesis (Chen et al., 2014; Cheng et al., 2010; Elhadi et al., 2015; Haudecoeur & Boumendjel, 2012; Lawrence et al., 2003; Manjulatha et al., 2012; Sim, Hong May et al., 2011; Sim, Hong-May et al., 2008; Tsou et al., 2010; Václavíková et al., 2006; Zhang et al., 2010), we also demonstrated the potential of aurones as inhibitors of cell metastasis. In summary, a series of fourteen aurone derivatives were synthesized, and several of these compounds displayed inhibitory activity against cancerous cell lines with high levels of selectivity. This growth inhibition was found to be associated with induction of apoptosis in cancer cells. Interestingly, although the growth inhibitory activity of the aurones were moderate, they showed remarkable inhibition on the motility of lung cancer cells A549 at a concentration as low as 6.25 μM. Analysis of the structure-activity relationship revealed that the aurone derivatives based upon five-membered heteroaromatic rings exhibited the most significant anti-cancer activity. Thus, aurone derivatives devoid of the unusual oxygenation found in the benzofuranone fragment could be potential leads for new anti-cancer agents.

5. Acknowledgements

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Chapter 3

THE RESVERATROL OLIGOMERS *CIS*- AND *TRANS*-GNETIN H INHIBIT HUMAN CANCER CELLS BY INDUCTION OF APOPTOSIS AND OXIDATIVE STRESS

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Abstract

***Background*:** The oligostilbenes *cis*- and *trans*- gnetin H were previously studied for their ability to inhibit cancer cell proliferation and induce apoptosis. However, an in-depth understanding of how this this process is regulated remains poorly understood.

***Methods*:** In this study, we tested the ability of *cis*- and *trans*-gnetin H to act as an antioxidant by scavenging one of the stable free radicals DPPH. We also tested the ability of both compounds to generate oxidative stress by elevating the reactive oxygen species ROS that causes apoptosis using lung cancer cell line A549. Also, immunoassay enzyme-linked immunosorbent assays (ELISAs) were performed to test the levels of the major proteins that induce apoptosis using a triple negative breast cancer cell line MDA-MB-231.

Results: *Cis*- and *trans*-gnetin H treatment possessed a high percentage of radical scavenging activity RSA%=25% at the lowest concentration 12.5μM ($p \leq 0.0001$) and elevated the levels of ROS in A549 to 50% and 45%, respectively ($p \leq 0.001$). In addition, *cis*- and *trans*- gnetin H induced early apoptosis in A549 cell line by 59% and 38.9%, respectively, at the highest concentration of 25μM compared to the untreated control ($p \leq 0.0001$). Results from apoptosis protein array showed certain up-regulated proteins such as Bid, Bad, cytochrome c, FasL, TRAIL1-4 and down-regulated proteins such as XIAP, surviving, Hsp60, suggesting inducing apoptosis was facilitated by cross talk between intrinsic and extrinsic pathway through the TRAIL pathway.

Conclusion: These observations suggested that *cis*- and *trans*-gnetin H induce apoptosis in human lung and breast cancer cells *in vitro* by elevating the levels of oxidative stress and directly affecting the primary apoptosis regulatory proteins.

Key words

Resveratrol, *cis*- and *trans*- gnetin H, cancer apoptosis, reactive oxygen species (ROS), oxidative stress.

1. Introduction

The oligostilbenes, *cis*- and *trans*- gnetin H, are plant-based polyphenolic compounds derived from resveratrol (3, 5, 4'-trihydroxystilbene). Resveratrol is a phytoalexin found in about 72 plant species, particularly in grape skin (Dercks & Creasy, 1989; Goldberg et al., 1995; Jeandet et al., 1991). Resveratrol has been well studied for its pharmacological properties, including its antioxidant, anti-inflammatory properties and anti-tumor abilities (Deigner & Kinscherf, 1999). Many studies have demonstrated the ability of resveratrol to induce p53 dependent apoptosis (Huang et al., 1999).

Cis- and *trans*- gnetin H were previously isolated from the seeds of *Paeonia suffruticosa* and were screened for their cytotoxicity against different cancer cell lines (Kim et al., 2002). They were subsequently tested for their abilities to induce apoptosis by releasing cytochrome c, caspase 3/7, inhibiting the NF-kB pathway as well as arresting cancer cells at G0-G1 phase (Gao et al., 2015a) and for their anti-metastasis effect (Gao & He, 2017).

Apoptosis, also known as programmed cell death, plays a crucial role in maintaining normal tissue homeostasis, and any imbalance between cell death and proliferation can lead to tumor formation (Lowe & Lin, 2000). Specific morphological changes can identify apoptosis; this includes cell shrinkage, chromatin condensation, plasma membrane blebbing, DNA fragmentation, and eventually the breakdown of the cells into

small pieces known as apoptotic bodies (Earnshaw, 1995). Apoptosis can be initiated through the death receptor or the mitochondria to activate the effector caspase, followed by the executioner caspases (Hengartner, 2000). Triggering apoptosis through the death receptor can occur by stimulating proteins involved in the tumor necrosis factor superfamily, such as CD95 (Apo-1/Fas) or through the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) that eventually activates caspase 8 and cleavage of the effector caspases (Ashkenazi, 2002; Walczak & Krammer, 2000). Triggering apoptosis through mitochondria is initiated through the release of the apoptogenic factors such as cytochrome *c*, triggering the release of caspase 3 and forming the apoptosome complex from cytochrome c/ apaf-1/ caspase-9, also known as "the wheels of death."

In cancer cells, reactive oxygen species (ROS) that are produced by the mitochondria have a crucial role in cell proliferation via inducing apoptosis. Once mitochondria is activated, it will result in the loss of its membrane potential ($\Delta\Psi m$), initiating the release of crucial apoptotic factors such as cytochrome *c* and other pro-apoptotic proteins into the cytoplasm, which eventually allows the execution of cell death via the caspases. The role of ROS could be a double-edged sword depending on the levels of both ROS and other antioxidants. The redox state of a specific cell is essential in terms of its susceptibility for an apoptosis stimuli; where an overproduction of the ROS to toxic levels in cancer cells while lowering the levels of an antioxidant can eventually lead to oxidative stress and death of the cancer cells (Sharma et al., 2007).

This study aims to understand the molecular process by which *cis*- and *trans*- gentin H compounds inhibit the proliferation of A549 cancer cells via triggering the induction of

apoptosis through ROS while identifying the proteins involved in this process to determine the apoptosis induction pathway.

2. Materials and Methods.

2.1. Cell culture.

Human cancer cell lines A549 (lung carcinoma) and MDA-MB-231 (human breast adenocarcinoma) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). A549 cells were cultured using RPMI-1640 medium (Sigma-Aldrich), MDA-MB-231 cell was cultured using DMEM medium (Sigma-Aldrich). All cells were maintained in medium containing 10% fetal bovine serum (FBS), 1% antibiotic (penicillin/ streptomycin), and incubated in 5% CO2 at a 37°*C* incubator.

2.2. Cytotoxicity assay and IC_{50} determination.

Alamar blue (Invitrogen, Frederick, MD, USA) was used to determine the cytotoxicity of the compounds based on the fluorescence. Briefly, cells were seeded in a 96-well microplate at 5×10^{3} density and allowed to attach overnight before treatment. Next day, replace the old medium with media containing *cis*- or *trans*- gnetin H compounds using different concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.78 μM, cells were kept in the incubator for 48h. DMSO (1%) was used as a negative control. After 48h, 10% Alamar blue was added and kept in the CO2 incubator for 3-4h. The fluorescent intensity was measured using Spectra Max 5M microplate reader (Molecular Devices,

LLC, Sunnyvale, CA, USA) at Excitation/ Emission (Ex/Em) 555,590 nm. Cytotoxicity was measured using the IC50, which is the concentration that inhibits 50% of the cell growth. The IC50 was determined using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA).

2.3. Measuring the radical scavenging activity using anti-oxidant DPPH.

DPPH (2, 2-diphenyl-1-picrylhydrazyl) is used to measure the ability of a compound to scavenge free radicals in terms of hydrogen donating ability. This will reduce the purple DPPH into a yellow color (DPPH-H) resulting in an absorbance decrease. Briefly, DPPH was prepared at a 0.1mM concentration using ethanol and *cis*- or *trans*- gnetin H were prepared in different concentration of 50, 25, and 12.5µM using DMSO. Ascorbic acid (10mg/ml) was used as a positive control. The reaction mixture was prepared using DPPH and the compound and incubated for 30min at RT in a dark condition and absorbance was read using Spectra Max 5M microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) at 517nm. The % radical scavenging activity (% RSA) of the compounds was calculated using the formula:

$\%\ RSA = (Abs_{control} - Abs_{Sample}/Abs_{control}) \times 100.$

Where the $Abs_{Control}$ and Abs_{Sample} are the absorbance values of the control and test sample at 517nm.

2.4. Oxidative stress assay.

To determine the generation of reactive oxygen species (ROS), Hikit oxidative stress kit was used (Thermo Scientific, Waltham, MA, USA). Briefly, cells were seeded in a 96-

well microplate at 5×10^3 cell density and allowed to attach overnight before treatment. Next day, replace the old medium with medium containing *cis*- or *trans*- gnetin H compounds using different concentrations of 100, 32, 10 μM for 24h. Next, cells were fixed using 3.7% formaldehyde and stained with Hoechst dye and dihydroethidium (DHE) and incubated for 30 min at 37°C and 5% CO2. Doxorubicin (DOX) was used as a positive control at a 1μM concentration while cells treated with vehicle only served as a negative control. Arrayscan VTI High content screening reader (HCS) (Thermo Fisher Scientific, Inc.) was used to measure the ROS generation by measuring the fluorescence intensity of the ethidium within the nuclei. Images and data were acquired and analyzed using the vHCS scan software, and P-values were determined using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA).

2.5. Apoptosis assay using high-content analysis.

A549 cells were seeded at 5×10^3 density in a 96-well plate and incubated overnight. Cells were treated with *cis*- or *trans*- gnetin H compounds at different concentrations (25, 12.5 and 3.125) for 20 h. Next, the cells were stained with a mixture of three dyes (Hoechst, FTIC Annexin V and PI) for 15 min. The Excitation/Emission (nm) for Hoechst, FTIC-Annexin V and PI are 350/461, 494/518 and 535/617, respectively. Staurosporine was used as a positive control at 1μM concentration (Sigma-Aldrich), and cells treated with the vehicle served as a negative control. Cells were imaged using Arrayscan VTI High-Content Screening (HCS) reader with appropriate filters, and data were analyzed using vHCS Scan software.

2.6. Apoptosis protein array.

To evaluate apoptotic protein expression, The Human Apoptosis Antibody Array kit (RayBiotech, inc., Norcross GA, USA) was used. Briefly, A549 cells were seeded at 8×10^3 cells/well into a 96-well plate. The cells were treated using *cis*- or *trans*- gnetin H at a 50μM concentration for 6h. Cells were lysed using protease inhibitors and concentrated using a protein concentration column (EMD Millipore) to yield a total protein concentration 2mg/ml. The sample was diluted 10-fold using an assay buffer and incubated on the array membrane for 2h at room temperature. Cells were washed with washing buffer five times before adding a cocktail of biotin-conjugated antibody mix and incubated overnight at 4°C. Cells were then incubated with HRP-conjugated streptavidin for 2h at room temperature and a chemiluminescent substrate was added to detect the signal. The intensity of each array dot, was quantified using Image Studio software (LI-COR Biotechnology, Lincoln, NE, USA).

2.7. Statistical analysis

Statistical analysis of results were analyzed using GraphPad Prism 8.0 software (GraphPad Software Inc), and mean ±SD was calculated using unpaired t-test. Statistical differences of $p < 0.05$ were only considered significant.

3. Results

3.1. IC_{50} cytotoxicity determination of *cis*- and *trans*- gnetin H.

The cytotoxicity of both *cis*- and *trans*- gnetin H was previously evaluated on several different cancer cell lines, including the A549 lung cancer cell line and the BT20 and

MCF-7 breast cancer cell lines. To confirm these results we evaluated the cytotoxicity of both *cis*- and *trans*- gnetin H on the A549 lung cancer cell line and the MDA-MB-231 (ER^- PR^- $HER2^-$) basal B cell line. Supporting previous findings, *trans*- gnetin H had a lower IC50 (3.09-3.90μM) than *cis*-gnetin H (4.63-8.69μM) on both MDA-MB-231 and A549, respectively (Figure 6).

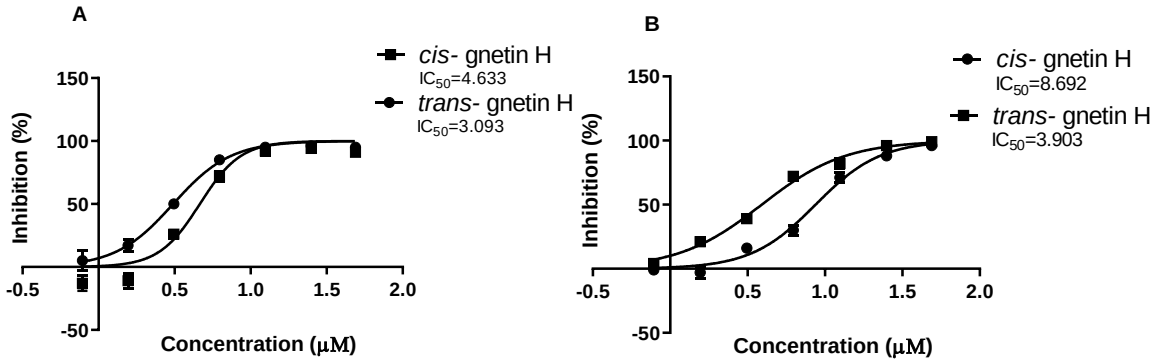

Figure 6: Determination of the IC_{50} values *cis*- and *trans*- gnetin H using two different cell lines.

(A) Breast cancer cell line MDA-MB-231. (B) Lung cancer cell line A549.

3.2. Measuring the radical scavenging activity of *cis*- and *trans*- gnetin H using the antioxidant DPPH.

The free radical DPPH was used to evaluate the RSA of both *cis*- and *trans*-gnetin H. The antioxidant (ascorbic acid) was used as a control and had a RSA of 96% at a concentration of 10mg/ml (****$p \leq 0.0001$). Both *cis*- and *trans*-gnetin H showed similar RSAs in a concentration-dependent matter. *Cis*-gnetin H had an RSA of 25.2%, 35% and

52% at 12.5, 25 and 50 μM, respectively (****$p \leq 0.0001$, Fig 7A). Likewise, *trans*-gnetin H had an RSA of 25.68%, 38.2% and 55.7% at 12.5, 25 and 50 μM, respectively (****$p \leq 0.0001$, Fig 7B)

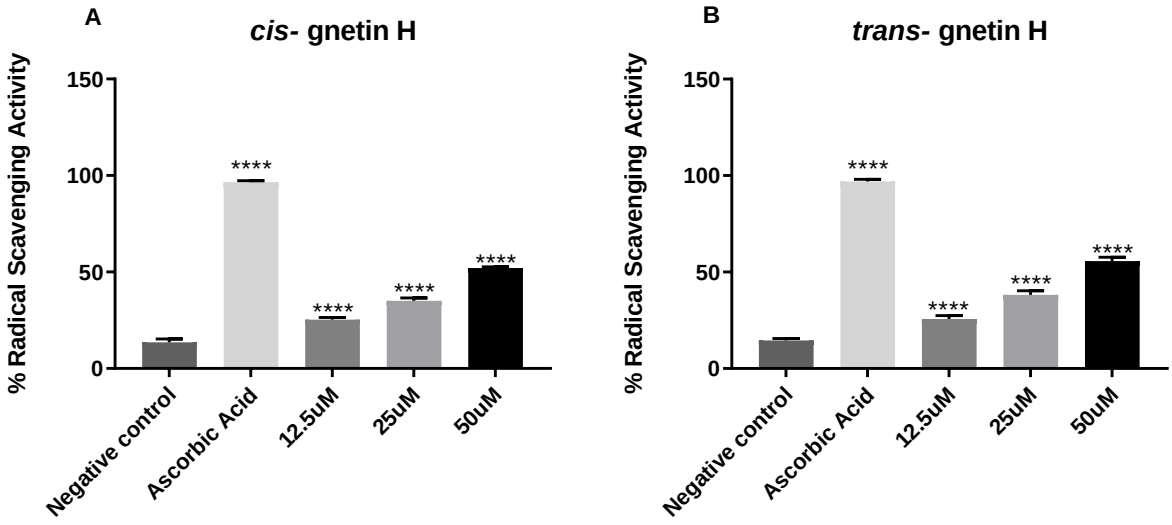

Figure 7. Scavenging activity using DPPH for both *cis*- and *trans*-gnetin H.

The reaction mixture was kept at RT for 30 min and the absorbance was measured at 517 nm. The error bar represents the standard deviation for the four replicates. *P≤0.05, **P≤0.01; ***P≤0.001; ****P≤0.0001.

3.3. Measuring ROS generation by *cis*- and *trans*- gnetin H.

ROS generation by *cis*- and *trans*- gnetin H was evaluated in A549 cells. A significant increase in the intracellular mitochondrial ROS was observed and measured using HCS to directly quantify the conversion of non-fluorescent DHE dye into fluorescent ethidium within the nuclei (Figure 8A). Doxorubicin was used as a positive control and increased ROS by 111.32% (***$p \leq 0.001$) (Fig 8B). *Cis*- gnetin H induced ROS generation at all

concentrations tested, 10, 32, and 100μM in a dose-dependent manner and ROS levels were increased by 50, 67 and 97%, respectively compared to the negative control, while *trans*-gnetin-H was able to induce ROS at the 32 and 100μM concentration by 45 and 85%, respectively (*$p \leq 0.05$, **$p \leq 0.01$, or ***$p \leq 0.001$) (Fig 8B). To determine whether or not the levels of ROS were truly associated with inducing OS, A549 cells were treated with 10mM of the anti-oxidant N-acetyl-L-cysteine (NAC) using the same concentrations for inducing OS. NAC was able to attenuate the OS caused by treating A549 with both compounds at all concentrations, (*$p \leq 0.05$, **$p \leq 0.01$ or***$p \leq 0.001$) (Fig 8C).

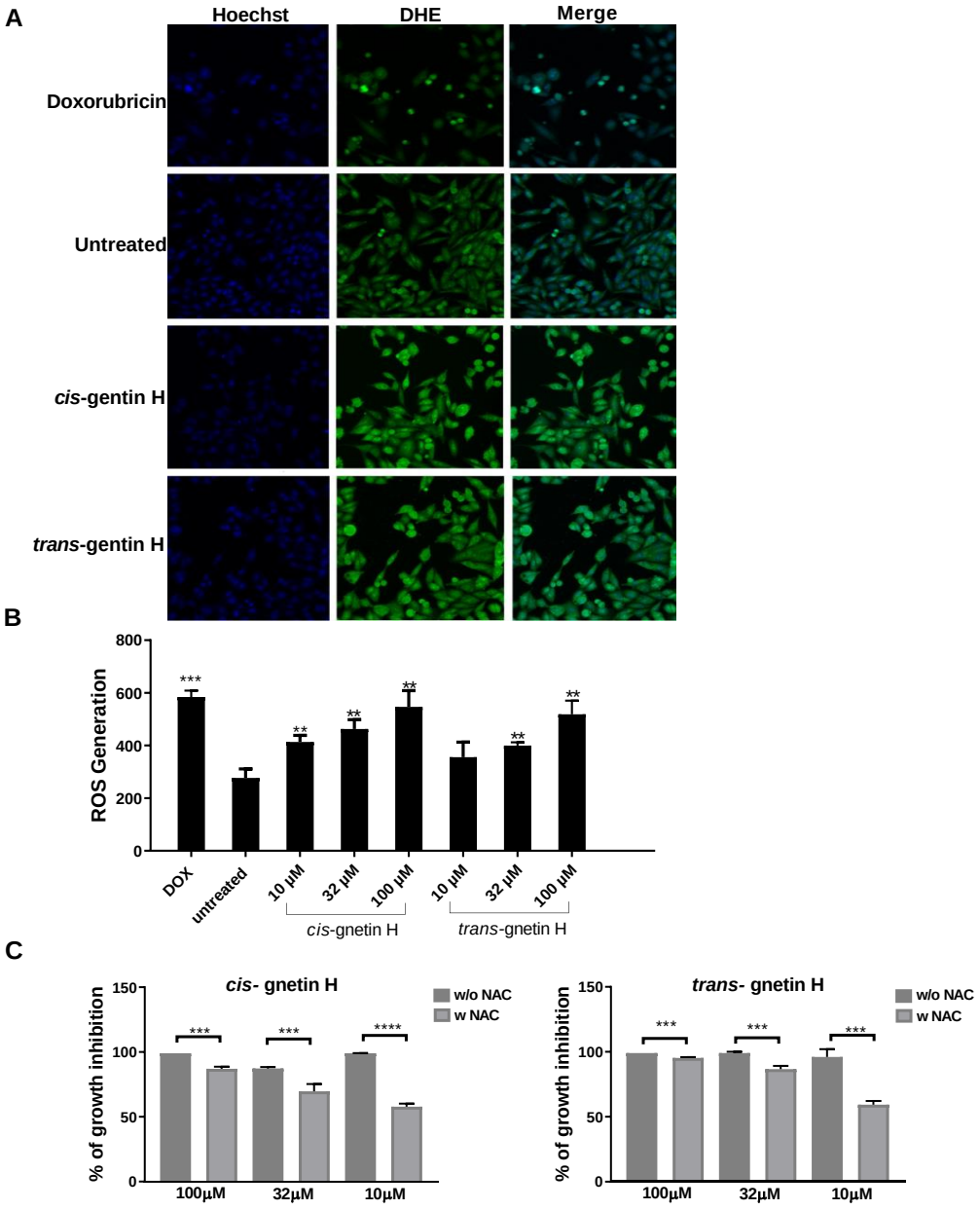

Figure 8. Induction of oxidative stress by *cis*- and *trans*- gnetin H in A549 cell.

A549 cells were treated with *cis*- or *trans*- gnetin H at various concentration for 24h, then cells were stained with Hoechst and DHE dye. Cells treated with Doxrubricin served as a positive control and cells treated with vehicle only served as a negative control. ROS levels were measured by the fluorescence intensity of DHE oxidation to ethidium bromide in the nucleus. (A) HCS fluorescent cells images. (B) A549 cells treated with various concentrations (10, 32 and 100 µM) of *cis*- or *trans*- gnetin H. (C) The NAC anti-

oxidant was used to attenuate the cell death caused by treatment with both compounds. The error bars indicates the standard deviation SD (n=4) *P≤0.05, **P≤0.01, ***P≤0.001.

3.4. Using HCS to measure the induction of apoptosis by *cis*- and *trans*- gnetin H.

Apoptosis can be observed as cell shrinkage leading to chromatin condensation and eventually resulting in cell death. These cellular changes can be easily identified by HCS using Hoechst dye, FITC-Annexin V dye and propidium iodide (PI) dye. Hoechst dye specifically stains the nucleus. FITC-Annexin V dye binds to certain phospholipids such as phosphatidylserine (PS). The phospholipids are normally found in the inner part of the cell wall which becomes exposed during the first stages of apoptosis. Thus FITC-Annexin V dye can be used to differentiate live versus dead cells. PI binds to DNA and can only get inside the cell when the membrane is fully disrupted, which happens in the last stage of apoptosis.In this study, *cis*- and *trans*- gnetin-H were tested for their ability to induce apoptosis in A549 cells (Figure 9A). *Cis*- gnetin H induced early apoptosis after treatment with different concentrations 3.125, 12.5 and 25μM by 11.6%, 23.6% and 59.2%, respectively. *Trans*- gnetin H also induced early apoptosis after treatment at 3.125, 12.5 and 25μM by 9.9%, 44.9% and 38.9%, respectively (**$p \leq 0.01$, ***$p \leq 0.001$ or ****$p \leq 0.0001$) (Fig 9B). Few of the concentrations induced late apoptosis detected by PI. *Cis*- and *trans*- gnetin H induced late apoptosis at only the highest concentration of

25μM (**$p \leq 0.01$ or ***$p \leq 0.001$) (Fig 9C).

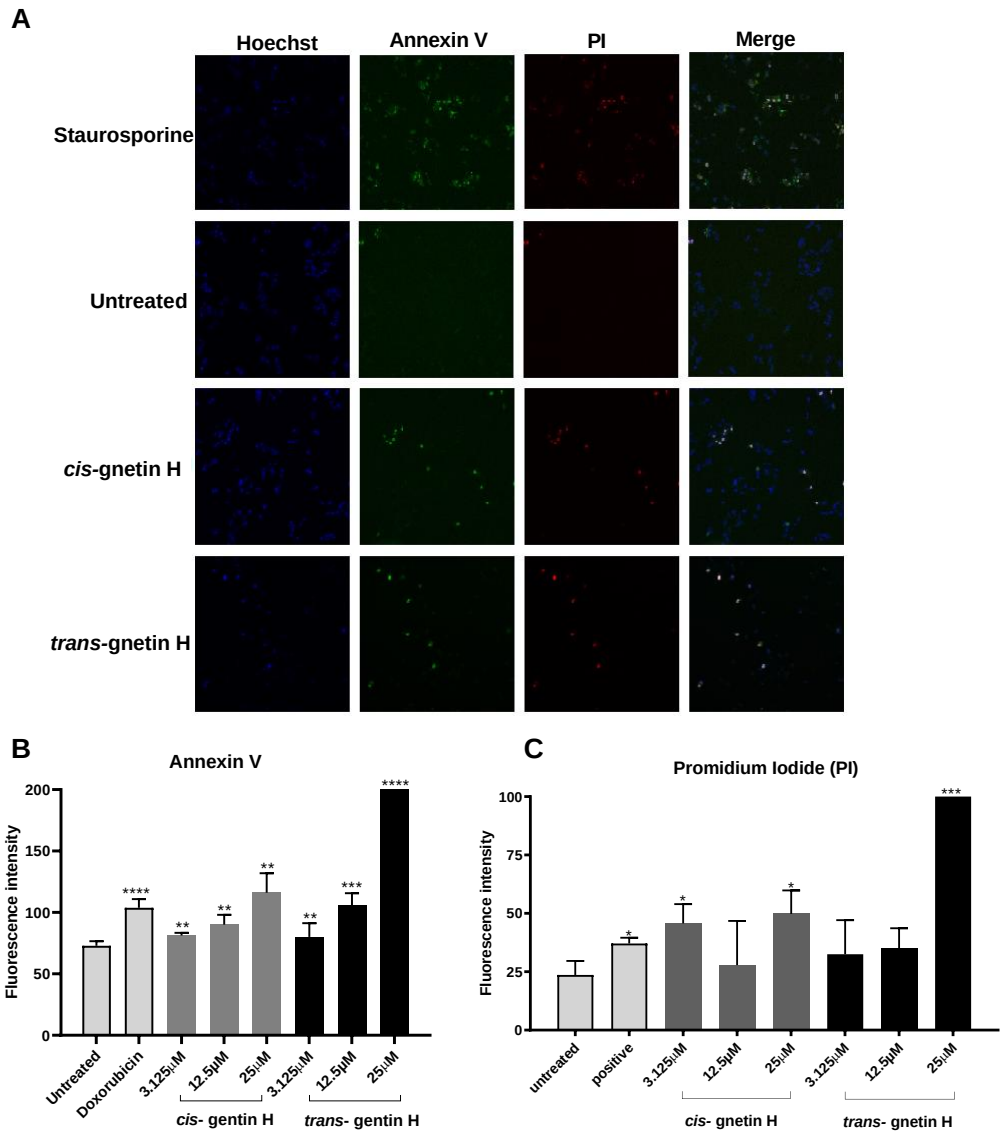

Figure 9. Induction of apoptosis in A549 cell by treating with *cis*- and *trans*- gnetin H.

Cells were treated with test compounds at different concentrations for 20h, then stained with Hochest, Annexin V and PI dyes. Staurosporine was used as a positive control, and cells treated with vehicle only served as a negative control. (A) HCS images represent 3 different channels; Hoechst channel to identify the nuclei (stained blue), Annexin V channel identifies phosphatidylserine **(**PS) indicating early apoptosis (stained green) and

Promidium iodide (PI) channel to identify plasma membrane disruption (stained red). The last channel represents all three channels merged together. (B) Quantification analysis of cells stained with Annexin V indicated an early apoptosis. (C) Quantification analysis for cells stained with PI indicated late apoptosis. (n=3) *P≤0.05, **P≤0.01, ***P≤0.001.

3.5. Apoptosis protein array.

Proteins mainly affecting apoptosis were analyzed using the protein profile array, as shown in Figure 10. The results show proteins inhibiting apoptosis from the inhibitor protein family (IAP) such as the X-linked inhibitor of apoptosis protein (XIAP), survivin, and the heat shock protein (Hsp60) were significantly down-regulated after treatment with *cis*- and *trans*-gnetin H(****p≤0.00001) (Fig 10B). However, other pro-apoptotic proteins were significantly up-regulated after treatment with *cis*- and *trans*-gnetin H. Bad, cytochrome C, FasL and TRAIL-1 were up-regulated after treatment with *cis*-gnetin H only, while the BH3 interacting-domain death agonist BID, IGFBP-4, TRAIL-2, TRAIL-3 and TRAIL-4 were also up-regulated after treatment with both *cis* and *trans*-gnetin H (*p≤0.05, **p≤0.001, ***p≤0.0001 or ****p≤0.00001) (Fig 10A).

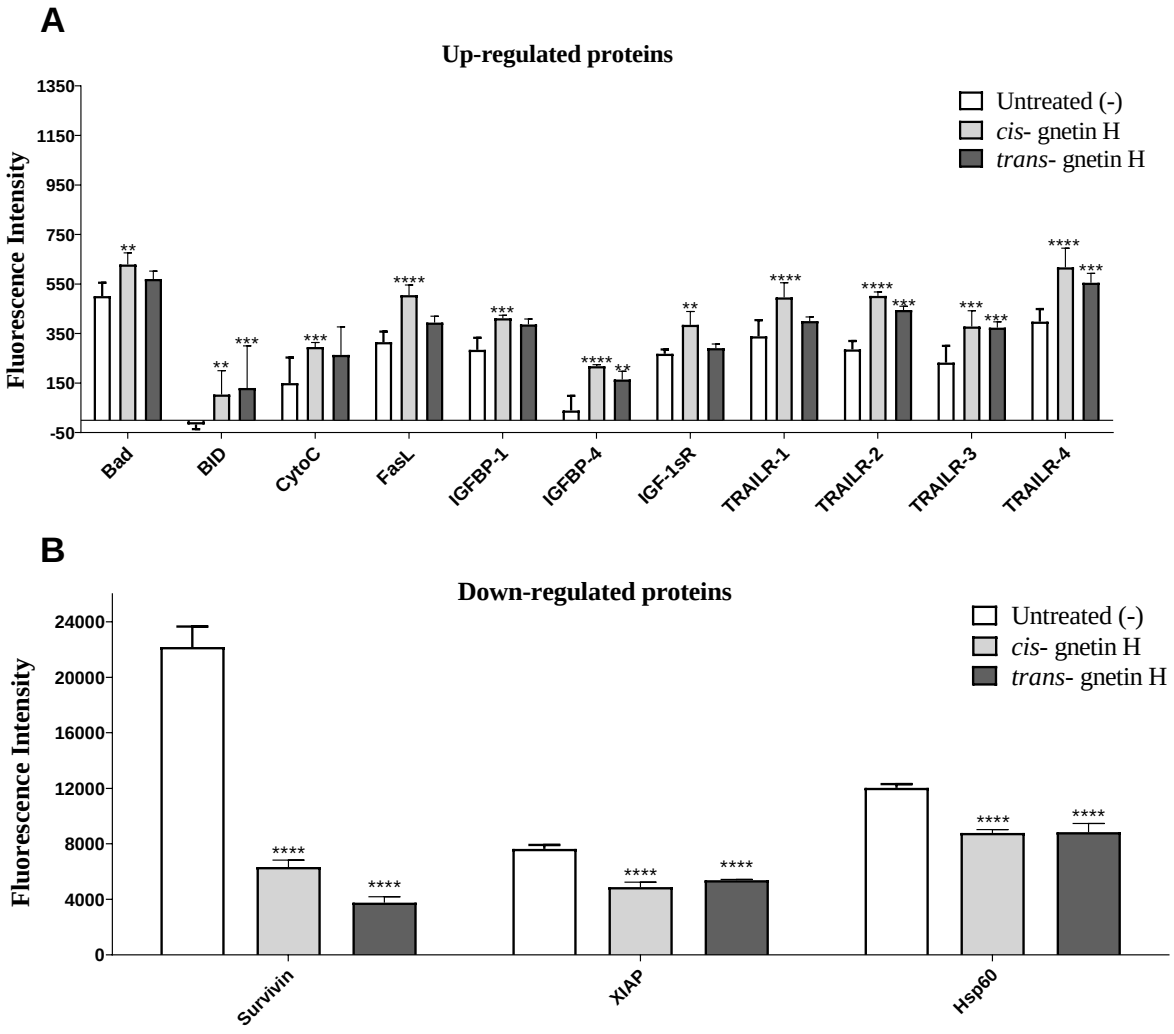

Figure 10. Effect of *cis*- or *trans*- gnetin H on key regulatory proteins of apoptosis in A549 cell line.

(A) Up-regulated proteins after treating with *cis*- or *trans*- gnetin H (B) Down-regulated proteins after treating with *cis*- or *trans*- gnetin H. Data are represented as mean ± SD. (n=4) *p≤0.05, **p≤0.01, ***p≤0.001 or ****p≤0.0001.

4. Discussion

Previously we showed that *cis*- and *trans*-gnetin H had highly selective cytotoxicity against different cancer cells versus normal cells and could induce apoptosis with anti-metastatic effect (Gao et al., 2015b; Gao & He, 2017). The purpose of this study was to better ascertain how *cis*- and *trans*-gnetin H induce apoptosis in cancer cells. Apoptosis refers mainly to cell changes; this includes many morphological changes comprising nuclear condensation as a result of chromatin compression, developing apoptotic bodies and plasma membrane blebbing leading to an opening in the cell surface (Hockenbery, 1995). The ability of *cis*- and *trans*- gnetin H to induce apoptosis was confirmed earlier by (Gao et al., 2015c) using flow cytometry; both compounds showed a significant induction od apoptosis in a dose-dependent manner (**$P \leq 0.010$). Gao et al., also measured cell injury caused upon treatment; this was observed as a loss of the mitochondrial potential indicating early apoptosis after 24h, while changes in the nuclear size as well as the loss of the plasma membrane permeability indicated severe cell injury after 48h treatment.

Resveratrol is produced within the plant by the resveratrol synthase enzyme as a part of plant defense mechanisms against pathogen attack, including UV or stress damage, which is consistent with anti-oxidant activity of resveratrol. In this study, *cis*- and *trans*- gnetin H were assessed for their anti-oxidant ability to scavenge free radicals using the DPPH free radical and demonstrated a high RSA % in a dose-dependent manner (Fig. 7). Since *cis*- and *trans*- gnetin H are trimers of the resveratrol, this finding shows that large molecular weight compounds can have a positive correlation with respect to DPPH

oxidation compared to the smaller compound from which they are derived (Limmongkon et al., 2017).

In this study, we further examined the anti-cancerous ability of *cis*- and *trans*- gnetin H by testing their ability to induce apoptosis using HCS. Annexin V dye was used to measure early apoptosis by detecting exposed PS while PI dye was used to measure late apoptosis by detecting the disruption of the plasma membrane. Both *cis*- and *trans*-gnetin H induced early apoptosis at all concentrations tested and induced late apoptosis at the highest concentration tested suggesting that 25μM might be a toxic concentration for both compounds inducing other types of cell death (Fig. 9).

Mitochondria are known as the main generator of ROS. In this study, we detected the mitochondria's ability to significantly increase the OS levels after treatment with *cis*- or *trans*- gnetin H, therefore, decreasing the mitochondrial potential. Gao et al., 2015 showed that treatment with *cis*- or *trans*-gnetin H caused the release of cytochrome C and the activation of caspases 3 and 7. Thus most likely the disruption of the mitochondrial membrane facilitates the pro-apoptotic protein cytochrome C to travel from the mitochondria into the cytosol and allows the initiation of cellular destruction by promoting the activation of caspases 3 and 7, causing the final stage of apoptosis. Gao et al., 2015 also showed that *cis*- and *trans*- gnetin H inhibit the translocation of NF-κB by inhibiting the production of TNF-α, therefore suppressing cell proliferation and inducing apoptosis. Consistent with this finding in this study both *cis*- and *trans*- gnetin H increased the ROS generation in cancer cell line (Fig. 8).

The blockage of the NF-kB pathway may also be associated with inhibiting of cytokines by high levels of ROS. We observed that an x-linked inhibitor of apoptosis (XIAP), which is one of the inhibitor apoptosis proteins (IAPs), was inhibited for both *cis*- and *trans*- gnetin H, hindering its ability to bind to the caspases and blocking their apoptotic signaling via either the intrinsic or extrinsic pathway. Furthermore, heat shock protein (HSP 60) was also inhibited. Hsp60 is involved in the maturation of caspase 3 and upon apoptosis activation both Hsp60 and procaspase 3 will be dissociated before release into the cytosol (Tang et al., 2016). Moreover, as cancer cells continue to grow, survivin continues to be elevated at high rates allowing cancer to escape apoptosis by interacting with procaspases and blocking their activation (Mobahat et al., 2014). Upon treatment with *cis*- or *trans*-gnetin H, survivin levels were drastically down-regulated facilitating cell apoptosis and elevating the levels of caspase 3 and 7. Recently, the HSP60-survivin complex has been shown to be elevated in many cancer types and the biological functions of the complex depends on where this interaction takes place (Huang & Yeh, 2020).

In contrast, *cis*- and *trans*-gnetin H caused an increase in the death ligand FasL and TNFα- related apoptosis inducing ligand (TRAIL), suggesting that *cis*- and *trans*-gnetin H activate the extrinsic pathway by binding to the cell surface receptor. Unlike other death receptors, TRAIL does not cause any systemic toxicity making it an attractive target for cancer therapy, especially since most tumors are resistant to TRAIL activation during treatment (Fulda & Debatin, 2005; LeBlanc & Ashkenazi, 2003). This binding engages the extrinsic pathway to the intrinsic pathway through cleaving of BH3-only proteins such as Bad, Bid, and Bim, which are known as the pro-apoptotic proteins. These proteins were seen to be elevated upon treatment with *cis*- and *trans*- gnetin H

supporting this hypothesis. Other proteins involved in inducing apoptosis include the insulin-like growth factors binding proteins (IGFBPs), such as IGFBP-4. The results of previous studies are not clear as to whether IGFBP-4 induces or suppresses apoptosis (Durai et al., 2007; Mai et al., 2018). Our results showed that both *cis*- and *trans*- gnetin H cause elevated levels of IGFBP-4 by treatment coinciding with the induction of apoptosis, strongly suggesting that IGFBP-4 acts to promote apoptosis.

In conclusion, our study demonstrates that *cis*- and *trans*-gnetin H have both anti-oxidant and anti-cancerous activities. *Cis*- and *trans*-gnetin H induced apoptosis by elevating the ROS causing high level of OS leading to activating both the intrinsic pathway (mitochondrial pathway) and the extrinsic pathway (death receptor pathway) through the TRAIL pathway.

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CHAPTER 4

THE RESVERATROL OLIGOMERS *CIS*- AND *TRANS*-GNETIN H SUPPRESS HUMAN LUNG AND BREAST CANCER CELL METASTASIS BY REGULATION OF MMP FAMILY PROTEINS

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Abstract

***Background*:** The antitumor compounds *cis*- and *trans*- gnetin H derived from *Paeonia suffruticosa* Andrews (PSE) have been known to exhibit many biological activities including antioxidant, anti-inflammatory and antitumor effect. In addition to induction of apoptosis and nuclear condensation, changes in cell permeability and disruption of the mitochondrial transmembrane potential *in vitro*, *cis*-gnetin H has been demonstrated to significantly suppresses the growth of xenograft tumors *in vivo*.

***Methods*:** In this study, we tested the potential of *cis*- and *trans*-gnetin H to inhibit lung cancer cell A549-GFP motility. We also tested the ability of both compounds to inhibit adhesion, migration, and invasion, critical steps in cancer metastasis; utilizing the extracellular matrix (ECM) proteins. In addition, Enzyme linked Immunosorbent Assay (ELISA) was performed to test the levels of matrix metalloproteinase MMP-1 and its inhibitors TIMP-1 and TIMP-2 using a highly invasive Triple negative breast cancer cell line MDA-MB-231.

Results: *Cis*- and *trans*-gnetin H treatment caused a decrease in the motility of A549-GFP cells to 43% and 51%, respectively, ($p < 0.001$) and a reduction in A549 cell migration through a filter membrane to 73% and 89%, respectively ($p < 0.001$). Likewise, A549 cell invasion through a Matrigel matrix layer was also decreased by 82% and 87%, respectively, ($p < 0.001$) after *cis*- and *trans*-gnetin H treatment in a dose-dependent manner. In addition, *cis*- and *trans*-gnetin H treatment caused a 70% and 65% reduction of A549-GFP cell adhesion to human vascular endothelial cells (HUVEC), respectively ($p < 0.001$). After treatment with *cis*- or *trans*-gnetin H, MMP-1 was drastically reduced in combination with

both TIMP-1 and TIMP-2, which suggests an independent effect of the TIMPs on the MMP-1 function.

Conclusion: These observations suggest that *cis*- and *trans*-gnetin H inhibits the metastasis of human lung and breast cancer cells *in vitro* through reduction of cancer cell adhesion, migration, and invasion by inhibiting MMP family proteins MMP-1, TIMP-1 and TIMP-2.

Key words

Resveratrol, *cis*-gnetin H, *trans*-gnetin H, cancer metastasis, matrix metalloproteinase (MMP), tissue inhibitor metalloproteinase (TIMP).

1. Introduction

Resveratrol (3,5,4'-trihydroxystilbene) is a naturally occurring stilbene present in grape skin, peanuts, soybeans, and many other vegetables. It plays a defensive role within the plant in response to many stress factors including injury (Langcake & Pryce, 1976) or fungal attacks (Adrian et al., 1997). Recently, resveratrol has been used extensively as a dietary supplement and gained a lot of interest due to its chemo-preventive properties against cancer initiation, promotion, and progression (He et al., 2010a; Jang et al., 1997). It has been revealed that resveratrol can inhibit the growth of various cancer cells *in vivo* and *in vitro* by mediating different signaling pathways (Buhrmann et al., 2019; Liu et al., 2018; Rossi et al., 2018); (Sinha et al., 2016). Additionally, it has been demonstrated that

resveratrol inhibits migration and invasion of the MCF-7 cell line through the PI3K/AKT and Wnt/ß-catenin signaling pathways (Tsai et al., 2013).

The oligostilbenes, *cis*- and *trans*-gnetin H, have been previously isolated from the dried seeds of *P. suffruticosa* and studied along with other oligostilbenes for their cytotoxicity against various cancer cell lines (Kim, H. J. et al., 2002). They have been demonstrated to have anti-proliferative and apoptotic activities against different cancer cell lines by releasing cytochrome c, activating caspases 3/7, inhibiting NF-κB activation, and arresting cells at the G0–G1 phase (Gao, Y., He., 2015a). *Cis*- and *trans*-gnetin H have also been shown to have anti-metastatic activity against MDA-MB-231 cell line (Gao & He, 2017). As both *cis*- and *trans*-gnetin H showed much more potent cytotoxic and apoptotic effects in human cancer cells compared to resveratrol, we wanted to further investigate the anti-metastatic potential of these two oligostilbenes.

Cancer metastasis, the dissemination of cancer cells from the primary location to a distant site, is a very complicated process that explicitly defines malignant from benign tumors. Despite the general effort in detection, diagnosis, surgical techniques and treatments, 90% of all cancer deaths occur due to cancer metastasis rather than the growth of tumors (Folgueras et al., 2004). This is due to the obstacles that limit the development of anti-metastatic drugs, such as the limitation of a useful high throughput screen for compounds or not using proper animal models for pre-clinical studies (Gao, Y. & He, 2017). For this reason, more efforts are needed to overcome these limitations to develop a proper anti-metastatic drug.

The metastasis of cancer cells require degradation of the extracellular matrix (ECM), which is primarily influenced by enzymes known as the matrix metalloproteinases (MMPs). It has been demonstrated that MMPs play a major role in cancer adhesion, invasion, and metastasis (Mook et al., 2004). MMPs are a group of enzymes that are responsible for degrading many different forms of proteins in the ECM. Based on their structures, MMPs are divided into six different groups: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs and other non-classified MMPs (Jabłońska-Trypuć, et al., 2016). Since high levels of MMPs have shown a close relationship with the progression of cancer, anticancer treatment by inhibiting MMPs would be an effective approach (Winer et al., 2018). One of the main strategies for inhibiting the activity of MMPs is through their nuclear transcription factor by blocking the cytokine-receptor interactions that are known to up-regulate the MMP genes such as: NF-κB, IL-1 or epithelial growth factor (EGF) receptors (Folgueras et al., 2004). One of the most widely expressed MMPs is MMP-1, an interstitial collagenase, which is known to be overexpressed in many cancers leading to poor prognosis (Shin et al., 2015). MMP-1 is also known to be upregulated in the triple negative breast cancer cell line (TNBC) MDA-MB-231 with no PR, ER and HER2 hormone expression making it more aggressive neoplasm due to lack of effective treatments (de Ruijter et al., 2011). Therefore, overexpression of MMPs can be regulated by blocking the interaction of certain cytokines, such as TNF-α, IL-1 or epithelial growth factor with their receptors leading to the inhibition in cancer progression (Gao, Y. & He, 2017).

Tissue inhibitor metalloproteinases (TIMPs) are a group of proteins known to inhibit the MMPs and consist of TIMP-1, 2, 3 and 4 (de Ruijter et al., 2011). TIMP-1 in particular has been known to be overexpressed in multiple cancers including prostate, lung and breast cancer (Gouyer et al., 2005; Oh et al., 2011; Yoshiji, Gomez, & Thorgeirsson, 1996). Originally, when TIMPs were discovered, they were thought to inhibit MMP activity and facilitate ECM degradation. However, it is now clear that TIMPs are a multi-protein family that participate in many biological activities independent of the inhibition of MMPs, such as facilitating cancer cell proliferation, migration, invasion and apoptosis in multiple cell lines (Luparello et al., 1999; Porter et al., 2004; Stetler-Stevenson, 2008a), allowing both MMPs and TIMPs to play a dual role when it comes to tumor progression by affecting both pro- and anti-tumorigenic activities (Stetler-Stevenson, 2008b). TIMP-2 was primarily known to be overexpressed with the presence of low MMP activity, inhibiting the cancer invasion and metastasis (DeClerck et al., 1992). In contrary, recent studies suggest overexpression of these proteins could also be associated with the progression of breast cancer. High levels of TIMP-2 were found to be related to poor prognosis and overall survival associated with high TIMP mRNA levels in breast cancer (Ree et al., 1997; Remacle et al., 2000).

Previously, both *cis*- and *trans*-gnetin H were evaluated for their ability to inhibit MDA-MB-231 metastasis (Gao, Y. et al., 2015a). In this study, we further examined the anti-metastatic activity of *cis*- and *trans*-gnetin H against the human lung cancer cell line A549 to identify the mechanism of the action by which this activity occurs.

2. Materials and Methods

2.1. Cell culture

A549 (human lung carcinoma) cell line, MDA-MB-231 (human breast adenocarcinoma) and Huvec (Human umbilical vein endothelial cells) cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The A549 cell line expressing green fluorescent protein (A549-GFP) was purchased from Cell BioLabs Inc. (San Diego, CA, USA). A549 and A549-GFP cells were cultured in RPMI-1640 medium (Sigma-Aldrich), MDA-MB-231 cells were cultured in DMEM medium (Sigma-Aldrich). Huvec (Human umbilical vein endothelial cell) were cultured in vascular cell Basal Medium supplemented with Endothelial cell Growth Kit-BBE (ATCC, Manassas, VA, USA). All cells were maintained in medium containing 10% fetal bovine serum (FBS) and 1% antibiotic (penicillin/ streptomycin) and incubated in 5% CO_2 at 37˚C.

2.2. Cell motility assay

A cell motility assay was conducted to measure the tracks generated by migrating cells using a 96-well collagen plate (Corning, NY, USA) coated with blue fluorescent beads (Life Technologies, Eugene, OR, USA). Briefly, fluorescent beads were centrifuged at 14,000 g for 1 min and washed twice with PBS. Subsequently, 75 µL beads were added to each well and incubated for 1 h in the 37˚C incubator. Beads were washed five time with PBS before adding the A549-GFP cells at 500 cells/well. Cells were allowed to attach for 1h before they were treated with various concentrations of *cis*- or *trans*-gnetin H

containing 10% FBS for 18 h. Cells treated with 10% FBS served as a positive control while cells treated with serum free medium served as a negative control. Tracks generated by motile cells were imaged using Arrayscan VTI High Content Screening (HCS) analyzer (Thermo Fisher Scientific) and the data were analyzed using vHCS Scan software.

2.3.Migration and invasion assay

The migration and invasion assays were performed as described previously (Gao, Y. & He, 2017). Briefly, a 24-well cell culture plate containing 0.5 mL cell culture medium supplemented with 10% FBS in each well. Millicell® Cell Culture Inserts (EMD Millipore) with 8-µm pore size were placed in each well. A549 cells (2 x 10^5 cells/mL) in 0.5 mL serum-free medium were seeded into the insert. In this assay, we tested the ability of the compounds to inhibit migration using three concentrations (50, 25 and 12.5 µM). *Cis*- or *trans*-gnetin H was added to the medium in both the insert and the well to the desired concentration (50, 25 and 12.5 µM). Cells treated with Doxycycline at 100 µM (Sigma-Aldrich) were used as a positive control while cells treated with vehicle only were used as a negative control. After 16 h incubation, cells were removed from the filter insert using a sterile cotton swab and 10% AlamarBlue dye was added to the well. Therefore, the A549 cells that have migrated through the 8-µm pore filter were detected. After incubation at 37˚C for 3 h, the plates were read in a SpectraMax M5 microplate reader at Ex/Em wavelengths of 550/590 nm.

For the cell invasion assay, the procedure was the same except that the filter inserts were pre-coated with a thin layer of Matrigel matrix (100 µL at 5 mg/mL; Corning Inc).

2.4. Adhesion assay

An adhesion assay was conducted to determine the effect of *cis*- or *trans*-gnetin H on the adhesion of highly metastatic cancer cell line (A549-GFP) to human umbilical vein endothelial cells (HUVECs). Briefly, HUVECs were seeded in a 96-well plate to achieve a 100% confluent monolayer. Meanwhile, A549-GFP cells were also seeded and allowed to attach overnight before the treatment. Subsequently, A549-GFP cells were pretreated for 3 h using various concentrations (50, 25 and 12.5 μM*)* of *cis*- or *trans*-gnetin H, then stimulated A549-GFP by adding TNF-α 10 ng/ml for 5 h. Next, A549-GFP cells were trypsinized and resuspended with complete medium into a density of 10,000 cells/mL. The medium of HUVECs was removed and replaced with 100 μL of pretreated A549-GFP cells. After the HUVECs and A549-GFP cells were incubated together for 1 h at 37‘C CO_2. Cell medium was removed and cells were gently washed with serum-free medium. Attached A549-GFP cells were imaged using Arrayscan VTI HCS analyzer (Thermo Fisher Scientific) and the data were analyzed using vHCS Scan software. Cells treated with doxycycline (100 μM) were used as a positive control while cells treated with vehicle only were used as a negative control.

2.5.MMP and TIMP protein assay

An ELISA was performed according to manufacturer’s protocol to assess the levels of MMPs and TIMPs. Briefly, MDA-MB-231 cells were seeded in a 96-well microplate at 5×10^{3} cells/well density and allowed to attach overnight before treatment. Cell culture medium was replaced with fresh one containing *cis*- or *trans*- gnetin H compounds at

various concentrations (50, 25 and 12.5 μM). After incubation for 3 h, the supernatant was collected for further analysis. Cells treated with doxorubicin (100 μM) were used as a positive control, while cells treated with vehicle only were used as a negative control.

2.6. Cytokine assay

To assess cytokine response after treating with *cis*- or *trans*- gnetin H. An ELISA was performed according to manufacturer's protocol. Briefly, cells were seeded in a 96 well microplate at 5×10^3 density and allowed to attach overnight before treatment. Cell culture medium was replaced with a new medium containing *cis*- or *trans*- gnetin H compounds using different concentrations: 50, 25 and 12.5 μM for 24h. Next, supernatant was collected for further analysis. Doxorubicin 100μM was used as a positive control, while cells treated with a vehicle only served as a negative control.

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0 software (GraphPad Software Inc). All data were reported as mean ±SD and were analyzed with unpaired t-test. Differences are considered statistically significant if $p<0.05$.

3. Results

3.1. *Cis*- and *trans*-gnetin H suppresses the motility of A549-GFP cancer cells

To test the effect of *cis*- and *trans*-gnetin H on the motility of A549-GFP cancer cells, we measured the tracks that were generated by each moving cell (Fig. 11). The track generated was proportional to the magnitude of cell movement as it pushed away the coated beads (Fig. 11A). In this assay, A549-GFP cells treated with 10% serum (positive control) showed a 55.6 % increase in cell motility compared to the cells that were not treated with serum (negative control) (***$p < 0.001$). *Cis*-gnetin H reduced the motility of A549-GFP cells by 43%, 41% and 43% compared to the positive control at 10, 25 and 50 μM, respectively (**$p < 0.01$ or ***$p < 0.001$) (Fig. 11B). Similarly, *trans*-gnetin H reduced the cancer cell motility by 37.9%, 35% and 46.2% at 10, 25 and 50 μM, respectively (**$p < 0.01$ or ***$p < 0.001$) (Fig. 11C).

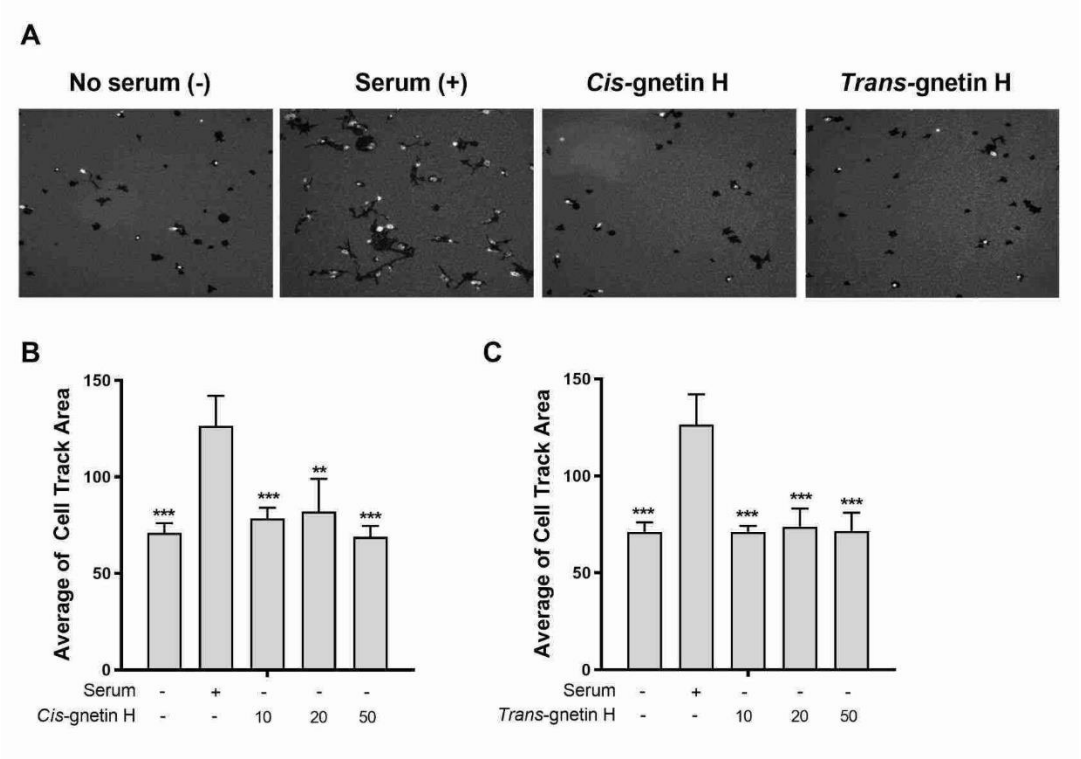

Figure 11. Effects of *cis*- and *trans*-gnetin H on cell motility of A549 cells.

A 96-well collagen plate was coated with fluorescent beads and seeded with A549-GFP cells. After cells were treated with *cis*- or *trans*-gnetin H for 18 h, cell movement was evaluated by measuring the track area. Cells treated with serum-free medium served as a negative control and cells treated with medium containing 10% serum served as a positive control. **A)** Track areas generated by moving cells. **B), C)** Track areas of cells treated with *cis*- or *trans*-gnetin H. The error bars indicate the standard deviation (n = 4). *$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

3.2. *Cis*- and *trans*-gnetin H inhibit the migration and invasion of A549 lung cancer cells

The effect of *cis*-and *trans*-gnetin H on the migration and invasion of A549 lung cancer cells was investigated at multiple concentrations and following 16 h of treatment, both compounds showed inhibition of the migration and invasion of A549 cells *in vitro* (Fig. 12). In the migration assay, *cis*-and *trans*-gnetin H significantly reduced the number of A549 cells that migrated through the pores of the filter insert. *Cis*-gnetin H inhibited the number of migrated A549 cells by 28.4% and 74% compared to the negative control at 25 and 50 μM, respectively (*$p < 0.05$ or ***$p < 0.001$) (Fig. 12A). *Trans*-gnetin H inhibited the number of migrated A549 cells by 35.4%, 89.9%, and 89.7% compared to the negative control at 12.5, 25 and 50 μM, respectively (*$p < 0.05$ or ***$p < 0.001$) (Fig. 12B).

In the invasion assay, only those cells that passed through the Matrigel matrix layer and the filter membrane were detected. The inhibition rates of *cis*-gnetin H on the invasion of A549 cells were 68.1%, 85.6% and 82.1% compared to the negative control at 12.5, 25 and 50 μM, respectively (**$p < 0.01$ or ***$p < 0.001$) (Fig. 12C). The inhibition rates of *trans*-gnetin H on the invasion of A549 cells were 77.6%, 82.9% and 87.9% compared to the negative control at 12.5, 25 and 50 μM, respectively (***$p < 0.001$) (Fig. 12D).

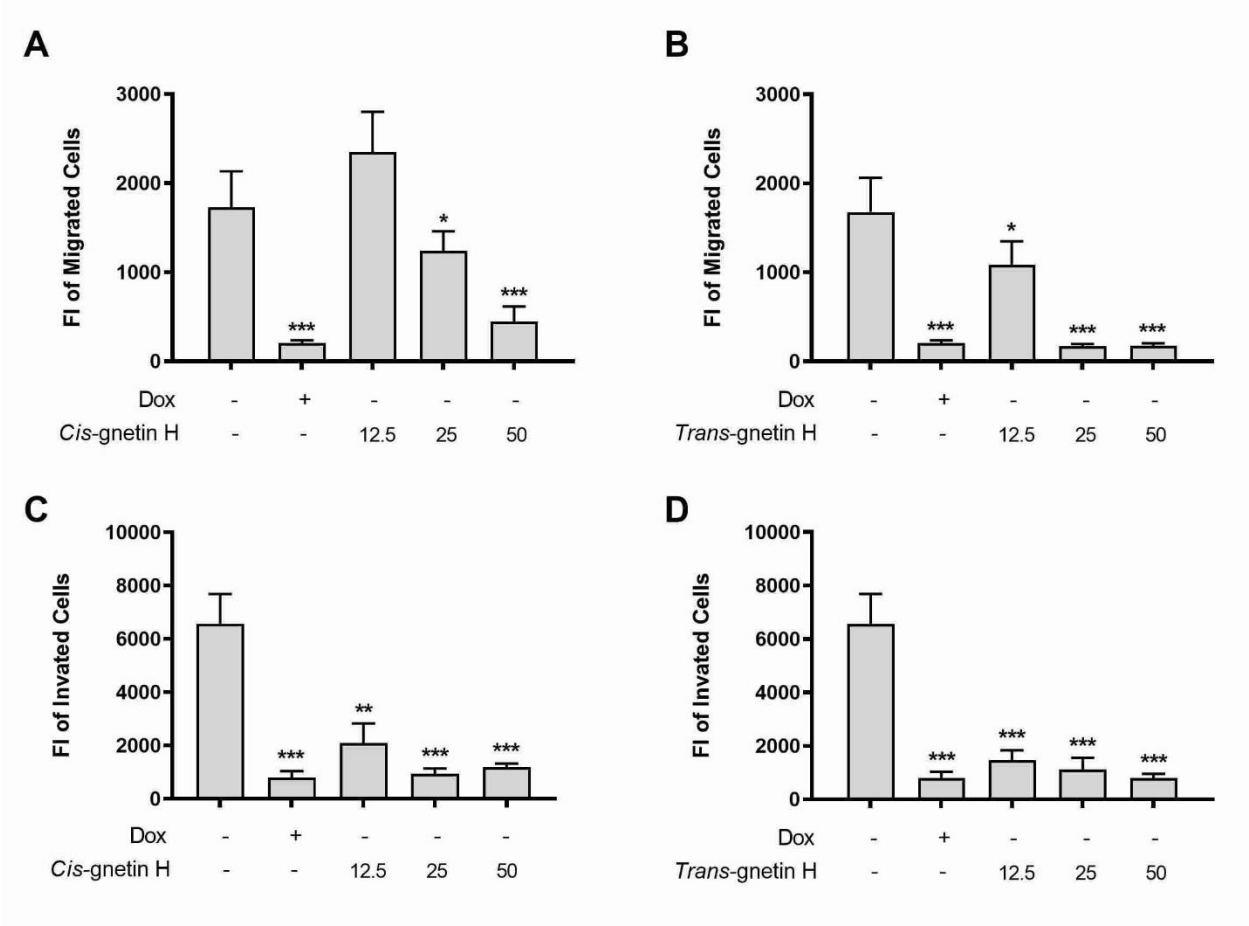

Figure 12. Effects of *cis*- and *trans*-gnetin H on cell migration and invasion of A549 cells.

A549 cells were treated with 50, 25 and 12.5 µM of *cis*- or *trans*-gnetin H for 16 h. Cells treated with doxycycline (100 µM) served as a positive control and cells treated with vehicle only served as a negative control. Results are presented as the % of the inhibited cells using AlamarBlue staining after passing through the Millicell culture inserts with the presence of Matrigel (invasion) or without Matrigel (migration). **A), B)** Inhibition of cell migration after treatment with *cis*-gnetin H (right) or *trans*-gnetin H (left). **C), D)** Inhibition of cell invasion after treatment with *cis*-gnetin H (right) or *trans*-gnetin H (left). Data are presented as mean ± standard deviation (SD) (n = 4). *$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

3.3.*Cis*- and *trans*-gnetin H reduce the adhesion of A549-GFP cancer cells onto HUVECs

The metastatic process of malignant tumor cells involves the adhesion of the tumor cell to the vascular endothelial cell as well as attachment of the tumor cell to the extracellular matrix (ECM) (Welch & Hurst, 2019). In the adhesion assay, A549-GFP cancer cells were pre-activated with TNF-α and treated with *cis*- or *trans*-gnetin H before they were incubated with the HUVEC cells. HCS results showed less adherent green fluorescent A549 cells compared to A549 cells that were only treated with TNF-α (Fig. 13A). A549-GFP cells pre-activated with TNF-α showed a 94% increase in cell adhesion compared to the non-activated cells (negative control) (***$p \leq 0.001$). A549-GFP cells treated with doxycycline (positive control) after pre-activation showed a 76% decrease in cell adhesion compared to the untreated cells (***$p \leq 0.001$). Similar to the positive control, *cis*- and *trans*-gnetin H remarkably decreased the adhesion of A549-GFP cells to HUVECs in a dose dependent manner. At the concentrations of 12.5, 25 and 50 μM, *cis*-gnetin H decreased the number of attached A549-GFP cells to HUVECs by 17%, 68% and 69% (Fig. 13B); At these same concentrations *trans*-gnetin H decreased the number of attached A549-GFP cells by 20%, 64% and 62% (Fig. 13C). (**$p \leq 0.01$ or ***$p \leq 0.001$)

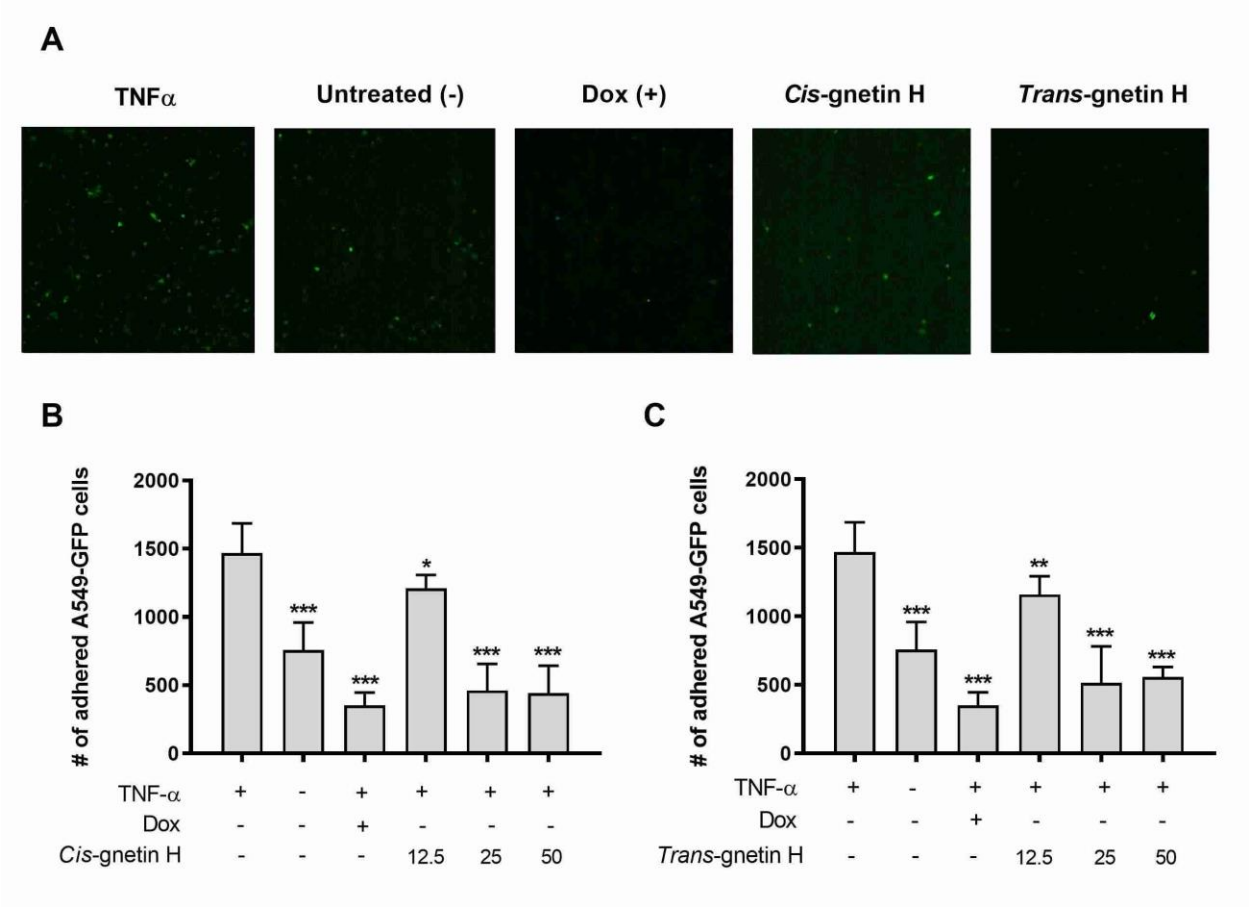

Figure 13. Effects of *cis*- and *trans*-gnetin H on l A549-GFP lung cancer cell line adhesion to Human Umbilical Endothelial Cells (HUVECs) upon activating the cells with TNF-α.

This was measured by (**A**) high content screening HCS using a 96-well, collagen plate for HUVEC attachment of A549-GFP (green). (**B, C**) Fluorescent quantification for A549-GFP adhesion. Data are presented as mean ± SD (n = 3). *$p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

3.4.*Cis*- and *trans*-gnetin H decrease the expression of MMP and TIMP proteins in MDA-MB231 cells

An ELISA assay was performed to assess the response of MMPs and TIMPs in cancer cells after treatment with *cis*- or *trans*-gnetin H. MMP-1, TIMP-1, and TIMP-2 secretion was measured after MDA-MB-231 cells were treated with *cis*- or *trans*-gnetin H at various concentrations (50, 25 and 12.5 μM). Doxorubicin, a well-known anti-cancer drug, was used as a positive control. Doxorubicin showed a decrease in the MMP-1, TIMP-1 and TIMP-2 secretion by 94%, 56%, and 83%, respectively, compared to the negative control. *Cis*- and *trans*-gnetin H also inhibited the expression of all three proteins MMP-1, TIMP-1 and TIMP-2. At these treated concentrations, 12.5, 25 and 50μM, MMP-1 secretion was reduced by *cis*-gnetin H by 81%, 95%, and 95% and was reduced for *trans*-gnetin H by 60%, 88% and 97%, respectively (Fig. 14A). TIMP-1 was inhibited by *cis*-gnetin H by 91%, 92% and 93% and inhibited by *trans*-gnetin H by 92, 92 and 96%, respectively (Fig. 14B). In addition, TIMP-2 was inhibited by *cis*- gnetin H by 89%, 92% and 92% while inhibited for *trans*-gnetin H by 85%, 92% and 92%, respectively (Fig. 14C).

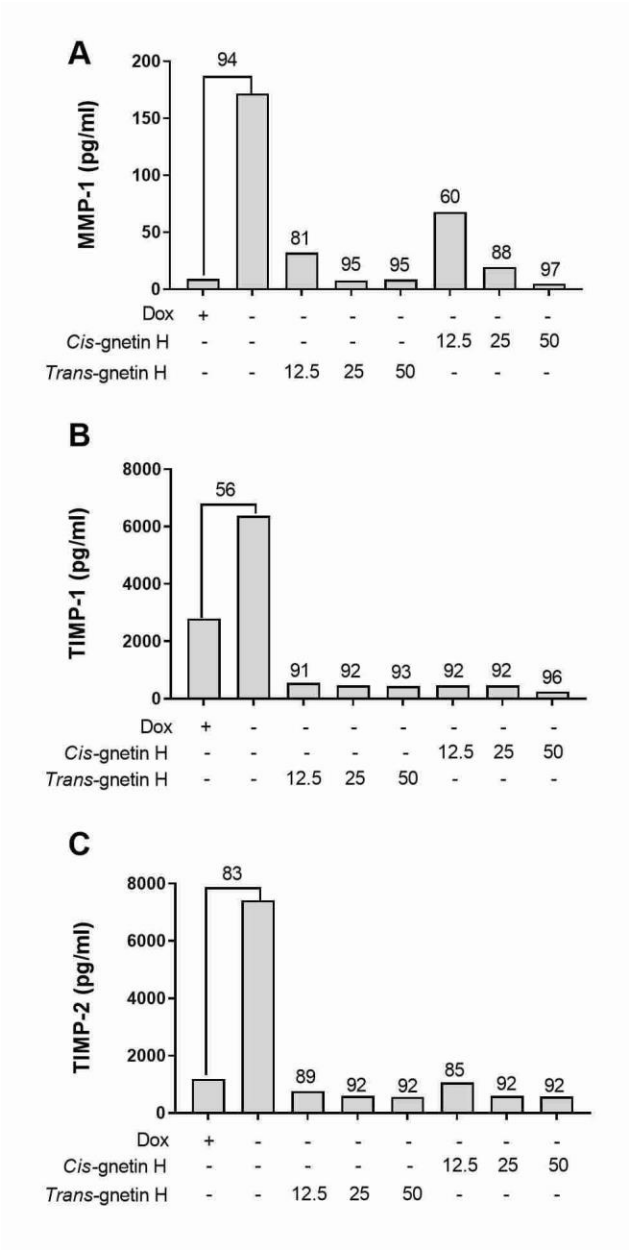

Figure 14. Protein expression of MMP-1, TIMP-1 and TIMP-2 in MDA-MB-231 cells.

MDA-MB-231 cells were pretreated with 50, 25, 12.5 μM of *cis*-gnetin H or *trans*-gnetin H for 24 h. The secretion of: A) MMP-1, B) TIMP-1 and C) TIMP-2 was determined by ELISA. The results show the inhibition percentage % of each concentration in (pg/mL) compared to the negative control.

3.5.*Cis*- and *trans*-gnetin H decreases the expression of inflammatory cytokines

We studied the effects of *cis*- and *trans*-gnetin H on the expression of inflammatory cytokines IL-2, IL-6, IL-8, IL-13, TNF-α, MCP-1, and INFγ in MDA-MB-231 cells. As shown in Fig. 15A-15G, doxorubicin (positive control) at 100 μM showed an inhibition of all cytokine expression tested compared with the untreated control. *Cis*- and *trans*-gnetin H showed inhibition on the expression of most inflammatory cytokines including IL-2, IL-8, IL-13, TNF-α, MCP-1, and INF-γ. Only the highesr concentration *trans*-gnetin H (50 μM) showed an inhibition of the expression of IL-6 (Fig. 15B). *Cis*- and *trans*-gnetin H showed the most significant inhibition against MCP-1. At the lowest concentration (12.5 μM), *cis*- and *trans*-gnetin H decreased MCP-1 expression by 91% and 92%, respectively (Fig. 15F).

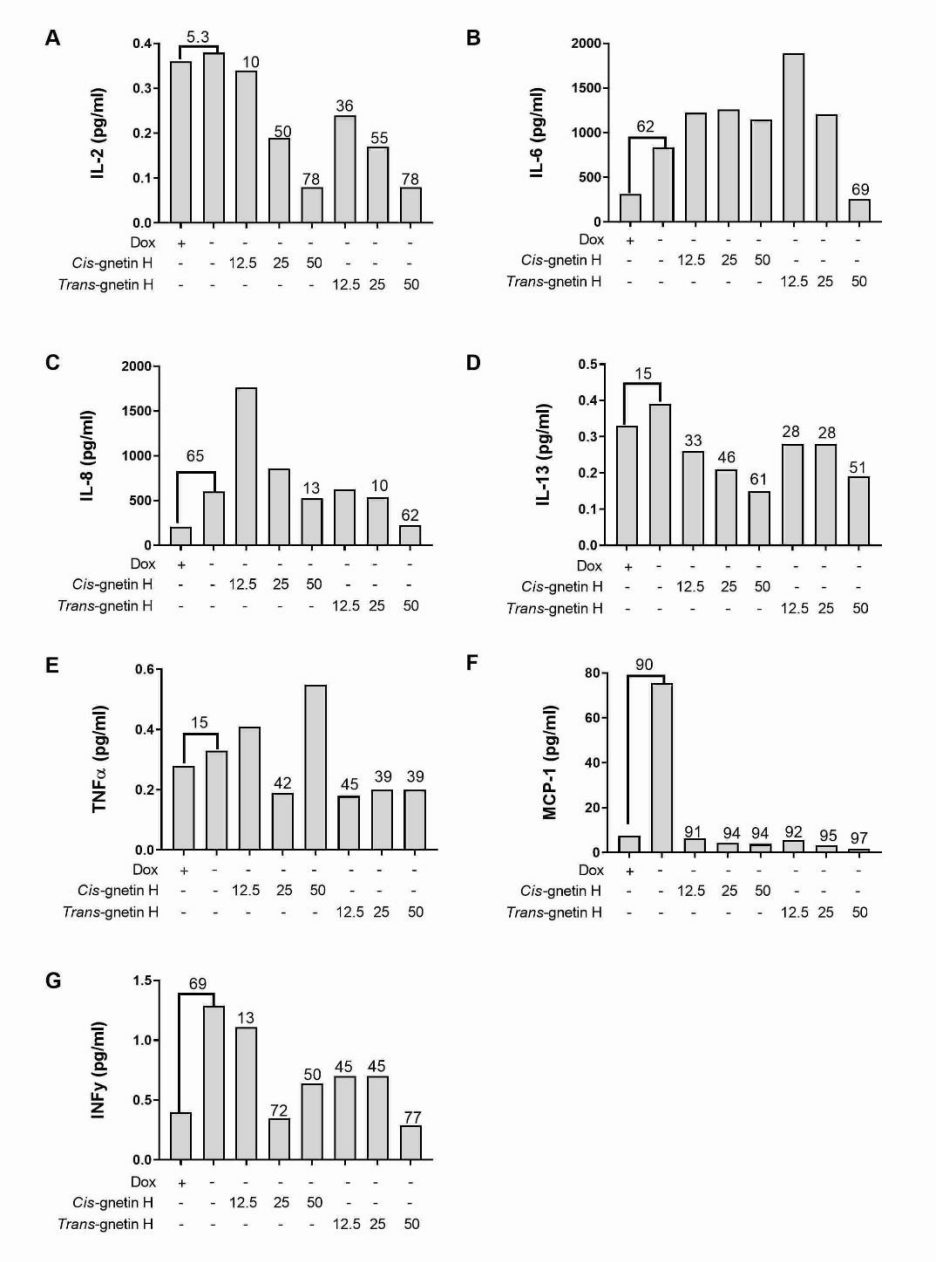

Figure 15. *Cis*- and *trans*-gnetin H inhibits IL-2, IL-6, IL-8, IL-13, TNF-α, MCP-1 and INFγ secretion in MDA-MB-231 cells.

MDA-MB-231 pretreated with 50, 25, 12.5 μM of *cis*-gnetin H and *trans*-gnetin H for 24 h. The secretion of IL-2, IL-6, IL-8, IL-13, TNF-α, MCP-1 and INFγ was determined by ELISA. The results are presented as the inhibition percentage at each concentration in (pg/ml) compared to the negative control. The numbers in the columns show the percent of inhibition (%) in the cytokine levels.

4. Discussion

Resveratrol is a well-known naturally-occurring chemo-preventative agent that can inhibit the proliferation of cancer cells with little toxicity toward normal cells (Tsai et al., 2017; Varoni et al., 2016). Previously, we reported that *cis*- and *trans*-gnetin H, trimers of resveratrol, both showed potent cytotoxicity that was 20-fold more effective than the resveratrol monomer in cancer cells (Gao & He, 2017). *Cis*-and *trans*-gnetin H showed an inhibition of different cancer cell lines with the lowest IC50 ranging between (2.8-2.6) μM and (9.7-7.1) μM for A549 and MDA-MB-231 cell lines, respectively. Moreover, in an *in vivo* study, *cis*- and *trans*-gnetin H were able to reduce the volume size of A549 lung tumors by over 70% in a xenograft mouse model with minimum toxic effect (Gao et al., 2015a). In the present study, the effects of *cis*- and *trans*-gnetin H on the motility, migration, invasion and adhesion of human lung cancer cells were investigated.

Cancer cell metastasis is initiated by cell dissemination, which depends on cell adhesion, migration and invasion, steps that are different but related (Guan, 2015). Generally, cancer cell adhesion to the extracellular matrix (ECM) facilitates their migration and invasion to distant organs or within the same organ. In this study, we demonstrated the ability of both *cis*- and *trans*- gnetin H to suppress cancer cell motility through regulation of MMP-1 along with TIMP-1 and TIMP-2 proteins. We have shown that both *cis*-and *trans*-gnetin H, reduced A549-GFP lung cancer cells motility and adhesion to HUVEC endothelial cells as well as migration and invasion of lung cancer A549 cell through a Matrigel matrix layer. Also, *cis*-and *trans*-gnetin H down-regulated the expression of both MMP-1, TIMP-1 and TIMP-2 while reversing the effect of major pro-inflammatory cytokines. *Cis*- and *trans*-

gnetin H significantly inhibited the mobility of the lung cancer cell line A549 after 18 h treatment at all concentrations that were tested. The effect of inhibiting motility was also seen in the ability of both compounds to inhibit the cells migration/ invasion through polycarbonated filter inserts. It has been suggested that insulin like growth factor-1 (IGF-1) can act as a major cell stimulant for cell migration/ invasion for the MDA-MB-231 cell line and that resveratrol can significantly inhibit IGF-1, and thus inhibit cancer metastasis (Tang et al., 2008). Many studies have been conducted on the effect of resveratrol in inhibiting the migration and invasion of different cancer cells. In pancreatic cancer cells, resveratrol suppressed the migration and invasion by inhibiting the Hh signaling pathway as well as MALAT1 in colorectal cancer cells or through inactivating the Akt and ERK1/2 signaling pathways in renal cancer cells (Ji et al., 2013; Li, et al., 2016; Zhao et al., 2018). Moreover, Sun *et al*. found that resveratrol can suppress migration in MDA-MB231 cells through reversing TGF-ß1 induced Epithelial-Mesenchymal Transition (EMT), as well as inhibiting its metastasis to lung cancer using a xenograft mouse model (Sun et al., 2019).

Cancer cell adhesion to endothelial cells is an essential step to facilitate cancer cell migration and invasion (Honn & Tang, 1992). Once cancer cells adhere to the endothelial cells, blood vessels can form, facilitating tumor growth through angiogenesis (Igura et al., 2001; Pellegatta et al., 2003). We demonstrated the ability of *cis*- and *trans*- gnetin H to inhibit the adherence of TNF-α stimulated A549-GFP cells to HUVEC cells. Activating cancer cells with TNF-α stimulates certain cell-surface adhesion molecules (CAMs) such as ICAM-1, VCAM-1, and ECAM-1, which can also play an important role in cancer metastasis by activating the NF-κB pathway (Aggarwal et al., 2004). It has been demonstrated that blocking the NF-κB pathway can block cancer cell adhesion by

inhibiting the activated E-selectin in colorectal cancer (Kobayashi et al., 2000). Moreover, Ferro *et al*. showed that resveratrol could inhibit both ICAM-1 and VCAM-1 in HUVEC and HSVEC cell lines (Ferrero et al., 1998).

High levels of TIMP-1 and TIMP-2 are strongly associated with the progression of breast cancer cells, especially amongst triple-negative breast cancer (TNBC), which represents about 15% of aggressive breast cancer subtypes and in many breast cancer cases that are known for their poor prognosis (Ree et al., 1997). It was observed that upon treatment with *cis*- or *trans*- gnetin H, both levels of TIMP-1 and TIMP-2 were inhibited by over 90% at almost all of the tested concentrations. Previous studies have shown that TIMPs act as a protease inhibitor, while recent studies showed that TIMPs could also affect cell proliferation and survival through MMP independent path (Chirco et al., 2006; Ree et al., 1997). Our finding supports many previous studies showing an increase in the levels of TIMP-1 and TIMP-2 in MDA-MB-231 cell line, and upon treating with both *cis*- and *trans*-gnetin H the levels of expression were drastically reduced, suggesting that TIMP-1 in TNBC cells might induce cell cycle arrest at G1 phase and decrease cyclin D1 levels. This can be associated with the overall survival (OS) of TNBC patients compared to other types of breast cancer patients, as well as reducing cancer colony formation (Ree et al., 1997). To further identify the mechanism of action, Cheng *et al.* showed that knocking down the TIMP-1 levels in TNBC cells reduced both the Akt and NF-κB signaling pathways, suggesting that the Akt signaling pathway is involved in the breast cancer proliferation (Cheng, G. et al., 2016). High levels of TIMP-2 in MDA-MB-231 cells is usually associated with high levels of MMP-2 and it has been suggested that proMMP-2 activation mediated by the MT-1 MMP/TIMP-2 complex allowing cancer cells

to invade through the basement membrane and causing tumor progression (Chirco et al., 2006). Generally, high levels of TIMP-2 in MDA-MB-231 cells is known to be associated with low MMP activities; this is thought to be mediated by TIMP-2 binding to the cell surface using its N-terminal domain rather than the C-terminal domain (Sounni et al., 2010). Interestingly, we observed that treating MDA-MB-231 cells with both *cis*- and *trans*- gnetin H caused a decrease in TIMP-1, TIMP-2, and MMP-1 levels, and this was associated with a reduction of both migration and invasion. These findings suggest that TIMP-2 might use the C-terminal domain instead of the N-terminal in to binding to the cell surface and exerting its inhibitory effect toward MMPs (Sounni et al., 2010).

Pro-inflammatory cytokines have an essential role in cancer progression, including metastasis (Esquivel-Velázquez et al., 2015). Specific pro-inflammatory cytokines, such as TNFα, IL-1, IL-6, IL-11 and transforming growth factor ß (TGFß) are usually elevated in breast cancer cells. At the same time, other angiogenic cytokines such as TNF-α, IFN-γ, IL-8 and monocyte chemoattractant protein MCP-1 also stimulate the release of certain cytokines that can play an important role in tumor progression and metastasis by degrading the ECM (Wilson et al., 2010; Zu et al., 2012). The NFkß pathway regulates many of the pro-inflammatory cytokines and many studies suggest that the inhibition of these cytokines could lead to potential cancer treatment (Bishayee et al., 2010). In particular, TNF-α plays a critical role in inducing inflammation by releasing certain interleukins. Our study showed the ability of *trans*- gnetin to effectively suppress TNF-α at all treated concentrations at a higher percentage than *cis*-gnetin H. Moreover, both compounds inhibited the release of pro-inflammatory cytokines such as: IL-2, IL-6, IL-8, IL-13 and INFγ. Although treatment with *cis*- and *trans*- gnetin H showed different inhibition results amongs the pro-

inflammatory / angiogentic cytokines, MCP-1 was inhibited the most by *cis*- and *trans*-gnetin H suggesting that this protein may be a key regulator affecting breast cancer metastasis.

In one of many studies conducted on resveratrol having an anti-inflammatory properties by inhibiting pro-inflammatory cytokines, Bishayee *et al*. used a hepatocellular cancer cell line, where resveratrol suppressed the elevated levels of HSP70, COX-2, and NFkß (Bishayee et al., 2010). Another study show an inhibition in hypoxia-induced factor-1 α (HIF-1α) protein in human tongue squamous cell carcinoma (SCC-9) as well as an inhibition in Vascular Endothelial Growth Factor (VEGF) expression in hepatoma cells (HepG2), both which are important in the metastasis of tumor cells (Zhang, Q. et al., 2005). This was combined by a decrease in the levels of MMP-1, an interstitial collagenases known to be elevated in breast cancer cell lines. It is thought that bone morphogenetic protein-6 (BMP-6) can act as an MMP-1 inhibitor in breast cancer metastasis, which is mediated through BMP-6/ δEF1/MMP-1 cascade (Hu et al., 2016). Our findings demonstrated the ability of both *cis*- and *trans*-gnetin H to drastically inhibit the levels of MMP-1 at greater than 90% at the highest treated concentrations.

The results of this study show that both *cis*-and *trans*-gnetin H inhibited the motility, adhesion, migration and invasion of cancer cells, the key steps in tumor metastasis by inhibiting TIMP-1, TIMP-2 and MMP-1 independently, indicating their potential use as a therapeutic compounds for cancer metastasis.

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OVERALL CONCLUSION

The importance of natural products in the field of cancer therapy has gained considerable interest in recent years. Although it is estimated that there are over 200,000 different plant species, only about 5,000 of them have been studied for their therapeutic potential (Wink, 2006). It is suggested that a combination of clinical trials, molecular pharmacological analysis and advanced technologies needs to accompany each other to accelerate the overall investigating of the majority of the plants, this approach will help in providing a better overview to the potential treating properties of plants toward cancer (Efferth et al., 2007). Although many efforts are dedicated to developing a novel treatments to inhibit cancer cell growth and metastasis, many have major side effects and have weak selectivity that results in inhibiting normal cells but not completely affecting cancer cells. Therefore, it is important to develop new anticancer agents that mainly inhibit the targeted cancer cells yet do not affect normal cells to minimize the side effects and have a better prognosis (Zhong et al., 2017).

In particular, one of the most abundant classes within the plant kingdom are the flavonoids and their isomers, the aurones. In this study, we assessed the potential anti-cancer activity of fourteen semi-synthetic aurone derivatives with different substitution patterns in the B-ring against two cancer cell lines, human breast cancer cell lines BT20, MCF7 and lung cancer cell line A549. Aurones are a subset of the flavone family and they play an important role in the pigmentation of some flowers such as snapdragon, coreopsis or dahlia. Aurones have emerged as a potential anticancer agent that utilize a different mechanism of action towards cancer (Alsayari et al., 2019), as well as having

other antibiological properties including anti-inflammatory (Hassan et al., 2014), antimicrobial (Jardosh & Patel, 2017) and antimalarial properties (Carrasco et al., 2014). Upon synthesizing aurone derivatives, “drug-likeness” was tested to examine the physio-chemical parameters of each aurone. This provides an estimation of the fate of the tested compounds once they are consumed orally which is very important for biological activity and a predictor for their eligibility as a candidate for clinical trials.

Results from this study showed that out of the fourteen aurone derivatives, only four of them A3, A5, A10 and A14 inhibited the growth of the A549 lung cancer cell line by over 90% with minimum inhibition against the normal lung cell line HPL1a. Moreover, A3 and A10 inhibited the growth of the BT20 and MCF7 breast cancer cell lines by over 90% with minimum inhibition against the normal breast cell line HMEC. Collectively, these four aurones were selected to assess their ability to induce A549 cell apoptosis as well as inhibiting A549-GFP metastasis as part of their cytotoxicity effect. The result of this test suggests that aurone derivatives devoid of the unusual oxygenation found in the coumaranone fragment are potential leads for new anticancer agents. Also, the analysis of the structure-activity relationship revealed that the aurone derivatives based upon five-membered heteroaromatic rings exhibited the most significant anti-cancer activity. More in-depth study should be conducted to better understand the molecular mechanism by which aurones affect both apoptosis and cell motility.

We also examined *cis*- and *trans*-gnetin H, two oligostilbene derivatives of resveratrol, one of the most well-known potential anticancer agents that has been characterized from plants to date. Resveratrol has been reported to have cytotoxic effect on different cancer

cells and studies have mainly focused on its apoptotic, anti-proliferation and anti-inflammatory properties (Aluyen et al., 2012; Varoni, 2016). *Cis*- and *trans*- gnetin H were isolated from the seeds of *Paeoniea suffruticosa* along with ten other oligostilbenes (He et al., 2010b). *Cis*- and *trans*- gnetin H was further characterized by Gao *et al*, and showed to possess high selectivity and cytotoxicity against cancer cells versus normal cells and induced apoptosis (Gao, Y., He, et al. 2015b).

In our current studies, more in-depth investigations were done to understand the mechanism of action by which *cis*- and *tran*- gnetin H induce apoptosis and inhibit metastasis in cancer cells. Cancer is mainly caused by the dysfunction of certain anti-apoptotic proteins, inhibitor apoptosis proteins (IAPs) and growth factors (Millimouno et al., 2014), even with therapeutic treatments, cancer can develop secondary drug-resistance and alternate their survival pathway. Therefore, recent studies have suggested using phytochemicals with multiple target molecules rather than pharmaceutical drugs with monotarget molecules as an attractive approach in regulating tumor growth (Khan et al., 2015). Both *cis*- and *trans*- gnetin H were able to induce apoptosis by activating both pathways, the intrinsic pathway through elevating the levels of ROS mediated by mitochondria, as well as the extrinsic pathway by elevating the levels of TRAIL. Elevating the levels of ROS can selectively inhibit cancer cells by inducing apoptosis through arresting the cell division cycle (Khan et al., 2012), and both *cis*- and *trans*- gnetin H can also affect this important criteria. Both compounds were able to affect major apoptotic proteins by down-regulating anti-apoptotic proteins, such as survivin, Hsp60, and XIAP while elevating the levels of pro-apoptotic proteins, such as Bad, Bid, cytochrome c, caspase 3, 7 and IGFBP-4.

Cancer metastasis is one of the major causes of mortality amongst all cancers (Emon et al., 2018).The process of cancer metastasis is regulated by many growth factors, cytokines, and modifications of the extra cellular matrix (ECM) that changes the cell-cell and cell-ECM interactions through ECM-enzymes, such as the MMPs. Cancer metastasis is an essential junction for tumor progression. Therefore, it has been considered to be an attractive therapeutic target to eliminate cancer.

Our studies showed that *cis*- and *trans*- gnetin H were able to inhibit cancer cell motility by reducing the adhesion of cancer cells to endothelial cells, as well as inhibiting cancer cell invasion and migration, all properties that are essential for cancer metastasis. Both *cis*- and *trans*-gnetin H were able to inhibit MMP-1, TIMP-1 and TIMP-2 relatively at the same efficacy suggesting that both compounds possess the same effect. MMP-1 is an important player in cancer progression and its expression can be regulated by certain members of the mitogen-activated protein kinase (MAPK) super family, such as extracellular signal-related kinases 1 and 2 (ERK1/2), c- Jun V-terminal kinase/stress protein kinase (JNK/SAPK) and p38 (Kim et al., 2010). Therefore, inhibiting MMP-1 might also inhibit these major signaling pathways. In addition to our previous finding that *cis*- and *trans*-gnetin H inhibited the NFkB pathway by blocking TNF-α translocation from the nucleus into the cytoplasm (Gao, Y. et al., 2015b), we found that *cis*- and *trans*-gnetin H inhibited the NFkB cytokine mediators IL-2, IL-6, IL-8, IL-13, TNF-α, MCP-1 and INFγ. In particular *cis*- and *trans*- gnetin H showed the highest inhibition percentage toward MCP-1. Since MCP-1 plays an important role in the mesenchymal- epithelial transition that regulates cancer invasion and migration and cell-cell adhesion facilitating

tumor progression (Li et al., 2017), this may play a role in *cis*- and *trans*-gnetin H is inhibiting motility.

In conclusion, the present studies demonstrated that the aurone derivatives A3, A5, A10, A14, and the oligostillbenes *cis*- and *trans*-gnetin H have anticancer properties. Moreover, since these compounds are based on natural products from the plant, it is also suggested that they may also exhibit multiple targets affecting many different cancer hallmarks that have not yet been studied.

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