Study of resveratrol-salicylate hybrid analogues: design, synthesis and their biological evaluation as potential multi-target chemopreventive agents

by

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ABSTRACT

Nature is an important source of bioactive molecules with favorable biological properties. Resveratrol is a natural compound found in grapes and berries and is among other constituents of red wine. This polyphenol has been studied and extensively reported in the literature. Resveratrol exerts a wide range of activities including but are not limited to anti-inflammatory, cardio-protective, antioxidant, chemopreventive and anticancer. It is considered a "multi-target" agent that modulates many cellular signaling pathways. However, its poor pharmacokinetic profile and a track record of contradicting evidence have slowed down (or even precluded) its clinical use. Furthermore, the observed biphasic effects produced by this stilbene in which desirable/undesirable effects are concentration-dependent have raised many questions about its efficacy. Consequently, there are many reports in the scientific literature describing different approaches aimed at improving resveratrol's pharmacological profile by medicinal chemistry concepts.

Acetylsalicylic acid (aspirin) has been used for more than a century and it represents one of the most successful analgesic and anti-inflammatory agents. Despite its notoriously simple chemical formula, aspirin has proven high efficacy not only as an anti-inflammatory agent, but most recently as a potential chemopreventive agent in human cancers, particularly in colon cancer.

Resveratrol and aspirin target similar (complementary) enzymes and receptors involved in the inflammation-to-cancer process. Therefore, we hypothesized that the chemical combination of resveratrol with a salicylate moiety would provide a new "hybrid" scaffold combining the biological properties of both parent compounds, and possibly, a novel series of resveratrol-salicylate derivatives with synergistic effects. The design of the new resveratrol-salicylate hybrids was based on the addition of a carboxylic acid (or carboxylate) group at position 3'- in

resveratrol's chemical structure. The biological evaluation (target selection) was based on literature reports describing (a) the role of these targets (COX-2, NF-κB, NO and ROS) in the inflammation-to-cancer process and (b) the effect of the parent resveratrol and/or aspirin.

I conducted the synthesis of ten resveratrol-salicylate derivatives by three different chemical reactions, namely the Wittig reaction, the Horner-Wadsworth-Emmons reaction, and the Heck coupling. The Heck coupling produced the desired *trans* isomer as a major product (55% yield) while the Wittig reaction produced a mixture of both *cis* and *trans* isomers.

The biological evaluation of the resveratrol-salicylate analogues started with an *in vitro* enzyme inhibition screening on the CYP1A1 enzyme. We observed a modulatory effect exerted by the test drugs in which some of them were inactive, some produced the desired CYP1A1 inhibition, but others seemed to increase CYP1A1's enzymatic activity, which was somewhat unexpected. In this regard, compound **3** was the most potent derivative decreasing the activity and the expression (mRNA) of CYP1A1. Using the EROD assay in HT-29 cells, resveratrol at 5μM *enhanced* TCDD-induced CYP1A activity (unfavorable), showing a clear difference between the hybrid and the parent molecule, resveratrol.

In the second study, we evaluated the activity of hybrid drugs to inhibit the catalytic activity of the DNA-methyltransferase (DNMT) enzymes DNMT-1 and DNMT3. Compounds **10** and **9** showed a selective inhibition of the DNMT-3 isoform. In this regard, resveratrol was weaker than the hybrids and it was non-selective. Compounds **3**, **4** and **10** showed an *in vitro* cancer cell proliferation inhibition on three human cancer cells. However, **3** showed a significant cytotoxicity on the non-cancerous MCF 10A which is considered a serious side effect. To support the enzymatic inhibition of CYP1A1 and DNMT by hybrid resveratrol-salicylate

molecules, we conducted series of molecular modeling (docking) studies. We observed key binding interactions within the active sites of these two enzymes, correlating the *in vitro* activity with computer-assisted drug simulations.

Finally, we carried out screening assays using compound **10** to evaluate its ability to suppress inflammation both in vitro and in vivo. In these studies, compound **10** exhibited an *in vitro* inhibition of COX-2 and NF-κB and a significant decrease in ROS production. Furthermore, compound **10** induced cell apoptosis in Jurkat cells. When tested *in vivo*, compound **10** reduced carrageenan-induced peritonitis and carrageenan-induced paw edema in mice, which were higher than that exerted by resveratrol and its natural analogue, TMS. Compound**10** reduced the myeloperoxidase (MPO) activity in the inflamed tissue, particularly at a low dose (10mg/kg). Despite its relatively low stability profile, compound **10** is a promising candidate for future experiments. The favorable activities produced by compound **10** support the overall design of the hybrid resveratrol-salicylate analogues.

PREFACE

This thesis is an original work by Fahad Aldawsari. The animal experiment protocols included in this work were conducted in collaboration with a research group in Brazil and were approved by the Ethical Committee for Animal Experimentation (State University of Maringá, Brazil, protocol number: CEAE/UEM 017/2013).

The literature review presented in Chapter 2 is published as **Aldawsari, FS** and Velázquez-Martínez, CA, "3,4',5-*trans*-Trimethoxystilbene; a natural analogue of resveratrol with enhanced anticancer potency," in *Invest. New Drugs*, **2015**, 33(3), 775. I performed the literature search and prepared the manuscript. Velázquez-Martínez, CA was the supervisory author and was involved in the manuscript organization.

Chapter 4 is published as **Aldawsari, FS**, Elshenawy, OH, El Gendy, MA, Aguayo-Ortiz, R, Baksh, S, El-Kadi, AO, Velázquez-Martínez, CA., "Design and synthesis of resveratrol-salicylate hybrid derivatives as CYP1A1 inhibitors," in *J. Enzyme Inhib. Med. Chem.*, **2015**, 30(6), 884. I carried out the chemical synthesis, and I wrote the manuscript. Elshenawy, OH performed the mRNA experiment, El Gendy, MA conducted the EROD assay, and Aguayo-Ortiz, R conducted the docking study inside the CYP1A1 active site. All of the co-authors contributed to manuscript edits. Velázquez-Martínez, CA was the supervisory author and was involved in the manuscript organization.

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L. Medina-Franco, Arno G. Siraki and Carlos A. Velázquez-Martínez, ”Anti-inflammatory and antioxidant properties of a novel resveratrol-salicylate hybrid analogue” in *Bioorg. & Med. Chem. Lett.*, **2016**, 26(5), 1411. I performed the chemical synthesis, in vitro COX enzyme inhibition, and the antioxidant assays, and I wrote the manuscript. Chattopadhyay, M. performed the NF-κB inhibition in the HT-29 cells, Salla, M. conducted the NF-κB inhibition in the HCT-116 cells, Goldhahn, K. performed apoptosis in the Jurkat cells, Aguiar, R.P. and Wiirzler, L.A.M. conducted the in vivo anti-inflammatory experiments, and Aguayo-Ortiz, R. conducted the docking study inside the COX enzymes. All of the authors contributed to the manuscript edits. Velázquez-Martínez, CA was the supervisory author and was involved in the manuscript organization.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES AND ILLUSTRATIONS

LIST OF ABBREVIATIONS

$(Ac)_2O$ acetic anhydride

$(CH_3)_2SO_4$ dimethyl sulfate

5-LOX 5-Lipoxygenase

AhR aryl hydrocarbon receptor

AKT protein kinase B

AlI_3 aluminum iodide

AP-1 activator protein 1

BBr_3 boron tribromide

Bcl-2 B-cell lymphoma 2

Bcl-X_L B-cell lymphoma extra large

BSA bovine serum albumin

CAT catalase

CD_3OD deuterated methanol

$CDCl_3$ deuterated chloroform

CDKs cyclin dependent kinases

CH_2Cl_2 dichloromethane

CH_3CN acetonitrile

CH_3COCl acetyl chloride

COX cyclooxygenase enzyme

COX-1 cyclooxygenase-1

COX-2 cyclooxygenase-2

CRC colorectal cancer

CYP1A1 cytochrome P450, family 1, member A1

DFMO difluoromethylornithine

DMBA 7,12-dimethylbenz[a]anthracene

DMEM Dulbecco's Modified Eagle Medium

DMF dimethyl formamide

DMSO dimethyl sulfoxide

DMSO-d_6 deuterated dimethyl sulfoxide

DNMT DNA methyltransferase

EGFR epidermal growth factor receptor

EMT epithelial mesenchymal transition

EROD ethoxyresorufin O-deethylation

ESI-MS electrospray ionisation mass spectroscopy

Et_3N triethylamine

EtOAc ethyl acetate

FBS fetal bovine serum

FDA US Food and Drug Administration

GSH glutathione

GSK glycogen synthase kinase

H_2O_2 hydrogen peroxide

HCl hydrochloric acid

HDAC histone deacetylase

HO-1 heme oxygenase-1

HPLC high pressure liquid chromatography

HSA human serum albumin

HWE Horner-Wadsworth-Emmons

i.p. intraperitoneal

i.v. intravenous

ICAM-1 intercellular adhesion molecule

IKK IκB kinase

IL-12 interleukin 12

iNOS inducible nitric oxide synthase

IκB inhibitory kappa B unit

JNK c-Jun N-terminal kinase

K_2CO_3 potassium carbonate

LAH lithium aluminum hydride

LDL low density lipoproteins

LPS lipopolysaccharide

MAPK mitogen-activated protein kinase

MeOH methanol

MMP metalloproteinase

mRNA messenger ribonucleic acid

Na_2SO_4 sodium sulfate

NaH sodium hydride

$NaHCO_3$ sodium bicarbonate

NaOH sodium hydroxide

n-BuLi n-butyl lithium

NF-κB nuclear factor kappa-light-chain-enhancer of activated B cells

NMR nuclear magnetic resonance

NQO1 NAD(P)H dehydrogenase quinone 1

Nrf-2 nuclear factor (erythroid-derived 2) like-2

NSAIDs nonsteroidal anti-inflammatory drugs

PAH polyaromatic hydrocarbons

PBr_3 phosphorus tribromide

PBS phosphate buffered saline

$Pd(OAc)_2$ palladium(II) acetate

PGD_2 prostaglandin D_2

PGE_2 prostaglandin E_2

$PGF_{2\alpha}$ prostaglandin $F_{2\alpha}$

PGI_2 prostaglandin I_2

Ph_2S_2 diphenyl disulfide

PI3K Phosphoinositide 3-kinase

PPAR-γ peroxisome proliferator activated receptor gamma

PPh_3 triphenyl phosphine

r.t. room temperature

R_f retention factor

RNS reactive nitrogen species

ROS reactive oxygen species

SAH *S*-adenosyl-*L*-homocysteine

SAM *S*-adenosyl-*L*-methionine

SD standard deviation

SEM standard error of the mean

Sirt1 Sirtuin type 1 (silent information regulator type 1)

SOD superoxide dismutase

STAT signal transducer and activator of transcription

STS stilbene synthase

$t_{½}$ half-life

TCDD 2,3,7,8-tetrachlorodibenzo-p-dioxin

tert-BuOK potassium tert-butoxide

THF tetrahydrofuran

TLC thin layer chromatography

TMS 3,4',5-*trans*-trimethoxystilbene

TNF tumor necrosis factor

TPA 12-O-tetradecanoylphorbol-13-acetate

TxA_2 thromboxane A_2

UV ultraviolet

VCAM-1 vascular cell adhesion protein 1

VEGF vascular endothelial growth factor

XRE xenobiotic response element

Chapter 1: Introduction

1.1 Cancer as a complex disease

According to experimental studies in animals, cancer is traditionally divided into three stages: initiation, promotion, and progression. These stages are controlled by genetic and epigenetic mechanisms [1]. Initiation involves biological changes such as DNA damage and/or mutations, inactivation of tumor suppressor genes, and activation of oncogenes [1]. The promotion step comprises the expansion of initiated stem cells as well as benign tumor development [1]. In the progression stage, proteolysis starts in parallel to adhesion, migration, and metastasis [1]. During the different carcinogenesis stages, normal biological anticancer mechanisms use several approaches to try to inactivate or remove the origin of the tumor. These mechanisms include but are not limited to enhancement of antioxidant protein functions (e.g. CAT, SOD, GSSG), DNA damage repair mechanisms (e.g. base excision repair (BER) and homologous recombination (HR)), tumor suppressor gene activation, activation of natural inhibitors of angiogenesis (e.g. interferon-α and IL-12) and tumor immunesurveillance [1]. In addition to the normal physiological anti-tumor mechanisms, anti-carcinogenic molecules present in the human diet demonstrate beneficial roles not only in interfering with cancer progression but also in cancer prevention.

1.2 Interplay between inflammation and cancer

Clinical and epidemiological studies have showed that chronic unresolved inflammation can promote and exacerbate numerous types of cancers [2]. Statistics have shown an association between inflammation and cancers. For example, colorectal cancer risk is found to be ten-fold greater in inflammatory disorders such as Crohn's disease and ulcerative colitis [2]. Furthermore, the risk of respiratory system cancers is directly proportional to the severity and duration of the associated inflammation [2]. Importantly, chronic inflammation is involved in multiple stages of carcinogenesis such as proliferation, transformation, apoptosis, survival, angiogenesis, and metastasis [2]. Additionally, inflammation contributes to the process of tumorigenesis through the generation of reactive oxygen and nitrogen species which can damage DNA [2]. To that end, inflammation produces numerous pro-carcinogen products such as TNF-α, cytokines, interleukins, iNOS, COX-2, 5-LOX, MMPs, and NF-κB [2].

1.3 Cancer chemoprevention

Cancer chemoprevention can be defined as the inhibition, reversal, or prevention of carcinogenesis [3]. This intervention is achieved by the intake of one or more non-toxic chemical entities such as synthetic agents or medicinal agents present in the human diet [3]. There are no accurate classifications for chemopreventive agents due to the lack of precise mechanisms by which these agents alter tumorigenesis [3]. Furthermore, many chemopreventive molecules interfere with numerous targets (multi-target) and exert more than one mechanism of action [3]. Nevertheless, chemopreventive agents can be classified into three categories, depending on the interference of tumor stage (initiation, progression and metastasis). These categories are [1] inhibitors of carcinogen formation; [2] inhibitors that block tumor initiation; and [3] inhibitors that suppress tumor promotion/progression [3].

1.4 Phytochemicals in cancer treatment and prevention

According to www.clinicaltrials.gov (accessed Sept. 15th, 2015), there are about 778 registered clinical studies involving the use of dietary supplements in patients already diagnosed with cancer, as well as in patients at high risk of developing cancer. It is estimated that about 50-60% of patients diagnosed with cancer in the US use drugs that originate from plants or nutrients, either exclusively or in addition to another therapeutic regimen such as radiation and/or chemotherapy [4]. Interestingly, it is estimated that about 7-31% of worldwide cancers can be potentially reduced by the intake of diets enriched with fruits and vegetables [5]. These active dietary ingredients include but are not limited to curcumin from turmeric, tea polyphenols such as epigallocatechin gallate (EGCG) from green tea, genistein from soybeans, sulforaphane from broccoli, resveratrol from grapes, isothiocyanates from cruciferous vegetables, diallyl disulfide from garlic, silymarin from milk thistle, lycopene from tomatoes, gingerol from gingers, hydroxytyrosol from olive oil, and rosmarinic acid from rosemary [4].

1.5 Targets for chemoprevention

Experimental studies of synthetic and natural molecules revealed numerous proteins that are considered potential targets for chemoprevention. These include but are not limited to apoptotic mediators (e.g. bcl-2, bax, c-myc, caspases, cytochrome C and TRAIL), cell cycle targets (e.g. Cdk4, cyclins), inflammatory mediators (COXs, NF-κB, cytokines, PGE_2, LOXs and NO),

epigenetic enzymes (DNMTs, HDAC), metabolizing enzymes (CYPs, NQO1), antioxidant proteins (GSH, SOD and Nrf-2), kinases (Akt, PKC, PI3k, MAPK, STAT, JNK), and migration/invasion targets (ICAM, VCAM, VEGF and MMPs) [6, 7].

1.6 Selected targets for chemoprevention

1.6.1 CYP1A1

CYP1A1 is a member of the CYP1 subfamily of CYP450 enzymes (phase I metabolizing enzymes). CYP1A1 is predominantly expressed in the extra-hepatic tissues [8]. CYP1A1 plays a crucial role in the metabolism of endogenous (e.g. 17β-estradiol) as well as exogenous (e.g. environmental carcinogens) substrates [8]. The confirmed high expression of CYP1A1 in some cancers (compared to normal tissues) is linked to tumor resistance, as demonstrated by the CYP1A1 rapid metabolism of certain chemotherapeutic drugs [8]. Additionally, CYP1A1 biotransformation of environmental polyaromatic hydrocarbons (PAHs) can generate reactive intermediates that damage the DNA and may ultimately initiate tumors [8]. The co-administration of CYP1A1 inhibitors (either natural or synthetic) is capable of improving cancer drugs' efficacy as well as interfering with tumor initiation [8]. For that reason, CYP1A1 is considered a promising chemopreventive target [8].

Mechanistically, CYP1A1 is transcriptionally regulated by the aryl hydrocarbon receptor (AhR) [8]. During its inactive state, AhR exists in the cytosol where it associates with a homodimer of heat shock protein 90 (HSP-90), a p53 chaperone, and an immunophilin-related protein XAP-2 [8]. After substrate (e.g., PAHs) binding, the coupled proteins dissociate and the remaining AhR-substrate complex is then translocated to the nucleus [8]. In the nucleus, the complex binds to an AhR nuclear translocator (ARNT) which then binds to the xenobiotic response element (XRE), which subsequently initiates the expression of the corresponding genes (such as CYP1A1) [8].

Numerous plant extracts as well as isolated phytochemicals have been experimentally proven to inhibit CYP1A1 [8]. These "chemoprotectants" showed different mechanisms *in vitro* and *in vivo* by using several models of pro-carcinogens and carcinogens [8]. Clinical studies (even though limited) have also investigated the beneficial effects of natural agents to protect against inducers (e.g., smoking) of the AhR signaling pathway [8, 9].

1.6.2 DNMT

"Epigenetics" refers to the study of heritable modifications to gene expression, without altering the DNA nucleotide sequence [10]. These modifications include DNA methylation; alterations of chromatin structure (by methylation or histone acetylation); and the small, non-coding micro-RNAs that can degrade mRNA and/or inhibit its translation [11]. Epigenetic regulations are essential for normal biological functions and can be adjusted to adapt to changes in the environment (e.g., chemical exposure, radiation, smoking, diet) [11]. Unlike genetic modifications that are challenging to reverse, epigenetic changes are *reversible,* and this advantage opens an opportunity for interventions [10].

In addition to the physiological role of epigenetic mechanisms, the aberrant methylation of certain genes or so-called "*gene silencing*" is associated with multiple diseases such as Alzheimer's disease, schizophrenia, autoimmune diseases, Crohn's disease, atherosclerosis, and cancers [12]. Silencing of genes (including tumor suppressor genes) caused by epigenetic modifications is experimentally evident in all three stages of cancer (initiation, promotion and progression). This silencing of genes occurs parallel to the overexpression of some methylation enzymes such as DNA methyltransferase (DNMTs) [10, 13]. Consequently, targeting otherwise overexpressed DNMTs is considered a potential strategy for cancer prevention.

DNMTs are a group of enzymes that catalyze the transfer of the methyl group from the methyl donor *S*-adenosyl-L-methionine (abbreviated as SAM or AdoMet) to the cytosines, particularly at the 5'-position [11]. This reaction mostly takes place at the cytosine adjacent to a guanine (CpG sites) and produces 5-methylcytosine and *S*-adenosyl-L-homocysteine (SAH or AdoHcy) [11]. Three active DNMTs (DNMT1, DNMT3a, and DNMT3b) have been identified so far, along with one regulatory domain (DNMT3L), which lacks the catalytic activity [14]. DNMT1 is considered a "maintenance methyltransferase" that preserves methylation patterns during DNA replication, while DNMT3a/3b are "*de novo*" enzymes that catalyze methylation of previously unmethylated DNAs [11].

1.6.3 COX

Cyclooxygenases (COXs) are a group of enzymes that modulate inflammation by catalyzing prostaglandins' (PGs) synthesis from the substrate, arachidonic acid [15]. There are three COX

isoforms, among which two enzymes are extensively studied, and their roles are well-established (COX-1 and COX-2). Additionally, there is a third enzyme (COX-3) which is expressed in only specific tissues such as the brain and spinal cord. Its functions have yet to be explored [15]. Although both COX-1 and COX-2 produce the same PGs (such as PGE_2, $PGF2_{\alpha}$, PGD_2, PGI_2 and thromboxane A_2 (TxA_2)), they control very distinct biological processes [15]. COX-1 is constitutively expressed in numerous tissues to ensure normal physiological functions such as hemostasis regulation, gastric mucosal protection, and renal-water balance [15]. COX-2 is commonly silent but expressed in response to stimuli such as pathogens, cytokines, growth factors, cellular mitogens, and cellular stress [16], and is predominantly involved in inflammation [15].

In addition to inflammation-mediated COX-2 expression, COX-2 induction may exceed physiological control, resulting in pathological conditions such as autoimmune disorders (e.g., systemic lupus erythematosus and rheumatoid arthritis), diabetes, and cancers [15]. In the previous examples, the observed over-expression of COX-2 has been associated with poor prognosis in these diseases [15]. Particularly in cancer, a frequent incidence of chemotherapeutic failure was associated with cases of colon, breast, and prostate cancers in which elevated expression of COX-2 is reported [15].

Experimental models for carcinogenesis have suggested that COX-2 plays a crucial role in early tumor promotion, chemoresistance development, and metastasis [15]. Epidemiological studies have shown a two- to 50-fold increase in COX-2 expression in about 40% of colorectal adenoma patients and 80-90% of colorectal cancer patients [16]. Consequently, multiple efforts have been made to design novel agents that modulate COX-2 functions [15].

1.6.4 NF-κB

The nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a transcription factor that has conserved, very crucial roles in the immune system [17]. Besides these functions, NF-κB influences the gene expression of modulators of cell survival, cell differentiation, and cell proliferation [17, 18]. Consequently, the dysregulation of NF-κB can lead to severe pathophysiological disorders [17]. As a universal regulator of essential elements of cell survival, and by functioning through multiple signaling, NF-κB can link physiology to pathology [17].

Indeed, NF-κB has been proven to form an etiological link between obesity and inflammation [19] and between inflammation and malignancy [17, 20].

NF-κB presents in numerous different cells and represents a group of structurally related proteins (called Rel family) [18]. In mammals, there are five members of the Rel family: p65 (Rel A), p50/p105 (NF-κB1), RelB, c-Rel, and p52/p100 (NF-κB2), which exist as homo- or heterodimers bound to the inhibitory protein (IκB) [18]. NF-κB activation is controlled by several positive and negative regulatory elements. In the inactive state, the NF-κB dimer is held in the cytoplasm via the association with IκB proteins to form a complex [18]. Upon stimulation, a complex termed as IκB kinase complex (IKK) is activated, which phosphorylates IκB, after which the IκB proteins are ubiquitinated and degraded [17, 18]. Then, the released NF-κB dimer translocates to the nucleus, binds to a specific DNA sequence, and initiates the transcription of target genes [17].

NF-κB can be activated by multiple agents (e.g., cytokines, UV and γ-radiations, hypoxia, or bacterial infections). This induced NF-κB is implicated in the regulation of more than 400 different genes [21]. Following the findings that many human disorders are associated with abnormal NF-κB expression, numerous research projects focused on the quest for NF-κB inhibitors. It is estimated that more than 700 NF-κB inhibitors have been developed by research groups and pharmaceutical companies [21]. Among the United States Food and Drug Administration (FDA) approved drugs, aspirin (NSAID), simvastatin (lipid lowering agent), docetaxel (anti-mitotic agent), and raloxifene (estrogen receptor modulator) were shown to inhibit the NF-κB signaling pathway [21]. To that end, the proteasome inhibitor bortezomib which is clinically approved to treat multiple myeloma modulates the NF-κB pathway via the reversible regulation of 26S proteasome activity [21].

1.6.5 ROS/RNS

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are involved in cellular defense against the invasion by foreign bodies. They participate in crucial biological processes such as cell proliferation, cell-cell adhesion, transcription, signal transduction, inflammation, and apoptosis [5]. However, their accumulation beyond physiological needs leads to damage to macromolecules as well as modifications in cell signaling, resulting in multiple disorders [22, 23].

ROS (e.g., superoxide anion, hydrogen peroxide, and hydroxyl radicals) are considered "unavoidable" by-products generated at low levels by normal cellular functions [5]. Uncontrolled ROS production can trigger DNA damage by initiating a series of metabolic reactions inside the cell that can cleave the DNA backbone and cause DNA base modifications or replication errors that may lead to tumor initiation [24, 25]. Additionally, ROS production is a common characteristic feature of tumor promoters (e.g., TPA, TCDD, phenobarbital, and UV) and it is implicated in the multistep processes of carcinogenesis [5]. Furthermore, ROS can contribute to carcinogenesis by modulating numerous signaling pathways that include but are not limited to AP-1, MAPK cascades, PI3K /Akt pathway, NF-κB, p53, Jak/Stat pathway, and heat shock proteins (HSP) [26]. To that end, the biological system counteracts the harmful effects of ROS/NOS through a dynamic system of enzymatic antioxidants (e.g., superoxide dismutase (SOD), catalase, glutathione reductase, glutathione peroxidase), and non-enzymatic antioxidants (e.g. vitamins C and D, glutathione) [23, 25].

1.7 Combination chemoprevention approach

In recent years, research has focused on the use of two more chemopreventive agents to reduce cancer incidence/progression, particularly for colorectal cancers (CRC) [27]. This approach seems attractive because it produces fewer side effects, which is especially useful as chemoprevention is a long-term intervention [27]. The ornithine decarboxylase inhibitor difluoromethylornithine (DFMO) in combination with sulindac (NSAID), has shown an improved reduction in CRC occurrence compared to each agent alone [27]. A clinical combination of statins and aspirin reduced the risk of CRC by 37%, again, a percentage that was higher than each agent alone [27]. Recently, the use of two or more "multi-target" natural chemopreventive agents to alleviate tumor-promoting inflammation has been highlighted [2].

In complex disorders such as inflammation and cancer, it is increasingly evident that when it comes to efficacy and side effects, interfering with multiple targets is more beneficial than focusing on a single target [28]. Additionally, these complex disorders are regulated by numerous signaling cascades that require interaction between a wide range of cellular responses [28]. Currently, combination therapy is emerging as one way to improve clinical efficacy in inflammation and cancer [28]. However, there are three main disadvantages to this approach [28]. First, patient compliance is reduced, particularly in the elderly population, in which

multiple disorders are often common [28]. Second, combination therapy addresses only a limited number of targets but not the overall disease-associated signalling pathways [28]. Third, clinicians may deal with complex pharmacokinetics in the combination therapy. Such pharmacokinetics require extensive trials to assess side effects and possible drug-drug interactions [28].

1.8 Multi-target approach

The concept that one drug acts through a single receptor is not as effective as predicted from the reductionism theory of the lock and key model [29]. There is increasing evidence supporting the move towards computational and experimental multi-target approaches [29]. Polypharmacology is relatively a new term that emphasizes designing a single molecule that can interact with multiple targets simultaneously [30]. Ideally, the multi-target agent may enhance the efficacy (either synergically or additively) while being less susceptible to drug resistance [30]. Nowadays, it is recognized that several clinically used drugs demonstrate their therapeutic response via complex polypharmacology, an observation that was identified retrospectively [30]. Identification of the most suitable combination of targets is critical and challenging, and requires taking into consideration the homology of certain proteins [30].

1.9 What is resveratrol? A definition, and background on its natural occurrence

Polyphenols comprise a wide class of natural antioxidants which include but are not limited to flavonoids, phenolic acids, lignans, anthocyanins, and stilbenes [31]. Resveratrol (3,4',5-*trans*-trihydroxystilbene) is among the stilbenes produced by many plant species under stress conditions such as UV radiation and fungi attacks [31]. It is classified as a defense molecule, "phytoalexin," that protects plants from insults [31]. In plants, resveratrol presents in forms of conjugates (to mask phenol groups) such as glucosides, sulphate, or methyl conjugates [31]. Plants synthesize resveratrol by the condensation of *p*-coumaroyl residue with three units of malonyl-CoA, with the aid of stilbene synthase (**Figure 1-1**) [31]. Resveratrol is found in many edible natural products, mainly as 3-O-β-D-glucosides (called piceids), which include but are not limited to grapes, peanuts, berries, and rhubarb (**Table 1-1**) [31]. It is also present in non-edible plants such as wild berries, mulberry and red sandalwood [31].

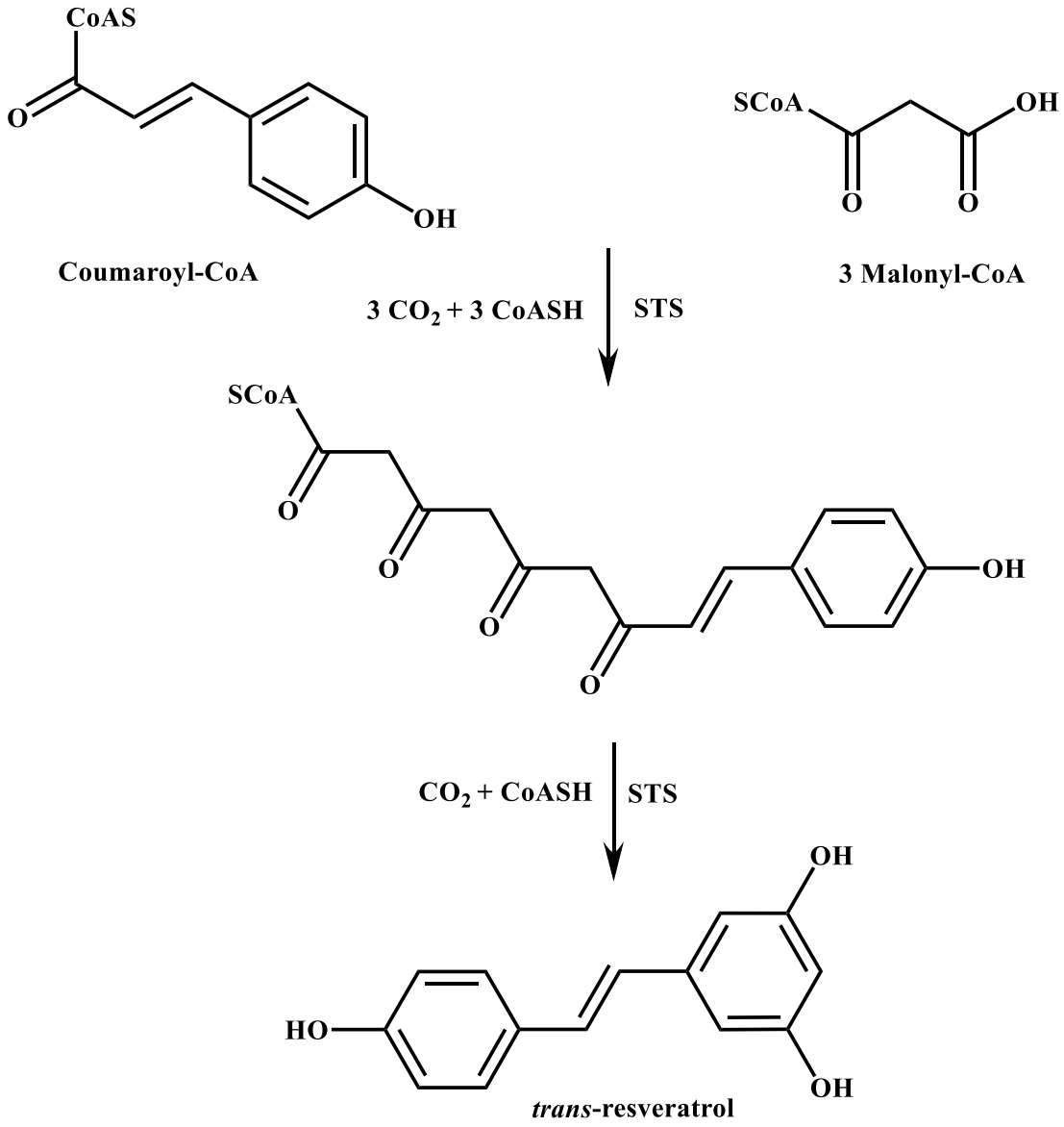


Figure 1-1: Plant's biosynthesis of resveratrol. Adapted from [32], STS: stilbene synthase.

Dietary sources	*trans*-resveratrol concentrations
Grapes	0.16-3.54 µg/g
Red wine	0.1-14.3 mg/L
Peanut butter	0.3-0.4 µg/g
Cranberry	0.2 mg/L
Pistachio	0.09-1.67 µg/g
Blueberries	32 ng/g
Hops	0.5-1 µg/g

Table 1-1: Examples of some dietary sources of resveratrol [33]

1.10 Resveratrol isolation and the first therapeutic evidence

Resveratrol was isolated for the first time from hellebore roots (*Veratrum grandiflorum* Loes. fil.) in 1940, by Takaoka [34]. More than 20 years later, in 1963, it was isolated from the Japanese knotweed *Polygonum cuspidatum* [35]. In the 1970s, in what was called the "French Paradox," epidemiological studies showed an inverse correlation between cardiovascular disorders and red wine consumption in France [31]. Later on, resveratrol was identified among the antioxidant constituents of red wine and showed favorable cardioprotective actions. These activities demonstrated by the inhibition of low-density lipoprotein (LDL) oxidation, platelet aggregation inhibition, thrombogenic potential reduction, and thus resveratrol was thought to be the missing link in the French paradox. In 1997, Jang et al. reported the first *in vivo* study showing that resveratrol could reduce tumor mass in rodents. [31]. Authors showed that resveratrol is effective in inhibiting tumor initiation, promotion, and progression [31]. Since then, several studies have showed that resveratrol has a wide spectrum of biological activities which include but are not limited to anti-inflammatory [36], antidiabetic [37], antibacterial [38], antihypertensive [39], estrogen receptor modulation [40], chemopreventive [41] and antioxidant [42] properties.

1.11 Pharmacokinetics of resveratrol

Initial human pharmacokinetic studies of resveratrol after a single dose of 25 mg failed to detect the parent polyphenol in plasma [43]. However, higher doses (25-5000 mg) revealed that the maximum plasma concentration reached around 500 ng/mL, which clearly shows the limited pharmacokinetic profile of this polyphenol [43]. Furthermore, the chemopreventive properties of resveratrol in experimental animals were demonstrated after long exposure to this stilbene (as long as 15-20 weeks of treatment) [43]. Resveratrol has a low water solubility (< 0.05 mg/mL), which may alter its tissue distribution [44]. After resveratrol is absorbed, it rapidly conjugates (particularly at position 3- and 4'-) to glucuronides as main conjugates [43]. The phase II metabolizing enzyme SULT1A1 has shown sulfates conjugates of resveratrol, particularly at 4'-position [43].

Resveratrol uptake into cells was demonstrated to be occurred through a carrier-mediated process and passive diffusion in HepG2 cells [44]. Additionally, studies have shown that resveratrol accumulates significantly in rat livers after being administered orally, a phenomenon that was thought to be correlated with specific toxicity in malignant hepatocytes [44].

Due to its low hydrophilic nature, resveratrol binds to serum proteins and remains at considerably high concentrations in the circulation [44]. Indeed, resveratrol has demonstrated a slower passive transport in serum-containing cultures compared to its transport in serum-free HepG2 cells [44]. Adding resveratrol to bovine serum albumin (BSA) decreased resveratrol uptake by cells, suggesting that albumin can be considered "a natural polyphenol reservoir" [44]. To this end, in addition to BSA, resveratrol has demonstrated binding properties to human serum albumin (HSA) but only at high concentrations [44].

Studies have shown that resveratrol's bioavailability is independent of the dose in rats [45]. A single oral dose (50 mg/kg) showed an elimination half-life ($t_{½}$, h) and bioavailability (F%) of 11.8 and 29.8% respectively, while a (150 mg/kg) dose exhibited 3.6 and 19%, for $t_{½}$ and F% respectively [45]. The same observation was found after continuous administration (14 consecutive days) [45]. In humans, a 25 mg oral resveratrol dose revealed rapid absorption (about 70%) but with negligible oral bioavailability due to its extensive metabolism ($t_{½}$ of

resveratrol metabolites of 9-10 hours) [32]. Resveratrol bioavailability has showed high inter-individual variability, even though gender and age were found to have no direct effects [46].

1.12 Isomers of resveratrol and derivatives

In plants, resveratrol exists as both *trans*- and *cis*- isomers. However, the *trans* one is the major and most stable isomer compared to the corresponding *cis*-isomer [44]. A trans-isomer can be converted to a cis-isomer upon exposure to sunlight or UV radiation [44]. The majority of studies have used *trans* isomers of resveratrol and its analogues due to their enhanced stability compared to their *cis*-counterparts. However, recent reports have showed that *cis*-isomers for some resveratrol analogues demonstrate enhanced anticancer effects compared to the corresponding *trans* isomers, specifically when resveratrol derivatives are tested against human HT-29 [47] and mouse metastatic B16 F10 cells [48].

1.13 Recent controversy about resveratrol

After nearly two decades of *in vitro* and *in vivo* research, many questions and concerns have emerged about the efficacy of resveratrol. For example, *in vitro* effects of free resveratrol might be irrelevant as this polyphenol is absorbed as conjugates [46]. Additionally, resveratrol doses used in clinical trials as well as in the *in vitro* studies were far higher than resveratrol amounts consumed in diets [46]. There is one factor that might add complexity to these controversial results of resveratrol: the "hormetic-like" properties of this stilbene. Hormesis is a term used by scientists to describe a U-shaped dose-response curve characterized by beneficial effects at low doses, and toxic or inhibitory actions at high doses [49]. Resveratrol at low concentrations has demonstrated an *enhancement* of tumor cell proliferation while at high concentrations it has showed an *inhibition* of tumor growth [49]. This biphasic effect has led some authors to call resveratrol a "two-edged sword" indicating that resveratrol concentration (or dose) will determine its specific effects [49].

1.13.1 Resveratrol is active over a broad range of doses

In the literature, resveratrol has been used at low doses (which correspond to its dietary constituents) and high doses (either isolated from plants or chemically synthesized) [50]. Of note, resveratrol's efficacy in rodents has been proven with both low and high doses [50].

Human clinical trials conducted to determine a safely tolerated dose have used as low as 25 mg and as high as 5 g in either capsule or caplet forms [51]. Resveratrol was reported to be safe due to the absence of serious side effects detected by hematological, clinical, and biochemical tests during the intervention and follow-up periods [51]. Additionally, gastrointestinal side effects (mainly diarrhea, nausea, and abdominal pain) were observed with doses exceeding 1 g [51]. The resveratrol content after moderate consumption of red wine is estimated to be around 1.25 mg/kg [50].

The human-equivalent corresponding dose used in preclinical carcinogenesis models in rodents has ranged from 2.5 mg to 1520 mg. The available literature reports utilized rodents suggest that resveratrol is active over a wide range of doses [50]. This suggests that the inhibition of carcinogenesis in different tissues could be influenced by the underlying mechanism of action as well as concentrations of resveratrol in the given organ [28].

1.13.2 Low dose resveratrol is better than high dose for chemoprevention

A recent study found that a low dose of resveratrol (representing its dietary content) is more efficacious than a high dose [52]. The chemopreventive effects of low doses were confirmed *in vivo* (using Apc^{Min} mice as a colorectal cancer model) as well as *ex vivo* (using human colorectal tissues). Specifically, a 0.07 mg/kg/day dose reduced the tumor burden more significantly than did a 200-fold higher dose (14 mg/kg/day) [52]. In parallel with these findings, the same group reported that colorectal patients undergoing surgery who took a low dose of resveratrol (5mg daily for one week) had a greater increase in their cytoprotective enzyme NQO1 levels than did patients who received a 1g dose [52]. The authors warn that the idea “more is better” is not likely the case for resveratrol [52].

1.14 Resveratrol and chemoprevention

The evidence of resveratrol in cancer chemoprevention has been explicitly studied *in vitro* as well as *in vivo*. In cultured cells, resveratrol inhibited the proliferation of a wide range of tumor cell lines such as A549, SW480, HepG2, HeLa, HL-60, LNCaP, HT-29 and MDA-MB-231 [53], to name a few. *In vivo*, oral resveratrol in rats and mice decreased DMBA-induced and 12-*O*-tetradecanoylphorbol-13-acetate-(TPA)-promoted skin tumors, suppressed 1,2-

dimethylhydrazine-induced colon cancers, inhibited DMBA-triggered mammary carcinogenesis, and reduced N-nitrosomethylbenzylamine-induced esophageal cancers [54].

1.15 Aspirin and chemoprevention

Colorectal cancer (CRC) is, perhaps, the most studied cancer in which aspirin has showed positive chemoprevention actions. Epidemiological studies that included both men and women revealed that aspirin reduced the incidence of CRC by about 70%. This percentage increases with prolonged duration of aspirin intake [55]. Additionally, clinical studies have demonstrated a decrease in the mortality rate by up to 48% after continuous aspirin intake in already diagnosed CRC patients [55]. It is believed that aspirin, as well as other NSAIDs, exerts its anticancer/chemopreventive actions through COX-dependent and COX-independent mechanisms. COX-independent mechanisms include but are not limited to apoptosis induction, angiogenesis inhibition, NF-κB modulation and polyamine synthesis alterations [55].

1.16 Both aspirin and resveratrol eliminate tetraploid cells

Tetraploid cells represent genomically stable cells that are often found in pre-neoplastic lesions as a result of the inactivation of tumor suppressors [56]. These types of cells contain twice as many chromosomes as their normal, diploid counterparts [56]. Normally, once such variations in diploid cells occur, apoptosis becomes activated to eliminate these abnormal cells [56]. Tetraploid cells have been detected in early stages of multiple human cancers that include but are not limited to prostate, ovarian, colorectal, mammary, and gastric cancers [56]. In a recent screening of potential compounds capable of eliminating these tetraploid cells, both resveratrol and aspirin (and its metabolite, salicylate) showed interesting results [56]. Specifically, resveratrol and salicylates selectively killed tetraploid cells while sparing their parental diploid counterparts [56]. These chemopreventive actions of resveratrol and salicylates were confirmed *in vitro* (in $p53^{-/-}$HCT116 cells), *ex vivo* (primary cells from $p53^{-/-}$ mice), and *in vivo* using an $Apc^{Min/+}$ mice model to represent human familial adenomatous polyposis (FAP) patients [56].

1.17 Resveratrol analogues

Resveratrol's wide range of therapeutic properties and its poor pharmacokinetic profile prompted research for new resveratrol analogues with improved pharmacokinetic and/or biological effects.

Using a simple search in the "PubMed" database, and typing "resveratrol derivatives synthesis" between 2006 and 2015 revealed 469 publications, which translates to an average of 47 publications annually. These analogues of resveratrol are varied in their chemical structure nature as well as their targets. Examples include methoxylated and hydroxylated resveratrol analogues that inhibited ribonucleotide activity in HT-29 cells [57], resveratrol imine derivatives as potential agents to alleviate Alzheimer's disease [58], 4'-methylthio-resveratrol analogues as CYP1 inhibitors [59], and fluorinated resveratrol analogues as anti-proliferative agents [60].

An interesting approach to designing resveratrol analogues was to hybridize the resveratrol structure (or stilbenes in general) with another active moiety to enhance the desired activity. Examples of this hybridization strategy are resveratrol-avobenzone [61], resveratrol-nitroxide [62], resveratrol-vitamin E [26], resveratrol-diclofenac conjugates [24], and resveratrol-coumarin analogues [22] (**Figure 1-2**).

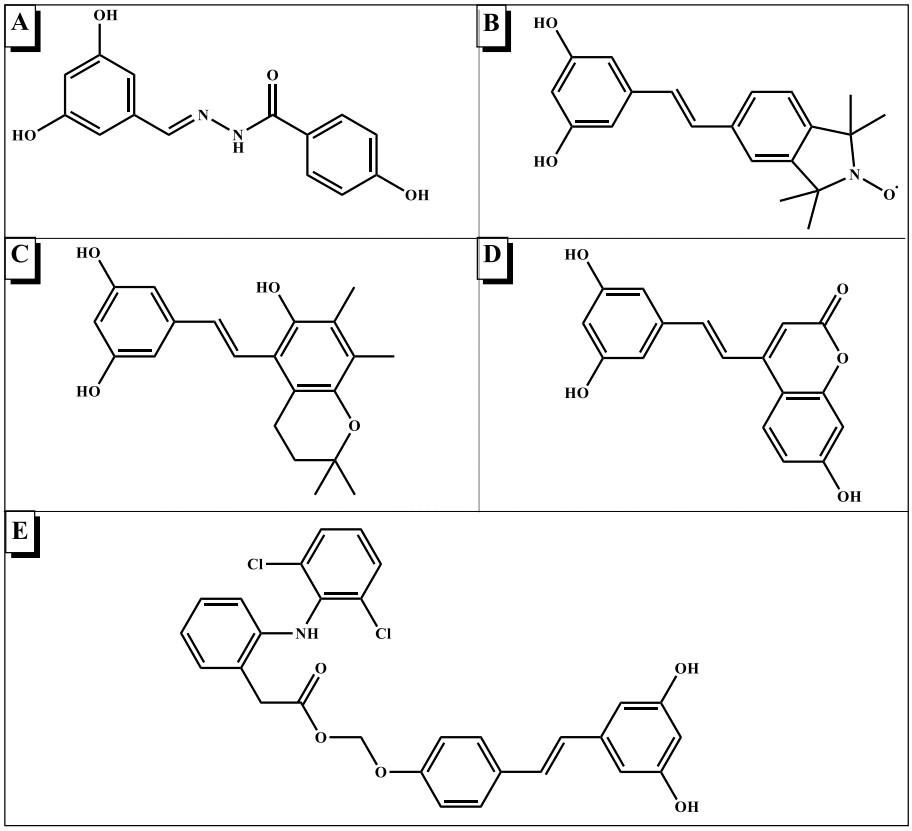


Figure 1-2 : Chemical structures of representative examples for the reported hybrid resveratrol analogues. **A**: resveratrol-avobenzone, **B**: resveratrol-nitroxide, **C**: resveratrol-vitamin E, **D**: resveratrol-coumarin, and **E**: resveratrol-diclofenac derivatives.

1.18 Resveratrol-aspirin prodrugs

The attractive cancer chemoprevention properties of aspirin and resveratrol led to a recent work by Zhu, Yingdong et al. [63] in which novel resveratrol-aspirin prodrugs have been synthesized and evaluated as safe anticancer agents. These agents were *in vivo* metabolized to release the parent molecules, aspirin (or salicylic acid) and resveratrol, in addition to other metabolites [63]. Some of these resveratrol-aspirin prodrugs showed an interesting chemopreventive profile demonstrated by their inhibition of colony formation in HCT-116 and HT-29 cells, and their induction of cell cycle arrest and apoptosis [63].

1.19 Molecular targets of resveratrol and aspirin

The broad spectrum of the biological activities of resveratrol requires this molecule to interact with dozens of molecular targets. There are numerous validated targets for resveratrol actions. A few examples of these target proteins are illustrated in (**Figure 1-3**). Of note, **Figure 1-3** shows that resveratrol interferes with key biological processes such as cell cycle, apoptosis, and cell invasion. Aspirin exhibits cancer prevention by modulating certain proteins (via COX-dependent and COX-independent mechanisms). **Table 1-2** shows proteins modulated by aspirin (or salicylates). Interestingly, there are shared proteins that are targeted by both aspirin and resveratrol, and hence some of these targets were included in this study.

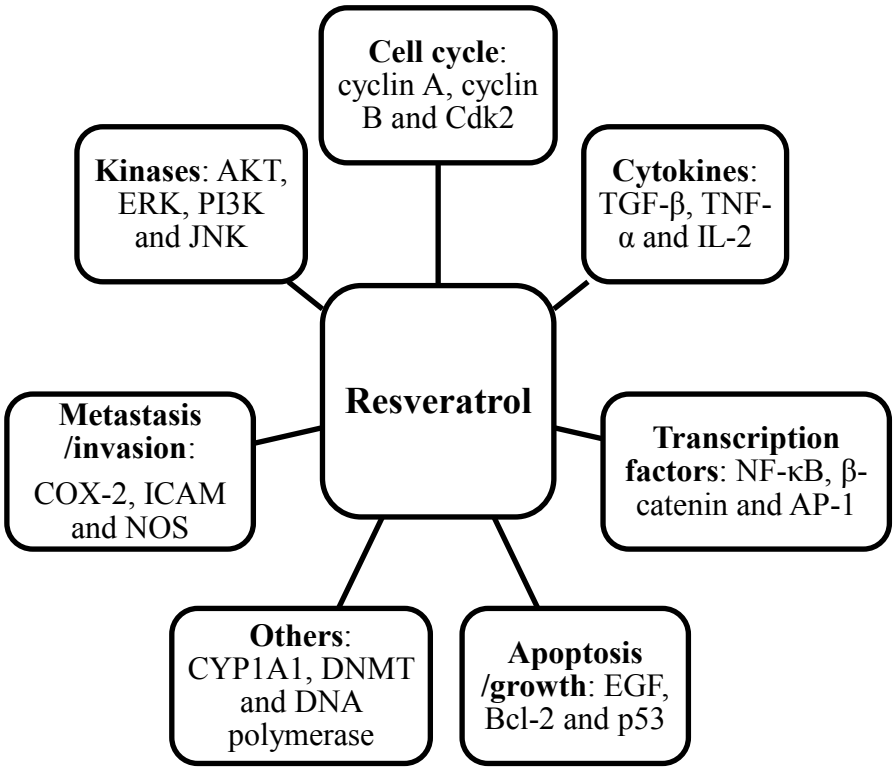


Figure 1-3: Some of the important target proteins modulated by resveratrol.

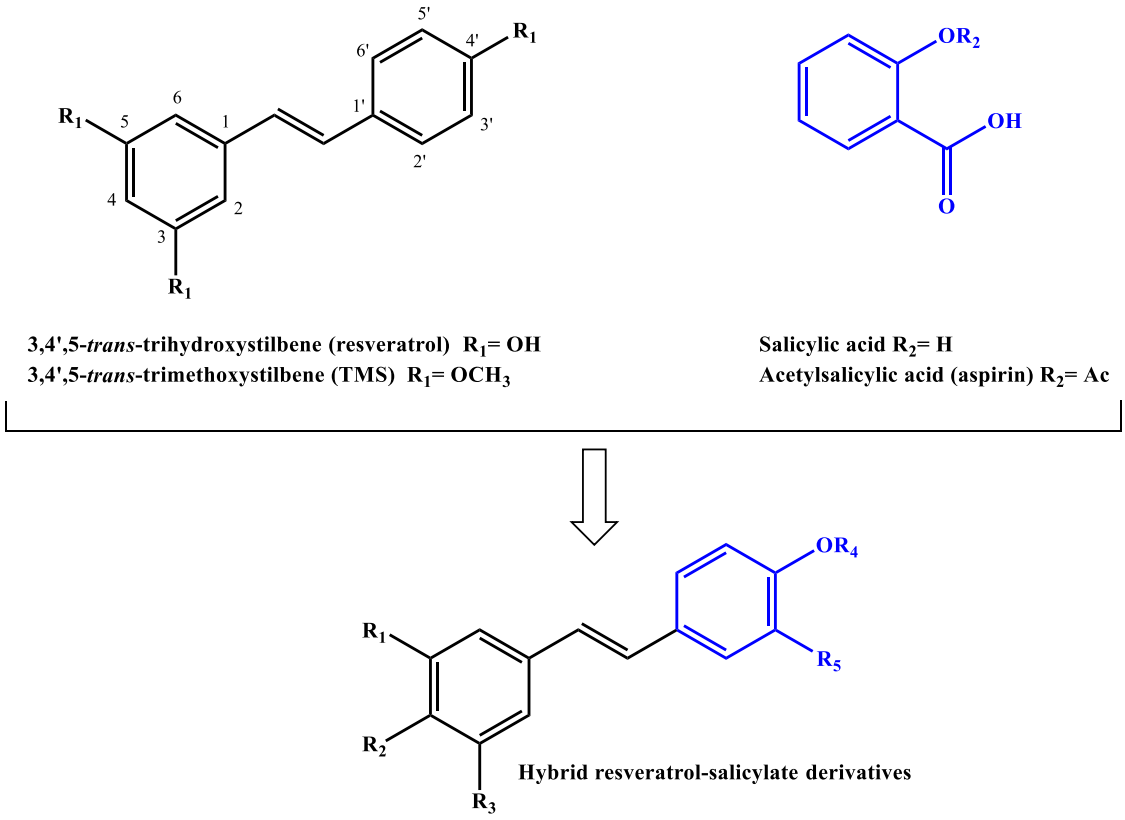
Molecular targets	Reference
COX-1 and COX-2	[64], [65]
NF-κB	[65-68]
c-Jun-N-terminal Kinase (JNK)	[69]
P38	[70]
Wnt/β-catenin	[71]
CYP1A1	[72]
DNA methylation	[73]

Table 1-2: Molecular targets involved in carcinogenesis that are altered by aspirin and salicylates.

1.20 Drug design

Inspired by the multi-target properties of resveratrol, and the shared chemopreventive targets between resveratrol and aspirin (or salicylates), we aimed to design hybrid molecules containing resveratrol and salicylate scaffolds. We designed these hybrid molecules based on the addition of a single carboxylic acid group at the position of 3'- in resveratrol's chemical structure (**Figure 1-4**).

Furthermore, we hypothesized that protecting phenol groups might enhance the biological activities of these new derivatives, according to recent observations that methylated [74] and acetylated [75] resveratrol analogues demonstrated improved anticancer potencies. Accordingly, we designed and synthesized ten resveratrol-salicylate analogues that include hydroxylated, methoxylated, and acetylated derivatives. Additionally, we included in this small library set one derivative that is void of the carboxylic group at position 3'- for the purpose of structure-activity relationships. We predicted that the new hybrid resveratrol derivatives would demonstrate improved biological activities compared to the parent, resveratrol.



Compound	R_1	R_2	R_3	R_4	R_5
3	H	OCH_3	H	CH_3	$COOCH_3$
4	H	OCH_3	H	Ac	$COOCH_3$
5	OCH_3	H	OCH_3	Ac	OCH_3
6	OCH_3	H	OCH_3	Ac	$COOCH_3$
7	OCH_3	H	OCH_3	CH_3	$COOCH_3$
8	H	OH	H	H	COOH
9	OH	H	OH	H	COOH
10	OH	H	OH	H	$COOCH_3$
11	H	OAc	H	Ac	COOH
12	OAc	H	OAc	Ac	COOH

Figure 1-4: The design and chemical structures of resveratrol-salicylate derivatives.

1.21 Aims and objectives

This study aims to develop and evaluate a new group of resveratrol derivatives which possess improved chemopreventive and anti-inflammatory properties. The new derivatives will be evaluated against CYP1A1 and DNMTs enzyme inhibition. Modulations of these analogues will be assessed against selected inflammatory mediators (COXs, NF-κB and ROS).

Activities in this research project include designing, chemically synthesizing, and in vitro testing of these derivatives in cultured cells and experimental animals to evaluate the potential therapeutic effects of these analogues.

1.21.1 Hypothesis

Adding the carboxylic group at position 3'- in the resveratrol structure will produce a series of hybrid resveratrol-salicylate analogues with enhanced anti-inflammatory and chemopreventive properties compared to those of resveratrol. The main therapeutic target of this study is colorectal cancer chemoprevention however, the current resveratrol-salicylate derivatives could be also applicable in the chemoprevention of other cancers.

1.21.2 Research objectives

Objective one: to chemically synthesize resveratrol-salicylate analogues by exploring Wittig, Horner-Wadsworth-Emmons (HWE), and Heck reactions.

Objective two: to test the potential of these resveratrol analogues to modulate the enzyme CYP1A1.

Objective three: to test the potential of these new derivatives to inhibit DNMT enzymes.

Objective four: to test the anti-inflammatory and antioxidant properties of these analogues.

1.22 References

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Chapter 2: Literature Review

This chapter is published as **Fahad S. Aldawsari**, Carlos A. Velázquez-Martínez. "3,4',5-*trans*-Trimethoxystilbene; a natural analogue of resveratrol with enhanced anticancer potency". *Invest. New Drugs*, **2015**, 33(3):775-786. I performed the literature search and prepared the manuscript. Velázquez-Martínez, CA was the supervisory author and was involved in the manuscript organization.

2.1 Introduction

Resveratrol (3,4',5-*trans*-trihydroxystilbene, **Figure 2-1**) is a natural polyphenol which has shown a plethora of biological activities. These activities are attributed to its interference with multiple signalling pathways which include but are not limited to inflammatory mediators (e.g. COX-1/2, iNOS, TNF), transcription factors (e.g. NF-κB, β-catenin, STAT3, PPAR-γ), cell cycle regulatory genes (e.g., cyclins, CDKs, p53), angiogenic genes (e.g., VEGF, MMPs, ICAM-1), apoptotic genes (e.g., survivin, Bcl-2, Bcl-X_L), antioxidant enzymes (e.g., SOD, CAT, HO-1), and protein kinases (e.g., AKT, PI3K, JNK) [1]. Most of these targets are associated with different stages of carcinogenesis, which is why resveratrol has been recognized as an inhibitor of cancer initiation, progression, and metastasis.

In 1997, Jang et al. documented the first cancer-preventive properties of resveratrol [2]. Since then, a considerable amount of work has been conducted to elucidate resveratrol's mechanism of action. There are several reviews describing the *in vivo* and *in vitro* biological effects exerted by resveratrol [3-6], but despite about 18 years of continuous research into its chemopreventive effects, there are still many questions and concerns about its potency, efficacy, and safety [7-9]. Furthermore, despite the relatively high commercial success of several "alternative" products containing resveratrol, its low chemical stability [10], low bioavailability [10], high metabolic rate [10], and the lack of properly controlled clinical studies, make the use of this polyphenol controversial, to say the least, as an effective chemopreventive, adjuvant, or chemotherapeutic agent [7, 11]. Moreover, questions have been raised about whether the dose of resveratrol that has been proven to be somewhat promising in animal models can be reliably extrapolated to humans [7, 8]. All these issues have prompted searches for improved resveratrol analogues in addition to establishing efficient delivery systems for resveratrol itself [12, 13].

3,4',5-*trans*-Trimethoxystilbene (TMS) (**Figure 2-1**), is a natural analogue of resveratrol which has been found to exhibit antitumor activities, superior to those of resveratrol, on multiple targets involved in carcinogenesis. TMS has been identified in the literature by different names, including "resveratrol trimethyl ether" (RTE) [14, 15], MR-3 [16, 17], M-5 [18], BTM-0521 [19], trimethoxy resveratrol [20], trimethylated resveratrol [21], and, as noted at the beginning of this paragraph, TMS [22-24].

Comparatively speaking, TMS has been under the scientific community's radar for many years. In this literature review, we report the findings of a comprehensive literature search in which we compiled publications from 2002-2015 (**Figure 2-1**) describing potential anticancer properties of TMS. These publications were considerably lower than those of TMS's hydroxylated analogue, resveratrol. Consequently, we considered it is essential to carry out a comprehensive literature search to collect available (and applicable) information to address the question of whether or not this methoxylated derivative could constitute a suitable alternative to resveratrol. In the current review, we discuss available reports in which TMS has been studied (screened) as an anticancer or chemopreventive agent, regardless of whether or not it was compared to resveratrol. At the end of this review, we submit two key findings. First, the more lipophilic and cell membrane-permeable TMS is not sufficiently studied, considering that its chemical structure only differs from that of resveratrol for having three methyl groups (small, lipophilic, and naturally occurring). Second, the potential anticancer profile of TMS is promising enough (despite the low number of publications) to merit additional studies, opening the door for future research projects in which this compound can be used and compared to resveratrol.

2.2 Materials and methods

A literature search was conducted in PubMed and Web of Science databases by searching keywords such as "TMS," "trimethoxy resveratrol," and "trimethoxy stilbene." Then, results were filtered to make sure they were specific for the compound 3,4',5-*trans*-trimethoxystilbene, and not for other related stilbenes. A chemical structural search was also performed using the "SciFinder" database. Only articles in English were retrieved from all databases. The first study to describe the anticancer effects of TMS was published in 2002. For comparative purposes, we searched for "resveratrol anticancer" in PubMed between 2002 and 2015. The search revealed 445 papers. The total number of TMS anticancer publications in this review was 58 articles. The individual number of publications per year can be found in **Figure 2-1**.

2.3 TMS sources

The first documented natural source of TMS was reported in 1969 by Blair, G.E. and coworkers [25]. A petroleum ether extract of the plant *Virola cuspidate* (species from this plant genus were used by South American Indians to prepare narcotic snuffs and as an arrow poison [25, 26]), was

used to isolate TMS [25]. TMS was subsequently isolated from different plant genera [27-33]. Recent analytical studies of dietary sources such as grape berries [34], grapevine [35], almonds [36] and peanuts [37, 38] have revealed that they all contain resveratrol but not TMS. The majority if not all of the TMS used in literature studies was chemically synthesized in laboratories. The common synthetic procedures employed to obtain TMS involve chemical reactions such as a Wittig reaction [39], Heck coupling [40], a Horner-Wadsworth-Emmons (HWE) reaction [41] and a Perkin reaction [42], all of which produced TMS in moderate to good yields.

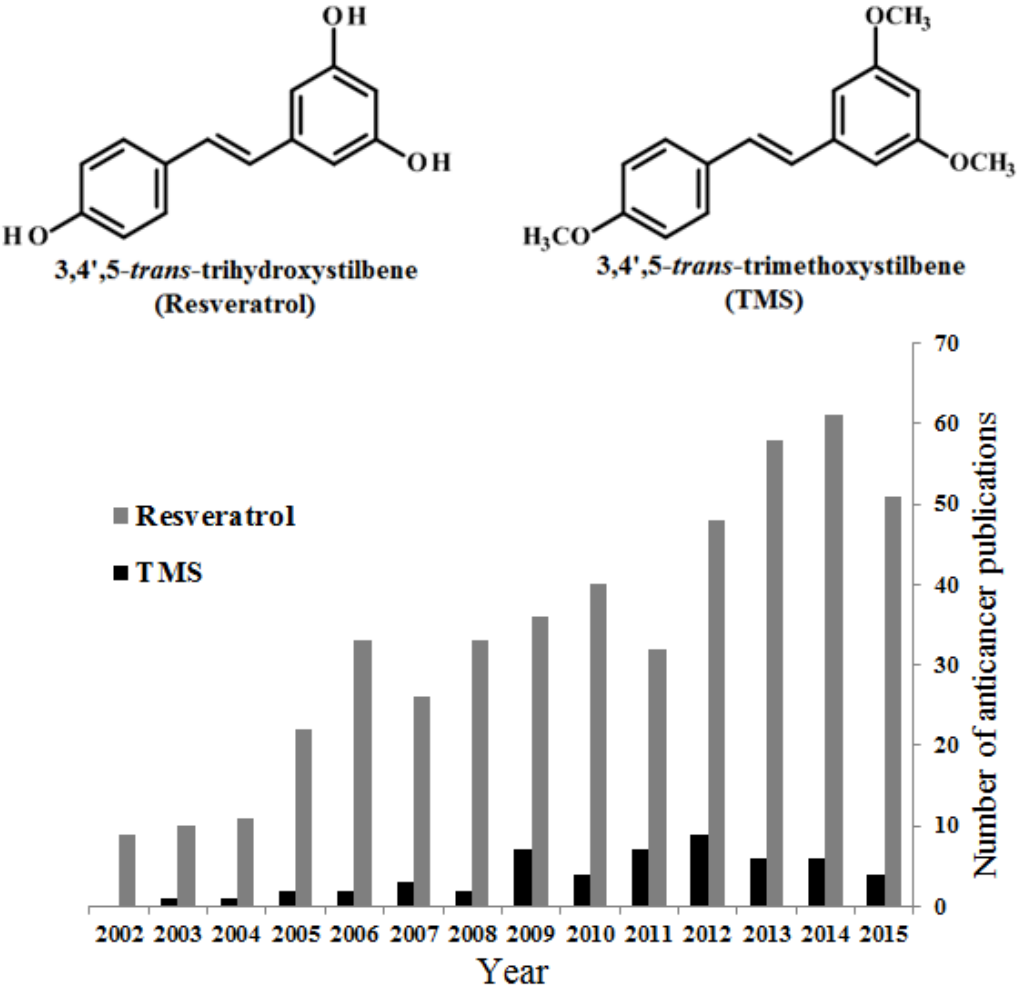

Figure 2-1 : Comparison between anticancer publications of resveratrol and TMS.
The graph shows the number of publications describing the potential anticancer properties of both TMS (black) and resveratrol (grey), between 2002-2015.

2.4 *In vitro* anti-proliferative effects

Even though resveratrol and TMS have very similar chemical structures, TMS has showed a higher cell proliferation inhibition than resveratrol against numerous cancer cell lines using *in vitro* cytotoxic assays (**Table 2-1**). As shown in **Table 2-1**, TMS's potency to inhibit cancer cell proliferation varies according to the literature source, ranging from nearly equipotent efficacy compared to resveratrol [20], to up to seven-fold higher than resveratrol [18]. Perhaps the only exceptions are the SW480 [43] and PC-3 cells [44], in which resveratrol was more potent than TMS. TMS's enhanced *in vitro* anticancer effect can be partially attributed to two main reported features. First, the three methoxy groups in the TMS structure enhance the lipophilic character of this molecule (calculated LogP values: TMS = 3.85 while resveratrol = 3.06) [44], increasing its cell membrane permeability, and ultimately its intracellular concentration [14, 20, 45]. Second, TMS had been reported to destabilize microtubule formation in cancer cells when administered at concentrations as low as 1.0 μM, whereas resveratrol did not exert this effect to a significant extent [46].

In regard to the conformational structure of TMS, it is a fact that the corresponding *cis* isomer of TMS has shown higher cancer cell proliferation inhibition than the *trans*-isomer. This is particularly true for human colon cancer cells (HT-29 and Caco-2 [47, 48]) and mouse melanoma B16 F10 cells [49]. However, it is important to point out that even though the *trans*-resveratrol has been, by far, the preferred isomer cited in anticancer studies, the *trans*-TMS isomer has followed a similar pattern (most studies in the literature were carried out with the *trans*-isomer of TMS).

An interesting observation related to the effects produced by resveratrol against cancer cell lines *in vitro* is illustrated by a concentration-dependent biphasic effect. This phenomenon has been reported in several tumor cell lines including breast, colon, lung, prostate, and liver [51], as well as in leukemia [50]. This biphasic ("hermetic-like") action exerted by resveratrol is characterized by the inhibition of cancer cell proliferation at high concentrations, whereas at low concentrations, resveratrol seems to *enhance* cancer cell proliferation [50]. To the best of our knowledge, this hermetic effect has not been reported for TMS, and it constitutes a highly promising research area, considering that it could be one essential area in which TMS could potentially have an advantage over resveratrol.

Cell line	Origin	Species	Resveratrol IC_{50} (µM)	TMS IC_{50} (µM)	Ref.
DU-145	Prostate	Human	42.3	9.7	[20]
LNCaP	Prostate	Human	12.7	2.5	[20]
PC-3M	Prostate	Human	31.5	23.3	[20]
PC-3	Prostate	Human	0.6 ± 0.01	3.6 ± 0.9	[44]
22Rv1	Prostate	Human	149.92	9.45	[52]
M-14	Skin	Human	31.0 ± 3.1[a]	12.1 ± 1.7[a]	[53]
WI38VA	Skin fibroblast	Human	50	25-50	[54]
KB	Nasopharyngeal	Human	>80	10.2 ± 0.5	[55]
A549	Lung	Human	6.9[b]	0.8[b]	[56]
CH27	Lung	Human	Not reported	92	[16]
HT-29	Colon	Human	45.3 ± 4.4	16.1 ± 5	[47]
Caco-2	Colon	Human	24.35 ± 0.2	11.95 ± 2.9	[47]
SW480	Colon	Human	20 ± 3	54 ± 8	[43]
MCF-7 (RF.M [erbB2])	Breast	Human	47.7	6.6	[18]
MDA-MB-231	Breast	Human	20.5 ± 2.6	1.2 ± 0.2	[57]
Hepa1c1c7	Liver	Mouse	>25	5.2	[58]
HepG2	Liver	Human	38.9 ± 6	11.99 ± 1.9	[59], [60]
Vero	Kidney	Monkey	73.6	26.7	[33]
HeLa	Cervical	Human	No activity at 100 µM	13.3	[33]
SK-OV-3	Ovarian	Human	113	55.5	[33]
BXPC-3	Pancreas	Human	3.3[c]	0.35[c]	[61]
HL-60	Blood	Human	5 ± 2	2.5 ± 0.6	[62], [63]

Table 2-1: *In vitro* cytotoxic potencies of TMS versus resveratrol against several tumor cells; [a] Reported as GI_{50} (µM). [b] The IC_{50} was reported as (g/mL). [c] Reported as GI_{50} (µg/mL).

2.5 *In vivo* anti-proliferative effects (xenograft models)

The *in vivo* anticancer effects exerted by resveratrol have been studied in xenograft models using a wide range of cancer cells; a comprehensive summary is shown in **Table 2-2**.

Resveratrol (dose, mg/kg)	Route of administration	Cell line	Tissue origin	Ref.
100	i.v.	SUM159	Breast	[64]
40	Oral gavage	MCF-10A-Tr	Breast	[65]
50	Oral gavage	MDA-MB-231	Breast	[66]
50, 100, 200	i.p.	SKOV3	Ovary	[67]
50, 100	i.p.	PA-1	Ovary	[68]
30	Oral gavage	PC-3	Prostate	[69]
20	Oral gavage	PC-3M-MM2	Prostate	[70]
50	Oral gavage	LNCaP-Luc	Prostate	[20]
40	Oral gavage	MIA-PaCa2	Pancreas	[71]
10, 50	Oral gavage	MIA-PaCa2	Pancreas	[72]
15, 30, 60	i.v.	A549	Lung	[73]
20	i.p.	A549/VC	Lung	[74]
20	i.p.	CNE-2Z	Nasopharynx	[75]
10, 50	Oral gavage	FaDu	Pharynx	[76]
40	Oral gavage	BGC-823	Stomach	[77]
20, 40	Oral gavage	HNC-TIC	Head-neck	[78]
20	i.p.	Mz-ChA-1	Bile duct	[79]
20	i.p.	T24	Bladder	[80]
150	Oral gavage	HCT-116	Colon	[81]
10, 20, 30	Not specified	A431	Skin	[82]

Table 2-2: **Xenograft models used to confirm the *in vivo* anticancer properties of resveratrol**. In these studies, resveratrol significantly reduced tumor cell growth compared to the control.

Out of twenty *in vivo* studies, eight used injections (i.v. or i.p.) as the main route of administration, as this method assures complete absorption. These studies reported a significant anticancer profile as determined by a decrease in tumor size and weight [64, 67, 68, 73-75, 79, 80]. However, when resveratrol is administered orally, it seems that the preferred method of study is to pre-treat the animals with resveratrol (chemopreventive approach) before the subcutaneous injection of cancer cells [20, 70, 72]. One study that followed this protocol used as high as 150 mg/kg of resveratrol (oral dose) [81] to achieve the significant antiproliferative effect, which highlights that bioavailability of this polyphenol represents a critical issue in these models.

It has been difficult to reconcile the different results reported in the literature on the efficacy and potency of resveratrol in prostate cancer, since there is conflicting evidence showing that

resveratrol in some cases is an effective inhibitor of cancer cell proliferation [20, 69, 70], whereas in other studies it shows negligible activity compared to the control group [83, 84]. These discrepancies could be attributed to differences in cell type, dose, route of administration, or the different treatment protocols (chemopreventive vs. chemotherapeutic). In addition to these discrepancies, resveratrol has also been associated with a decrease ("worsening") in the survival rate of SCID mice in which prostate cancer cells (LAPC-4) were injected [85].

In agreement with the experiments conducted using prostate cancer cells, similar findings have been reported for other cancer cells such as melanoma cells [DM738 ([84], and DM443 [86]]. In these studies, authors showed that resveratrol had weak anticancer activity compared to the control group. Furthermore, resveratrol *enhanced* tumor cell proliferation in xenograft models using melanoma MDA-MB-435 cells, relative to the effect observed in the control [87]. This lack of significant activity was also reported in xenograft models using acute lymphoblastic leukemia (SEM cells), in which detailed analysis showed no significant difference between control- and resveratrol-fed mice [88]. Possible explanations for these variations among the *in vivo* anticancer effects of resveratrol are the resveratrol dose (considering the "hermetic-like" characteristic mentioned earlier in this review), the integrity and identity of the resveratrol-mixed diet, or the sex of the experimental animals (taking into consideration that resveratrol is a phytoestrogen).

TMS has been much less studied in xenograft experiments. Therefore, an objective comparison between TMS and resveratrol is difficult at this point. Nevertheless, one of the few reports describing the *in vivo* anticancer profile of TMS involved prostate cancer cells (LNCaP-Luc) injected into nude mice [20]. In this study, the authors report that the oral administration of TMS (50 mg/kg) led to a significant decrease in tumor formation and tumor progression compared to the control group [20]. In a different study, TMS (50 mg/kg, i.p.) produced a significant anticancer effect on colon cancer cells (COLO 205) when injected three times per week for 23 days into nude mice [89]. TMS showed an evident reduction of tumor growth accompanied by a significant inhibition of tumor/body weight ratio [89]. In a complementary study, TMS (10 mg/kg, i.p.) led to a significant reduction of both tumor weight (21% decrease) and tumor volume (45% decrease) of colon cancer cells (HT-29) in mice [47].

These results suggest that TMS has a higher anticancer profile than that exerted by resveratrol. However, it is also evident that to date, the literature data is limited and merits further investigation.

2.6 TMS and apoptosis

An early study by Weng et al. [16] proposed similar apoptotic effects exerted by resveratrol and TMS. The authors reported that human lung carcinoma cells (A549 and CH27) experienced a significant degree of apoptosis when incubated in the presence of these stilbenes at concentrations ranging from 10 to 100 μM [16]. Using flow cytometry and staining (Annexin V-FITC and PI), TMS and resveratrol increased the number of cells undergoing apoptosis in a dose-dependent fashion. However, the increasing inhibition of cell proliferation exerted by TMS in CH27 cells was not correlated with the extent of apoptosis [16]. Therefore, the authors suggested that additional mechanisms other than apoptosis could be involved in the anticancer effects exerted by TMS in these cells [16]. This study is one of the early distinguishing features of TMS compared to resveratrol.

In another study, both TMS and resveratrol induced apoptosis in a clone of the MCF-7 breast cancer cell line into which a mutant p53 gene had been inserted [18]. According to this report, the "I_pC_{50}" values (expressed as the concentration of a compound needed to inhibit cell proliferation by 50%), for TMS and resveratrol were 6.9 μM and 27 μM respectively [18]. This indicates a four-fold enhanced activity of TMS compared to resveratrol. However, in the wild-type MCF-7 cells, TMS and resveratrol showed more or less the same potency with I_pC_{50} values of 7.5 and 9.2 μM respectively. The authors suggested that the increased antiproliferative effect exerted by TMS might be p53-independent [18]. In the same report, the authors provided additional evidence to suggest a significant difference in the mechanism of antiproliferative activity between TMS and resveratrol; they used other two variants of the cloned MCF-7 cell line possessing the mutant p53 protein. Those variants had been modified to be resistant to 2'-deoxy-5-fluorouridine and arabinosylcytosine. In this model, TMS showed a 2.5-fold higher potency than resveratrol [18].

The hypothesis that TMS could terminate cancer cell proliferation through a p53-independent mechanism is supported by two studies of Hsieh et al. In the first paper, the authors explained

that TMS and resveratrol did not change p53 mRNA levels in LNCaP cells [90]. However, in the second study, in which the authors used MCF-7 cells, TMS decreased the expression of a downstream target of p53, namely the transcription factor p53R2, whereas resveratrol had the opposite effect [91]. Of note, resveratrol upregulation of p53 has been previously reported in mouse skin exposed to the carcinogen DMBA [92] as well as in the MTA1-silenced human prostate cancer DU145 and LNCaP cells [93]. These differences in p53 regulation by resveratrol and TMS further confirm the different mechanisms by which these natural agents alter tumor cell proliferation.

Additional evidence supporting a significant difference between TMS and resveratrol is seen in the work published by Daniele et al., in which they tested the *in vitro* apoptotic effects of several stilbenes (including resveratrol and TMS) on myeloblastic acute leukemia cells (HL-60), using the Annexin *V* test and morphological examination. They observed that the apoptotic effect (expressed as AC_{50}) induced by TMS was found to be 10-12 times higher than that of resveratrol (4.0 ± 2.1 μM and 50 ± 6 μM respectively) [63]. Additionally, TMS (at 10 μM) showed a higher pro-apoptotic potential than resveratrol in human neutrophils stimulated with TPA [24].

The human androgen-responsive prostate cancer cell (LNCaP) has been used to evaluate the effects of several resveratrol analogues on cell cycles and apoptosis [94]. Using flow cytometry, Wang et al. reported that resveratrol induced cell cycle arrest (at G1/S) after 72 hours post-treatment, whereas TMS produced a significant effect at the G2/M phase much sooner than resveratrol (as early as 24 hours post-treatment) [94]. In the same study, cell cycle arrest was also assessed by measuring the expression of the cyclin inhibitors CDKN1A and CDKN1B (mRNA level). In this regard, resveratrol showed a significant upregulation of both cyclin inhibitors at 25 μM. Of note, TMS upregulated both CDKN1A and CDKN1B at much lower concentrations (1 μM and 5 μM respectively) [94]. Additionally, it was observed that resveratrol exhibited weak apoptotic effects while TMS showed a significant dose-dependent action at concentrations as low as 5 μM [94]. Importantly, during the evaluation of apoptosis-associated caspase 3/7 activation, TMS (but not resveratrol) led to about a six-fold induction in caspase activity compared to the control [94]. The authors commented that despite the similarity in chemical structures between resveratrol and TMS, each stilbene exerts different effects on LNCaP cells [94]. TMS did not induce ceramide accumulation (as a pro-apoptotic marker) in MDA-MB-231 cells, despite

showing a high antiproliferative effect [57], whereas resveratrol showed a significant ceramide accumulation [57].

2.7 TMS and angiogenesis

Alex D. et al. [95] studied the anti-angiogenic properties of TMS and resveratrol in two models: an *in vitro* model using HUVEC cells, and an *in vivo* model of blood vessel formation in transgenic Zebrafish embryos [95]. The results of the *in vivo* experiment showed that resveratrol had a negligible effect on blood vessel formation at the highest tested concentration (100 μM) [95]. In contrast, TMS exhibited a significant inhibition of angiogenesis at 10 and 30 μM, compared to the control (untreated) group [95]. The authors suggested that TMS might target EGFR, which could explain the reduction in neovascularization [95]. Indeed, they found that TMS (at 100 μM) caused about a four-fold downregulation of VEGFR-2 mRNA compared to the control [95]. In a similar study, Belleri M. et al. [46] studied the anti-angiogenic properties of TMS and resveratrol using different endothelial cells originated from murine, bovine, and humans [46]. TMS was at least 30 times more potent than resveratrol in the all tested assays (type-I collagen gel invasion, morphogenesis on Matrigel, sprouting within fibrin gel, and endothelial cell proliferation) [46].

2.8 TMS and autophagy

Autophagy (self-eating) is a conserved cellular process initiated under certain conditions such as stress and starvation. As a biological process, it results in the sequestration of cellular organelles such as proteins and membranes [96]. Autophagy plays a crucial role in cell differentiation, growth and death. The dysregulation of this process has been associated with many disorders such as cardiovascular diseases and cancers [96]. Resveratrol has been known to modulate autophagy through multiple mechanisms such as mTOR, Akt, and AMPK [97, 98]. Perhaps the only study describing TMS's potential to induce autophagy is the one recently published by Lu et al. [96]. In this study, using HUVEC cells and in the presence of serum, TMS was observed to induce significant autophagy in an mTOR-dependent mechanism. Furthermore, a transient receptor potential canonical channel 4 (TRPC4) was found to be an important gene that is upregulated after TMS treatment [96]. Due to the lack of additional studies related to TMS

potential in autophagy, further investigations are needed to augment the beneficial therapeutic properties of this stilbene.

2.9 TMS and cancer metastasis

In a recent study, Yang et al. reported the anti-metastatic properties of TMS using a human lung cancer cell line (A549), by measuring the effects of this compound on MMP, MAPK, NF-κB, and AP-1 [17]. In this regard, TMS (at 5 μM) significantly decreased the migratory, adhesive, and invasive properties of the A549 cells by 39%, 34%, and 22 % respectively. TMS also decreased both the activity and mRNA levels of the MMP-2 protein in a time-dependent manner [17]. A possible mechanism for TMS's downregulation of MMP-2 was studied by assessing the phosphorylation pattern of JNK and p38 proteins; TMS reduced the phosphorylation levels in both JNK and p38 [17]. In the same study, the authors also reported the effects of TMS on the transcriptional factors NF-κB and AP-1, which are two of the major proteins associated with multiple pathophysiological disorders such as inflammation, angiogenesis, cancer cell migration, invasion, and metastases. Treating human lung cancer cells (A549) with TMS led to a time-dependent reduction in the protein levels of NF-κB (p65 subunit), as well as AP-1 in the nucleus [17].

Consistent with the Yang et. al study, TMS's ability to downregulate the AP-1 protein in cancer cells was further confirmed by Deck et al. in a report using human embryonic kidney cells (293T/AP-1-luc) [99]. The incubation of these cells with TMS (at 15 μM) resulted in a significant reduction in AP-1 activation (calculated IC_{50} = 3.8 μM) [99]. In contrast, resveratrol (at 15 μM) and under the same experimental conditions has showed more than a one-fold induction of AP-1 compared to TPA-treated cells [99].

In another study, Weng et al. investigated the anti-invasive properties exerted by both TMS and resveratrol on hepatocarcinoma HepG2 and Hep3B cells [100]. The authors found that TMS and resveratrol decreased the activities of MMP-9 and MMP-2 in Hep3B cells in a dose-dependent manner [100]. Also, incubating HepG2 cells with TMS and resveratrol led to a marked decrease in the invasion of these cells by about 60% and 80% respectively [100]. Similar results were obtained with Hep3B cells [100]. It is noteworthy that the reported anti-invasive potency

determined for TMS in Hep3B cells (IC_{50} = 1 μM) was significantly higher than that determined in HepG2 cells (IC_{50} = 50 μM) [100].

The epithelial-mesenchymal transition (EMT) is an important mechanism by which primary cancer cells can invade (metastasize) other tissues and organs [101]. E-Cadherin is a receptor which plays a critical role in cell adhesion. The decreased expression of this protein is a characteristic feature of a tumor cell undergoing EMT [101]. In this regard, it has been observed that EMT-associated transcriptional factors such as snail and slug reduce the expression of E-cadherin. Tsai et al. studied the modulations in EMT-related markers in MCF-7 cells incubated with resveratrol and TMS [101]. They observed that both stilbenes were able to significantly increase the level of E-cadherin in MCF-7 cells treated with these compounds. The concentrations used in this experiment were relatively not toxic to the cells (20 μM) [101]. TMS and resveratrol decreased the levels of the EMT-related protein snail [101]. Interestingly, after the MCF-7 cells were transfected with an E-cadherin promoter gene, TMS (but not resveratrol) showed a significant effect reinstating the epithelial marker E-cadherin activity [101].

Another interesting example of how the naturally occurring stilbenes resveratrol and TMS can inhibit cancer metastasis is the study of the β-catenin protein. This protein, along with E-cadherin, works to maintain proper cell-to-cell adhesion and epithelial integrity [101]. The elevation of free β-catenin in cytoplasm activates the Wnt/β-catenin signalling pathway, and ultimately, initiates EMT in certain cancers [101]. Moreover, the Wnt/β-catenin signalling pathway modulates several other genes including c-myc and cyclin D1 [101]. In this regard, and as a regulatory mechanism, the protein GSK-3β is one of the main components that proteolytically degrades β-catenin and helps to maintain its normal levels [101]. Tasi et al. observed that the incubation of MCF-7 cells with TMS significantly decreased GSK-3β phosphorylation, resulting in a significant accumulation of free (active) GSK-3β [101]. The authors also reported that TMS displayed three key changes in the β-catenin signalling pathway in MCF-7 cells. First, TMS decreased the level of β-catenin in a dose-dependent manner, along with a marked decrease in its nuclear translocation [101]. Second, TMS triggered β-catenin ubiquitination, and consequently, it promoted significant β-catenin degradation [101]; and third, TMS exerted a substantial reduction in the mRNA levels of the β-catenin target genes c-myc and

cyclin D1 [101]. Taken together, these results provide a strong case to suggest that TMS could have an important function in restoring normal epithelial characteristics in cancer cells [101].

Metastasis-associated protein 1 (MTA1) is one member of the nucleosome remodeling and deacetylating co-repressor complex (NuRD) which is involved in protein deacetylation and transcriptional regulations [102]. Recently, MTA1 was found to be upregulated in numerous cancers such as breast, head and neck, ovarian, gastrointestinal, and lung [103]. Elevated MTA1 expression is associated with angiogenesis, poor prognosis, and high tumor grade [103]. Resveratrol has showed a dose-dependent reduction in MTA1 protein level in both DU145 and LNCaP cells [93]. Perhaps the only report to investigate the effects of TMS on MTA1 is the study by Kun Li and coworkers [102]. In this report, TMS significantly downregulated the MTA1 protein level in PC-3M cells (ED_{50}= 55.1 μM) while TMS effects were less pronounced in LNCaP cells (ED_{50} >100 μM) [102]. Under the same experimental conditions, resveratrol was active in reducing the MTA1 level in PC-3M and LNCaP cells with ED_{50} of 74.5 and 35.1 μM respectively [102].

2.10 TMS and radical scavenging/antioxidant findings

The anticancer/chemopreventive actions of natural antioxidants are partially attributed to their ability to scavenge reactive oxygen species (ROS) [104]. This scavenging mechanism is catalyzed by antioxidant proteins such as CAT, SOD, HO-1 and peroxidase enzymes. In this particular case, the evidence for a difference in the antioxidant mechanisms exerted by resveratrol, and its methylated analogue, TMS, requires detailed analysis. There are literature reports describing the failure of TMS to induce these antioxidant enzymes. For example, Basini et al. reported that TMS, at concentrations of up to 100 μM, did not increase the activity of the free radical-scavenging enzymes peroxidase, catalase, or SOD in swine granulosa cells [105]. In another study, Li et al. reported that TMS showed only "weak" antioxidant activity by limited scavenging effects on the superoxide anion (O_2^-), and the hydroxyl radical (OH^-), as evaluated by an ethanol-induced gastric mucosal injury assay in rats [19].

In accordance with the reports mentioned in the previous paragraph, Kim et al. reported a time- and concentration-dependent increase in the expression of the antioxidant enzyme HO-1, in

murine neuronal HT22 cells, when incubated in the presence of resveratrol [23]. In contrast, TMS did not increase the expression (or the activity) of HO-1 [23]. Son et al. found similar results, and also observed a similar effect in a different setting, in this case using RAW264.7 cells; they reported that resveratrol increased the expression and the activity of HO-1, but not TMS [106].

Another variable adding to the complexity of the role of antioxidant compounds on cancer treatment/prevention, is the observation that resveratrol has been reported to exert pro-oxidant effects which could lead to DNA damage [104, 107]. In this regard, Rossi et al. found that TMS exerted an improved protective profile compared to resveratrol, as determined by the ability of these compounds to prevent H_2O_2-induced DNA damage in CHO cells (comet assay) [107]. In a different paper, using an ethidium bromide binding assay, Zheng et al. demonstrated that TMS did not induce oxidative DNA damage in calf DNA when incubated in the presence of Cu (II), whereas resveratrol exerted "minor" DNA damage [104].

In a study published in 2014, Liu and coworkers reported the effects of resveratrol and TMS on the reduction of H_2O_2 levels in a hypoxia-induced pulmonary artery hypertension (PAH) rat model [22]. In this study, resveratrol and TMS showed a nearly equipotent effect, by causing a significant decrease in hydrogen peroxide levels as measured both in plasma and in lung tissue. Liu et al. suggested that TMS's ability to decrease H_2O_2 levels confirms its antioxidant properties, despite not having the characteristic free phenol groups of resveratrol and other antioxidant phenolic compounds [22]. Studies have shown that polyphenols' antioxidant scavenging ability is associated with the presence of free hydroxyl groups in the aromatic rings of stilbenes [107]. It has also been hypothesized that the hydroxyl groups present in resveratrol are an essential structural feature to (1) induce HO-1 expression [23, 106], and (2) scavenge free radicals through a hydrogen transfer mechanism [108].

This leads to questions about why and how TMS exerts an antioxidant profile comparable to that of resveratrol. The answer has been partially described in three different papers published by Zheng et al. [104], Malgorzata et al. [24] and Rossi et al. [107]. In these papers, the authors proposed that TMS might scavenge hydroxyl radicals via an electron transfer process rather than a hydrogen transfer mechanism seen in resveratrol [104, 107]. Furthermore, it was found that TMS (at 100 μM) increased GSH levels in human TPA-stimulated neutrophils [24]. However, at

this point it is evident that TMS's radical scavenging/antioxidant profile needs the support of complementary studies, specifically a side-by-side comparison between TMS and its hydroxylated analogue resveratrol.

2.11 Pharmacokinetics of TMS

It has been well established that resveratrol undergoes extensive phase II metabolism after it is absorbed, yielding both sulfate and glucuronide conjugates as major metabolites [11]. In addition to these conjugates, resveratrol is metabolized by phase I (CYP450 enzymes) as well, producing piceatannol, which has an additional phenol group adjacent to the 4-hydroxyl group of the parent compound [109, 110]. It is noteworthy that piceatannol is also produced by some plants. In one study, Rivera et al. suggested that TMS could be a resveratrol prodrug [21]. However, this assumption needs further testing and many more supporting studies.

There have been a few complementary pharmacokinetic studies, in which several research groups have tried to correlate anticancer effects observed with stilbenes and their plasma concentrations. In one study, the injection of resveratrol into nude mice using an osmotic mini pump (adjusted to administer about 50 mg over 14 days), was followed by measurements of resveratrol serum concentration in 10 different tumor samples [84]. In this study, the authors found that only two out of 10 samples had a "detectable" level of free resveratrol, whereas resveratrol sulfate and resveratrol glucuronide metabolites were detected in all samples [84].

In another study, Dias et al. reported that oral administration of resveratrol or TMS into nude mice (50 mg/kg dose, administered every-other-day, for 52 days), resulted in an average serum concentration of resveratrol and TMS around 0.02 ± 0.01 μg/mL and 0.94 ± 0.55 μg/mL respectively [20]. This data clearly shows a greater extent of metabolic degradation experienced by resveratrol, compared to TMS [20].

Finally, in a recent study, Lin et al. assessed the pharmacokinetic profile of TMS in rats. In this report, it was found that after a single i.v. dose (5 mg/kg) of TMS, this compound displayed a half-life of 8.5 ± 2.2 h [14]. In agreement with the study by Dias (previous paragraph), the calculated clearance for resveratrol was eight- to nine-fold higher (faster elimination) than that

calculated for TMS [14]. In this regard, the authors also made an interesting observation; TMS had a negligible bioavailability (<1.5%) when it was administered orally in a suitable vehicle, whereas its bioavailability was significantly increased (up to 46.5 ± 4.8 %) when it was administered using a solution of methylated-β-cyclodextrin [14].

These observations suggest that for any subsequent studies carried out with TMS, it will be essential to consider not only the intrinsic physicochemical and pharmacological properties, but also the use of suitable excipients that modulate and increase the oral bioavailability of this promising, and so far, understudied stilbene.

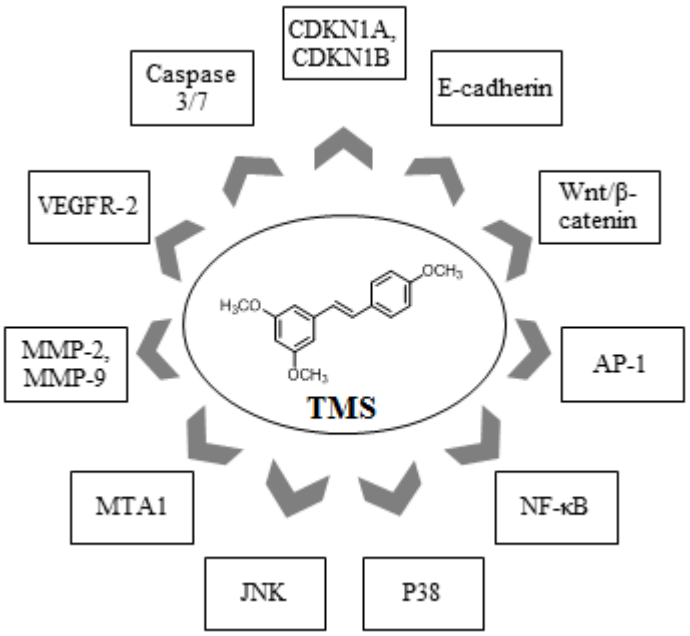

Figure 2-2: Summary of carcinogenesis targets that are modulated by TMS.

2.12 Conclusion

TMS, the naturally occurring methoxylated analogue of resveratrol, is a promising natural candidate displaying enhanced anticancer properties. It inhibits cancer cell proliferation in numerous *in vitro* assays to a greater extent compared to resveratrol. Furthermore, TMS showed a unique and different anticancer profile, distinguishing it from the parent polyphenol; *in vitro* screening assays demonstrate that TMS is capable of inducing cycle arrest and apoptosis by

different mechanisms of action compared to those observed for resveratrol, and in some cases, with an improved potency and efficacy. The overall targets through which TMS interferes with carcinogenesis are summarized in (**Figure 2-2**).

However, due to the limited number of studies on the anticancer properties of TMS, the pharmacological potential of this compound is still somewhat limited. We realize that this is not at all a disadvantage, but a window of opportunity in which many potential research projects could address the ultimate question of whether or not TMS represents a better candidate than resveratrol. The evidence so far seems to suggest this premise.

2.13 References

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Chapter 3: Chemical Synthesis

3.1 Introduction

The literature describes several synthetic pathways by which resveratrol derivatives are synthesized and ultimately characterized. Examples of organic reactions used to prepare stilbenes include but are not limited to Wittig [1], Heck [2], Horner-Wadsworth-Emmons (HWE) [3], Perkin [4], and McMurry [5] reactions. These chemical reactions produce either a single or mixed ratio of isomers (*trans* and *cis*) depending on the type of reaction and/or conditions used. We performed a simple search for the frequent and the most used pathway to synthesize resveratrol analogues (**Figure 3-1**). We found that the most common synthetic approaches were Wittig, Heck, and HWE reactions. Additionally, the Wittig reaction provides access to both *cis* and *trans* isomers [6] while the HWE reaction [7] and Heck coupling [2] are known for their greater *trans* selectivity.

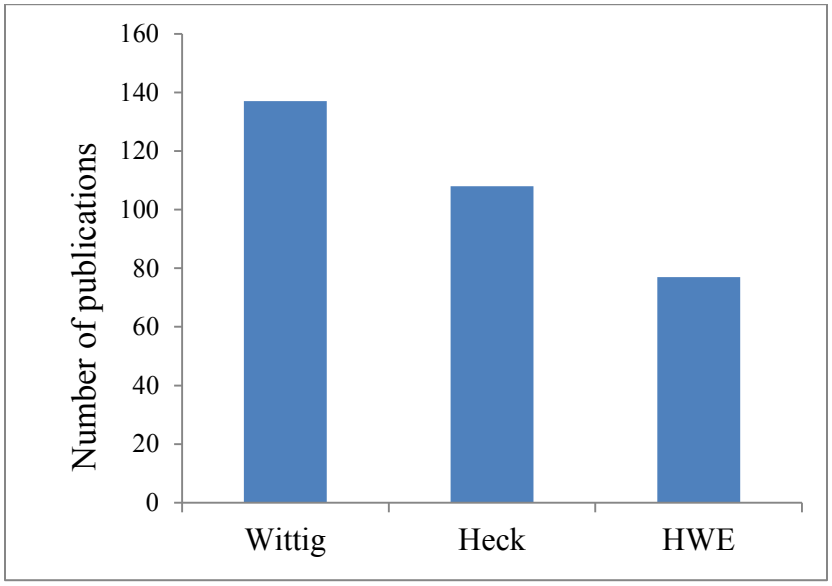


Figure 3-1: Number of publications describing resveratrol derivatives and the utilized synthetic approaches. The number of studies was obtained from the "ScienceDirect" database using keywords "resveratrol derivatives synthesis Wittig." The same keywords were used for the Heck and the HWE searches.

The Wittig reaction is the reaction between an aldehyde (or ketone) and triphenylphosphonium ylide to produce an alkene product and phosphine oxide as a by-product (**Figure 3-2**) [8, 9]. The

Wittig reaction ylide is typically formed by having phosphonium salts react with a suitable base [9]. The ylides can be classified into three categories: non-stabilized (or reactive), semi-stabilized, and stabilized ylides [10]. Mechanistically, the Wittig reaction involves two intermediate species: betaine and oxaphosphetane (**Figure 3-2**) [9], although recent observations suggest that oxaphosphetane is the only intermediate in certain Wittig reactions [10].

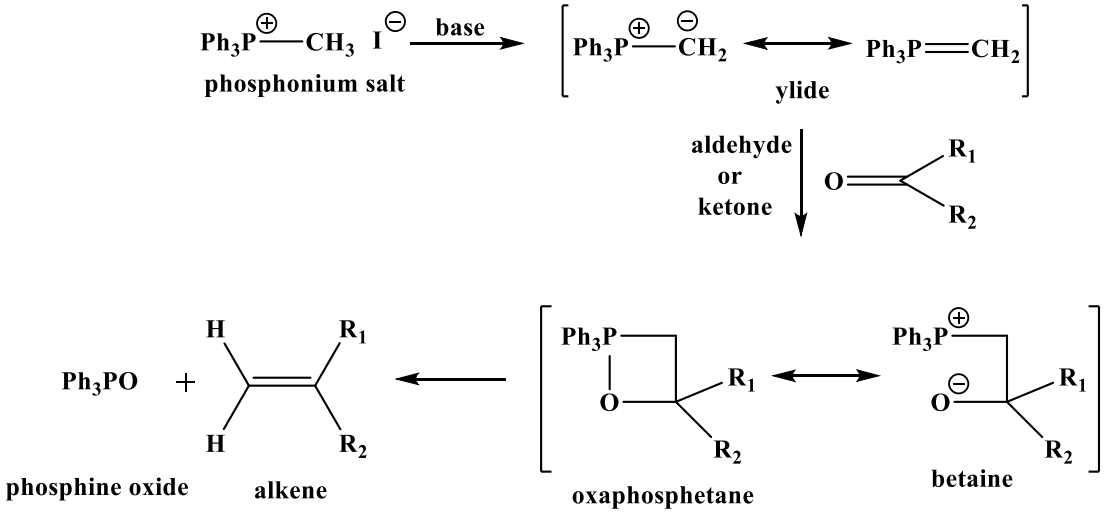


Figure 3-2: Wittig reaction. The scheme was adapted from [9].

The stereoselectivity of the Wittig reaction is controlled by various factors, particularly the intermediate oxaphosphetane (exists in *cis* and *trans* configurations) that is eliminated to produce *Z*- and *E*-alkenes respectively [9]. The *Z*-alkene appears to predominate under kinetic conditions while the *E*-alkene is produced under thermodynamic conditions in which the two oxaphosphetane configurations equilibrate with reactants to generate the most stable *E*-alkene [9]. Other factors that influence the *E*/*Z* isomer ratio are the type of ylide, lithium salts, reaction temperature, substituents on the aldehyde or ketone and aprotic conditions [9].

The Horner-Wadsworth-Emmons (HWE) reaction is a modified version of the Wittig reaction in which a phosphonate-stabilized carbanion reacts with a carbonyl substrate to generate alkenes [9, 11]. The phosphonate used in the HWE reaction can be readily prepared using a Michaelis-Arbuzov reaction of the desired phosphite compound with an organic halide [9]. The mechanism of the HWE reaction involves the formation of an oxyanion intermediate under reversible

conditions. The oxyanion ultimately decomposes through a four-centred intermediate to produce alkenes (**Figure 3-3**) [9, 11].

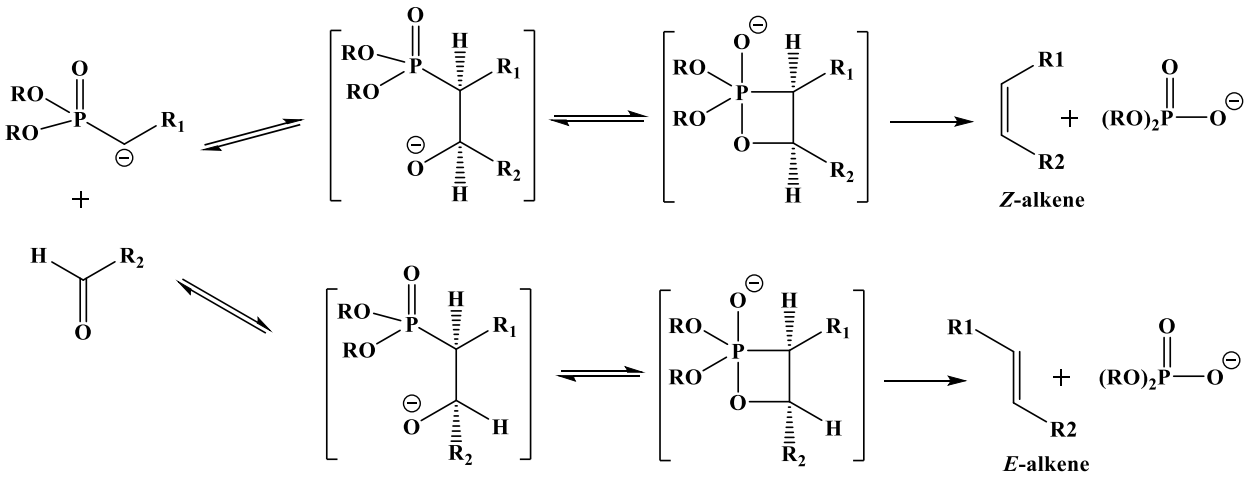
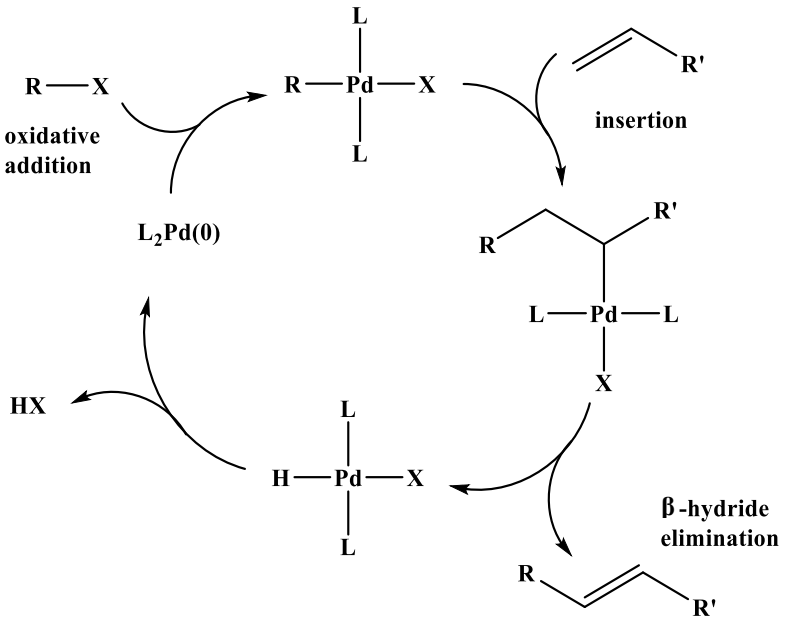


Figure 3-3: HWE reaction. Scheme was adapted from reference [9].

The stereoselectivity in the first carbon-carbon bond formation step along with the reversibility of the intermediates determines the stereochemistry of the HWE alkene product [11]. The last step, that is intermediate decomposition, is irreversible and leads to either an *E*-alkene from a *threo* diastereomer collapse or a *Z* alkene from an *erythro* counterpart [12].

The Heck reaction (also called the Mizoroki-Heck reaction) is a palladium (Pd)-catalyzed coupling between alkenes and halides that produces substituted alkenes by forming a new carbon-carbon bond [13].

Figure 3-4: catalytic cycle for Heck reaction. The scheme was adapted from [14].



The Heck coupling starts with the oxidative addition of the halide to Pd(0) with the cleavage of a halide covalent bond and the formation of two new bonds (**Figure 3-4**) [13, 14]. The next step is the insertion (also called carbometallation) of the alkene to the Pd-complex, which requires *cis* coordination [13, 14]. This step is thought to be reversible [13, 14]. After insertion, there is a "β-hydride elimination" in which hydrogen is eliminated from the carbon at the β-position and transferred to the Palladium in the Pd-complex, generating a Pd hydride (H-Pd-X) and an alkene [13]. Finally, H-Pd-X produces Pd(0) and HX by reductive elimination with the aid of a suitable base [13]. The *E*-isomer is predominant in the Heck reaction, which gives this reaction a stereospecific advantage over the Wittig reaction [15].

3.2 Objectives

The aim of this study is to synthesize resveratrol and its derivatives (**3**-**12**). Particular focus will be to investigate the possibilities of the Wittig reaction, HWE reaction and Heck coupling to produce reasonable yields of the desired *trans* isomers of resveratrol-salicylate derivatives.

3.3 Materials and methods

3.3.1 Chemistry

All melting points were determined with an Electrothermal Mel-Temp® melting point apparatus (Dubuque, IA, USA), and are uncorrected. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on either a Bruker AVANCE 600, or a Bruker AM-300 NMR spectrophotometer; coupling constants (*J*) are reported in Hertz (Hz), and the corresponding chemical shifts are represented as δ units (ppm), using tetramethylsilane (TMS) as an internal standard. ESI-MS spectra were recorded using a Water's micromass ZQ-4000 single quadruple mass spectrometer. Final compounds (**3-12**) showed a single spot on RediSep® silica gel glass plates (UV_{254}, 0.2 mm) using high, medium, and low polarity solvent mixtures. No residue remained after combustion, indicating a purity higher than 95%. Column chromatography was performed on a CombiFlash Retrieve®, or CombiFlash Rf® system using RediSep Rf silica gel® (40-60 μm) cartridges, or pre-packed RediSep Gold® columns. Microwave-assisted reactions were performed using a Biotage Initiator (Biotage, Charlotte, NC, USA) with (0.5-2.0 mL) and (2-5.0 mL) reaction vessels. All reagents were purchased from either Sigma-Aldrich (Milwaukee, IN) or TCI America (Portland, OR) and were used without further purification.

3.3.1.1 General procedure for the synthesis of compounds 1a, 2a, and 2h

In a round-bottom flask equipped with a magnetic stirrer, benzoic acid derivative (1.5-32 mmol) was dissolved in methanol (20-100 mL) with a catalytic amount of sulfuric acid. Then, the reaction flask was refluxed for 15-19 hours. After that, the reaction mixture was evaporated under a vacuum to provide a solid residue that was dissolved in EtOAc (20 mL) and extracted with a saturated $NaHCO_3$ aqueous solution (3 X 60 mL). Then, the organic layer was dried over Na_2SO_4 and evaporation of solvent furnished the ester product. In case of **2h**, column chromatography using hexane/EtOAc (95:5) was performed to isolate the **2h** from the unreacted starting material.

3.3.1.1.1 Methyl 3,5-dihydroxybenzoate (1a)

3,5-Dihydroxybenzoic acid (1.0 g, 6.5 mmol) was used as a starting material following the procedure described above. **1a** (1.05 g, 96%) was obtained as a white solid, m.p. (163-164°C).

^{1}H-NMR (300 MHz, DMSO-d_6): δ 9.34 (s, 2H, OH), 6.85 (d, *J* = 1.83 Hz, 2H, H-2 & H-6), 6.45 (t, *J* = 1.83 Hz, 1H, H-4), 3.81 (s, 3H, CH_3).

3.3.1.1.2 Methyl 5-formyl-2-hydroxybenzoate (2a)

5-Formylsalicylic acid (0.50 g, 3.0 mmol) was used as a starting material following the procedure described above. **2a** (0.29 g, 54%) was obtained as yellow crystals, m.p. (77-79°C). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 11.37 (s, 1H, CHO), 9.89 (s, 1H, OH), 8.39 (d, *J* = 1.83 Hz, 1H, H-6), 8.01 (dd, *J* = 8.52 & 1.83 Hz, 1H, H-4), 7.11 (d, *J* = 8.52 Hz, 1H, H-3), 4.02 (s, 3H, CH_3O).

3.3.1.1.3 Methyl 5-iodo-2-hydroxybenzoate (2h)

5-Iodosalicylic acid (3.2 g, 12 mmol) was used as a starting material following the procedure described above. **2h** (1.4 g, 42%) was obtained after chromatography as a pale yellow solid, m.p. (69-72°C). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 10.72 (s, 1H, OH), 8.13 (d, *J* = 2.4 Hz, 1H, H-6), 7.69 (dd, *J* = 8.5 & 2.4 Hz, 1H, H-4), 6.78 (d, *J* = 8.5 Hz, 1H, H-3), 3.96 (s, 3H, CH_3).

3.3.1.2 General procedure for the synthesis of compounds 1b, 1g, 1j, 2b, 2f, and 2g

In a round-bottom flask equipped with a magnetic stirrer, an aromatic compound bearing either free phenol group(s) and/or free carboxylic acid (1.7-31 mmol) was stirred with potassium carbonate (1.5 Eq.) at room temperature in acetone (15-200 mL) for 1 hour before the addition of dimethylsulfate (1.5 Eq. per each OH). The reaction flask was then refluxed for 1-3 hours and the reaction progress was monitored by TLC. After the reaction was complete, the solvent was evaporated and the resulting liquid was mixed with water (60 mL) and extracted with EtOAc (3 X 20 mL). In the case of **1g**, acidic water (pH = 3) was mixed with the residue after evaporation, followed by extraction with EtOAc. The combined organic layers were dried over Na_2SO_4 and the evaporation of the solvent afforded the target solid products.

3.3.1.2.1 Methyl 3,5-dimethoxybenzoate (1b)

1a (5.2 g, 31 mmol) was used as a starting material following the procedure described above. **1b** (5.9 g, 98%) was obtained as a white solid, m.p. (40-42°C). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.18 (d, *J* = 2.43 Hz, 2H, H-2 & H-6), 6.64 (t, *J* = 2.43 Hz, 1H, H-4), 3.91 (s, 3H, CH_3OCO), 3.82 (s, 6H, CH_3O).

3.3.1.2.2 4-Methoxybenzyl alcohol (1g)

4-Hydroxybenzylalcohol (1.0 g, 8 mmol) was used as a starting material following the procedure described above. **1g** (1.05 g, 94%) was obtained as a golden liquid. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.30 (d, *J* = 8.5 Hz, 2H, H-2 & H-6), 6.90 (d, *J* = 9.15 Hz, 2H, H-3 & H-5), 4.62 (s, 2H, CH_2), 3.81 (s, 3H, CH_3).

3.3.1.2.3 4-Methoxybenzaldehyde (1j):

4-Hydroxybenzaldehyde (0.30 g, 2.5 mmol) was used as a starting material following the procedure described above. **1j** (0.32 g, 95%) was obtained as a pale yellow oil. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 9.89 (s, 1H, CHO), 7.84 (d, *J* = 9.15 Hz, 2H, H-2 & H-6), 7.01 (d, *J* = 8.55 Hz, 2H, H-3 & H-5), 3.90 (s, 3H, CH_3).

3.3.1.2.4 Methyl 5-formyl-2-methoxybenzoate (2b)

2a (0.31 g, 1.7 mmol) was used as a starting material following the procedure described above. **2b** (0.3 g, 90%) was obtained as a white solid, m.p. (88-90°C). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 9.93 (s, 1H, CHO), 8.34 (d, *J* = 1.83 Hz, 1H, H-6), 8.03 (dd, *J* = 9.15 Hz & 1.83 Hz, 1H, H-4), 7.12 (d, *J* = 9.15 Hz, 1H, H-3), 4.01 (s, 3H, OCH_3), 3.93 (s, 3H, CO_2CH_3).

3.3.1.2.5 4-Methoxyiodobenzene (2f)

4-Hydroxyiodobenzene (0.6 g, 2.7 mmol) was used as a starting material following the procedure described above. **2f** (0.61, 95%) was obtained as a light brown solid, m.p. (49-51°C). ^{1}H-NMR (600 MHz, $CDCl_3$): δ 7.55 (d, *J* = 8.4 Hz, 2H, H-2 & H-6), 6.68 (d, *J* = 9.0 Hz, 2H, H-3 & H-5), 3.96 (s, 3H, CH_3).

3.3.1.2.6 Methyl 5-iodo-2-methoxybenzoate (2g)

5-Iodosalicylic acid (1.56 g, 5.9 mmol) was used as a starting material following the procedure described above. **2g** (1.62 g, 94%) was obtained as colorless needles, m.p. (49-51°C). ^{1}H-NMR (600 MHz, $CDCl_3$): δ 8.07 (d, *J* = 2.4 Hz, 1H, H-6), 7.73 (dd, *J* = 8.4 & 2.4 Hz, 1H, H-4), 6.75 (d, *J* = 8.4 Hz, 1H, H-3), 3.88 (s, 6H). ^{13}C-NMR (600 MHz, $CDCl_3$): 165.15, 158.97, 142.0, 140.02, 122.15, 114.33, 81.71, 56.15, 52.26.

3.3.1.3 Procedure for the synthesis of 3,5-dimethoxybenzyl alcohol (1c)

In a round-bottom flask equipped with a magnetic stirrer, **1b** (0.7 g, 3.6 mmol) was added, and the flask was flushed several times with nitrogen gas. Then, lithium aluminum hydride (LAH)

(0.14 g, 3.7 mmol) was added, followed by addition of dry THF (10 mL) while keeping the reaction flask under N_2 gas. After that, the reaction was stirred at room temperature for two hours and the reaction progress was monitored by TLC. After the reaction was complete, it was quenched by the careful addition of water (60 mL). The reaction mixture was then acidified (Conc. HCl) to pH=3. Then, the mixture was extracted with EtOAc (3 X 20 mL), and the combined organic layer was dried over Na_2SO_4. The evaporation of EtOAc afforded the title compound (0.54 g, 90%) as a light brown solid, m.p. (45-47°C). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 6.53 (d, *J* = 1.83 Hz, 2H, H-2 & H-6), 6.39 (t, *J* = 2.43 Hz, 1H, H-4), 4.64 (s, 2H, CH_2), 3.79 (s, 6H, 2 X CH_3).

3.3.1.4 Procedure for the synthesis of 3,5-dimethoxybenzaldehyde (1d)

For the synthesis of **1d**, we reproduced the method reported by Varssev, G.N. [16]. Briefly, **1c** (0.46 g, 2.7 mmol) was dissolved in CH_2Cl_2 (8 mL) in a round-bottom flask equipped with a magnetic stirrer. Then, pyridinium dichromate (1.2 g, 3.2 mmol) was added to the reaction flask, and the reaction was allowed to stir at room temperature for 18 hours. After the reaction was completed (determined by TLC), the mixture was filtered out and the solid residue was trapped after the filtration was washed with CH_2Cl_2 (20 mL). Then, this organic layer was washed three times, first with 5% NaOH (10 mL), then with 5% HCl (10 mL), and finally with an $NaHCO_3$ saturated aqueous solution. After that, the organic layer was dried over Na_2SO_4 and the evaporation of solvent produced **1d** (0.38 g, 84%) as a white solid, m.p. (48-50°C). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 9.91 (s, 1H, CHO), 7.01 (d, *J* = 2.43 Hz, 2H, H-2 & H-6), 6.71 (t, *J* = 2.43 Hz, 1H, H-4), 3.85 (s, 6H, 2 X CH_3).

3.3.1.5 General procedure for the synthesis of compounds 1h and 1e

The benzylic alcohol derivative **1g** or **1c** (1.6-6.5 mmol) was placed in a dry round-bottom flask equipped with a magnetic stirrer before the addition of dry CH_2Cl_2 (7-25 mL). Then, the reaction flask was kept under N_2 gas and placed in an ice-bath before the addition of PBr_3 (1.2 Eq.) dropwise over 5-9 minutes. After the complete addition of PBr_3, the reaction was allowed to stir in the ice-bath for 2 hours and at room temperature for one hour. Meanwhile, the reaction progress was monitored by TLC. After the reaction was complete, it was quenched by cold water (100 mL) and extracted with CH_2Cl_2 (2 X 20 mL). The organic layer was then filtered off, and the clear filtrate was evaporated to produce the target compounds.

3.3.1.5.1 4-Methoxybenzyl bromide (1h)

1g (0.2 g, 1.6 mmol) was used as a starting material following the procedure described above. **1h** (0.29 g, 90%) was obtained as a light brown liquid. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.32 (d, *J* = 8.52 Hz, 2H, H-2 & H-6), 6.86 (d, *J* = 8.52 Hz, 2H, H-3 & H-5), 4.50 (s, 2H, CH_2), 3.80 (s, 3H, CH_3).

3.3.1.5.2 3,5-Dimethoxybenzyl bromide (1e)

1c (1.1 g, 6.5 mmol) was used as a starting material following the procedure described above. **1e** (1.27 g, 84%) was obtained as a brown solid, m.p. (69-71°C). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 6.54 (d, *J* = 2.46 Hz, 2H, H-2 & H-6), 6.39 (t, *J* = 2.43 Hz, 1H, H-4), 4.42 (s, 2H, CH_2), 3.79 (s, 6H, 2 X CH_3).

3.3.1.6 General procedure for the synthesis of compounds 1i and 1f

The benzyl bromide derivative **1h** or **1e** was placed in a round-bottom flask equipped with a magnetic stirrer before the addition of toluene (4-50 mL). Then, PPh_3 was added (1.2 Eq.), and the reaction flask was refluxed for 18 hours. The completion of the reaction was confirmed by the disappearance of the starting material using TLC. After that, the white solid produced from this reaction was filtered off and washed with toluene two times (15 mL). The white solid obtained was dried in a vacuum-oven, and the title compounds were later confirmed by measuring their melting point.

3.3.1.6.1 4-Methoxybenzyltriphenylphosphonium bromide (1i)

1h (0.18 g, 0.9 mmol) was used as a starting material following the procedure described above. **1i** (0.33 g, 80%) was obtained as an off-white solid, m.p. 229-231°C (reported 235-237°C [17]).

3.3.1.6.2 3,5-Dimethoxybenzyltriphenylphosphonium bromide (1f)

1e (1.13 g, 4.9 mmol) was used as a starting material following the procedure described above. **1f** (2.18 g, 90%) was obtained as an off-white solid, m.p. 269-272°C (reported 275°C [17]).

3.3.1.7 General procedure for the synthesis of compounds 2c, 2d and 2i

In a round-bottom flask equipped with a magnetic stirrer, the compounds bearing the hydroxyl group (0.9-3.3 mmol) and Et_3N (1.2 Eq.) were mixed into dry THF (8-50 mL). Then, CH_3COCl (1.2 Eq.) was added dropwise to the reaction flask over 2-4 minutes. After that, the reaction was allowed to stir at room temperature for 1-2 hours. Upon reaction completion (by TLC), the solid

produced from this reaction was filtered out, and the clear organic filtrate was dried over Na_2SO_4 before evaporation of the solvent. After the evaporation, we obtained the target solid products. They showed high purity and we used them without further purification in the subsequent reactions.

3.3.1.7.1 Methyl 2-acetoxy-5-formylbenzoate (2c)

2a (0.16 g, 0.90 mmol) was used as a starting material following the procedure described above. **2c** (0.18 g, 93%) was produced as yellow crystals, m.p.(34-36 °C). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 10.03 (s, 1H, CHO), 8.53 (d, *J* = 1.83 Hz, 1H, H-6), 8.09 (dd, *J* = 8.52 Hz &1.83, Hz, 1H, H-4), 7.29 (d, *J* = 8.55 Hz, 1H, H-3), 3.92 (s, 3H, CH_3OCO), 2.38 (s, 3H, CH_3CO). ^{13}C-NMR (600 MHz, $CDCl_3$) δ 190.09, 169.02, 163.80, 155.09, 134.01, 133.94, 133.85, 124.99, 124.05, 52.49, 20.89. ESI-MS *m/z*; 245.0 $[M+Na]^+$.

3.3.1.7.2 4-Acetoxy-3-methoxybenzaldehyde (2d)

3-Methoxy-4-hydroxybenzaldehyde (vanillin) (0.5 g, 3.3 mmol) was used as a starting material using the procedure described above. **2d** (0.58 g, 91%) was produced as pale yellow crystals, m.p. (77-79°C). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 9.95 (s, 1H, CHO), 7.50 (d, *J* = 1.83 Hz, 1H, H-2), 7.48 (dd, *J* = 7.32 Hz & 1.83 Hz, 1H, H-6), 7.22 (d, *J* = 7.32 Hz, 1H, H-5), 3.91 (s, 3H, CH_3O), 2.35 (s, 3H, CH_3CO).

3.3.1.7.3 2-Acetoxy-5-formylbenzoic acid (2i)

5-Formylsalicylic acid (0.5 g, 3.0 mmol) was used as a starting material following the procedure described above. **2i** (0.52 g, 83%) was produced as a pale yellow liquid. The product is unstable and decomposes at room temperature (as we observed impurities in ^{1}H-NMR upon leaving it on the bench). ^{1}H-NMR (300 MHz, DMSO-d_6): δ 9.96 (s, 1H, CHO), 8.50 (d, *J* = 1.83 Hz, 1H, H-6), 8.01 (dd, *J* = 7.92 & 1.83 Hz, 1H, H-4), 7.19 (d, *J* = 7.95 Hz, 1H, H-3), 2.29 (s, 3H, CH3CO).

3.3.1.8 Wittig reaction for the synthesis of *cis*/*trans* isomers of TMS and resveratrol derivatives 3-7

In a heat-dried three-neck round bottom flask, the corresponding phosphonium bromide (**1i** or **1f**, 1.1 Eq.) was added, and the flask was immediately flushed with nitrogen gas to remove oxygen and moisture. Using a bath of dry ice and acetone, the flask was cooled down to about -60 °C, at which time (15-50 mL) of dry THF was added. After a few minutes of stirring, *n*-butyllithium

(1.1 Eq.) was added. The resulting red suspension was stirred for 30-45 minutes before a solution of the corresponding aldehyde (**1d**, **1j** or **2b-2d**, 0.6-2.5 mmol) was added in (2-10 mL) of dry THF, dropwise, over 20 to 30 minutes. After that, the dry ice bath was removed to allow the reaction mixture to reach room temperature. The mixture was then stirred for about 15-18 hours (until the aldehyde was completely consumed, as monitored by TLC). After the reaction was complete, it was quenched by adding water (90 mL). It was then extracted with EtOAc (3 X 30 mL). The combined organic layers were dried using Na_2SO_4, and the solvent was evaporated under a vacuum to obtain the crude liquid product, which was then purified by silica gel column chromatography, using a solvent mixture of hexane/ethyl acetate (60:40) to afford the *E*/*Z* mixture of stilbenes as an oily liquid.

3.3.1.9 General procedure for *trans*-isomerization

To obtain the pure *E*-isomer from the purified isomeric mixtures obtained above, the stilbene (0.1-6.3 mmol) was dissolved in dry THF (50 mL/1.46 mmol stilbene), and diphenyl disulfide (0.2-0.7 Eq.) was added as previously described [18]. This reaction mixture was stirred under a nitrogen atmosphere until all the *Z* stilbene re-isomerized to the *E* isomer (on average, the *Z* isomer R_f value was 0.64, while that of the *E* isomer was 0.51). Finally, the solvent was evaporated under a vacuum, and the product was purified by column chromatography using a mixture of petroleum ether/ethyl acetate (70:30) to afford the pure *E*-isomer.

3.3.1.9.1 (*E*)-3,4',5-Trimethoxystilbene (TMS)

This compound was obtained from the reaction between aldehyde **1d** (0.64 g, 3.9 mmol) and the salt **1i** (1.85 g, 4.0 mmol) using the procedure described above. *Trans*-TMS (0.61 g, 61%) was produced after reaction with Ph_2S_2 as colorless needles, m.p. 55-56 °C (reported 55-57 °C [19]). ^{1}H-NMR (600 MHz, $CDCl_3$) δ 7.44 (d, J = 8.4 Hz, 2H, H-2' & H-6'), 7.04 (d, J = 16.2 Hz, 1H, alkene), 6.90 (d, J = 16.8 Hz, 1H, alkene), 6.89 (d, J = 8.4 Hz, 2H, H-3' & H-5'), 6.65 (d, J = 2.4 Hz, 2H, H-2 & H-6), 6.37 (t, J = 2.4 Hz, 1H, H-4), 3.83 (s, 9H, 3 X CH_3O). ^{13}C-NMR (300 MHz, $CDCl_3$) δ 160.98, 159.42, 139.72, 129.97, 128.76, 127.77, 126.62, 114.16, 104.39, 99.68, 55.36.

3.3.1.9.2 (*E*)-4,4'-Dimethoxy-3-(methoxycarbonyl)stilbene (3)

This compound was obtained from the reaction between aldehyde **2b** (0.12 g, 0.64 mmol) and the salt **1i** (0.31 g, 0.65 mmol) using the procedure described above. **3** (0.68 g, 68%) was

produced after the Ph_2S_2 reaction as a white solid, m.p.133-134 °C. ^{1}H-NMR (600 MHz, $CDCl_3$) δ 7.93 (d, J = 2.4 Hz, 1H, H-2'), 7.58 (dd, J = 8.4, 2.4 Hz, 1H, H-6'), 7.43 (d, J = 9.0 Hz, 2H, H-2 and H-6), 6.98 (d, J = 16.2 Hz, 1H, alkene), 6.97 (d, J = 8.4 Hz, 1H, H-5'), 6.91 (d, J = 18.3 Hz, 1H, alkene), 6.89 (d, J = 9 Hz, 2H, H-3 and H-5), 3.92 (s, 3H, CH_3O), 3.92 (s, 3H, CH_3OCO), 3.83 (s, 3H, CH_3O). ^{13}C-NMR (300 MHz, $CDCl_3$) δ 166.61, 159.20, 158.25, 131.03, 130.07, 130.04, 129.36, 129.20, 127.52, 127.38, 125.01, 120.16, 114.13, 112.30, 56.16, 55.32, 52.09. Exact mass (298.12), found mass using ESI-MS m/z = 321.0 $[M+Na]^+$. Anal. calcd for $C_{18}H_{18}O_4$: C 72.47, H 6.08, found: C 72.49, H 6.19.

3.3.1.9.3 (*E*)-4-Acetoxy-4'-methoxy-3-(methoxycarbonyl)stilbene (4):

This compound was obtained from the reaction between aldehyde **2c** (0.42 g, 1.9 mmol) and the salt **1i** (1.02 g, 2.2 mmol) using the procedure described above. **4** (0.59 g, 59%) was produced after the Ph_2S_2 reaction as a white solid, m.p. 123-125 °C. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.11 (d, J = 2.43 Hz, 1H, H-2'), 7.62 (dd, J = 8.55, 2.43 Hz, 1H, H-6'), 7.44 (d, J = 9.15 Hz, 2H, H-2 and H-6), 7.07 (d, J = 7.95 Hz, 1H, H-5'), 7.06 (d, J = 15.87 Hz, 1H, alkene), 6.93 (d, J = 16.47 Hz, 1H, alkene), 6.90 (d, J = 9.15 Hz, 2H, H-3 and H-5), 3.90 (s, 3H, CH_3OCO), 3.81 (s, 3H, CH_3O), 2.36 (s, 3H, CH_3CO). ^{13}C-NMR (300 MHz, $CDCl_3$): δ 169.55, 164.72, 159.43, 149.24, 135.71, 130.87, 129.47, 129.39, 129.14, 127.74, 124.29, 123.88, 123.05, 114.03, 55.13, 52.06, 20.83. Exact mass (326.12), found mass using ESI-MS m/z = 348.9 $[M+Na]^+$.

3.3.1.9.4 (*E*)-4'-Acetoxy-3,3',5-trimethoxystilbene (5)

This compound was obtained from the reaction between aldehyde **2d** (0.48 g, 2.5 mmol) and the salt **1f** (1.28 g, 2.6 mmol) using the procedure described above. **5** (0.76 g, 76%) was produced after the Ph_2S_2 reaction as white crystals, m.p. = 118-120 °C (reported 120-122 °C [20]). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.09 (dd, J = 9.15 & 1.83 Hz, 1H, H-6'), 7.06 (d, J = 15.84 Hz, 1H, alkene), 7.03 (d, J = 1.83 Hz, 1H, H-2'), 7.02 (d, J = 8.55 Hz, 1H, H-5'), 6.97 (d, J = 15.87 Hz, 1H, alkene), 6.67 (d, J = 2.4 Hz, 2H, H-2 & H-6), 6.41 (t, J = 2.4 Hz, 1H, H-4), 3.89 (s, 6H, 2 X CH_3O), 3.83 (s, 3H, CH_3O), 2.32 (s, 3H, CH_3CO). ^{13}C-NMR (300 MHz, $CDCl_3$) δ 169.00, 160.96, 151.17, 139.36, 139.07, 136.20, 128.95, 128.53, 122.91, 119.30, 110.11, 104.58, 100.11, 55.89, 55.38, 20.70. Exact mass (328.12), found mass using ESI-MS m/z = 351.0 $[M+Na]^+$.

3.3.1.9.5 (*E*)-4'-Acetoxy-3,5-dimethoxy-3'-(methoxycarbonyl)stilbene (6)

This compound was obtained from the reaction between aldehyde **2c** (0.39 g, 1.8 mmol) and the salt **1f** (0.94 g, 1.9 mmol) using the procedure described above. **6** (0.67 g, 67%) was produced after the Ph_2S_2 reaction as colorless needles, m.p. 97-99 °C. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.12 (d, J = 1.83 Hz, 1H, H-2'), 7.80 (dd, J = 8.55, 1.83 Hz, 1H, H-6'), 7.19 (d, J = 16.47 Hz, 1H, alkene), 7.13 (d, J = 8.55 Hz, 1H, H-5'), 7.12 (d, J = 16.47 Hz, 1H, alkene), 6.73 (d, J = 2.46 Hz, 2H, H-2 and H-6), 6.41 (t, J = 2.40 Hz, 1H, H-4), 3.87 (s, 3H, CH_3OCO), 3.80 (s, 6H, 2 X CH_3O), 2.30 (s, 3H,CH_3COO). ^{13}C-NMR (300 MHz, $CDCl_3$) δ 169.67, 164.75, 160.96, 149.77, 138.70, 135.30, 131.32, 130.10, 129.66, 127.06, 124.09, 123.26, 104.65, 100.36, 55.36, 52.25, 20.96. Exact mass (356.13), found mass using ESI-MS m/z = 378.9 $[M+Na]^+$.

3.3.1.9.6 (*E*)-3,4',5-Trimethoxy-3'-(methoxycarbonyl)stilbene (7)

This compound was obtained from the reaction between aldehyde **2b** (0.33 g, 1.7 mmol) and the salt **1f** (0.88 g, 1.8 mmol) using the procedure described above. **7** (0.59 g, 59%) was produced after the Ph_2S_2 reaction as a white solid, m.p. = 102-103 °C. ^{1}H-NMR (300 MHz, CD_3OD) δ 7.92 (d, J = 1.83 Hz, 1H, H-2'), 7.72 (dd, J = 8.55, 2.46 Hz, 1H, H-6'), 7.12 (d, J = 9.15 Hz, 1H, H-5'), 7.11 (d, J = 16.29 Hz, 1H, alkene), 7.00 (d, J = 16.47 Hz, 1H, alkene), 6.70 (d, J = 2.43 Hz, 2H, H-2 and H-6), 6.38 (t, J = 2.46 Hz, 1H, H-4), 3.89 (s, 3H, CH_3OCO), 3.87 (s, 3H, CH_3O), 3.80 (s, 6H, 2 X CH_3O). ^{13}C-NMR (300MHz, $CDCl_3$) δ 166.32, 160.80, 158.46, 139.08, 131.21, 129.58, 129.31, 127.58, 127.41, 120.03, 112.15, 104.26, 99.76, 99.65, 55.96, 55.16, 51.91, 29.57. Exact mass (328.13), found mass using ESI-MS m/z = 351.0 $[M+Na]^+$.

3.3.1.10 Procedure for the synthesis of diethyl (3,5-dimethoxybenzyl) phosphonate (1k)

1e (0.5 g, 2.2 mmol) and triethyl phosphite (2.0 Eq.) were mixed in a round-bottom flask equipped with magnetic stirrer before heating the reaction flask at 130°C for 2 hours. Then, the reaction mixture underwent column chromatography using hexane/EtOAc (30:70) to remove the unreacted triethyl phosphite. **1k** (0.44 g, 70%) was obtained as a colorless liquid. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 6.46 (t, J = 2.43 Hz, 2H, H-2 & H-6), 6.35 (d, J = 2.43 Hz, 1H, H-4), 4.03 (quint, J = 7.32 Hz, 4H, O$\underline{CH_2}CH_3$), 3.77 (s, 6H, OCH_3), 3.09 (d, J = 21.99 Hz, 2H, CH_2P), 1.26 (t, J = 7.32 Hz, 6H, $OCH_2\underline{CH_3}$).

3.3.1.11 Wittig reaction for the synthesis of intermediates 1l and 1m to be used in the Heck coupling

1d or 4-hydroxybenzaldehyde, methyltriphenylphosphonium bromide (4.0 Eq.), and sodium hydride (4.5 Eq.) were added to a dry round-bottom flask equipped with a magnetic stirrer. Then, the flask was flushed with N_2 gas and kept under an inert atmosphere. After that, dry THF (5-25 mL) was added and the flask was stirred at room temperature for 6 to17 hours. Upon reaction completion (monitored by TLC), cold water (50 mL) was added, followed by extraction with EtOAc (3 X 15 mL). In the case of **1m**, mixture acidification (pH = 3) was performed before extraction. Then, the combined organic layer was dried over Na_2SO_4. When the solvent evaporated, it produced a liquid mixture which was purified by silica gel column chromatography using an eluent mixture of hexane/EtOAc (80:20).

3.3.1.11.1 3,5-Dimethoxystyrene (1l)

1d (0.8 g, 4.8 mmol) was used to prepare **1l** using the procedure described above. **1l** (0.73 g, 93%) was obtained as colorless oil. ^{1}H-NMR (600 MHz, $CDCl_3$): δ 6.64 (dd, *J* = 17.4 & 10.8 Hz, 1H, alkene), 6.56 (d, *J* = 1.8 Hz, 2H, H-2 & H-6), 6.38 (t, *J* =1.8 Hz, 1H, H-4), 5.73 (d, *J* = 17.4 Hz, 1H, alkene), 5.25 (d, *J* = 10.8 Hz, 1H, alkene), 3.80 (s, 6H, 2 X CH_3).

3.3.1.11.2 4-Hydroxystyrene (1m):

4-Hydroxybenzaldehyde (1.0 g, 8.2 mmol) was used to prepare **1m** using the procedure described above. **1m** (0.77 g, 78%) was obtained as colorless oil. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.29 (d, *J* = 8.55 Hz, 2H, H-2 & H-6), 6.80 (d, *J* = 8.52 Hz, 2H, H-3 & H-5), 6.65 (dd, *J* =17.70 & 10.98 Hz, 1H, alkene), 5.73 (broad s, 1H, OH), 5.59 (d, *J* = 17.70 Hz, 1H, alkene), 5.11 (d, *J* = 10.38 Hz, 1H, alkene).

3.3.1.12 General procedure for the HWE reaction

In a round-bottom flask equipped with a magnetic stirrer, a phosphonate derivative (**1k**) (1.0 Eq.) was added to the aldehyde (**1j** or **2b**) or aldehyde-devoid (**2e**) (1.3 mmol) solution in DMF (10-30 mL). The base was either added completely or gradually to the reaction mixture (according to each specified reaction). Then, the reaction was allowed to stir at room temperature while the reaction process was monitored by TLC. Using sodium methoxide as a base, **1k** was first added

with the base in DMF, after which the aldehyde was gradually added, and the reaction was then heated at reflux. When the reaction was completed (or as per the specified time), it was quenched by the addition of an aqueous ammonium chloride solution (60 mL), followed by extraction with EtOAc (3 X 20 mL). The combined organic layer was dried over Na_2SO_4. When the solvent evaporated, it produced a liquid that was purified by silica gel chromatography using hexane/EtOAc (80:20).

3.3.1.13 General procedure for the Heck coupling

Styrene **1m** or **1l** (0.1-4.5 mmol), iodo-derivative **2f, 2g or 2h** (0.1-4.5 mmol), palladium acetate (1% mmol), triethyl amine (0.2-1.5 mL), and triphenyl phosphine (4.5% mmol) were added in either a dry microwave vial or in a round-bottom flask equipped with a magnetic stirrer (to be used for traditional reflux). Then, acetonitrile (0.5-6.0 mL) was added, and the reaction vessel was sealed and was either refluxed or heated in the microwave for the specified temperatures and time. After the reaction was completed, (TLC monitored), it was quenched by the addition of cold water (70 mL) and extracted with EtOAc (3 X 20 mL). The combined organic layer was dried over Na_2SO_4. When the solvent evaporated, it produced a solid mixture that was purified by silica gel chromatography using petroleum ether:EtOAc (80:20). After chromatography, the *trans* isomer was recrystallized from a mixture of hexane and EtOAc.

3.3.1.13.1 3,3'-dicarbomethoxy-4,4'-dimethoxybiphenyl (13)

This compound was identified as a by-product during Heck coupling to produce resveratrol derivative **7**. Compound **13** was obtained as colorless crystals, m.p. 110-112°C (reported 115-117°C [21]). ^{1}H-NMR (600 MHz, $CDCl_3$): δ 7.99 (d, J = 2.4 Hz, 2H, H-2 & H-2'), 7.66 (dd, J = 8.4 Hz & 2.4 Hz, 2H, H-6 & H-6'), 7.04 (d, J = 8.4 Hz, 2H, H-5 & H-5'), 3.94 (s, 6H, 2 X OCH_3), 3.92 (s, 6H, 2 X $COOCH_3$). ^{13}C-NMR (600 MHz, $CDCl_3$): 50.26, 54.31, 54.42, 110.62, 118.43, 127.93, 129.61, 130.00, 156.50, 164.73. ESI-MS *m/z*; 331.0 $[M+H]^+$ (reported *m/z* = 330 by EI-MS [21]).

3.3.1.14 General procedure for the synthesis of resveratrol, 8, 9, and 10

The corresponding *E*-stilbene (**TMS**, **3** or **7**) was dissolved in dry dichloromethane using a heat-dried three-neck round-bottom flask equipped with a magnetic stirrer. Using dry ice and acetone,

the solution was cooled to about -60°C. Then a solution of boron tribromide (1 M in CH_2Cl_2) was added dropwise over 1-3 minutes. After adding BBr_3, the ice bath was removed to allow the reaction mixture to warm up to room temperature, and it was stirred for 1- 4 hours. The reaction was quenched by adding water (60 mL; very slowly), and the product mixture was extracted with dichloromethane (3 X 20 mL). The combined organic phases were dried using Na_2SO_4, and the solvent was evaporated under vacuum. Finally, the product was purified by silica gel column chromatography using a mixture of EtOAc/methanol (80:20) to afford the corresponding hydroxylated stilbenes.

3.3.1.14.1 (*E*)-3,4',5-Trihydroxystilbene (resveratrol)

Trans-TMS (0.053 g, 0.19 mmol) was used to prepare resveratrol using the procedure described above. Resveratrol (0.03 g, 67%) was obtained as a light red solid, m.p. 168-171°C (reported 172-174°C [17]). ^{1}H-NMR (300 MHz, CD_3OD): δ 7.34 (d, J = 8.55 Hz, 2H, H-2' & H-6'), 6.95 (d, J = 15.87 Hz, 1H, alkene), 6.79 (d, J = 16.47 Hz, 1H, alkene), 6.76 (d, J = 8.55 Hz, 2H, H-3' & H-5'), 6.44 (d, J = 2.43 Hz, 2H, H-2 & H-6), 6.15 (t, J = 2.43 Hz, 1H, H-4).

3.3.1.14.2 (*E*)-4,4'-Dihydroxy-3'-(hydroxycarbonyl)stilbene (8)

3 (0.26 g, 0.87 mmol) was used to prepare derivative **8** utilizing 6.0 Eq. of BBr_3 and following the procedure described above. **8** (0.13 g, 60%) was obtained as an off-white solid, m.p. = 237-240 °C (reported 241-243 °C [22]). ^{1}H-NMR (300 MHz, CD_3OD) δ 7.94 (d, J = 2.46 Hz, 1H, H-2'), 7.66 (dd, J = 8.55 Hz & 2.43 Hz, 1H, H-6'), 7.35 (d, J = 8.55 Hz, 2H, H-2 & H-6), 6.96 (d, J = 16.4 Hz, 1H, alkene), 6.90 (d, J = 8.52 Hz, 1H, H-5'), 6.89 (d, J = 15.87 Hz, 1H, alkene), 6.75 (d, J = 8.55 Hz, 2H, H-3 & H-5). Exact mass (256.07), found mass using ESI-MS m/z = 256.8 $[M+H]^+$.

3.3.1.14.3 (*E*)-3,4',5-Trihydroxy-3'-(hydroxycarbonyl)stilbene (9)

7 (0.61 g, 1.9 mmol) was used to prepare derivative **9** utilizing 8.0 Eq. of BBr_3 and following the procedure described above. **9** (0.25 g, 50%) was obtained as a pale yellow solid, m.p. = 213-215 °C. ^{1}H-NMR (300 MHz, CD_3OD) δ 7.95 (d, J = 2.46 Hz, 1H, H-2'), 7.68 (dd, J = 8.55 Hz & 2.43 Hz, 1H, H-6'), 6.98 (d, J = 16.47 Hz, 1H, alkene), 6.91 (d, J = 8.52 Hz, 1H, H-5'), 6.86 (d, J = 16.47 Hz, 1H, alkene), 6.46 (d, J = 1.83 Hz, 2H, H-2 & H-6), 6.16 (t, J = 2.46 Hz, 1H, H-4).

^{13}C-NMR (300 MHz, CD_3OD) δ 173.34, 162.65, 159.69, 140.78, 134.07, 130.24, 129.60, 128.62, 128.17, 118.62, 113.96, 105.93, 103.02. Exact mass (272.07), found mass using ESI-MS *m/z* = 271.2 $[M-H]^-$.

3.3.1.14.4 (*E*)-3,4',5-Trihydroxy-3'-methoxycarbonylstilbene (10)

7 (0.50 g, 1.5 mmol) was used to prepare derivative **10** utilizing 4.50 Eq. of BBr_3 and following the procedure described above. **10** (0.33 g, 76%) was obtained as a pale yellow solid, mp = 80-84 °C. ^{1}H-NMR (300 MHz, CD_3OD) δ 7.93 (d, *J* = 2.43 Hz, 1H, H-2'), 7.70 (dd, *J* = 8.55 Hz & 2.43 Hz, 1H, H-6'), 6.97 (d, *J* = 16.47 Hz, 1H, alkene), 6.94 (d, *J* = 8.55 Hz, 1H, H-5'), 6.86 (d, *J* = 15.84 Hz, 1H, alkene), 6.46 (d, *J* = 1.83 Hz, 2H, H-2 & H-6), 6.17 (t, *J* = 1.83 Hz, 1H, H-4), 3.97 (s, 3H, CH_3OCO). ^{13}C-NMR (300 MHz, CD_3OD) δ 171.60, 162.08, 159.69, 140.69, 134.29, 130.50, 129.05, 128.89, 127.98, 118.83, 113.63, 105.96, 103.09, 52.93. Exact mass (286.08), found mass using ESI-MS *m/z* = 285.3 $[M-H]^-$.

3.3.1.15 General procedure for the synthesis of analogues 11 and 12

In a round-bottom flask, compound **8** or **9** was dissolved at room temperature in pyridine (0.5-1.0 mL). Acetic anhydride (13 Eq.) was then added drop-wise to the reaction flask. The reaction mixture was stirred until the stilbene starting material was consumed as tested by TLC (about 3-4 h). Then, the reaction was quenched by adding water (25 mL) and the mixture was acidified to pH =3 using conc. HCl. After that, the product was extracted by adding EtOAc (8 mL) two times; then the combined organic layer was dried with Na_2SO_4. Evaporation of the solvent under vacuum provided the crude product, which was purified by crystallization from a mixture of petroleum ether/EtOAc.

3.3.1.15.1 (*E*)-4,4'-Diacetoxy-3'-(hydroxycarbonyl)stilbene (11)

8 (0.061 g, 0.24 mmol) was used to prepare derivative **11** using the procedure described above. **11** (0.062 g, 76%) was obtained as a white solid, m.p. = 193-196°C. ^{1}H NMR (600 MHz, CD_3OD) δ 8.16 (d, *J* = 2.4 Hz, 1H, H-2'), 7.79 (dd, *J* = 8.4 Hz & 2.4 Hz, 1H, H-6'), 7.61 (d, *J* = 8.4 Hz, 2H, H-2 & H-6), 7.22 (d, *J* = 16.2 Hz, 1H, alkene), 7.19 (d, *J* = 16.2 Hz, 1H, alkene), 7.12 (d, *J* = 8.4 Hz, 1H, H-5'), 7.10 (d, *J* = 8.4 Hz, 2H, H-3 & H-5), 2.28 (s, 3H, CH_3CO), 2.27 (s, 3H, CH_3CO).^{13}C-NMR (600 MHz, DMSO-d_6) δ 169.72, 169.66, 166.06, 150.56, 149.77, 135.52, 134.95, 131.44, 129.73, 129.10, 128.10, 128.03, 127.28, 124.69, 122.62, 21.36, 21.34. Exact mass (340.09), found mass using ESI-MS *m/z* = 363.1 $[M+Na]^+$.

3.3.1.15.2 (*E*)-3,4',5-Triacetoxy-3'-(hydroxycarbonyl)stilbene (12)

9 (0.166 g, 0.61 mmol) was used to prepare derivative **12** using the procedure described above. **12** (0.075 g, 31%) was obtained as a pale yellow solid, m.p. = 160-165 °C. ^{1}H-NMR (600 MHz, CD_3OD) δ 8.16 (d, J = 1.8 Hz, 1H, H-2'), 7.80 (dd, J = 8.4 Hz & 2.4 Hz, 1H, H-6'), 7.25 (d, J = 17.4 Hz, 1H, alkene), 7.24 (d, J = 1.8 Hz, 2H, H-2 & H-6), 7.19 (d, J = 16.8 Hz, 1H, alkene), 7.13 (d, J = 8.4 Hz, 1H, H-5'), 6.84 (t, J = 1.8 Hz, 1H, H-4), 2.29 (s, 3H, CH_3CO), 2.28 (s, 6H, 2 X CH_3CO). ^{13}C-NMR (600 MHz, DMSO-d_6) δ 169.71, 169.48, 165.98, 151.65, 150.08, 139.64, 135.08, 131.73, 130.02, 129.18, 128.27, 124.93, 124.79, 117.83, 115.70, 21.33, 21.29. Exact mass (398.10), found mass using ESI-MS m/z = 421.1 $[M+Na]^+$.

3.4 Results and discussion

3.4.1 Wittig reaction

3.4.1.1 Preparation of 3,4',5-*trans*-trimethoxystilbene (TMS)

At the beginning of the Wittig reaction, we tried to reproduce the reported procedure to synthesize the resveratrol analogue 3,4',5-*trans*-trimethoxystilbene (TMS) [17]. This reaction utilizes the two Wittig starting materials: 3,5-dimethoxybenzaldehyde and 4-methoxybenzyltriphenylphosphonium bromide. Accordingly, we initiated the synthesis of 3,5-dimethoxybenzaldehyde from the commercially available 3,5-dihydroxybenzoic acid (**Figure 3-5**). First, we esterified the carboxylic acid group using Fischer esterification by methanol and conc. sulphuric acid. Then, we wanted to mask the two phenol groups before the following step (reduction). We tried iodomethane and sodium hydroxide in water [23], iodomethane and potassium carbonate in acetonitrile [24], iodomethane and sodium hydroxide in DMSO [24], iodomethane and cesium carbonate in acetonitrile [24], and, finally, dimethylsulfate and potassium carbonate in acetone [25]. We found that the last two reactions were efficient to produce our desired methyl-protected product in good yields while the first reactions produced by-products. In an attempt to do esterification and methyl protection of the phenol group in one reaction (i.e., obtaining **1b** directly from 3,5-dihydroxybenzoic acid), we set up a reaction between 3,5-dihydroxybenzoic acid and potassium carbonate and dimethylsulfate in acetone according to previous methods [16, 26]. We noticed that these concentrated solutions (0.65 mmol reactant/mL acetone) produced a mixture of spots on TLC, which correspond to partially protected products. In contrast, as reported by others [25], diluted solutions (0.07 mmol reactant/mL acetone) produced only one spot that corresponds to the desired product, methyl-3,5-dimethoxybenzoate (**1b**).

The next step involved the reduction of **1b** to form a 3,5-dimethoxybenzyl alcohol (**1c**) (**Figure 3-5**). For that purpose, we used lithium aluminum hydride (LAH) in dry THF as previously reported [27]. This reaction produced the desired product in a good yield (90%). After that, **1c** was successfully oxidized to 3,5-dimethoxybenzaldehyde (**1d**) using pyridinium dichromate according to previous work [16]. It was reported that in a one-pot reaction, carboxylic acids can be reduced and subsequently oxidized to the corresponding aldehyde derivative using LAH and pyridinium chlorochromate [28]. We tried this approach by reacting

3,5-dimethoxybenzoic acid with LAH and pyridinium chlorochromate. However, this reaction did not proceed and we only obtained unreacted starting material (tested by TLC).

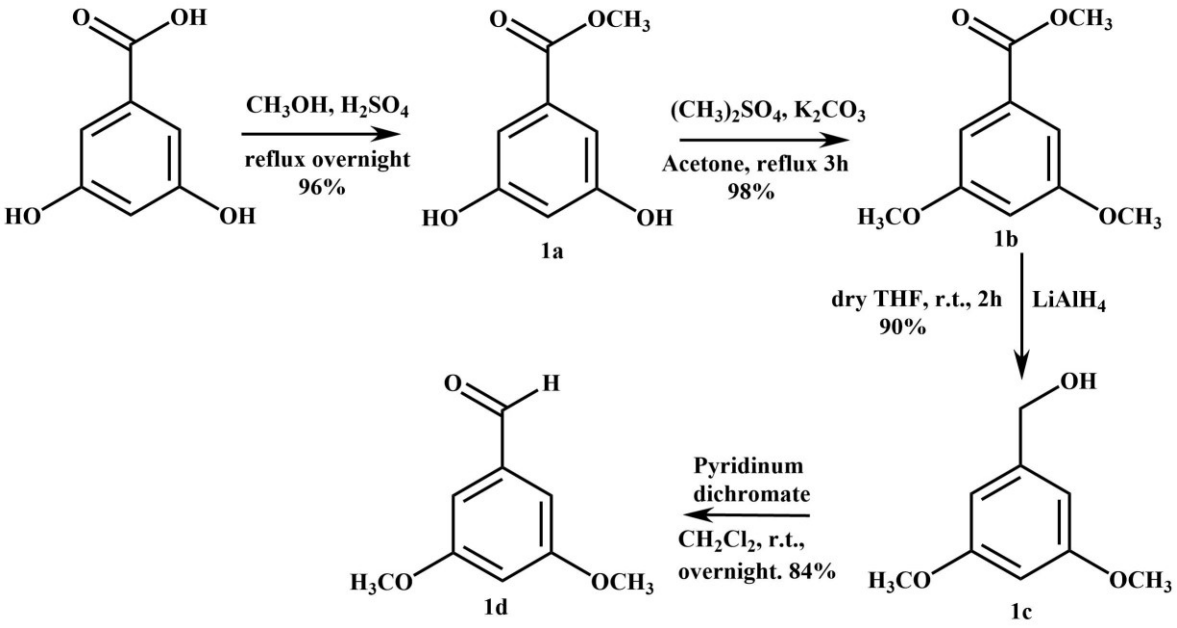


Figure 3-5: Synthesis of 3,5-dimethoxybenzaldehyde starting from commercially available 3,5-dihydroxybenzoic acid.

The other substrate for a Wittig reaction is the phosphonium salt. In the case of TMS synthesis, the salt is 4-methoxybenzyltriphenylphosphonium bromide (**1i**). The synthesis of this salt started from the commercially available 4-hydroxybenzyl alcohol that was protected using $(CH_3)_2SO_4$ and K_2CO_3 in acetone to produce 4-methoxybenzyl alcohol (**1g**), which was then brominated using phosphorus tribromide in CH_2Cl_2 to afford 4-methoxybenzylbromide (**1h**) (**Figure 3-6**) [23]. The latter was refluxed in toluene with triphenylphosphine to produce **1i** in an 80% yield.

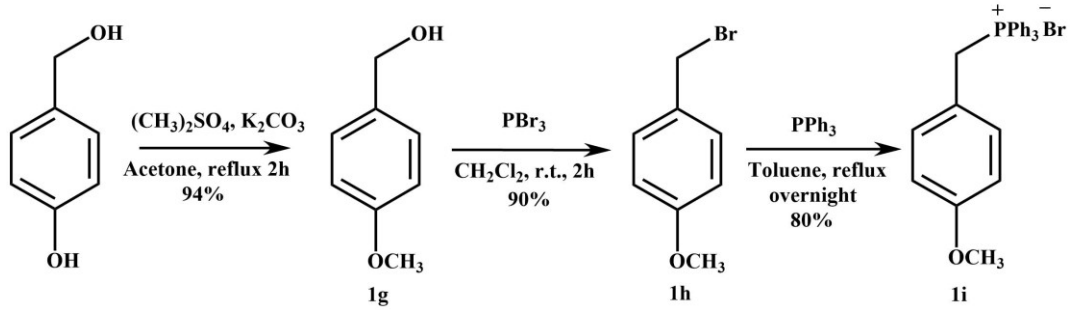


Figure 3-6: Synthesis of 4-methoxybenzyltriphenylphosphonium bromide from 4-hydroxybenzyl alcohol.

In the first attempt to obtain 3,4',5-*trans*-trimethoxystilbene (TMS), we reacted **1d** with **1i** using *n*-butyllithium in dry THF (**Figure 3-7**) [17]. This reaction produced *cis*/*trans* mixtures of TMS in about a (40:60) ratio (determined by ^{1}H NMR of the crude product). As a general trend in the literature, usually *cis* isomers of stilbenes elute first from column chromatography, followed by *trans* isomers [17]. We tried to purify the *trans* isomer using silica gel chromatography but these attempts were unsuccessful because the *cis* isomer impurity co-eluted with the *trans* isomer. We varied column sizes, decreased the flow rate and/or reduced the polarity of solvents, but the issue persisted.

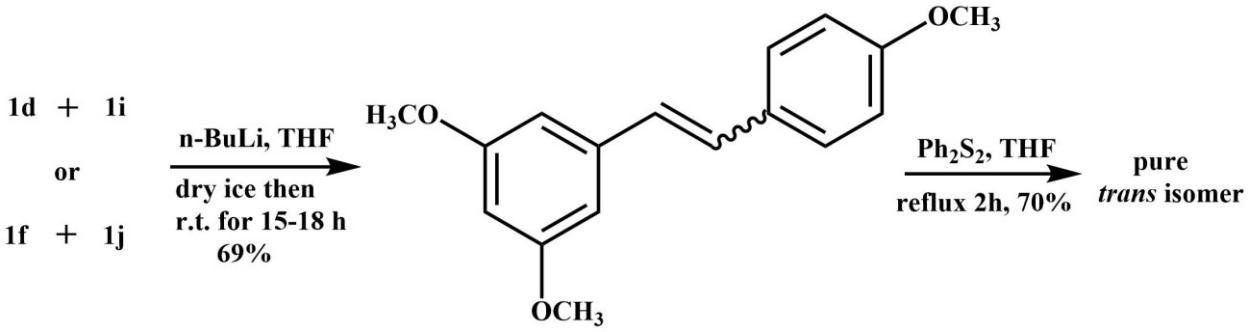


Figure 3-7: Synthesis of *trans*-TMS using either (1d and 1i) or (1f and 1j).

In addition to obtaining a mixture of isomers of TMS by reacting **1d** and **1i** using the Wittig reaction, we also changed the substitution on both substrates of the Wittig so that the aldehyde was mono-methoxylated and phosphonium salt was dimethoxylated. Accordingly, we prepared 4-methoxybenzaldehyde (**1j**) from the commercially available 4-hydroxybenzaldehyde by protecting the phenol group using $(CH_3)_2SO_4$ and K_2CO_3 in acetone (**Figure 3-8**). Furthermore, 3,5-dimethoxybenzyltriphenylphosphonium bromide (**1f**) was prepared by brominating **1c** to produce 3,5-dimethoxybenzyl bromide (**1e**), which was subsequently refluxed with triphenylphosphine in toluene to produce **1f** in 90% yield (**Figure 3-8**).

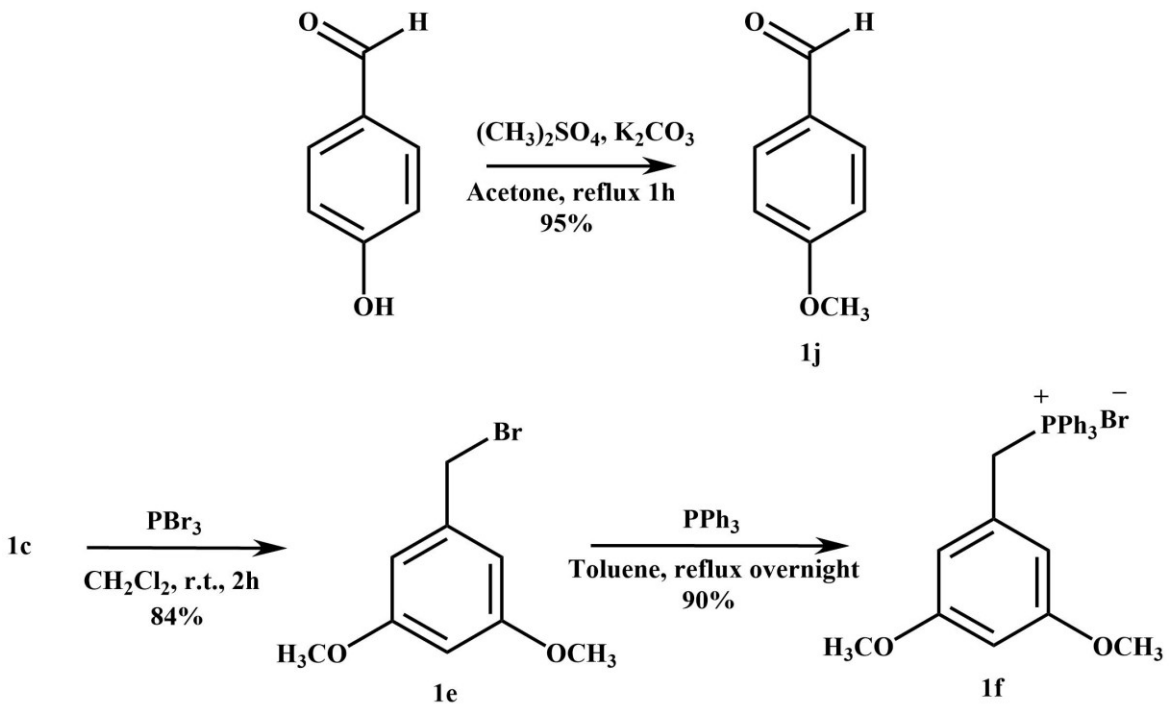


Figure 3-8: Preparation of 1j and 1f as an alternative approach for the synthesis of the isomer mixture of TMS.

We looked in the literature for possible reactions by which we could react the mixture of isomers (*cis* and *trans*) so that we would end up with only the *trans* isomer. We found that iodine in hexane [29], AlI_3 in acetonitrile [30], and diphenyl disulfide in dry THF [18] were documented methods. First, we tried iodine in hexane [29] to *trans* isomerize TMS. However, when we used this approach, the isomer ratio did not change, even after 72 hours of refluxing. Next, we tried AlI_3 in acetonitrile and this reaction produced many products (observed by TLC), but none corresponded to the *trans* isomer of TMS. We decided to move to the diphenyl disulfide approach [18]. When we performed this reaction, we were successfully able to convert the *cis*/*trans* mixture of TMS to only a *trans* isomer with about a 70% yield (**Figure 3-7**). It should be noted that this reaction has two main limitations. First, it has been reported to proceed under diluted conditions (e.g., 50 mL THF per 1.46 mmol stilbene) [18] and consequently a large quantity of stilbenes would require a high THF volume while maintaining inert/dry conditions. The second limitation is the reflux time: this reaction can take as long as 22 hours under reflux [31]. To that end, we tried to conduct the reaction using a smaller volume of THF for large quantities of stilbene mixtures. However, these isomerization attempts were unsuccessful.

3.4.1.2 Preparation of hybrid salicylate-resveratrol derivatives

After we investigated the Wittig reaction for the synthesis of *trans*-TMS, we moved to the synthesis of salicylate-resveratrol analogues. To synthesize the compounds (**3-7**), we utilized the previous two phosphonium salts (**1i** and **1f**) described in TMS synthesis as one substrate for the Wittig reaction. In the case of compounds (**3**, **4**, **6**, **7**), the aldehyde substrates were either methyl-5-formyl-2-methoxybenzoate (**2b**) or methyl-5-formyl-2-acetoxybenzoate (**2c**) (**Figure 3-9**). The synthesis of **2b** and **2c** started from the 5-formylsalicylic acid that was esterified to produce methyl-5-formyl-2-hydroxybenzoate (**2a**). Then, **2a** was either methoxylated using $(CH_3)_2SO_4$ and K_2CO_3 in acetone to produce **2b** or acetylated using acetyl chloride in the presence of triethylamine to produce **2c** (**Figure 3-9**).

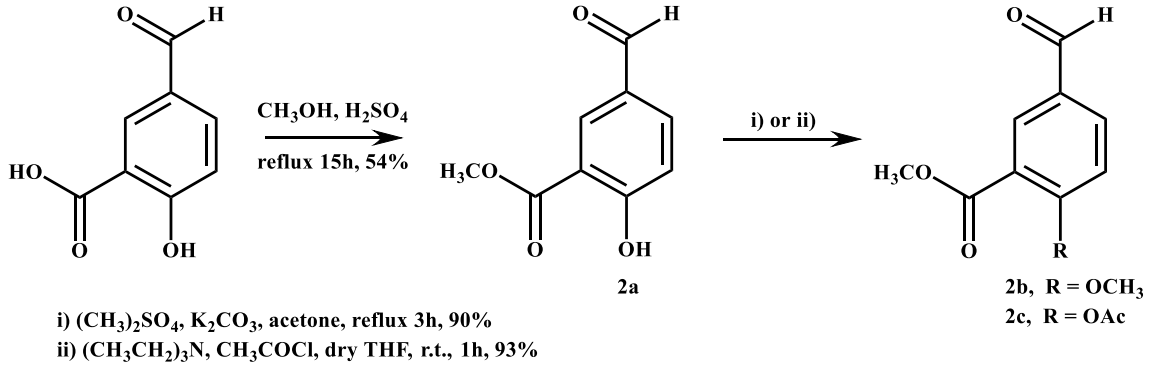


Figure 3-9: Preparation of 2b and 2c.

We used 4-hydroxy-3-methoxybenzaldehyde (vanillin) as a starting aldehyde to synthesize the aldehyde substrate for the synthesis of compound **5** (a compound that lacks carboxylic acid group at position 3'). Vanillin was acetylated using acetyl chloride and triethylamine in THF to produce 4-acetoxy-3-methoxybenzaldehyde (**2d**) (**Figure 3-10**).

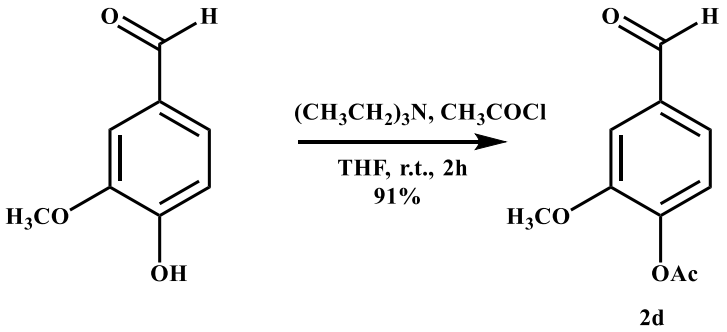
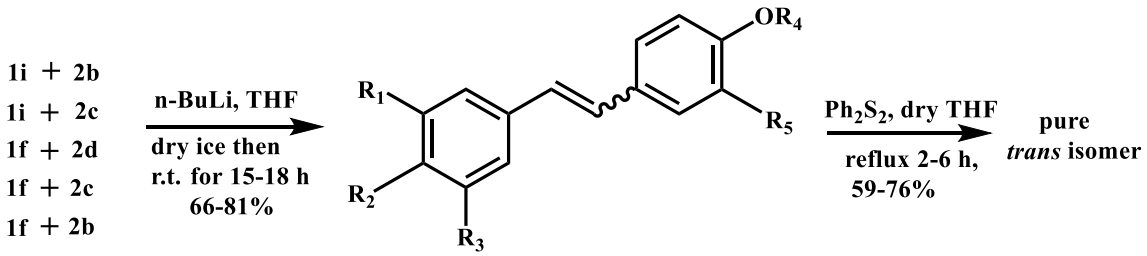


Figure 3-10: Preparation of 2d from vanillin.

As observed in the *trans*-TMS synthesis, the Wittig reactions used to produce compounds (**3-7**) yielded isomer mixtures of the corresponding hybrid derivatives of resveratrol (**Figure 3-11**). Our observation that a mixture of *E*/*Z* isomers was produced after using the phosphonium salts **1i** and **1f** is in agreement with reported observations, in which semi-stabilized ylides produced low selectivity for *E* isomers [10]. Likewise with TMS, we had to isomerize every Wittig reaction product to generate only an *E* isomer of the corresponding stilbenes.



1i + 2b ⟶ *Z*/*E* (3), R_1=R_3=H, R_2=OCH_3, R_4=CH_3, R_5=CO_2CH_3

1i + 2c ⟶ *Z*/*E* (4), R_1=R_3=H, R_2=OCH_3, R_4=Ac, R_5=CO_2CH_3

1f + 2d ⟶ *Z*/*E* (5), R_1=R_3=OCH_3, R_2=H, R_4=Ac, R_5=OCH_3

1f + 2c ⟶ *Z*/*E* (6), R_1=R_3=OCH_3, R_2=H, R_4=Ac, R_5=CO_2CH_3

1f + 2b ⟶ *Z*/*E* (7), R_1=R_3=OCH_3, R_2=H, R_4=CH_3, R_5=CO_2CH_3

Figure 3-11: Wittig reaction and subsequent isomerization to obtain compounds 3-7.

Among our proposed list of salicylate-resveratrol derivatives are compounds that possess a free carboxylic acid group (**8**, **9**, **11** and **12**). We thought of performing a Wittig reaction with a standard phosphonium bromide. However, instead of using ester aldehydes (**2b** or **2c**), we used aldehyde bearing a free carboxylic acid (2-acetoxy-5-formyl benzoic acid, **2i**) (**Figure 3-12**). When we conducted this reaction, we ended up with many products (tested by TLC) which were difficult to purify and identify. It is noteworthy that one report documented a low yield (17%) of stilbenes with free carboxylic acid after a Wittig reaction was performed between aldehyde bearing a free carboxylic acid and a phosphonium salt [32].

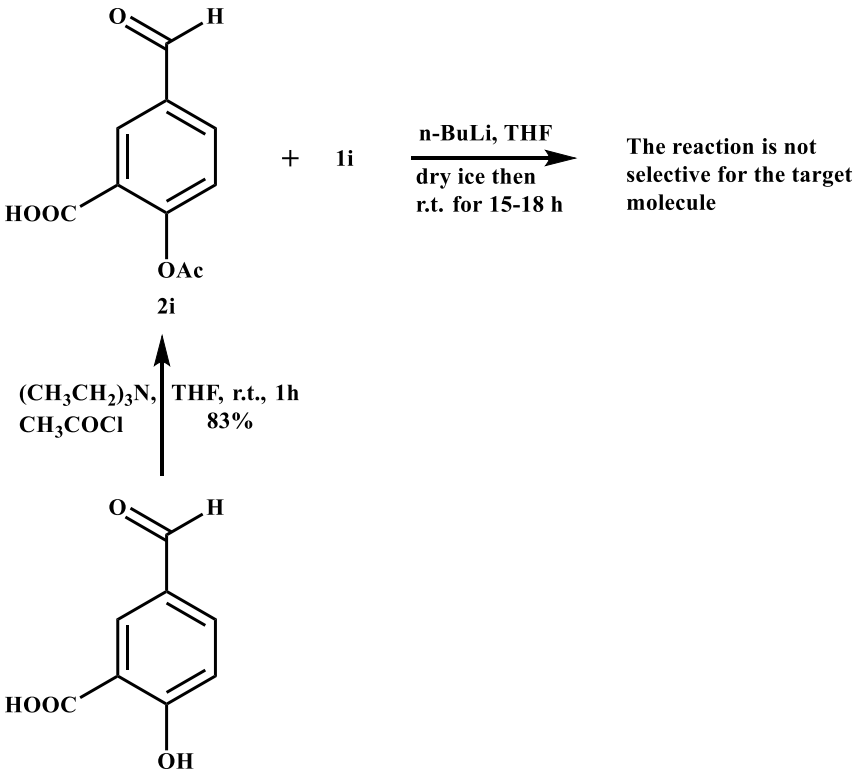


Figure 3-12: An attempt to synthesize stilbenes having a free carboxylic acid using the Wittig reaction.

3.4.2 Horner-Wadsworth-Emmons (HWE) reaction

As the HWE reaction is known for its *trans* selectivity [7], we tried this reaction for TMS as well as for salicylate-resveratrol analogues. First, we aimed to reproduce the reported work [3, 33] to synthesize a *trans*-TMS using the HWE approach. We used **1e** to prepare diethyl [3,5-di(methoxy)benzyl]phosphonate (**1k**) according to **Figure 3-13**. Then, **1k** and **1j** were reacted in the presence of *tert*-BuOK and THF at room temperature (**Figure 3-13**). We successfully obtained a *trans* isomer of TMS in a 75% yield.

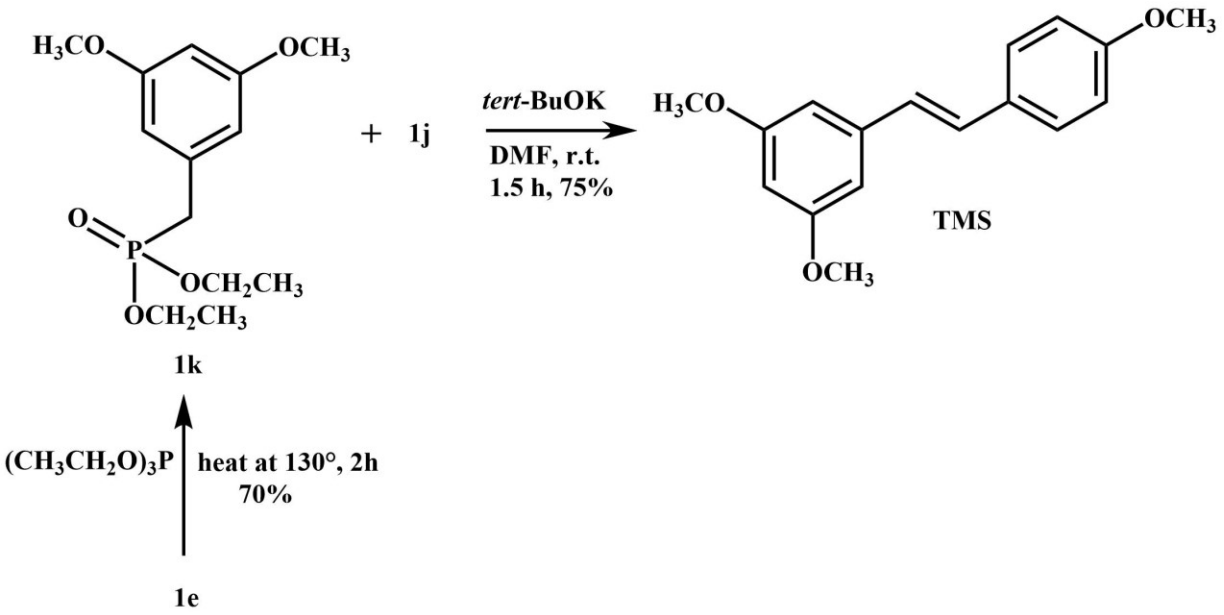


Figure 3-13: HWE reaction to obtain *trans*-TMS.

The good *trans* selectivity of TMS using the HWE reaction prompted us to use this reaction for the synthesis of resveratrol derivatives. Consequently, the phosphonate **1k** was reacted with the aldehyde **2b.** However, this reaction yielded many products (tested by TLC) in addition to the desired stilbene product, compound **7**. We were able to obtain **7** in only a 25% yield by crystallization from hexane/petroleum ether (**Figure 3-14**).

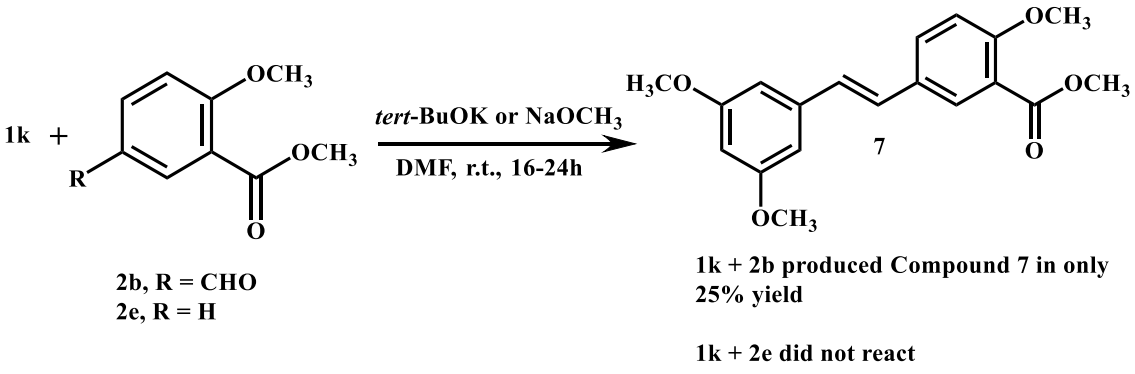


Figure 3-14: An attempt to synthesize compound 7 using HWE reaction.

The low yield of **7** using the HWE reaction, in addition to the formation of other impurities, led us to search for underlying explanations. It was observed that phosphonates can react not only with aldehydes and ketones, but also with carbonyl groups such as esters [34]. The fact that the aldehyde that we used in the HWE (**2b** in **Figure 3-14**) contains an ester group led us to suspect that the phosphonate **1k** may react at the ester group in **2b** in addition to the aldehyde functionality. To address this concern, in a different reaction, **1k** was reacted with methyl-2-methoxybenoate (**2e** in **Figure 3-14**) (a molecule which mimic **2b** but lacks the aldehyde group). When we performed this reaction, we found that the phosphonate did not react with **2e** (**2e** was intact as observed by TLC) even after being stirred for 16 hours at room temperature. Next, we changed the base from *t*-BuOK to a milder base (sodium methoxide) according to the method of Roman, BI et al. [35], which involved refluxing the reaction mixture. Using this modified approach, the same issue persisted because we obtained **7** in an even lower yield (20%).

Finally, we went back to our initial reaction (using *t*-BuOK) and re-investigated the progress of the reaction (by TLC) while adding the base gradually. The reference we followed [3] had used 2.5 Eq. of the base for the synthesis of stilbenes, and we successfully reproduced this reaction to synthesize *trans*-TMS (**Figure 3-13**). We repeated the reaction in **Figure 3-13** but by slowly adding the base (*t*-BuOK) to monitor the changes in product formation (by TLC). We started by adding only one equivalent of the base that produced a minor product of our *trans* isomer while we noticed a major unreacted starting material (tested by TLC). Adding 0.5 Eq. also increased the intensity of our product while the starting material was getting consumed. Up to 1.5 Eq., we observed that this reaction was clean and produced only our *trans* isomer of **7**. After we added another 0.25 Eq. of the base, we started to see new unknown impurities even though the starting material (aldehyde) was still not fully consumed. This observation suggests that **7** was being degraded and other new products were formed. As a result, we decided to stop using this approach and moved to the Heck coupling.

3.4.3 Heck coupling

3.4.3.1 Synthesis of *trans*-TMS

Organic chemists are increasingly looking to Heck coupling, especially to prepare resveratrol derivatives [2, 36-38]. Recent applications involved performing a Heck reaction in microwave vessels [39, 40]. We tried to explore obtaining resveratrol derivatives using this approach. First, we aimed to reproduce the reported synthesis of *trans*-TMS using Heck coupling [2]. Accordingly, we synthesized the substrates for Heck coupling, 3,5-diemthoxystyrene (**1l**) and 4-methoxyiodobenzene (**2f**), as illustrated in **Figure 3-15**.

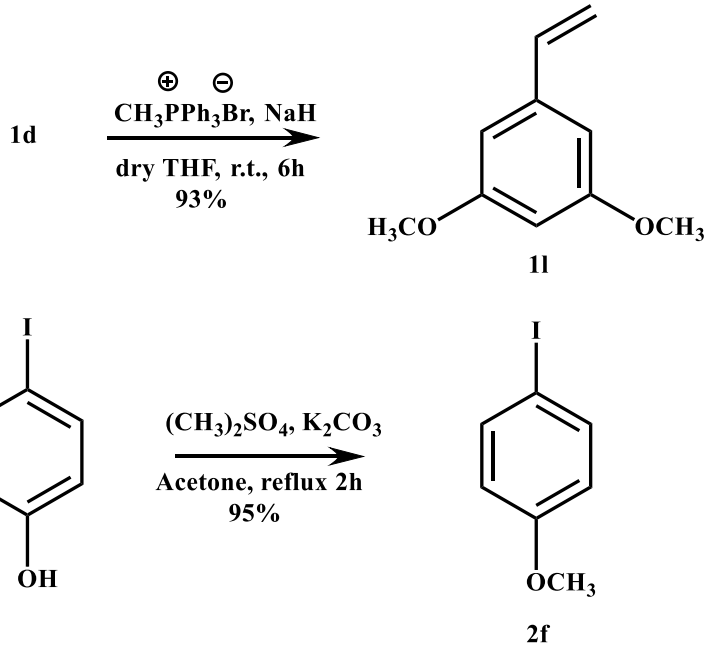


Figure 3-15: Preparation of 1l and 2f for the synthesis of *trans*-TMS using Heck coupling.

When we used the reported reagents (PPh_3, $Pd(OAc)_2$ and Et_3N in acetonitrile) of this Heck coupling [2], we were able to produce *trans*-TMS in a 48% yield after column chromatography (**Figure 3-16**).

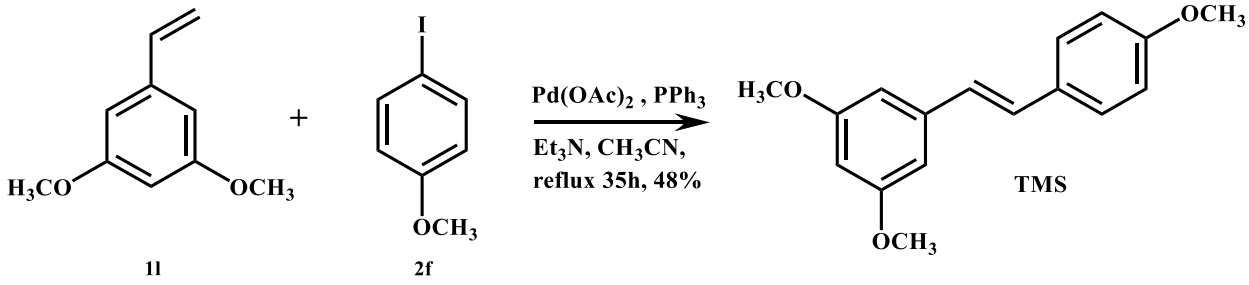


Figure 3-16: Preparation of *trans*-TMS using Heck coupling.

3.4.3.2 Synthesis of resveratrol derivative 7

In light of the reported lengthy time for a Heck reaction (it took 34 hours under traditional reflux to produce the *trans*-TMS [17]), and the advantage of a microwave instrument to proceed reactions at high temperatures (and in less time), we decided to explore the possibility of attaining resveratrol-derivative **7** using this technique. We prepared the methyl-5-iodo-2-methoxybenzoate (**2g**) from the commercially available 5-iodosalicylaic acid (**Figure 3-17**).

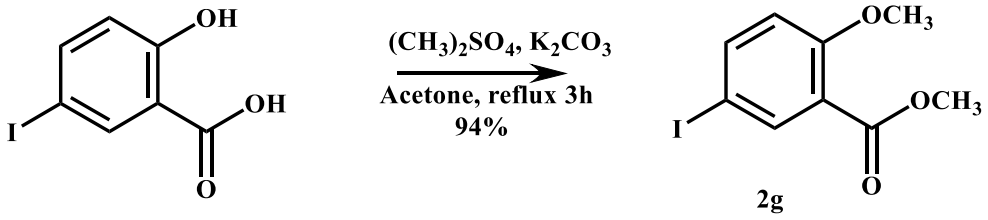
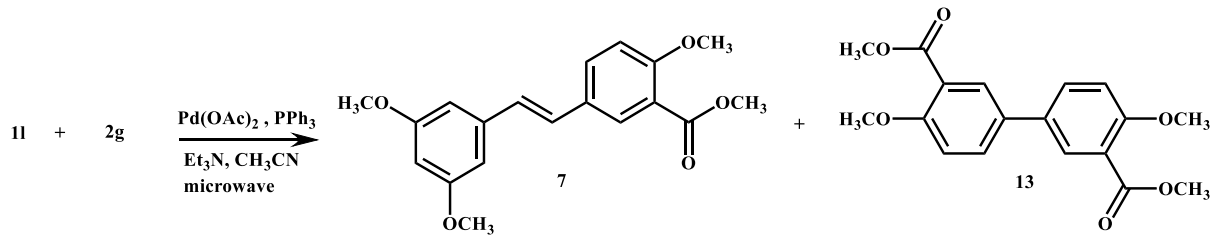


Figure 3-17: Preparation of 2g from 5-iodosalicylic acid for use in Heck coupling to produce 7.

Then, we conducted pilot experiments to optimize the microwave conditions to be used to synthesize **7**. As a general trend, we observed that a long reaction time at a lower temperature improves product formation (tested by TLC). We also found that acetonitrile is better than THF

in terms of reactant homogeneity in the reaction vessel. Based on that, we performed a set of microwave reactions based on decreasing the reaction temperature and increasing the reaction time using acetonitrile, starting at 150°C for one hour (**Figure 3-18**).



Temprature (°C)	Time (h)	Product yield[1] (%)	Isomer ratio[2] (*E*/*Z*)
150	1	23	69:31
140	4	27	ND[3]
130	8	30	ND[3]
120	12	37	70:30
110	16	46	ND[3]
105	20	50	ND[3]
90	24	55	71:29

Figure 3-18: Synthesis of compound 7 using Heck coupling. [1]The yield of the *E* isomer after chromatography and crystallization from petroleum ether/ethyl acetate. [2]Determined by ^{1}H-NMR of the crude product. [3]Not determined.

For the synthesis of **7** using a Heck reaction under microwave heating, we could see that the isomer ratio did not change significantly at high, medium or low temperatures because the selectivity for the *E* isomer remains favorable over that of the *Z* isomer (about 70:30). This finding is in agreement with the stereoselectivity of the Heck reaction [15]. However, regarding the *trans* isomer yield at these various temperatures/times we noticed that lower yields were obtained at high temperatures while the yield increased when the temperature decreased and the reaction time increased (**Figure 3-18**). We tried to find an explanation, and we noticed that along with stilbene **7**, a by-product was formed that was enhanced at high temperatures (TLC tested). After comparing the melting point, ^{1}H NMR, mass spectra, and ^{13}C NMR with a reference [21], we identified that by-product as 3,3'-dicarbomethoxy-4,4'-dimethoxybiphenyl (**13**).

Our Heck reactions in the microwave to obtain **7** revealed that the maximum yield we could obtain was about 55% when we conducted the reaction at 90°C for 24 hours (**Figure 3-18**). We decided to repeat the reaction again but using traditional reflux instead of the microwave system (so the reaction temperature would decrease and hence the yield might be increased). Similarly, when we performed a Heck coupling under regular reflux for 24 hours, we produced **7** with a 54% yield. Additionally, the by-product (**13**) was produced by Heck coupling using conventional reflux. Synthesizing **7** using a Heck coupling improved the yield in only a one-step reaction. This contrasted with the Wittig reaction, which generated a 41 % yield for **7** in a two-step reaction (Wittig followed by *trans*-isomerization).

3.4.3.3 Attempts to produce resveratrol derivative 8 using a Heck reaction

Resveratrol-salicylate analogue **8** contains free phenol groups. Since the Heck coupling was previously utilized to produce stilbenes with free phenols [2] even at low yields, we investigated using this reaction to generate our resveratrol analogues possessing free phenols. Consequently, we prepared 4-hydroxystyrene (**1m**) and methyl-2-hydroxy-5-iodobenzoate (**2h**) according to **Figure 3-19**. We then conducted a Heck reaction as we had for compound **7**. We obtained *E*/*Z* isomer mixtures of **8** in an 18 % yield. We tried to purify the *trans* isomer from the *cis* isomer, but our attempt was unsuccessful. Next, we tried *trans*-isomerization using Ph_2S_2 as we had in the case of the Wittig reaction products. However, we were unable to convert the isomer mixtures completely to only *trans* isomers. We observed a slight change in the isomer ratio upon refluxing with Ph_2S_2 (tested by TLC), but no complete *trans*-isomerization occurred. It was proposed that electron withdrawing groups on stilbenes reduce the *trans*-isomerization process using Ph_2S_2 and often need prolonged heating (for example 10 hours) [41]. In such cases, 4,4'-dimethoxydiphenyldisulfide has been proven to accelerate and favors *trans*-isomerization in a shorter time (for example 4 hours) [41]. Consequently, we used 4,4'-dimethoxydiphenyldisulfide instead of Ph_2S_2 for the *trans*-isomerization of *E*/*Z* mixtures of stilbene **8**. However, we noted a minimal change in the *E*/*Z* isomer ratio after this reaction, but no complete *trans*-isomerization occurred.

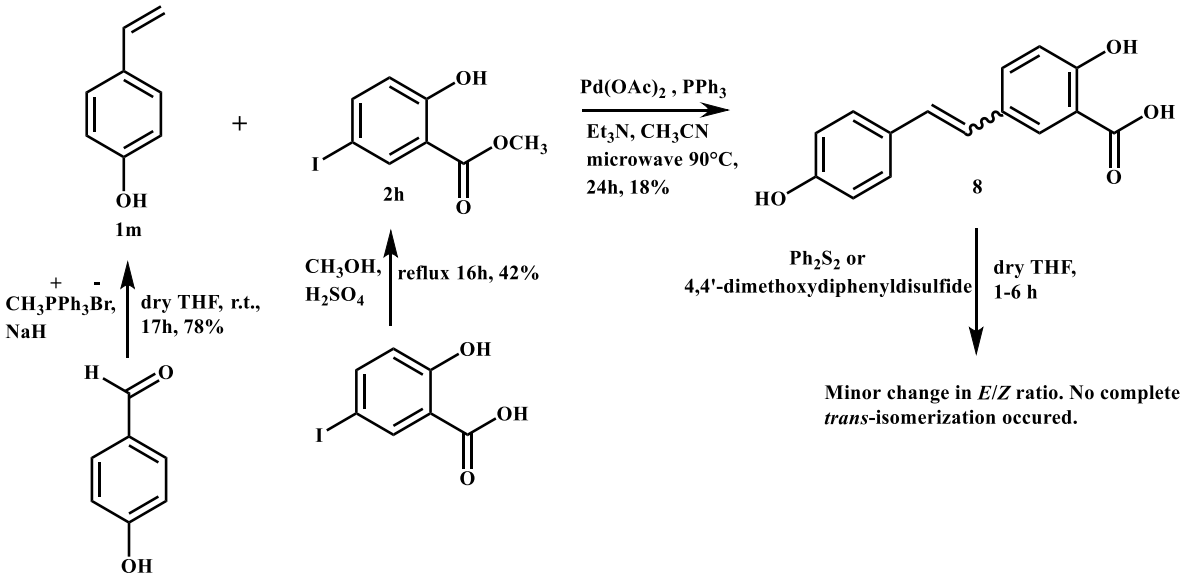


Figure 3-19: An attempt to synthesize 8 using Heck coupling.

3.4.4 Demethylation reactions to produce resveratrol and derivatives 8-10

After we obtained compounds **3-7** (which are protected by either methyl or acetyl groups), we moved to the deprotection step to produce stilbenes with free phenol groups. In particular, the Heck reaction to produce pure *trans*-stilbenes with free phenols did not work according to our settings. We looked into the literature for methyl deprotection strategies, and found that boron tribromide (BBr_3) is a common reagent [17-19]. We started the demethylation reaction by trying to reproduce the reported deprotection reactions of *trans*-TMS to produce resveratrol [17, 19]. Using a BBr_3 solution in CH_2Cl_2, the demethylation of TMS afforded a 67% yield of *trans*-resveratrol (**Figure 3-20**). We noticed that resveratrol is very unstable: discoloration of this product appears instantly, even when it is stored at -20°C covered with foil. Resveratrol's low stability has already been reported [42-44]. For that reason, we purchased resveratrol from commercial sources, and used it in the biological studies discussed in this project.

Demethylation reactions to obtain resveratrol derivatives **8-10** were conducted using compounds **3** and **7** as starting materials. We used the reported standard conditions for performing such reactions [17, 19], which involved cooling the reaction down to about -60°C while adding BBr_3 to the reaction flask, followed by warming the reaction to room temperature (**Figure 3-20**). We used around 2.0 Eq. of BBr_3 per each OCH_3 group and we obtained compounds **8** and **9** from **3** and **7** respectively. Our lab and others have found that masking carboxylic acid functionality in some NSAIDs decreases ulcer side effects while maintaining the drugs' anti-inflammatory properties [45, 46]. Accordingly, we aimed to reduce the equivalence of BBr_3 during derivative 7 demethylation so that the carboxylate methyl ester group would be intact in the produced free phenol-containing stilbene. Indeed, using about 4.5 Eq. of BBr_3 produced derivative **10,** which retained the methyl ester moiety. During the demethylation reaction using BBr_3, we did not observe any transformation in the starting *trans*-isomer (i.e. no *trans*→*cis* isomerization observed by TLC).

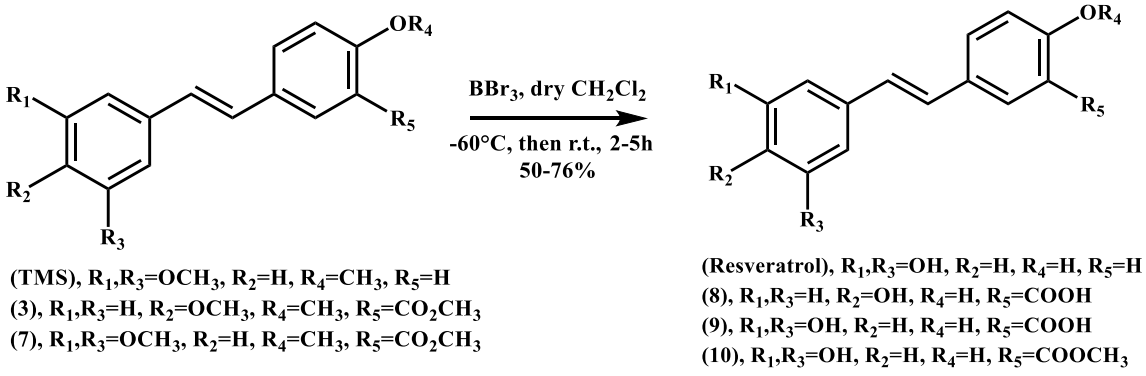
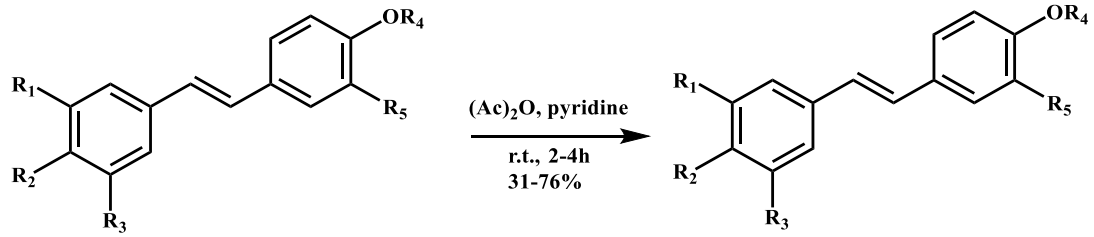


Figure 3-20: Demethylation reactions to produce free phenol derivatives including resveratrol.

3.4.5 Acetylation reactions to produce derivatives 11 and 12

The final reaction pathway involved the acetylation of derivatives **8** and **9** to produce compounds **11** and **12** respectively. These acetylated derivatives are thought to possess an aspirin-like scaffold, as they both have an acetylsalicylic acid structure on one stilbene ring (**Figure 1-4**). To prepare these acetylated derivatives, we initially tried the acetylation of **8** using acetyl chloride

and triethylamine in dry THF. In addition to some impurities, this reaction produced major product(s) which showed isomers trend (two closely separated spots on TLC where they were not well-separated using low, medium or high polar solvent systems). After we noted these products, we predicted that this reaction likely alters the *trans*-isomer configuration of the products and/or the starting materials. Then, we tried the acetylation using the reported method for resveratrol acetylation [47] which utilized acetic anhydride and pyridine. We used this approach for acetylating **8** and **9** to produce compounds **11** and **12** respectively (**Figure 3-21**).



(8), R_1,R_3=H, R_2=OH, R_4=H, R_5=COOH
(9), R_1,R_3=OH, R_2=H, R_4=H, R_5=COOH

(11), R_1,R_3=H, R_2=OAc, R_4=Ac, R_5=COOH
(12), R_1,R_3=OAc, R_2=H, R_4=Ac, R_5=COOH

Figure 3-21: Acetylation reaction of 8 and 9 to obtain compounds 11 and 12.

3.5 Summary and conclusions

A new class of resveratrol derivatives possessing a carboxylic acid group was synthesized based on Wittig, HWE and Heck reactions. For methoxylated stilbenes, the Heck and Wittig reactions produced the desired analogues but in different *trans*/*cis* isomer ratios: the Wittig produced mixtures of isomers and the Heck produced mainly *trans* isomers. Diphenyl disulfide emerged as an efficient catalyst to transform isomer mixtures to only *E*-isomers, a phenomenon that was true for methoxylated analogues but not for their hydroxylated counterparts. The Heck coupling under microwave irradiation appeared to be not the ideal choice for the synthesis of these resveratrol-salicylate derivatives because it produced by-products at high temperatures. However, the Heck coupling using microwaves at low temperatures, as well as under regular reflux, produced both methoxylated and hydroxylated isomers of target stilbenes. The Heck reaction also produced an enhanced yield compared to the Wittig for the synthesis of derivative **7**

(55% versus 41% respectively). Despite being effective at synthesizing *trans*-TMS, the HWE reaction appeared problematic and/or resulted in low yields for the synthesis of methoxylated resveratrol derivatives possessing a carboxylate function group. Further modifications to this synthetic approach may provide a greater understanding and help to solve the selectivity, making it possible to produce target molecules. Of note, demethylation (using BBr_3) and acetylation (using $(Ac)_2O$) reactions appeared to produce the lowest yield (average of 58%) among all the reactions for the synthesis of resveratrol-salicylate analogues. Further development of these reaction pathways may improve the overall yield of these analogues.

3.6 References

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Chapter 4: Inhibition of CYP1A1 enzyme

This chapter is published as **Fahad S. Aldawsari**, Osama H. Elshenawy, Mohamed A. M. El Gendy, Rodrigo Aguayo-Ortiz, Shairaz Baksh, Ayman O. S. El-Kadi, and Carlos A. Velázquez-Martínez. "Design and Synthesis of Resveratrol-Salicylate Hybrid Derivatives as CYP1A1 Inhibitors". *J. Enzyme Inhib. Med. Chem.* **2015**, 30(6): 884-895. I performed the cell culture treatment and I wrote the manuscript. Elshenawy, OH performed the mRNA experiment, El Gendy, MA conducted the EROD assay, and Aguayo-Ortiz, R conducted the docking study inside the CYP1A1 active site. All of the co-authors contributed to manuscript edits. Velázquez-Martínez, CA was the supervisory author and was involved in the manuscript organization.

4.1 Introduction

Cytochrome P450 (CYP) is a large family of constitutive and inducible enzymes associated with the metabolic transformation of endogenous molecules, as well as external xenobiotic substrates [1]. CYP450 plays a significant role in carrying out phase I metabolism reactions, which include (but are not limited to) oxidative dehalogenation, oxidative cleavage, hydroxylation, epoxidation, dealkylation, and alcohol/aldehyde oxidation [2]. However, aside from the physiological detoxification mechanism assisted by CYP enzymes, CYPs are also involved in activating procarcinogenic compounds into carcinogenic agents [1]. The overexpression of different CYP enzymes in cancer tissues has been found to be a crucial step in cancer progression [3].

The CYP1A1 enzyme is a member of the CYP1 family, which is mainly expressed in extra hepatic tissues such as the lung, skeletal muscle, and thyroid gland [4]. CYP1A1 is one of the main CYP enzymes involved in the metabolic activation of several environmental contaminants, such as polycyclic aromatic hydrocarbons (PAH) [5]. CYP1A1 is primarily regulated by the aryl hydrocarbon receptor (AhR) pathway. Its carcinogenic potential involving the bio-activation of several polyaromatic hydrocarbons (PAH) has been well documented [6]. Furthermore, CYP1A1 expression (transcriptional level) is significantly higher in cancer than in normal tissues [3], which makes the CYP1A1 isozyme a potential drug target. In this regard, numerous efforts have been made to develop novel CYP1A1 inhibitors [7].

There is a significant body of evidence supporting the potential chemopreventive properties of natural polyphenols and NSAIDs, both *in vitro* and *in vivo*. This favorable chemopreventive profile is attributed to the ability of these two classes of compounds to modulate, simultaneously, several cell targets linked to cancer initiation and cancer progression, such as cyclooxygenase (COX)-2, NF-κB, iNOS, 5-LOX, VEGF, and CYP450 enzymes, among many others [8]. Some of these proteins are targeted by both NSAIDs (particularly aspirin) and polyphenols (specifically resveratrol), and some proteins are targeted by only one type of drug. Consequently, *the idea of forming a hybrid molecule combining the pharmacological profiles of aspirin and resveratrol is promising*. It is clear that resveratrol (3,4',5-trihydroxystilbene) is a naturally occurring polyphenol capable of exerting significant *in vitro* inhibition of the CYP1A1 enzyme [9, 10], but the data on aspirin (and salicylates in general) is still controversial. For example, salicylic acid only shows CYP1A1 inhibition at relatively high concentrations (≥ 2.5 mM) when

tested in MCF-7 cells [11], while other reports have shown that neither aspirin nor salicylic acid significantly inhibit CYP1A1 activity [12]. Adding a salicylate moiety to resveratrol's chemical structure would suggest a significant decrease in this polyphenol's ability to inhibit CYP1A1's enzymatic activity. Nevertheless, preliminary results that our group generated with a molecular modeling (docking) study using the recently reported crystal structure of human CYP1A1 (described in the experimental section), seem to suggest that it might be possible for the new hybrid molecules to inhibit this enzyme. Encouraged by these *in silico* (preliminary) results, we decided to assess the ability of hybrid resveratrol-salicylate derivatives (synthesized in Chapter 3) to inhibit CYP1A1 catalytic activity.

As part of interdisciplinary research work aimed at developing new anticancer/chemopreventive agents, we report in this chapter the effects of adding a salicylate (or an acetylsalicylate) moiety on resveratrol's ability to modulate CYP1A1's activity and expression. We tested hybrid resveratrol derivatives using the 7-ethoxyresorufin-O-deethylation (EROD) assay to measure the catalytic activity of CYP1A1 in the presence of each test drug. The results obtained in this experiment showed different degrees of *in vitro* modulation of enzyme activity: some compounds inhibited CYP1A1, while others increased it. Among the group of inhibitors, we identified compound **3** as the most promising resveratrol analogue, as it showed a suitable inhibitory profile by reducing 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-mediated induction of CYP1A1 catalytic activity in HepG2 cells. We also observed that this effect was associated with a reduction of TCDD-mediated induction of CYP1A1 mRNA levels. We submit that compound **3** is a novel hybrid resveratrol-salicylate derivative with promising modulatory effects on CYP1A1, and that it could protect against CYP1A1 activation of environmental carcinogens.

4.2 Materials and methods

4.2.1 Cell culture

Human hepatoma HepG2 cells (ATCC HB-8065, Manassas, VA) and human colon adenocarcinoma HT-29 cells (ATCC HTB-38, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% heat-inactivated fetal bovine serum, 2 mM *l*-

glutamine, 100 IU/mL penicillin and 100 µg/mL streptomycin. Cells were grown in 75-cm^2 tissue culture flasks at 37°C in a 5% CO_2 humidified incubator.

4.2.2 Determination of CYP1A1 enzymatic activity (24 h incubation)

The CYP1A1-dependent 7-ethoxyresorufin O-deethylase (EROD) activity was performed on intact living cells, using 7-ethoxyresorufin (7ER) as a substrate, following a previously reported procedure [13]. Briefly, cells (10^4 cells per well) were seeded onto 96-well microtiter cell culture plates until they reached 70-80% confluency. Resveratrol (or its analogues) dissolved in DMSO was added to the cells (5 µM final concentration) before TCDD addition (1 nM final concentration) and the cells were incubated for 24 h. After that, the cells were washed with PBS, and 200 µL of 7ER solution (2 µM) were added to each well. The amount of resorufin formed in each well at each time-point (every 5 min) was detected by fluorescence spectroscopy for each treatment per minute by comparison with a standard curve of known concentrations (excitation, 545 nm; emission, 575 nm; Baxter 96-well fluorometer) [14]. The CYP1A1 enzymatic activity was normalized to cellular protein content using a modified fluorescence method [15]. Results are presented as the mean ± SEM, and statistical differences between treatment groups were determined using one-way ANOVA followed by a Student-Newman-Keuls post-hoc test, using the SigmaStat 3.5 program for Windows, Systat Software Inc. (San Jose, CA).

4.2.3 Determination of CYP1A1 enzymatic activity (1 h incubation)

To test the direct inhibitory effect of resveratrol and its analogues on the CYP1A1 enzyme, a method similar to an EROD assay was performed, with a slight modification as described previously [16, 17]. Briefly, HepG2 cells were incubated with TCDD (1 nM) for 24 h. The media was then removed, and the cells were washed three times with PBS. One mM of resveratrol or its analogues in an assay buffer [Tris (0.05 M), NaCl (0.1 M), pH 7.8] was added to the cells for 60 min before 7ER (2 µM final concentration) was added as a substrate for the EROD measurement. The CYP1A1 enzymatic activity was normalized to cellular protein content using a modified fluorescence method [15]. Results are presented as mean ± SEM. Statistical differences between treatment groups were determined using one-way ANOVA, followed by a Student-Newman-Keuls post-hoc test using the SigmaStat 3.5 program for Windows, Systat Software Inc. (San Jose, CA).

4.2.4 CYP1A1 mRNA expressions

4.2.4.1 RNA extraction and cDNA synthesis

Six hours after being incubated with the test compounds, cells were collected and total RNA was isolated using a TRIzol reagent (Invitrogen) according to the manufacturer's instructions, and quantified by measuring the absorbance at 260nm. After that, first-strand cDNA synthesis was performed using the High-Capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. Briefly, 1.5 μg of total RNA from each sample were added to a mix of a 2.0 μl of 10 x reverse transcription (RT) buffer, 0.8 μl of 25 x dNTP mix (100mM), 2.0 μl of 10 x RT random primers, 1.0 μl of MultiScribe reverse transcriptase, and 4.2 μl of nuclease-free water. The final reaction mix was kept at 25 °C for 10 min, heated to 37 °C for 120 min, heated to 85 °C for 5 min, and finally cooled to 4 °C.

4.2.4.2 Quantification by real-time PCR

Quantitative analysis of CYP1A1 mRNA expression was performed by real time PCR by subjecting the resulting cDNA to PCR amplification using 96-well optical reaction plates in the ABI Prism 7500 system (Applied Biosystems). Twenty-five-microliter reactions contained 0.1 μl of 10 μM forward primer and 0.1 μl of 10 μM reverse primer (40nM final concentration of each primer), 12.5 μl of SYBR Green Universal Master mix, 11.05 μl of nuclease-free water, and 1.25 μl of a cDNA sample (equivalent to 0.20 ng/mL). The primers used in the current study were chosen from a previous study [18]; human *CYP1A1*: forward primer 5'-CTATCTGGGCTGTGGGCAA-3', reverse primer 5'-CTGGCTCAAGCACAACTTGG-3' and human *β-actin*: forward primer 5'-CTGGCACCCAGCACAATG-3', reverse primer 5'-GCCGATCCACACGGAGTACT-3'. Assay controls were incorporated into the same plate: namely, no-template controls to test for the contamination of any assay reagents. After the plate was sealed with an optical adhesive cover, the thermocycling conditions were initiated at 95 °C for 10 min, followed by 40 PCR cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. A melting curve (dissociation stage) was performed by the end of each cycle to ascertain the specificity of the primers and the purity of the final PCR product.

4.2.4.3 Real-time PCR data analysis

The real-time PCR data were analyzed using relative gene expression, i.e., the $\Delta\Delta C_T$ method, as described in Applied Biosystems *User Bulletin No.2* and explained further by Livak and Schmittgen [19]. In brief, the primers used in this study were tested to avoid primer dimers, self-priming formation, or non-specific amplification. To ensure the quality of the measurements, each plate included, for each gene, a negative control and a positive control. For each sample, a threshold cycle (CT) was calculated based on the time (measured by the number of PCR cycles) at which the reporter fluorescence emission increased beyond a threshold level (based on the background fluorescence of the system). The triplicate measurements for each sample were averaged to give an average C_T value for each group, after removing the outliers [20]. The samples were diluted in such a manner that the C_T value was observed between 15 and 30 cycles. Results were expressed using the comparative C_T method as described in *User Bulletin No.2* (Applied Biosystems). Briefly, the ΔC_T values were calculated in every sample for each gene of interest as $C_{T\ \text{gene of interest}} - C_{T\ \text{reporter gene}}$, with β-actin as the reporter gene. The calculation of relative changes in the expression level of one specific gene ($\Delta\Delta C_T$) was performed by subtracting the ΔC_T of the control (untreated cells) from the ΔC_T of the corresponding treatment groups. The values and ranges given in the **Figure 4-3** were determined as follows: $2^{-\Delta\Delta CT}$ with $\Delta\Delta C_T$ + SE and $\Delta\Delta C_T$ - SE, where SE is the standard error of the mean of the $\Delta\Delta C_T$ value (*User BulletinNo.2*; Applied Biosystems).

Data are presented as the mean ± SEM. Control and treatment measurements were compared using a one-way ANOVA followed by a Student-Newman-Keuls post-hoc test, using the SigmaStat 3.5 program for Windows, Systat Software Inc. (San Jose, CA).

4.2.5 Molecular Modeling

Protein. The crystal structure of the human CYP1A1 (PDB ID: 4I8V) [21] was downloaded from the Protein Data Bank (PDB) [22]. The chains B and C, crystallographic water molecules, and 2-phenyl-4*H*-benzo[*H*]chromen-4-one (inhibitor co-crystalized with the protein) were removed manually. Subsequently, all hydrogens and electrostatic charges were added, and protonation sites were corrected. The atomic partial charges of the heme group were computed using the Gasteiger charge method [23]. Finally, the structure was submitted to a geometry optimization

with 500 steepest descent steps, and 100 conjugate gradient steps, using the AMBER99SB force field implemented in UCSF Chimera 1.9 [24].

Ligands. The compounds **3** to **12**, TMS and resveratrol were constructed and submitted to a geometry optimization using the AMBER99SB force field in UCSF Chimera 1.9.

Docking. Docking calculations were carried out using AutoDock 4.2 software [25]. A grid box of 70 x 70 x 70 points with a grid spacing of 0.375 Å, and centered on the heme group was used to calculate the atom types needed for the calculation. The Lamarckian genetic algorithm was used as a search method with a total of 30 runs (maximum of 20,000,000 energy evaluations; 27,000 generations; initial populations of 150 conformers). The best binding mode of each molecule was selected based on both the lowest binding free energy, and the largest cluster size.

4.3 Results and discussion

4.3.1 Determination of CYP1A1 enzymatic activity

We measured the induction of the CYP1A1 enzyme by TCDD using HepG2 and HT-29 cells; this model has been extensively used to study the effects of resveratrol on CYP1A1 activity and expression [26]. In this regard, Beedanagari et al. reported that TCDD induced the expression of CYP1A1 mRNA in HepG2 cells, but not CYP1B1 [26]. We selected the EROD assay to test the ability of compounds **3-12** to inhibit the catalytic activity of the CYP1A1 enzyme, but first, we conducted a concentration-dependent cell viability assay (MTT assay) to determine the maximum concentration at which enzymatic inhibition occurred without causing significant cell death. Based on this assay, we determined that the final concentration for subsequent screening assays with compounds **3-12** was 5 μM, at which we consistently observed cell viabilities higher than 80% (data not shown).

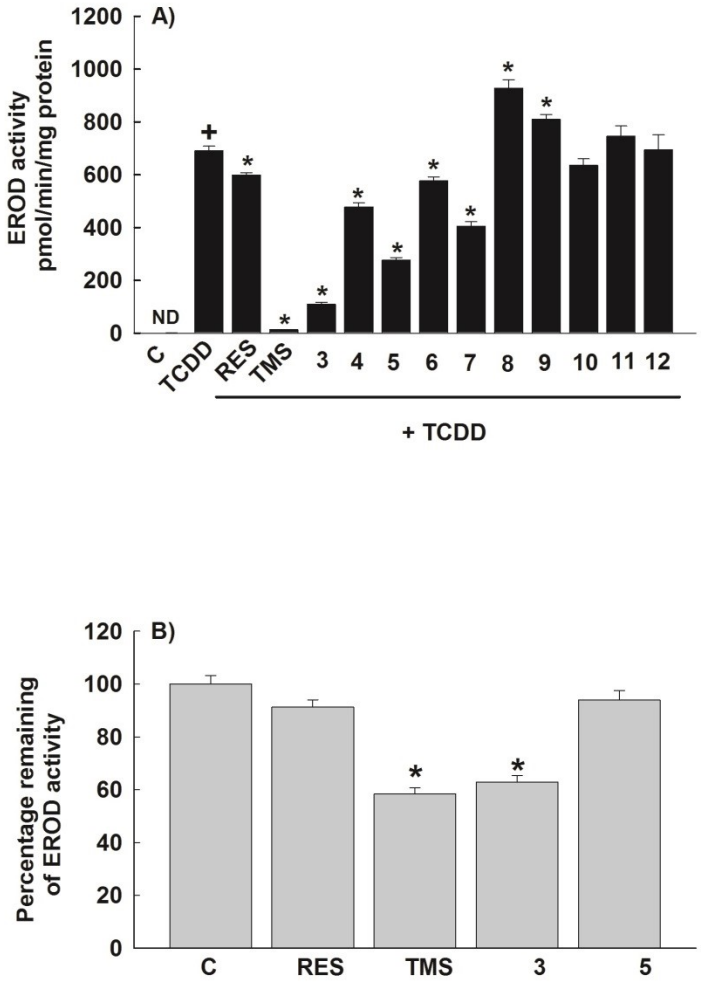

Figure 4-1: Effect of resveratrol analogues on TCDD-mediated induction of CYP1A1 catalytic activity in HepG2 cells. (A) Cells were pre-incubated with 5 µM of resveratrol (Res) or its analogues for 30 min before TCDD (1nM) was added for an additional 24 h. The CYP1A1 activity level was determined using a CYP1A1-dependent EROD assay. Values represent mean activity ± SEM. (n = 8). (+) $P < 0.05$ compared to the control (C), (*) $P< 0.05$ compared to TCDD. (B) The direct inhibitory effects of resveratrol and its analogues on the CYP1A1 enzyme. HepG2 cells were pre-treated with TCDD (1nM) for 24 h, thereafter, media were removed, washed three times with PBS, and 5 µM of the tested compounds in an assay buffer [Tris (0.05 M), NaCl (0.1 M), pH 7.8] were added for 60 min before of 7ER (2 µM final concentration) was added for the the EROD measurement. Results are expressed as a percentage of the remaining EROD activity (mean ± SEM, n = 8). (*) $P< 0.05$ compared with the control (C). ND: not detected.

4.3.2 CYP1A1 catalytic inhibition (24 h incubation) in HepG2 cells

As expected, we did not observe any CYP1A1 activity in DMSO-treated cells (negative control), whereas cells treated with TCDD (positive control) showed a significantly higher enzyme activity, which was about 700 pmol/min/mg protein, indicating TCDD's ability to induce the expression and activity of this enzyme in HepG2 cells (**Figure 4-1, A**). In the same experiment, resveratrol and its methylated derivative, trimethoxystilbene (TMS), inhibited TCDD-mediated induction of CYP1A1 by 15% and 98%, respectively. This dramatic difference between the hydroxylated and methoxylated stilbenes is likely due to TMS's enhanced lipophilicity and improved cell membrane permeability. This is consistent with previous reports which have demonstrated that replacing hydroxyl groups with methoxy groups in resveratrol's structure increased inhibitory potency on CYP1A1 catalytic activity, as well as on other CYP450 enzymes [27, 28].

Figure 4-1, A also shows that there are four hybrid resveratrol-salicylate compounds that significantly decreased TCDD-induced CYP1A1 activity, namely, compounds **3**, **4**, **6**, and **7** which inhibited CYP1A1 activity by 84%, 29%, 20%, and 43% respectively. These derivatives share two common structural features: first, they possess a methyl ester at the carboxyl group at position 3'- ; second, they have either a methoxy group at position 4-, or two methoxy groups at positions 3- and 5-. These observations are consistent with previous reports in which it was observed that methoxylation of the stilbene scaffold resulted in an enhanced CYP1A1 inhibition [27, 29]. Additional structure-activity relationship (SAR) analysis shows that hybrid compounds with an acetoxy group at position 4'- (compounds **4** and **6**), are weaker CYP1A1 inhibitors than those with methoxy groups (compounds **3** and **7**) at the same position. Apparently, the acetyl group decreases the ability of these stilbenes to inhibit CYP1A1's catalytic activity.

Additional data about the effect of acetylation on the inhibitory activity exerted by stilbenes is provided by the results obtained with derivative **5**, which is structurally similar to 3,3',4,5'-tetramethoxystilbene, a synthetic analogue of piceatannol (CYP1A1 IC_{50} = 750 nM [30]). Compound **5** showed about a 60% inhibition of CYP1A1 (**Figure 4-1, A**). The structural difference between this molecule and 3,3',4,5'-tetramethoxystilbene (not tested) is the presence of an acetoxy group at position 4'- instead of a methoxy group. This supports the observation that acetoxy groups decrease the CYP1A1 inhibitory activity exerted by methoxystilbenes.

Compounds with a free carboxylic acid (**8**, **9**, **11** and **12**) did not inhibit CYP1A1 activity at all (**Figure 4-1, A**). These compounds possess either a salicylic acid or an acetylsalicylic acid (aspirin) moiety. In fact, we observed that compounds **8** and **9** produced a slight increase in CYP1A1 activity as measured by the EROD assay. These findings seem to be consistent with previous reports showing that both aspirin and salicylic acid failed to decrease EROD levels even when tested at concentrations as high as 1 mM in HepG2 cells [31]. Consequently, we observed that stilbenes acted as modulators of CYP1A1 activity; in other words, depending on the chemical structure, some of the stilbenes inhibited CYP1A1 activity while others increased it. From a therapeutic point of view, an increased CYP1A1 activity in cells with significantly increased metabolic rates (such as cancer cells) might be regarded as an unwanted side effect, because an increased CYP1A1 activity would potentially increase the rate and the extent at which some pro-carcinogens can be converted to carcinogens.

When we analysed the structure-activity relationship for derivatives **3**, **4**, **6**, and **7**, we observed that compounds with a single methoxy group at position 4- are better CYP1A1 inhibitors than those with a 3,5-dimethoxy moiety. This observation is not consistent with the findings of Mikstacka et al., who reported the synthesis and biological evaluation of a series of methylthiostilbenes as CYP1A1, CYP1A2, and CYP1B1 inhibitors [32]. Mikstacka et al. observed that 3,5-disubstituted compounds were more potent than those with only a 4-methoxy group [32]. Nevertheless, we realize that our observation requires further validation by testing a more expanded library of compounds using the same assay conditions.

4.3.3 CYP1A1 catalytic inhibition after (1 h incubation) in HepG2 cells

The main mechanism of TCDD-mediated carcinogenicity involves activating AhR and subsequently inducing CYP1A1 through transcriptional and translational mechanisms [33]. Resveratrol is a reported AhR antagonist [34], which has the ability to inhibit CYP1A1 activity by directly inhibiting CYP1A1 activity [7]. To investigate whether our hybrid resveratrol-salicylate analogues exhibit “direct” CYP1A1 inhibition, we tested the inhibitory effects of the most active compounds (TMS, **3** and **5**) by incubating HepG2 cells in the presence of these compounds for 1h. Briefly, first we treated cells with TCDD for 24 hours to induce the expression of the CYP1A1 enzyme (as described in materials and methods). Then, after changing the medium, we incubated the cells with the corresponding test compound for 1 h

(before adding the substrate for CYP1A1). Finally, we measured the catalytic activity of CYP1A1 as described in the experimental section.

Our results (**Figure 4-1, B**) demonstrate that, unlike the 24 h incubation experiment described previously, resveratrol did not significantly inhibit CYP1A1 activity. Resveratrol concentrations previously used to illustrate resveratrol antagonism on AhR were higher than 10 μM [35-37]. This may explain why we did not observe any significant effect of resveratrol using this experiment (at 5 μM resveratrol). Similarly, compound **5** had no direct inhibitory effect on the CYP1A1 enzyme, even though this compound exerted about 60% enzyme inhibition when tested over a 24 h-incubation period (**Figure 4-1, A**). In contrast, the new compound **3** showed a moderate inhibitory effect on CYP1A1 enzyme activity (about 38% inhibition), which was very similar to that obtained with TMS (42%, **Figure 4-1, B**). We also observed that compound **3** was a more potent CYP1A1 inhibitor than the parent compound resveratrol, which suggests that the hybrid resveratrol-salicylate derivative **3** can significantly inhibit the carcinogen-activating enzyme CYP1A1 after 1 and 24 hours incubation.

The significance of the 1 h incubation, compared to the 24 h incubation experiment, is the fact that the assay carried out by incubating cells for 1 h shows how the drug affected CYP1A1 at the post-translational level (direct inhibition), rather than (or in addition to) showing the potential effects at the transcriptional level (modulation of CYP1A1 mRNA expression).

4.3.4 CYP1A1 catalytic inhibition after (24 h incubation) in HT-29 cells

To further confirm our finding in cultured HepG2 cells, we repeated the EROD experiment using HT-29 cells. The result of this experiment is presented in (**Figure 4-2**). We used the most potent compounds observed in HepG2 cells (TMS, **3** and the parent molecule, resveratrol). In the absence of TCDD, none of the compounds (including resveratrol) significantly altered the EROD activity compared to DMSO-treated cells (data not shown). Figure 4-2 shows that EROD was detected in these cells without TCDD induction, an observation which is consistent with the literature showing that HT-29 cells constitutively express CYP1A that can be detected by an EROD assay [38]. The main finding in HT-29 cells is that instead of decreasing EROD activity, resveratrol *enhanced* the toxicity of TCDD as it caused about a two-fold induction in EROD activity compared to TCDD-treated cells (**Figure 4-2**). The elevation in EROD activity

attenuated as the resveratrol concentration increased (data not shown). This finding can be explained by three recent literature observations. The first is that resveratrol was observed to be a partial agonist of AhR, as it induced CYP1A1 and CYP1A2 mRNA in HepG2 cells [39, 40]. The second is that resveratrol stimulated CYP1A1 activity (by EROD assay) in Caco-2 cells after 24 hours incubation [41]. The third is that resveratrol increased the CYP1A1 activity in HaCaT cells along with reducing the clearance of the endogenous AhR ligand FICZ, which prolonged the activation of CYP1A1 [42]. Unlike resveratrol, TMS and compound **3** did not enhance TCDD-induced EROD activity, which may suggest different modulations of CYP1A1 exerted by these stilbenes compared to resveratrol.

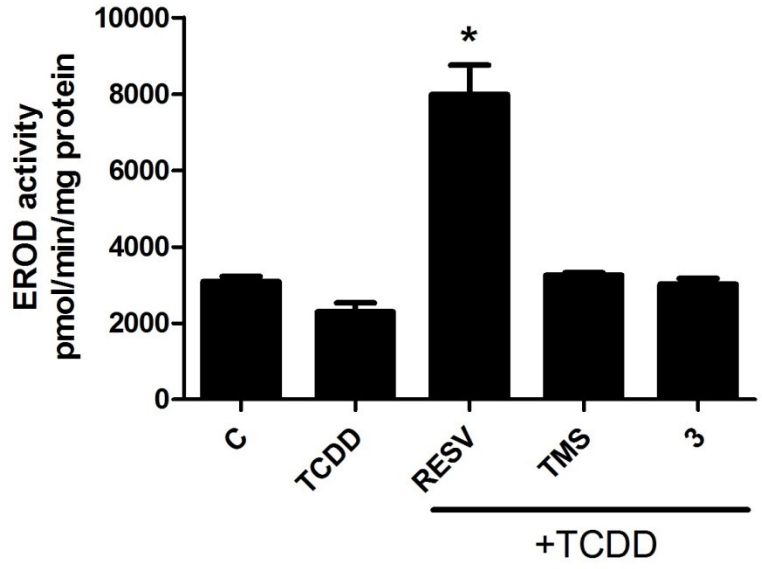

Figure 4-2: Effect of resveratrol, TMS, and compound 3 on TCDD-mediated CYP1A1 catalytic activity in HT-29 cells. Cells were pre-incubated with 5 μM of resveratrol (Res) or its analogues for 30 min before TCDD (1nM) was added for an additional 24 h. The CYP1A1 activity level was determined using a CYP1A1-dependent EROD assay. Values represent mean activity ± SEM. (n = 8). (*) $P < 0.05$ compared with TCDD.

4.3.5 Effects on the expression of CYP1A1 mRNA

To investigate if the decrease in enzyme activity exerted by the test compounds was due to a classical inhibition of the CYP1A1 active site, or to a decrease in CYP1A1 transcription, we also measured CYP1A1 mRNA levels in HepG2 cells in the presence of the test compounds (5 μM), for six hours as described previously [16]. We selected compound **3** (the most promising hybrid stilbene-salicylate analogue), and compared it to TMS under the same experimental conditions. The results of this experiment are shown in **Figure 4-3**. DMSO-treated cells (control) showed a negligible induction of CYP1A1 mRNA, whereas TCDD-treated cells exhibited a significant increase in CYP1A1 mRNA levels (about a 295-fold increase compared to the control cells). Cells co-treated with both TCDD and TMS showed a dramatic decrease of CYP1A1 mRNA expression compared to those treated with TCDD alone, which is consistent with the results observed after both (1 and 24 h incubation) EROD assays in HepG2 cells. Similarly, cells co-incubated with TCDD and compound **3** also showed a significant decrease in the expression of CYP1A1 mRNA levels, although this effect was not as strong as that obtained with TMS. This suggests that compound **3** exerts its modulatory effect on CYP1A1 by both inhibiting CYP1A1's catalytic activity and decreasing its mRNA levels.

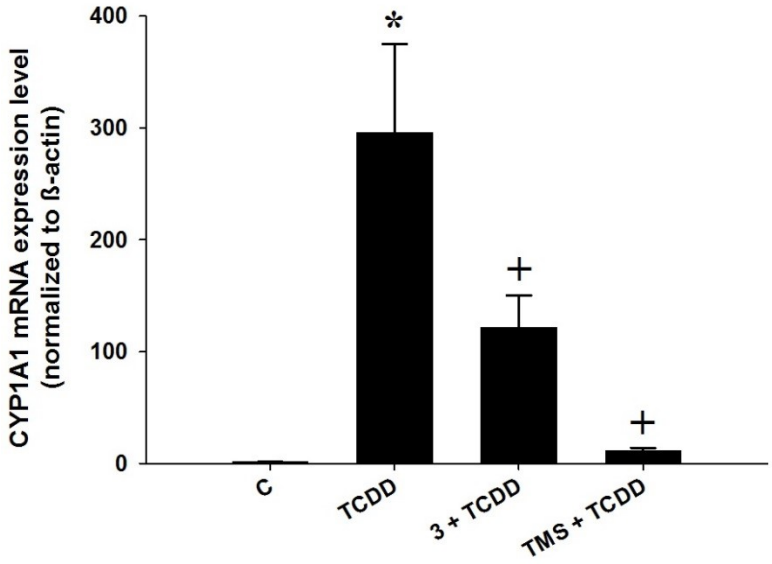

Figure 4-3: Effects of stilbenes on CYP1A1 mRNA level in HepG2. Cells were co-treated with 1 nM TCDD plus compound 3 or TMS at 5 μM for 6 hours. First-strand cDNA was synthesized from total RNA (1.5 μg) extracted from HepG2 cells. cDNA fragments were amplified and quantitated using the ABI 7500 real-time PCR system as described in the material and methods section. Duplicate reactions were performed for each experiment, and the values presented as the mean ± SEM. (n= 6). (*) $P < 0.05$ compared to DMSO-control (C). (+) $P < 0.05$ compared to TCDD alone.

4.3.6 Molecular Modeling

To evaluate the ability of the test compounds to interact with the catalytic site of human CYP1A1, we carried out a molecular modeling (docking) experiment, in which we assessed the ability of all compounds to exert binding interactions with key amino acid residues in the CYP1A1 active site. Similar studies have been reported previously, but these had used the CYP1A2 template (PDB code: 2HI4) instead, and from these predictions the authors assumed potential binding interactions between experimental drugs, and the CYP1A1 active site (homology models) [7]. Nevertheless, considering that the crystal structure of human CYP1A1 was recently reported (PDB code: 4I8V [21]), we decided to use this template in our docking study. To the best of our knowledge, our work is the first to correlate *in vitro* CYP1A1 activity data with docking studies using the recent crystal structure of human CYP1A1.

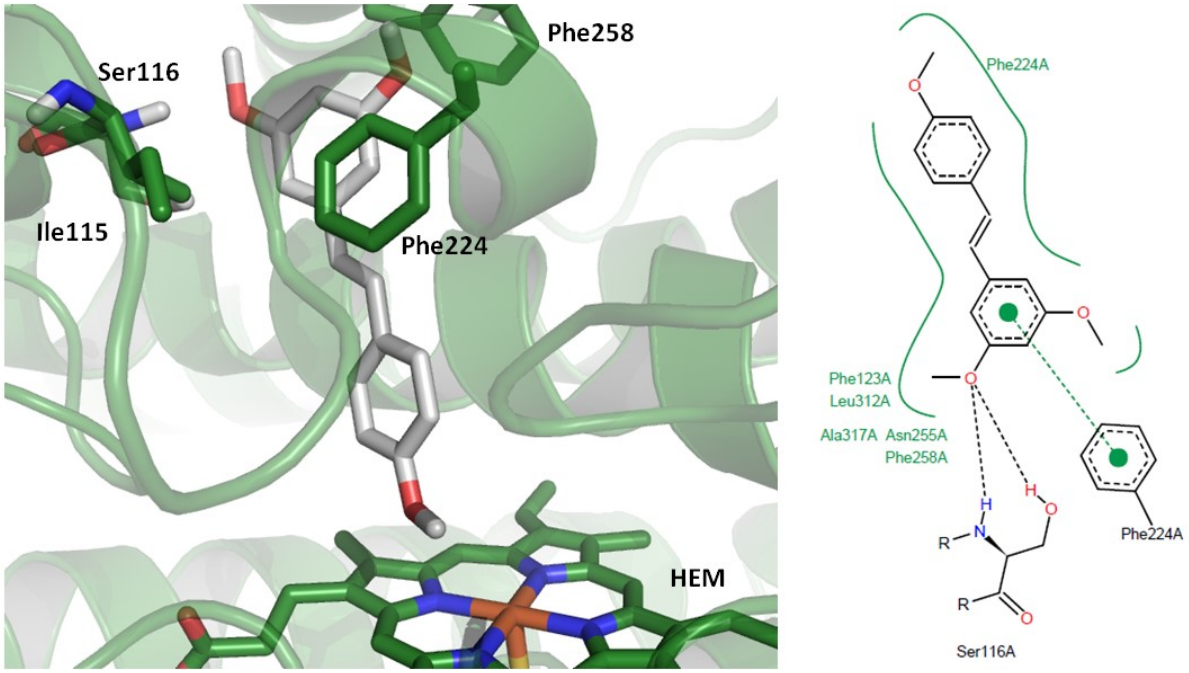

Figure 4-4: Binding mode for TMS in the active site of CYP1A1. The 3D figure was generated using a PyMOL Molecular Graphics System (DeLano Scientific LLC, Palo Alto, CA, 2007); the 2D figure was generated according to a procedure described in the literature [43].

Figure 4-4 shows the binding mode for TMS within the active site of human CYP1A1. This molecule exerts a π-π interaction with Phe224, which has been reported by others in the *in silico* studies with resveratrol analogues [44]. **Figure 4-5** shows the binding modes calculated for all the test compounds (**3-12**); it is interesting to note that compounds **3**, **4**, **5,** and **6** exert a similar binding profile (**Figure 4-5**) to that observed for TMS, which may account for their *in vitro* inhibitory activity. On the other hand, compound **7** showed a slight shift within the active site of CYP1A1, which allows its carbonyl group to interact with the iron atom within the *heme* group, and at the same time, to produce a π-π interaction with both Phe224 and Phe258.

Compounds **3-7** showed low free energy values and at the same time, showed high cluster size values (**Table 4-1**). These data suggest a high probability of exerting binding interactions with the CYP1A1 active site, having the actual conformation calculated and reported for these drugs. However, compounds **8** and **9** showed a significant binding interaction between their corresponding carboxylate groups and the iron atom present in the *heme* group of human CYP1A1, which resulted in a loss of the binding interaction with Phe224 (**Figure 4-5**).

Despite the low free binding energy values calculated for compounds **8** and **9**, their low cluster size values suggest that these two hybrid salicylate-resveratrol derivatives would likely adopt a slightly different conformation than that determined by our molecular modeling protocol. In other words, there is a significant probability that these compounds will bind with the CYP1A1 active site by a different conformation than that predicted theoretically. Compound **10** showed the highest free binding energy and the lowest cluster size among the tested compounds. The predicted binding mode for this particular molecule does not show the π-π interaction with Phe224 observed for some of its analogues (**Figure 4-6**). This would explain why this compound was practically inactive *in vitro*. Finally, compounds **11** and **12** showed the lowest free binding energies and the highest cluster size values, which would account for their inability to *in vitro* inhibit the CYP1A1 enzyme. However, the observed interaction of the carboxylic acid group present in these molecules with the iron atom in the *heme* group of CYP1A1 (**Figure 4-5**) could be related to the observed slight increase (even though not significant) in enzymatic activity (activation), rather than enzyme inhibition. This observation could also be linked to the increased enzymatic activity of CYP1A1 in the case of compounds **8** and **9** (**Figure 4-1**).

Compound	$\Delta G_{binding}$ (kcal/mol)	Cluster size	Compound	$\Delta G_{binding}$ (kcal/mol)	Cluster size
TMS	-8.97	30/30	**8**	-12.07	18/30
3	-9.42	30/30	**9**	-11.74	16/30
4	-10.67	27/30	**10**	-8.17	10/30
5	-10.57	29/30	**11**	-15.01	30/30
6	-10.06	23/30	**12**	-14.70	27/30
7	-9.71	22/30	-	-	-

Table 4-1: Calculated binding energies for resveratrol derivatives inside CYP1A1 active site.

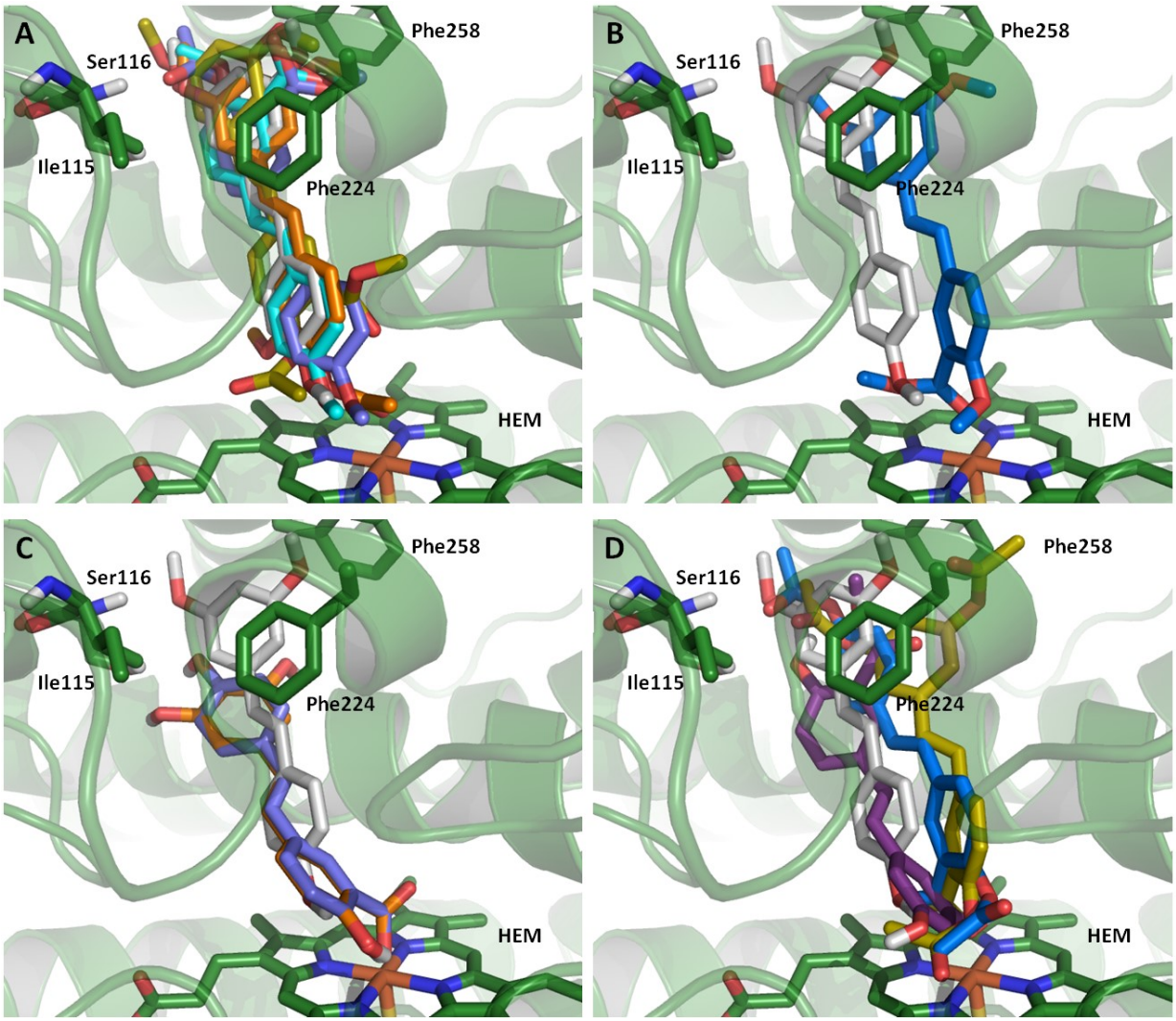

Figure 4-5: Comparison of different binding modes inside human CYP1A1 exerted by resveratrol derivatives. TMS (shown in grey) is compared to that of compounds **(A) 3** (cyan), **4** (purple), **5** (orange), **6** (yellow); **(B) 7**; **(C) 8** (purple), **9** (orange); **(D) 10** (purple), **11** (blue) and **12** (yellow) in the active site of human CYP1A1. The 3D figures were generated using a PyMOL Molecular Graphics System (DeLano Scientific LLC, Palo Alto, CA, 2007).

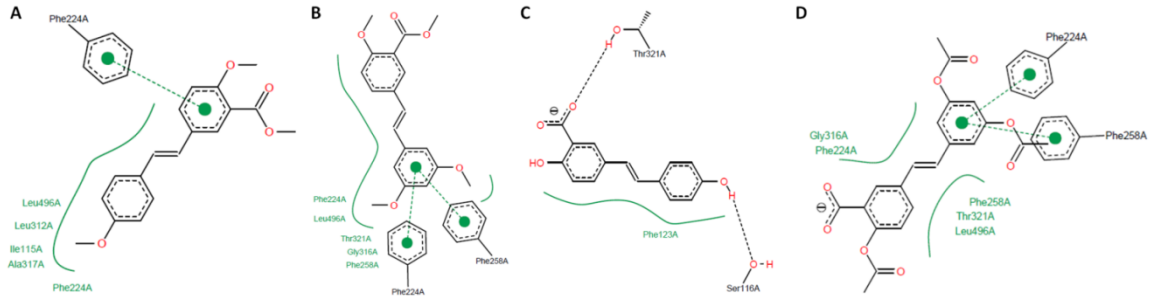

Figure 4-6: 2-D representation of binding interactions inside CYP1A1. Compounds **(A) 3**; **(B) 7**, **(C) 8** and **(D) 12** are displayed. The 2D figures were generated according to a procedure described in the literature [43].

It is noteworthy that the vast majority of previous literature reports have described only the inhibitory profile of *trans*-stilbenes on CYP1A1, and not much attention has been paid to the corresponding *cis*-isomers. One of the future directions in this regard could involve the biological evaluation of hybrid resveratrol-salicylate derivatives possessing the *cis* configuration, especially because *cis*-isomers have been shown to exert significant anticancer activity [45], even though this therapeutic profile has not been correlated with *in vitro* CYP1A1 inhibition studies. We have identified (by molecular modeling) at least one hybrid derivative that could exert potent CYP1A1 inhibition (results not shown), and which constitutes the focus of one of our future research projects.

4.4 Conclusion

The addition of a carboxylate group at position 3'- in the resveratrol structure yielded a new series of aspirin-resveratrol analogues, which modulated the *in vitro* activity of the carcinogen-activating CYP1A1 enzyme. Compounds with methyl esters showed a moderate inhibitory profile of CYP1A1 activity, compared to the parent resveratrol; however, compounds with a free carboxylic acid group (including those with an acetylsalicylic acid group) increased the enzyme's catalytic activity. The potential chemopreventive effect exerted by compound **3** (the most active compound) obtained in this series is supported by the observation that it also exerts a decrease in the expression of CYP1A1 mRNA in HepG2 cells at concentrations as low as 5 μM. Importantly, **3** (in addition to TMS) did not potentiate TCDD-mediated CYP1A in HT-29 cells. The implications of these results are significant, considering the potential synergistic profile of the new hybrid resveratrol-salicylate analogue(s) on many other targets relevant to chemoprevention. This study also showed that differences in the chemical structure of different stilbene derivatives may potentially switch CYP1A1 inhibition to CYP1A1 induction, which may not be useful in a long-term chemopreventive setting, but it might find potential applications in short-term states where CYP1A1 activity is required but compromised.

The results of this study provide preliminary evidence supporting the design of hybrid molecules combining the chemical features of two well-known chemopreventive agents, resveratrol and aspirin (salicylates). According to our findings, the previously reported unfavorable effects of a salicylate moiety did not void the ability of some hybrid resveratrol analoguess to inhibit the catalytic activity of CYP1A1, or its effects on CYP1A1 mRNA *in vitro*. Finally, compound **3** represents a new potential hybrid chemopreventive agent which combines the structural features of two well-known chemopreventive compounds.

4.5 References

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Chapter 5: DNMT enzymes inhibition, cell proliferation, and stability studies.

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5.1 Introduction

Resveratrol (**Figure 5-1**) is a naturally occurring polyphenol with a wide variety of biological properties. It has been regarded as a phytoalexin (plant antibiotic), and is produced at low concentrations by several plant species [1]. The biological effects of resveratrol have been extensively studied *in vitro* and *in vivo* [2-6]; some of its reported effects include but are not limited to anti-inflammatory [7], anticancer [8], antioxidant [9], cardio-protective [10], modulation of the estrogen receptor [11], and chemopreventive activity [12]. In this regard, resveratrol possesses an attractive chemopreventive profile, because it inhibits the proliferation of cancer cells *in vitro* without exerting significant cytotoxicity to normal cells [13]; it induces cancer cell apoptosis in several cell lines from different tissue types [14-16], and it significantly decreases tumor size *in vivo* using different cancer cells in xenograft models of rodents [17, 18]. The mechanisms of action associated with the chemopreventive profile of resveratrol are varied and rather complex. In accordance with the current paradigm involving the design of "multi-target" drugs, and the relatively new concept known as polypharmacology [19], there is evidence supporting the multi-target profile of resveratrol. In this regard, resveratrol downregulates the expression and/or inhibits the activity of key enzymes and transcription factors involved in carcinogenesis, including (but are not limited to) cyclooxygenase (COX) enzymes, inducible nitric oxide synthase (*i*NOS), lipoxygenase (LOX), PI3-kinases, NF-κB, PPARγ, Sirt1, and DNA-methyltransferases (DNMTs) [20].

DNMTs are a group of enzymes expressed by mammals in three active isoforms: DNMT1, DNMT3A, and DNMT3B (and one more regulatory enzyme identified as DNMT3L) [21]. Under normal physiological conditions, DNMTs are crucial for DNA methylation at cytosine residues [21]; specifically, DNMT3 functions as an initial (*de novo*) methylator, while DNMT1 is responsible for "maintenance" of the methylation during cell division [21]. However, aberrant methylation patterns (referred as "epigenetic") affecting certain genes and/or overexpression of DNMTs have been associated with many cancer types including lung, colorectal, prostate, breast, endometrial, gastric, hepatocellular, cervical, and pancreatic [22, 23].

Experimentally, the selective inhibition of different DNMT enzymes has provided important clues to determine their role in physiology and pathophysiology. For example, it has been

observed that DNMT inhibition reactivates "silenced" or hypermethylated genes, particularly tumor suppressor genes (genes associated with the expression of proteins that prevent tumor formation) [24, 25]. Another important observation is that the concomitant incubation of DNMT inhibitors with chemotherapeutic agents [26, 27], as well as radiotherapy [28], have showed significant synergistic effects of both of these therapeutic strategies. Finally, the inhibition of DNMT1 and DNMT3B has been shown to abrogate hepatitis C infection in hepatocellular carcinoma cells [29]. Consequently, it has been proposed that targeting the aberrant enzymatic activity of DNMTs could restore otherwise hypermethylated tumor suppressor genes, which is considered a promising strategy to prevent cancer initiation and cancer development [30, 31].

The chemical structure features required for a given molecule to display DNMT inhibition are described in the literature. According to the chemical structure, DNMT inhibitors can be classified into two main groups: the nucleos(t)ide and the non-nucleos(t)ide DNMT inhibitors [32-34]. Azacitidine (Vidaza®, Celgene) and Decitabine (Dacogen®, Astex) are two US FDA clinically-approved nucleoside DNMT inhibitors [34], whereas the compound MG98 represents an oligonucleotide. Representative examples of the non-nucleos(t)ide class of DNMT inhibitors are a tryptophan derivative (RG108), quinoline derivatives (SGI-1027), alkyne derivatives, cyclopenta- and cyclohexathiophene derivatives, procainamide derivatives, genistein (natural flavonoid), curcumin, Psammaplin A (a marine natural compound), and hydralazine (see **Figure 5-2** for chemical structures) [34].

Based on the observation that DNA methylation can be reversed by specific DNA repair mechanisms, the inhibition of hypermethylation of tumor suppressor genes is a promising strategy to prevent cancer initiation and development. This inhibition may take place over a long period of time after administering either synthetic [35] or naturally occurring chemopreventive drugs [36]. Computational approaches have demonstrated the ability to identify DNMT inhibitors or compounds with demethylating properties that have novel scaffolds [33, 37]. A recent work published by Kuck et al. [38] reported the docking-based, virtual screening, and *in vitro* evaluation of more than 26,000 compounds from the National Cancer Institute (NCI) database on DNMT enzymes. In that paper, authors reported a series of small molecules with relatively high biochemical selectivity towards individual human DNMT enzymes. Using a

multistep docking approach of lead-like compounds with a homology model of the catalytic site of DNMT1, followed by experimental testing, Kuck et al. identified seven new molecules with detectable DNMT1 inhibitory activity. The molecules identified in the study had diverse scaffolds, some were not previously reported as DNMT inhibitors. These included a series of methylenedisalicylic acids, among which the compound NSC 14778 (**Figure 5-1**) was one of the most potent compounds tested on DNMT1 and DNMT3B enzymes [38]. We analyzed the chemical structure of the new scaffold present in methylenedisalicylic acids, and compared it to that of our recently reported resveratrol-salicylate analogues (**Table 5-1**). After adding a carboxylic acid group to one of the aromatic rings present in the polyphenol [39], we hypothesized that, in addition to the CYP1A1 inhibitory activity reported previously, these hybrid drugs could also inhibit the enzymatic activity of DNMT enzymes.

To the best of our knowledge, there are no reports in the literature describing the direct inhibitory effect of resveratrol on DNMT enzymes. The only paper we could find with a similar focus, by Qin et al., looked at the effects of resveratrol on the expression of DNMT enzymes [40]. As part of ongoing research aimed at developing new cancer chemopreventive agents, we are reporting in this chapter the *in vitro* biological evaluation and molecular modeling (docking) studies of new resveratrol-salicylate derivatives against DNMT enzymes. Our hypothesis is based on the idea that adding a carboxylic acid or its methyl ester attached *ortho* to one of the phenol groups present in hydroxystilbenes might confer resveratrol with a novel DNMT inhibitory profile, similar to that exerted by methylenedisalicylic acids described above. In this chapter, we identified compound **10** as the most active analogue which showed greater than four-fold potency compared to resveratrol in inhibiting the DNMT3A enzyme. Additionally, compound **10** exerted cell proliferation inhibition on three different human cancer cell lines (HT-29, HepG2, and SK-BR-3), suggesting that this chemical compound is more effective than the parent resveratrol under the same experimental conditions. Finally, stability studies of this molecule showed that this stilbene is being hydrolyzed in plasma to produce compound **9**, a molecule that also exhibited significant DNMT3 inhibitory activities.

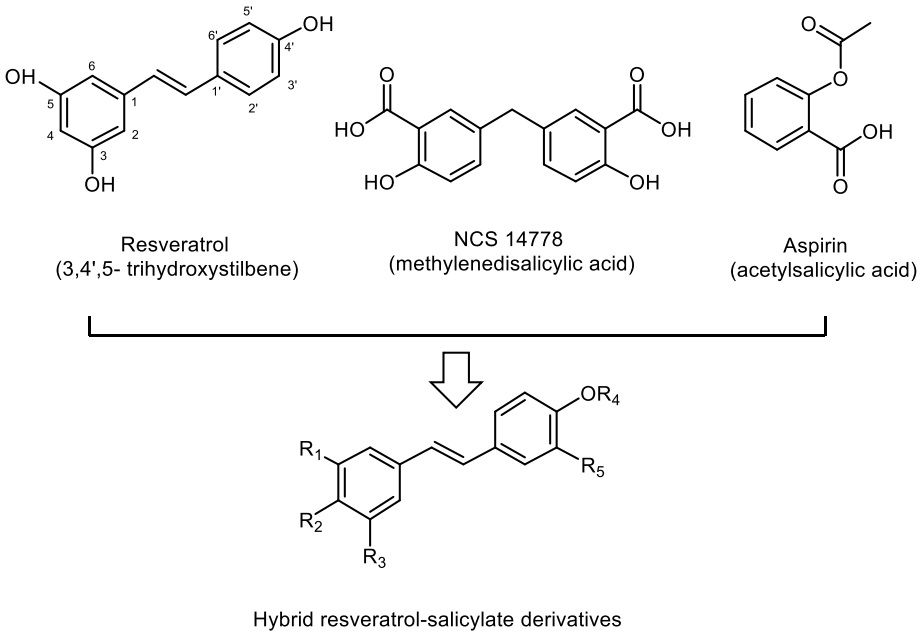


Figure 5-1: Chemical structures of resveratrol, NSC 14778, and aspirin. The hybrid resveratrol-salicylate derivatives have the combined chemical features of these three different types of agents.

	R_1	R_2	R_3	R_4	R_5
Resveratrol	OH	H	OH	H	H
TMS	OCH_3	H	OCH_3	CH_3	H
3	H	OCH_3	H	CH_3	$COOCH_3$
4	H	OCH_3	H	Ac	$COOCH_3$
5	OCH_3	H	OCH_3	Ac	OCH_3
6	OCH_3	H	OCH_3	Ac	$COOCH_3$
7	OCH_3	H	OCH_3	CH_3	$COOCH_3$
8	H	OH	H	H	COOH
9	OH	H	OH	H	COOH
10	OH	H	OH	H	$COOCH_3$
11	H	OAc	H	Ac	COOH
12	OAc	H	OAc	Ac	COOH

Table 5-1: Different substitutions on the hybrid resveratrol-salicylate derivatives.

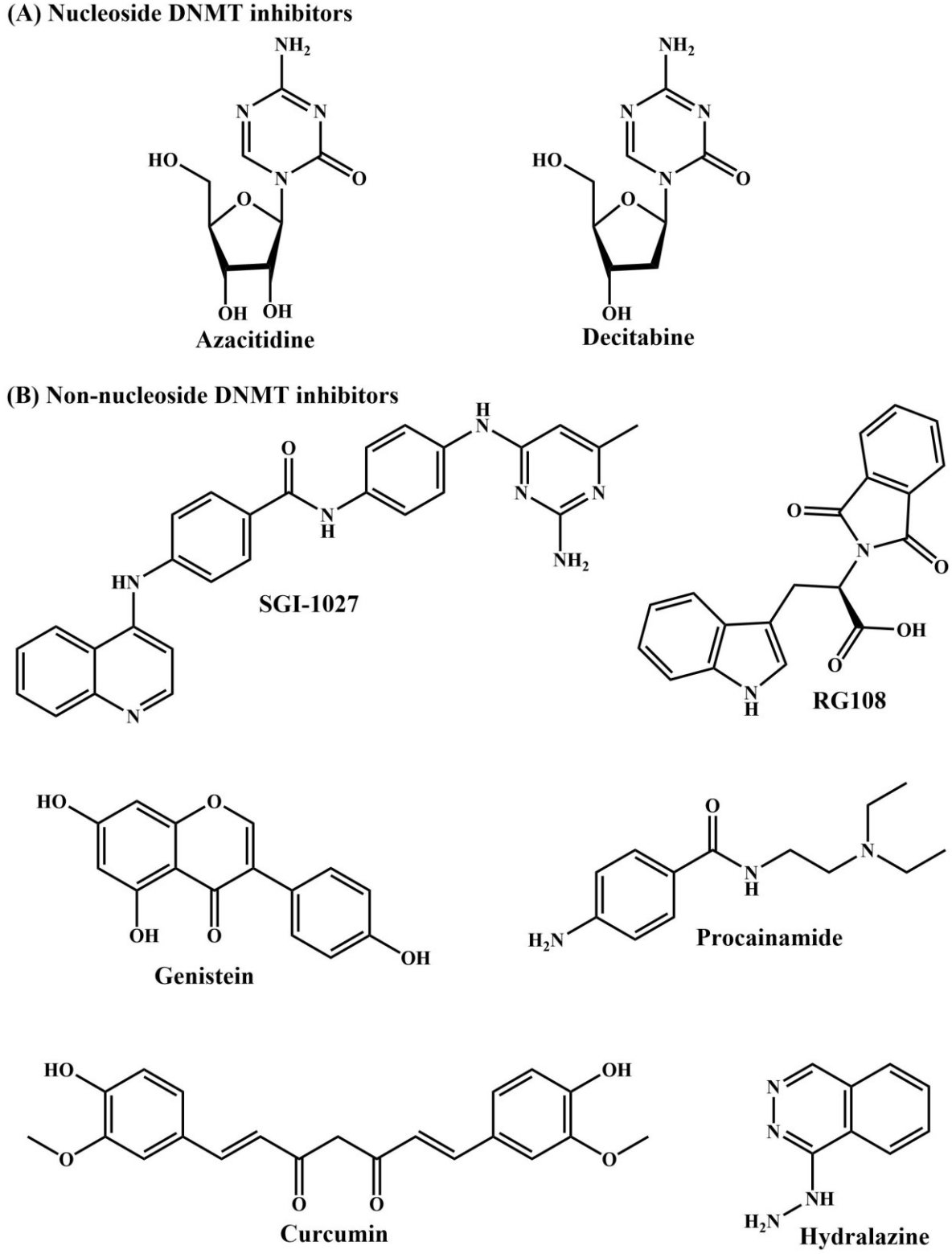


Figure 5-2: Chemical structures of representative examples of (A) nucleoside and (B) non-nucleoside DNMT inhibitors.

5.2 Materials and methods

5.2.1 Chemistry

We carried out the synthesis of hybrid resveratrol-salicylate derivatives **3**-**12** as described in Chapter **3**.

5.2.2 Inhibition of DNMT enzymes

The catalytic domains of DNMT3A/3B and full-length DNMT 3L were purified as previously described [41]. Full-length DNMT1 was purified as previously described [42]. The dose-response experiments were performed against DNMT1 and DNMT3A/3B using the radiometric assay described by Hemeon et al. [41]. Briefly, the assay was conducted in the buffer containing 50 mM HEPES, 50 mM KCl, 5% glycerol and 1 mM DTT, pH = 8.0. The inhibitors were preincubated in the buffer containing 1 μM of the corresponding enzyme or enzyme complex for 30 min, and the reaction was initiated by the addition of the substrate mix (1 μg dIdC substrate and 1.83 μM ^{3}H-*S*-adenosyl-*L*-methionine). The methylation reactions were allowed to proceed at an ambient room temperature of 22 °C for 4 h (DNMT3B/3L) and overnight (DNMT3A/3L). Subsequently, 6 μL of the reaction were spotted on 1.2 cm x 1.2 cm DE81 Anion Exchanger exchange filter paper squares. Each reaction was spotted three times. The filter paper was allowed to dry for 15 minutes, and washed twice with 0.2 M ammonium bicarbonate, followed by deionized double distilled water and ethanol. The filter paper was then put in scintillation vials, to which was added 0.5 mL of deionized double distilled water followed by 5 mL of scintillation fluid. The signal was monitored using a Liquid Scintillation Analyzer (Perkin Elmer Tri-Carb 2910 TR) and the percent of inhibition was calculated as previously described [43].

5.2.3 Molecular Modeling

Proteins. The crystal structures of human DNMT1 (PDB ID: 3SWR) and DNMT3A (PDB ID: 2QRV) were retrieved from the Protein Data Bank (PDB). For the DNMT3B structure, we used the homology model that we had previously published for this isozyme [38]. The structures were prepared and submitted to a geometry optimization protocol (OPLS force field) using the default settings on the Protein Preparation Wizard protocol of Schrödinger software [44].

Ligands. Compounds **3** to **12**, 3,4',5-*trans*-trimethoxystilbene (TMS) as well as resveratrol were built and submitted to a geometry optimization protocol using the AMBER99SB force field in UCSF Chimera 1.9 [45].

Docking. Molecular docking studies were performed using AutoDock 4.2 software [46]. In these studies, we evaluated the compounds in the DNMT catalytic site in the presence and absence of the co-factor. We used a grid box of 80 x 80 x 80 points with a grid spacing of 0.375 Å that covers the catalytic pocket and the co-factor binding site. The Lamarckian genetic algorithm was used as a search method. Twenty runs were carried out with a maximal number of 5,000,000 energy evaluations and an initial population of 150 conformers. The best binding modes for each molecule were selected for the analysis. We had previously used AutoDock to model DNMT inhibitors [38].

5.2.4 Cell proliferation inhibition (MTT assay)

Human colorectal adenocarcinoma HT-29 cells (ATCC HTB-38, Manassas, VA), human hepatoma HepG2 cells (ATCC HB-8065, Manassas, VA), and human mammary gland/breast SK-BR-3 cells (ATCC HTB-30, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin. Human mammary gland MCF 10A (ATCC CRL-10317) and human umbilical vein endothelial HUVEC cells (kindly provided by the Davidge Lab., Department of Obstetrics/Gynecology and Physiology, University of Alberta) were used as non-cancerous cell line models. MCF 10A cells were maintained in MEGM media (Lonza, MD, USA) while HUVEC cells were maintained in EGM media (Lonza, MD, USA). Cells were grown in 75-cm^2 tissue culture flasks at 37°C in a 5% CO_2 humidified incubator. To evaluate the antiproliferative effects of resveratrol analogues, we carried out a series of MTT assays using a published procedure [47] with minor modifications. All test compounds were dissolved in DMSO and tested at a final concentration range of 0.03 to 125 µM, over a 24-hour incubation period. The final concentration of DMSO in culture media was fixed at 0.5% (v/v). The corresponding IC_{50} values were calculated from the cell proliferation inhibition curves using GraphPad Prism software (IC_{50} values represent the mean ± S.D. of three different experiments, in triplicate).

5.2.5 Stability study

5.2.5.1 Mass spectroscopy

Electrospray ionisation (ESI) mass spectroscopy (negative mode) was used to qualitatively study the stability of compound **10** in the same media used in the above cell proliferation inhibition studies. A stock solution of **10** in DMSO (100 mM) was diluted into culture media to a final concentration of 500µM and was incubated in the dark at 37°C. Samples were taken at 0, 1, 6, and 24 hours and were diluted in acetonitrile for mass analysis.

5.2.5.2 HPLC analysis

5.2.5.2.1 Sample preparation

Stock solutions of compound **10** were prepared at 15 mM in DMSO and diluted in either culture media or rat plasma (Innovative Research Inc., MI, USA) to a final concentration of 125 µM. Samples were incubated in the dark at 37°C for 0, 1, 3, 6, 12, and 24 hours. Samples of **10** in culture media were diluted in HPLC-grade acetonitrile/water (50:50) in a ratio of 1:2. The volume of the sample injected into the HPLC column was 100µl. For compound **10** samples in plasma, three volumes of acetonitrile were added to one volume of the plasma sample. The mixture was then vortexed and centrifuged at 5000 x g for 5 min at 4°C. Finally, 200µl of the supernatant was added to the auto-sampler vial, and only 100µl was injected into the column.

5.2.5.2.2 HPLC conditions

An HPLC system (Shimadzu Prominence, Mandel Scientific, Guelph, ON, Canada) was used in this study. The system consists of an LC-20AT dual pump, SIL-20A auto-sampler, DGU-20A5 degasser, CTO-20AC column oven, UV-vis dual wavelength detector, and a system controller. The system was controlled by a personal computer using Shimadzu Class VP 7.4 version software.

Method A: We used the chromatographic conditions reported by Lin H-S et al. [48], but with a minor modification. Briefly, the separation was performed using a reversed-phase HPLC column (Agilent Zorbax Eclipse Plus C18: 250 x 4.6 mm i.d., 5µM), protected by a guard column (Agilent Zorbax Eclipse Plus C18: 12.5 x 4.6 mm i.d., 5µM). Separation time was 12.5 min

using a gradient solvent mixture of acetonitrile/water at a flow rate of 1.5 mL/min with the column temperature set at 50 °C. The gradient schedule was as follows: (i) 0-4.5 min, acetonitrile 65%; (ii) 4.6-5.5 min, acetonitrile 65→90%; (iii) 5.6-9.0 min, acetonitrile 90%; (iv) 9.1-12.5 acetonitrile 65%. Ultraviolet (UV) absorbance was recorded at 305 nm (maximum UV absorption for compound **10**).

Method B: We used the HPLC method reported by Omar J.M. et al. [49] with some modifications. We used the same C18 and guard columns described in Method A. The column temperature was set at 35°C. The chromatographic run time was 22 min with a flow rate of 1.0 mL/min. The mobile phase consisted of HPLC-grade water (containing 0.1% formic acid) and HPLC-grade methanol (containing 0.1% formic acid) as follows: (i) 0-10.0 min starting with water/methanol (50:50), linearly changed to 100% methanol; (ii) 10.1-15.0 min held at 100% methanol; (iii) 15.1-22.0 min water/methanol (50:50). UV absorbance was recorded at 305 nm, and the auto-sampler was operated at room temperature.

5.3 Results and discussion

During the last decade, the evolution of epigenetics and its validated association with many disorders such as cancer [50], Alzheimer's disease [51], cardiovascular diseases [52], and diabetes [53] have been the subject of scientific research. These epigenetic mechanisms are regulated by multiple proteins including DNA methyltransferase enzymes (DNMTs) [54]. DNMTs catalyze the transfer of a methyl group from the substrate S-adenosylmethionine (SAM) to DNA cytosine residues (called "CG sites") [21]. DNMT1 specifically methylates hemi-methylated DNA, while DNMT3A and DNMT3B bind to unmethylated DNA to carry out *de novo* methylation [21]. It has been proposed that small molecule inhibitors of DNMT enzymes can bind either at the catalytic binding pocket binding site of (DNA) or at the binding site of the cofactor S-adenosylhomocysteine (SAH) [55], or both, depending on the inhibitor's structure. The latter is particularly applicable if the inhibitor's structure has a "long" scaffold, as does SGI-1027 [56].

Compound NSC14778, a methylenedisalicylic acid, was reported by Kuck et al., who had implemented a virtual screening protocol on more than 26,000 compounds from the NCI

database, and found that NSC14778 provided a useful lead scaffold to design new molecules with potential DNMT inhibitory activity [38]. Along the same lines, we recently reported the chemical synthesis and CYP1A1 inhibitory profile of a new series of hybrid resveratrol-salicylate analogues with promising chemopreventive activity [39]. We identified some structural similarities between NSC14778 and our recently reported salicylate-resveratrol analogues, so it was reasonable to predict a certain degree of DNMT inhibition by our molecules (however, please note that we did not start the design of our salicylate-resveratrol derivatives based on the docking pose of NSC14778). We recognized a potentially useful pattern by replacing the central methylene group in NSC14778 for the ethylene (CH=CH) moiety present in stilbenes. Consequently, we hypothesized that these compounds could exert significant inhibition of DNMT enzymes, for two reasons.

First, the new salicylate moiety on resveratrol would resemble the salicylate group in NSC14778, which has been reported as "essential" for DNMT inhibition [38]; second, the literature has showed that resveratrol is capable of reducing the expression of DNMT enzymes, reactivating previously hypermethylated tumor-suppressor genes [24, 25, 35].

Compounds	IC_{50} (μM)		
	DNMT3A/3L	DNMT3B/3L	DNMT1
3	282	>300	NI[1]
4	>300	>300	NI[1]
5	>300	>300	NI[1]
6	>300	>300	NI[1]
7	>300	>300	NI[1]
8	281	156	NI[1]
9	40	52	NI[1]
10	25	62	NI[1]
11	186.6	190	NI[1]
12	100	215	NI[1]
TMS	>300	>300	NI[1]
Resveratrol	105	65	>300
SAH	ND[2]	0.25[3]	2

Table 5-2: Concentration (μM) of test compounds required to inhibit by 50% the enzymatic activity of DNMT1, DNMT3A, and DNMT3B. Results are expressed as IC_{50} values using a cell-free biochemical assay. To generate the enzyme inhibition curves, duplicate reactions were performed for each concentration; IC_{50} values were calculated using the GraphPad Prism v6 software. [1] NI = no inhibition at the maximum test compound concentration (300 μM). [2] ND = not determined. [3] Taken from reference [38].

5.3.1 *In vitro* DNMT inhibition

To study the *in vitro* DNMT inhibition exerted by the salicylate-resveratrol analogues reported previously [39], we used a filter-paper-based Scintillation Proximity Assay (SPA) [43] with the well-known DNMT inhibitor (although structurally unrelated) *S*-adenosyl-*L*-homocysteine (SAH) [57, 58] as the standard which showed IC_{50} = 2 μM on DNMT1. For comparison purposes, we also used the parent stilbene resveratrol, which showed inhibition on DNMT3B (IC_{50} = 65 μM) and DNMT3A (IC_{50} = 105 μM), but no activity on DNMT1 (IC_{50} higher than 300 μM, **Table 5-2**). These results suggest that the parent polyphenol shows selective inhibition of the DNMT3B isozyme. This observation is somewhat related to a recent study reported by Qin et al. [40] in which his group reported a significant reduction in the expression of DNMT3B after a 21-week treatment period with resveratrol into rats. Interestingly, this treatment did not

significantly reduce the expression of the DNMT1 enzyme [40]. It is important to distinguish that our study measured enzyme activity, whereas that of Qin et al. measured protein expression.

Another compound that could be a potential DNMT inhibitor is the methylated version of resveratrol, or 3,4',5-*trans*-trimethoxystilbene (TMS), which has displayed an enhanced anticancer profile compared to resveratrol [47, 59]. We observed no significant inhibition of DNMT enzymes at the highest test compound concentration (300 μM). This suggests that the free hydroxyl groups present in resveratrol are essential to exert inhibition of the DNMT enzymatic activity. As far as we are concerned, our study is the first to report TMS's apparent lack of activity on DNMT1, DNMT3A, and DNMT3B enzymes. A similar effect (i.e., loss of enzymatic inhibitory activity with DNMT1 upon methylation of a hydroxyl group), was noted for a sulfonamide DNMT inhibitor recently identified by high-throughput screening [60, 61].

Once we analyzed the inhibitory profile of the compounds described above, we started the screening evaluation of the new hybrid salicylate-resveratrol derivatives. According to our results, derivatives possessing methoxy groups (**3**-**7**) at any position on the stilbene structure were practically inactive at the highest test compound concentration (300 μM), which is consistent with the results obtained for TMS. However, we also observed that derivatives possessing free hydroxyl groups (**8**-**10**) were significantly more potent than their methoxylated counterparts, but their inhibitory profile was significant only on the DNMT3A and DNMT3B enzymes, not on DNMT1. The most active compounds in the series were compounds **9** and **10**, which significantly inhibited both DNMT3A (IC_{50} = 40 μM and 25 μM respectively), and DNMT3B (IC_{50} values = 52 μM and 62 μM respectively) (**Table 5-2**).

Compounds possessing an acetyl group (mimicking an acetylsalicylic acid moiety), either on a 4'- or a 3,5- pattern, are not as potent as those with free hydroxyl groups. Interestingly, the negative effect of adding acetyl groups on DNMT3 inhibition is milder than that of adding methoxy groups, given that compounds **11** and **12** still showed some degree of inhibition on both DNMT3A and DNMT3B enzymes (IC_{50}'s in the 100-215 μM range). The observation that methoxy groups reduce DNMT inhibition seems to be consistent with a recent report by Rilova et al., which reported that dimethoxytriazine groups decreased the DNMT3A inhibitory activity of quinolone-based DNMT inhibitors [62].

A detailed comparison of molecules **8** and **9**, both possessing free hydroxyl groups, suggests that two phenol groups at positions 3- and 5- (compound **9** IC_{50} values = 40 μM –on DNMT3A–, and 52 μM –on DNMT3B–) exert a better enzyme inhibitory profile than having only one at the 4-position (compound **8** IC_{50} values = 281 μM –on DNMT3A–, and 156 μM –on DNMT3B–).

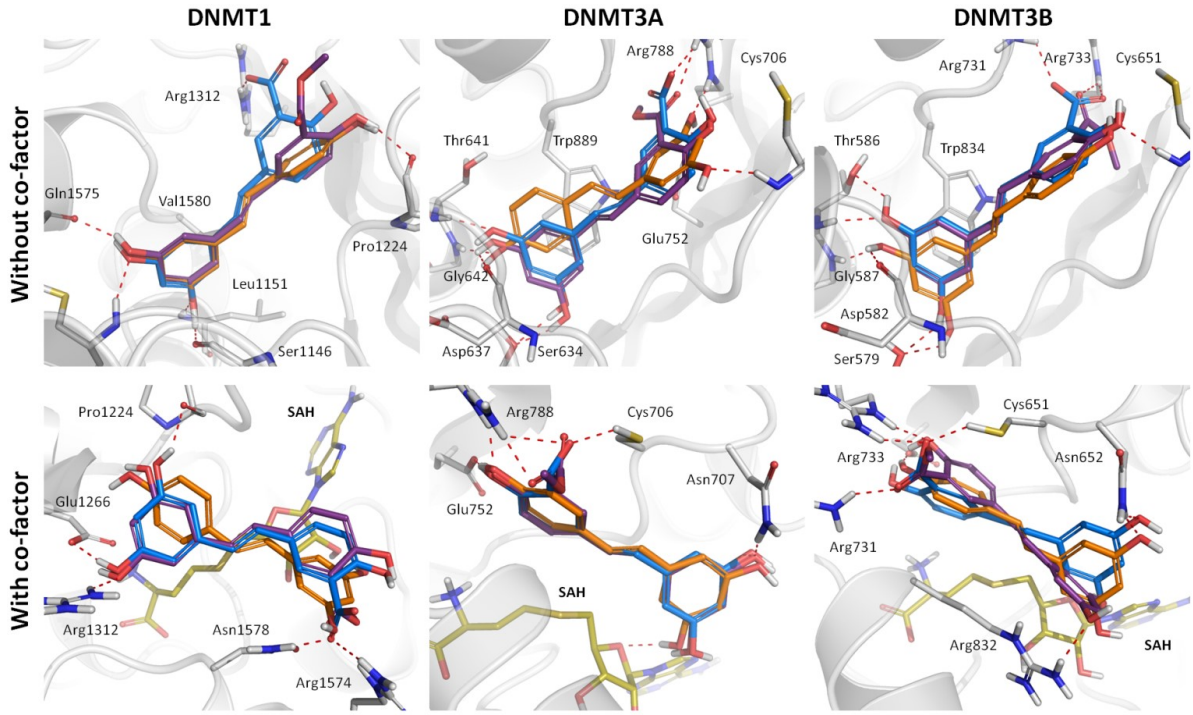
To complement the structural analysis of compounds **3**-**12**, we studied the effects of the carboxylic acid group on the stilbene scaffold which, according to previous reports, seems to be an essential requirement for DNMT1 inhibition. This requirement has been described in different molecules. Analogue series of drugs with a carboxyl group are, in general, more potent DNMT1 inhibitors than those not having it [35, 63]. This observation has been studied using molecular modeling (docking) studies. It has been predicted that carboxylate anions can form hydrogen bonds with important amino acid residues in the active site of DNMT1 [35, 63]. Nevertheless, according to our results and experimental conditions we used for DNMT3A and DNMT3B, the presence of the carboxylic acid group on the stilbene scaffold seems to be significant only when the phenol groups are free. As can be observed with our small library of hybrid salicylate-resveratrol derivatives, compounds with a free carboxylic acid (**8**, **9**, **11** and **12**), a carboxylate methyl ester (**3**, **4**, **6**, **7** and **10**), or no carboxylic acid at all (compound **5**) did not show any inhibition on DNMT1, even at concentrations as high as 1 mM (results not shown). A recent study by Asgatay et al. showed that a *N*-phthaloyl-L-tryptophan derivative, in which a carboxylate group was replaced by an amide function, could still display some activity towards DNMT1. Therefore, the authors proposed that the essential role of the carboxyl group is still "inconclusive" [64].

As far as DNMT3A/3B inhibition is concerned, it is still not clear if the presence of a carboxyl group is required for a drug to exert binding interactions in the active site of DNMT3 enzymes. Nevertheless, recent developments with small molecule inhibitors have showed that *in vitro* DNMT3A inhibition is possible without the presence of carboxylate groups [62]. Results of this study showed that, against both DNMT3 enzymes, compounds bearing either a free carboxylic acid (**9**) or a carboxylate methyl ester (**10**) exerted a better inhibitory profile than resveratrol (**Table 5-2**).

5.3.2 Molecular Modeling

To test *in silico* the ability of the test compounds to interact with the catalytic site of DNMT enzymes, we carried out molecular modeling (docking) simulations, in which we assessed the ability of hybrid salicylate-resveratrol derivatives to exert binding interactions with key amino acid residues in the enzyme's active site. We did these experiments in the presence and absence of the co-factor SAH, according to a previously reported protocol [55].

Figure 5-3 shows the binding mode for the parent compound (resveratrol), and the active compounds **9** and **10** within the human DNMT enzyme binding sites, in the presence and absence of the co-factor. The table below **Figure 5-3** summarizes the calculated binding free energies for each binding mode. The binding free energies as calculated by Autodock, and the binding modes of the remaining compounds, are reported in **Table 5-3** and **Figure 5-4**, respectively. According to our molecular modeling results, the docking scores calculated for resveratrol, compound **9,** and compound **10** in the active sites of both DNMT3A and DNMT3B (in the presence and absence of the co-factor) are, overall, more favorable (more negative), than those values obtained with DNMT1 (**Figure 5-3**). Despite the well-known number of approximations considered in calculating docking scores [65], this is in *good qualitative agreement* with the trend observed experimentally. In the docking study performed in the absence of the co-factor, the presence of a π-π interaction with Trp889 and Trp834 was observed in the DNMT3A and DNMT3B structures, respectively. It is noteworthy that the tryptophan is absent in the structure of the DNMT1, which may explain the differences in binding energies and the lack of activity on the DNMT1 isoform.



Compound	DNMT1		DNMT3A		DNMT3B	
	Without co-factor	With cofactor	Without co-factor	With cofactor	Without co-factor	With cofactor
9	-8.13	-7.67	-9.66	-8.17	-8.85	-7.54
10	-7.43	-6.42	-9.27	-7.01	-8.99	-8.82
Resveratrol	-7.74	-5.83	-9.03	-6.57	-7.95	-6.22

Figure 5-3: Comparison of the binding modes calculated inside the active site of DNMT1, DNMT3A, and DNMT3B enzymes. Compounds **9** (blue), **10** (purple), and resveratrol (orange) in the DNMTs active site in the presence and absence of the co-factor SAH (yellow), as predicted by AutoDock 4.2.

In the study carried out in the presence of the co-factor, we observed interactions of the ligands with the catalytic cysteine, glutamic acid, and arginine in both DNMT3A (Cys706, Glu752, and Arg788) and DNMT3B (Cys651, Glu697, and Arg733) active sites, which in previous studies have been proven to be a primary interaction for enzyme inhibition. In this regard, Cys651 has previously shown to be a key site for binding interactions between the antibiotic Nanaomycin and the DNMT3B enzyme [66]. These docking results allowed us to hypothesize that regardless of the operating inhibition mechanism (with or without the co-factor), these binding interactions may offer a plausible explanation for the observed selectivity toward DNMT3 enzymes by the test compounds, including the parent resveratrol. It is noteworthy that the presence of the 3,5-dihydroxyphenyl group (also called resorcinol) is important for the interaction of the ligands in both studies, suggesting that the test compounds should have this group to inhibit DNMT3 isoforms.

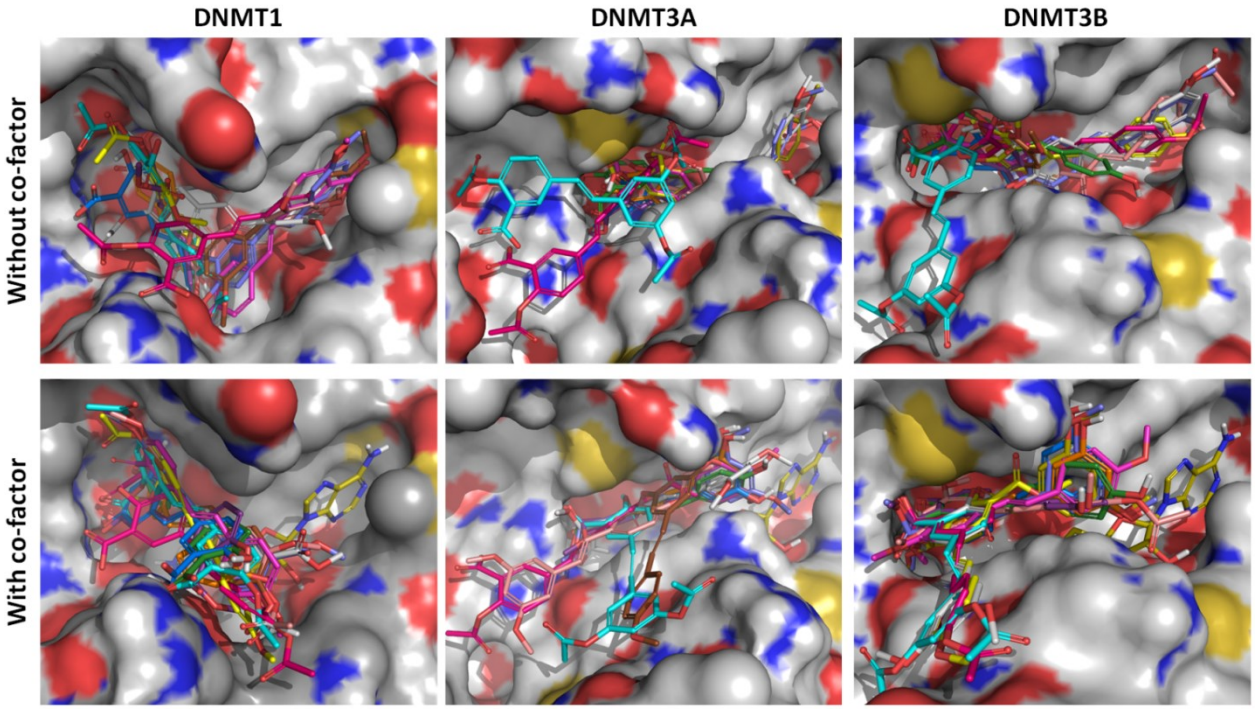


Figure 5-4: Comparison of the different binding modes in the presence and absence of co-factor SAH of DNMT1, DNMT3A, and DNMT3B. Compounds **3** (magenta), **4** (yellow), **5** (pink), **6** (gray), **7** (violet), **8** (green), **9** (blue), **10** (purple), **11** (pink), **12** (cyan), **TMS** (brown), and resveratrol (orange) as predicted by AutoDock 4.2.

Compound	DNMT1		DNMT3A		DNMT3B	
	Without cofactor	With cofactor	Without cofactor	With cofactor	Without cofactor	With cofactor
3	-7.52	-6.58	-8.11	-7.08	-7.32	-7.16
4	-8.47	-5.83	-8.99	-5.84	-8.02	-6.78
5	-8.26	-6.26	-8.52	-6.27	-8.76	-6.17
6	-7.05	-6.39	-5.06	-6.29	-8.28	-7.02
7	-7.44	-6.14	-8.40	-5.76	-7.82	-7.85
8	-8.37	-6.91	-9.41	-7.84	-9.05	-8.54
9	-8.13	-7.67	-9.66	-8.17	-8.85	-7.54
10	-7.43	-6.42	-9.27	-7.01	-8.99	-8.82
11	-8.94	-7.21	-7.65	-7.72	-9.33	-8.23
12	-7.95	-8.96	-8.42	-7.50	-7.88	-8.44
TMS	-7.36	-6.02	-7.95	-5.67	-8.23	-7.53
Resveratrol	-7.74	-5.83	-9.03	-6.57	-7.95	-6.22

Table 5-3: Calculated free binding energies of resveratrol-salicylate analogues in human DNMTs.

In a previous study [38], NSC14778 was predicted to form multiple hydrogen bonds with key amino acids, particularly Arg174 and Cys88, inside the active site of DNMT1. These residues have played an essential role in explaining the mechanism of DNA methylation [38]. Additionally, NSC14778 was thought to block the active site of DNMT1 by non-covalent interactions [38]. The fact that our resveratrol-salicylate analogues showed less favorable binding energies on DNMT1 (**Table 5-3)** and inactivity against DNMT1 *in vitro* (**Table 5-2)** might suggest that the mechanism of action of resveratrol-salicylate derivatives is different from that of methylenedisalicylic acid NSC14778.

Compounds	IC_{50} (μM)[1]				
	HT-29	HepG2	SK-BR-3	MCF 10A	HUVEC
3	33.1 ± 40.3	>100	6.4 ± 2.1	1.6 ± 0.46	30.3 ± 9.2
4	40.1 ± 24.0	52.2 ± 11.9	64.3 ± 35.1	ND[2]	ND[2]
5	28.9 ± 15.4	>100	ND[2]	ND[2]	ND[2]
6	>100	>100	ND[2]	ND[2]	ND[2]
7	>100	>100	ND[2]	ND[2]	ND[2]
8	>100	>100	ND[2]	ND[2]	ND[2]
9	>100	>100	ND[2]	ND[2]	ND[2]
10	44.8 ± 15.8	19.1 ± 6.1	11.3 ± 2.5	27.5 ± 16.3	>100
11	>100	>100	ND[2]	ND[2]	ND[2]
12	>100	>100	ND[2]	ND[2]	ND[2]
Resveratrol	>100	54.9 ± 3.3	>100	>100	>100
TMS	16.6 ± 12.6	>100	>100	83.5 ± 75.4	80.0 ± 50.4

Table 5-4: [1]Concentration (μM) of the test compounds required to inhibit cell proliferation by 50 % (IC_{50}) using the MTT assay. Each IC_{50} value represents the mean ± S.D. of three different experiments in triplicate. To generate the cell proliferation inhibition curves, six concentrations (in the 0.03 to 125 μM range) were used for each compound. IC_{50} values were generated using GraphPad v6 Prism software. [2]ND = not determined.

5.3.3 Cell proliferation inhibition in culture cells

The promising DNMT inhibitory profile observed for these resveratrol-salicylate analogues, along with our previous finding on their abilities to inhibit the CYP1A1 enzyme [39], and the observation that epigenetic modifications in cancer cells are essential for cell proliferation led us to evaluate the effects of these compounds on *in vitro* cell proliferation. We used three different human cancer cell lines: HT-29 cells (colorectal), HepG2 cells (liver), and SK-BR-3 cells (breast). DNMT-mediated epigenetic regulations have been recently confirmed in these cell lines [26, 67, 68]; the results are summarized in (**Table 5-4**). Interestingly, compound **10** exerted a stronger cell proliferation inhibition than that demonstrated by the parent resveratrol in all three cancer cells, and it was more active than TMS on HepG2 and SK-BR-3 cells. In this regard, it has been reported that TMS, being a more lipophilic stilbene than resveratrol (and consequently, more likely to cross cell membranes), demonstrated a higher cell proliferation inhibition than

resveratrol in cancer cells [47, 59]. In non-cancerous cultured cells, **10** showed considerable toxicity in MCF 10A (IC_{50} = 27.5 μM, which is considered a side effect) and showed an IC_{50} higher than 100 μM in HUVEC cells. This difference in the way in which compound **10** affects primary cells might indicate that it exerts a certain tissue-specific toxicity. However, additional studies are needed to further establish its safety profile.

Resveratrol's ability (as well as the ability of compound **10**) to inhibit DNMT3 activity does not exclude other mechanisms by which it could decrease cell proliferation. In fact, there is a considerable evidence backing up the multi-target profile exerted by resveratrol. This may be applicable to its salicylate hybrid **10**; nevertheless, further studies are required to illustrate other potential anti-proliferative mechanisms modulated by this compound.

In Chapter Four, we discussed the CYP1A1 inhibitory profile of compounds **3**-**12**, and elaborated on the chemical features required for hybrid molecules to exert inhibitory activity on CYP1A1. Compound **10** was not as effective as other molecules in inhibiting the CYP isoform; however, the binding interactions make this molecule an effective DNMT3 inhibitor, despite its lack of activity on CYP enzymes. These observations suggest that the overall design of hybrid salicylate-resveratrol analogues is flexible enough to offer preferential inhibition against at least these two proteins (CYP1A1 and DNMT3).

5.3.4 Stability studies

5.3.4.1 Mass analysis

After we had observed the significant cancer cell proliferation inhibition exerted by the DNMT3 inhibitor (compound **10**), we aimed to qualitatively study the stability of this stilbene in the culture media with the aid of mass spectroscopy. Initially, the same maximum concentration used in the cell proliferations assays (125 μM) was used in this stability study. However, using this concentration, and after 24 hours incubation, we observed that only media masses were detected with no signs of stilbene mass (data not shown). This could be attributed to the technique's sensitivity, particularly since the sample is routinely diluted before mass detection

(so that the final concentration before detection would be less than the typical mass sample concentration (1µg/mL)). After this observation, we increased the concentration in the media to 500 µM instead of 125 µM. The results from this assay can be seen in (**Figure 5-5**). At time zero, a mass of *m/z* = 284.9 was detectable, which corresponds to compound **10** $[M-H]^-$. After one hour of incubation in the media, another signal was observed at a mass of *m/z* = 270.8 in addition to the initial mass of stilbene **10**. After four hours (the spectrum after four hours is not shown), we observed that the compound **10** mass was undetectable. However, the mass at *m/z* = 270.8 was detected and it was visible even after 24 hours incubation (see spectra after 6 and 24 hours in **Figure 5-5**). It should be noted that the masses of *m/z* = 284.9 and 270.8 $[M-H]^-$ correspond to compounds **10** and **9** respectively, which we identified previously [39]. After this stability assay, we suspected that compound **10** was likely unstable and is probably being hydrolyzed to produce compound **9**. However, the extent of hydrolysis and the exact hydrolyzed products were not precisely identified, and this is the reason for moving to the quantitative technique, HPLC.

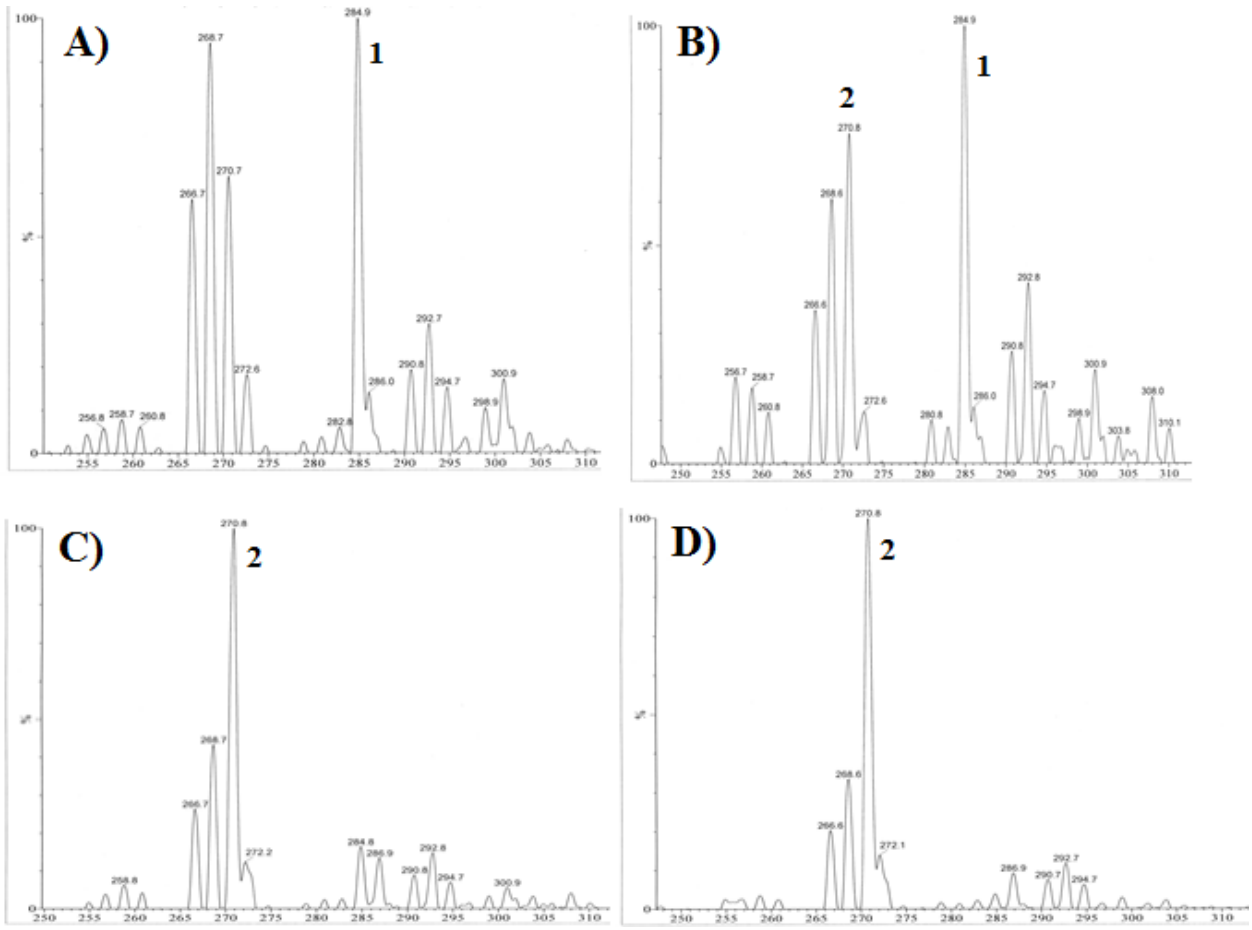


Figure 5-5: Mass spectra of compound 10 incubated in culture media at 37°C after time zero **(A),** 1 hour **(B),** 6 hours **(C)** and 24 hours **(D).** Compound **10** (dissolved in DMSO) was added into the culture media at a final concentration of 500 µM. Mass (1) is for compound 10 at m/z = 284.9 $[M\text{-}H]^-$ and mass (2) is for compound 9 at m/z = 270.8 $[M\text{-}H]^-$. The masses for the two molecules were identified previously.

5.3.4.2 HPLC analysis

To quantitatively evaluate the stability of compound **10**, we initially used the reported HPLC method for TMS [48, 69]. We reproduced these chromatographic conditions (method A) to study

the stability of compound **10** in culture media. To make sure that this method is reproducible, we first used it for TMS. Using method A, we found that TMS had a retention time of 7.4 min, similar to what had already been reported (7.0 min [48]) (data not shown). After that, we tested the stability of compound **10** in culture media using the same conditions. The results are shown in **Figure 5-6**. This method was developed for relatively lipophilic molecules such as TMS [48, 69], and for polar molecules such as **10**, we suspected that this stilbene might elute sooner than TMS under these conditions. Indeed, compound **10** (dissolved in acetonitrile) showed a retention time of 2.3 min when it was injected into the HPLC column.

After we had identified the compound **10** peak, we incubated **10** in a culture media at 37°C and tested the remaining percentage after 0, 1, 3, 6, and 12 hours. We observed that **10** seems to decompose after as soon as one hour because the percentage of the stilbene remaining after one hour of incubation was 73%. These results are consistent with the above mass analysis study. After six hours, only 11% of this compound remained. By 12 hours, the peak had completely disappeared. We tried to investigate the product(s) of **10**'s decomposition. By analyzing the chromatogram after 12 hours, we could see only the signals of the media with no additional peaks.

We suspected that the product(s) of **10** degradation could be hidden under the media peaks. For that reason we decreased the polarity of the solvent mixture and increased the separation time. Unfortunately, these changes in chromatographic conditions did not sufficiently separate the media peaks or even delay molecule **10**'s retention time (data not shown). We concluded that this method is likely not ideal to analyze such molecules. Our reasons are as follows: First, our ultimate goal is to investigate the stability of **10** in plasma. However, plasma peaks have been reported to be around 1.8 to 2.5 min using this method [48], which would likely interfere with **10**'s peak and/or its degradation products. Second, **10** is a relatively polar and from a structural perspective, it is similar to resveratrol. Based on that information, we knew that the HPLC methods described in the literature for resveratrol could be applied to **10**. Therefore, we moved to method B.

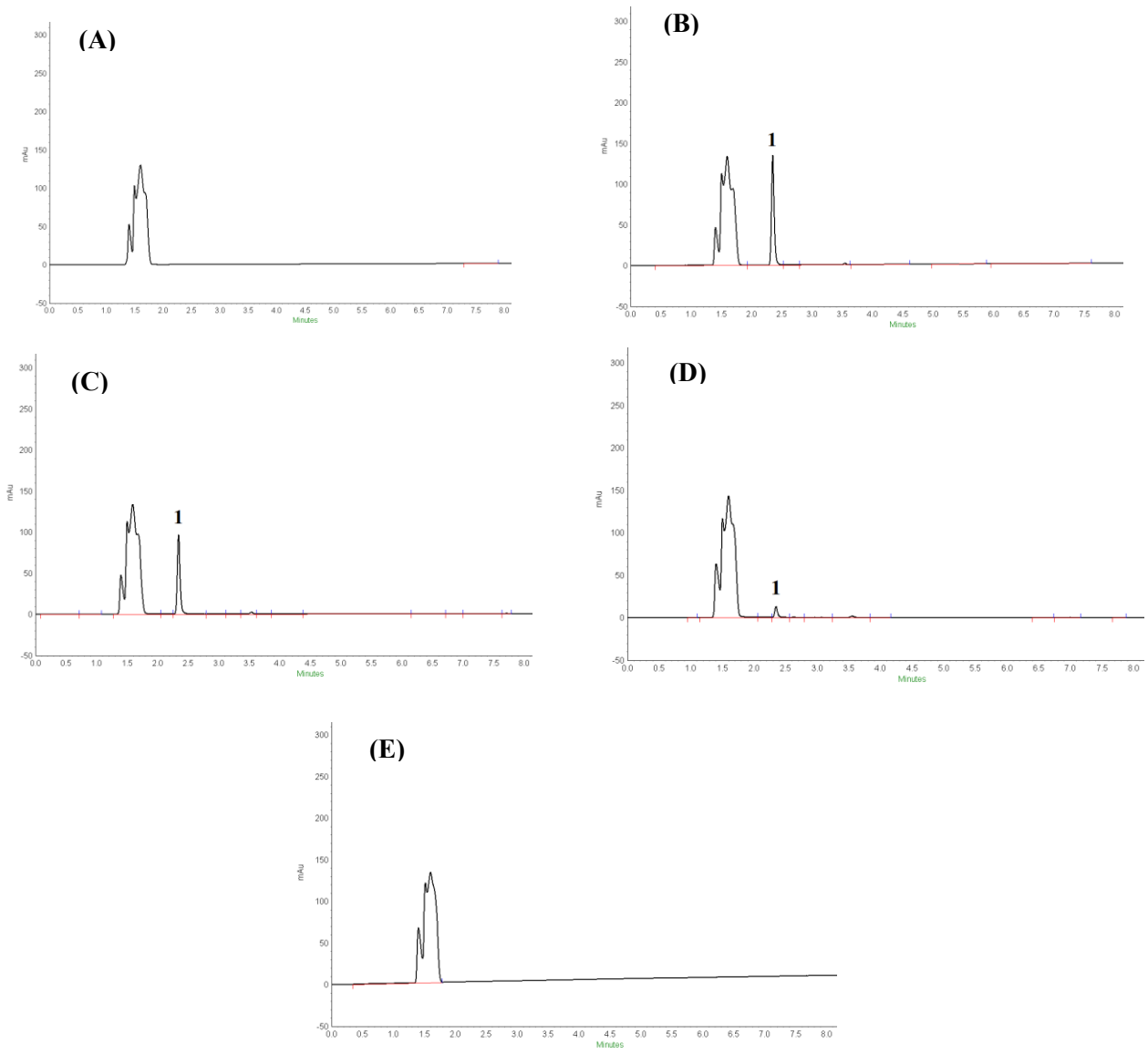


Figure 5-6: chromatograms of compound 10 (125 µM) after incubation in culture media at 37°C. (A) is blank media with only the vehicle (DMSO); **(B), (C), (D), (E)** are compound **10** samples at 0, 1, 6, and 12 hours respectively. Peak (1) is compound **10**.

Method B has been recently developed and applied for resveratrol analysis in commercial sources [49]. It uses methanol and water (containing 0.1% formic acid) as a mobile phase and the chromatography is performed at 35°C (see method B in material and methods). Using this method, we obtained a resveratrol peak at 5.3 min (reported 5.8 min [49]) (data not shown). Next, we injected a pure sample of **10,** which showed a retention time of 10.8 min (**Figure 5-7**). After that, we incubated **10** in rat plasma at 37°C and measured the percentage of compound remaining after 0, 1, 3, 6, 12, and 24 hours. The percentages of stilbene remaining after 1, 3, 6, 12, and 24 hours were 88, 60, 32, 9 and 0.8% respectively. Additionally, we observed a new peak (at around 8.1 min), which was detectable after one hour of incubation (**Figure 5-7**). This peak increased in intensity within time, and was visible even after 24 hours of incubation. Compound **10** is a methyl ester and because of our previous results using mass spectroscopy suggested the hydrolysis of compound **10** to produce **9**, we suspected that this new peak might corresponds to the stilbene bearing free carboxylic acid (compound **9**). Indeed, when we injected an authentic sample of stilbene **9** in acetonitrile (as well as in rat plasma), it showed the same retention time observed for the new peak (8.1 min). This suggests that compound **10** has a relatively low stability profile in plasma. However, despite its low stability, it still produced compound **9** (which also exhibited DNMT3 inhibitory activity) after hydrolysis in rat plasma.

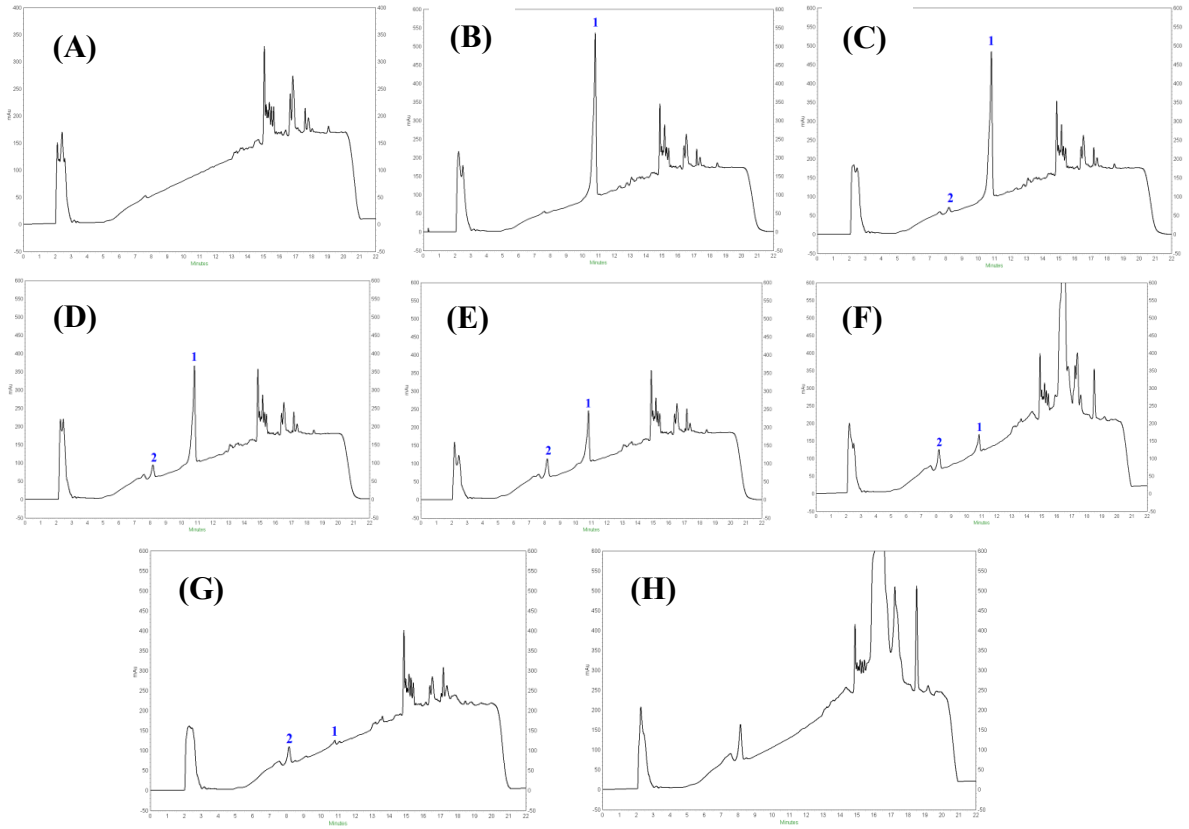


Figure 5-7: chromatograms of compound 10 (125 µM) after incubation in rat plasma at 37°C. (A) Blank plasma sample spiked with only the vehicle (DMSO) while **(B), (C), (D), (E), (F),** and **(G)** are plasma samples of **10** after 0, 1, 3, 6, 12, and 24 hours respectively. **(H)** Fresh plasma sample spiked with 50 µM of pure compound **9.** Peak (1) is compound **10** and peak (2) is the degradation product which, after being compared to a pure sample of compound **9** in plasma (chromatogram (**H),** is identified as compound **9**.

5.4 Conclusion

We showed that the hybrid salicylate-resveratrol scaffold is a promising alternative to the parent stilbene resveratrol and its methylated analogue TMS as a DNMT inhibitor. Derivatives **9** and **10** showed a significant and selective inhibitory profile on DNMT3A and DNMT3B enzymes, which is 2-4 times more potent than that exerted by resveratrol under the same experimental conditions. Structure-activity relationships showed that free hydroxyl groups are required to exert DNMT3 inhibition. This pattern is better in analogues with phenols in positions 3- and 5- of a stilbene. The salicylate group in resveratrol's structure produced an enhanced inhibitory profile only when free phenol groups were on the stilbene. The stability of **10** in rat plasma seems to be limited, but this stilbene is hydrolyzed to compound **9,** which has DNMT inhibitory properties. Compound **10** showed an improved *in vitro* cell proliferation inhibition compared to resveratrol and TMS on at least two human cancer cells, suggesting that compound **10**, and possibly compound **9**, are promising candidates worth evaluating *in vivo*, to further elucidate their potential anticancer/chemopreventive properties.

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Chapter 6: Anti-inflammatory and antioxidant properties.

This chapter is published as **Fahad S. Aldawsari**, Rafael Pazzinatto Aguiar, Luiz Alexandre Marques Wiirzler, Rodrigo Aguayo-Ortiz, Naif Aljuhani, Roberto Kenji Nakamura Cuman, José L. Medina-Franco, Arno G. Siraki and Carlos A. Velázquez-Martínez. "Anti-inflammatory and antioxidant properties of a novel resveratrol-salicylate hybrid analog." *Bioog. & Med. Chem. Lett.*, **2016**, 26(5), 1411. I performed the in vitro COX enzyme inhibition, the antioxidant assays, and I wrote the manuscript. Chattopadhyay, M. performed the NF-κB inhibition in the HT-29 cells, Salla, M. conducted the NF-κB inhibition in the HCT-116 cells, Goldhahn, K. performed apoptosis in the Jurkat cells, Aguiar, R.P. and Wiirzler, L.A.M. conducted the in vivo anti-inflammatory experiments, and Aguayo-Ortiz, R. conducted the docking study inside the COX enzymes. All of the authors contributed to the manuscript edits. Velázquez-Martínez, CA was the supervisory author and was involved in the manuscript organization.

6.1 Introduction

Chronic inflammation and deregulated cellular oxidative stress are two of the conditions commonly associated with the pathophysiology of cancer, atherosclerosis, diabetes, Alzheimer's disease, obesity, pulmonary disease, and many others [1]. Additionally, prolonged exposure to reactive oxygen species (ROS) can trigger the overexpression of numerous transcription factors that in turn activate many genes involved in cell cycle regulation, inflammation, and chemotaxis [1]. Polyphenols and other natural agents can counteract stress-induced tissue injury by acting as antioxidant and anti-inflammatory agents [2].

Resveratrol is a natural phytoalexin produced by many plant species as a defensive agent against insults such as fungi and UV radiation. The effects of resveratrol in mammalian cells also exert a protective role. Resveratrol exerts its pharmacological actions through several mechanisms regulating many biological processes, including but are not limited to hemostasis [3], glucose metabolism [4], cell division [5], neuroprotection [6], free radical scavenging [7], and inflammation [2]. The scientific literature describes resveratrol as a "multi-target" compound [8]. There is a significant amount of research establishing the anti-inflammatory activity of resveratrol, both *in vitro* and *in vivo* [2]. Moreover, resveratrol can modulate inflammatory mediators such as NF-κB, TNFα, nitric oxide (NO) production, COX-2, IL-1, PGE_2, MPO, ROS, and HO-1 [9].

Nevertheless, despite the considerable number of research studies published over the last two decades, the therapeutic potential of resveratrol remains elusive, at least in part due to its low oral bioavailability and significant metabolic decomposition [10]. In recent years there has been a debate about the efficacy of resveratrol [11, 12], limiting its clinical application. Consequently, most of the medicinal chemistry projects in this field have concentrated on developing resveratrol derivatives with better pharmacokinetic profiles and enhanced potencies. In this regard, cancer is a common target for these newly designed resveratrol derivatives. There are only a few reports describing concerted efforts in medicinal chemistry aimed at improving resveratrol's anti-inflammatory profile. However, due to the proven association between cancer and inflammation [1], the development of new resveratrol derivatives could very well have implications for both areas of research. Some representative resveratrol derivatives with potential anti-inflammatory activity are triple-bond resveratrol derivatives [13], the resveratrol-related

stilbenes piceatannol and triacetoxystilbene [14], synthetic analogues RSVA405 and RSVA314 [15], and the resveratrol trimer α-Viniferin [16].

Another important group of drugs that has been used to target the inflammation-to-cancer process is non-steroidal anti-inflammatory drugs (NSAIDs) [17]. Because of the observation that key inflammatory mediators are overexpressed in cancer tissues [18], the use of NSAIDs was a logical step forward. The prototypical example that illustrates this approach is the use of aspirin to prevent or delay the prevalence and severity of colorectal cancer [17]. Aspirin targets similar pathways to those modulated by resveratrol, for example, the inhibition of the cyclooxygenase-1 (COX-1) and COX-2 enzymes, and modulation of the NF-κB pathway. Nevertheless, it should be noted that those targets modulated by both aspirin and resveratrol respond to the pharmacological effects of these drugs but at different concentrations.

In two of our recent publications, we described the synthesis and biological evaluation of a new series of hybrid salicylate-resveratrol derivatives, designed to combine the chemical and pharmacological features of the parent counterparts. This "multi-target" approach is described in the literature [8] and is based on the premise that modulating several pathways underlying a disease state is better than targeting a single receptor or enzyme. In one of our previous publications, we described the inhibitory profile of ten different salicylate-resveratrol derivatives on the enzymatic activity of two distinct proteins, CYP1A1 [19] and DNMT [20]. As part of interdisciplinary research aimed to develop multi-target agents with potential anti-cancer activity, we report in this chapter the anti-inflammatory and antioxidant properties of these salicylate-resveratrol hybrids.

In this chapter, we evaluated whether these compounds can reduce the concentration of free radicals (using the DPPH assay) and intracellular reactive oxygen species (ROS). Furthermore, we assessed the *in vitro* inhibitory profile of hybrid molecules on both COX-1 and COX-2 enzymes, as well as their ability to downregulate NF-κB activity. Then, we studied the potential of these compounds to reduce the carrageenan-induced inflammatory response in two mouse models. The most active compound, compound **10**, was compared to resveratrol and the methyl analogue 3,4',5-*trans*-trimethoxystilbene (TMS) that previousely demonstrated improved

anticancer potency compared to resveratrol [21]. Our results demonstrated that compound **10** is a promising analogue of resveratrol that possesses significant anti-inflammatory and antioxidant properties.

6.2 Materials and methods

6.2.1 Chemistry

The chemical synthesis of resveratrol-salicylate analogues was conducted as described in Chapter 3.

6.2.2 Cell culture

HepG2 (Human hepatoma cells (ATCC HB-8065), VA, USA), HCT-116 (human colorectal carcinoma cells (ATCC CCL-247), VA, USA) and HT-29 (human colorectal adenocarcinoma cells (ATCC HTB-38), VA, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/mL penicillin, and 100 µg/mL streptomycin. Jurkat cells were kindly provided by K Schmetterer (Medical University, Vienna, Austria) and were cultured in a RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin (Life Technologies, MA, USA). Cells were grown in 75-cm^2 tissue culture flasks at 37°C and under 5% CO_2 in a humidified incubator.

6.2.3 Radical scavenging using DPPH method

We used the colorimetric method of DPPH (2,2-diphenyl-1-picrylhydrazyl), as this technique is routinely used to study radical scavenging properties of potential molecules [22]. DPPH is a mixture of commercially stable nitrogen radicals which have UV-vis absorption at 517 nm. Upon reduction, the solution color fades. This change can be monitored using a spectrophotometer [22]. Twenty µl of resveratrol or its analogues in methanol were added to 180 µl of DPPH (400 µM) in a 96-well microtitre plate (final concentrations ranged from 0.06 to 200 µM). The plate was then incubated in the dark at 37 °C for 30 minutes, and the absorbance change at 517 nm was measured. Methanol was used as a negative control and the percent of radical scavenging was calculated as follows:

Percent of radical scavenging activity (%) = $(A_{blank}-A_{sample})/A_{blank}$ x 100

Where A_{blank} and A_{sample} are the absorbances of the reaction mixture without and with resveratrol analogues respectively. The percent of scavenging is expressed as the mean ± S.D. of three different experiments, in triplicate.

6.2.4 Measurement of intracellular ROS

The intracellular ROS scavenging properties of resveratrol analogues was evaluated using the oxidant-sensitive fluorescent probe 2',7'-dichlorofluorescein diacetate (DCF-DA), using a recently reported method [23] with minor modifications. Briefly, HepG2 cells (about 4 x 10^4 cells/well) were seeded into a 96-well plate and allowed to attach overnight. Then, resveratrol analogues dissolved in DMSO were added to the cells at final concentrations of 2.5, 5 and 10 μM and incubated for 20 hours (DMSO concentrations were adjusted to be < 0.5% v:v). After that, cells were washed with PBS before stimulation with H_2O_2 (1 mM final concentration) and incubated for 30 minutes at 37 °C. Then, cells were washed two times with PBS followed by treatment with DCF-DA (20 μM final concentration) and incubated for another 30 minutes. Finally, the fluorescence change was measured at excitation and emission wavelengths of 485 and 528 nm respectively. In a different experiment, the effect of resveratrol analogues on HepG2 cells without H_2O_2 stimulation was performed. The above procedure of cell treatment was followed by direct DCF-DA (20 μM) treatment. Then, fluorescence was measured after 30 minutes of incubation. The data are expressed as the percentage of the ROS level relative to H_2O_2- and vehicle-treated cells for the first and second experiment respectively. The results are expressed as the mean ± SEM (n = 8).

6.2.5 COX screening assay

Resveratrol-salicylate derivatives were evaluated for their ability to inhibit COX-1/COX-2 enzymes *in vitro* using the COX Inhibitor Screening Kit provided by Cayman Chemicals (Catalog No.560131) according to the manufacturer's instructions. Briefly, 10 μL of resveratrol derivatives dissolved in DMSO (final concentrations range of 0.02 to 100 μM) were incubated with 10 μL of either ovine COX-1 or human recombinant COX-2 in a reaction buffer (0.1 M Tris–HCl, pH = 8.0) and 10 μL of heme for 10 minutes at 37 °C. The reaction was initiated by the addition of 10 μL of arachidonic acid and incubated for 2 minutes to produce PGE_2. Then, the

reaction was quenched by the addition of 10 μL of 1 M HCl followed by the addition of 20 μL of saturated stannous chloride to reduce the PGE_2 to the most stable $PGF_{2\alpha}$. This was quantified using an ELISA assay, which was compared to a standard curve of PG performed on the same plate. IC_{50} values were calculated from the mean of two determinations, and the dose-response curve was analyzed using GraphPad Prism software.

6.2.6 Docking study

Homology modeling: Several three-dimensional structures homologous to the *Homo sapiens* COX isoforms are deposited in the Protein Data Bank (PDB) [24]. In this study, we carried out the homology modeling process with a Swiss-Model server [25], using the amino acid sequence of *H. sapiens* COX-1 (UniProt: P23219) and COX-2 (UniProt: P35354) and employing the *Ovies aries* COX-1 (PDB: 4O1Z) [26] and *Mus musculus* COX-2 (PDB: 4FM5) [27] crystallographic structures as templates, respectively. The final models were evaluated by the QMEAN4 and GMQE scores [28] to obtain an estimation of the global and local quality of the model, whereas the quality of the protein geometry was assessed using MolProbity [29]. Finally, the models were prepared and submitted to a geometry optimization with 1000 steepest descent steps, and 100 conjugate gradient steps, using the AMBER99SB force field implemented in the UCSF Chimera 1.9 [30].

Ligands: Compound **10** and resveratrol were built and submitted to a geometry optimization protocol employing the AMBER99SB force field in UCSF Chimera 1.9.

Docking: Molecular docking studies were performed using AutoDock 4.2 software [31]. In these studies, we evaluated the compounds in the COXs' catalytic domains. We used a grid box of 65 x 65 x 65 points with a grid spacing of 0.375 Å that covers the catalytic pocket. The Lamarckian genetic algorithm was used as a search method. Thirty runs were carried out with a maximal number of 50,000,000 energy evaluations and an initial population of 150 conformers. The best binding modes with the lowest binding free energy values for each molecule were selected for the analysis.

6.2.7 NF-κB inhibition

6.2.7.1 In HCT-116 cells

HCT-116 cells were transfected with an NF-κB plasmid for 48 hours before the treatment with resveratrol analogues (20μM) for 24 hours. After that, cells were stimulated with LPS for 4 hours and then the luciferase activities were measured using a dual luciferase reporter assay kit (Promega, WI, USA) according to the manufacturer's instructions. The NF-κB luciferase-construct used was pGL3-3X (Promega) while the normalization plasmid was pRnull-Renilla (Promega). Results are expressed as mean ± SEM of triplicate experiments.

6.2.7.2 In HT-29 cells

HT-29 cells ($5x10^6$ cells/well) were seeded overnight in 10 cm dishes followed by incubation with various concentrations of compound **10** for 24 hours. Then, the nuclear protein was extracted using an extraction kit (Cayman Chemical Co, MI, USA) followed by protein concentration determination using a Bio-Rad reagent (Bio-Rad Laboratories). The nuclear extracts were stored at -80°C until use. NF-κB p65-DNA binding activity was determined using an NF-κB (p65) transcription factor assay kit (Cayman Chemical Co.) in which a specific double-strand DNA sequence containing an NF-κB response element was immobilized in a 96-well plate. Briefly, fifty μg of nuclear proteins were added to wells prefilled with a transcription factor buffer and were incubated overnight at 4°C. NF-κB binding was detected by adding an NF-κB primary antibody followed by incubation at room temperature for one hour. After that, wells were washed and the secondary antibody (conjugated to horseradish peroxidase) was added, followed by incubation at room temperature for another hour. Finally, 100μL of developing solution was added followed by incubation for 45 minutes with gentle shaking, after which a "stop" solution was added and the absorbance was recorded at 450 nm. The change in the percentage of activity of NF-κB p65-DNA was calculated in each sample relative to the untreated sample. Results are expressed as mean ± SEM of two determinations.

Antibodies against NF-κB p65 (catalogue number D14E12) were purchased from Cell Signalling Technology (MA, USA) and used to immunodetect the NF-κB p65 protein in the nuclear extract.

6.2.8 Cytotoxicity in Jurkat cells

A CellTox™ Green Cytotoxicity Assay (Promega, WI, USA) was used in this experiment. Cells ($1x10^5$ cells/well) were cultured in a media containing 0.2% CellTox green dye in a 96-well plate. Resveratrol, TMS, or compound **10** was incubated with cells at different concentrations for 24 hours before the fluorescence was measured at excitation and emission wavelengths of 485 and 520 nm respectively, using GloMax (Promega, USA). The results are expressed as mean ± SEM of triplicate experiments.

6.2.9 Apoptosis in Jurkat cells

Apoptosis was assessed in Jurkat cells using Annexin-V/7-amino-actinomycin D (7-AAD) staining. Briefly, Jurkat cells ($1x10^5$ cells/well) were grown for 24 hours in the presence or absence of different concentrations of resveratrol, TMS, or compound **10**. Then, the cells were harvested and 100 μL of cell suspension were labelled in the dark for 20 minutes with a Muse™ Annexin-V and Dead cell reagent (Merck Millipore, Germany). Subsequently, detection of Annexin-V/7-AAD positive cells was performed using a Muse™ Cell Analysis (Merck Millipore). Cells stained with Annexin-V only were identified as early apoptotic while double-stained (Annexin-V and 7-AAD) cells were identified as late apoptotic. Results are expressed as mean ± SEM of duplicate experiments.

6.2.10 Animals

Swiss or BALB/c mice (weighing 20-25 g) were provided by the Central Animal House of the State University of Maringá, Brazil. The animals were housed at 22 ± 2 °C under a 12h/12h light/dark cycle. Prior to the experiments, the animals were fasted overnight, with water provided *ad libitum*. The experimental protocols were approved by the Ethical Committee in Animal Experimentation at the State University of Maringá, Brazil (CEAE/UEM 017/2013).

6.2.11 MTT assay to determine cytotoxicity on leukocytes

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-2H-tetrazolium bromide) assay is based on the mitochondrial enzymatic reduction of tetrazolium dye by viable cells. This assay is frequently used to measure cell viability. Leukocytes were obtained from the peritoneal cavity of male Swiss mice, 4 h after being injected with zymosan (1 mg/cavity, i.p.). Briefly, leukocytes (5

× 10^5 cells/well) were treated with resveratrol, TMS, and compound **10** at different concentrations (10, 30, 60, and 90 μg/mL), dissolved in DMSO for 90 min at 37 °C in 5% CO_2. Then, 10 μL of MTT (5 mg/mL) were added to each well. After 2 h, 150 μL of the supernatant were removed, and 100 μL DMSO were added to each well. The cells were incubated at 25°C for an additional 10 minutes, and absorbance was measured using a Biochrom Asys Expert plus microplate reader (Asys) at a wavelength of 540 nm. The percentage of viable cells was determined using the following formula:

$$\%\text{Viable Cells (CV\%)} = (A_{treatment} - A_{blank})/(A_{control} - A_{blank}) \times 100$$

Where $A_{treatment}$ is the absorbance of treated cells, A_{blank} is the absorbance of blank wells and $A_{control}$ is the absorbance of control cells (vehicle treated). The data are expressed as mean ± SEM of triplicate experiments.

6.2.12 Carrageenan-Induced Peritonitis

BALB/c mice, in a group of four animals each, were treated with the experimental compounds using two doses (10 and 20 mg/kg). Resveratrol, TMS, and compound **10** were dissolved in a mixture of DMSO:water in a ratio of (10:90) and orally gavaged into mice 30 min before the intraperitoneal injection of the carrageenan solution (500µg/mice). The animals were euthanized 4 h later and the peritoneal cavity was washed with 2 mL of phosphate-buffered saline (PBS) containing ethylenediaminetetraacetic acid (EDTA). The leukocyte count was determined in the fluid recovered from the peritoneal cavity. The results are expressed as mean ± SEM for each group.

6.2.13 Carrageenan-induced paw edema

We used the original method of Winter, C.A. et al. [32] with minor modifications. BALB-c mice, in a group of four animals each, were orally treated with resveratrol, TMS, and compound **10** at 10 and 20 mg/kg doses. The derivatives were dissolved in a mixture of DMSO and methylcellulose (MC) [4 mL of DMSO + 0.5 mL of MC] and administered 30 min before the induction by intraplantar injection (intradermally) of carrageenan solution (500 µg/paw), dissolved in 0.9% saline into the left hind paw and the same volume of the vehicle (0.9% saline) into the right hind paw. The volume of both paws was measured using a plethysmometer (Ugo Basile 7140, CA, USA) 1, 2, 4, and 6 hours after the irritant agent was injected. The increase in

the volume of the paw was calculated by subtracting the volume of the paw injected with saline (control paw) from that of the paw injected with the phlogistic agent. The data are expressed as mean ± SEM for each group.

6.2.14 Measurement of Nitric Oxide (NO)

The level of NO production was monitored by measuring the nitrite level in the culture medium or paw tissues using a Griess reaction. The supernatant of homogenate (50 µL) was incubated with equal volumes of Griess reagent mixtures (1% sulfanilamide in phosphoric acid (5%) and N-1-naphthylethylenediamine dihydrochloride (0.1%) in water) at room temperature for 10 min. The absorbance was measured at 550 nm using a microplate reader. NO concentrations were calculated from the sodium nitrite standard curve performed on the same day. Data are expressed as the µM concentration of NO^{2-} and presented as mean ± SEM for each group.

6.2.15 Measurement of Myeloperoxidase (MPO) Activity

The MPO activity was assessed in the supernatant of tissue homogenates, obtained from the plantar tissue of mice paws (control and treated), according to the technique described by Bradley et al. [33]. The tissues were placed in a 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethyl-ammonium bromide (1 mL per 50 mg of tissue) in a Potter homogenizer. The homogenate was vortexed and then centrifuged for 20 minutes at 6000 g at 4 °C. Ten microliters of the supernatant obtained were placed in a 96-well plate (in duplicate) and added to a 200 µL of buffer containing o-dianisidine dihydrochloride (16.7 mg), double distilled water (90 mL), a potassium phosphate buffer (10 mL) and H_2O_2 1% (50 µL). The enzyme activity was determined using an end-point technique by measuring the absorbance at 460 nm in a microplate reader (ASYS - Biochrom Expert Plus - G020150). Data is expressed as mean ± SEM for each group.

6.2.16 Statistical analysis

The analysis of results was performed using GraphPad Prism software (GraphPad Software Inc., CA, USA). A one-way analysis of variance (ANOVA) followed by Tukey's post-test was used to determine the significance between treated and untreated (control) groups. The difference was considered significant when $p < 0.05$.

6.3 Results and discussion

Nature is an important reservoir for bioactive molecules. Resveratrol is one of the many different polyphenols targeting many cellular pathways and, therefore, an important lead molecule with potential therapeutic applications. One of the main drawbacks of resveratrol is its unfavorable pharmacokinetic profile [10], which has prompted significant research efforts to develop drug candidates with similar structures. One of the many different approaches described in the literature is to chemically modify resveratrol to produce "hybrid" molecules harnessing the bioactive scaffold of the polyphenol with that of another drug with a synergistic profile. Some examples are the resveratrol-octyl methoxycinnamate [34], resveratrol-nitroxide derivatives [35], a combination of resveratrol with vitamin E [36], and resveratrol-coumarin hybrids [37].

Our group has recently designed a new class of resveratrol hybrids based on adding a carboxylic acid group (-COOH) adjacent to one of the phenol groups present in resveratrol, to obtain salicylate-like chemical structures. We also proposed an additional acetylation of the phenol group in the newly formed salicylate moiety, to produce aspirin-like resveratrol derivatives. Some of these molecules exert a potential chemopreventive activity by inhibiting the activity of CYP1A1 (a carcinogen-activating CYP450 protein), and the DNA methyltransferase (DNMT) enzyme, the latter of which is responsible for inactivating tumor suppressor proteins [38, 39]. In this chapter, we evaluated the anti-inflammatory profile of these resveratrol-salicylate hybrids, as well as their ability to decrease intracellular oxidative stress [1].

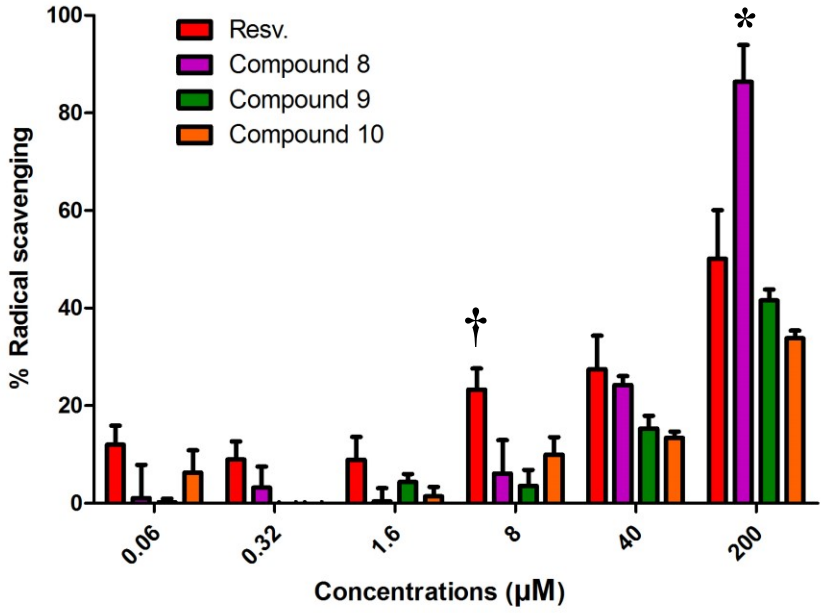


Figure 6-1: DPPH radical scavenging activities of resveratrol-salicylate analogues. The above indicated concentrations of resveratrol analogues in methanol were incubated with DPPH (400 μM) for 30 minutes at 37 °C before the absorbance was read at 517 nm. Results are expressed as mean ± S.D. of three different experiments, in triplicate. †$P < 0.05$ compared to compound **8** at the same concentration. *$P < 0.05$ compared to resveratrol at the same concentration.

6.3.1 Antioxidant activity

6.3.1.1 DPPH assay

The mechanism by which resveratrol reduces intracellular ROS is associated with the expression and activation of antioxidant proteins, and, to a lesser extent, to the direct quenching (scavenging) of free radicals [40]. The compound 2,2-diphenyl-1-picrylhydrazyl (DPPH) is a relatively stable nitrogen radical that is frequently used in free radical scavenging assays, to determine the ability of test drugs to exert a potential antioxidant activity [22]. We used this *in vitro* assay to investigate whether resveratrol-salicylate analogues could quench free radicals using a previously reported procedure [22]. **Figure 6-1**shows the results of the DPPH assay. We screened all the ten resveratrol-salicylate derivatives. **Figure 6-1** displays the only ones that showed activity.

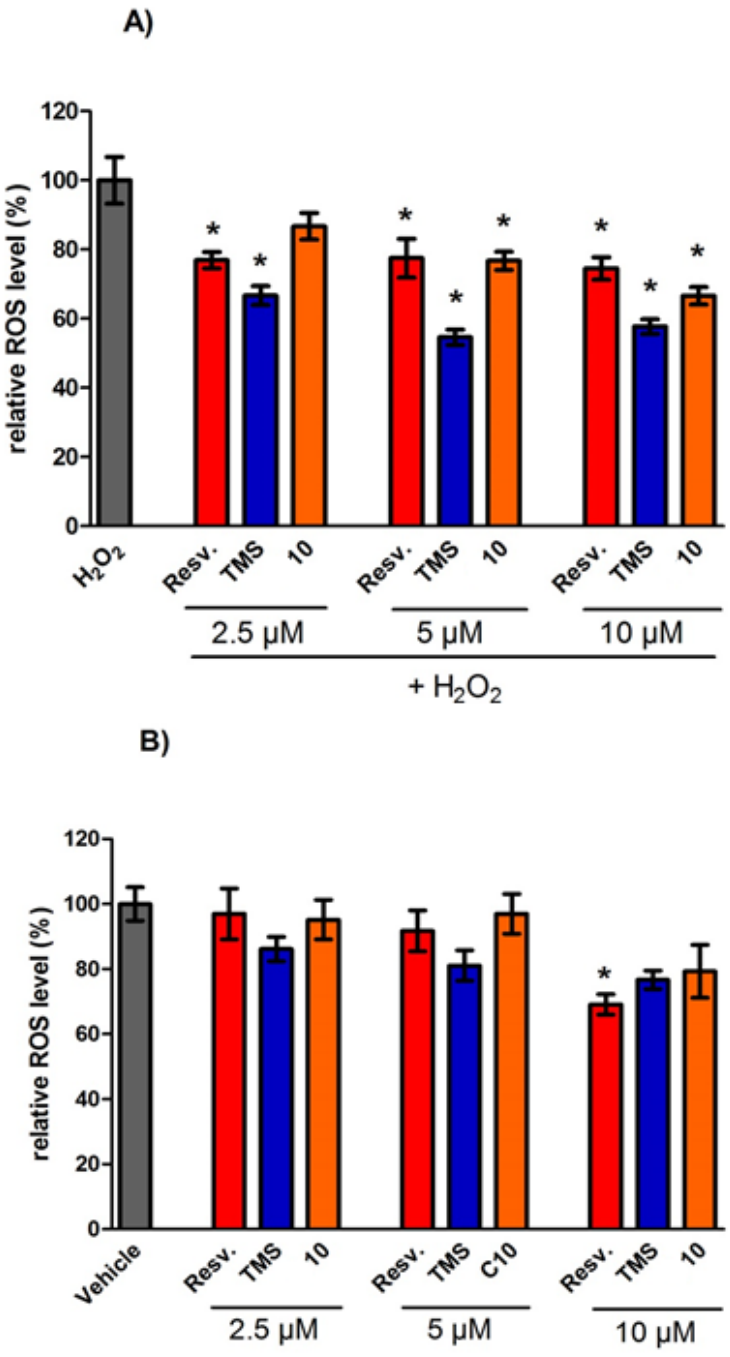


Figure 6-2: Effects of resveratrol analogues on intracellular ROS levels in HepG2 cells stimulated with A) H_2O_2 and B) non-stimulated cells.

HepG2 cells were seeded in a 96-well plate before treatment with non-toxic concentrations of resveratrol analogues (2.5, 5 and 10 µM) for 20 hours. Then, the cells were stimulated with H_2O_2 (1 mM) and incubated at 37° C for 30 minutes followed by incubation with DCF-DA (20 µM) for 30 minutes at 37°. Fluorescence was then measured at $\lambda_{ex.}$ and $\lambda_{em.}$ of 485 and 528nm respectively. For graph (B), the same procedure was followed but without H_2O_2. Results are expressed as mean ± SEM. (n = 8). *P < 0.05 compared to H_2O_2-only cells in (A) and vehicle-only cells in (B).

Compound **8** demonstrated the highest free radical scavenging activity, which was higher than that of resveratrol only at the maximum tested compound concentration (200 μM). Analogue **8** is a resveratrol-salicylate hybrid that has a 4,4'-dihydroxy substitution pattern, as opposed to the parent compound resveratrol, which has a 3,4',5-trihydroxy substitution pattern. This observation is consistent with a previous report by Fan et al. in which they reported that 4,4'-dihydroxystilbene scavenged galvinoxyl radicals to a greater extent than resveratrol [7]. Compounds **9** and **10** showed moderate free radical-scavenging properties, a bit lower than those obtained for resveratrol (**Figure 6-1**). Finally, 3,4',5-*trans*-trimethoxystilbene (TMS, a natural resveratrol derivative) did not show significant free radical-scavenging properties (not shown).

6.3.1.2 Intracellular ROS

Oxidative stress is a broad term describing the imbalance between the production of ROS or radicals in general, and their removal by endogenous antioxidant proteins [1]. The continuous exposure to ROS is associated with many disorders including cancers, obesity, cardiovascular diseases, and inflammation [1].

Ladurner et al. showed that resveratrol exerts a dual concentration-dependent antioxidant/pro-oxidant profile that also depends on the "oxidative status" of cells and tissues [40]. For example, resveratrol at a concentration of 50 μM enhanced the production of ROS by HepG2 cells [41], whereas at 10 μM it decreased ROS levels in HUVEC cells [42].

We evaluated the effects of resveratrol, TMS, and compound **10** on intracellular ROS levels in HepG2 cells, using the fluorescent DCF-DA probe in the presence and the absence of H_2O_2 [23]. We selected compound **10** based on its significant inhibitory effect on two enzymes, the DNMT enzymes (results of a previous report [20]), and the cyclooxygenase (COX) enzyme (next section). For comparison purposes, we also tested the parent resveratrol and TMS; we summarize these results in **Figure 6-2**.

We screened the test compounds at three concentrations (2.5, 5.0, and 10.0 μM), based on the observation that these concentrations did not significantly decrease the viability of the HepG2 cells after a 20 hour incubation period with the drugs (MTT assay; data not shown). When the cells were exposed to an oxidative stress insult after the addition of H_2O_2 (1 mM), we observed that all of the test drugs (resveratrol, TMS and **10**) significantly decreased the concentration of

intracellular ROS (**Figure 6-2, A**). In this regard, at 10 μM, resveratrol decreased ROS levels by 25% (compared to H_2O_2-treated cells), TMS exerted a 42% decrease, and compound **10** diminished ROS by 33%. In the absence of H_2O_2, we observed that only resveratrol (10 μM) significantly reduced the concentration of ROS (by about 30%) (**Figure 6-2, B**).

Compound	COX-1 IC_{50} (μM)	COX-2 IC_{50} (μM)	Selectivity for COX-2[1]
3	>500	>500	--
4	>500	>500	--
5	>500	>500	--
6	>500	>500	--
7	>500	>500	--
8	421.2	>500	--
9	3.2	10.3	0.31
10	4.1	1.0	4.1
11	>500	>500	--
12	>500	>500	--
Resveratrol	0.535[2]	0.996[2]	0.54
TMS	1.228[2]	1.667[2]	0.74

Table 6-1: Calculated IC_{50} values (in μM) of resveratrol analogues against ovine COX-1 and human recombinant COX-2. We used an enzyme immunoassay kit (Catalog No.560131, Cayman Chemicals Inc., Ann Arbor, MI, USA). The IC_{50} values were calculated from sigmoidal concentration-inhibition curves of two determinations for each concentration. The concentrations used ranged from 0.02 to 100 μM. Prism software was used to generate IC_{50} values.[1] Selectivity index = COX-1/COX-2. [2] Obtained from reference [43].

6.3.2 Anti-inflammatory activity

6.3.2.1 *In vitro* COX-1 and COX-2 inhibition

Initially, we were interested in determining whether the hybrid resveratrol-salicylate derivatives could inhibit the enzymatic activity of the inflammation-associated COX-1 and COX-2 enzymes,

so we tested the drugs using an *in vitro* screening assay [43]. We calculated the potency of the test compounds as the corresponding IC_{50} values. We present the results in **Table 6-1**.

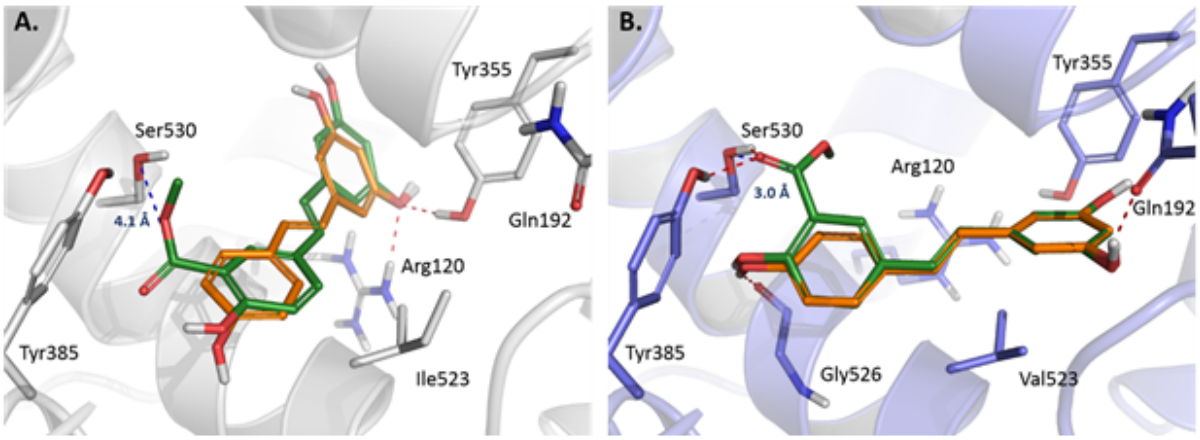
We observed that methoxylated analogues (**3-7**), as well as acetylated ones (**11** and **12**), were practically inactive against both COX enzymes. This finding is consistent with a previous report that described methoxylated resveratrol analogues as "poor" inhibitors of the COX-2 enzyme [43]. According to the literature, both resveratrol and its methoxylated derivative, TMS, exert significant inhibitory activity on COX-1 as well as COX-2 (non-selective COX inhibitors), with selectivity ratios (COX-1/COX-2) = 0.5 and 0.7 respectively [43]. Compound 8 did not show considerable activity (COX-1 IC_{50} = 421, COX-2 $IC_{50} \geq 500$ μM), whereas compounds **9** and **10** exerted significant inhibition of both enzymes. Generally speaking, **9** and **10** were almost equipotent (fairly similar IC_{50} values on COX-1), but compound **10** was more potent (10-fold) than **9** on COX-2, and more selective toward this enzyme as well (**Table 1**).

Parameter	Goal	COX-1	COX-2
QMEAN4	> -2.00	-1.08	-1.61
GMQE	> 0.70	0.97	0.92
Poor rotamers	< 1.00 %	3.99 %	5.82 %
Ramachandran outliers	< 0.05 %	0.09 %	0.09 %
Ramachandran favored	> 98.00 %	95.01 %	94.73 %
Cβ deviations >0.25Å	0.00 %	0.00 %	0.19 %
Bad backbone bonds	0.00 %	0.91 %	0.77 %
Bad backbone angles	< 0.1 %	0.27 %	0.29 %

Table 6-2: Results of protein quality and geometry of COX-1 and COX-2 models.

6.3.2.2 Molecular modeling (docking) studies

To support the observations of the *in vitro* screening assay performed on the COX enzymes, we carried out molecular modeling (docking) simulations. The goal was to understand the COX-2 selectivity exerted by compound **10**. Because the human COX isoforms are not available in the Protein Database Bank, we generated homology models for the two COX enzymes, using known three-dimensional structures. We observed that the sequence alignment of the human COX enzymes showed an acceptable identity with the COX-1 (ID: 92.97 %) and COX-2 (ID: 86.75 %) template sequences. **Table 6-2** displays the quality and the geometry results obtained with the Swiss-Model and MolProbity servers for both models. Additionally, the statistics of the protein geometry, as well as the QMEAN4 and GMQE scores, showed values that make it acceptable to consider these models for molecular docking studies.



	COX-1		COX-2	
	Score (ΔG_{bind})	Cluster size	Score (ΔG_{bind})	Cluster size
10	-7.54	10/30	-8.68	8/30
Resveratrol	-6.72	30/30	-7.89	30/30

Figure 6-3: Comparison of the binding modes calculated for compound 10 (green) and resveratrol (orange) as predicted by AutoDock 4.2, in the active site of human (A) COX-1 and (B) COX-2 models.

Figure 6-3 shows the binding mode for the parent molecule (resveratrol), and the hybrid derivative **10** within the homology model of human COX binding sites. The table below **Figure 6-3** summarizes the calculated binding free energies and cluster size values for each binding mode. The docking scores calculated for resveratrol (-7.89) and compound **10** (-8.68) in the active site of COX-2 are more favorable (more negative) than those values obtained with COX-1 (-6.72 and -7.54 respectively). These values support the observation that compound **10** is capable of exerting better binding interactions with the larger COX-2 active site.

The molecular features required to exert strong and selective inhibition of COX enzymes are widely known in the literature. It is generally accepted that the presence of a small Val523 within the COX-2 active site, compared to an isoleucine at the same position in the COX-1 active site, enables COX-2 to have a "secondary" pocket, capable of hosting bigger ligands [44]. Additioanlly, the calculated volume of the COX-2 active site is about 20% larger than that of the COX-1 enzyme [44].

Interestingly, we observed that the binding modes of these two compounds in the COX-2 active site are different from those observed in COX-1. The smaller Val523 in COX-2 allowed the stilbene to interact with the catalytically active Ser530, Tyr385, and Gln192. Nevertheless, in the COX-1 active site, we observed that the bigger Ile523 residue blocked this alternate binding pose and directed the 3,5-dihydroxyphenyl group of the stilbene toward the Arg120 and Tyr355 residues. Moreover, the carbonyl group present in compound **10** was located closer to the OH oxygen of Ser530 in COX-2 (3.0 Å), than in COX-1 (4.1 Å, **Figure 6-3**) , supporting the observed selectivity determined *in vitro*. These results are consistent with previous reports describing the binding mode for NSAIDs with both COX enzymes [44].

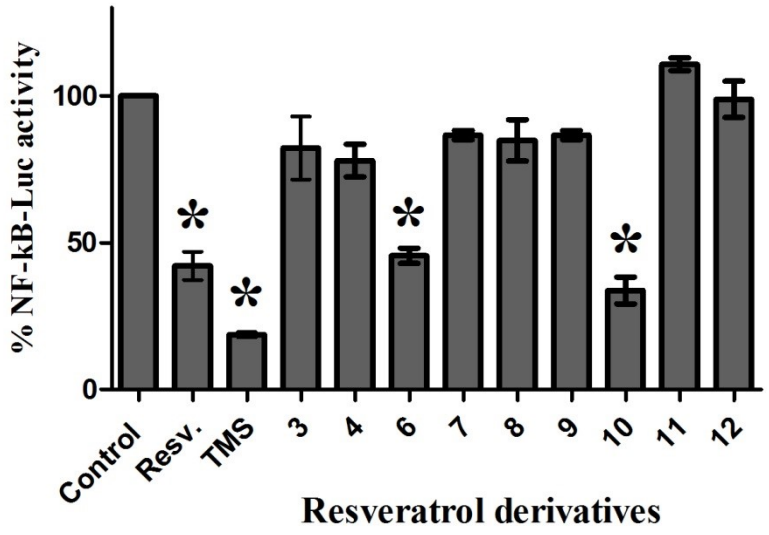


Figure 6-4: Screening of resveratrol-salicylate hybrid derivatives against NF-κB inhibition in HCT-116 cells. Cells were transfected with an NF-κB plasmid for 48h, then treated with compounds at a final concetration of 20 μM for 24h. Then, cells were challenged with LPS for 4h, after which chemiluminescence was measured. Results are expressed as mean ± SEM of triplicate experiments. * $P < 0.05$ compared to control.

6.3.2.3 Screening of resveratrol-salicylate derivatives against NF-κB inhibition

NF-κB is a master transcriptional factor involved in cellular responses to stimuli such as stress, ROS, cytokines, UV, or bacterial antigens [45]. This transcription factor is persistently active in numerous disorders such as arthritis, asthma, heart diseases, chronic inflammation, and cancers [45]. Molecules capable of reducing NF-κB activation have a potential therapeutic promise, and consequently this transcription factor is considered an important therapeutic target [45]. We screened all resveratrol-salicylate derivatives against NF-κB in HCT-116 cells transfected with a NF-κB-luciferase plasmid. We initially tested the viability of these cells after incubation with resveratrol derivatives for 24 hours to determine the maximum non-toxic concentrations to be used in the NF-κB reporter assay. We found that at a concentration of 20 μM, compounds showed more than 70% cell viability (data not shown) and accordingly we decided to use this concentration in the reporter assay. The results of this experiment are shown in **Figure 6-4**.

Resveratrol, TMS, **6,** and **10** reduced NF-κB activity by 58, 82, 55, and 66% respectively. The highest NF-κB inhibition exerted by TMS (82%) could be related to the observed potent proliferation inhibition in these cells displayed by this molecule (data not shown).

HT-29 cells have been shown to constitutively express high levels of active NF-κB when tested among a panel of human cancer cells [46, 47]. We followed the same recently published procedure [48] when we used this model to investigate whether resveratrol analogue **10** can interfere with this transcription factor. The results in **Figure 6-5** showed that compound **10** has the potential to downregulate the basal level of NF-κB in HT-29 cells. This effect was observed at the IC_{50} value of **10** as well as at the double IC_{50}, an effect that may be attributed to the cell death rather than the compound effect. Results from HCT-116 and HT-29 cells suggest that compound **10** is able to modulate NF-κB function. However, the exact mechanism needs to be investigated further.

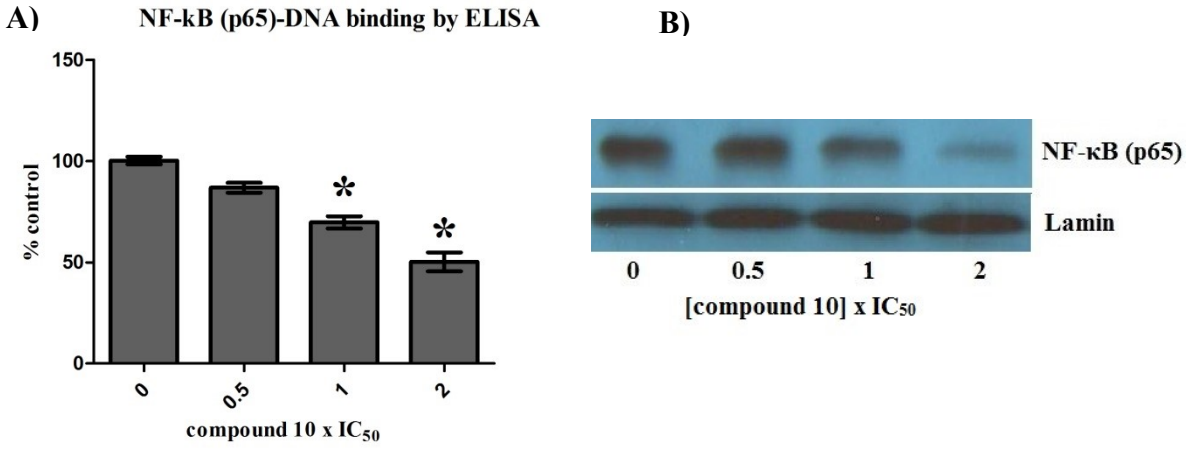


Figure 6-5: Effects on constitutive NF-κB in HT-29 cells treated with either vehicle or compound10 for 24 hours. A) After harvesting of nuclear proteins, DNA binding of NF-κB was determined from the nuclear extract. The reduction in NF-κB activity relative to the vehicle (100%) was determined. Results are expressed as mean ± SEM of two determinations. **P* < 0.05 compared to control. **B)** Western blot analysis of nuclear protein extract after immunoblotting for NF-κB (p65) protein.

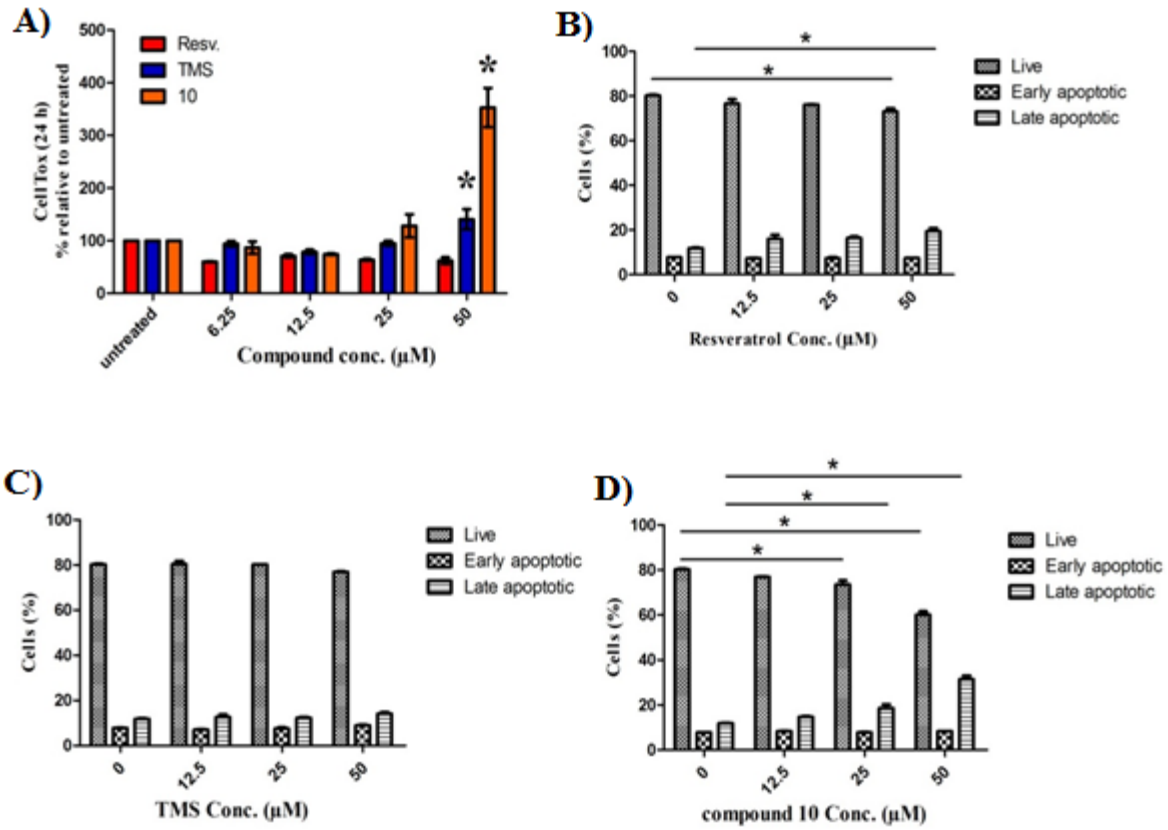


Figure 6-6: Effects of resveratrol, TMS, and compound 10 on cytotoxicity and apoptosis in Jurkat cells. A) Cytotoxicity of compounds after 24 h incubation using CellTox green cytotoxicity assay. Results are expressed as mean ± SEM of triplicate experiments. **P* < 0.05 compared to untreated cells. Apoptosis was evaluated by staining with Annexin-V/7-AAD after incubating Jurkat cells with **B)** resveratrol, **C)** TMS, and **D)** compound **10** at different concentrations. Results are expressed as mean ± SEM of duplicate experiments. **P* < 0.05 compared to untreated cells.

6.3.2.4 Study of apoptosis in Jurkat cells

At the beginning of our assays in Jurkat cells, we used a CellTox green cytotoxicity assay (Promega, WI, USA) [49, 50] to measure the cells' cytotoxicity after 24 hours of incubation. This assay is based on staining the DNA of damaged (dead) cells with a fluorescent dye, which is excluded from viable cells. Thus, the fluorescent intensity is directly proportional to the

cytotoxicity of a given compound. Resveratrol analogues were used at final concentrations ranging from 6.25 to 50 μM. Our results in (**Figure 6-6, A)** demonstrated that resveratrol did not exhibit toxicity on Jurkat cells over the concentration range used here. It was reported that resveratrol had an IC_{50} of 58 μM in Jurkat cells after 24 hours of incubation [51]. The fact that we did not observe any cytotoxicity of resveratrol even at the maximum concentration used (50 μM) is likely attributable to the difference in the assay used, as the IC_{50} reported for resveratrol was achieved through an Alamar blue assay and here we used a CellTox assay. On the other hand, we observed that TMS and **10** exhibited a significant cytotoxicity at 50 μM, while below this concentration there were no statistically significant effects.

Resveratrol has been shown to induce apoptosis in multiple cell lines. Specifically, resveratrol showed an induction in the intracellular calcium concentration as an early apoptotic event in breast cancer cells [52]. In this study, we investigated apoptosis induced by resveratrol analogue **10**, TMS, and resveratrol in Jurkat cells. In contrast to previous reports indicating resveratrol's ability to initiate early apoptosis [52, 53], we did not observe significant effects of resveratrol on early apoptosis in Jurkat cells. Nevertheless, we found that signs of late apoptosis induced by resveratrol were evident at only the highest tested concentration (50 μM) (**Figure 6-6, B**). Surprisingly, the natural analogue of resveratrol (TMS), which was reported to exert strong apoptotic effects at low concentrations [54, 55], did not show significant effects on apoptosis using this model (**Figure 6-6, C**). The reason could be related to the mechanism by which this stilbene exerts its apoptotic effects.

Interestingly, resveratrol analogue **10** displayed significant apoptotic effects starting at 25 μM and later at 50 μM (**Figure 6-6, D**). These effects of **10** were predominantly illustrated by its action on late apoptosis, where it was similar to resveratrol in not showing detectable effects on early apoptosis. Moreover, these apoptotic actions of **10** correlated with its cytotoxicity in Jurkat cells. Resveratrol-salicylate analogue **10'**s ability to induce apoptosis in Jurkat cells at lower concentrations than resveratrol is a positive sign; it suggests that **10** has a potential therapeutic role in inflammatory-associated disorders, as apoptosis is essential for terminating inflammation [56].

6.3.2.5 *In vivo* anti-inflammatory activity (peritonitis model)

Based on the COX inhibitory profile (COX-1 IC_{50} = 4.1 µM; COX-2 IC_{50} = 1.0 µM) exerted by compound **10**, its ability to scavenge free radicals (please see **Figure 6-1** and **Figure 6-2**), and its potential to downregulate NF-κB, we decided to evaluate its potential *in vivo* anti-inflammatory profile using a mouse model.

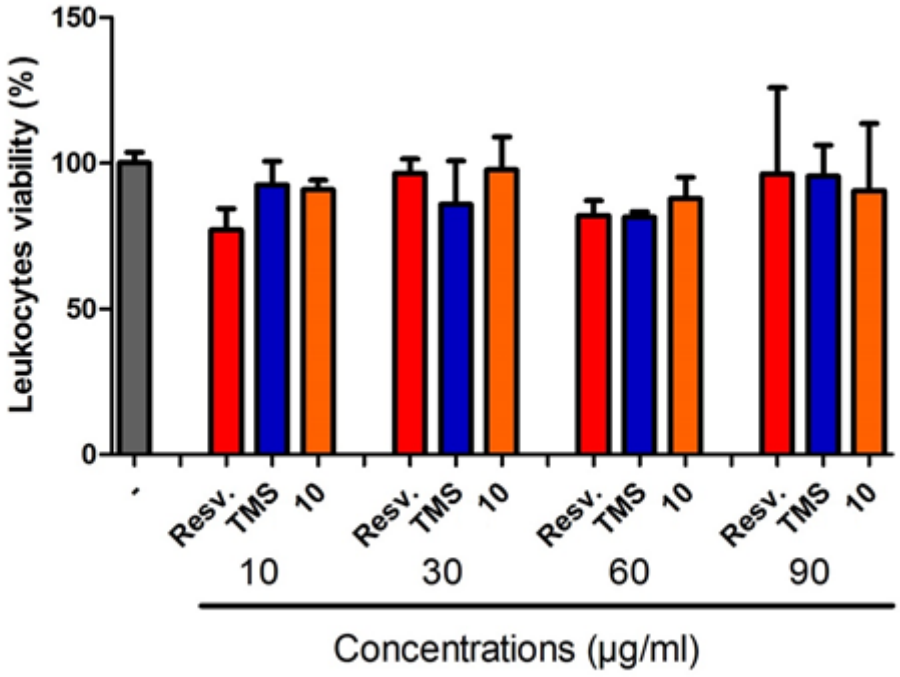


Figure 6-7: Leukocytes' cell viability (by MTT assay) after 90 minutes of incubation with different concentrations of the experimental compounds. The concentration range (10-90 µg/mL) presented here is equivalent to 30-400 µM. Results are presented as mean ± SEM of triplicate experiments.

First, we assessed the cytotoxicity exerted by compound **10** on leukocytes isolated from mice, using the standard MTT assay. This step is essential for determining a safe dose at which we could evaluate the *in vivo* anti-inflammatory profile without affecting the leukocyte viability [57]. In this regard, we observed that resveratrol, TMS, and compound **10** did not exert significant cytotoxicity at any of the test drug concentrations (see **Figure 6-7**). The overall cell viability was higher than 77 % within this concentration range (30-400 µM).

Based on previous reports, a relatively safe dose for resveratrol and, therefore, its hybrid salicylate-like derivatives, is 10-20 mg/kg body weight [58-60]. To determine the potential anti-inflammatory effects of the test drugs, first we tested the compounds by i.p. administration; this method is frequently reported in the literature based on the limited oral absorption of resveratrol [38, 61].

We observed that administering 10 mg/kg of resveratrol (i.p.) to mice, following an acute model of inflammation, produced an elevated leukocyte count as well as increased concentrations of nitric oxide. These two observations would suggest that rather than exerting an anti-inflammatory effect, resveratrol exacerbated the effects of the carrageenan-induced inflammatory insult (data not shown). These results are not consistent with previous reports in which resveratrol (10 mg/kg, i.p.) decreased the count of neutrophils in the same model (peritonitis model in mice [61]). However, it should be pointed out that we used a different inducer of inflammation (carrageenan) than that reported by Issuree et al. (the compound "C5a," which is a strong chemoattractant).

In a second experiment, we decided to change the route of administration from i.p. to p.o. (oral), to determine if this change could counteract the apparent irritant effect exerted by resveratrol. The results of the new experiment are shown in (**Figure 6-8**). Based on previous reports in which the oral dose of resveratrol was as high as 200 mg/kg [62, 63], we assumed that lower doses (10-20 mg/kg) were relatively safe for oral administration of the test compounds.

Initially, we measured both the number of leukocytes and the concentration of nitric oxide in peritoneal exudates as relevant endpoints to determine the potential anti-inflammatory effects of the drugs (**Figure 6-8**). We observed that both doses of test compounds (10 and 20 mg/kg) exerted similar effects, with a modest increase in activity at 20 mg/kg compared to 10 mg/kg. The resveratrol natural analogue TMS and the hybrid salicylate-resveratrol derivative **10** exerted a dose-dependent decrease in the number of leukocytes in samples of peritoneal exudates (**Figure 6-8, A**). Unlike the results obtained with the i.p. administration described above, the reduction in white blood cells (50-80 % decrease) produced by oral administration of resveratrol (and its derivatives) suggests that *the route of administration makes a difference when evaluating the effects of these drugs*.

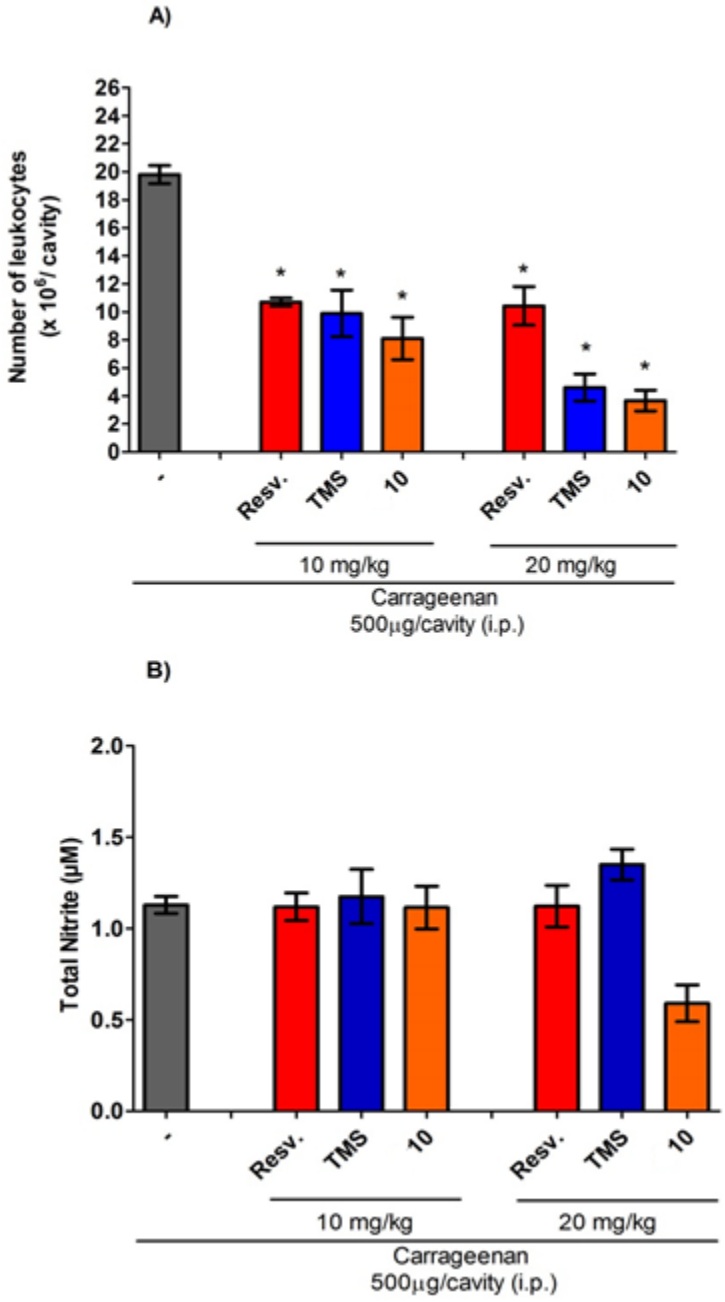


Figure 6-8: Effects of oral administration of resveratrol analogues (at 10 and 20 mg/kg doses) on A) total leukocytes and B) NO level using carrageenan-induced peritonitis in mice. Animals were orally gavaged 30 minutes before i.p. injection of carrageenan. After four hours, animals were euthanized and leukocytes and NO levels were assessed in the fluid obtained from the peritoneal cavities. Results are expressed as the mean ± SEM (n = 4) for each group. **P* < 0.05 compared to the untreated (carrageenan only) group.

In the same experiment, we also evaluated the concentration of nitric oxide (as sodium nitrite) in peritoneal exudates from animals in different groups (**Figure 6-8, B**). At 10 mg/kg p.o., compared to the carrageenan-treated group, none of the tested compounds significantly decreased the concentration of nitric oxide. This effect was similar when we administered the test drugs at 20 mg/kg. Compound **10** showed an increased anti-inflammatory effect, but not strong enough to produce a statistically significant difference compared to that observed in the control group.

6.3.2.6 *In vivo* anti-inflammatory activity (paw edema model)

To provide additional evidence supporting a potential anti-inflammatory effect produced by the test compounds resveratrol, TMS, and compound **10**, we also carried out a carrageenan-induced mice paw-edema assay. In this complementary experiment, we administered the same oral doses (10 and 20 mg/kg) of the test compounds used in the peritonitis model described above. The results of this experiment are shown in **Figure 6-9**.

As expected, animals in the carrageenan-treated group experienced the highest paw volume (time-dependent). At six hours (last time point determined), animals in the control group showed, on average, an increase of paw volume = 123 μL. However, the paw volume determined for animals in all drug-treated groups suggested a dose-dependent, anti-inflammatory effect, which was statistically significant for compound **10** at 10 mg/kg, and TMS and **10** at 20 mg/kg (**Figure 6-9, A** and **B**). These results are consistent with those obtained in the peritonitis assay described above.

Specifically, six hours after the initial carrageenan stimulus, we observed that resveratrol and TMS (10 mg/kg) reduced mice paw volumes by 34% and 19% respectively, but this reduction was not statistically significant. This was not the case for compound **10**, which at a dose = 10 mg/kg significantly attenuated paw swelling (59% reduction) compared to the control group. This anti-inflammatory effect was more evident at 20 mg/kg (p.o.), where we observed a 74% reduction of mice paw volume exerted by **10** after six hours. Additionally, we observed that at 20 mg/kg, resveratrol significantly decreased (52%) the paw volume. The methylated resveratrol analogue, TMS, exerted a 34% reduction in paw volume (not statistically significant).

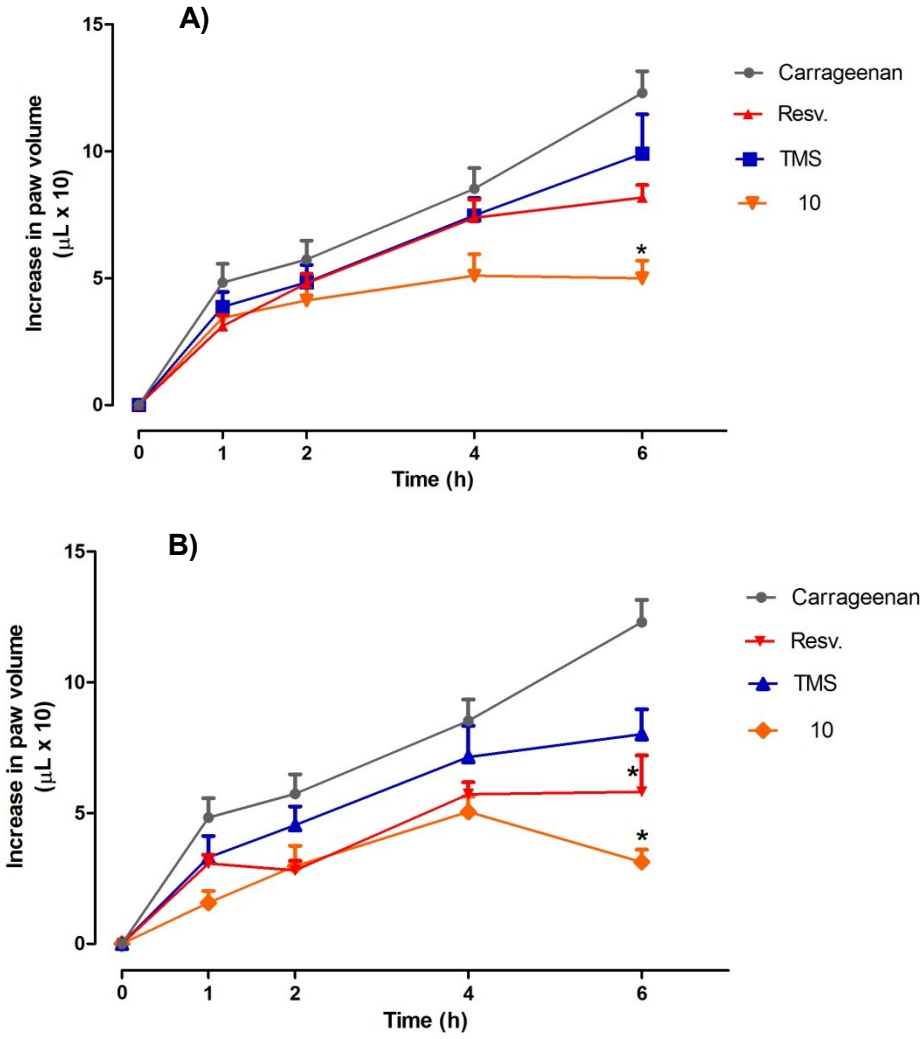


Figure 6-9: The reduction in paw volume after oral administration of resveratrol analogues at doses of A) 10 mg/kg and B) 20 mg/kg using carrageenan-induced paw edema in mice. Animals were orally treated with resveratrol analogues 30 minutes before intradermal injection of carrageenan in the left paw while the right paw was injected with the vehicle only (0.9 % saline). The change in paw volume was calculated at the indicated times by subtracting the right paw (vehicle) from the left paw (carrageenan) for each mouse. Results are expressed as mean ± SEM (n = 4) for each group. *$P < 0.05$ compared to the untreated (carrageenan only) group.

Finally, we observed that none of the test compounds produced a statistically significant response within the first four hours of the experiment, suggesting that the drugs are effective only after a possible delayed absorption/biodistribution, or maybe after an *in vivo* time-dependent metabolic activation. This observation is consistent with a previous report by Xu et al. in which resveratrol significantly reduced the mouse paw volume three to six hours post-carrageenan injection [62]. At this time we do not have evidence supporting a delayed absorption profile or a metabolic activation of the test compounds; these premises require validation by appropriate pharmacokinetic experiments that extend beyond the scope of the current study.

6.3.2.7 Measurement of myeloperoxidase activity

The expression and activity of the myeloperoxidase (MPO) enzyme is one of several markers of neutrophil infiltration at inflammation sites [64]. Therefore, MPO constitutes a good indicator of inflammation-induced chemotaxis. Additionally, MPO catalyses the oxidations of a broad range of substrates which results in increased concentrations of numerous reactive radical species [56]. Consequently, inhibiting MPO activity in mice paw exudates would provide additional evidence supporting the anti-inflammatory effects exerted by the test compounds.

We observed that resveratrol significantly decreased MPO activity (53% decrease; $P < 0.05$) only at a 20 mg/kg dose. This was not the case for TMS, which despite the modest effect observed at 20 mg/kg, did not produce a significant change compared to the control group (**Figure 6-10, B**). The phenol-containing compound **10** was an effective inhibitor of MPO activity at 10 and 20 mg/kg (around 60% decrease; $P < 0.05$), suggesting that the presence of free aromatic alcohols is essential for MPO enzyme inhibition by stilbenes.

Previously, Dey et al. showed that resveratrol exerts a concentration-dependent biphasic modulation of MPO activity. In this regard, Dey's group determined that low doses (2 mg/kg) of resveratrol decreased MPO activity in the ulcerated gastric tissues of mice, whereas high doses (10 mg/kg) increased MPO activity [64].

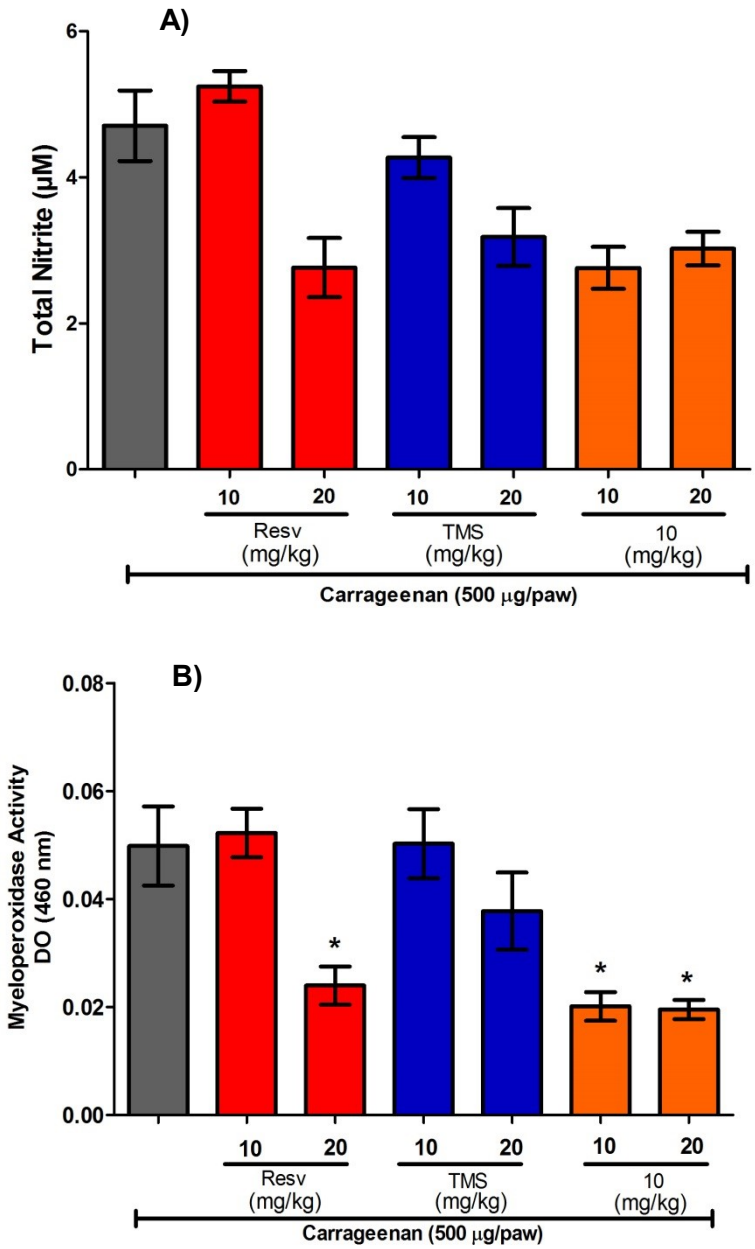


Figure 6-10: Effects of resveratrol analogues (10 and 20 mg/kg doses) against A) NO levels and B) myeloperoxidase activity tested in plantar tissues of mice paws after carrageenan-induced paw edema experiment. Results are expressed as mean ± SEM (n = 4) for each group. *$P < 0.05$ compared to the untreated (carrageenan only) group.

Along with MPO activity, we also determined the concentration of nitric oxide (NO) in mice paw exudates (**Figure 6-10, A**). Previous reports have shown that resveratrol can reduce the expression of inducible nitric oxide synthase (*i*NOS) [59, 65]. Also, resveratrol decreased NO levels in LPS-stimulated macrophage RAW.264.7 cells [66-68]. In our experiments, we did not observe that any of the drugs significantly decreased the concentration of NO in the mice paw exudates at two different doses (10 and 20 mg/kg).

Two recent reports described the benefits of low resveratrol concentration/dose against inflammation, oxidative stress, and cancers. In the first report, a low concentration of resveratrol (10 μM) was found to be more effective than a high concentration (60 μM) in alleviating the toxic effects of H_2O_2 in myoblast cells (C2C12) [69]. In the second report, a low dose of resveratrol (0.07 mg/kg) reduced the tumor burden more significantly than did a 200-fold higher dose (14 mg/kg) using Apc^{Min} mice as a colorectal cancer model [70]. The fact that compound **10** demonstrated better anti-inflammatory properties than resveratrol at low doses may give **10** an advantage over resveratrol. However, at this point we believe that further studies are needed to support this hypothesis.

6.4 Conclusions

The insertion of an additional carboxylic acid group into the chemical structure of resveratrol yields a hybrid resveratrol-salicylate derivative **10**, with enhanced anti-inflammatory properties (compared to the parent polyphenol). This was demonstrated *in vitro* by inhibiting the COX-2 enzyme and downregulating NF-κB, as well as *in vivo* using two different inflammatory models in mice (carrageenan-induced peritonitis and paw edema assays). Adding a carboxylate group increased the selectivity of compound **10** toward COX-2 inhibition *in vitro*. This structural modification did not alter **10**'s ability to decrease the concentration of ROS *in vitro*, suggesting that hybrid salicylate-resveratrol derivatives may offer a better therapeutic profile compared to resveratrol. Additionally, compound **10** showed apoptotic effects in Jurkat cells at concentrations lower than those of resveratrol. These results, along with those reported in previous investigations by our group, provide evidence to support the multi-target profile (especially at

low doses) of the new hybrid scaffold. Finally, this chapter complements the potential chemotherapeutic profile of hybrid salicylate-resveratrol derivatives, by showing that these compounds maintain (or even improve) resveratrol's anti-inflammatory effects.

6.5 References

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Chapter 7: Summary, Conclusion and Future Directions

7.1 Summary of results

1- Introduction

Cancer is a complex disease in which various signalling pathways are modulated. Instead of targeting one protein overexpressed in cancer, the "multi-target" approach is expanding within the scientific community [1]. In fact, this feature is probably the reason for the success of natural agents (e.g. resveratrol and aspirin) as promising chemopreventive agents. Theoretically, the multi-target strategy is supposed to provide enhancement (synergism) of therapeutic response, producing fewer side effects and being less susceptible to drug resistance [1]. However, the selection of targets is crucial due to the high degree of homology of certain protein families.

Resveratrol has been extensively studied in the last two decades as a potential chemopreventive agent. However, its low stability, high metabolic rate and the discrepancies among the results of *in vitro* and *in vivo* studies on it initiated the search for resveratrol derivatives [2]. One approach for resveratrol derivatization is to hybridize the resveratrol structure with another active moiety in order to enhance the desired therapeutic response. In this project, we designed hybrid resveratrol derivatives based on hybridization of the resveratrol structure with aspirin (or salicylate).

2- Literature review

As resveratrol has its own disadvantages, the quest was initially started for studying "natural" analogues of resveratrol and comparing their efficacy with the parent. These naturally occurring stilbenes include piceatannol [3], pterostilbene [4] and 3,4',5-*trans*-trimethoxystilbene (TMS). In regards to the first two analogues, there are literature reviews describing their properties [3, 5], which is not the case for TMS. From that perspective, we compiled available reports which investigated the anticancer properties of this stilbene.

We found that TMS exhibits significant inhibition of carcinogenesis through acting on apoptosis, proliferation, angiogenesis and metastasis. Additionally, it represents debatable antioxidant properties that require further investigations. Nevertheless, its pharmacokinetic properties appear to be better than those of resveratrol. Importantly, this stilbene is far less studied in the literature

compared to resveratrol. This natural resveratrol analogue is promising and needs to be evaluated against the majority (if not all) of targets considered previously for resveratrol.

3- Chemical synthesis

For the synthesis of methoxylated resveratrol derivatives, we explored Wittig, Horner-Wadsworth-Emmons (HWE), and Heck reactions to produce the desired *trans* isomers. It appears that the Heck reaction can produce *trans* isomers in a better yield than the Wittig reaction (e.g., the case of compound **7**). Nevertheless, the Wittig reaction gives access to *cis* isomers which could be evaluated in future studies (see future directions). Ph_2S_2 seems to be an efficient catalyst to convert an isomeric mixture to only a *trans* isomer. However, this statement was only true for methoxylated analogues, but not their hydroxylated counterparts. Further modifications to the deprotection and acetylation reactions may improve the overall yield of these derivatives.

4- CYP1A1 inhibition

Compound **3** showed promising inhibitory activity against TCDD-induced CYP1A1 activation by reducing EROD activity and decreasing CYP1A1 mRNA. Additionally, **3** exhibited direct (after one hour of incubation) inhibition of the CYP1A1 enzyme, which is supported by a docking study inside the recent crystal structure of the CYP1A1 active site. Importantly, compounds **8** and **9** showed an enhancement of TCDD-induced CYP1A1 activation after 24 hours of incubation in HepG2 cells, which is considered an unfavorable effect. Finally, in HT-29 cells, resveratrol at a concentration of 5 μM induced EROD activity at a higher level than that of TCDD alone, an observation which seems to confirm the reported biphasic effect of this polyphenol.

5- DNMTs inhibition

Resveratrol is reported to modulate the methylation pattern of certain genes' "epigenetic modulator". We used a biochemical assay to assess the potential of current resveratrol-salicylate analogues to inhibit DNMTs. Interestingly, compounds **10** and **9** inhibited DNMT3B and 3A enzymes with an activity higher than that of resveratrol. Compound **10** demonstrated lower IC_{50} values against cancer cell proliferation of three human cancer cell lines (HepG2, HT-29 and SK-

BR-3). Despite not showing apparent proliferation inhibition on the HUVEC cells, the low IC_{50} value of **10** against MCF 10A highlights the need for additional studies to evaluate this molecule's safety to normal cells.

6-Anti-inflammatory and antioxidant properties

In this set of experiments, compound **10** demonstrated enhanced anti-inflammatory properties compared to resveratrol and TMS. Additionally, **10** retained the antioxidant profile similar to that exerted by the parent resveratrol. Importantly, **10** was active *in vivo* (particularly at low dose) in reducing MPO activity in the inflamed mice tissue. Along with its safety profile in leukocytes, **10** represents a promising candidate for further experimental studies related to inflammation-promoted cancers.

7.2 Conclusion

Adding the carboxylate functional group to the resveratrol scaffold (at position 3'-) produced multi-target molecules capable of modulating certain proteins involved in carcinogenesis. These derivatives varied in their structure-activity relationship according to the protein of interest. Despite not showing CYP1A1 inhibitory activity, compound **10** showed NF-κB, COX-2, and ROS inhibitory activities. Moreover, it displayed *in vivo* anti-inflammatory activities in two carrageenan-induced inflammation models, activities that were higher than those for TMS and resveratrol. Resveratrol-salicylate derivatives represent a novel class of resveratrol analogues which require additional investigation into their role in cancer prevention. Other targets modulated by resveratrol, aspirin, or both might be the focus of future projects in which resveratrol-salicylate derivatives can be evaluated.

7.3 Future directions

There are numerous proteins which are modulated by either resveratrol or aspirin or both (**Figure 1-3** and **Table 1-2**). These proteins might be potential targets for the current resveratrol-salicylate derivatives. In order to narrow down these targets, we are providing here a list of potential future projects (some of which have already started):

1- Resveratrol's pharmacokinetic properties are unfavorable [6]. In the current project, the most promising derivative was compound **10** which showed that in rat plasma it was hydrolyzed to

produce **9** (a derivative that showed DNMT and COX enzymes inhibition). It will be necessary to evaluate the pharmacokinetic characteristics of these hybrids and compare them to that of resveratrol.

2- Testing the *in vivo* chemopreventive profile of the most promising compounds. Perhaps **10** and **3** could be potential candidates. This experiment could be carried out by utilizing APC10.1 cells (derived from APC^{Min} mouse adenoma) which have been suggested as a novel *in vitro* model to study the chemopreventive potential of candidate molecules [7]. Alternatively, an *in vivo* model of an APC^{Min} mouse model can be used.

3- Preliminary *in silico* screening of *cis* isomers of the current resveratrol-salicylate analogues showed an enhanced CYP1A1 binding affinity (data not shown in this thesis). *Cis*-isomers can be synthesized using a Wittig reaction and tested against this enzyme. Additionally, methylthiostilbenes were recently designed and assessed as CYP1A1 and CYP1B1 inhibitors [8]. The current resveratrol-salicylate derivatives can be modified to their methylthio-counterparts and evaluated against CYP1A1 and CYP1B1 enzymes.

4- The demethylation potential of **10** (and perhaps **9**) can be further confirmed using cell-based systems as recently reported [9]. Additionally, re-activation of silenced genes can be investigated (perhaps *in vivo*), similar to the report recently published for resveratrol [10].

5- Mercaptostilbene analogues were recently synthesized and assessed as "extraordinary" antioxidant agents [11]. Future work could involve modifying the current resveratrol-salicylate derivatives so that they can possess a thiol moiety. Additionally, an electron paramagnetic resonance (EPR) instrument can be used to assess the antioxidant properties of these analogues.

6- Chronic inflammation (such as rheumatoid arthritis) might be a potential target for these analogues. In fact, we have already started a collaboration with Dr. Burkhard Kloesch (Ludwig Boltzmann Institute for Rheumatology and Balneology, Vienna, Austria) to evaluate the anti-inflammatory properties of these analogues in chronic inflammation.

7- Cardioprotective and/or antiplatelet properties may be assessed, as these properties are among the earlier observation of resveratrol therapeutic potential (French Paradox).

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