eman ta zabal zazu

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Antitumoral actions of natural stilbenes derived from *Vitis vinifera*

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A Jesús, Carmen, Coral y Hugo

<< La vie est courte· Transgressez les règles, pardonnez rapidement, embrassez lentement, aimez véritablement, riez sans contrôle et, ne regrettez jamais quelque chose qui vous a fait sourire >>

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Ahora puedo decir, que bailó la pena.

Iris

The doctoral thesis work presented has been organized as follows:

1. Introduction. Polyphenols and specifically stilbenes are described, as well as their main biological effects. The human hepatocarcinoma is described as a study model used; briefly exposing its etiology and the treatments that currently exist. Finally, the most relevant molecular mechanisms involved in its formation and progress are detailed, such as inflammation, free radical formation and cell death. The revision made in this section allows establishing the bases from which the hypothesis and the objectives are defined.

2. Hypothesis and Objectives. The starting hypothesis is proposed and the objectives to be developed are described.

3. Results and Discussion. The results and discussion are presented in 4 chapters, which have been developed by grouping several aspects of the thesis. Each chapter corresponds to an original research article. (Published, pending submission).

3.1 Chapter I. In the first chapter novel stilbenes from the woody parts of *Vitis vinifera* are isolated and test their cytotoxic activity is tested in HepG2.

3.2. Chapter II. A screenning with 20 natural stilbenes is performed to analyze its ability to reduce the inflammatory effect induced in murine macrophages by stimulation with LPS. Its cytotoxic activity is analyzed, as well as its ability to reduce the production of reactive oxygen species (ROS), and nitric oxide (NO). Finally, its influence on the production of inflammatory cytokines such as TNF-α and IL-1β is analyzed.

3.3. Chapter III. In this chapter, a screening with 6 stilbenes is performed to analyze its effect on the HepG2 and Hep3B human hepatoma lines and on the HH4 non-transformed hepatocyte line. Derived from this first screening, stilbene with the greatest cytotoxic potential in tumor cells is selected without being in non-tumor cells and studies are initiated to determine its mechanism of action. To do this, we proceed to analyze its effect on the cell cycle, the production of ROS and O_2^-, the activation of caspases and, finally, the ratio of pro- and anti-apoptotic proteins Bax and Bcl2.

3.4. Chapter IV. In the last chapter, studies continue to try to detail the mechanism of action of R2-viniferin on the human hepatocarcinoma HepG2 line. To this end, p53 transfection assays, lactate dehydrogenase (LDH) release, H_2O_2, and analysis of possible proteins involved by western blotting are performed. Finally, the possible effects of the pretreatment of these cells with stilbene on cell viability, the ability to form colonies and their ability to migrate are analyzed.

4. Conclusions. This section defines the final conclusions drawn from the results of the 4 chapters of the doctoral thesis.

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ABBREVIATIONS

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AP-1	Activator protein 1
ATM	Ataxia-telangiectasia mutated
BCLC	Barcelona clinic liver cancer
BSA	Bovine serum albumin
CDKs	Cyclin-dependent kinases
CHD	Coronary heart disease
COX	Cyclo-oxygenase
CPC	Centrifugal partition chromatography
DCF	2´,7´-Dichlorofluorescein
DISC	Death-inducing signaling complex
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DR	Death receptors
DTT	Dithiothreitol
EASL	European Association for the Study of the Liver
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Egtazic acid
ERK	Extracellular-signal-regulated kinase
Fas-L	Fas ligand
FBS	Fetal bovine serum
FDA	Food and drug administration
GAPDH	3 Glyceraldehyde
GSH	Glutathione
H_2DCF-DA	2´7´-dichlorodihydrofluorescein diacetate
H_2O_2	Hydrogen peroxide
HBV	Hepatitis B-virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C-virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPCs	Hepatic progenitor cells
HPLC	Reverse phase high-performance liquid chromatography
HSCs	Hepatic Stellate Cells
IAPs	Inhibitors of the apoptosis proteins
IC30	30% maximal inhibitory concentration
IC50	Half maximal inhibitory concentration
IKK	IKB kinase
IL-1	Interleukin-1
IL-1α	Interleukina-1α
IL-1β	Interleukin-1β
IL-6	Interleukina-6
IL-8	Interleukina-8
iNOS	Nitric oxide synthase
IκB	Inhibitor of NF-κB
KCs	Kupper Cells
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
LPS	Lipopolysaccharide
MLKL	Mixed lineage kinase domain-like pseudokinase protein
MTT	Thiazolyl blue tetrazolium bromide
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcohol steatosis
NF-κβ	Nuclear factor kappa β
NO	Nitric oxide
NO_2	Nitrogen dioxide
NO_2^-	Nitrite
NO_3^-	Peroxynitrite
Nrf2	Nuclear factor erythroid 2-realted factor 2
O_2^-	Superoxide anion
OH^-	Hydroxyl
PBS	Phosphate buffer saline
PInsP2	Phosphatidylinositol 4,5-bisphosphate

PInsP3	Phosphatidylinositol (3,4,5)-trisphosphate
ROS	Reactive oxygen species
scRNA	Scrambled siRNA
SE	Standard error
Sirt1	Sirtuin 1
SOD	Superoxide dismutase
TMS	Trans-3,5,4´-trimethoxystilbene
TNF-α	Tumor necrosis factor-α
TRAIL	TNF- related apoptosis-inducing ligand
UPLC	Ultra-performance liquid chromatography

SUMMARY/RESUMEN/RÉSUMÉ

SUMMARY

The use of natural compounds for cancer treatment has increased considerably in recent years. In fact, about half of the molecules tested as potential chemotherapeutic agents are compounds derived from plants. The secondary metabolism of plants, also called specialized metabolism, produces a large number of molecules, one of their main objectives being the plant protection from both biotic and abiotic stress (climatic factors, pathogens, etc.). In their secondary metabolism, plants produce innumerable compounds whose purpose is to protect them from stressful agents (pathogens, ultraviolet light). The structural diversity of these chemical compounds is enormous, polyphenols being a very large group, They are classified according to their chemical structure into flavonoids, phenolic acids, lignans and stilbenes, the latter being the object of study in this thesis. Stilbenes are polyphenols widely distributed in the plant kingdom, present in our diet, those that come from the domestic vine (*Vitis vinifera L.*), such as those present in wine and grapes, being an important source. The basic structure of stilbenes is composed of two phenolic rings linked by a double bond, from which monomers, dimers, trimers and tetramers are generated. The most studied stilbene is resveratrol, of which antioxidant and potentially antitumor properties, among others, have been described in the scientific literature. However, the analysis of the possible benefits of the rest of the oligomers present in *Vitis vinifera* is very scarce. The structural diversity of stilbenes can explain the different activity exerted by each of them in the different biological models used. Several of these compounds show higher effects than resveratrol.

Hepatocarcinoma represents approximately 90% of all primary types of liver cancer, and is therefore considered a worldwide problem. To date, Sorafenib is the drug with which the best results have been achieved in the treatment of advanced hepatocarcioma. Sorafenib is a multi-kinase inhibitor that increases the

patient's life expectancy from 3 to 5 months, but on many occasions it produces side effects that require discontinuation of therapy.

Hepatocarcinoma is a complex process in which many mechanisms are involved. Liver cirrhosis stands out as the main risk factor associated with the development of this tumor; liver cirrhosis results from chronic inflammation following continued damage. In this chronic inflammation inflammatory mediators such as TNF-α or IL-1β are released, and oxidative stress is established as a consequence of the imbalance between the production and elimination of free radicals. All these events initiate the necessary mechanisms to provoke phenotypic changes in the liver and damage the healthy cell, causing its death and creating a microenvironment that favors the development and growth of tumor cells.

The HYPOTHESIS of this work is that *Vitis vinifera* contains bioactive stilbenes, some of which show cytotoxic activities against human hepatoma cells higher than resveratrol, the most studied stilbene.

The main OBJECTIVE of the present Doctoral Thesis was first to carry out a preliminary screening of a series of novel stilbenes from *Vitis vinifera* vine for their cytotoxic and anti-inflammatory potential, and second to select the most potent one to establish its mechanism of action.

The following OPERATIVE OBJECTIVES were raised:

1. To isolate novel stilbenes from the woody parts of *Vitis vinifera* and test their cytotoxic activity in HepG2.

2. To screen 20 stilbenes for their anti-inflammatory activity in a cellular model of lipopolysacharide-stimulated murine macrophages.

3. To analyze in the murine model the anti-inflammatory effects of the most potent stilbenes in terms of the production of nitric oxide, reactive oxygen species (ROS), and pro-inflammatory TNFα and IL- cytokines.

4. To screen the cytotoxicity for seven oligoestilbenes from *Vitis vinifera* vine in two human hepatoma cell lines with different p53 status (wild-type p53 HepG2 and p53-null Hep3B), and in non-transformed human hepatocytes.

5. To stablish the involvement of p53 in the toxic effects of the selected stilbene (R2-viniferin) in the hepatoma cells.

6. To unravel the cytotoxic mechanisms of R2-viniferin in HepG2, studying specifically the production of superoxide anion, hydrogen peroxide, cell death pathways, and the influence of the stilbene on cell cycle, migration, and capacity of colony formation.

To achieve the objectives, 4 cell lines are used. The study of the anti-inflammatory effect of stilbenes is carried out on the RAW 264.7 murine macrophage cell line. The HepG2 and Hep3B human hepatocarcinoma lines and the HH4 untransformed human hepatocyte line are the models for studying the antitumor potential of stilbenes. All the stilbenes used for the development of this doctoral thesis have a purity of at least 95% and are extracted from a natural extract rich in stilbenes, called Vineatrol®30, made from vine shoots, except resveratrol (Sigma) , oxyresveratrol and piceatannol (TCI chemicals).

Statistical analysis of the data was carried out using the statistical programs SPSS 19.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6 (San Diego, CA, USA). Data were expressed as the mean ± standard error of at least three independent experiments. The IC50 or IC30 (concentration that inhibits 50% or 30% cell growth) was derived from the semi-log dose-response curve. The statistical significance of the differences in the means was estimated by the Student t test or

ANOVA followed by the Dunnett post hoc test. Differences between means were considered statistically significant for $P < 0.05$.

The stilbenes used for the analysis of antitumor activity comprise 8 monomers (resveratrol, piceide, piceatannol, astringin A, pterostilbene, oxyresveratrol, isorhapontine and isorhapontigenine), 7 dimers (ε-viniferin, ω-viniferin, δ-viniferin, ampelopsin A scirpusin A, pallidol and vitisinol C), a trimer (miyabenol C) and 4 tetramers (hopeaphenol, isohopeaphenol, R2-viniferin and R-viniferin). All were tested in the LPS-activated murine macrophage line, analyzing both their cytotoxicity and their ability to inhibit NO production. From these experiments, their IC30 or IC50, respectively, were calculated. The stilbenes selected based on the results obtained were piceatanol, ε-viniferin, δ-viniferin, hopeaphenol and isohopeaphenol, with which the effect that they exert on the release of inflammatory cytokines and the production of ROS was studied. Hopeaphenol and piceatannol inhibit ROS production, and only δ-viniferin does not decrease the release of cytokines IL-1β and TNF-α. It is concluded that the derivatives of resveratrol piceatanol, ε-viniferin, hopeaphenol and isohopeaphenol have interesting effects on the inflammatory response produced by the activation that LPS exerts on murine macrophages. This finding justifies continuing the study in more complex models given its possible usefulness in the treatment of diseases with an inflammatory basis.

For the study of antitumor activity, the IC50 obtained after treatment with 6 stilbenes from the 2 hepatoma lines were compared with the line of non-transformed hepatocytes. Resveratrol (monomer), ampelopsin A and ε-viniferin (dimers) and the tetramers hopeaphenol, isohopeaphenol, R2-viniferin and R-viniferin were analyzed. The R2-viniferin tetramer shows the highest cytotoxic activity in HepG2 (IC50 <10 μM), without being toxic at this concentration for HH4. Hep3B cells are notably more resistant to R2-viniferin (IC50 48 μM). Stilbene produced increased HepG2 cells in SubG0, without alteration in Hep3B.

The different response to this stilbene turned out to be due to the different status of p53, since silencing p53 in HepG2 cells increased their resistance to treatment, while transfection of p53 into Hep3B made them more sensitive. R2-viniferin causes phosphorylation of p53 in HepG2.

The activation of caspase 3 and 9, the increase in the ratio of Bax / Bcl2 proteins and the release of intracellular LDH evidenced cell death by apoptosis and necrosis caused by that stilbene, which can be modulated by the PI3K/Akt and ERK1/2 on HepG2. Furthermore, the expression of histone γ-H2AX showed the damage that treatment with R2-viniferin produces in DNA. The key role that free radicals play in this entire process was elucidated due to the increased production of ROS and H_2O_2 and the increase in Mn-SOD activity. It was also shown that R2-viniferin decreases the migration and colony formation capacity of HepG2.

It is concluded that the R2-viniferin tetramer exerts its anti-tumor activity through apoptosis mechanisms linked to p53 and is a promising compound with potential effects for chemoprevention and treatment of liver cancer.

RESUMEN

El uso de compuestos naturales para el tratamiento del cáncer ha sufrido un aumento considerable en los últimos años. De hecho, aproximadamente la mitad de las moléculas ensayadas como potenciales agentes quimioterapéuticos son compuestos derivados de plantas. El metabolismo secundario de las plantas, también denominado metabolismo especializado, produce gran cantidad de moléculas, siendo uno de sus objetivos principales protegerlas de los agentes estresantes tanto bióticos como abióticos (factores climáticos, patógenos, etc.). La diversidad estructural de estos compuestos químicos es enorme, suponiendo un grupo muy numeroso los polifenoles, que se clasifican atendiendo a su estructura química en flavonoides, ácidos fenólicos, lignanos y estilbenos, siendo estos últimos objeto de estudio en la presente tesis. Los estilbenos son polifenoles distribuidos ampliamente en el reino vegetal, presentes en nuestra dieta, siendo una importante fuente los que provienen de la viña doméstica (*Vitis vinifera L.*), como los presentes en el vino y las uvas). La estructura básica de los estilbenos está compuesta por dos anillos fenólicos unidos por un doble enlace, a partir de los cuales se generan monómeros, dímeros, trímeros y tetrámeros. El estilbeno más estudiado es el resveratrol, del que se han descrito en la literatura científica, entre otras, propiedades antioxidantes y potencialmente antitumorales. Sin embargo, el análisis de los posibles beneficios del resto de oligómeros presentes en *Vitis vinifera* es muy escaso. La diversidad estructural de los estilbenos puede explicar la diferente actividad ejercida por cada uno de ellos en los distintos modelos biológicos empleados. Algunos de estos compuestos presentan efectos superiores a los ejercidos por el resveratrol.

El hepatocarcinoma representa aproximadamente el 90% de todos los tipos de cáncer de hígado primarios, por lo que es considerado como un problema mundial. A día de hoy el Sorafenib es el fármaco con el cual se han conseguido los mejores resultados en el tratamiento del hepatocarcioma avanzado. El

sorafenib es un inhibidor multiquinasa que aumenta la esperanza de vida del paciente de 3 a 5 meses, pero en muchas ocasiones produce efectos secundarios que obligan a suspender la terapia.

El hepatocarcinoma es un proceso complejo en el cual están implicados muchos mecanismos. La cirrosis hepática destaca como principal factor de riesgo asociado con el desarrollo de este tumor; la cirrosis hepática es consecuencia de una inflamación crónica tras un daño continuado. En esta inflamación crónica se liberan mediadores inflamatorios como TNF-α o IL-1β, y se instaura un estrés oxidativo como consecuencia del desequilibrio entre la producción y eliminación de radicales libres. Todos estos eventos inician los mecanismos necesarios para provocar cambios fenotípicos en el hígado y dañar la célula sana, provocando su muerte y creando un microambiente que favorece el desarrollo y crecimiento de las células tumorales.

La HIPÓTESIS de este trabajo fue que *Vitis vinifera* contiene estilnos bioactivos, algunos de los cuales posee mayor actividad citotóxica contra las células de hepatoma humano que el resveratrol, el estilbeno más estudiado.

El OBJETIVO principal de la presente Tesis Doctoral fue en primer lugar, realizar una evaluación preliminar de una serie de nuevos estilbenos de la vid *Vitis vinifera* por su potencial citotóxico y antiinflamatorio, y segundo seleccionar el más potente para establecer su mecanismo de acción.

Los OBJETIVOS OPERATIVOS los siguientes:

1. Aislar aislar nuevos estilbenos de las partes leñosas de *Vitis vinifera* y testar su actividad citotóxica en HepG2.

2. Examinar la actividad antiinflamatoria de 20 estilbenos en un modelo celular de macrófagos murinos estimulados con lipopolisacárido.

3. Analizar en el modelo murino los efectos antiinflamatorios de los estilbenos más potentes en términos de producción de óxido nítrico, especies reactivas de oxígeno (ROS) y citocinas proinflamatorias TNFa e IL-1β.

4. Detectar la citotoxicidad de siete oligoestilbenos de la vid *Vitis vinifera* en dos líneas celulares de hepatoma humano con diferente estado de p53 (HepG2 p53 natural y Hep3B nulo p53) y en hepatocitos humanos no transformados.

5. Establecer la implicación de p53 en los efectos tóxicos del estilbeno seleccionado (R2-viniferina) en las células de hepatoma.

6. Descifrar los mecanismos citotóxicos de R2-viniferina en HepG2, estudiando específicamente la producción de anión superóxido, peróxido de hidrógeno, vías de muerte celular y la influencia del estilbeno en el ciclo celular, la migración y la capacidad de formación de colonias.

Para la consecución de los objetivos se emplean 4 líneas celulares. El estudio del efecto antiinflamatorio de los estilbenos se lleva a cabo en la línea celular de macrófagos murinos RAW 264.7. Las líneas de hepatocarcinoma humano HepG2 y Hep3B y la línea de hepatocitos humanos no transformados HH4 son los modelos para el estudio del potencial antitumoral de los estilbenos. Todos los estilbenos usados para el desarrollo de esta tesis doctoral poseen una pureza de al menos el 95% y se extraen de un extracto natural rico en estilbenos, llamado Vineatrol®30, elaborado a partir de sarmientos de vid, excepto el resveratrol (Sigma), oxiresveratrol y piceatanol (TCI chemicals).

El análisis estadístico de los datos se llevó a cabo mediante los programas estadísticos SPSS 19.0 (SPSS Inc., Chicago, IL, USA) y GraphPad Prism 6 (San

Diego, CA, USA). Los datos se expresaron como la media ± el error estándar de al menos tres experimentos independientes. El IC50 o IC30 (concentración que inhibe un 50% o un 30% el crecimiento celular) se derivó de la curva semilogarítmica dosis-respuesta. La significación estadística de las diferencias de las medias se estimó por el test de la t de Student o ANOVA seguido del test post hoc de Dunnett. Las diferencias entre las medias se consideraron estadísticamente significativas para $P<0,05$.

Los estilbenos utilizados para el análisis de la actividad antitumoral comprenden 8 monómeros (resveratrol, piceido, piceatanol, astringina A, pterostilbeno, oxiresveratrol, isorhapontina e isorhapontigenina), 7 dímeros (ε-viniferina, ω-viniferina, δ-viniferina, ampelopsina A, escirpusina A, palidol y vitisinol C), un trímero (miyabenol C) y 4 tetrámeros (hopeafenol, isohopeafenol, R2-viniferina y R-viniferina). Todos se ensayaron en la línea de macrófagos murinos activados por LPS, analizando tanto su citotoxicidad como su capacidad para inhibir la producción de NO. A partir de dichos experimentos se calcularon sus IC30 o IC50, respectivamente. Los estilbenos seleccionados en base a los resultados obtenidos fueron piceatanol, ε-viniferina, δ-viniferina, hopeafenol e isohopeafenol, con los que se estudió el efecto que ejercen sobre la liberación de citoquinas inflamatorias y la producción de ROS. Hopeafenol y el piceatanol inhiben la producción de ROS, y únicamente δ-viniferina no disminuye la liberación de las citoquinas IL-1β y TNF-α. Se concluye que los derivados del resveratrol piceatanol, ε-viniferina, hopeafenol e isohopeafenol poseen interesantes efectos sobre la respuesta inflamatoria producida por la activación que el LPS ejerce en los macrófagos murinos. Este hallazgo justifica continuar el estudio en modelos más complejos ante su posible utilidad en el tratamiento de enfermedades con base inflamatoria.

Para el estudio de la actividad antitumoral, se compararon los IC50 obtenidos tras el tratamiento con 6 estilbenos de las 2 líneas de hepatoma con la línea de

hepatocitos no transformados. Se analizaron el resveratrol (monómero), ampelopsina A y ε-viniferina (dímeros) y los tetrámeros hopeafenol, isohopeafenol, R2-viniferina y R-viniferina. El tetrámero R2-viniferina presenta la mayor actividad citotóxica en HepG2 (IC50 < 10 μM), sin resultar tóxico a dicha concentración para HH4. Las células Hep3B son sensiblemente más resistentes a R2-viniferina (IC50 48 μM). El estilbeno produjo aumento de células HepG2 en SubG0, sin alteración en Hep3B. La diferente respuesta a este estilbeno resultó ser debida al diferente estatus de p53, puesto que al silenciar p53 en las células HepG2 aumentaba su resistencia al tratamiento, en tanto que la transfección de p53 en Hep3B las hicieron más sensibles. R2-viniferina provoca la fosforilación de p53 en HepG2.

La activación de caspasa 3 y 9, el incremento del ratio de las proteínas Bax/Bcl2 y la liberación de LDH intracelular evidenciaron la muerte celular por apoptosis y necrosis producida por dicho estilbeno, la cual puede ser modulada por las rutas de PI3K/Akt y ERK1/2 en HepG2. Además, la expresión de la histona γ-H2AX mostró el daño que el tratamiento con R2-viniferina produce en el DNA. El papel clave que juegan los radicales libres en todo este proceso fue dilucidado debido al incremento de la producción de ROS y H_2O_2 y al aumento de la actividad Mn-SOD. También se evidenció que la R2-viniferina disminuye la capacidad migratoria y de formación de colonias de HepG2.

Se concluye que el tetrámero R2-viniferina ejerce su actividad antitumoral a través de mecanismos de apoptosis ligados a p53 y es un prometedor compuesto con potenciales efectos para la quimioprevención y tratamiento de cáncer de hígado.

RÉSUMÉ

L'utilisation de composés naturels pour prévenir ou lutter contre le cancer a connu un engouement considérable ces dernières années. En effet, près de la moitié des molécules testées comme agents chimiothérapeutiques potentiels sont des composés issus de plantes. Le métabolisme secondaire des plantes, appelé également métabolisme spécialisé, produit de nombreuses molécules dont l'un des buts est de les protéger de facteurs de stress biotiques ou abiotiques (facteurs climatiques, agents pathogènes, etc.). La diversité structurale de ces composés chimiques est très grande. Parmi ces molécules les composés phénoliques occupent une place singulière. Ces composés, en fonction de leur structure chimique, sont classés en différentes familles : flavonoïdes au sens large, acides phénoliques, lignanes, stilbènes. Cette dernière famille fait l'objet du présent travail. Les stilbènes sont des polyphénols présents dans différentes sources végétales. Bien que présents dans certains fruits rouges, ceux de notre alimentation proviennent principalement de la vigne domestique (*Vitis vinifera* L.) par la consommation de raisin et de vin. La structure de base des stilbènes est composée de deux cycles phénoliques reliés par une double liaison. A partir de laquelle sont formés différents monomères, dimères, trimères et tétramères. Le stilbène le plus étudié est le resvératrol, dont les propriétés antioxydantes et potentiellement antitumorales sont bien décrites dans la littérature scientifique. Cependant, l'analyse des propriétés biologiques des autres stilbènes de la vigne reste un domaine de recherche relativement vierge. La diversité structurale des stilbènes peut expliquer les différentes activités exercées par chacun d'eux sur divers modèles biologiques. Certains de ces composés présentant des effets supérieurs à ceux du resvératrol.

Le carcinome hépatocellulaire représente près de 90% des cancers du foie, ce qui en fait un problème de santé publique majeur à l'échelle mondiale. Aujourd'hui, le sorafénib est le médicament avec lequel les meilleurs résultats ont été obtenus

dans le traitement du carcinome hépatocellulaire avancé. Le sorafénib est un inhibiteur multi-kinase qui augmente l'espérance de vie du patient de 3 à 5 mois, mais à de nombreuses reprises, il produit des effets secondaires qui nécessitent l'arrêt du traitement.

Le carcinome hépatocellulaire est un processus complexe dans lequel de nombreux mécanismes sont impliqués. La cirrhose du foie est le principal facteur de risque associé au développement de cette tumeur; la cirrhose du foie résulte d'une inflammation chronique suite à des dommages continus. Dans cette inflammation chronique, des médiateurs inflammatoires tels que le TNF-α ou l'IL-1β sont libérés, et un stress oxydant survient en réponse au déséquilibre entre la production et l'élimination des radicaux libres. Tous ces événements provoquent des changements phénotypiques dans le foie et endommagent les cellules saines, induisant sa mort et créant un micro-environnement qui favorise le développement et la croissance des cellules tumorales.

Compte tenu de ce contexte, l'HYPOTHÈSE de base de ce travail est que d'autres stilbènes présents dans *Vitis vinifera* puissent avoir une plus grande activité cytotoxique sur des cellules du carcinome hépatocellulaire que le resvératrol, le stilbène de référence pour la communauté scientifique.

L'OBJECTIF PRINCIPAL de cette thèse de doctorat était d'abord de réaliser une sélection préliminaire d'une série de nouveaux stilbènes de *Vitis vinifera* vine pour leur potentiel cytotoxique et anti-inflammatoire, et ensuite de sélectionner le plus puissant pour établir son mécanisme d'action.

Pour cela, les OBJECTIFS OPÉRATIONNELS suivants sont proposés:

1. Isoler de nouveaux stilbènes des parties ligneuses de *Vitis vinifera* et tester leur activité cytotoxique dans HepG2.

2.. Analyse de la capacité cytotoxique et inhibitrice de la production d'oxyde nitrique de 20 stilbènes issus d'extraits de *Vitis vinifera* sur des macrophages murins activés par le LPS, et sélection de ceux ayant le potentiel anti-inflammatoire le plus élevé.

3. Étude de la capacité anti-inflammatoire des stilbènes sélectionnés sur des macrophages murins, en termes de libération de cytokines inflammatoires, telles que IL-1β et TNF-α, et production d'espèces réactives oxygénées (ERO).

4. Analyse de l'activité cytotoxique de 7 stilbènes sur deux lignées cellulaires d'hépatome humain (HepG2 et Hep3B) ayant un statut p53 différent, en utilisant des hépatocytes humains non transformés (HH4) comme modèle de référence.

5. Étude de l'influence du statut de p53 sur la réponse aux stilbènes ayant la plus grande activité antitumorale (R2-viniférine).

6. Analyse du mécanisme d'induction de la cytotoxicité sur des cellules HepG2 par la R2-viniférine en termes de production de EROs, la production d'anion superoxyde et le peroxyde d'hydrogène, mécanismes de mort cellulaire, l'influence du stilbène sur le cycle cellulaire,migration et capacité de formation de colonies.

Pour atteindre les objectifs, 4 lignées cellulaires sont utilisées. L'étude de l'effet anti-inflammatoire des stilbènes est réalisée sur la lignée cellulaire macrophage murine RAW 264.7. Les lignées de carcinome hépatocellulaire humain HepG2 et Hep3B et la lignée d'hépatocytes humains non transformés HH4 sont les modèles utilisés pour étudier le potentiel antitumoral des stilbènes. Tous les stilbènes utilisés pour l'élaboration de cette thèse de doctorat ont une pureté d'au moins 95% et sont extraits d'un extrait naturel riche en stilbènes, appelé Vineatrol®30, issu de sarments de vigne, à l'exception du resvératrol (Sigma), oxyresveratrol et piceatannol (TCI chemicals).

L'analyse statistique des données a été réalisée en utilisant les programmes statistiques SPSS 19.0 (SPSS Inc., Chicago, IL, USA) et GraphPad Prism 6 (San Diego, CA, USA). Les données ont été exprimées comme « moyenne ± erreur standard » d'au moins trois expériences indépendantes. La CI50 ou la CI30 (concentration qui inhibe la croissance cellulaire de 50% ou 30%) a été dérivée de la courbe dose-réponse semi-logarithmique. La signification statistique des différences dans les moyennes a été estimée par le test t de Student ou ANOVA suivi du test post hoc de Dunnett. Les différences entre les moyennes ont été considérées comme statistiquement significatives pour $P < 0,05$.

Les stilbènes utilisés pour l'analyse des activités biologiques comprennent 8 monomères (resvératrol, picéide, piceatannol, astringine A, pterostilbene, oxyresveratrol, isorhapontine et isorhapontigenine), 7 dimères (ε-viniférine, ω-viniférine, δ-viniférine, ampélopsine A, scirpusine A, pallidol et vitisinol C), un trimère (miyabenol C) et 4 tétramères (hopeaphenol, isohopeaphenol, R2-viniférine et R-viniférine).

Concernant les effets anti-inflammatoires, tous les stilbènes ont été testés sur une lignée de macrophages murins activés par du LPS, en analysant à la fois leur cytotoxicité et leur capacité à inhiber la production de NO. Leurs CI30 ou CI50 ont été calculées. A partir de ces travaux, 6 composés ont été sélectionnés : piceatannol, ε-viniférine, δ-viniférine, hopéaphenol et l'isohopéaphenol. Sur ces composés, la libération de cytokines inflammatoires et la production de ROS a été étudié. L'hopeaphenol et le piceatannol inhibent la production de ROS. La δ-viniférine ne diminue pas la libération des cytokines IL-1β et TNF-α. Il a été montré que le piceatannol, l'ε-viniférine, l'hopeaphenol et l'isohopeaphenol ont un effet sur la réponse inflammatoire produite par l'activation que le LPS exerce sur les macrophages murins. Cette observation justifie de poursuivre l'étude dans des modèles plus complexes étant donné son utilité possible dans le traitement des maladies à base inflammatoire.

Pour l'étude de l'activité anti-inflammatoire, la CI50 obtenue après traitement par 6 stilbènes (monomère : resvératrol ; dimères : ampélopsine A et ε-viniférine ; tétramères : hopeaphenol, isohopeaphenol, R2-viniférine et R-viniférine) de 2 lignées d'hépatomes a été comparée à une lignée d'hépatocytes non transformés. La R2-viniférine présente l'activité cytotoxique la plus élevée sur la lignée HepG2 (CI50 <10 μM), sans être toxique à cette concentration pour la lignée HH4. Les cellules Hep3B sont nettement plus résistantes à la R2-viniférine (CI50 48 μM). Le stilbène a produit une augmentation des cellules HepG2 en phase SubG0, sans altération des cellules Hep3B. La réponse différenciée des lignées cellulaires à l'action de ce stilbène peut être due au statut différent de p53. En effet, l'extinction de p53 dans les cellules HepG2 a augmenté leur résistance au traitement, tandis que la transfection de p53 dans les cellules Hep3B les a rendues plus sensibles. La R2-viniférine provoque la phosphorylation de p53 sur les lignées HepG2.

L'activation des caspases 3 et 9, l'augmentation du rapport des protéines Bax/Bcl2 et la libération de LDH intracellulaire ont mis en évidence la mort cellulaire par apoptose et nécrose provoquée par l'action de la R2-viniférine. Dans les lignée HepG2, Cette action est modulable par PI3K/Akt et ERK1/2. De plus, l'expression de l'histone γ-H2AX a montré les dommages que le traitement par la R2-viniférine produit sur l'ADN. Le rôle clé que jouent les radicaux libres dans tout ce processus a été observé en raison de l'augmentation de la production de ROS et de H_2O_2, et de l'augmentation de l'activité Mn-SOD. Il a également été démontré que la R2-viniférine diminue la capacité de migration et de formation de colonies de cellules HepG2.

Ces résultats montrent que la R2-viniférine, un tétramère du resvératrol, exerce une activité antitumorale par le biais de mécanismes d'apoptose liés à p53. Ce composé est un agent prometteur avec des effets potentiels pour la chimioprévention et le traitement du cancer du foie.

INTRODUCTION

1. Polyphenols

Plant-derived compounds have gained more interest in cancer chemotherapy because they usually exert less toxicity to non-tumoral cells, perhaps as a consequence of they have evolved with the target sites in biological systems (Mishra and Tiwari 2011). In fact, between 1940 and 2014, nearby half of the small molecules tested as chemotherapy agents were natural products, compared to the 17% amounting the synthetic agents (Newman and Cragg 2016).

Among natural products with antitumor properties, secondary metabolites stand out. Secondary metabolites are small organic molecules produced by plants which are no essential for the growth, development and reproduction of the organism, but that are considered as phytoalexins because of their involvement in defense against ultraviolet radiation or aggression by pathogens (Seca and Pinto 2018).

1.1 Biosynthesis

According to the pathway by which they are synthesized, secondary metabolites can be classified in phenolic compounds, terpenoids/isoprenoids and alkaloids/glucosinolates (Aharoni and Galili 2011).

Natural polyphenols can be originated through two pathways that can occur independently or together. One pathway involves the binding of two-carbon units to form polyketides, which undergo subsequent cyclisation into polyphenols (Cutrim and Cortez 2018). But the major mechanism by which most polyphenols are biosynthesized is shikimate pathway. Via this pathway, the derived carbohydrate precursors from the glycolysis and pentose phosphate pathways are converted to the aromatic amino acids phenylalanine, tyrosine and tryptophan. The deletion of an ammonia molecule to form cinnamic acid and the

subsequent conversion to *p*-OH-cinnamic acid, followed by the sequential addition of hydroxyl and methoxy groups, give rise to the formation of caffeic and ferulic acids, which are precursors of the largest group of polyphenols, that is flavonoids (O'Connell and Fox 2001; Hopkins, W.G Hüner N.P 2004; Taiz, L. & Zeigner, E. 2002).

1.2 Classification

The structure of polyphenols consists of a benzene ring with at least one hydroxyl group attached to it. Attending to the number of phenol rings presented and to the structural components that bind these rings together, polyphenols can be broadly divided in four categories: flavonoids, phenolic acids, stilbenes and lignans (Manach et al. 2004).

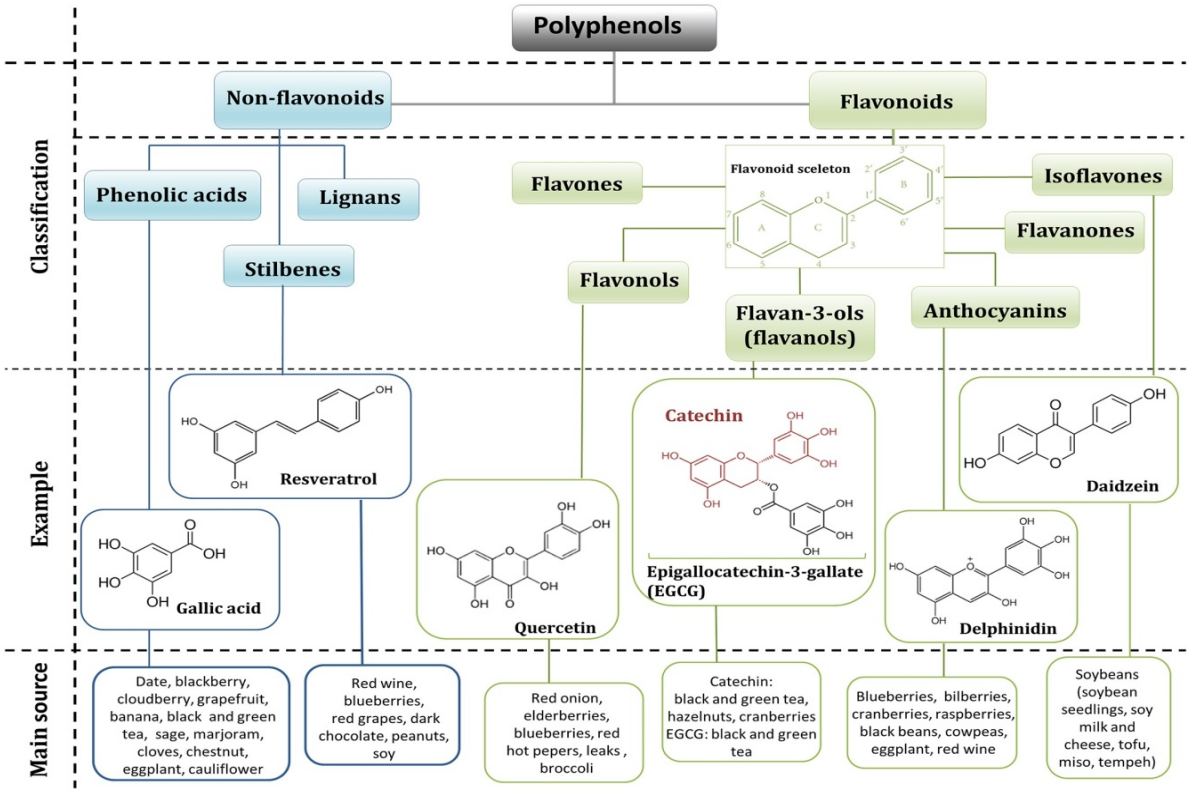


Figure 1. Chemical structures and classification of polyphenols (Goszcz et al. 2017).

1.2.1 Stilbenes

Stilbenes, or stilbenoids, are polyphenols whose basic structure is formed by two phenol rings. Ring A carries two hydroxyl groups in the metha-position, while ring B can carry hydroxy and methoxy groups in the *ortho-* (R2), *metha-* (R3) and/or *para-* (R4) position. Their precursors are cinnamic acid derivatives, and the substitution pattern of the acid determines the substitution pattern in the ring B of the stilbene (Cassidy, Hanley, and Lamuela-Raventos 2000).

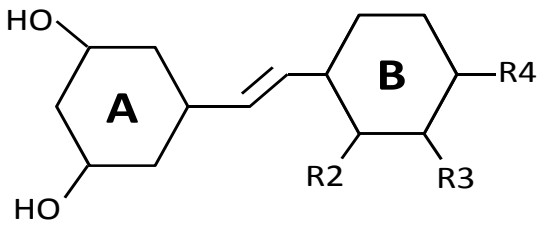


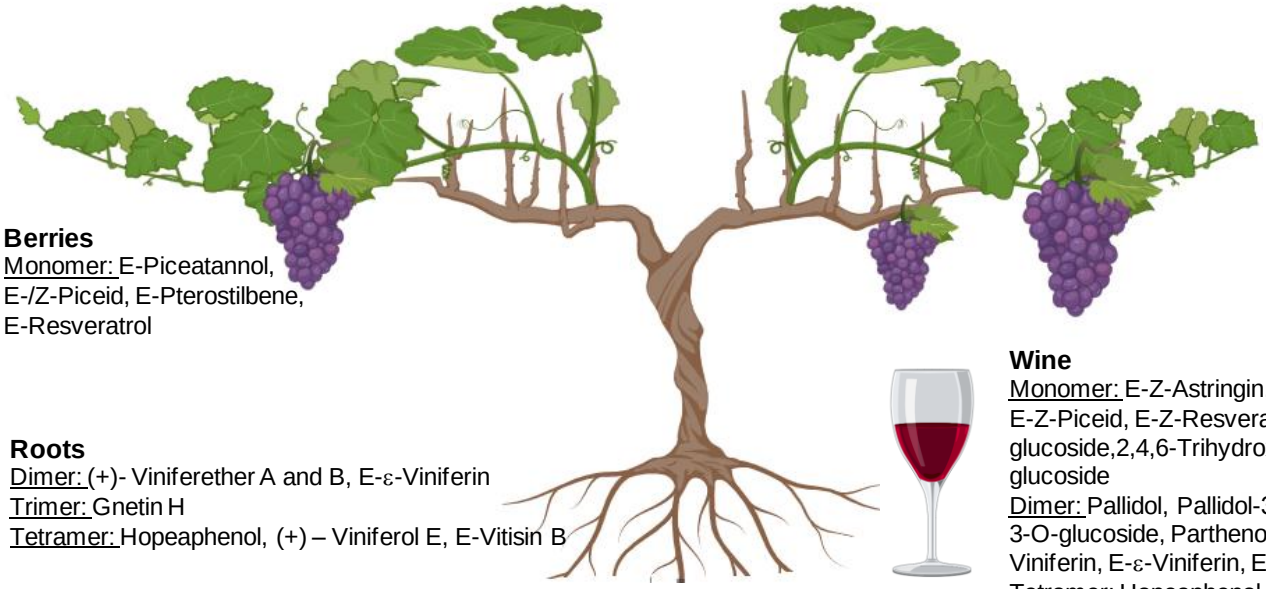
Figure 2. *Trans*-stilbene structure.

Stilbenes can be found in a wide range of plant families, as Celastraceae, Cyperaceae, Dipterocarpaceae, Fabaceae, Gnetaceae, Iridaceae, Moraceae, Paeoniaceae and Vitaceae (Rivière, Pawlus, and Mérillon 2012). They paly a protective role against biotic and abiotic stress factors. Despite of its wide distribution in plants, in human diet is limited to a few foods, being the most important western dietary source of stilbenes the derivatives of *Vitis vinifera L.*, as wine (98.4% of the intake) and berries and juice grapes (1.6%), followed in a lower quantity by peanuts and other berries (<0.01%) (Zamora-Ros et al. 2008).

In the classification proposed by Pawlus et al. among 100 stilbenes identified in the genus *Vitis*, only 17 monomers, 24 dimers, 6 trimers and 14 tetramers resulted to be present in *Vitis vinifera,* being diverse its distribution in the different parts of the vine, and only some of them present in wine **(Figure 3)**.

Leaves
Monomer: E-Piceid, E-Pterostilbene, E-/Z-Resveratrol
Dimer: Ampelopsin D, Pallidol, Quadrangulin A, E-δ-Viniferin, Z-ε-Viniferin, (+)-E-ε-Viniferin, E-ε-Viniferin, E-/Z-ω-Viniferin
Trimer: E-/*Z-trans*- Miyabenol C, E-*cis*-Miyabenol C, α-Viniferin
Tetramer: Ampelopsin H, Hopeaphenol, Isohopeaphenol, Vaticanol C isomer

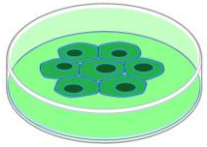
Stems
Monomer: E-Piceatannol, E-/Z- Piceid, E-/Z- Resveratrol, E- Resveratrol-2-C-glucoside
Dimer: (+) – Ampelopsin A and F, (-) – Malibatol A, Pallidol, Scirpusin A, Viniferifuran, (+)-E-ε-Viniferin, E-ε-Viniferin
Trimer: E-*trans*-Miyabenol C, (+)-Viniferol D
Tetramer: Hopeaphenol, Isohopeaphenol, (+)-Viniferol A, B and C, (+)-Vitisifuran A and B,Vitisin A, E-Vitisin B and C



Berries
Monomer: E-Piceatannol, E-/Z-Piceid, E-Pterostilbene, E-Resveratrol

Roots
Dimer: (+)- Viniferether A and B, E-ε-Viniferin
Trimer: Gnetin H
Tetramer: Hopeaphenol, (+) – Viniferol E, E-Vitisin B

Wine
Monomer: E-Z-Astringin, E-Piceatannol, E-Z-Piceid, E-Z-Resveratrol, E-Resveratrol-2-C-glucoside,2,4,6-Trihydroxyphenanthrene-2-O-glucoside
Dimer: Pallidol, Pallidol-3,3"-diglucoside, Pallidol-3-O-glucoside, Parthenocissin A,E-δ-Viniferin, Z-ε-Viniferin, E-ε-Viniferin, E-/Z-ε-Viniferin- diglucoside
Tetramer: Hopeaphenol

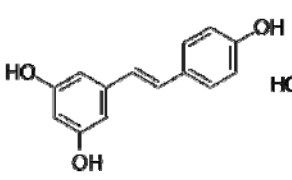


Cell suspension culture
Monomer: E-/Z-Astringin, E-/Z- Piceid, E-/Z-Resveratrol, E-Z- Resveratrol-3,4´- O-β-diglucoside, E-/Z- Resveratrol-3,5-O-β-diglucoside, Z-Resveratrol-3,5,4´-O-β-triglucoside, E-/Z-Resveratroloside
Dimer: Pallidol, E-δ-Viniferin, E-δ-Viniferin-11-O- β-D-glucopyranoside, E-δ-Viniferin-11´-O- β-D-glucopyranoside

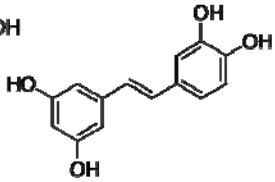
Figure 3. Stilbenes in cell suspension culture, berries, stems, leaves, roots and wine of *Vitis vinifera* according to (Pawlus et al. 2012)**.**

Resveratrol is the most studied stilbene. Its synthesis is catalyzed by stilbene synthase in three different condensation reactions among three molecules of malonyl-CoA and one molecule of coumaroyl-CoA (Soleas, Diamandis, and Goldberg 1997). There are a high number of resveratrol analogues and oligomers, differing in the number and position of substituents (hydroxyl, methoxyl, halogenated, glycosylated, esterified), the presence of stilbenic double bonds and different stereoisomers (Perrone et al. 2017). Oligomers occur as dimers, trimers, and tetramers in plants. Dimers are almost universally generated by an oxidative radical coupling, and the others oligomers seem to be the product of oxidative dimerizations (Langcake and Pryce 1977). Trimers are formed by three resveratrol monomers, and tetramers are originated from four monomers, or two different dimmers, or a monomer and a trimer (Xue et al. 2014).

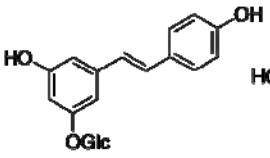
MONOMERS



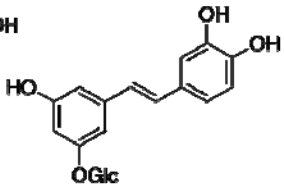
Resveratrol



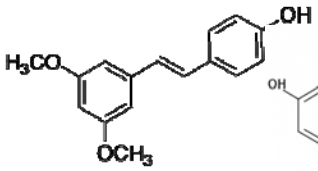
Piceatannol



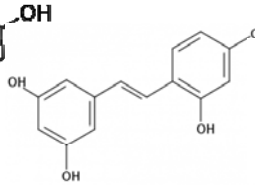
Piceid



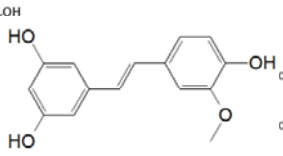
Astringin A



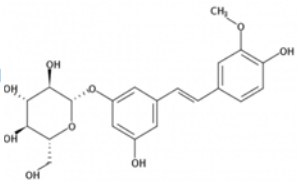
Pterostilbene



Oxyresveratrol

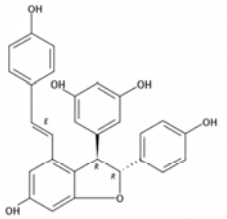


Isorhapontigenin

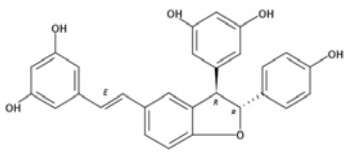


Isorhapontin

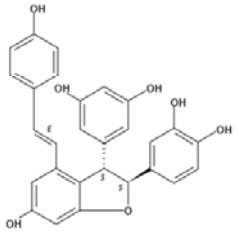
DIMERS



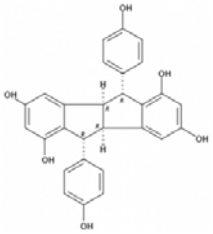
ε-Viniferin



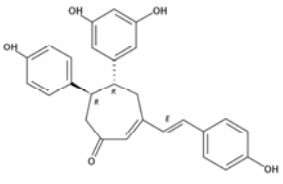
δ-Viniferin



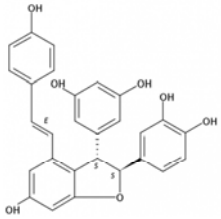
ω-Viniferin



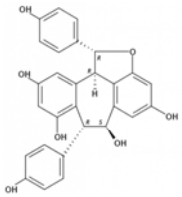
Pallidol



Vitisinol C

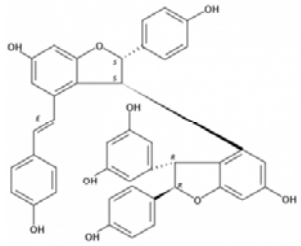


Scirpusin A



Ampelopsin A

TRIMER



Miyabenol C

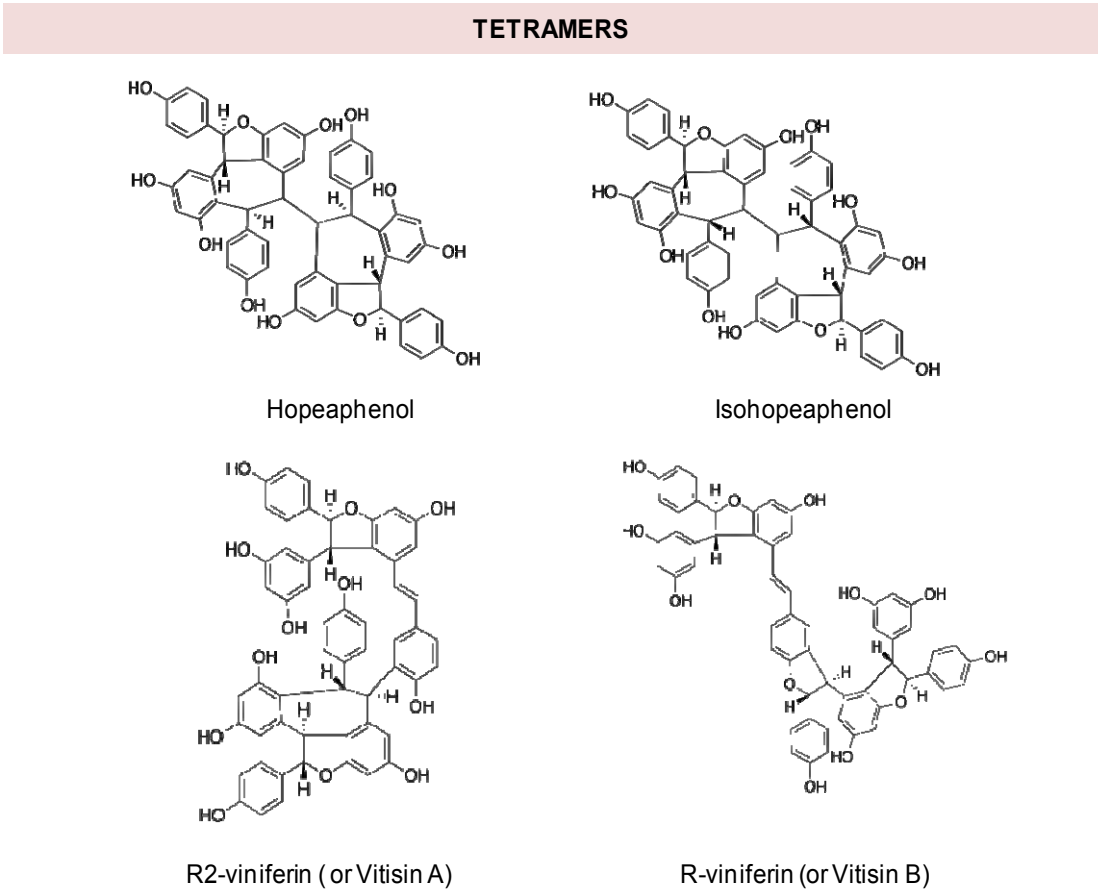


Figure 4. Chemical structure of stilbenes. Obtained from https://mib-polyphenol.eu/

The characterization of the total stilbene content from raw grapevine shoot extract rendered the identification of 13 compounds (Biais et al. 2017). Stilbene amounted 48.8% of the raw material, and ε-viniferin was the most abundant stilbene (16.5%), followed by resveratrol (9.3%), isohopeaphenol (4.4%), ampelopsin A (3.9%) and R2-viniferin (also named as Vitisin A) (3.2%) (Biais et al. 2017).

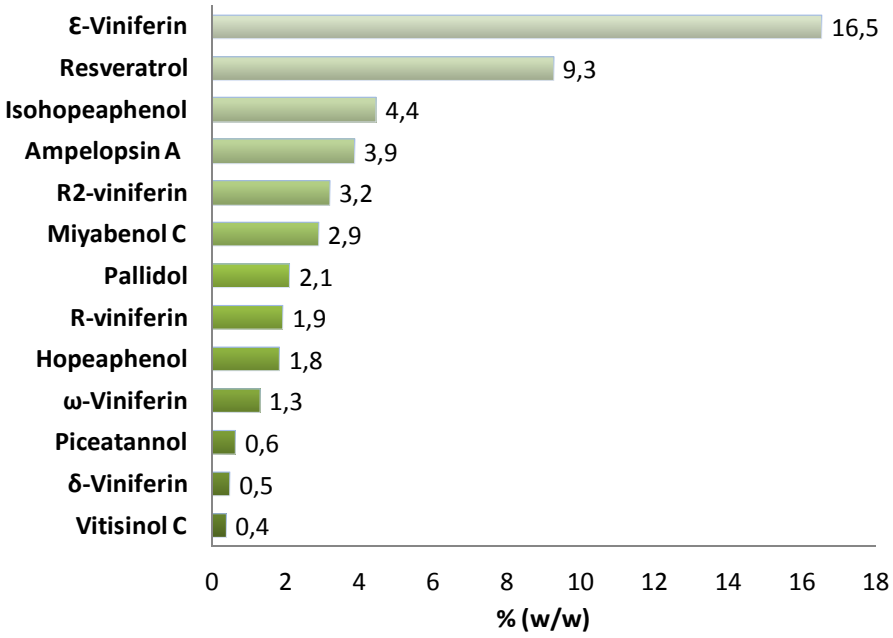


Figure 5. Stilbene content in raw grapevine shoot extract. Data adapted from (Biais et al. 2017).

The degree of oligomerization is an important factor in terms of bioavailability, absorption and distribution, so each stilbene has a different pattern, presenting monomers the highest rates. While vitisin B (also named as R2-viniferin) is not able to reach the bloodstream after an oral administration, viniferins do. Better rates are reach by monomers oxyresveratrol, gnetol and pinostilbene (around 20%), presenting the highest bioavailavility the pterostilbene (around 60%). Moreover, pterostilbene and its metabolites have been found in different organs, so they are well distributed (El Khawand et al. 2018). The presence of two methoxy groups in the pterostilbene structure makes it more lipophilic and thus more bioavailable (Kapetanovic et al. 2011). The same authors (El Khawand et al. 2018) hypothesized the possibility that oligomers exert a local action in the intestine while monomers present a systemic action.

1.2.2 Biological effects

It could be said that interest in the human health benefits of stilbenes arose from the year 1958, when Bronte-Stewart 1958 publish a study in which shown that in spite the European countries had comparables amounts of dietary fat intakes, France had the lower mortality rates due to coronary heart disease (CHD). In 1979, Leger, Cochrane, and Moore (1979) described the same phenomena comparing France to other development countries such as the United Kingdom and United States.

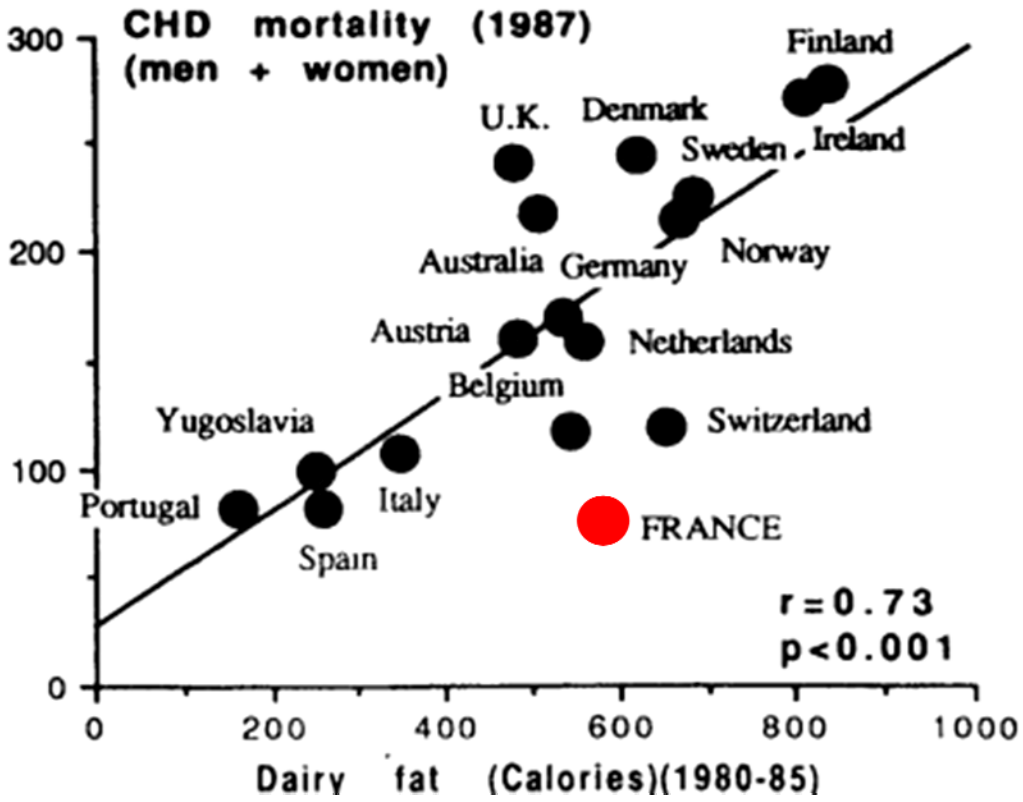


Figure 6. Relation between age-standardised death rate from CHD (mean for the men and women) and compsumption of dairy fat in countries reporting wine consumption. (Renaud and de Lorgeril 1992)

But it was in 1992 when Renaud and de Lorgeril tried to explain the French Paradox postulating that it might be due to the ability of wine consumption to counteract the negative effects of fat intake. From there, biological activities of

stilbenes, particularly for their ability to prevent various diseases associated with oxidative stress, like cancers, cardiovascular and neurodegenerative diseases, have been extensively studied (Akinwumi, Bordun, and Anderson 2018).

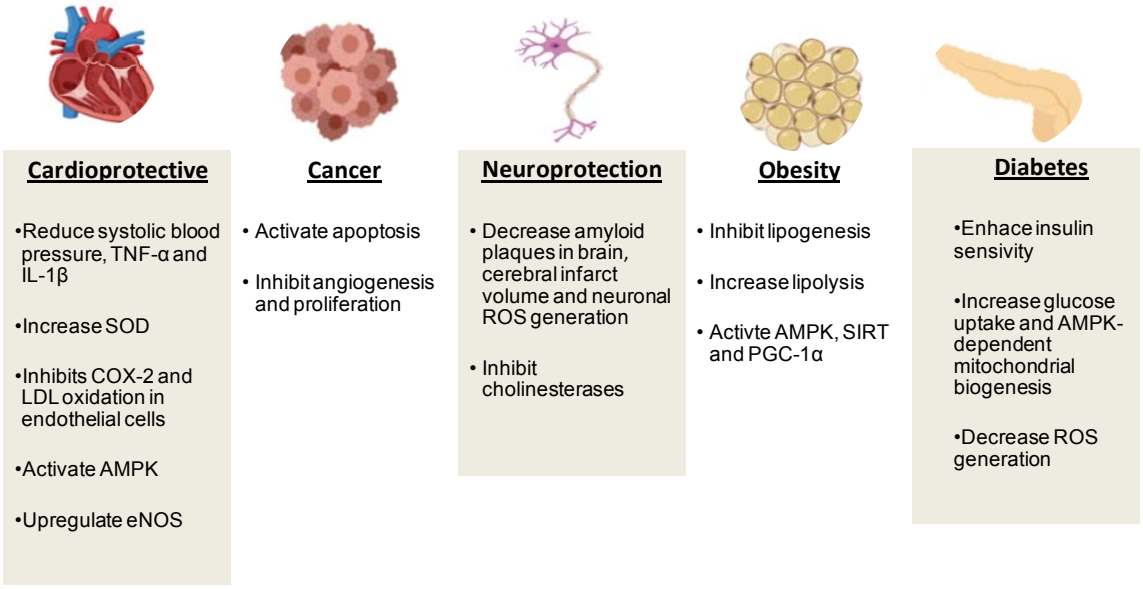


Figure 7. Biological activities of stilbenes. Adapted from (Akinwumi, Bordun, and Anderson 2018).

2. Hepatocellular carcinoma

According to the last European Association for the Study of the Liver (EASL) Clinical Practice Guidelines (Galle et al. 2018), hepatocellular carcinoma (HCC) is considered a global health problem that represents about 90% of primary liver cancers worldwide. Attending to the geographical distribution providded by GLOBOCAN 2018, a striking imbalance is observed, with the highest incidence rates in Asia (72.5%), followed by Europe (9.8%), Africa (7.7 %) and North America (5%), where the incidence is lower.

In last years the incidence of HCC has suffered a significant growth, standing out that new cases of HCC increased by 75% between 1990 and 2015, mainly due to changes in the population's age pyramid and the demographic increase (Akinyemiju et al. 2017).

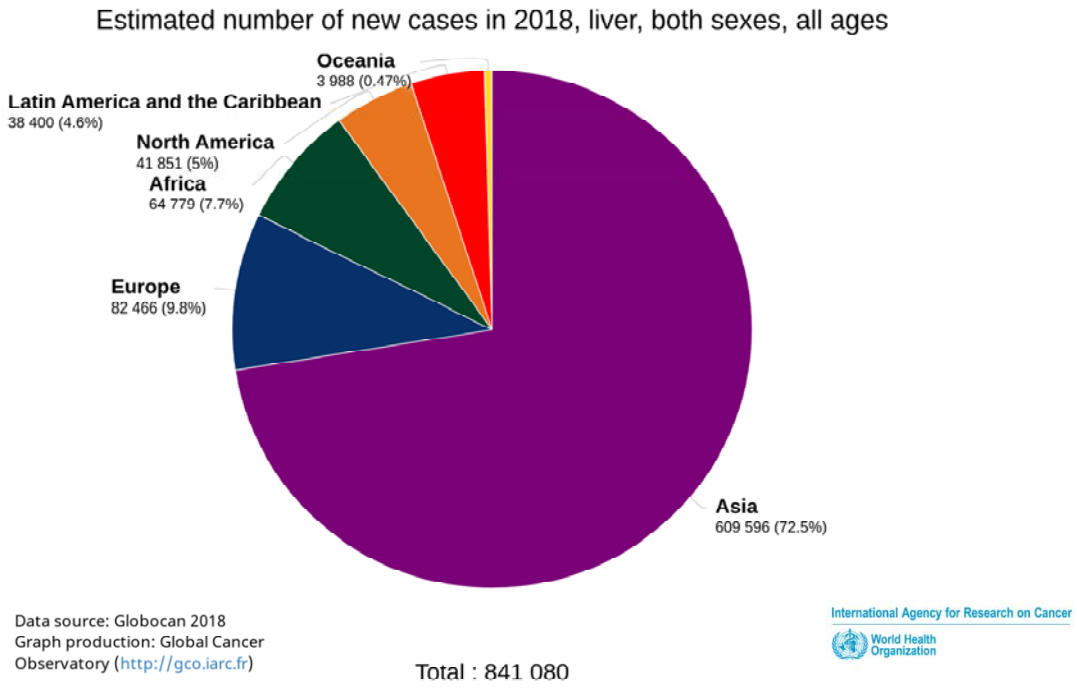


Figure 8. Geographical distribution of HCC. Obtained from GLOBOCAN.

2.1 Aethiology

The main risk factor for the development of HCC is liver cirrhosis associated with chronic hepatitis B (HBV) and hepatitis C (HVC) infections, nonalcoholic fatty liver disease (NAFLD), aflatoxin B exposure, alcohol intake, and several metabolic disorders (Tretiakova et al. 2010; Leung 2015).

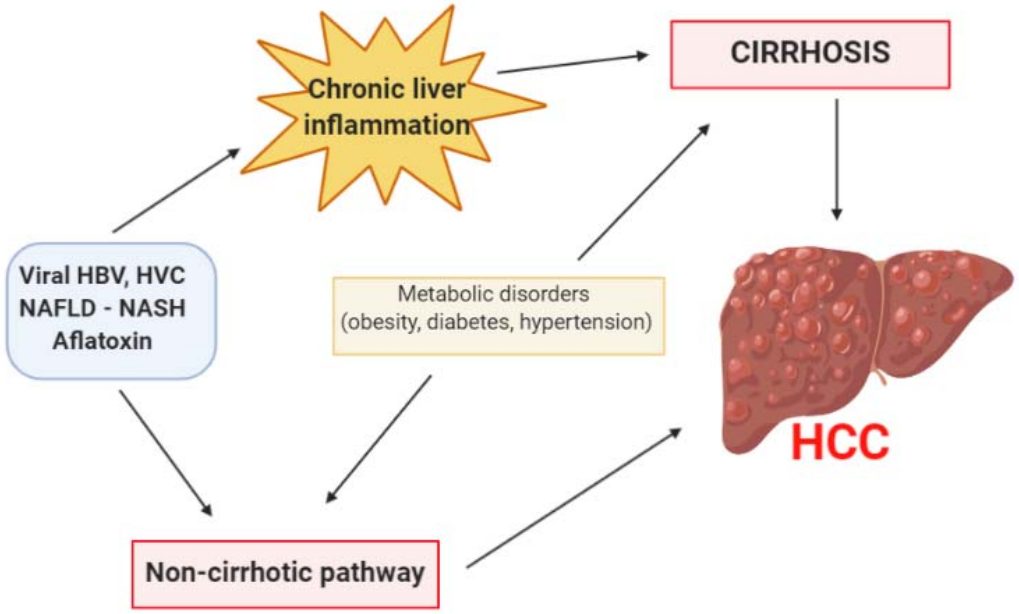


Figure 9. Risk factors for the HCC development.

Cirrhosis is a result of continuous damage to the liver and enduring inflammation, both important promoters of carcinomas as HCC (Tang et al. 2018). This chronic inflammation is accompained with the release of cytokines, such as tumor necrosis factor-α (TNF-α) or interleukin-1 (IL-1) and reactive oxygen species (ROS). The release of these mediators and ROS, also produced by kupffer cells (KCs), induce the activation of hepatic stellate cells (HSCs) to a collagen synthesizing phenotype (Li et al. 2008; Campo, Gallego, and Grande 2018). These mediators also can cause mitochondrial dysfunction, partly via oxidative damage to lipids (peroxidation), oxidation of respiratory chain proteins, and DNA damage, triggering cell death due to necrosis or apoptosis (Ademowo et al. 2017).

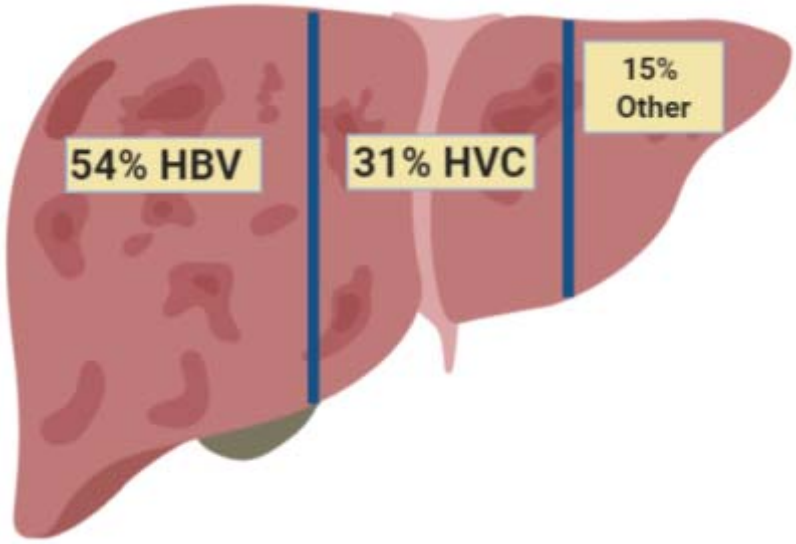


Figure 10. Most frequent causes of HCC

Approximately 54% of cases can be attributed to HBV infection worldwide, while 31% can be attributed to HCV infection, and the remaining 15% associated with other causes (Tang et al. 2018). Both hepatitis viruses promote the development of HCC by oncogenic viral protein expression that carry on the mutations and malignant transformation of infected cells. In addition, HVC induces hepatic inflammation and fibrosis, both of which contribute to carcinogenesis and apoptosis, as mentioned before (J. D. Yang et al. 2011; Lemon and McGivern 2012). In 2018, 54.5% of HCC worldwide cases were attributable to HBV infection, while 21.2% were to HCV infection (de Martel et al. 2020). The risk of development HCC among those with chronic HVB or HVC infection increases with co-infection, male sex, older age, chronic alcohol or tobacco use, aflatoxin exposition, diabetes and obesity (Rapti 2015).

Despite alcohol abuse, non-alcoholic fatty liver disease (NAFLD) is a major risk also for HCC development. In patients with NAFLD an excess of triglycerides accumulates in liver cells and, along other manifestations such as obesity and

type-2 diabetes mellitus, results in non-alcohol steatosis (NASH) and, finally, in cirrhosis development (Massarweh and El-Serag 2017).

Aflatoxins are secondary metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus* that contaminate a wide variety of maize, groundnuts, wheat, soy and rice, especially is countries with a warm and humid environment (Valdes 2017). Aflatoxin is mainly metabolized in the liver, and generates hypermutation in p53 tumor suppression gene, that could lead the HCC development (Adam et al. 2017).

2.2 HCC treatments

The actual EASL Clinical Practice Guidelines (Galle et al. 2018), propose a modified Barcelona Clinic Liver Cancer (BCLC) staging system and treatment strategy, in which patients with HCC are classified into five stages according to pre-established prognosis variables, and therapies are allocated according to treatment-related status.

During the early stages of HCC, surgical approaches like local ablation, liver resection or liver transplantation, and nonsurgical approaches such as chemoembolization, are the most common treatments. However, in patients with advanced cancer-related symptoms like macroscopic vascular invasion, lymph node involvement or metastases, other approaches are necessary. In 2007, the Food and Drug Administration (FDA) approved the use of Sorafenib (a multi-tyrosine kinase inhibitor) in patients with advanced HCC. The great results of Sorafenib supported the searching and evaluation of other first-line therapies as Lenvatinib, second-line therapies as Regorafenib, Cabozantinib, Ramucirumab

and immune checkpoint inhibitors as Nivolumab or Pembrolizumab (Montironi, Montal, and Llovet 2019).

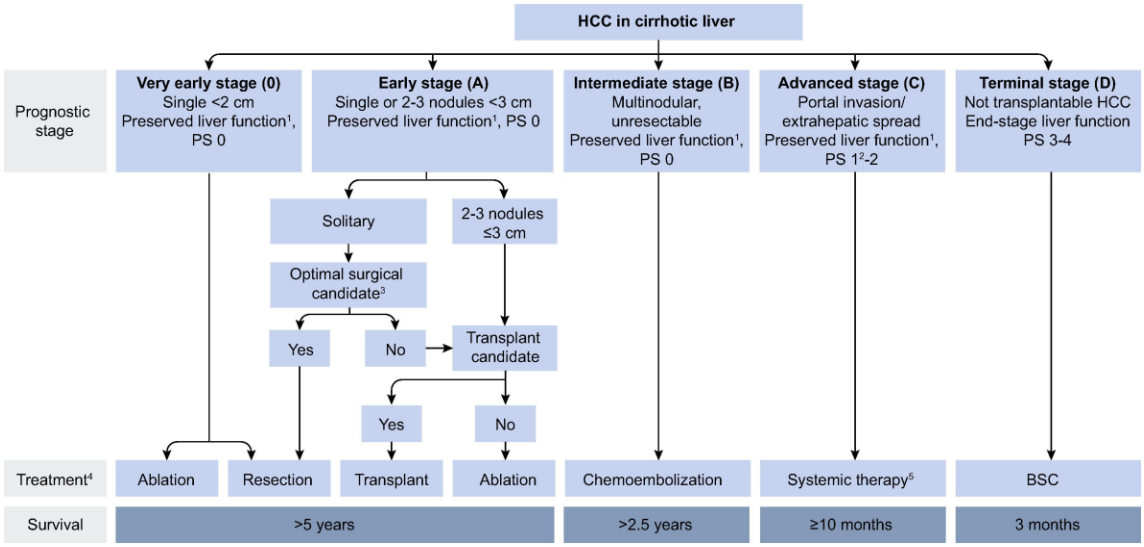


Figure 11. Modified BCLC staging system and treatment strategy. EASL Clinical Practice Guidelines.

In spite of being the standard treatment for advanced HCC, Sorafenib efficacy is moderate, with a tumor response of around 30%, and an average survival rate increased by 3-5 months (Llovet et al. 2008). Generally, it is well-tolerated, but some patients show severe toxicities, such as skin reactions, diarrhea, fatigue or liver dysfunction and in rare cases, but fateful, cardiovascular events, arterial thromboembolic events and bleeding (Llovet et al. 2008; Kane et al. 2009; Philip 2009). Due to the side effects, in many cases the Sorafenib treatment is dose-reduced or discontinued (Philip 2009) and no alternative effective therapeutics are available after the failure of treatment with Sorafenib (Bahman et al. 2018).

3. Molecular mechanisms of HCC

HCC is a multistep process and the molecular basis of HCC progression may differ depending on diverse factors and a number of mechanisms might be involved (Alotaibi et al. 2016). As mentioned, in majority of cases, HCC occurs as result of cirrhosis. The cirrhotic nodules support a favorable microenvironment for the malignant transformation of hepatocytes which leads to HCC development through the successive accumulation of genetic and epigenetic changes (Alqahtani et al. 2019).

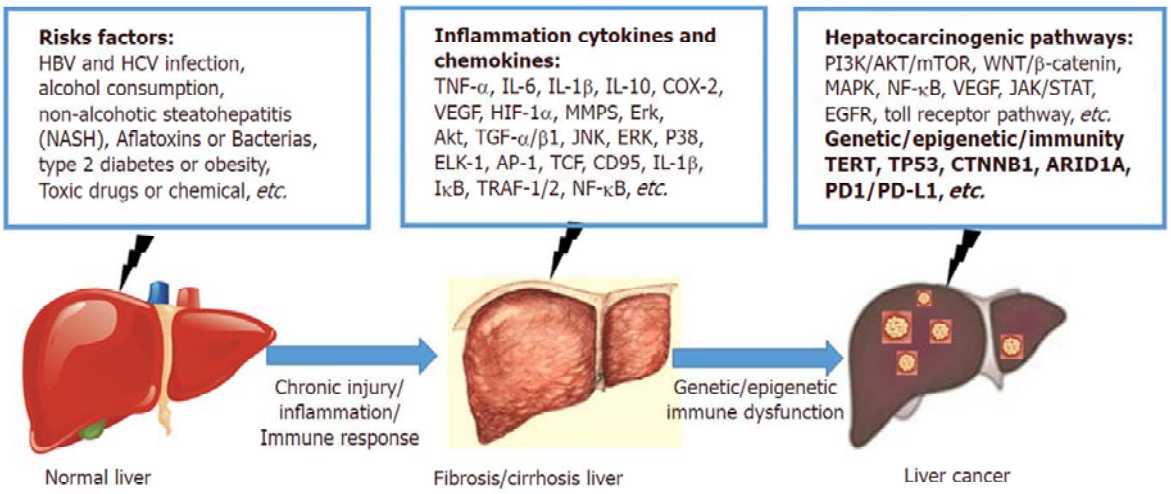


Figure 12. The multistep process of the development of liver cancer. The risk factors, inflammatory cytokines and chemokines, and hepatocarcinogenic pathways are related to the inflammation-cancer transformation during the development of primary liver cancer. From (Chen et al. 2018).

3.1 Inflammation

Exposure to HVB or HVC viruses, aflatoxins or lipopolysaccharide (LPS), triggers hepatic inflammation. As result of this chronic damage, the homeostasis of liver parenchymal cells is upset and macrophages and mast cells immediately triggers the inflammatory process by releasing soluble mediators as cytokines, chemokines, ROS and bioactive mediators as histamine, which induced leukocytes mobilization and infiltration at the site of injury (B. Vendramini-Costa

and E. Carvalho 2012). Chronic inflammation leads to excessive tissue remodeling, loss of tissue architecture and modifications on DNA and proteins as consequence of oxidative stress, enhancing the risks for cancer development (Chen et al. 2018).

Many of the immune cells, including macrophages, neutrofils and eoisinofils, are involved directly or indirectly in pathology of chronic inflammation by cytokine production. Tumor necrosis factor-α (TNF-α) and interleukin family (IL-6, IL-1β) are well known cytokines. IL-6 is produced by activated kupffer cells in chronic heptatitis. It promotes local inflammatory response and activates hepatocyte proliferation leading to cancerous hepatocytes (Naugler and Karin 2008). IL-1β is found to promote hepatic stellate cell proliferation, activation and transdifferentiation into the myofibroblastic phenotype (Han et al. 2004). This cytokine can promote hepatic inflammation by inducing the production of C-reactive protein, a sensitive marker of infection and inflammation, independently of IL-6 (Weinhold and RüTher 1997, 6). It has been identified that TNF-α causes DNA damage through the induction of ROS (Montfort et al. 2019). Recently, (Jing et al. 2018), have described that the overexpression of TNF-α promotes HCC through the activation of hepatic progenitor cells (HPCs); the knocking down of TNF-α inhibited the HPC activation and proliferation, which reduces tumor incidence. All these data confirmed that TNF-α plays an important role in liver injury and prognosis.

3.1.1 NF-κB pathway

NF-κB is a transcription factor that regulates innate immunity and inflammation. It has been demonstrated to play an essential role in the regulation of inflammatory signaling pathways in the liver (Xiao and Ghosh 2005) (Muriel

2009), being a critical link between inflammation and cancer (Taniguchi and Karin 2018).

NF-κB complex is constituted by five proteins: RelA (p65), c-Rel, RelB, NF-κB1 (p50/p105), and NF-κB2 (p52/p100) (Ghosh and Karin 2002). In physiological conditions, the dimmer formed by the p50 and p65 subunits rests inactive in the cytoplasm through binding to the inhibitory protein of NF-κB (IκB) (Hoffmann and Baltimore 2006). In response to pro-inflammatory stimuli, including inflammatory cytokines (such as TNF-α and IL-1β), the IκB kinase (IKK) complex phosphorylates IκB and promotes its degradation by the proteasome (Karin and Ben-Neriah 2000; West et al. 2006). Consequently, the activated NF-κB dimmer is released and translocates to the nucleus. There, binds specific DNA sequences, and stimulates transcription of several target genes involved in inflammation, immune responses, cell proliferation, and cell survival (Pahl 1999; Ghosh and Karin 2002).

3.2 Free radicals: ROS and RNS

Free radicals are molecules containing unpaired electrons in their orbitals, and are capable of have independent existence. They act as redox signaling molecules and they are extremely unstable and highly reactive. Cells produce two different types of redox signaling molecules: reactive oxygen species (ROS), such as the superoxide anion radical ($O^{\bullet -}_2$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\bullet}OH$) and reactive nitrogen species (RNS), such as the nitric oxide radical ($^{\bullet}NO$), the nitrogen dioxide radical ($^{\bullet}NO_2$), and peroxynitrite ($ONOO^-$) (Nathan and Ding 2010).

3.2.1 ROS

Reactive oxygen species (ROS) are oxygen-containing radicals that are produced by organisms as a result of normal cellular metabolism. They have an important role in physiological cell processes. Under normal physiological conditions, antioxidants are able to modulate the internal levels of ROS. However, several types of stress, as cytokines produced by chronic inflammation, leads that the ROS production overpasses the capacity to scavenge them. In such cases, they trigger detrimental modifications to lipids, proteins and DNA, leading a pathological cell process. This unbalance between oxidant and antioxidant capacity is known as "oxidative stress."

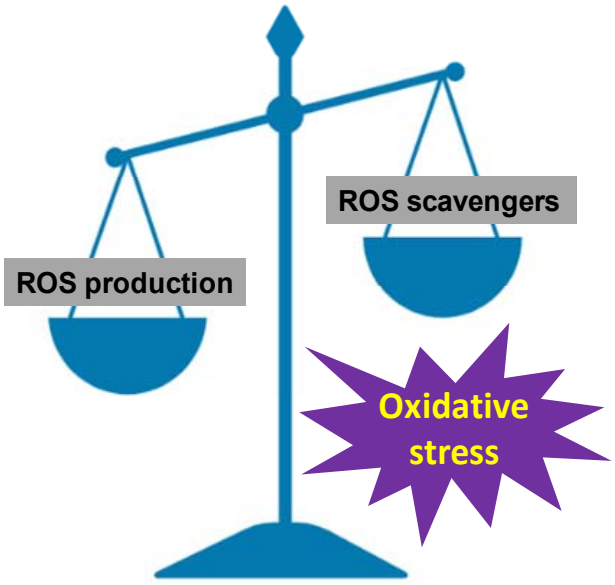


Figure 13. Imbalance between ROS production and ROS scavengers produce oxidative stress, which is dangerous for the fate of the cell.

The superoxide anion radical ($O^{\bullet -}_2$) hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\bullet}OH$) are the more common ROS. These molecules are produced as by-products during the mitochondrial electron transport, or by oxydoreductase

enzymes and metal catalyzed oxidation. Cells have a variety of defense mechanisms to ameliorate their harmful effects. Among antioxidants are included enzymatic scavengers as superoxide dismutase (SOD), catalase and glutathione peroxidase, as well as non-enzymatic scavengers such as vitamins C and E, glutathione (GSH), lipoic acid, carotenoids and iron chelators (Redza-Dutordoir and Averill-Bates 2016).

SOD is the responsible of catalyze the transformation of two superoxide anions into a molecule of hydrogen peroxide (H_2O_2) and oxygen (O_2). In eukaryotic peroxisomes, the enzyme catalase converts H_2O_2 to H_2O and O_2, and thus concludes the detoxification initiated by SOD. Glutathione peroxidase is a family of enzymes containing selenium, which also catalyze the degradation of hydrogen peroxide, as well as break down organic peroxides to alcohol.

In addition to ROS, RNS also play an important role. In physiological conditions these reactive species are detoxified, however, when they are overproduced, as occurs in many diseases related to chronic inflammation, these reactive species can cause nitrative and oxidative DNA damage (Cerutti and Trump 1991).

Nitric oxide (NO) is an important mediator between chronic inflammation and hepatocarcinoma, which is produced by hepatic parenchymal and non-parenchymal cells through the inducible nitric oxide synthase (iNOS) (Bishayee 2014). The expression of iNOS is induced in a wide variety of tissues in response to endotoxin, endogenous mediators of inflammation, and other stimuli such as hypoxia (Weigert and Brüne 2008). In the presence of important level of $O_2^{\cdot-}$, the NO generated by iNOS may react with the radical in a n-enzymatic reaction to generate peroxynitrite ($ONOO^-$). All the reactive species, at a non-counterbalanced concentration, damage macromolecules, and ultimately drive cells to death by processes such as apoptosis and/or necrosis (Halliwell 2011).

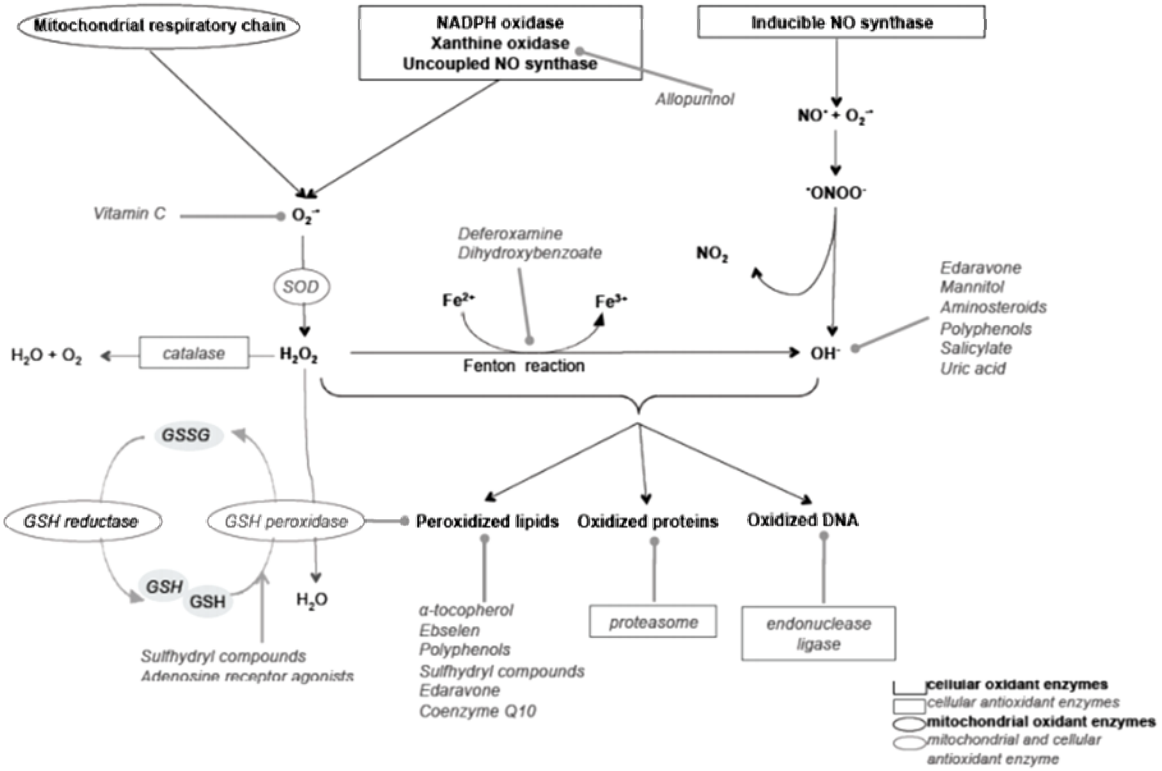


Figure 14. Major pathways of cellular ROS/RNS generation and detoxification. Antioxidant elements are represented in italics characters. Blunted grey arrows represent an inhibitory effect against oxidative stress by antioxidant enzymes or by antioxidant compounds. (L. Poirrier et al. 2010).

3.3 Cell death

Cell death includes two broadly defined mechanisms: programmed cell death and necrosis (Fuchs and Steller 2011). While necrosis is the result of an unordered cellular explosion in response to severe and irresistible trauma (Jan and Chaudhry 2019), programmed cell death is a genetically programmed process of cell suicide in response to particular signals (Gorski and Marra 2002; Fuchs and Steller 2011). It is an extended error to equate programmed cell death with apoptosis, despite of existing non-apoptotic forms of programmed cell death. Therefore, programmed cell death and apoptosis could not be used as synonymous (Jan and Chaudhry 2019).

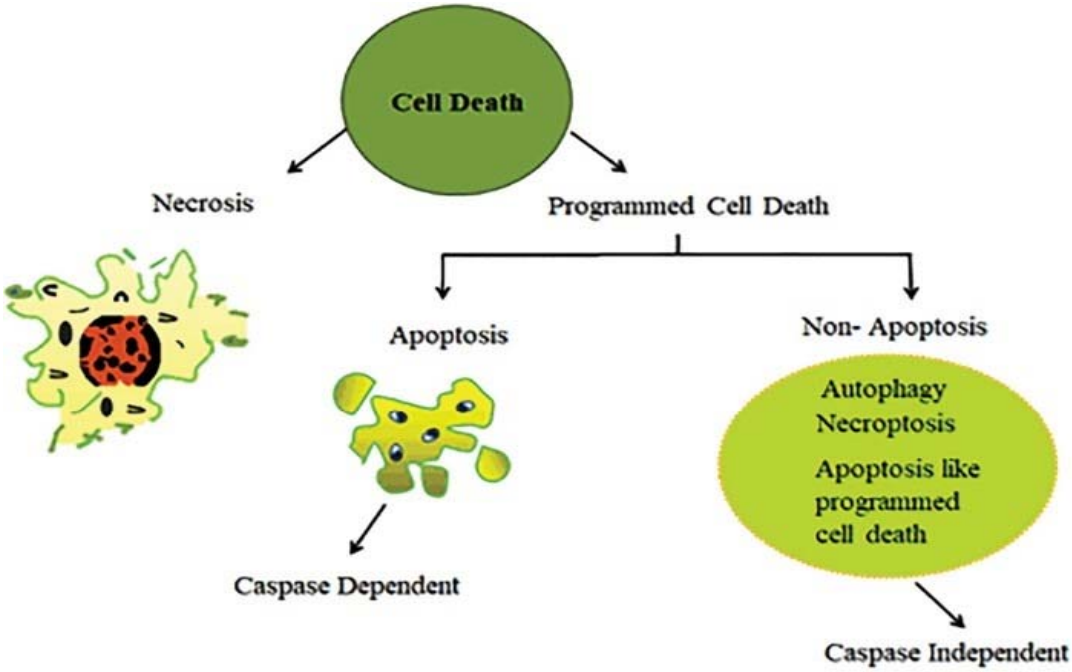


Figure 15. General mode of cancer cell death. Cancer cell death involves two broadly defined mechanisms: programmed cell death and necrosis. Programmed cell death mainly apoptosis and nonapoptosis base cell death, such as autophagy, necroptosis and apoptosis like programmed cell death. (Jan and Chaudhry 2019).

3.3.1 Apoptosis

Apoptosis is a complex process that proceeds through at least two main pathways, extrinsic and intrinsic pathways. Both of them converge on the execution pathway which is initiated by the activation by cleaving of caspase-3, resulting in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, crosslinking of proteins, formation of apoptotic bodies, expression of ligand for phagocytic cell receptors and, finally, uptake by phagocytic cells (Elmore 2007).

Caspases are the main effectors of apoptosis and belongs to a family of proteins with proteolytic activity because they are able to cleave proteins in aspartic acid residues (McIlwain, Berger, and Mak 2013). Caspases are widely expressed as zymogens in most cells, and once proteolytically processed can activate other procaspases, leading to a cascade by which the apoptotic signal is amplified and

results in cell death promotion (Elmore 2007). These enzymes are classified in three types according to their functions. Caspases-1, 5, 12 and 14 have an important role as inflammation mediators. In relation with apoptosis, capases-2, 8, 9 and 10 play a role as apoptosis initiators, while caspases-3, 6 and 7 are executioner caspases (Behzadi and Ranjbar 2015).

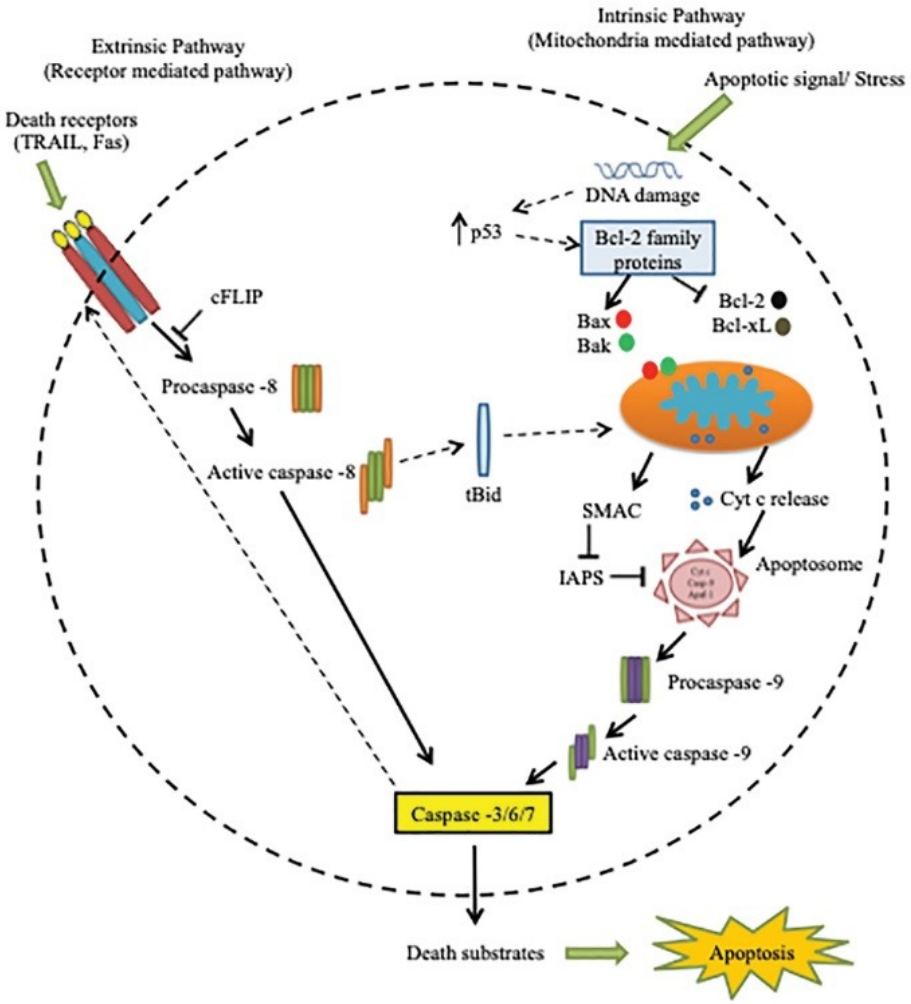


Figure 16. Pathways of apoptosis. Apoptosis consists of two main pathways. Extrinsic pathway triggered by external stimuli or ligand molecules and particularly involved death receptors (DRs). The intrinsic pathway is mediated by Bax/Bak insertion into mitochondrial membrane. Subsequently, cytochrome c is released, which combines with Apaf–1 and procaspase-9 to produce apoptosome followed by the activation of caspase-3 cascade of apoptosis. TNF related apoptosis inducing ligand (TRAIL), cellular FLICE inhibitory proteins (cFLIP), truncated bid (tBid), B-cell lymphoma protein 2 (Bcl-2), Bcl-2 homologue splice variants (Bcl-xL), cytochrome (Cyt C), second mitochondrial activator of caspases (SMAC), inhibitor of apoptosis proteins (IAPs). (Jan and Chaudhry 2019).

3.3.1.1 Extrinsic pathway

This apoptotic pathway is activated when extracellular ligands such as TNF-α, Fas ligand (Fas-L) and TNF-related apoptosis-inducing ligand (TRAIL) recognize the extracellular domain of the death receptors (DR) and active them. This activation results in the formation of a death-inducing signaling complex (DISC) (J. W. Kim, Choi, and Joe 2000). Stimulation of death receptors results in activation of the initiator caspase-8, which can propagate the apoptosis signal by direct cleavage of downstream effector caspases such as caspase-3 (Walczak and Krammer 2000). Death receptors mediated apoptosis can be inhibited by a protein named c-FLIP (Guicciardi and Gores 2009). There is a link between the extrinsic and intrinsic pathway that starts when active caspase-8 cleaves Bid and truncated Bid interacts with mitochondria. From this point is not possible distinguish between the two apoptotic pathways (Kantari and Walczak 2011).

3.3.1.2 Intrinsic pathway

This apoptosis pathway is mediated mainly by mitochondria. The intrinsic pathway is triggered by extra and intra-cellular stresses like oxidative stress, irradiation and treatment with cytotoxic drugs (Jan and Chaudhry 2019). These stimuli causes changes in the inner mitochondrial membrane that leads the Bax/Bak insertion into mitochondrial membrane and subsequently, cytochrome c release from the mitochondrial membrane space into the cytosol (R. Kim 2005). In non-apoptotic conditions, the anti-apoptotic proteins (members of Bcl-2 family) prevents the release of cytochrome c (Cory and Adams 2002). The tumor suppressor p53 has a critical role in regulation the expression of Bcl-2 family proteins. p53 increases the ratio pro-apoptotic (Bax, Bad, Bid)/anti-apoptotic (Bcl-2, Bcl-x, Bcl-XL) proteins triggering the release of cytochrome c, caspase activation and finally apoptosis (Fridman and Lowe 2003). The release of

cytochrome c into the cytosol triggers caspase-3 activation through formation of the cytochrome c/Apaf-1/caspase-9-containing apoptosome complex, whereas Smac/DIABLO promote caspase activation through neutralizing the inhibitory effects to the inhibitors of the apoptosis proteins (IAPs) (Fulda and Debatin 2006).

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HYPOTHESIS AND OBJECTIVES

HYPOTHESIS AND OBJECTIVES

The interest in identifying new chemotherapeutic agents has greatly increased in recent years, specifically natural products derived from plants, because of their anti-inflammatory and antitumor activities. Among these natural products, those with a phenolic structure have focused a great attention. Stilbenes are polyphenols produced by plants as secondary metabolites against infections. The most studied stilbene is resveratrol, which has shown properties against diseases such as cancer. Other members of the stilbene family include those based on the resveratrol structure, with different number and position of hydroxyl groups, sugars, methyl or methoxy groups, and oligomers (dimers, trimers and tetramers). Grapes and red wine are the main sources of resveratrol, but other parts of the grapevine (wood, canes and roots), which are waste in the wine industry, are important and cheap sources for bioactive stilbenes. Despite being present at high quantities, there are few reports of the antitumoral activities of these resveratrol-derived stilbenes.

The HYPOTHESIS of this work is that *Vitis vinifera* contains bioactive stilbenes, some of which show cytotoxic activities against human hepatoma cells higher than resveratrol, the most studied stilbene.

The main OBJECTIVE of the present Doctoral Thesis was first to carry out a preliminary screening of a series of novel stilbenes from *Vitis vinifera* vine for their cytotoxic and anti-inflammatory potential, and second to select the most potent one to establish its mechanism of action.

The following OPERATIVE OBJECTIVES were raised:

1. To isolate novel stilbenes from the woody parts of *Vitis vinifera* and test their cytotoxic activity in HepG2.

2. To screen 20 stilbenes for their anti-inflammatory activity in a cellular model of lipopolysacharide-stimulated murine macrophages.

3. To analyze in the murine model the anti-inflammatory effects of the most potent stilbenes in terms of the production of nitric oxide, reactive oxygen species (ROS), and pro-inflammatory TNFα and IL- cytokines.

4. To screen the cytotoxicity for seven oligoestilbenes from *Vitis vinifera* vine in two human hepatoma cell lines with different p53 status (wild-type p53 HepG2 and p53-null Hep3B), and in non-transformed human hepatocytes.

5. To stablish the involvement of p53 in the toxic effects of the selected stilbene (R2-viniferin) in the hepatoma cells.

6. To unravel the cytotoxic mechanisms of R2-viniferin in HepG2, studying specifically the production of superoxide anion, hydrogen peroxide, cell death pathways, and the influence of the stilbene on cell cycle, migration, and capacity of colony formation.

RESULTS AND DISCUSSION

CHAPTER I

Unusual stilbene glucosides from *Vitis vinifera* roots

This chapter has been published in:

Iris Aja, Grégory Da Costa, Eric Pedrot, Marie-Laure Iglesias, Antonio Palos-Pinto, Josep Valls, Nassima Chaher, M. Begoña Ruiz-Larrea, Jean-Michel Mérillon, Djebbar Atmani, José Ignacio Ruiz-Sanz and Tristan Richard. "Unusual stilbene glucosides from Vitis vinifera roots". *OENO One* 2019, 3, 573-579 Doi:10.20870/oeno-one.2019.53.3.2462

Abstract

Aim: Stilbenes are well-known phytoalexins present in vine and wine. Stilbene glucosides have been identified in wine; however, other than piceid, these compounds have never been reported to be present in the woody parts of grapevine. The aims of this study were to investigate the presence of stilbene glucosides in the woody parts of the vine and to evaluate their cytotoxic activity, in comparison with that of resveratrol, in human hepatoma HepG2 cells.

Methods and results: Stilbene glucosides were isolated from a *Vitis vinifera* root extract. The extract was partitioned with ethyl acetate and fractionated by polyamide gel column chromatography. Pure compounds were obtained by semipreparative high-performance liquid chromatography. These were then identified by mass spectrometry and nuclear magnetic resonance (NMR) analyses, including analysis of two-dimensional NMR spectra. In addition to resveratrol, five stilbene glucosides were found: resveratroloside, resveratrol rutinoside, *trans*-ε- viniferin diglucoside, *cis*-ε-viniferin diglucoside and piceid. Of these, the first four showed cytotoxic effects against HepG2 cells when the crystal violet assay was used to determine cell viability.

Conclusion: In addition to resveratrol and piceid, this is the first report of the presence of four glucosylated derivatives of resveratrol (resveratroloside, resveratrol rutinoside, and *trans*- and *cis*-ε-viniferin diglucosides) in the woody parts of vine. These compounds showed significant cytotoxicity against HepG2 cells.

Significance and impact of the study: Stilbenes are well-known biological compounds. Grapevine is one of the main sources of this family of polyphenols. Other than piceid, stilbene glucosides have been identified in wine but never in the woody parts of vine. This is the first study in which five glucosylated derivatives of resveratrol were isolated from woody parts of vine. They were also shown to exert antiproliferative effects in human hepatoma HepG2 cells.

Keywords: antiproliferative effects, cytotoxicity, HepG2 cells, phytoalexins, resveratrol, stilbene glucosides

1. Introduction

Stilbenes are natural compounds present in several plants (Rivière *et al.*, 2012). One of their main vegetal sources is grapevine. They are present throughout the plant; however, the woody parts (e.g. wood and roots) are significantly richer in stilbenes than the vegetative and generative parts (Gabaston *et al.*, 2017). Stilbenes are known particularly for their antimicrobial properties (Schnee *et al.*, 2013); they also act as grapevine phytoalexins (Langcake and Pryce, 1977; Jeandet *et al.*, 2002).

Members of the stilbene family are based on the resveratrol structure (3,5,4′-trihydroxystilbene), differing in the number and position of hydroxyl groups, sugars, and methyl or methoxy groups. Furthermore, oxidation of resveratrol leads to the formation of various oligomers, including ε-viniferin, which is a dimer of resveratrol and one of the main stilbenes in grapevine stem extract (Biais *et al.*, 2017). In grapevine berries, stilbene glucosides are usually found in significantly higher concentrations than those of stilbene aglycones (Pawlus *et al.*, 2012). However, other than piceid, no stilbene glucoside has been reported in the woody parts of grapevine.

Resveratrol is a well-known bioactive polyphenol with many biological activities associated with life extension and some of the health benefits of wine (Vang *et al.*, 2011). Several studies have shown that resveratrol exerts, via diverse mechanisms, proapoptotic and antiproliferative effects in a number of different cancer cell lines (Ko *et al.*, 2017). Piceid is a resveratrol glucoside and the major resveratrol derivative in grape juices and wine (Neveu *et al.*, 2010). It may have similar beneficial actions against various pathologies to those of resveratrol (Fabris *et al.*, 2008). Su and colleagues have compared the antioxidant and antiproliferative effects of resveratrol and piceid in different cell lines (Su *et al.*, 2013). Their results have shown that piceid has stronger radical-scavenging activity than resveratrol and inhibits

proliferation in the human hepatoma HepG2 and human breast cancer MDA-MB-231 and MCF-7 cell lines.

Our aims in carrying out the present study were to investigate the presence of stilbene glucosides in grapevine root extracts and to evaluate the antiproliferative activity of these compounds *in vitro*, using the hepatoma HepG2 cell line.

2. Materials and methods

2.1. Plant material and reagents

Grapevine roots were kindly provided by Actichem S.A. (Montauban, France). Healthy grapevine roots, namely SO4 rootstocks (*Vitis riparia ´ Vitis berlandieri*), were harvested in the *Saint Christoly de Blaye* vineyard in the Bordeaux area of France. The roots were dried at room temperature for 2 months under conditions of no light, and then crushed in powder. Extraction was carried out using an ethanol–water mixture (85:15, v/v) under agitation at 60 °C. The ethanol was removed by evaporation *in vacuo*. The aqueous phase was then lyophilized, producing a brown powder.

Analytical-grade methanol, formic acid and ethyl acetate were supplied by Fisher Scientific (Waltham, MA, USA) or Sigma–Aldrich (St Louis, MO, USA). Acetonitrile for ultra–high- performance liquid chromatography (UPLC)–mass spectroscopy (MS) was of high- purity grade and purchased from Sigma–Aldrich. Deuterated solvents were purchased from Eurisotop (Saint-Aubin, France).

2.2. Extraction and isolation procedures

The crude root extract (10 g) was partitioned with ethyl acetate (100 mL). After removal of the ethyl acetate, the precipitate (3 g) was reconstituted in water (30 mL). Column chromatography was carried out using a glass column loaded with polyamide gel. The sample solution was loaded at the top of the polyamide and eluted with 500 mL of methanol, at a flow rate of 10 mL/min; this was done eight times. The eight fractions thereby obtained were subjected to evaporation *in vacuo*. They were screened for the presence of stilbenes by reverse- phase high-performance liquid

chromatography (HPLC) and ultra-performance liquid chromatography (UPLC)–MS in the negative-ion mode (Gabaston *et al.*, 2017). Fraction 3 (440 mg) was found to contain five stilbene glucosides.

This fraction was rechromatographed on a semipreparative HPLC Varian Pro Star equipped with an Agilent Zorbax C18 column (7 μm, 250 ´ 25 mm) (Agilent, Santa Clara, CA, USA). The solvent programme was a gradient systemA, water with 0.1 % formic acid; B, acetonitrile with 0.1 % formic acid. The elution programme at 15 mL/min was as follows: 10 % B (0–5 min); 10–20 % B (5–10 min); 20–30 % B (10–20 min); 30–35 % B (20–25 min); 35–60 % B (25–35 min); 60–100 % B (35–45 min); and 100 % (45–50 min). Chromatograms were monitored at 280 and 306 nm. Final purification yielded resveratroloside (3 mg), resveratrol rutinoside (1 mg), piceid (4 mg), *trans*-viniferin diglucoside (2 mg) and *cis*-viniferin diglucoside (1 mg).

2.3. NMR and mass spectrometry

Nuclear magnetic resonance spectra were recorded on a Bruker Avance III 600 NMR spectrometer (Bruker, Billerica, MA, USA). Mass spectra were recorded by an Agilent 1290 series UHPLC apparatus connected to an Esquire LC-ESI-MS/MS from Bruker Daltonics. Mass spectra were recorded in negative mode, with the capillary set at 1500 V, the end plate at –500 V, the capillary exit at –120.4 V, dry gas at 330 °C, gas flow at 11 L/min, the nebulizer at 60 p.s.i., target mass at *m*/*z* 500, scan range from *m*/*z* 100 to 3000, helium as the collision gas, and MS/MS fragmentation amplitude at 1.0 V. An analytical C18 column (Zorbax C18, 100 ´ 2.1 mm, 1.8 μm, Agilent) was used, with a flow rate of 0.4 mL/min (solvent system: 0.1 % [v/v] formic acid [A], acetonitrile [B]). Gradient started over 0 min at 5 % B, 1.7 min at 10 % B, 3.4 min at 20 % B, 5.1 min at 30 % B, 6.8 min at 30 % B, 8.5 min at 35 % B, 11.9 min at 60 % B, 15.3 min at 100 % B and 17.0 min at 100 % B, and resulted in 17.3

min at 10% B.

2.4. Cell culture and cell viability assay

The human hepatoma cell line HepG2 was purchased from the American Type Culture Collection (Manassas, VA, USA). The HepG2 cells were cultured in Eagle's Minimum Essential Medium (Sigma-Aldrich) supplemented with 10 % heat-inactivated fetal bovine serum, 2 mM L-glutamine, 0.1 mg/mL streptomycin and 100 U/mL penicillin (Sigma-Aldrich). The cells were grown at 37 °C in a humidified 5 % CO_2 atmosphere, with the medium replaced every 2–3 days. When the culture reached approximately 80 % confluence, the cells were detached by a solution of 0.1 % trypsin–0.04 % EDTA and harvested for use in the cell viability assays.

The HepG2 cells were seeded onto 96-well plates (5×10^3 cells/well), 24 h before treatment. Solutions of stilbenes (dissolved in culture medium) were added to the wells at increasing concentrations. After at least 72 h, the viability of the cells was determined using the crystal violet assay. The assay procedure was as follows. First, the medium was removed. The cells were then washed once with phosphate- buffered saline (PBS), before being fixed by exposure to a 3.7 % formaldehyde solution for 15 min at room temperature. Next, the cells were washed twice with PBS, before being stained by exposure to a 0.25 % crystal violet solution (Merck, Darmstadt, Germany) for 20 min in the dark. The microplates were then washed with running water and dried at 37 °C. After the crystal violet had been dissolved by adding 150 μL of a 33 % acetic acid solution, a Synergy HT microplate reader (BioTek, Winooski, VT, USA) was used to measure absorbance at 590 nm.

2.5. Statistical analysis

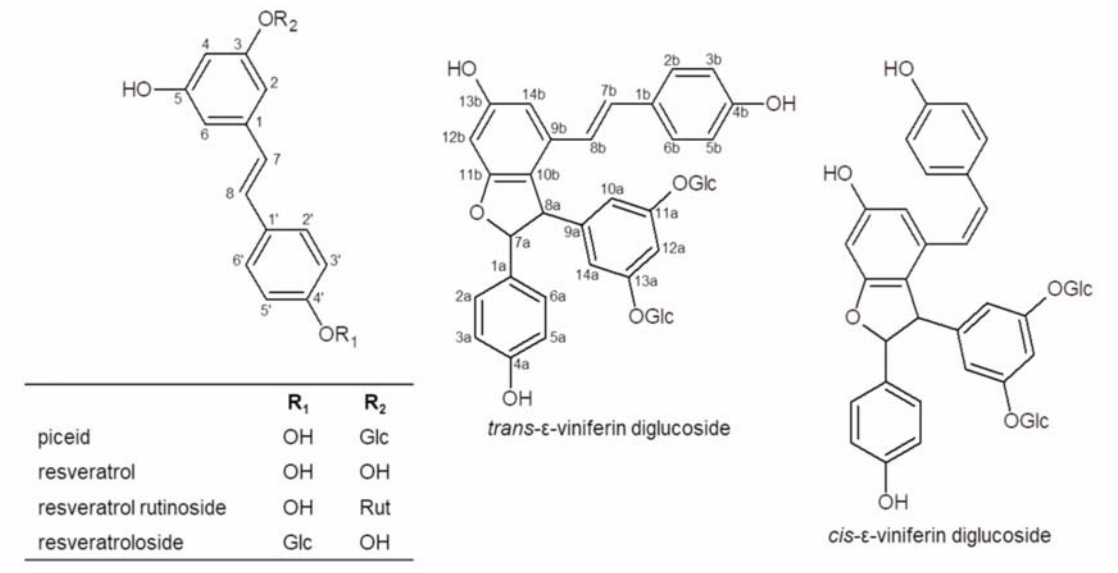
Means (± standard error, SE) were calculated from data yielded from at least three independent experiments. Means of related groups were compared by paired-samples Student's *t*-test, using the SPSS 17.0 statistical package (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

The main stilbenes in different grapevine extracts have been identified and quantified by previous studies (Biais *et al.*, 2017; Gabaston *et al.*, 2017). Thirteen stilbenes have been identified: ampelopsin A, hopeaphenol, isohopeaphenol, myabenol C, pallidol, piceatannol, resveratrol, δ-viniferin, ε-viniferin, ω-viniferin, vitisin A, vitisin B and vitisinol C. Additionally, their antioxidant and cytotoxic activities in PC12 cells have been evaluated using oxygen radical absorbance capacity (Biais *et al.*, 2017).

Surprisingly, liquid chromatography–MS analyses of extracts from different parts of grapevine, including canes, wood and roots, showed the presence of stilbene glucosides mainly in the root extracts. In an effort to isolate and identify these compounds, the grapevine root extract was fractionated further. First, the extract was partitioned with ethyl acetate. The precipitate was then subjected to column chromatography on a polyamide gel. Subsequent elution with methanol yielded eight fractions.

Each fraction was subjected to evaporation, reconstituted in water–methanol (1:1, v/v), and analysed by UPLC-MS or MS spectroscopy in negative mode. In addition to resveratrol, five putative glycosylated stilbenes were found to be present in fractions 3 and 4. These compounds were isolated and purified to enable elucidation of their structures. Purification was carried out using reverse-phase semipreparative HPLC. The structures were deduced from two-dimensional NMR experiments. Assignments of proton and carbon resonances were based on homonuclear and heteronuclear experiments, including COSY, HSQC, HMBC and ROESY experiments. The five stilbenes were identified as resveratroloside, resveratrol rutinoside, piceid, *trans*-viniferin diglucoside and *cis*-viniferin diglucoside. Their structures are shown in **Figure 1**.



	R_1	R_2
piceid	OH	Glc
resveratrol	OH	OH
resveratrol rutinoside	OH	Rut
resveratroloside	Glc	OH

Figure 1. Structures of resveratrol glucosides isolated from grapevine root extract.

1. Determination of the structure of the compounds

The structures of the stilbenes isolated are as follows:

- *Resveratroloside (3,5,4′-trihydroxystilbene-4′- O-b-glucoside)*: ESI-MS *m/z* 389 $[M - H]^-$; ^{1}H- NMR (DMSO-*d*6) δ 7.49 (2H, d, *J* = 8.7 Hz, H- 2′,6′), 7.00 (2H, d, *J* = 8.6 Hz, H-3′,5′), 6.98 (1H, d, *J* = 16.3 Hz, H-8), 6.91 (1H, d, *J* = 16.3 Hz, H-7), 6.39 (2H, d, *J* = 2.0 Hz, H-2,6), 6.11 (1H, brs, H-4), 4.86 (1H, d, *J* = 7.6 Hz, H-1″), 4.01 (1H, dd, *J* = 11.1, 4.0 Hz, H-6″a), 3.87 (1H, dd,*J* = 11.1, 6.5 Hz, H-6″b), 3.60–3.40 (4H, m, H-2″,3″,4″,5″) (Jayatilake *et al.*, 1993).

- *Resveratrol rutinoside (3,5,4′- trihydroxystilbene-3-O-b-rutinoside)*: ESI-MS *m/z* 578 $[M - H]^-$; ^{1}H-NMR (DMSO-*d*6): δ 7.38 (2H, d, *J* = 8.6 Hz, H-2′,6′), 7.01 (1H, d, *J* = 16.3 Hz, H-8), 6.82 (1H, d, *J* = 16.3 Hz, H- 7), 6.75 (2H, d, *J* = 8.6 Hz, H-3′,5′), 6.70 (1H, d, brs, H-2′), 6.53 (1H, d, brs, H-6′), 6.29 (1H, t, *J* = 2.1 Hz, H-4′), 5.12 (1H, d, *J* = 1.1 Hz, H-1′), 4.91

(1H, d, *J* = 7.4 Hz, H-1″), 3.90–3.20 (10 sugar protons), 1.19 (d, *J* = 6.2 Hz, OCH3) (Joseph *et al.*, 2007).

- *Piceid (3,5,4′-trihydroxystilbene-3-O-b- glucoside)*: ESI-MS *m/z* 389 [M – H]$^-$; ^{1}H-NMR (DMSO-*d*6): δ 7.36 (2H, d, *J* = 8.6 Hz, H-2′,6′), 7.02 (1H, d, *J* = 16.3 Hz, H-8), 6.85 (1H, d, *J* = 16.3 Hz, H-7), 6.79 (1H, br s, H-2), 6.77 (2H, d, *J* = 8.6 Hz, H-3′,5′), 6.62 (1H, brs, H-6), 6.46 (1H, t, *J* = 2.1 Hz, H-4), 4.89 (1H, d, *J* = 7.3 Hz, H-1″), 3.93 (1H, dd, *J* = 12.1, 2.5 Hz, H-6″a), 3.72 (1H, dd, *J* = 12.1, 5.6 Hz, H-6″b), 3.50–3.35 (4 sugar protons).

- *Trans-ε-viniferin-diglucoside*: ESI-MS *m/z* 777 [M – H]$^-$; ^{1}H-NMR (DMSO-*d*6): δ 7.16 (2H, d, *J* = 8.6 Hz, H-2a,6a), 7.14 (2H, d, *J* = 8.6 Hz, H- 2b,6b), 6.85 (1H, d, *J* = 16.3 Hz, H-8b), 6.74 (2H, d, *J* = 8.6 Hz, H-3a,5a), 6.68 (2H, d, *J* = 8.6 Hz, H-3b,5b), 6.61 (1H, t, *J* = 1.9 Hz, H- 14b), 6.59 (1H, d, *J* = 1.9 Hz, H-12a), 6.61 (1H, t, *J* = 1.9 Hz, H-14b), 6.56 (1H, d, *J* = 16.3 Hz, H-7b), 6.41 (2H, d, *J* = 1.9 Hz, H-10a,14a), 6.26 (1H, t, *J* = 1.9 Hz, H-12b), 5.34 (1H, t, *J* = 4.2 Hz, H-8a), 4.77 (2H, d, *J* = 7.5 Hz, H-1′,1″), 4.58 (1H, d, *J* = 4.2 Hz, H-7a), 3.64 (2H, dd, *J* = 11.4, 5.8 Hz, H-6a′,6a″), 3.43 (2H, m, H-6b′,6b″), 3.30–310 (8 sugar protons) (Baderschneider and Winterhalter, 2000).

- *Cis-ε-viniferin-diglucoside*: ESI-MS *m/z* 777 [M – H]$^-$; ^{1}H-NMR (DMSO-*d*6): δ 6.97 (2H, d, *J* = 8.6 Hz, H-2a,6a), 6.93 (2H, d, *J* = 8.6 Hz, H- 2b,6b) , 6.70 (2H, d, *J* = 8.6 Hz, H-3a,5a), 6.60 (2H, d, *J* = 8.6 Hz, H-3b,5b), 6.57 (1H, d, *J* = 1.9 Hz, H-12a), 6.21 (2H, d, *J* = 1.9 Hz, H- 10a,14a), 6.20 (1H, d, *J* = 12.2 Hz, H-8b), 6.19 (2H, brs, H-12b,14b), 5.94 (1H, d, *J* = 12.2 Hz, H-7b), 5.17 (1H, t, *J* = 5.3 Hz, H-8a), 4.77 (2H, d, *J* = 7.5 Hz, H-1′, 1″), 4.00 (1H, d, *J* = 5.3 Hz, H-7a), 3.66 (2H, dd, *J* = 11.4, 5.8 Hz, H-6a′,6a″), 3.44 (2H, m, H-6b′,6b″), 3.30–310 (8 sugar protons) (Baderschneider and Winterhalter, 2000).

Resveratroloside has previously been isolated from roots of *Polygonum cuspidatum* (Jayatilake *et al.*, 1993) and in cell cultures of *Vitis vinifera* (Waffo Teguo *et al.*, 1998). However, to the best of our knowledge, the present study is the first to find resveratroloside in the woody parts of grapevine. Resveratrol-3-*O*-b-rutinoside has previously been described in root bark of *Terminalia sericea* (Joseph *et al.*, 2007); this is the first report of the presence of resveratrol- rutinoside in the *Vitis* genus. Piceid has been identified in different parts of grapevine, including stems and berries (Pawlus *et al.*, 2012). Both the *trans*- and *cis*-ε-viniferin diglucosides have previously been identified in wine (Baderschneider and Winterhalter, 2000), but this is the first report of their presence in grapevine extract.

2. Cytotoxicity in human hepatoma HepG2 cells

The cytotoxic activity of resveratroloside, resveratrol rutinoside, and *trans*- and *cis*-ε- viniferin diglucosides in HepG2 cells was evaluated, using resveratrol as a positive control **(Figure 2).** The results for resveratrol showed this stilbene to be cytotoxic in a dose-dependent manner, with a half-maximal inhibitory concentration (mean ± SE) of 37.2 ± 9.9 μM at 72 h. It showed significant cytotoxicity at concentrations as low as 20 μM, well below previously reported cytotoxic concentrations (Su *et al.*, 2013). All the glucosylated stilbenes showed moderate but significant cytotoxicity against HepG2 cells. The most active compound was resveratrol rutinoside, which at the lowest concentration tested, 20 μM, decreased cell viability by 30%.

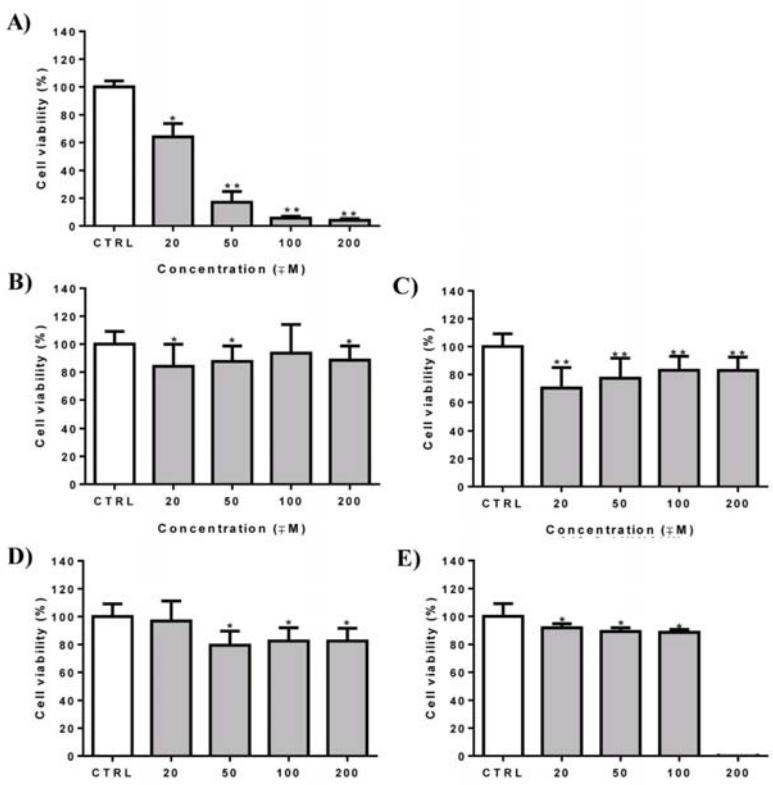


Figure 2. Effect of resveratrol (A), resveratroloside (B), resveratrol rutinoside (C), *trans*-ε-viniferin diglucoside (D) and *cis*-ε-viniferin diglucoside (E) on the viability of HepG2 cells. CTRL, negative control (no added stilbenes). Compared with control group: *$p < 0.05$, **$p < 0.001$

Although *trans*-ε-viniferin diglucoside was not active at 20 μM, it reduced viability significantly, by 20%, when assayed at higher concentrations (50–200 μM). The *cis*-ε-viniferin diglucoside isomer was less cytoxic, producing an 11% decrease in viability. These results highlight the importance of the *trans* stereoisomer in cytotoxicity. In the case of *trans* and *cis* isomers of resveratrol and piceid, the former are more thermodynamically stable and quantitatively more abundant in nature than the latter (Cicero *et al.*, 2019).

The finding that glucoside derivatives exert lower toxic effects than resveratrol could be due to the lower availability in the cell of the free active forms of these glucoside compounds, as has previously been suggested for piceid in comparison with resveratrol (Su *et al.*, 2013).

4. Conclusion

This is the first study to show the presence of glucosylated derivatives of resveratrol, other than piceid, in the woody parts of grapevine. In addition to piceid, four compounds were identified: resveratroloside, resveratrol rutinoside, and the *trans*- and *cis*-ε-viniferin diglucosides. All the compounds showed moderate cytotoxicity against the human hepatoma HepG2 cell line. However, they showed lower biological activity than resveratrol.

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CHAPTER II

Stilbenes at low micromolar concentrations mitigate the NO, TNF-α, IL-1β and ROS production in LPS-stimulated murine macrophages

This chapter has been sent for publication in:

Iris Aja Pérez, Stéphanie Krisa, Ruth Hornedo-Ortega, M. Begoña Ruiz Larrea, Jose I. Ruiz-Sanz, Tristan Richard, Arnaud Courtois. "Stilbenes at low micromolar concentrations mitigate the NO, TNF-α, IL-1β and ROS production in LPS-stimulated murine macrophages". Natural Product Research (submitted).

Abstract

Stilbenes, of which resveratrol is the most studied, were described as antioxidant and anti- inflammatory molecules. Presumably, other stilbenes have similar biological properties, but evidences are poorly documented. Therefore, 20 stilbenes from resveratrol monomers to tetramers were screened as anti-inflammatory and antioxidant agents in a cellular model of LPS-stimulated murine macrophages. Piceatannol (monomer), ε-viniferin and δ-viniferin (dimers), and hopeaphenol and isohopeaphenol (tetramers) were the most powerful compounds to inhibit NO and ROS generation upon LPS exposure. Among these compounds, hopeaphenol (5-10 μM) showed the highest efficiency. Nevertheless, ε-Viniferin was the most powerful to inhibit TNF-α release whereas isohopeaphenol was the greatest reducing IL-1β upon LPS stimulation. Thus, this work provided evidence that the chemical structure of stilbenes is highly relevant on the behavior of their activities. These results will allow the selection of potential bioactive stilbenes to develop further studies in order to elucidate their molecular mechanism of action.

Keywords: stilbenes, macrophages, anti-inflammatory, antioxidant

1. Introduction

Stilbenes are naturally occurring molecules that belong to the wide family of polyphenolic compounds. These compounds are present in various plants, but their presence in the human diet is limited to a few foods such as grapes, red wine and some types of nuts and berries (Neveu et al. 2010). These secondary metabolites are considered as phytoalexins, associated with the mechanisms of defense of plants. Moreover, they were described as antioxidant and anti- inflammatory agents, in particular the major stilbene studied, i.e. resveratrol. Indeed, in various cellular or animal models, antitumoral, anti-obesity, cardioprotective, and neuroprotective properties were also reported that highlight their interest for human health (K. Pandey and Rizvi 2009; Crozier, Jaganath, and Clifford 2009).

Resveratrol presents a chemical structure of two phenolic rings linked by a methylene bridge. Oligomers can be formed by the oxidative polymerization of two to eight resveratrol units. The biological activities of stilbenes are dependent on structural variations such as polymerization, glycosylation, prenylation, methoxylation, and various hydroxylation patterns (Coppa et al. 2011).

Inflammation is a first line of physiological defense response that appears to combat an external aggression upon exposure to infectious, chemical or physical agents. Macrophages are the predominant cell types in the early stage of inflammation response and have been identified as a key factor in the progression of tissue inflammation (Gordon and Plüddemann 2017). After bacterial infection, macrophages are stimulated and can secrete noxious agents, such as nitric oxide (NO), in order to destroy the infectious agent, and produce pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), or interleukin 1β (IL-1β) that participate in the regulation of the host-response against the pathogen. Inflammation thus appears as a beneficial response.

Nevertheless, when occurring chronically, it has some detrimental consequences. In fact, during inflammation, an enhanced reactive oxygen species (ROS) generation can damage lipids, proteins and nucleic acids. Moreover, chronic inflammation and oxidative stress are both involved in the physiopathological process of several human diseases such as asthma, arthritis, inflammatory bowel diseases, cardiovascular diseases, neurodegenerative disorders and cancer (Romier et al. 2009; Bush 2019; Libby and Kobold 2019; Caggiu et al. 2019).

The anti-inflammatory properties of stilbenes are widely described in *in vitro* and *in vivo* studies (Serino and Salazar 2018; Shahidi and Yeo 2018). It was demonstrated that resveratrol could inhibit the NO production through the inhibition of inducible nitric oxide synthase (iNOS) at protein and mRNA levels, and could inhibit the release of pro-inflammatory cytokines (Qureshi et al. 2012; Djoko et al. 2007; Martín et al. 2006; Bi et al. 2005). The anti-inflammatory activity of resveratrol is associated with the inhibition of the nuclear translocation of nuclear factor- kappa B (NF-κB) that regulates the expression of cyclo-oxygenase (COX) and iNOS (Szaefer et al. 2014; Cichocki et al. 2008; Martinez and Moreno 2000). Resveratrol could also modulate inflammation through the activation of sirtuin1 (Sirt1) which is a member of the group of histone deacetylases, and its activation is involved in cell survival and longevity in diverse species (Singh et al. 2010; Csiszar et al. 2008).

Other stilbenes were also described to exert some anti-inflammatory properties. As an example some monomers such as piceatannol (a hydroxylated derivative of resveratrol) or pterostilbene (a dimethoxy derivative of resveratrol) could suppress the LPS-induced inflammatory response in murine macrophages (Djoko et al. 2007; Islam et al. 2004; Pan et al. 2008). Moreover, piceatannol

suppressed the gene expression of LPS-induced TNF-α more significantly than resveratrol (Richard et al. 2005; Son, Chung, and Pae 2014). Several oligomers such as a resveratrol dimer (ε-viniferin) and resveratrol tetramers (vitisin A, vitisin B and hopeaphenol) could suppress NO production by inhibiting iNOS and COX mRNA, as it has been described in LPS-stimulated macrophages (Chang et al. 2017). Finally, roots extracts from *Vitis vinifera*, that contained a mix of resveratrol monomer, trimers and tetramers, were shown to prevent DNA damage induced by hydrogen peroxide in macrophages. This effect occurred through the induction of heme oxygenase-1 and γ- glutamylcysteine synthetase after the stimulation of the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway (Esatbeyoglu et al. 2016).

According to literature data, resveratrol is the stilbene that exhibits the most important quantity of results on anti-inflammatory properties. By contrast, it is suggested that other stilbenoids also have anti-inflammatory activities, but the evidence is poorly documented. Therefore, in the present study, we screened the anti-inflammatory properties of 20 stilbenes mainly isolated from *Vitis vinifera*, from monomers to tetramers, in a cellular model of LPS- induced inflammatory response in murine macrophages. Their effects on inflammatory markers such as NO, TNF-α and IL-1β production, and their potential to limit oxidative stress were described.

2. Materials and methods

2.1 Stilbene compounds

Resveratrol and 19 of its analogues were evaluated for their potential to inhibit NO production by LPS-activated RAW 264.7 macrophages. Oxyresveratrol and piceatannol were purchased from TCI chemicals (Paris, France); piceid and resveratrol were purchased from Sigma-Aldrich (Lyon, France). The others stilbenes were isolated and purified from *Vitis vinifera* shoots and *Epicea abies* in our laboratory. Their purity (at least ≥ 96%) was evaluated by Nuclear Magnetic Resonance on a Bruker Avance III 600 MHz spectrometer equipped with a 5-mm triple-resonance probe (Bruker-Daltonics, Billerica, MA, USA).

2.2 Reagents and material

Culture media, dimethyl sulfoxide (DMSO), thiazolyl blue tetrazolium bromide (MTT), phosphate-buffered saline (PBS), lipopolysaccharide (LPS), 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA), Griess reagent, and Neutral Red were purchased from Sigma-Aldrich (Lyon, France). IL-1β and TNF-α ELISA kits were obtained by Biolegend (London, UK) and InmunoTools (Friesoythe, Germany), respectively.

2.3 Cell culture

RAW 264.7 murine macrophage cells, obtained from the European Collection of Authenticated Cell Cultures, were cultured in Dulbecco's modified Eagle's medium high glucose supplemented with 10% heat-inactivated fetal bovine serum at 37ºC in a 5% CO2 humidified incubator. Sub-confluent cell cultures (80-90%) are split by gentle scraping twice a week.

For the cytotoxicity studies and the screening of the activity of the 20 stilbenes on NO and ROS production, RAW 264.7 were plated at a density of 20.000 cells per well in 96-well culture plates. For IL-1β production assay, cells were plated at a density of 200.000 cells in 48-well plates.

2.4 Cytoxicity assays

Three days after seeding, cells were treated with or without LPS (0.1 μg/mL) in the presence or in the absence of increasing concentrations of stilbenes (from 1 to 50 μM). After 24h of exposure, MTT and Neutral Red assays were performed. Briefly, the medium was discarded and cells were incubated with MTT solution (0.5 mg/mL) or Neutral Red solution (40 μg/mL) at 37ºC and 5% CO_2. After 3h of incubation, the medium was discarded and 100 μL of DMSO or 150 μL of Neutral Red dissolution solution (acetic acid/ethanol/water, 1/49/50) were added in order to dissolve MTT formazan product or reveal Neutral Red accumulation, respectively. Finally, the absorbance was measured using a microplate reader (Fluostar Omega; BMG Labtech, Offenburg, Germany) at 540 nm for MTT and at 490 nm for Neutral Red.

2.5 Measurement of NO production

Two days after seeding, RAW 264.7 were activated with LPS (0.1 μg/mL) in the absence or in the presence of increasing concentrations of stilbenes (from 1 to 50 μM). After 24h of exposure, culture media were analyzed for nitrite (NO_2) content by the Griess reaction.

Briefly, 50 μL of Griess reagent was mixed with an equal volume of cell culture supernatants and incubated at room temperature for 15min. Color development was measured at 550 nm in a microplate reader (Fluostar Omega; BMG Labtech,

Offenburg, Germany). The amount of NO_2 in media was calculated using a $NaNO_2$ standard curve.

2.6 Measurement of ROS production

Two days after seeding, RAW 264.7 were stimulated with LPS (0.1 μg/mL) in the presence or in the absence of increasing concentrations of stilbenes (from 1 to 50 μM). After 24h of exposure, medium was discarded and H2DCFDA (10 μM, in phosphate buffered saline) was added to the cells for 30 min at 37 °C. Fluorescence intensity (λEx 485 nm; λEm 550 nm) was measured using a microplate reader (Fluostar Omega; BMG Labtech, Offenburg, Germany).

2.7 Measurement of TNF-α and IL-1β production

Two days after seeding, RAW 264.7 were exposed to LPS (0.1 μg/mL) in absence or in presence of increasing concentrations of stilbenes (from 10 to 50 μM). After 24h of exposure, measurement of IL-1β and TNF-α concentration in culture media supernatants was performed by ELISA sandwich assay. Briefly, supernatants were collected and diluted to 1/10 for TNF-α but not for IL-1β and processed according to manufacturer's instructions. Capture and detection antibodies were diluted at 1:100 (TNF-α) or 1:200 (IL-1β) and then cytokines were detected with a biotinylated secondary antibody and an avidin peroxidase conjugate with TMB as detection reagent. After 30min, the reaction was stopped by addition of the stop solution. The absorbance was measured at 450 nm using a microplate reader (Fluostar Omega; BMG Labtech, Offenburg, Germany). The amount of TNF-α and IL-1β secreted by the macrophages was obtained by using TNF-α (0 - 3000 pg/mL) or IL-1β (0 - 2000 pg/mL) standard curves.

2.8 Statistical analysis

Data are shown as mean ± SEM obtained from at least three independent experiments. Statistical analysis of the data was carried out by one-way analysis of variance (ANOVA) followed by Dunnett´s *post hoc* test, using GradPad Prism Software 5.0 (San Diego, CA, USA). Statistical significance was set at *p<0.05, **p<0.01 and ***p<0.001.

3. Results

A total of 20 stilbenes (resveratrol and its 19 analogues) were screened for the measurement of their cytotoxicity and their ability to inhibit NO production in RAW 264.7 cells after 24h of stimulation with LPS (0.1 μg/mL). The 30% inhibitory concentration (IC30) for cell cytotoxicity was calculated according to the International Standard Organization (ISO) 10993-5:2009 for *in vitro* cytotoxicity tests. Indeed, if the cell viability for the highest tested concentration of the compound is superior or equal to 70% (corresponding to its IC30) of the control group, then the compound can be considered as non-cytotoxic. These values were determined directly from the obtained dose-response curves. In the same manner, the IC50 (concentration of stilbene required to inhibit the 50% of NO production) was calculated **(Table 1).**

Piceatannol was the only monomer that showed cytotoxicity measured with MTT assay according to the calculated IC30. The other monomers showed no cytotoxicity at concentrations as high as 50 μM. Treatment of RAW 264.7 cells with LPS did not affect the viability of cells but produced a significant increase in NO production (6.1 ± 3.8 and 25.4 ± 6.1 μM for control and LPS-treated cells, respectively, data not shown). Among monomers, resveratrol and piceatannol showed the lowest IC50 value for the NO production (28 and 7.3 μM, respectively). Piceid, astringin A and isorhapontin, three glycoside derivatives and also oxyresveratrol, the IC50 could not be determined since it was higher than 50 μM, whereas pterostilbene and isorhapontigenin, two methylated derivatives, showed an IC50 of approximately 30 μM **(Table 1)**.

Among the dimers, ε- and δ-viniferin showed the highest cytotoxicity according to their lowest IC30 measured with MTT assay. Vitisinol C and ω-viniferin were cytotoxic at 29.1 and 41.3 μM, respectively, whereas all others dimers (ampelopsin A, scirpusin A and pallidol) showed no cytotoxicity at concentration

up to 50 μM. Among the seven studied dimers, ε-viniferin and δ-viniferin showed the lowest IC50 values for inhibiting NO production (15.6 and 12.9 μM, respectively). Ampelopsin A, scirpusin A and pallidol showed no effects on NO production at concentrations up to 50 μM. Moreover, vitisinol C and ω-viniferin showed intermediate inhibitory effects against NO production with IC50 values of 33.6 and 27.3 μM, respectively **(Table 1)**.

The miyabenol C trimer showed any cytotoxic neither inhibitory effect on NO production at the concentrations tested up to 50 μM **(Table 1).**

Among the tetramers, vitisin A and hopeaphenol showed the highest cytotoxicity. Isohopeaphenol and vitisin B were cytotoxic at 16.3 μM and 20.1 μM, respectively. With regard to NO production, hopeaphenol and isohopeaphenol had the IC50 values of 2.6 and 8 μM, respectively. However, for vitisin A and vitisin B, the IC50 could not be calculated.

The effects for the most active molecules were explored on the production of inflammatory cytokines elicited by LPS, an inducer of an inflammatory reaction that is also known to induce an oxidative stress in stimulated macrophages. The bioactive stilbenes were selected according to two different criteria. Firstly, the stilbene has to reduce the NO production by 50% of the control at concentrations lower than 20 μM; and secondly, the stilbene should not be cytotoxic at that concentration. According to these criteria and the cytotoxicity results, five stilbenes were selected: one resveratrol monomer (piceatannol), two dimers (ε-viniferin and δ-viniferin) and two tetramers (hopeaphenol and isohopeaphenol).

Table 1. Inhibitory concentrations on cell viability and NO production for the 20 stilbenes in LPS stimulated murine macrophages.

Compound	Cell viability		NO production
	MTT assay IC30 (μM)[a]	**Neutral Red assay** IC30 (μM)[a]	**Griess assay** IC50 (μM)[b]
Monomers			
Resveratrol	>50	24.1	28
Piceid	>50	>50	>50
Piceatannol	25.3	31.8	7.3
Astringin A	>50	>50	>50
Pterostilbene	>50	>50	34.7
Oxyresveratrol	>50	>50	>50
Isorhapontin	>50	>50	>50
Isorhapontigenin	>50	>50	31.0
Dimers			
ε-Viniferin	19.1	18.3	15.6
ω-Viniferin	41.3	>50	27.3
δ-Viniferin	18.9	22.6	12.9
Ampelopsin A	>50	>50	>50
Scirpusin A	>50	>50	>50
Pallidol	>50	>50	>50
Vitisinol C	29.1	25.2	33.6
Trimers			
Miyabenol C	>50	>50	>50
Te*tramers*			
Hopeaphenol	12.3	16.8	2.6
Isohopeaphenol	16.3	18.5	10
Vitisin A	<10	10.7	nd
Vitisin B	20.1	36.9	nd

IC30 (μM) values for cell viability were analyzed by MTT or neutral red assays and IC50 (μM) values for NO production were analyzed by Griess reaction. The concentrations of stilbenes used were 10, 20, 30, 40 and 50 μM. **a**IC30: Stilbene concentration that induced 30% of cytotoxicity relative to the LPS control. **b**IC50: Stilbene concentration that reduced 50% the production of NO relative to the LPS control. Untreated cells produced 6.1 ± 3.8 μM NO whereas activated cells with 0.1 μg/mL of LPS produced 25.4 ± 6.1 μM NO. nd, not determined when the compound was toxic at the concentration which reduced the production of NO by 50%.

As shown in **Figure 1A**, all compounds significantly suppressed LPS-induced NO production. The most active stilbene was hopeaphenol, followed by piceatannol, isohopeaphenol, ε- viniferin and δ-viniferin. At 10 μM concentration, these compounds reduced the NO production of 91.8% for hopeaphenol, 73% for isopeaphenol, 63.6% for piceatannol, 37.4% for ε-viniferin, and 31.1% for δ-viniferin. Interestingly, hopeaphenol at 5 μM exhibited more than 90% inhibition of NO production.

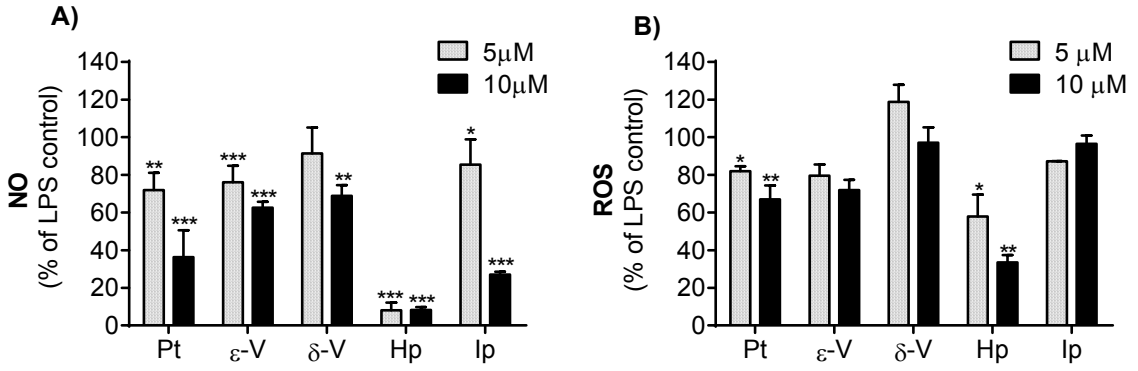

Figure 1. Effect of stilbenes on the NO production (**A**) or ROS formation (**B**) in LPS-activated RAW 264.7 cells. Data are represented as means (% relative to the LPS control) ± SEM of at least three independent experiments. *p<0.05, **p<0.01, ***p<0.001, significantly different from LPS-activated RAW 264.7 cells. Pt=Piceatannol, ε-V= ε-Viniferin, δ-V=δ-Viniferin, Hp=Hopeaphenol, Ip=Isohopephenol.

As well as for the NO production, treatment of cells with LPS (0.1 μg/mL) also induced a 1.8 folds increase in ROS levels as compared to the untreated cells. As displayed in **Figure 1B**, piceatannol and hopeaphenol significantly decreased LPS-induced ROS. The most active compound was hopeaphenol which could reduce the ROS generation (50 and 60% at 5 and 10 μM, respectively, as compared to the LPS treated cells). ε-viniferin, δ-viniferin and isohopeaphenol did not significantly decreased LPS-induced ROS formation in RAW 267.4 macrophages at the tested concentrations.

The levels of pro-inflammatory cytokines TNF-α and IL-1β in the culture supernatants of RAW 264.7 cells were measured by ELISA kits as described in Materials and Methods section. LPS treatment produced a significant increase of TNF-α production, around 10 folds (4016.8 ± 167.9 vs. 39513.7 ± 5007.5 pg/mL for control and LPS-treated cells, respectively) and IL-1β, around 2.3 folds (52.4 ± 2.2 vs. 118.3 ± 3.8 pg/mL for control and LPS-treated cells, respectively), as compared with the untreated control group. As illustrated in **Figure 2A**, piceatannol, ε-viniferin, hopeaphenol and isohopeaphenol significantly decreased LPS- induced TNF-α production in a dose-dependent manner. ε-Viniferin actively decreased TNF- α production (40 and 56% at 5 and 10 μM, respectively). By contrast, δ-viniferin showed no effect on LPS-induced TNF-α production. Piceatannol, ε-viniferin, hopeaphenol and isohopeaphenol decreased the LPS-induced IL-1β production in a dose dependent manner **(Figure 2B)**. Isohopeaphenol showed the highest inhibitory potency (30 and 52% at 5 and 10 μM, respectively). Similarly, to TNF-α production, δ-viniferin had no effect on LPS-induced secretion of IL-1β **(Figure 2B)**.

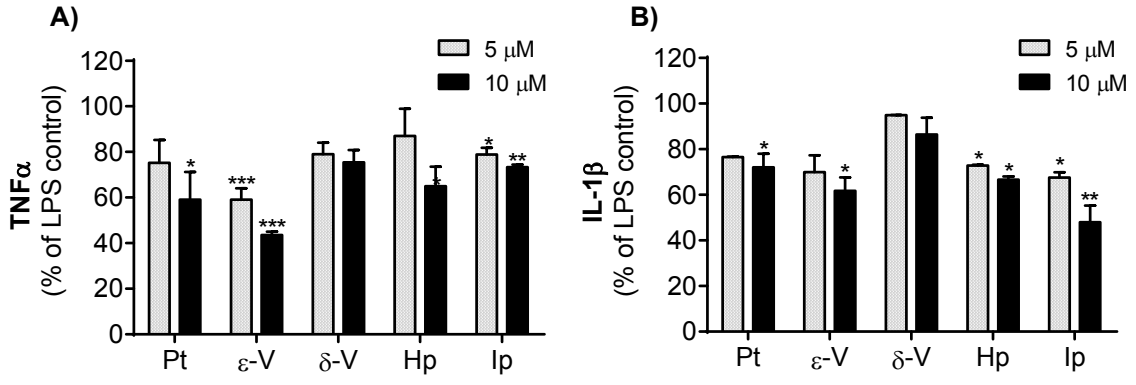

Figure 2. Effect of stilbenes on TNF-α (A) and IL-1β (B) secretion by LPS-activated RAW 264.7 cells. Data are represented as means (% relative to the LPS control) ± SEM of at least two independent experiments. *p<0.05, **p<0.01, significantly different from LPS-activated RAW 264.7 cells. Pt=Piceatannol, ε-V=ε-Viniferin, δ-V=δ-Viniferin, Hp=Hopeaphenol, Ip=Isohopephenol.

In the last set of experiments, we tried to understand if stilbenes could have additive effects on the inhibition of NO and ROS production in LPS treated RAW 264.7 macrophages. Each individual stilbene at the concentration of 1 μM did not reduce the LPS-induced production of markers of inflammatory response and oxidative stress. In addition, the treatment by all these stilbenes together in order to reach a cumulative concentration of 5 μM did not reduce the LPS-induced production of NO and ROS in RAW 264.7 macrophages (Data not shown).

The general effects of the five stilbenes are resumed in the **Table 2**. From that table it could be observed that the concentration of 10 μM showed the highest effect between the two concentrations tested. All stilbenes except δ-viniferin have important effects in the inhibition of NO, ROS and cytokines production triggered by LPS treatment. The most active stilbenes were the tetramers that showed the highest inhibitory activity.

Table 2. Resume of the inhibitory effects of the five more active stilbenes on the NO, reactive oxygen species, TNF-α and IL1-β production in LPS stimulated murine macrophages.

	Inhibition NO production		Inhibition ROS production		Inhibition TNF-α production		Inhibition IL-1β production	
	5 μM	10 μM	5 μM	10 μM	5 μM	10 μM	5 μM	10 μM
Piceatannol	↓	↓↓		↓	↓	↓		↓
ε-viniferin		↓		↓	↓	↓↓	↓	↓
δ-Viniferin		↓						
Hopeaphenol	↓↓	↓↓	↓	↓↓	↓	↓	↓	↓
Isohopeaphenol	↓	↓↓			↓	↓	↓	↓↓

Macrophages were activated by incubation with 0.1 μg/mL LPS and co-incubated with the addition of 5 or 10 μM of corresponding stilbene. After 24 h incubation period, NO, reactive oxygen species (ROS) and cytokines TNF-α and IL-1β production in cells were determined according to Materials and Methods. One arrow represents a reduction greater than 25% respect to control cells; two arrows represent a reduction greater than 50%.

4. Discussion

Chronic and low grade inflammation is involved in the pathophysiology of many diseases such as atherosclerosis, diabetes, rheumatoid arthritis and cancer (Dandona 2004). Therefore, in order to counteract this reaction, there is a growing interest to identify bioactive anti-inflammatory compounds. Stilbenes, a class of polyphenols, are known to possess some anti-inflammatory properties and due to their occurrence in our daily food, they could represent an interesting alternative to avoid the use of chronic medicine treatment.

In the present study we have demonstrated the anti-inflammatory and anti-oxidant activities of natural stilbenes present in *Vitis vinifera* and *Epicea abies*. We have tested resveratrol and 19 of its derivatives; seven monomers, seven dimers, one trimer and four tetramers for their anti-inflammatory and antioxidant properties in a murine cell model of LPS-activated macrophages. Among stilbenes, we found a strong variation concerning their bioactivity that in some cases can be explained taking into account the chemical structure and substituents of each molecule. However, when stilbenes are more complex (oligomers) there is no available information that may help to do a well-stablished hypothesis that relate the structure-activity relationship.

Among derivatives, the monomer piceatannol and the tetramers hopeaphenol and isohopeaphenol were the most active stilbenes that inhibited NO production highlighting that there is no correlation between their bioactivity and the degree of oligomerisation. It seemed that some chemical residues could modulate the activity of the compounds. Indeed, the presence of a glycoside moiety reduced the anti-inflammatory activity, as it has been observed for piceid (**Figure 3**), astringin A, and isorhapontin (Nassra et al. 2013). In addition, the position of the hydroxyl groups on B ring seems to be important for monomers activity. For example, piceatannol with hydroxyl groups at the *ortho* position, displayed anti-

inflammatory properties whereas oxyresveratrol with hydroxyl groups at *meta* position did not **(Figure 3).** These features were already observed in another model of microglial cells (Nassra et al. 2013). Literature data and our results indicate that glycosylation and the position of hydroxyl groups in the B ring constitute a key point for the anti-inflammatory activities of those monomers (Nassra et al. 2013). In fact, some of those features correspond to piceatannol a non-glycosylated compound with a hydroxyl group at the *ortho* position.

The monomer piceatannol, the two dimers ε-viniferin and δ-viniferin, and the two tetramers hopeaphenol and isohopeaphenol were selected among the 20 stilbenes because they were not cytotoxic at the effective concentrations able to decrease the NO production upon LPS exposure. Their structures are indicated in **Figure 3**.

In macrophages, NO is produced by iNOS and it plays a key role in the immune response to infectious agents. Therefore, NO production may reflect inflammation and allow the measurement of the potential activity of biological agents on inflammatory process. To further confirm the anti-inflammatory activity of the five tested stilbenes, we measured their potential to inhibit the release of the pro-inflammatory mediators TNF-α and IL-1β. These two cytokines exert pleiotropic effects on a variety of cells and play a crucuak roles in acute and chronic inflammatory and immune disorders (Akdis et al. 2016). After exposure to LPS, our results clearly showed that piceatannol, ε-viniferin, hopeaphenol and isohopeaphenol were able to diminish the secretion of pro-inflammatory cytokines in macrophages, although to different extent. These results again highlighted the different responses between molecules toward the inhibition of pro-inflammatory cytokines secretion, as it was already noticed for NO production.

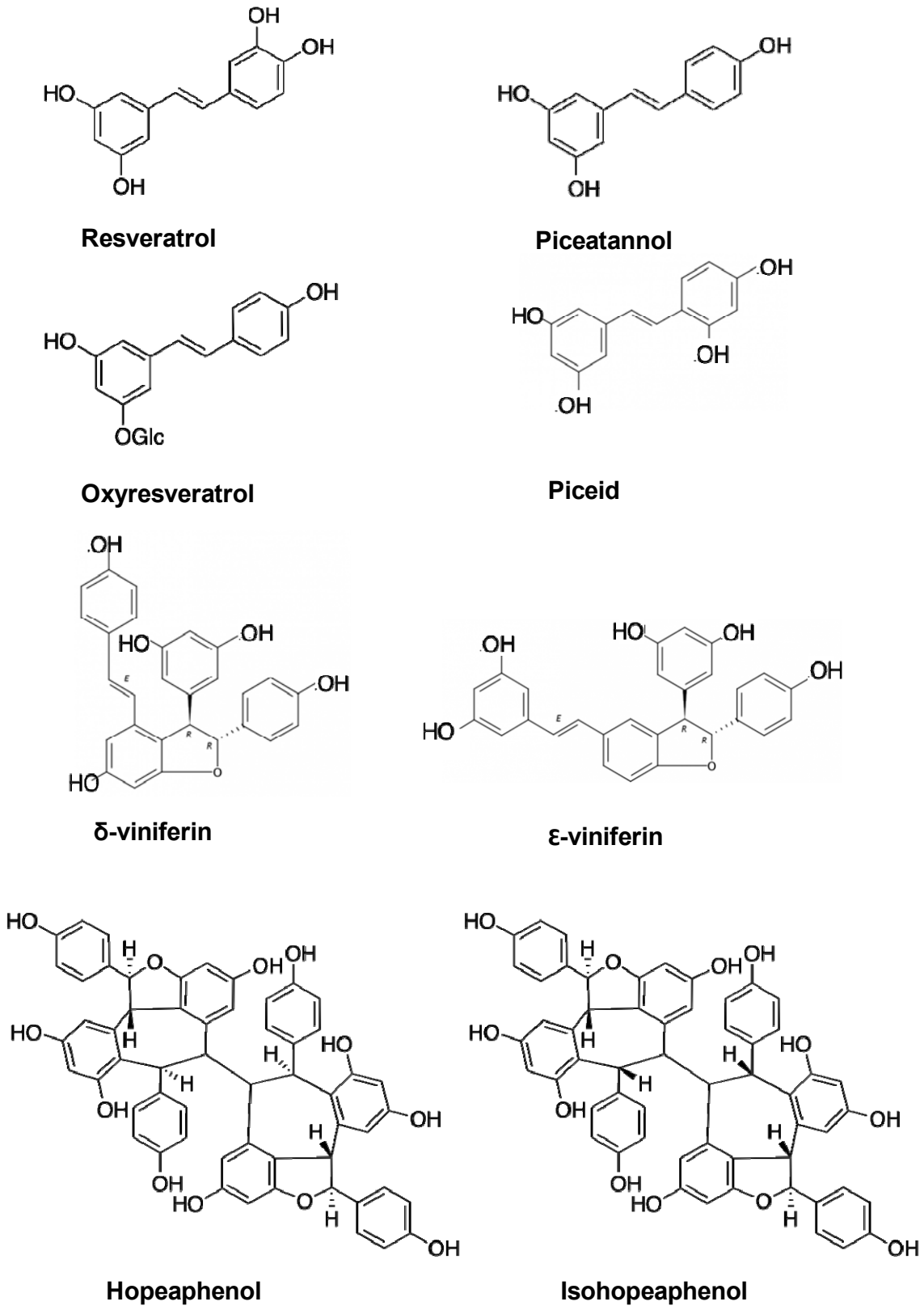

Figure 3. Chemical structures of some of the stilbenes studied

The most active molecule was the dimer ε-viniferin since it strongly suppressed the TNF-α and IL-1β secretion after LPS exposure. Surprisingly, the other dimer δ-viniferin did not exert any activity, whereas both dimers showed similar inhibitory activity toward NO production. This differential activity was already observed with other stilbenes. Indeed resveratrol, piceatannol and *trans*-3,5,4'-trimethoxystilbene (TMS) were all able to inhibit cytokine release upon LPS exposure, but only resveratrol and piceatannol could induce heme oxygenase-1 activity, whereas TMS did not (Son, Chung, and Pae 2014). Authors suggested that the hydroxyl groups on the phenyl rings could be one of the important structural features necessary to suppress LPS-induced cytokines production. However, further studies masking some hydroxyl groups by hemi- synthesis in order to obtain viniferin derivatives would be interesting in order to answer this issue.

As oxidative stress is closely associated with inflammation, antioxidant properties of stilbenes were further studied. A good correlation between their ability to inhibit NO production and decrease oxidative stress did occur except in the case of isohopeaphenol at 10 μM, which significantly inhibited NO production without decreasing oxidative stress. Polyphenols are well known to exhibit antioxidant properties by scavenging ROS, and according to previous experiments ε-viniferin was able to scavenge ROS in the ORAC acellular assay (Biais et al. 2017; Sáez et al. 2018).

Nevertheless, in the present study ε-viniferin did not produce any significant effect to limit ROS production in more biological context. Also, hopeaphenol, which possess a lesser *in vitro* scavenging activity than ε-viniferin was able to exert an antioxidant activity by limiting ROS production by macrophages upon LPS exposure. Thus, it appeared in the present study that the capacity of stilbenes to exert antioxidative properties may rely on their ability to modulate the antioxidant defence system rather than to directly scavenge ROS.

Other ways to counteract oxidative stress did occur through the induction of cellular defence mechanisms. Resveratrol or pterostilbene were described to induce the expression of anti- oxidant enzymes such as heme oxygenase-1 (Chiou et al. 2011). This induction occurred through the induction of the transcription factor Nrf2 that is able to bind to the anti-oxidant responsive element present in the promoter region of genes involved in the anti-oxidant defence mechanisms. In addition, it was shown that resveratrol inhibited the activity of transcription factors NF-κB and AP-1, two regulators of the expression and activity of cyclooxygenases and iNOS that produce pro-inflammatory and pro-oxidant mediators (C.-M. Yang et al. 2017). In our study the antioxidant and anti-inflammatory activities of the stilbenes did certainly occur through a similar mechanism. It was reported that the antioxidant and anti-inflammatory effect of stilbenes was associated with the induction of heme oxygenase-1 (Son et al. 2013; Truong, Jun, and Jeong 2018). It would be then interesting to study the effect of these stilbenes on the expression on heme oxygenase-because it would help us to identify stilbenes that exhibit antioxidant or anti-inflammatory properties, or both.

Several studies have shown an anti-inflammatory effect of whole extracts derived from plants or relative products such as wine (Chalons et al. 2018; Colombo et al. 2019; Harbeoui et al. 2019). These extracts are very complex and contain several molecules including numerous polyphenols. Sometimes the concentration of each molecule in the extract is below from the effective concentration when this molecule is studied alone. Considering that the combination of several molecules of the extract contributes to the activity of the whole extract, it is interesting to measure the potential synergic effects of a stilbene mixture; each molecule bringing its own effect or some molecule acting as synergistic way with others. For this reason, in our study we tried to test the combination of several stilbenes at an ineffective concentration in order to identify an additive or synergistic

effect. Unfortunately, the combination of five of the most active stilbenes did not produce any inhibitory effect on NO or ROS production. This suggests that stilbenes exert their activity on their own and did not act in an additive nor in a synergistic manner; moreover, they could trigger their anti-inflammatory action through the stimulation of different signalling pathways. In line with this, some studies have previously shown that resveratrol in combination with quercetin exhibited a synergistic effect on the inhibition of vascular smooth muscle cell proliferation (Kurin et al. 2012).

In conclusion this study provided evidence that monomers and oligomers of resveratrol as piceatannol, ε-viniferin, hopeaphenol and isohopeaphenol have potential interesting effects in the inhibition of an inflammatory response to LPS activation via inhibiting the NO, ROS, TNF- α and IL-1β production. Thus, our results indicate that these stilbenes may be of potential interest in treatment of inflammatory related diseases. However, more studies are required in order to elucidate their mechanism of action.

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CHAPTER III

Screening of natural stilbene oligomers from *Vitis vinifera* for anticancer activity on human hepatocellular carcinoma cells

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Abstract

The characterization of bioactive resveratrol oligomers extracted from *Vitis vinifera* canes has been recently reported. Here, we screened six of these compounds (ampelopsin A, *trans*-ε-viniferin, hopeaphenol, isohopeaphenol, R2-viniferin, and R-viniferin) for their cytotoxic activity to human hepatocellular carcinoma (HCC) cell lines p53 wild-type HepG2 and p53-null Hep3B. The cytotoxic efficacy depended on the cell line. R2-viniferin was the most toxic stilbene in HepG2, with inhibitory concentration 50 (IC_{50}) of 9.7 ± 0.4 μM at 72 h, 3-fold lower than for resveratrol, while Hep3B was less sensitive (IC_{50} of 47.8 ± 2.8 μM). By contrast, hopeaphenol (IC_{50} of 13.1 ± 4.1 μM) and isohopeaphenol (IC_{50} of 26.0 ± 3.0 μM) were more toxic to Hep3B. Due to these results, and because it did not exert a large cytotoxicity in HH4 non-transformed hepatocytes, R2-viniferin was selected to investigate its mechanism of action in HepG2. The stilbene tended to arrest cell cycle at G2/M, and it also increased intracellular reactive oxygen species (ROS), caspase 3 activity, and the ratio of Bax/Bcl-2 proteins, indicative of apoptosis. The distinctive toxicity of R2-viniferin on HepG2 encourages research into the underlying mechanism to develop the oligostilbene as a therapeutic agent against HCC with a particular genetic background.

Keywords: stilbene; resveratrol oligomers; natural product; polyphenol; anticancer activity; HepG2 cells.

1. Introduction

Hepatocellular carcinoma (HCC) is the most frequently diagnosed primary cancer of the liver (Mittal and El-Serag 2013) and the fourth most common cause of cancer-related death worldwide (J. D. Yang et al. 2019). There is currently no effective treatment for HCC due to the high heterogeneity of the cancer. Thus, extrinsic factors and different mutations contribute to the induction of liver cancer (S. Perwez Hussain and Harris 1998; Link and Iwakuma 2017). Among the genetic factors, mutations in the p53 tumor suppressor gene (TP53) are frequent, especially in HCC from populations exposed to environmental carcinogens, such as dietary aflatoxin B1 (S P Hussain et al. 2007). In these cases, anti-tumor strategies include p53 activation. However, in a high proportion of hepatocarcinomas, p53 is retained, and recently, it has been proven that depending on the specific isoform it could also have an oncogenic role. Thus, the specific p53 family isoform is the target of novel anticancer therapies against HCC (Kunst et al. 2016).

Several tumor cell lines with different phenotypes that resemble the various types of liver cancers have been used. These approaches are valid and allow the results to be extrapolated to an in vivo situation (Khoury, Zalko, and Audebert 2015).

Since the discovery of the natural antitumor drug paclitaxel in 1960s, the interest in identifying new natural chemotherapeutic agents, particularly those with phenolic structure, has greatly increased. Stilbenes are a class of polyphenols characterized by the presence of a 1,2-diphenylethylene moiety. They are produced as plant secondary metabolites and exert protective actions against environmental challenge, acting mainly as antifungal phytoalexins (K. B. Pandey and Rizvi 2009). Stilbenes are present in foods and beverages such as blueberries, peanuts, grapes, and red wine (El Khawand et al. 2018). Grapevine is one of the richest sources of stilbenes currently known. The cane and grape stems

and seeds show high concentrations of stilbenes (Pugajeva, Perkons, and Górnaś 2018; Loupit et al. 2020) which are also present in grape skin and juice and in red wines (Guerrero et al. 2020). Resveratrol is the most widely studied stilbene for its actions on human health. Different works have reported its properties against diseases such as diabetes (Zhu et al. 2017), cancer (Vervandier-Fasseur and Latruffe 2019), cardiovascular diseases (Cheng et al. 2020), and neurodegenerative diseases (Sun et al. 2010). However, in the biosynthetic pathway, as the result of various oxidative condensations of resveratrol monomer, several dimers, trimers and tetramers are formed. Despite the numerous works describing the beneficial effects of resveratrol on health, other natural stilbenes, particularly oligomers, have received far less attention. The wine industry generates a high quantity of waste (wood, cane, and root) and oligostilbens are the main stilbenes extracted from these wastes, which constitute a cheap source of bioactive products (Loupit et al. 2020). In this work, we have studied the cytotoxic potential of a range of resveratrol oligomers (dimers and tetramers), extracted and purified from the *Vitis vinifera* grapevine cane, on hepatoma cell lines. The effects were compared with those of resveratrol. After selecting the most active compound, we investigated the mechanism of its action.

2. Materials and methods

2.1. Stilbenes from *Vitis Vinifera*

Ampelopsin A, *trans*-ε-viniferin, hopeaphenol, isohopeaphenol, R2-viniferin, and R-viniferin were obtained from extracts of grapevine cane (*Vitis vinifera*), named Vineatrol®30. The extract was kindly provided by Actichem S.A. (Montauban, France). The isolation, characterization and purification of the stilbenes were carried out as described previously (Gabaston et al. 2018). The purity of the compounds was estimated to be ≥95%. *trans*-Resveratrol was purchased from Sigma-Aldrich (Lyon, France).

2.2. Cell Culture

The human hepatoma cell line HepG2 was obtained from the American Tissue Culture Collection (ATCC, Manassas, VA, USA). The human hepatoma cell line Hep3B was obtained from the European Collection of Authenticated Cell Cultures (ECACC, Porton Down, Salisbury, Wiltshire, UK). HH4 non-transformed human hepatocyte cell line (kindly provided by Dr. I. Fabregat, Molecular Oncology, Bellvitge Biomedical Research Institute, Spain) was established as described (Trepiana et al. 2017).

HepG2 and Hep3B cell lines were maintained in Eagle's Minimum Essential Medium and HH4 cell line was grown in Ham´s F12 Nutrient Mixture (Sigma-Aldrich, St Louis, MO, USA). Both culture media were supplemented with 10% heat inactivated fetal bovine serum, 2 mM L-glutamine and antibiotics (0.1 mg/mL streptomycin and 100 U/mL penicillin) (Sigma-Aldrich, St Louis, MO, USA). Cell cultures were grown in an incubator at 37 °C with 5% CO_2 atmosphere. After reaching approximately 80% of confluence, cells were detached in a solution of 0.1% trypsin and 0.04% EDTA and plated as required for further experiments.

2.3. Cell Viability Assay

Cell lines were seeded into 96-well plates at 5×10^3 (HepG2) and 3×10^3 (Hep3B and HH4) cells/well 24 h before treatment. Increasing concentrations of the monomer *trans*-resveratrol, the dimers ampelopsin A and *trans*-ε-viniferin and the tetramers isohopeaphenol, hopeaphenol, R2-viniferin and R-viniferin were then added and cells were incubated for 24, 48, and 72 h. Stilbenes were dissolved in dimethyl sulfoxide (DMSO), Sigma-Aldrich, St Louis, MO, USA) at a final concentration of 0.01%. The same amount of DMSO was added to control cells. After treatment, the cell viability was determined using the crystal violet assay (Trepiana et al. 2017). The absorbance was recorded at 590 nm in a Synergy HT microplate reader (BioTek, Winooski, VT, USA). Cell viability was calculated as the percentage of viable cells treated with stilbene versus untreated control cells using the following equation: Cell viability (%) = [OD (Treatment) − OD (Blank)]/[OD (Control) – OD (Blank)] × 100. The cytotoxic effect of stilbenes was determined by calculating IC_{50} values using non-linear regression analysis (GraphPad Prism 6, San Diego, CA, USA).

2.4. Cell Cycle Analysis

Exponentially growing cells were seeded in 6-wells plates (200,000 cells HepG2 and 180,000 cells Hep3B) 24 h before R2-viniferin treatment. On the day of treatment, the culture medium was replaced with culture medium containing R2-viniferin or vehicle solution (control).

After a treatment, cells were detached by trypsinization and fixed overnight at 4 °C in 70% ice-cold ethanol diluted in phosphate buffer saline (PBS). The ethanol was then discarded and the cells were washed with ice-cold PBS and finally stained with 25 μg/mL of propidium iodide (Sigma-Aldrich, St Louis, MO, USA) in the presence of 200 μg/mL ribonuclease A (Roche Biochemicals, Indianapolis, IN, USA) for 30 min at room temperature in the dark. Flow

cytometry analysis (Beckman Coulter Gallios) was carried out from a total number of 10,000 events acquired in the General Research Services SGIker of the UPV/EHU. Analysis of the data was performed using Summit 4.3 software (Dako, Glostrup, Hovedstaden, Denmark).

2.5. Intracellular ROS and Mitochondrial O_2^- Measurement

Intracellular levels of reactive oxygen species (ROS) were measured using the cell-permeant 2′,7′ dichlorodihydrofluorescein diacetate (H_2DCF-DA) probe (Molecular Probes, Eugene, OR, USA), which permeates living cells and is deacetylated and oxidized upon ROS exposure inside the cell, forming the fluorescent 2′,7′-dichlorofluorescein (DCF).

The mitochondrial superoxide anion (O_2^-) levels were measured using MitoSOX™Red reagent (Molecular Probes, Eugene, OR, USA), which permeates living cells where it selectively targets mitochondria and is oxidized by superoxide.

HepG2 cells were cultured in a 6-well cell culture plate at a density of 2 × 10^5 cells per well 24 h before starting the treatment with R2-viniferin. The medium was renewed and cells were incubated with R2-viniferin as described above. After treatment, cells were washed and incubated with 20 μM H_2DCF-DA or 4 μM MitoSOX™ for 30 min at 37 °C in the dark. The probe solution was then withdrawn and, after washing with PBS, the cells were trypsinized. The fluorescence intensity from living cells was measured by flow cytometry (DCF ⌾ex = 485/20 and ⌾em = 528/20, MitoSOX ⌾ex = 485/20 and ⌾em = 620/20) in a Beckman Coulter Gallios Flow cytometer in the General Research Services SGIker of the UPV/EHU. At least 10,000 events were detected. Data obtained from flow cytometry were analyzed using Summit 4.3 software (Dako, Hovedstaden, Denmark). Intracellular ROS and mitochondrial O_2^- were expressed as the

percentage of the fluorescence intensity in control cells at the same time of incubation.

2.6. Caspase-3 Activity

The activity of caspase-3 was measured using the specific synthetic tetrapeptide fluorogenic substrate Ac-DEVD-AMC (BD Pharmigen Biosciences, San Diego, CA, USA). The assay was carried out in 96-well plates in a total volume of 100 μL, with 37 μM of the substrate and 50 μg of protein in the assay medium in 100 mM HEPES, pH 7.4, containing 200 mM NaCl, 0.2% CHAPS, 2 mM EDTA, 20% glycerol, and 5 mM dithiothreitol (DTT).

In the presence of caspase-3, the Ac-DEVD-AMC substrate is hydrolyzed and the fluorogenic compound AMC is released. The activity was determined by continuous recording of the fluorescence at ⌾ex = 360 nm and ⌾em = 460 nm at 37 °C for 2 h every 5 min in a Synergy HT microplate reader (BioTek, Winooski, VT, USA). Results were expressed as the percentage of the fluorescence in control cells.

2.7. Western Blot Analysis

HepG2 cells were seeded in Petri dishes, incubated for 24 h and then treated with 5 and 10 μM R2-viniferin. Following 24 h of treatment, the cells were lysed in ice for 30 min with lysis buffer (20 mM HEPES pH 7.5, 1 mM NaF, 10 mM EGTA, 40 mM β-glycerophosphate, 1% NP-40, 2.5 mM $MgCl_2$, 2 mM orthovanadate, and 1 mM DTT) to which 10 μL/mL of a protease inhibitor cocktail (Sigma-Aldrich, St Louis, USA) was added just before use. Cellular fragments were removed by centrifugation at 12,000× *g* for 10 min at 4 °C, and total protein concentration was determined by Bradford assay [19]. The cellular protein extracts were boiled at 95 °C for 5 min in Laemmli buffer and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in 15%

polyacrylamide gels. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes by electro-blotting with constant amperage (1 mA/cm^2) for 2 h in a wet chamber. After blocking for 1 h in 5% bovine serum albumin (BSA) Tris-buffered saline containing Tween 20 at room temperature, membranes were incubated overnight at 4 °C with antibodies to Bax (Santa Cruz Biotechnology, 1:500), Bcl-2 (Santa Cruz Biotechnology, 1:500) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Abcam, 1:2000). After washing, membranes were probed with their corresponding secondary antibody conjugated to horseradish peroxidase for 1 h at room temperature. Specific proteins were detected using the enhanced chemiluminescence (ECL) substrate kit (Clarity Western ECL substrate, Bio-Rad, Hercules, California, USA) and the blots were imaged with the C-DiGit LI-COR blot scanner (Bonsai Advanced technologies S.L. Madrid, Spain). Intensities of target protein bands were determined by densitometry and normalized to the intensity of the loading control GAPDH protein.

2.8. Statistical Analysis

The statistical package SPSS 19.0 (SPSS Inc. Chicago, IL, USA) was used for data analysis. The results were expressed as mean ± standard error (SE) from at least three experiments. Statistical significance for the differences of the means was estimated by parametric Student's t-test. Differences between means were considered statistically significant for $p < 0.05$. IC_{50} values were derived from fitting the data to a sigmoidal dose-response curve with a three-parameter logistic model using GraphPad Prism 6.

3. Results

In this work, we have investigated the cytotoxicity of one stilbene monomer: *trans*-resveratrol; two stilbene dimers: ampelopsin A and *trans*-ε-viniferin; and four resveratrol tetramers: hopeaphenol, isohopeaphenol, R2-viniferin, and R-viniferin. Their chemical structures and that of resveratrol are shown in **Figure 1**.

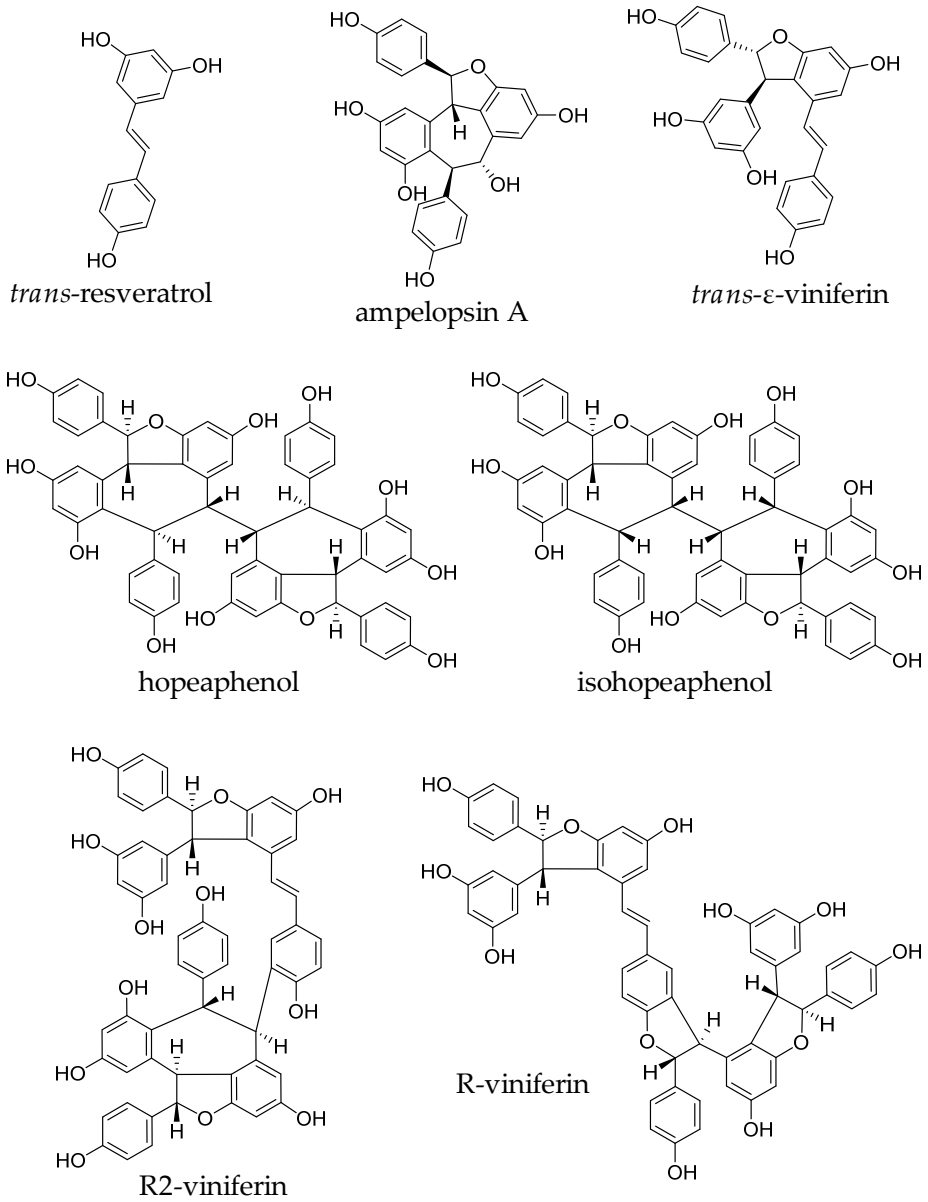


Figure 1. Chemical structures of resveratrol and resveratrol oligomers.

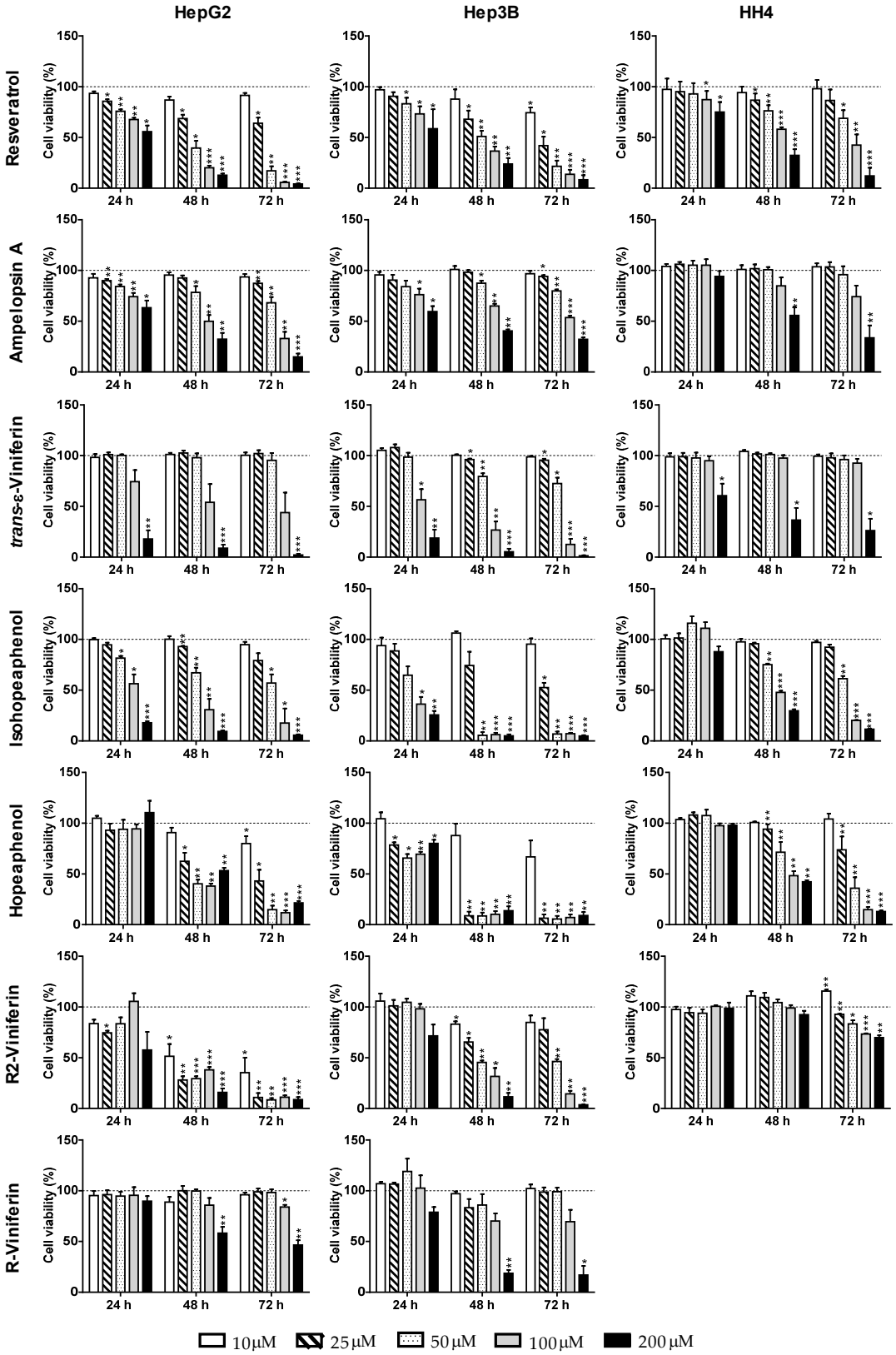


Figure 2. Effect of different stilbenoids on cell viability of HepG2, Hep3B and HH4. Cell viability was measured by crystal violet assay. Results are the mean + SE of n = 3–5 experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with control at the same time.

The cytotoxic activities of resveratrol oligomers were screened on HepG2, Hep3B and HH4. Cells were treated with increasing concentrations of each stilbene and after 24, 48, and 72 h, cell viability was measured by crystal violet assay **(Figure 2)**.

IC_{50} values were calculated from the inhibition curves (Table 1). The stilbenes induced a decrease of cell number in a dose- and time- dependent manner. Resveratrol, the reference stilbene, reduced the viability of HepG2 in a similar way to Hep3B, with IC_{50} values of 30 μM (in HepG2) and 21 μM (in Hep3B) at 72 h. In non-transformed hepatocytes, the concentration needed to reduce cell viability by 50% was markedly higher (93 μM).

Among dimers, ampelopsin A was more active in HepG2 (IC_{50} of 76 μM), while *trans*-ε-viniferin was more cytotoxic in Hep3B (IC_{50} 63 μM). In non-transformed hepatocytes, much higher concentrations of stilbene dimers were needed to induce toxicity. Among tetramers, hopeaphenol and isohopeaphenol were highly cytotoxic to Hep3B, with effective IC_{50} at 72 h of 13 μM and 26 μM, respectively. Their effects in HepG2 were less pronounced, with hopeaphenol (IC_{50} of 24 μM) being twice as potent as its geometric isomer isohopeaphenol (54 μM). R2-viniferin was the most toxic compound among all tested stilbenes in HepG2. The IC_{50} value was < 10 μM, more than three times lower than that found for resveratrol. In Hep3B the stilbene was not so efficient, showing an IC_{50} of 48 μM. Interestingly, in HH4 non-transformed hepatocytes the IC_{50} value was higher than 200 μM (Table 1). In the case of R-viniferin, containing one less free hydroxyl group than R2-viniferin, concentrations as high as 200 μM were necessary to induce cell death in HepG2 and Hep3B **(Figure 2)**.

Table 1. IC_{50}[a] values (μM) of different stilbenes against hepatocellular carcinoma cell lines.

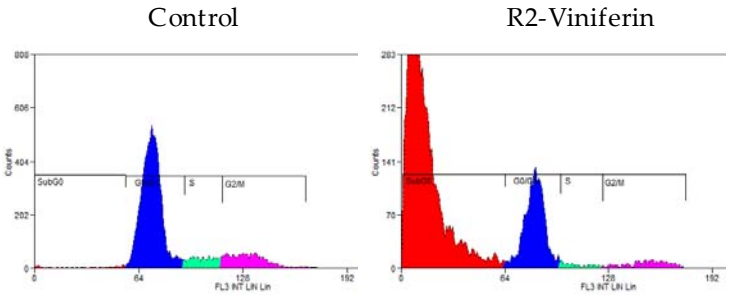
Compound	Time (h)	HepG2	Hep3B	HH4
Monomer				
trans-Resveratrol	24 h	>200	>200	>200
	48 h	40.3 ± 9.3	42.0 ± 11.6	135.0 ± 9.0
	72 h	30.3 ± 4.4	21.0 ± 16.8	93.0 ± 16.1
Dimer				
Ampelopsin A	24 h	>200	>200	>200
	48 h	98.6 ± 24.9	147.8 ± 14.4	178.3 ± 67.8
	72 h	75.5 ± 21.5	109.1 ± 7.3	133.8 ± 34.7
trans- ε-Viniferin	24 h	140.0 ± 39.7	108.1 ± 31.8	>200
	48 h	103.7 ± 19.2	73.9 ± 17.3	192.7 ± 21.1
	72 h	94.8 ± 28.3	63.1 ± 10.8	177.9 ± 20.5
Tetramer				
Hopeaphenol	24 h	>200	>200	>200
	48 h	27.0 ± 3.3	16.8 ± 2.3	92.0 ± 38.0
	72 h	24.4 ± 2.0	13.1 ± 4.1	37.6 ± 13.0
Isohopeaphenol	24 h	113.0 ± 33.0	86.6 ± 11.7	>200
	48 h	68.8 ± 31.0	37.0 ± 4.5	96.0 ± 5.5
	72 h	54.1 ± 34.0	26.0 ± 3.0	63.7 ± 3.7
R2-viniferin	24 h	>200	>200	>200
	48 h	10.2 ± 8.16	43.9 ± 3.6	>200
	72 h	9.7 ± 0.4	47.8 ± 2.8	>200
R-viniferin	24 h	>200	>200	n.d[b]
	48 h	>200	137.2 ± 19.8	n.d[b]
	72 h	192.0 ± 27.1	134.9 ± 35.7	n.d[b]

[a] mean ± standard error; [b] n.d., not determined.

In view of these results, R2-viniferin was selected to study the mechanisms of its cytotoxicity in hepatocellular carcinoma cells. The effect of R2-viniferin on cell cycle distribution in both HepG2 and Hep3B was studied by flow cytometry.

As can be seen in Figure 3, HepG2 treated with 10 μM R2-viniferin showed a progressive increase in the number of cells in subG0 phase over time. This effect was accompanied by a concomitant, though not significant, increase in the percentage of cells in the G2/M phase, suggesting an arrest in this phase. In Hep3B, however, R2-viniferin did not alter cell cycle at this concentration **(Figure 4).**

A)

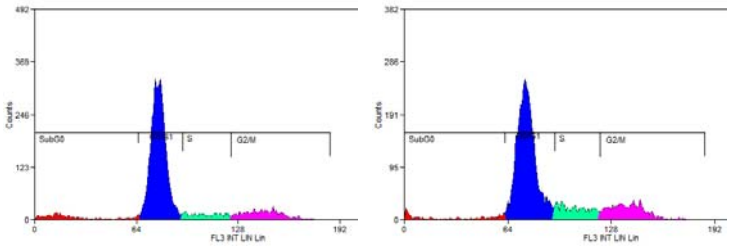


B)

		Control			R2-Viniferin		
		24 h	48 h	72 h	24 h	48 h	72 h
	SubG0	1.0 ± 0.2	1.1 ± 0.5	1.9 ± 0.6	8.5 ± 2.7	22.4 ± 11.0	71.0 ± 2.1*
Cell cycle	G0/G1	72.5 ± 3.2	78.5 ± 3.0	87.3 ± 1.7	65.6 ± 11.7	71.7 ± 10.9	75.5 ± 7.8
	S	12.4 ± 2.5	8.8 ± 1.4	3.0 ± 0.6	11.9 ± 3.2	9.0 ± 4.5	9.5 ± 5.6
	G2/M	15.1 ± 0.6	12.7 ± 1.6	9.7 ± 1.2	22.5 ± 8.5	19.3 ± 6.5	15.0 ± 2.2

Figure 3. Cell cycle distribution of HepG2 treated with R2-viniferin. **(A)** Representative graphs of cell cycle analysis by flow cytometry at 72 h. (**B**) Statistical analysis of cell cycle distribution. Data are expressed as the percentage (%) of cells at different stages of cell cycle. In the case of cells in subG0, data are expressed as the percentage of total cells. Results are the mean ± SE of three experiments. *p<0.001, significantly different from control.

A)



B)

		Control			R2-Viniferin		
		24 h	48 h	72 h	24 h	48 h	72 h
	SubG0	1.3 ± 0.4	1.0 ± 0.3	3.6 ± 1.3	2.0 ± 0.6	1.8 ± 0.4	3.3 ± 0.7
Cell cycle	G0/G1	62.8 ± 3.5	74.8 ± 3.6	82.2 ± 2.4	63.6 ± 8.0	73.7 ± 3.4	75.5 ± 3.9
	S	13.6 ± 1.1	9.6 ± 1.4	6.5 ± 1.6	15.7 ± 3.7	10.4 ± 1.6	9.8 ± 1.2
	G2/M	23.6 ± 3.1	15.6 ± 2.4	11.3 ± 1.2	20.7 ± 6.1	16.5 ± 2.0	14.7 ± 2.9

Figure 4. Cell cycle distribution of Hep3B treated with R2-viniferin. (**A**) Representative graphs of cell cycle analysis by flow cytometry at 72 h. (**B**) Statistical analysis of cell cycle distribution. Data are expressed as the percentage (%) of cells at different stages of cell cycle. In the case of cells in subG0, data are expressed as the percentage of total cells. Results are the mean ± SE of three experiments.

R2-viniferin increased the intracellular ROS concentration dose-dependently from the first time assayed **(Figure 5A)**. At the highest concentration used (10 μM), ROS remained significantly elevated up to 72 h. In the case of mitochondrial $O_2^{\cdot-}$, no significant effect could be observed at any of the stilbene doses or times assayed **(Figure 5B)**.

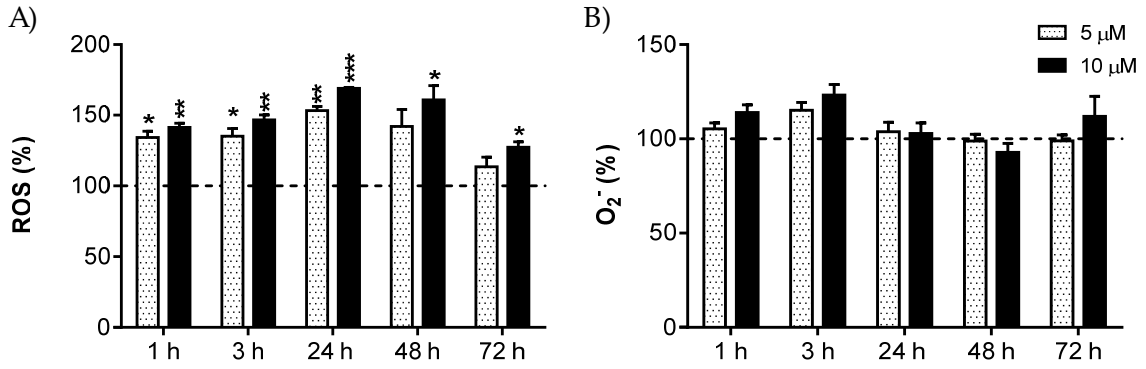


Figure 5. Effect of R2-viniferin on (**A**) intracellular ROS and (**B**) mitochondrial O_2^- levels in HepG2. Cells were treated with 5 and 10 μM of R2-viniferin for the indicated times. Reactive species were detected by flow cytometry. (**A**) ROS were determined by H_2DCF-DA assay. (**B**) O_2^- was determined by MitoSOX probe. Results are expressed as the percentage (%) of the control values at the same time, and are the mean ± SE of 3 experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

R2-viniferin (10 μM) increased the caspase-3 activity significantly from 24 h to 72 h. At 5 μM concentration the stilbene increased caspase-3 activity at 72 h **(Figure 6)**. These data indicate that R2-viniferin induces HepG2 death through a caspase-dependent mechanism, in which the executioner caspase-3 is involved.

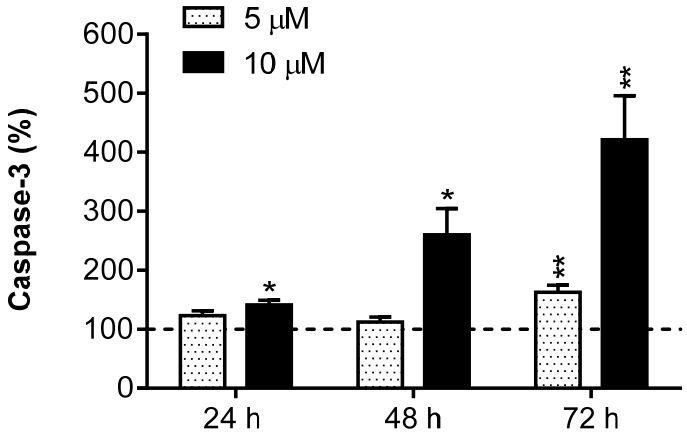


Figure 6. Effect of R2-viniferin on caspase-3 activity in HepG2. Cells were incubated with R2-viniferin (5 μM and 10 μM) at the indicated times. Results are expressed as the percentage (%) of the control values, and are the mean ± SE of 4 experiments. *$p < 0.05$, **$p < 0.01$, compared with controls.

We then analyzed by the effect of R2-viniferin on the apoptosis-related Bcl-2 protein family. The resveratrol oligomer upregulated the expression of the proapoptotic Bax protein and downregulated anti-apoptotic Bcl-2 proteins **(Figure 7)**. The Bax/Bcl-2 ratio increased dose-dependently (39% and 123%) over control at 5 μM and 10 μM, respectively.

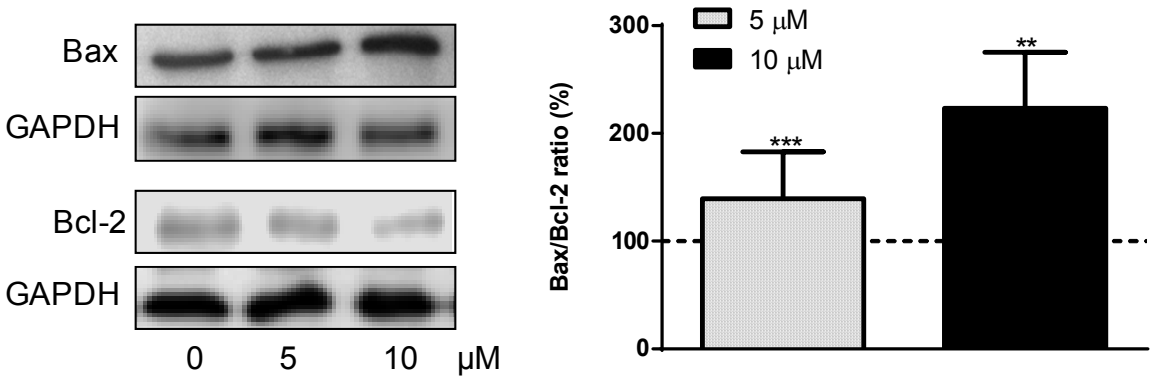


Figure 7. Western blot analysis of Bax and Bcl-2 expression in HepG2 cells incubated with R2-viniferin (5 and 10 μM). The bar graph shows the Bax/Bcl2 ratio, calculated by densitometric analysis of the anti-apoptotic proteins expressed as the percentage of the ratio (molecule of interest/GAPDH protein) detected in the control. Results are the mean + SE of $n = 6$ experiments. $^{**}p < 0.01$, $^{***}p < 0.001$, compared with control.

4. Discussion

The results presented herein show for the first time that several natural oligomeric products of resveratrol, in particular tetramers, are cytotoxic to human hepatocellular carcinoma cells, inhibiting cell proliferation and triggering death of the tumor cells. Resveratrol has been widely described to have antioxidant and protective actions against a wide range of diseases, particularly cardiovascular diseases (Cheng et al. 2020) and cancer (Vervandier-Fasseur and Latruffe 2019). However, other natural products obtained from the same source, the *Vitis vinifera* cane, in particular tetramers, have not been tested before for their antiproliferative activity against hepatoma cells. As resveratrol, some of these compounds, such as hopeaphenol, isohopeaphenol and R2-viniferin, were also identified in red wines and only recently R-viniferin and R2-viniferin could be detected at very low concentrations in certain red wines (Willenberg et al. 2012).

The dimer of resveratrol ε-viniferin was cytotoxic to various leukemia, HeLa cervix cancer, breast cancer, melanoma, and HepG2 cell lines (Espinoza and Inaoka 2017). The stilbene induced apoptosis in these cells and has been shown to inhibit topoisomerase IIa (Baechler et al. 2014). In HepG2, ε-viniferin showed slightly lower antiproliferative potential than resveratrol (Colin et al. 2008), and this was confirmed in the present work. The authors did not test toxicity in non-malignant liver cells. In our work, the resveratrol dimer also induced toxicity to HH4 cells, although the IC_{50} was high (178 μM). This result rules out the possibility to develop this product as a unique anticancer agent against hepatocellular carcinomas. However, the development of antitumor therapies could be established based on combinations of stilbenes at low concentrations with anticancer drugs, which may exert synergistic effects in the prevention or treatment of liver cancers, as it has been described for several cancer cells (Ivanova et al. 2019; Yen et al. 2018; Redondo-Blanco et al. 2017).

The results of this screening study showed that the cytotoxic effects of the natural stilbenes varied from one compound to another, and seemed to be dependent on the cellular model used. For example, in HepG2 hopeaphenol was two times more potent than its tetrameric geometric isomer isohopeaphenol. We do not know the cause of these differences in the cytotoxic activity of the two isomers. In a study reported by Loisruangsin et al. it was found that both tetramers acted as competitive inhibitors of sirtuin 1 (SIRT1), a key histone deacetylase in the regulation of cellular processes, suggesting that inhibitors could suppress the growth of tumor cells (Loisruangsin et al. 2019). Hopeaphenol proved to be a more efficient inhibitor than isohopeaphenol. The authors showed, by computer-assisted modeling, that there were differences in the bonds that were established in the enzyme complex resulting from the inhibition, with more hydrogen bonds being formed in the case of hopeaphenol than with isohopeaphenol. As regards the different efficacy depending of the cell line, hopeaphenol and isohopeaphenol, similar to the resveratrol monomer, were more toxic to Hep3B than to HepG2. By contrast, R2-viniferin, a tetramer formed from two dimers of ε-viniferin and ampelopsin B, was the most potent stilbene to induce cell death in HepG2 (with an IC_{50} three times lower than that of resveratrol), while Hep3B cells were less sensitive to this tetramer. The structural diversity of tested compounds could explain the variability of activity on different cellular models. To better understand these structural specificities, additional studies will be necessary to look at the structure–activity relationship.

Scientific reports regarding the R2-viniferin stilbene are quite scarce. There is great confusion about this oligostilbene, since R2-viniferin is also called vitisin A, a name that also refers to another compound with a pyranoanthocyanin structure found in red wines (Asenstorfer et al. 2003). Two studies reported that this oligostilbene induced cell death in prostate cancer (Shin et al. 2015) and leukemia cell lines (Mi Jeong Sung et al. 2009). In these latter cells, R2-viniferin induced cell

apoptosis, as well as the inhibition of ERK, p38, and NF-kB pathways. In our work, the differences in susceptibility to R2-viniferin and the other resveratrol oligomers may be related to differences in the genetic background of the hepatocarcinoma cell lines. Thus, HepG2 cells carry wild-type p53, while Hep3B are p53-null. Tumor protein p53 induces cell cycle arrest and apoptosis through the transcriptional regulation of *BAX* gene. The protein also upregulates Bak expression, and the activation of Bax and Bak induces activation of caspase-3 (Liao et al. 2018). In HepG2 cells, we have found that R2-viniferin tended to arrest cell cycle at G2/M phase, increased intracellular ROS levels, and the Bax/Bcl2 ratio in a dose-dependent manner. The induced effect on apoptosis is more dependent on the balance between Bcl-2 and Bax than on Bcl-2 quantity alone (Huang et al. 2012). When tested at the same concentration of 10 μM, R2-viniferin was unable to inhibit cell proliferation and induce cell cycle blockage in p53-null Hep3B cells. In a previous work, resveratrol was reported to inhibit HepG2 cell proliferation by blocking cell cycle at the G2/M transition (Delmas et al. 2000). Since cyclins and their cyclin-dependent kinases (CDKs) are key regulators of cell cycle (Johansson and Persson 2008), the cell cycle inhibition by resveratrol in cancer cells has been attributed to disturbances in cyclins-CDKs complexes. We propose a similar mechanism of cell proliferation inhibition for R2-viniferin.

We have seen that R2-viniferin increased intracellular ROS levels without affecting the mitochondrial O_2^- determined by the MitoSOX™Red fluorescent probe. We do not know the nature and source of these ROS. It has been described that resveratrol induces the expression of the transmembrane enzyme NADPH oxidase-5 (Nox5) in lung cancer cells. This enzyme generates O_2^- which is converted by action of superoxide dismutase (SOD) into hydrogen peroxide (Luo et al. 2013). In HepG2 resveratrol also causes the upregulation of SOD, without affecting glutathione peroxidase, which contributes to the formation and accumulation of hydrogen peroxide inside the cells (Khan et al. 2013). Although

speculative, R2-viniferin could exert similar effects in HepG2 in generating hydrogen peroxide, which would lead to cell apoptosis.

Our results show cytotoxic actions of stilbenes from *Vitis vinifera* cane in vitro. A major limitation of the study is the bioavailability of stilbenes in vivo. Several works have reported a weak bioavailability of resveratrol, mainly as a result of its low cell accessibility and its fast metabolism in the intestine and liver (Baur and Sinclair 2006; Walle 2011). Therefore, a series of synthetic resveratrol derivatives that are more hydrophobic and with higher cell permeability are being developed to test their biological activity (Snyder, Gollner, and Chiriac 2011; Matsuura et al. 2015; Keylor et al. 2016). To our knowledge, there are no reports on the bioavailability of the stilbene oligomers screened in this study, with the exception of the dimer ε-viniferin (El Khawand et al. 2018; J. Kim et al. 2017; Courtois et al. 2018), which showed by flow cytometry a cellular uptake kinetics similar to that of resveratrol (Colin et al. 2008). The bioavailability of stilbenes depends on many factors, among them their stability, the molecular size, the chemical structure, and the hydrophilicity/hydrophobicity properties of the compound (Gao and Hu 2010). Encapsulation into nanoparticles or liposomes of bioactive compounds with low water solubility may be a promising approach to facilitate their stability, absorption, transport to target cells and, therefore, their action. This challenge has recently been described for ε-viniferin in Caco-2 intestinal cells (Courtois et al. 2019).

5. Conclusions

We have described the cytotoxic activities of several natural resveratrol oligomers isolated from *Vitis vinifera* cane extracts against human hepatocellular carcinoma cells, and in comparison to HH4 non-transformed human hepatocytes. The cellular efficacies varied depending on the cell type. From the compounds tested, the tetramer R2-viniferin at concentrations below 10 μM was the most potent cytotoxic stilbene in p53-wilde type HepG2 cells, increasing intracellular ROS, and inducing cell apoptosis. The stilbene was innocuous in normal hepatocytes. These results suggest that R2-viniferin is a promising compound to develop in the chemoprevention and treatment of liver cancer. Further studies will be required in order to improve its bioavailability and to unravel its mechanism of action for potential clinical application in the treatment of liver cancer.

Abbreviations:

BSA Bovine serum albumin

CDKs Cyclin-dependent kinases

DCF 2′,7′-Dichlorofluorescein

DMSO Dimethyl sulfoxide

DTT Dithiothreitol

ECL Enhanced chemiluminescence

EDTA Ethylenediaminetetraacetic acid

EGTA Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′- tetraacetic acid

ERK Extracellular-signal-regulated kinase

GAPDH Glyceraldehyde-3-phosphate dehydrogenase

H_2DCF-DA 2′,7′ Dichlorodihydrofluorescein diacetate

HCC Hepatocellular carcinoma

HEPES 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid

NF-kB Nuclear factor kappa B

O_2^- Superoxide anion

PBS Phosphate buffer saline

ROS Reactive oxygen species

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CHAPTER IV

R2-viniferin induces toxicity in human hepatocellular carcinoma HepG2 through a p53-dependent mechanism

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Abstract

The natural R2-viniferin stilbene is a novel resveratrol tetramer very abundant in vine canes. Recently, we have reported that R2-viniferin caused toxicity in wild-type p53 HepG2 cells, while it was less toxic in p53-null Hep3B and innocuous in normal hepatocytes. In this work, we have investigated the mechanism by which R2-viniferin exerts toxic actions in HepG2 and specifically whether p53 is involved in this effect. To investigate the implication of this tumor suppressor, p53 was transiently silenced by siRNA in HepG2 and transfected with plasmid coding for the protein in Hep3B. Results showed an almost lack of effect in p53-silenced HepG2 (IC_{50} > 200 μM) and a higher sensitivity to R2-viniferin of p53-transfected Hep3B (40% reduction of IC_{50}). The oligostilbene induced the expression of phospho-p53, phospho-γ-H2AX, and p21, increased the intracellular H_2O_2 levels, and upregulated mitochondrial MnSOD in HepG2. The tetramer induced apoptosis through activation of caspase-9. Results by western blot also indicated alterations in ERK1/2 and PI3K/Akt signaling pathways, suggesting their implication in the apoptosis cascade. R2-viniferin eventually led to a permanent inhibition of clonogenicity and migration of HepG2. These results confirm the involvement of p53 in the toxic action of R2-viniferin and represent an important advance in research for the development of this natural compound in chemotherapies against p53-positive human hepatocellular carcinomas.

Keywords: stilbene; natural product; polyphenol; anticancer activity; p53: HepG2

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common types of cancer and has become a critical global health problem. The incidence of this cancer has progressively increased over time (Galle et al. 2018). The main cause of HCC is the cirrhosis produced by chronic infection with hepatitis B or C virus. Additional main risk derives from chronic inflammation such as alcoholic and non-alcoholic fatty liver disease (NAFLD), or from genetic factors (Muenzner et al. 2018). Mutations in the tumor suppressor gene TP53 are associated with HCC patients (Schulze et al. 2015). The TP53 family gene regulates a variety of cellular processes, involving cell cycle, cell death, apoptosis, senescence, and metabolism. The p53 protein product of the gene is a transcription factor that regulates a number of genes that are under its control. It stabilizes the genome by coordinating the response to repair the DNA when it is damaged. p53 induces cell cycle arrest, mainly mediated by the transcriptional activation of p21, until repair is completed and cells come back to the normal cell cycle. If the damage is too serious to being repaired, p53 activates the mechanism to induce cell death by programmed or non-programmed mechanisms. Therefore, mutations in TP53 results in deregulation of external challenge responses that normally maintain cell integrity. In contrast, the wild-type p53 is also present in numerous human HCC that retain functional p53, and specific isoforms of the family protein have been proposed to be oncogenic (Kunst et al. 2016; Kim et al. 2019). This oncogenic role of p53 has been recently reviewed (Jiang et al. 2019). Therefore, the p53 family is the target for therapeutic strategies in HCC.

The positive results of the treatment of advanced HCC patients with sorafenib, an oral multikinase inhibitor, triggered the search for and evaluation of other specific agents, especially the naturally occurring anti-cancer compounds. Resveratrol is the major natural stilbene that has been widely studied for its beneficial actions and anticancer activities in a large number of works, both in

vitro models and in clinical trials (Ramírez-Garza et al. 2018). R2-viniferin is a novel natural polyphenol, with tetrameric structure derived from resveratrol monomer, which is obtained in large quantities from vine canes. Recently, we have reported that R2-viniferin was highly toxic (at concentrations three-times lower than those observed for resveratrol) to the HCC wild-type p53 HepG2 cell line. The p53-null Hep3B was less sensitive to the stilbene (Aja et al. 2020). The results suggested a p53-dependent mechanism of toxicity. In this work, we have investigated in detail this mechanism, in particular whether p53 is involved in the antitumor activity of R2-viniferin.

2. Materials and methods

2.1. Reagents and antibodies

Vineatrol®30 was from Actichem S.A. (Montauban, France). The human hepatoma cell line HepG2 was purchased from the American Tissue Culture Collection (Manassas, VA, USA) and Hep3B from the European Collection of Cell Cultures (Porton Down, Salisbury, Wiltshire, UK). Amplex Red reagent, dimethyl sulfoxide, penicillin, and propidium iodide were from Sigma-Aldrich (St Louis, MO, USA), RNase A from Roche Biochemicals (Indianapolis, IN, USA), Lipofectamine RNAiMAX from Thermo Fisher Scientific (Waltham, MA, USA), scrambled siRNA from Santa Cruz Biotechnology Inc. (Dallas, TX, USA), and X-tremeGene 9 DNA Transfection Reagent from Roche Diagnostics (Mannheim, Germany). Human recombinant Cu,Zn-SOD and Mn-SOD proteins were purchased from ProSpec-Tany TechnoGene Ltd., Israel. The primary antibodies used were anti-ERK (sc-9102), anti-p53 (sc-126), and anti-p21 (sc-271610) from Santa Cruz Biotechnology (Dallas, TX), anti-caspase 9 (ab185719), anti-glyceraldehyde 3-phsphate dehydrogenase (GAPDH, ab8245), anti-◎-H2AX (ab22551), anti-MLKL (ab184718), anti-phospho-ERK (ab214362), and anti-phospho-MLKL (ab187091) from Abcam (Cambridge, MA), anti-Akt (cs-9272), anti-phospho-Akt (cs-9271) and anti-phospho-p53 (cs-9284) from Cell Signaling Technology (Danvers, MA), anti-MnSOD (06-984) from Millipore (Darmstadt, Germany), and anti-Cu/ZnSOD from Calbiochem (La Jolla, CA, USA).

2.2. Stilbene extraction from *Vitis vinifera*

R2-viniferin was obtained from Vineatrol®30, a vine (*Vitis vinifera*) shoot extract. The isolation, characterization and purification of the stilbene have been described previously (Gabaston et al., 2018). The estimated purity of R2-viniferin was ≥95%.

2.3. Cell cultures

Cells were seeded in Eagle's Minimum Essential Medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 0.1 mg/ml streptomycin and 100 U/ml penicillin and cultured in a humidified incubator with 5% CO_2 at 37 ºC. When the cells reached approximately 80% of confluence, they were detached using 0.1% trypsin-0.04% EDTA, and then harvested to perform subsequent assays. R2-viniferin was dissolved in dimethyl sulfoxide at a final concentration of 0.01%. Cell viability was determined by crystal violet assay as described previously (Trepiana et al. 2017).

2.4. Colony formation assay

HepG2 cells were plated in Petri dishes and treated with R2-viniferin (5 and 10 μM) for 24 h. After treatment, cells were trypsinized and 1,000-2,000 cells/well were seeded in 6 well plates with fresh culture medium for 10 days until visible colonies were formed. The colonies were fixed with 4% formaldehyde, stained with crystal violet, and then the number of colonies was manually counted.

2.5. Transwell migration assay

HepG2 cells were cultured in 24-well plates containing transwell inserts of 8 μm pore size, according to the manufacturer's instructions (Greiner bio-one, Switzerland). Briefly, 37,500 cells previously treated with R2-viniferin for 24 h were diluted in 0.4 ml serum free medium, placed into the insert immersed in 0.6 ml complete medium, and incubated for 24 and 48 h. After incubation, non-migrating cells were removed from the upper surface of the membrane by scrubbing, and migrating cells on the lower surface of the membrane were fixed in ice-cold 70% ethanol overnight. Subsequently, cells were stained overnight

with a solution containing 25 μg/ml propidium and 200 μg/ml RNasa A. The inserts were photographed under an Olympus Fluoview FV500 confocal microscope in the General Research Services SGIker of the UPV/EHU. The number of migrated cells was calculated using the ImageJ software (NIH, Bethesda, Maryland, USA).

2.6. Transient p53 silencing in HepG2 cells

HepG2 cells were seeded into 96 well plates at a density of 3,000 cells/well. Cells were transfected with 300 nM of p53 or scrambled siRNA (scRNA) using Lipofectamine RNAiMAX according to the protocol provided by the manufacturer. After 48 h of silencing, the medium was discarded and cells were treated with increasing concentrations of R2-viniferin (10-200 μM) for up to 72 h. Results were compared with those in cells transfected with scRNA.

2.7. Transient p53 transfection into Hep3B cells

The plasmid pCB6-p53, containing human wild-type p53 cDNA, was kindly provided by Dr. J.L. Zugaza (Achucarro Basque Center for Neuroscience, UPV/EHU, Spain). The transient transfection experiments were performed using the non-liposomal X-tremeGene 9 DNA Transfection Reagent according to the manufacturer's instructions. Hep3B cells were seeded (1,500 cells/well) in 96 well plates in complete culture medium without antibiotics and maintained at 37 °C in a humidified 5% CO_2 atmosphere. Non-stably transfection was conducted by the addition of 1 μg of plasmid DNA (p53-plasmid or empty vector). After 48 h, the medium was removed and cells were incubated for 72 h in fresh medium with increasing concentrations of R2-viniferin (10-200 μM). Results were compared with those in cells transfected with an empty vector.

2.8. Western blot analysis

Cells were seeded in Petri dishes, incubated for 24 h and then treated with 5 and 10 μM of R2-viniferin for 24, 48 and 72 h. Cell extracts were processed and the proteins were separated by western blot as described previously (Aja et al. 2020). The dilutions used for the primary antibodies to p53 and p21 were 1:500 and 1:1000 for the rest of proteins (Akt, phospho-Akt phospho-ERK, γ-H2AX, phospho-MLKL, MLKL, caspase-9, GAPDH, MnSOD, and Cu/ZnSOD). The protein concentration was determined as described by Bradford (Bradford 1976). GAPDH levels in the cell lysates were used as loading control. The membranes were image digitized by scanning with the C-DiGit LI-COR blot scanner (Bonsai Advanced technologies S.L., Madrid, Spain). For normalization, band intensity of each sample was determined by densitometry, and the intensity of the target protein was divided by the intensity of the loading control protein.

To quantify the absolute levels of cytosolic and mitochondrial SOD isoforms, we prepared standard curves with commercially available human recombinant SOD proteins. The ranges were 6.6-33 ng (Cu,Zn-SOD) and 1.6-7.9 ng (Mn-SOD). The intensity of the bands in the samples was translated into absolute amounts by interpolating it in the standard curve in each blot (Trepiana et al. 2018).

2.9. Lactate dehydrogenase release assay

HepG2 cells were grown in 6-well culture plates (180,000/cell) in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS for 24 h. Then, the cells were exposed to R2-viniferin in culture medium without phenol red for 24-72 h. After treatment, media were centrifuged for 5 min at 13,000xg at 4 °C. Lactate dehydrogenase (LDH) was measured in the supernatants. The cells adhered to the plates were detached by scrapping with 1.5 ml of milliQ H2O, lysed by two cycles of freezing-thawing in liquid nitrogen, and centrifuged for 5 min at 13,000 xg at 4°C. Supernatants were used for

measuring intracellular LDH.

2.10. Hydrogen peroxide measurement

H_2O_2 was determined in the culture medium using Amplex Red, as described before (Trepiana et al. 2018).

2.11. Statistical analysis

Data are shown as the mean ± standard error (SE) from at least three experiments. Statistical significance for the differences of the means was analyzed by parametric Student's t-test, using the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA), version 19.0. Differences were considered statistically significant for $p<0.05$.

3. Results

Previous studies from our research group have shown that R2-viniferin was cytotoxic to HepG2 at low micromolar doses (Aja et al. 2020). Here, we have used 5 and 10 μM for up to 48 h and have compared the induced toxicity to that obtained from a transient exposition to the stilbene. In this case, cells were challenged with R2-viniferin for 24 h and after thoroughly washing, cells were cultured in fresh medium for additional 24 and 48 h. As previously reported, continuous treatment with 10 μM R2-viniferin showed significant cytotoxicity at 48 h (approximately 50% cell viability reduction) **(Figure 1A).** The transient incubation of cells with the stilbene reduced viability by 30%, although this modification was not statistically significant **(Figure 1B).**

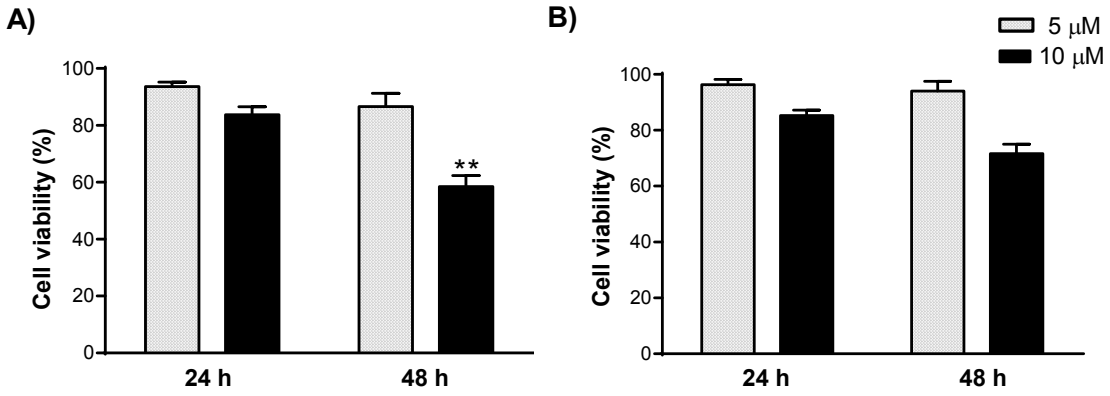

Figure 1. Effects of R2-viniferin on cell viability of HepG2 A) continuously and B) transiently exposed to the stilbene. In A) cells were treated with R2-viniferin for 24 and 48 h. B) Cells were treated with R2-viniferin for 24 h and after washing, viability was determined at 24 h and 48 h post-treatment. Cell viability was measured by crystal violet assay. Results are the mean + SE of 3 experiments. **p<0.01 compared with control at the same time.

We further analyzed the possible permanent effects of the transient exposure of HepG2 to R2-viniferin on the potential survival through the cellular colony-forming ability. The oligostilbene (10 μM) significantly ($p < 0.001$) reduced by 58% the number of colonies **(Figure 2).**

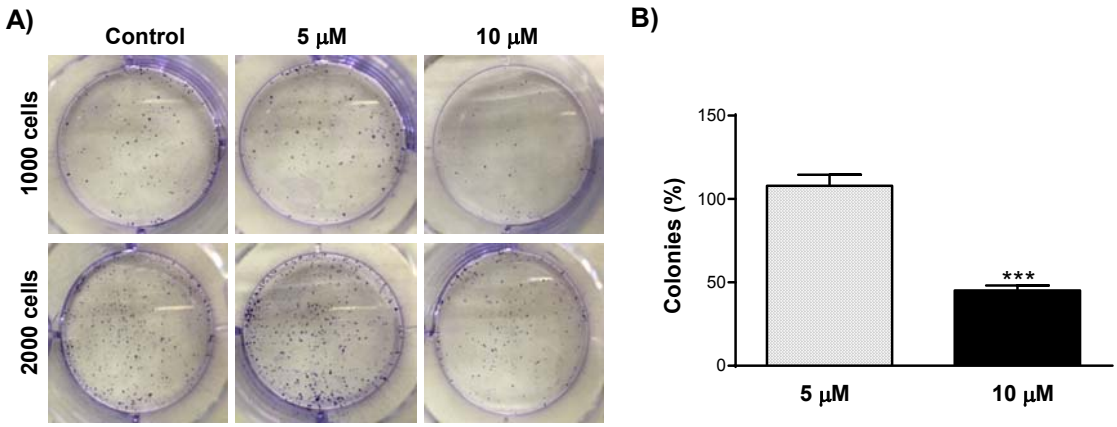

Figure 2. Colony-forming ability of HepG2 after pre-treatment with R2-viniferin. Cells were exposed to R2-viniferin (5 and 10 μM) for 24 h and after extensive washing, cells were cultured for 10 days before the colony count. A) Representative images of colony-forming assay. B) Rate (%) of colonies formed after R2-viniferin pre-treatment. ***p<0.001 compared with control (no additions).

Regarding modulation of cell migration, quantitative analysis showed that pre-treatment with 10 μM R2-viniferin partially abolished the migration ability of HepG2 at 24 h (44%) and 48 h (25%). Intriguing, a lower concentration of the stilbene temporarily conferred the cells a higher migrating ability, although not statistically significant **(Figure 3).**

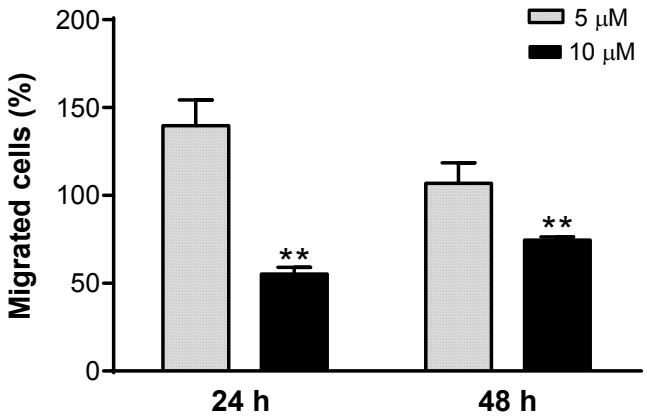

Figure 3. Effect of R2-viniferin pre-treatment on HepG2 cell migration. After 24 h of treatment with R2-viniferin (5 and 10 μM), cells were detached and seeded in the upper-chamber of transwell inserts of 8 μm pore size in the presence of serum free medium. Inserts were immersed in complete medium for 24 and 48 h, and then stained with propidium iodide and RNAse A. The number of migrating cells were photographed and counted. Results are the mean + SE of n=3 experiments **p<0.01 compared with control.

We have reported different sensitivities of hepatoma cell lines to R2-viniferin, as evidenced by the IC50 values (the concentration required to inhibit cell viability by 50%). In HepG2, R2-viniferin showed high toxicity, with IC50 < 10 μM, while in Hep3B the oligostilbene was less toxic, with IC50 = 47.5 μM (Aja et al. 2020). We addressed the involvement of p53 in the mechanism of toxicity by transient silencing of p53 with siRNAs in HepG2 and p53 transfection in Hep3B. The sensitivity to R2-viniferin of each cell line was compared with that of the corresponding unmodified cell line. As shown in **Figure 4A**, the siRNA silencing markedly reduced the expression of p53 protein, with recovering at the end of the assay. This silencing was successful in the time window that allowed a cytotoxicity assay. p53 silenced HepG2 revealed lower sensitivity (IC50 > 200 μM) to R2-viniferin than wild-type p53 cells (IC50 < 10 μM) **(Figure 4B).**

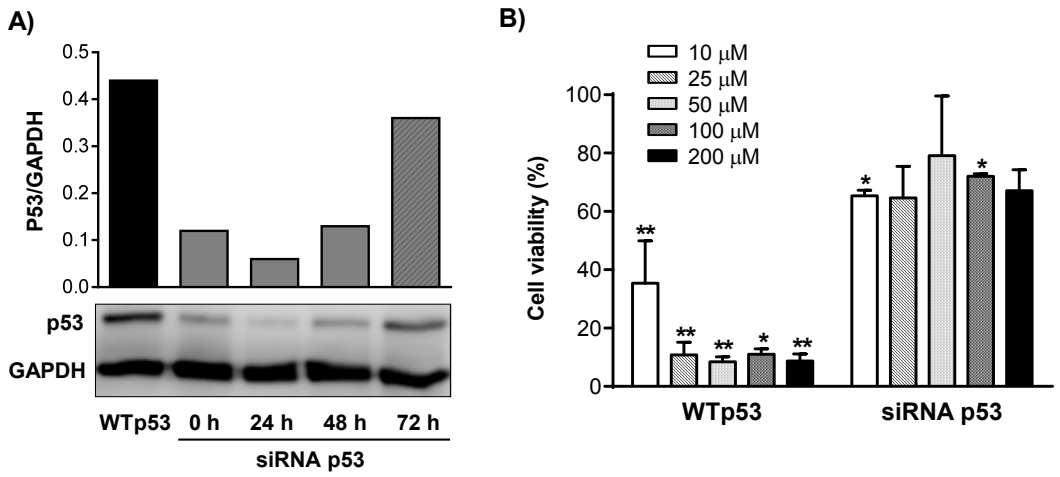

Figure 4. Effects of R2-viniferin on toxicity of wild-type and p53 silenced HepG2. A) Levels of p53 protein in wild-type and 24, 48 and 72 h after siRNA silencing. B) Cell viability at 72 h of wild-type and p53-silenced HepG2. Results are expressed as the percentage of the control values.

In the case of Hep3B, the p53 protein levels increased 6-fold at 24 h after transfection, progressively reverting to non-transfected cell levels over time **(Figure 5A)**. At 72 h of treatment with R2-viniferin, IC50 in p53-/- Hep3B was 59.4 μM, while p53 transfection made the cells more sensitive to the stilbene, decreasing IC50 to 37.5 μM.

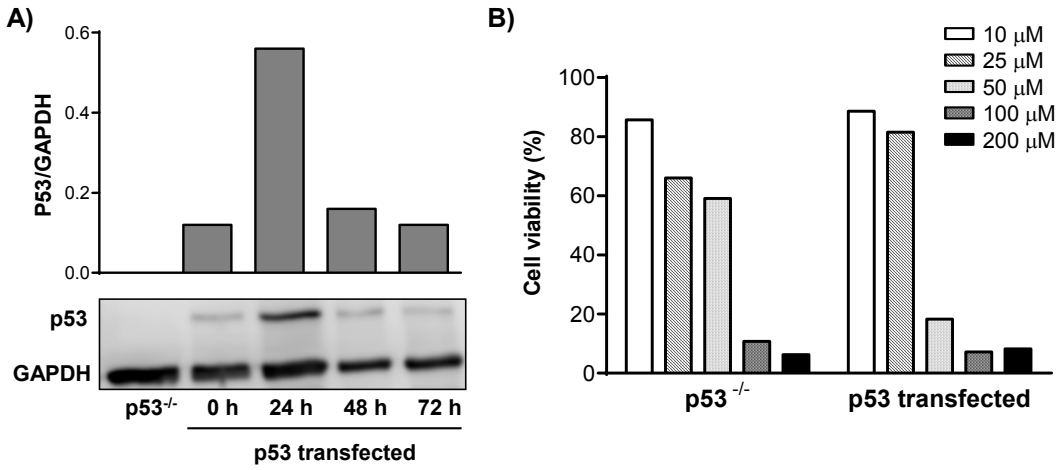

Figure 5. Effects of R2-viniferin on toxicity of wild-type and p53-transfected Hep3B. A) Levels of p53 protein in wild-type p53 Hep3B and 24, 48 and 72 h after transfection. B) Cell viability at 72 h of treatment of p53-null and p53-transfected Hep3B. Results are expressed as the percentage of the control values.

Under a cellular stress challenge, the p53 protein is subjected to numerous post-translational modifications, phosphorylation of serines and/or threonines being the most commonly reported. R2-viniferin increased significantly the phosphorylation of p53 on Ser15 at 24 h. Moreover, this effect was accompanied by an increase in the levels of p21, a protein with a key role in cellular apoptosis **(Figure 6)**.

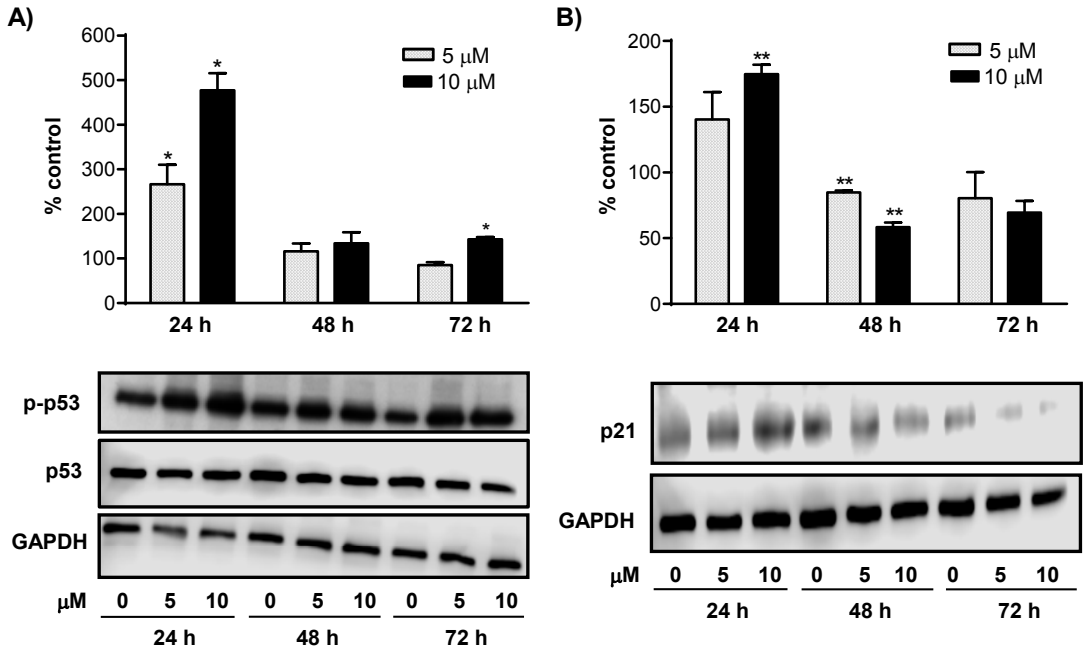

Figure 6. Effects of R2-viniferin on the protein expression of A) phosphorylated-p53 and B) p21 in HepG2. After exposure to R2-viniferin (5 and 10 μM) for 24, 48 and 72 h, protein expression was detected by western blot. A) The bar graph shows phospo-p53/p53 ratio compared to the control (no additions). B) The expression levels of p21 were normalized to glucose 6-phosphate dehydrogenase (GAPDH). Results are expressed as percentage values respecting control cells. Results are the mean + SE of n=3-7 experiments. *p<0.05; **p<0.01, compared with control.

We had found previously that R2-viniferin induced the activation of the executioner caspase-3 in HepG2 (Aja et al. 2020). In order to know the upstream caspases responsible of this activation, we analyzed the involvement of initiator caspases 2, 8 and 9. The activities of caspase-2 and 8 were tested by fluorometric assays, but we could not detect caspase activities (data not shown). Caspase-9 activation was analyzed by western blot. The enzyme was activated at 48 h of treatment and remained activated with time at the higher concentration of the stilbene **(Figure 7).**

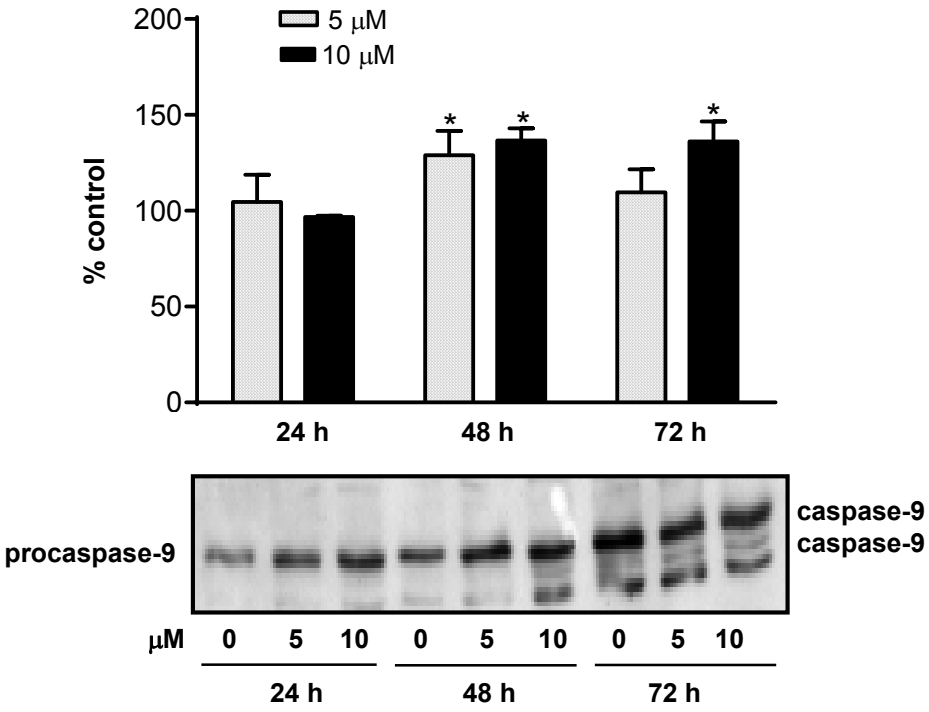

Figure 7. Effect of R2-viniferin on caspase-9 levels in HepG2. Cells were incubated without (control) and with R2-viniferin (5 and 10 μM) at the indicated times. Results are expressed as the percentage of the ratio cleaved caspase/procaspase, compared with control at the same time, and are the mean + SE of 3 experiments. *p<0.05, compared with control.

The death mechanism of necrosis was addressed by determining LDH activiy. R2-viniferin dose- and time-dependently decreased intracellular LDH activity, with a 70% reduction at the higher concentration at 72 h **(Figure 8).** These results indicate the rupture of the plasma membrane, reflecting a necrotic process.

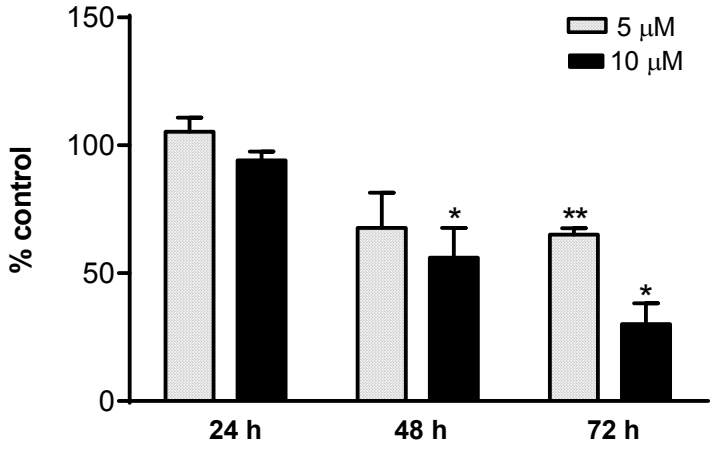

Figure 8. Effect of R2-viniferin on intracellular LDH activity. After treatment with R2-viniferin at the indicated times, the LDH activity in extra- and intracellular compartments was measured by monitoring the NADH decrease at 340 nm. Results are the mean + SE from 3 experiments. *p<0.05, **p<0.01.

As there is a cross-talk among the different cell death mechanisms, the regulated necrotic cell death named necroptosis was studied. We analyzed by western blot the phosphorylated statuts of the mixed lineage kinase domain-like pseudokinase (MLKL) protein, the terminal mediator in the necroptotic pathway. No significant modifications of the levels of phosphorylated MLKL were detected at any time or dose **(Figure 9).**

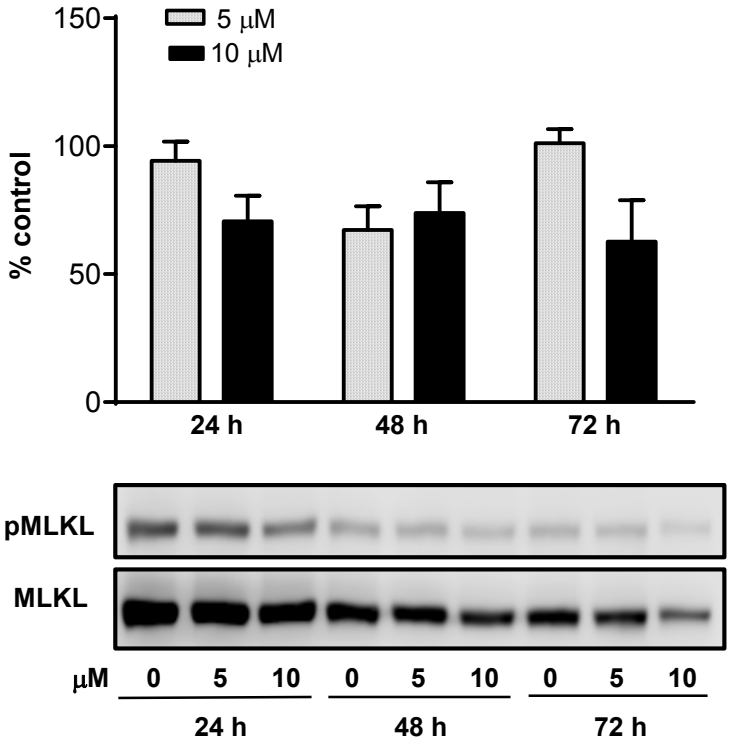

Figure 9. Effect of R2-viniferin on the protein expression of phosphorylated MLKL in HepG2. After treatment without (control) and with R2-viniferin at indicated times, phosphorylated and total MLKL levels were measured by western blot. The bar graph shows phospo-MLKL/MLKL ratio compared to the control. Results are the mean + SE of 3 experiments.

In order to determine the causes of the ROS increase induced by R2-viniferin in HepG2 (Aja et al. 2020), we analyzed the expression of the antioxidant enzymes Cu,Zn-SOD and Mn-SOD. The cytosolic isoform (basal levels, 2525 ± 649 ng/mg of protein) showed no significant changes in treated cells **(Figure 10A).** However, mitochondrial SOD (basal levels, 327 ± 111 ng/mg of protein) increased significantly by nearly 50% at 24 h and progressively returned to control values at 72 h **(Figure 10B).**

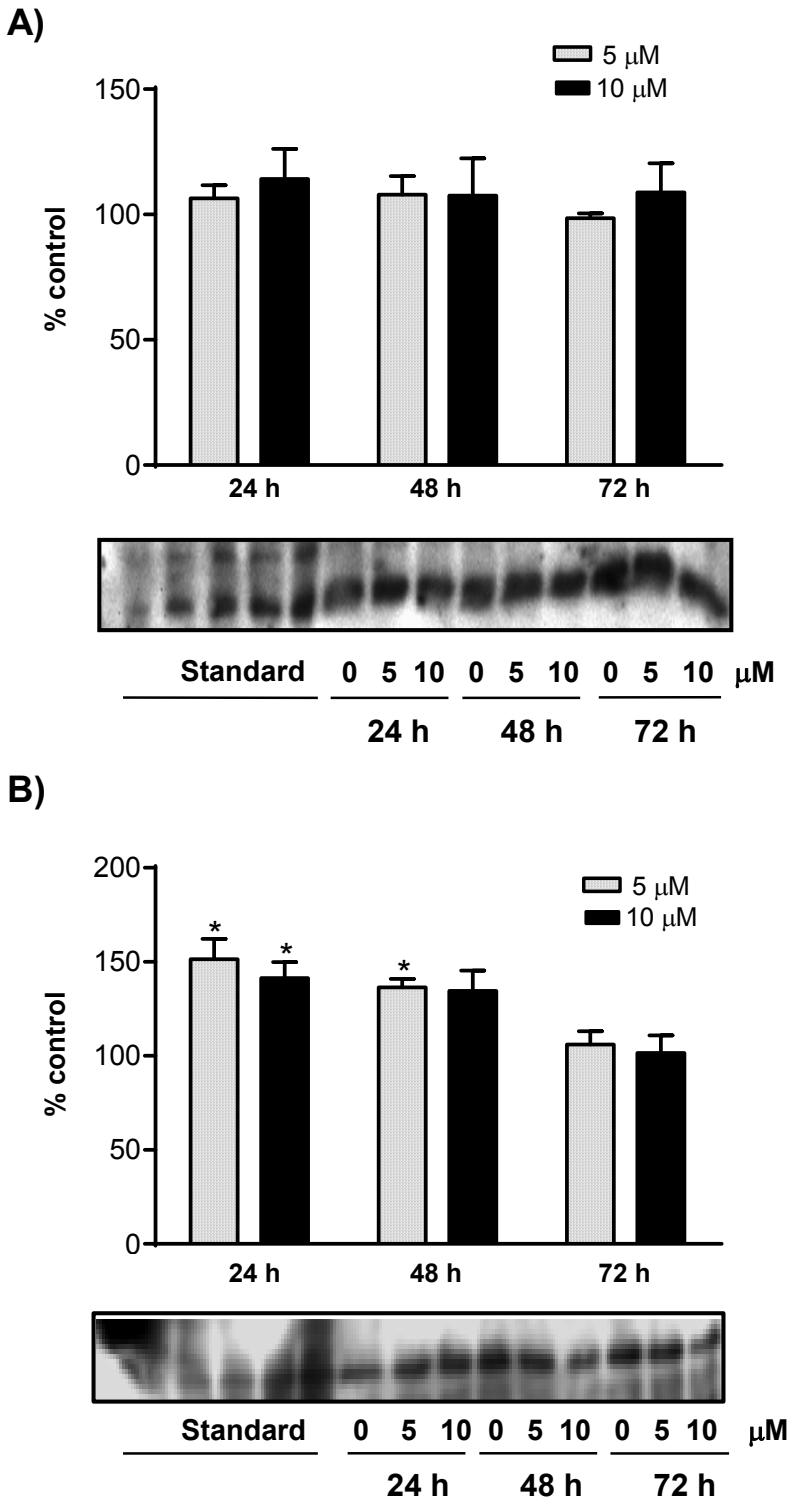

Figure 10. Effect of R2-viniferin on the protein levels of A) Cu,Zn-SOD and B) Mn-SOD in HepG2. Cells were incubated without (control) and with R2-viniferin (5 and 10 μM) for 24-72 h. The proteins were quantified by immunoblotting, Human recombinant proteins were used to construct standard curves (6.6-33 ng Cu,Zn-SOD and 1.6-7.9 ng Mn-SOD) to quantify absolute amounts of the proteins. Results are the mean + SE of 3 experiments. *$p<0.05$, different from control.

This remarkable upregulation of Mn-SOD could result in increased production of H_2O_2. Intracellular H_2O_2 was estimated by measuring its release into the medium, since the peroxide freely crosses the plasma membrane. R2-viniferin (10 μM) increased significantly H_2O_2 levels at 48 h (30 %) and 72 h (82 %) **(Figure 11).**

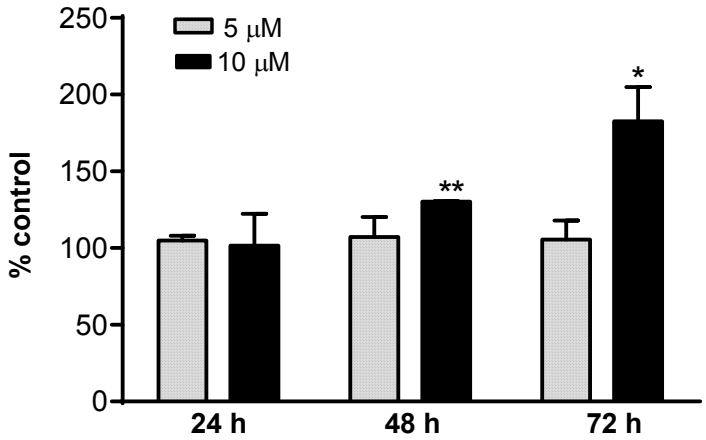

Figure 11. Effect of R2-viniferin on intracellular hydrogen peroxide in HepG2. Cells were incubated without (control) and with R2-viniferin (5 and 10 μM) for 24-72 h. After treatment, intracellular H_2O_2 was determined by the extracellular probe Amplex Red. Results are the mean + SE of 3 experiments. *p<0.05, **p<0.01.

The PI3K/Akt and Ras/MEK/ERK pathways have a critical role in the control of cell growth and proliferation in many different cell types (Saez-Rodriguez et al, 2015). Interaction between these two signal pathways may occur at several stages and could be either positive or negative. In order to study their implication in R2-viniferin-induced apoptosis, the phosphorylated and total Akt and ERK1/2 proteins were determined by western blot. The oligostilbene triggered dephosphorylation of Akt at 72 h, but increased ERK1/2 phosphorylation at the same time **(Figure 12).** These data suggest that R2-viniferin-induced apoptosis could be modulated by the PI3K/Akt and ERK1/2 pathways.

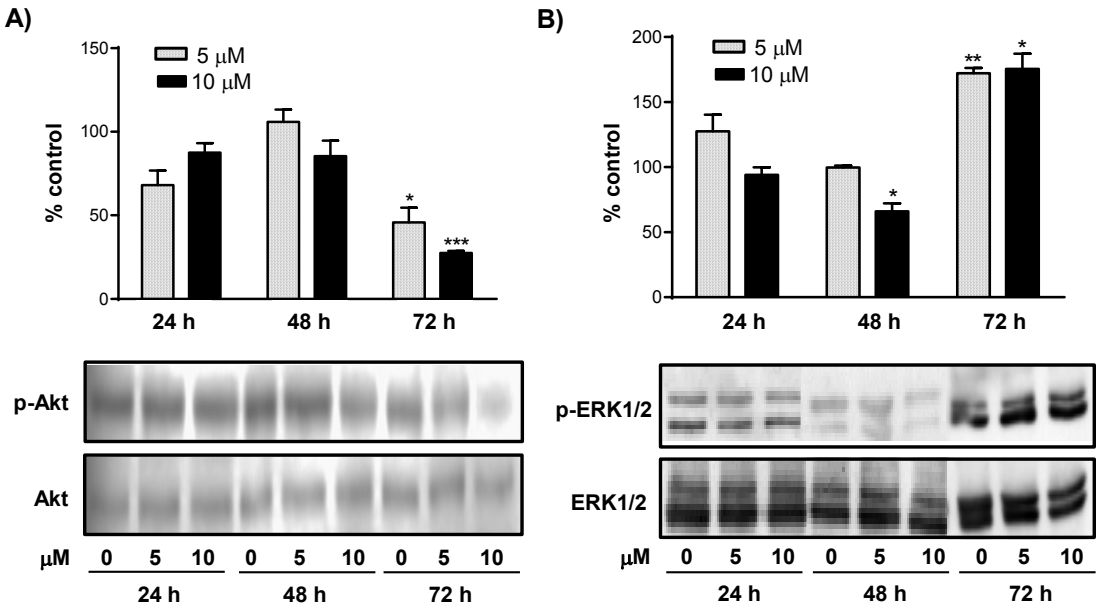

Figure 12. Effect of R2-viniferin on protein phosphorylation of A) Akt and B) ERK1/2 in HepG2. Cells were incubated without (control) and with R2-viniferin (5 and 10 μM) at the indicated times. The bar graph shows phospho-Akt/Akt or phospho-ERK/ERK ratio compared to the control. The bands shown are representative of at least 3 independent experiments. Results are the mean + SE of 3 experiments. *p<0.05, **p<0.01, ***p<0.001, compared with control.

γ-H2AX histone represents an early event in the cellular response against DNA double-strand breaks and plays a central role in sensing and repairing DNA damage. R2-viniferin prompted the increase γ -H2AX protein levels in a dose- and time-dependent manner **(Figure 13)**, suggesting that the stilbene induces DNA damage that is detected earlier than the apoptotic response.

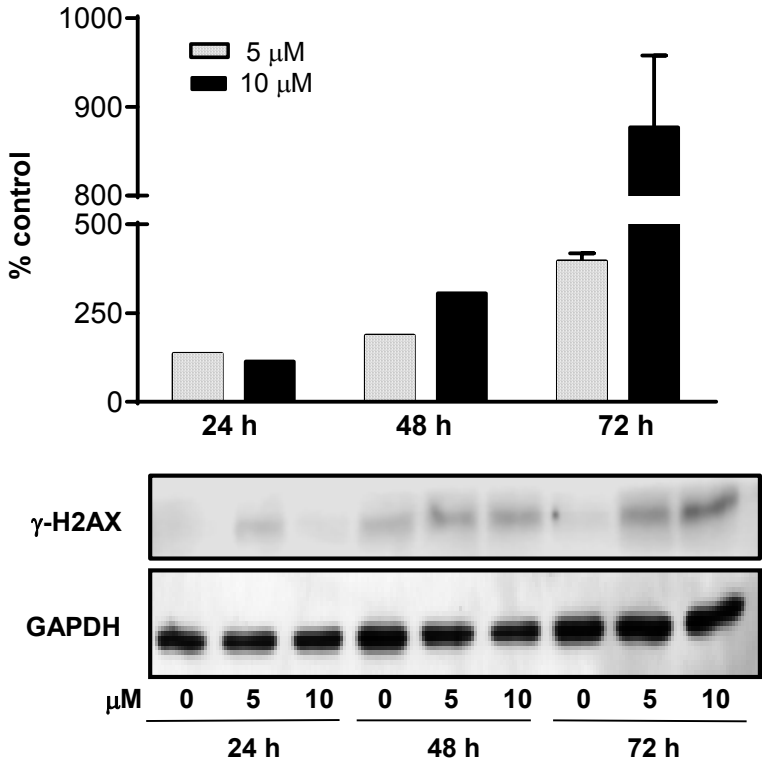

Figure 13. Effect of R2-viniferin on γ-H2AX levels in HepG2. Cells were incubated without (control) and with R2-viniferin (5 and 10 μM) at the indicated times. Proteins were analyzed by western blot.

4. Discussion

The aim of this work was to investigate the mechanism by which R2-viniferin exerts toxic actions in HepG2 and specifically whether p53 is mediating this effect. To investigate the implication of this tumor suppressor, p53 was transiently silenced by siRNA in HepG2 and transfected with plasmid coding for the protein in Hep3B. Results showed an almost lack of effect in p53-silenced HepG2 and a higher sensitivity to R2-viniferin of p53-transfected Hep3B. These results, together with a higher phosphorylation of the protein and the upregulation of p21, confirm that the induced toxicity is due, at least in part, to p53 status. p53 is subjected to numerous post-translational modifications (phosphorylation, modification with ubiquitin and other ubiquitin-like proteins, acetylation, methylation, glycosylation, farnesylation, hydroxylation and ADP ribosylation). These post-translational modifications, in concert with the interaction of p53 with a variety of protein-binding partners, regulate the subcellular localization and both its transcriptional and transcriptionally independent functions (Gu et al. 2012). p53 transcriptionally activates the expression of several pro-apoptotic Bcl-2 family proteins, and directly interacts with various pro-apoptotic and anti-apoptotic proteins in the cytoplasm and at the mitochondrial membrane (Green et al. 2009). Under pro-apoptotic conditions, p53 interacts with Bcl2, Bcl-X_L and Bak, de-repressing Bax and Bak (Chipuk et al. 2004). p53 also participates in cell repairing. Thus, following DNA damage, p53-activated cell cycle arrest can suspend cell cycle progression, allowing DNA lesions to be repaired. The main transcriptional target of p53 involved in establishing this process is *CDKN1A* (encoding p21), which can induce a temporary cell cycle arrest in addition to contributing to senescence. In response to DNA double-strand breaks the ataxia-telangiectasia mutated (ATM) kinase phophorylates γ-H2AX and p53 at Ser15 (Menolfi et al. 2020). We have shown that R2-viniferin triggered γ -H2AX phosphorylation and activation of p53, as

well as induced transcription of p21. In the previous study, we detected a cycle arrest at G2/M in cells treated with R2-viniferin (Aja et al., 2020). It should be considered that ATM-mediated phosphorylation of p53 and Chk2 leads to a series of cell cycle G1 and G2 checkpoints, preventing cell cycle progression and thus avoiding genomic instability after DNA damage (Matsuoka et al. 2000). A similar mechanism of toxicity could be the case of R2-viniferin in HepG2.

The Bcl-2 promotor contains a p53-negative response element (Seto et al. 1992), raising the possibility that Bcl-2 may be a direct target of p53-mediated transrepression (Wu et al. 2001). In this work, we have shown that p53 transfection in Hep3B rendered the cells more responsive to R2-viniferin and, by contrast, p53 silencing in HepG2 triggered resistance to the oligostilbene. The polyphenol treatment activated p53, as determined by Ser^{15} phosphorylation and p21 upregulation. These changes were accompanied by a highly significant decrease in Bcl-2 expression, rendering a Bax/Bcl-2 ratio more prone to apoptosis. We detected the activation of caspase-9, as shown by the procaspase-9 cleavage. p53 may trigger apoptosis through both mitochondrial and death-receptor pathways (Henry et al. 2012). We were unable to detect caspase-8 activity, so our results points to a p53-related activation of the intrinsic apoptotic pathway.

Activation of MLKL, a biochemical marker specific for necroptosis, is associated with LDH release presumably caused by cell membrane permeabilization. At the plasma membrane, phospho-MLKL sequesters the phosphoinositides, phosphatidylinositol 4,5-bisphosphate (PInsP2) and phosphatidylinositol (3,4,5)-trisphosphate (PInsP3), during assembly of a pore-forming cytotoxic complex. This precludes access to and activation of phosphoinositide-3 kinase, which may account for the reduced phosphorylation of Akt (Dondelinger et al. 2014). We have actually detected a decline in the phosphorylation status of Akt as a consequence of R2-viniferin treatment. However, this loss of pro-survival signaling was not accompanied by an

increased in MLKL phosphorylation. R2-viniferin prompted LDH release into the medium, pointing to a class of necrotic death, which would not be associated to necroptosis.

The early ROS accumulation induced by R2-viniferin previously reported (Aja et al., 2020) could be due to an overproduction of O_2^- that was converted into the H_2O_2 signaling molecule. In fact, in this work we demonstrated specifically the increase of the intracellular levels of H_2O_2 and a remarkable upregulation of the mitochondrial Mn-SOD enzyme, which would be responsible of the imbalance of H_2O_2 levels. As already suggested for the antitumor drug paclitaxel in human lung cancer cells, the early accumulation of mitochondrial H_2O_2 is a crucial event in the induced toxicity (Alexander et al., 2006), and this could be the case of R2-viniferin. Despite the recognized antioxidant properties both *in vitro* and *in vivo* models, polyphenols induce apoptosis increasing ROS production in different cancer cell lines, but not in normal cells (Hadi et al. 2010; Khan et al. 2014; Liu et al. 2019; Aja et al., 2020). This dual behavior is attributed in part to the pro-oxidant properties of polyphenols in the presence of transition metals, specifically iron and copper. Various reports indicate that elevated intracellular iron and copper levels is a hallmark in cancerous cells (Gupte et al. 2008; Ebara et al. 2000; Yoshida et al. 1993). The presence of high levels of these metals together with a higher production of H_2O_2 leads to Fenton reaction and lipid peroxidation. This will eventually cause cell death through ferroptosis, a type of programmed necrotic death (Dixon et al. 2019). Although not addressed in this work, it is conceivable that R2-viniferin, through the increased production of hydrogen peroxide, could trigger the activation of this programmed cell death.

In summary, we have shown that R2-viniferin exerts cytotoxic actions in human hepatoma HepG2 cells by a p53-dependent mechanism. It involves DNA damage, the phosphorylation and activation of p53 and the induction of p21 expression. The oligostilbene also causes induced apoptosis through caspase-9

activation, an increase in the intracellular H_2O_2 levels, and the upregulation of mitochondrial MnSOD. PI3K/Akt and ERK1/2 pathways are suggested to modulate R2-viniferin-induced apoptosis. The effects exerted by the oligostilbene leads eventually to a permanent inhibition of clonogenicity and migration. The proposed mechanism of action exhibited by R2-viniferin is outlined in **Figure 14.** The exact mechanism exerted by this novel stilbene represents an important advance in research for the development of this natural compound in chemotherapies against p53-positive human hepatocellular carcinomas.

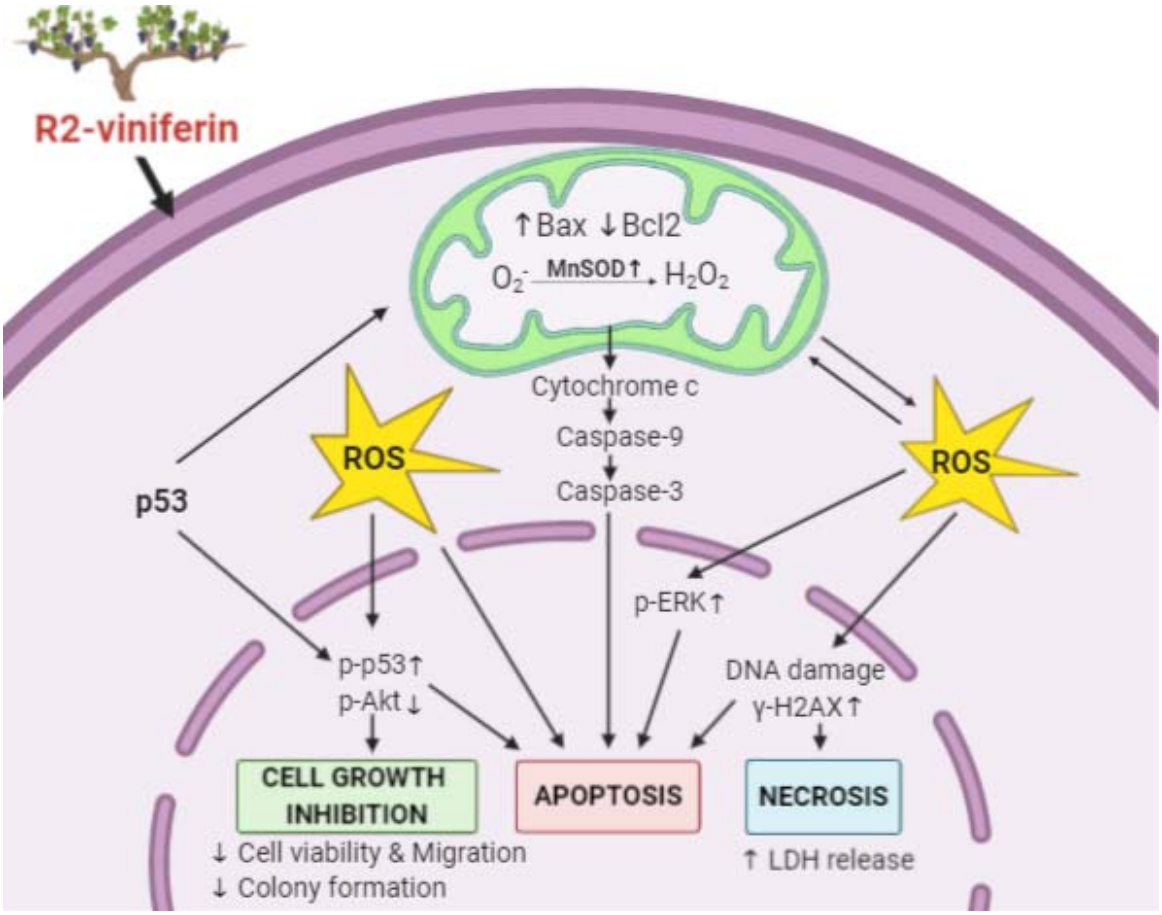

Figure 14. Proposed mechanism of toxicity exerted by R2-viniferin in HepG2.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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CONCLUSIONS

CONCLUSIONS

1. The woody parts (roots) of the *Vitis vinifera* grapevine contain the glucosylated derivatives of resveratrol: resveratroloside, resveratrol rutinoside, and *trans*- and *cis*-ε-viniferin diglucosides, which show moderate/low biological activity.

2. The piceatannol monomer and the resveratrol oligomers, ε–viniferin, hopeaphenol and isohopeaphenol, inhibit the inflammatory response to lipopolysacharide-stimulated murine macrophages, decreasing the levels of nitric oxide, reactive oxygen species, and the pro-inflammatory TNF-α and IL-1β cytokines.

3. From several natural resveratrol oligomers isolated from *Vitis vinifera* cane (ampelopsin A, *trans*-ε-viniferin, hopeaphenol, isohopeaphenol, R2-viniferin, and R-viniferin), the R2-viniferin tetramer is the most potent cytotoxic stilbene in inducing cell death of human HepG2 hepatoma cell line, without damaging non-transformed human hepatocyes.

4. R2-viniferin exerts cytotoxic actions in human hepatoma HepG2 cells by a p53-dependent mechanism. It involves DNA damage, the phosphorylation and activation of p53 and the induction of p21 expression. The oligostilbene also causes apoptosis through caspase-9 activation, an increase in the intracellular H_2O_2 levels, and the upregulation of mitochondrial MnSOD.

From the results, we conclude that *Vitis vinifera* cane is a source of R2-viniferin oligostilbene, a promising natural compound in the development of chemotherapies against p53-positive human hepatocellular carcinomas. Further studies will be required in order to improve its bioavailability and to unravel its mechanism of action both *in vitro* and *vivo* models for its potential clinical application in the treatment of liver cancer.