CHARACTERIZATION OF THE BIOLOGICAL EFFECTS OF NATURAL COMPOUNDS AGAINST INFLAMMATION, METABOLIC SYNDROME AND CANCER

Sara Tomás Hernández

UNIVERSITAT ROVIRA i VIRGILI

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SARAH TOMÁS HERNÁNDEZ

UNIVERSITAT ROVIRA I VIRGILI | DOCTORAL THESIS
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Characterization of the biological effects of natural compounds against inflammation, metabolic syndrome and cancer

Doctoral Thesis

Thesis supervised by Dr. Miquel Mulero Abellán and Dr. Santiago García Vallvé

Cheminformatics & Nutrition Research Group

Biochemistry & Biotechnology Department

UNIVERSITAT ROVIRA i VIRGILI

Tarragona 2017

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FEM CONSTAR que aquest treball, titulat "Characterization of the biological effects of natural compounds against inflammation, metabolic syndrome and cancer", que presenta Sarah Tomás Hernández per a l'obtenció del títol de Doctora, ha estat realitzat sota la nostra direcció al Departament de Bioquímica i Biotecnologia d'aquesta universitat i que compleix els requisits per poder optar a la menció internacional de doctorat.

HACEMOS CONSTAR que el presente trabajo, titulado "Characterization of the biological effects of natural compounds against inflammation, metabolic syndrome and cancer", que presenta Sarah Tomás Hernández para la obtención del título de Doctora, ha sido realizado bajo nuestra dirección en el Departamento de Bioquímica y Biotecnología de esta universidad y que cumple los requisitos para poder optar a la mención internacional de doctorado.

WE STATE that the present study, entitled "Characterization of the biological effects of natural compounds against inflammation, metabolic syndrome and cancer", presented by Sarah Tomás Hernández for the award of the degree of Doctor, has been carried out under our supervision at the Department of Biochemistry and Biotechnology of this university and that this thesis is eligible to apply for the international doctoral mention.

Tarragona, 26-6-2017

Els directors de la tesi doctoral

Miquel Mulero Abellán

Santiago García-Vallvé

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Als meus pares

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"La vida és un enigma que ens supera, ple de perspectives infinites; només cal mirar al cel o a través d'un microscopi per a adonar-se'n"

Manuel Baixauli

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Preface

This PhD work has been done in the Cheminformatics and Nutrition Research Group from the Biochemistry and Biotechnology department of the Universitat Rovira i Virgili. This group is quite characteristic due to the mixed expertise of its members. Half of them are chemists and are experts on the bioinformatic tools development and its applications. They are able to develop new *in silico* tools for target fishing and virtual screening, and they are also able to perform virtual screening assays and predict which molecules are more valuable due to its probability to interact with a selected protein target. On the other hand, the other part of the researchers of this group is composed by biologists and biochemists with long experience in the experimental work, both *in vitro* and *in vivo,* in several experimental fields such as metabolic pathologies, cancer, and neuroscience. As consequence, there are several ongoing experimental projects that are characterized by its "methodological nature". Hence, there are projects that are only based on an *in silico* approach and others that are characterized by its *in vitro* and *in vivo* techniques. Finally, there are projects that combine both abilities of the Cheminformatics and Nutrition research group; developing in consequence mixed works that are based on the combination of approaches (*in silico + in vitro +in vivo*).

Thus, this thesis reflects such work in this above mentioned context and has been done under the influence of several ongoing research projects. In this sense, this doctoral thesis is not focus on just one pathology or target since it is intended to cover some of the major diseases affecting worldwide such as metabolic syndrome, neurodegenerative diseases and cancer. This is the reason why this work is divided in three independent chapters addressed to different medical conditions but always aimed to the same purpose: the characterization of natural compound that could be useful for the treatment or prevention of such important diseases. Besides this doctoral thesis completely fulfils all the stages of any PhD process (scientific problem-hypothesis-results-conclusions).

Therefore, I have hardly worked with several target proteins (IKK-2, PPAR-γ, m-TOR, p70S6K) that have been previously or simultaneously worked from an *in silico* point of view. So, I have characterized the molecular effect of some of the novel predicted natural compounds (i.e. IKK2 inhibitors); but on the other hand I have also studied the modulation of the molecular pathways (i.e. apoptosis, ER-stress and autophagy) that are influenced by such natural compounds. Additionally, I have also established novel methodological methods to study such natural compounds effects (i.e. PPAR-γ CDK5 phosphorylation assay, microglial primary cell culture).

Finally, as a result of this PhD work I have written and published several research papers that will help to continue with the research of the group and will act as a "meeting point" of the *in silico* and *in vitro* fields. Furthermore, I humbly think that all my extensive work will reinforce the particular research identity of the Cheminformatics and Nutrition Research Group

Sarah Tomás-Hernández

June 2017

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[Summary]

Natural products (NPs) have been used for the treatment and prevention of many diseases and illnesses since early human history. The main goal of this doctoral thesis is to characterize selected bioactive natural compounds that could be used for the prevention or treatment of some major significant diseases, such as metabolic syndrome, neurodegenerative diseases and cancer. These medical conditions constitute an important health problem worldwide since they are included in the world leading causes of death.

Since numerous studies have shown that the chronic inflammation process is directly involved in the onset of metabolic syndrome and neurodegenerative diseases, we aimed to identify NP that could mitigate this harmful persistent inflammation state. In that sense, we investigated if the natural compound *o*-oresllinaldehyde might modulate the inflammatory response by acting as IKK-2 inhibitor. Our results confirm that *o*-orsellinaldehyde possesses strong anti-inflammatory properties suggesting that it may be a potential preventive or therapeutic candidate for the treatment of pathologies that deal with chronic inflammation, such as metabolic syndrome and neurodegenerative diseases.

Then, we examined the anti-diabetic effects of another natural compound kwon as 2,4,6-Trimethoxybenzophenone. Concretely, we analyzed the effect of this novel selective PPARγ modulator (SPPARγM) on the Cdk5-mediated phosphorylation of PPARγ as well as its influence on adipogenesis. Our data demonstrated that 2,4,6-Trimethoxybenzophenone would retain the benefits of improving insulin resistance since its able to inhibit Cdk5-meditated PPARγ phosphorylation at ser273, but minimizes the common side effects of existent drugs, such as adipogenesis, by alleviating PPARγ agonistic activity.

The last part of this thesis aimed to clarify the combined effect of the two natural compounds quercetin and resveratrol, on the autophagic process in HepG2 cancer cells, concluding that resveratrol potently counteracts quercetin starvation-induced autophagy and sensitizes HepG2 cancer cells to apoptosis.

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[Resum]

Els productes naturals han sigut àmpliament utilitzats per al tractament i prevenció de moltes malalties. L'objectiu principal d'aquesta tesi és caracteritzar compostos naturals bioactius que podrien ser utilitzats per a la prevenció o tractament d'algunes malalties rellevants com ara la síndrome metabòlica, les malalties neurodegeneratives i el càncer. Aquestes condicions mèdiques representen un problema de salut important ja constitueixen les principals causes de mort a nivell mundial.

Atès que nombrosos estudis han demostrat que el procés d'inflamació crònica està directament involucrat en l'aparició de la síndrome metabòlica i les malalties neurodegeneratives, hem intentat identificar compostos naturals que puguen mitigar aquest persistent i nociu estat d'inflamació. En aquest sentit, vam investigar si el compost natural *o*-orsellinaldehid podria modular la resposta inflamatòria actuant com a inhibidor d'IKK-2. Els nostres resultats confirmen que l'*o*-orsellinaldehid posseeix propietats antiinflamatòries potents suggerint que podria tractar-se d'un possible candidat preventiu o terapèutic per al tractament de patologies relacionades amb processos d'inflamació crònica com són la síndrome metabòlica i les malalties neurodegeneratives.

Posteriorment, estudiàrem els efectes antidiabètics d'un altre compost natural, la 2,4,6-Trimethoxibenzofenona. Concretament, vam analitzar els efectes d'aquest nou modulador selectiu de PPARγ en la inhibició de la fosforilació de la Ser273 de PPARγ duta a terme per l'enzim Cdk5, així com la seua influència en l'adipogènesi. Les nostres dades demostren que la 2,4,6-trimetoxibenzofenona podria millorar la resistència a la insulina ja que és capaç d'inhibir la fosforilació de PPARγ, i minimitza els efectes secundaris habituals dels fàrmacs existents, com l'adipogènesi, degut a la seua baixa activitat agonística.

L'última part d'aquesta tesi va tenir com a objectiu estudiar l'efecte combinat de la quercetina i el resveratrol sobre el procés autofàgic en cèl·lules HepG2, concloent que el resveratrol contraresta potentment l'autofàgia induïda per la quercetina sota una situació de restricció calòrica i sensibilitza les cèl·lules canceroses a l'apoptòsi.

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[Resumen]

Los productos naturales han sido ampliamente utilizados para el tratamiento y prevención de muchas enfermedades. El objetivo principal de esta tesis es caracterizar compuestos naturales bioactivos que podrían utilizarse para la prevención o tratamiento de algunas enfermedades relevantes como son el síndrome metabólico, las enfermedades neurodegenerativas y el cáncer. Estas condiciones médicas representan un problema de salud importante ya que constituyen las principales causas de muerte a nivel mundial.

Dado que numerosos estudios han demostrado que la inflamación crónica está directamente involucrada en la aparición del síndrome metabólico y las enfermedades neurodegenerativas, hemos intentado identificar compuestos naturales que puedan atenuar este persistente y nocivo estado de inflamación. Por ello, investigamos si el compuesto natural *o*-orsellinaldehido podría modular la respuesta inflamatoria actuando como inhibidor de IKK-2. Nuestros resultados confirman que el *o*-orsellinaldehido posee propiedades antiinflamatorias potentes sugiriendo que podría tratarse de un posible candidato preventivo o terapéutico para el tratamiento de patologías relacionadas con procesos de inflamación crónica como el síndrome metabólico y las enfermedades neurodegenerativas.

Posteriormente, estudiamos los efectos antidiabéticos de otro compuesto natural, la 2,4,6-Trimethoxibenzofenona. Analizamos los efectos de este nuevo modulador selectivo de PPARγ en la inhibición de la fosforilación de la Ser273 de PPARγ mediada por la enzima CdK5, así como su influencia en la adipogénesis. Nuestros datos demuestran que la 2,4,6-trimetoxibenzofenona podría mejorar la resistencia a la insulina ya que es capaz de inhibir dicha fosforilación, y minimiza los efectos secundarios habituales de los fármacos existentes, como la adipogénesis, debido a la su baja actividad agonística.

La última parte de la tesis tuvo como objetivo estudiar el efecto combinado de la quercetina y el resveratrol sobre el proceso autofágico en células HepG2, concluyendo que el Resveratrol contrarresta potentemente la autofagia inducida por la quercetina bajo una situación de restricción calórica y sensibiliza las cáncerosas a la apoptosis.

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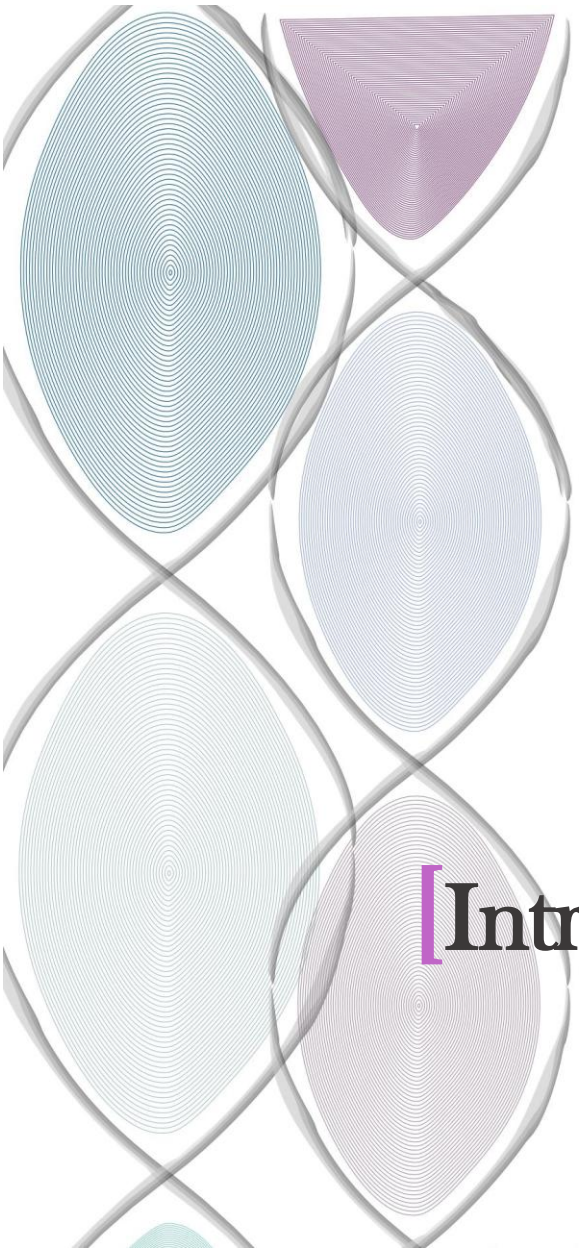
[List of Abbreviations]

AD	Alzheimer's disease
AMPK	AMP-activated protein kinase
Arg-1	Arginase 1
CADD	Computer-aided drug design
CMA	Chaperone-mediated autophagy
COX	Cyclooxygenase
CR	Caloric restriction
CRP	C-reactive protein
CVD	Cardiovascular disease
DM	Diabetes mellitus
DPP-4	Dipeptidyl Peptidase-4
eNOS	Endothelial nitric oxide synthase
FoxO	Forkhead box O
IDE	Insulin-degrading enzyme
IDF	International diabetes federation
IKK	IκB kinase
IL-4	Interleukin 4
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
IRS-1	Insulin receptor substrate-1
JNK	c-Jun N-terminal kinase
LAMP-2A	Lysosome-associated membrane protein type 2A
LBD	Ligand binding domain
LO	Lipoxygenase
LPS	Lipopolysaccharide
MetS	Metabolic syndrome

[LIST OF ABBREVIATIONS]

mTOR	mammalian target of rapamycin
MTT	3-(4,5 dimethyl thiazol-2yl)-2,5 diphenyl tetrazolium
NCEs	New chemical entities
NF-kB	Nuclear Factor Kappa Beta
NP	Natural products
PD	Parkinson's disease
PPAR- γ	Peroxisome proliferator activated receptor γ
PPREs	Peroxisome proliferator response elements
PTP1B	Protein tyrosine phosphatase 1B
QCT	Quercetin
RSV	Resveratrol
RXR	Retinoid X receptor
SPPARγM	Selective PPARγ modulators
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TZD	Thiazolidinedione
VS	Virtual Screening



[Introduction]

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1. NATURAL PRODUCTS AS SOURCE OF NEW DRUGS

Throughout the ages, natural products (NP) have been used for the treatment of many diseases and illnesses. Humans have relied on nature for the cure and prevention of a wide spectrum of diseases using traditional medicines, remedies, potions and oils with bioactive natural compounds. Many of these natural molecules have gone on to become current drugs [1–3].

Plants, in particular, have formed the basis of traditional medicine. The earliest records on medicinal applications of plants date back to 2600 bc, documentating the uses of approximately 1000 plant-derived substances in Mesopotamia [4]. Since then, numerous documents reported the use of natural products in the Egyptian, Chinese, Greek, Roman and Arabic medicine [5–7].

Until then, the use of medicinal plants was based on empirical evidences, without knowing anything about the pharmacological activities or its bioactive components. The basis for the rational clinical investigation of medicinal herbs is considered to have begun in 1785, when Withering introduced an extract of the foxglove plant for the treatment of edema [8]. Moreover, the first evidence of rational drug discovery can be attributed to Friedrich Sertürner. At the beginning of the 19th century, Sertürner was able to isolate a sleep-inducing and analgesic molecule from the opium plant, naming this compound morphium (morphine) after the Greek god of dreams, Morpheus. He published an extensive paper explaining its research and focusing on the pharmacological properties of the molecule that he had isolated [9]. From that moment, the concept of pure compound as drug emerged and several other bioactive compounds were isolated from medicinal herbs such as nicotine, codein, capsacine, cocaine, etc. The first commercial pure natural product introduced for therapeutic use is morphine marketed by Merck in 1826[10], and the first semi-synthetic pure drug, aspirin, based on a natural product salicin isolated from Salix alba, was introduced by Bayer in 1899[10].

Since then, natural products have continued to be a very important source for the development of new drugs [11]. It has been reported that by the end of 2015, of all the new chemical entities (NCEs) approved by the FDA, 18.4 % were NPs or semisynthetic NP derivatives. More concretely, a recent review that analyses all the NCEs approved worldwide from 1981-2014 revealed that 73% of antibacterial NCEs approved in this timeframe were NP or NP derivatives. These review also states that 75% of all anticancer drugs were NP based [12].

1.1 Biodiversity and complexity of natural products

NP have been isolated and characterized from a wide range of organisms. Although plants are still the main source for NP derived drugs, there are also other organisms with great importance on this field. Microorganisms, mainly bacteria and fungi, are a potent source of bioactive substances and had been widely used in traditional medicine as well as for the isolation of natural compounds with beneficial properties. Furthermore, despite the fact that marine organisms do not have a history of use in traditional medicine and are large unexplored [13] in the last 30 years this experimental field has significantly evolved. Since the beginning of the 1970s until now, marine environment has revealed to possess several novel bioactive compounds with unique structures not seen in terrestrial sources [14]. This great diversity of source material for natural products derived drugs is increasing with the study of unexplored areas like extreme ecosystems and deep oceans [15].

A consequence of the interaction of this wide variety of organisms among them and with their environment is the emergence of complex natural chemicals in these organisms that promote their survival [16]. This means that natural products isolated from them do not need further modifications since they have been already optimized through evolution, and due to its selectivity they have great chance for interacting specifically with biological target molecules.

1.2 Identification of new bioactive molecules. Computer aided drug design.

Due to the enormous variety of biocompounds above mentioned there are literally millions of natural chemical structures that could be candidates for pharmaceutical research. Thousands of chemicals must be evaluated and tested to find a proper hit against one specific target.

Unfortunately, bioactivity screening in extracts exclusively by *in vitro* or *in vivo* approaches is a complex and expensive process which is difficult to afford [17].In that sense, computer-aided drug design (CADD) methodologies like Virtual Screening (VS) have been successfully used to screen large NP databases in order to identify new bioactive molecules for specific targets [18,19]. The goal of VS techniques is to identify/predict the most promising candidates to afterwards focus the experimental efforts on them, by eliminating molecules that do not possess the required features. This kind of techniques play an essential role in lowering significantly the R&D expenses associated to the bioactive molecules identification. There are a lot of successful examples on the use of VS techniques in the discovery of bioactive compounds [20–22].

1.3 Functional Foods

As a working definition, a food can be regarded as functional if it is satisfactorily demonstrated that beneficially affects one or more target functions in the body, beyond adequate nutritional effects. This influence must be achieve in a way which is relevant to either the maintenance or promotion of a state of well-being of health, or the reduction of the risk of a pathologic process or a disease [23]. However, there is still controversy about the definition. The first step in the development of a functional food is the identification of a specific interaction between one or a few components of the food and a function in the organism that is potentially beneficial for the health [23]. Frequently, this healthy interaction is the result of a NP acting as a functional food component.

A significant difference between pharmaceutical industry and food industry lies in the kind of molecules that are involved. Thus, while any molecule is likely to have interest as a drug, when talking about functional food, the repertoire of molecules to be screened is mostly limited to those found in nature. Although natural products have been widely used as sources of new drugs[24,25], their use in functional food sector is more restricted. Therefore, as mentioned above, VS techniques can be very useful for the identification of undescribed bioactivities for known natural molecules, and, subsequently increase the repertory of ingredients that can be used in functional food development.

Moreover, the regulation 1924/2006 of the European Union states that only can be marketed as functional foods those foods that have been scientifically proven to have a positive effect on health[26]. In that sense, after bioinformatics prediction of the functional food component a further experimental validation is needed, generally by *in vitro* and *in vivo* techniques.

1.4. Development of new functional foods: Targeting the leading causes of death worldwide.

With the aim of developing new functional foods of great interest to the society it is necessary to first know which are the most prevalent diseases worldwide in order to direct scientific efforts towards a research activity focused on finding solutions to these important health problems.

According to World Health Organization, of the 56.4 million deaths worldwide in 2015, more than half were due to the top 10 causes being cancer, isquemic heart disease and stroke the three leading causes of death globally. Lower respiratory infections remained in the 4th position, causing 3.2 million deaths worldwide in 2015. These was followed by chronic obstructive pulmonary disease resulted in 3.2 million deaths in 2015. Diabetes harmed 1.6 million people in 2015, making it the 6th leading cause of global deaths in this year. Deaths due to dementias more than doubled between 2005 and 2015. The death

rate from diarrhoeal diseases significantly decreased since 2000 but still killed 1.4 million people in 2015. Similarly, despite tuberculosis prevalence also has been reduced it still caused 1.4 million deaths. HIV/AIDS is no longer among the world's top 10 causes of death, killing 1.1 million people in 2015 compared with 1.5 million in 2000 (Figure 1) [27,28].

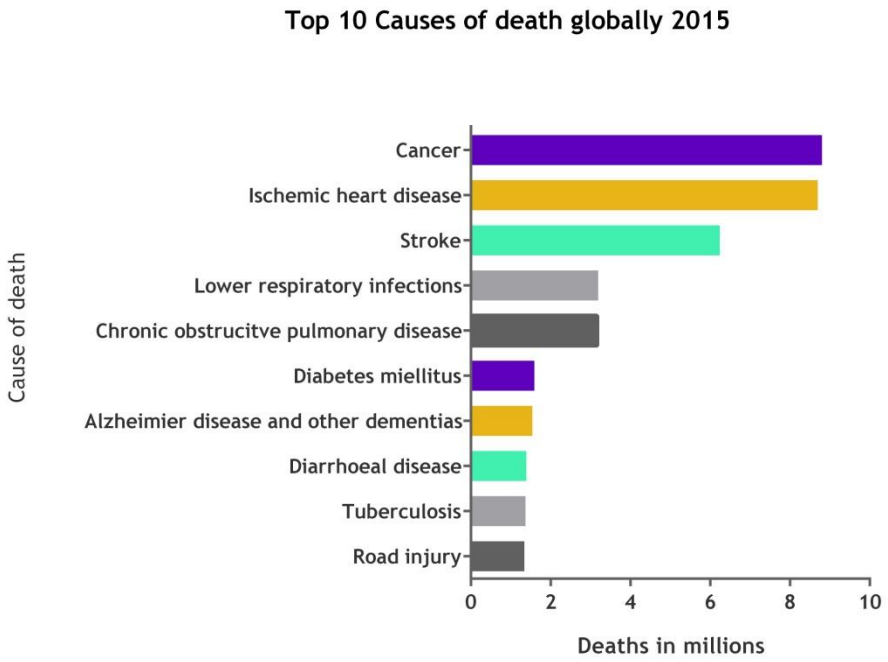


Figure 1.Top 10 Causes of death globally 2015. Adapted from The World Health Organization [27,28]

As we will explain later, the main goal of this doctoral thesis is the characterization of natural compounds that could serve for the prevention or treatment of some major significant diseases, such as metabolic syndrome, neurodegenerative diseases and cancer. In that sense, before starting to explain how this project was carried out it is necessary to better understand the main characteristics of this important conditions. Thus, in the following section we are going to get an overview of the mechanisms involved in metabolic syndrome,

neurodegenerative diseases and cancer, the main pathologies in which we have focused this doctoral thesis on.

1.4.1 Metabolic syndrome

Metabolic syndrome (MetS) is the name for a cluster of medical disorders that increase the risk of developing cardiovascular disease and type 2 diabetes mellitus (T2DM). Insulin resistance, hypertension, atherogenic dyslipidemia, elevated blood pressure and endothelial dysfunction are the main factors which constitute the syndrome[29–31].

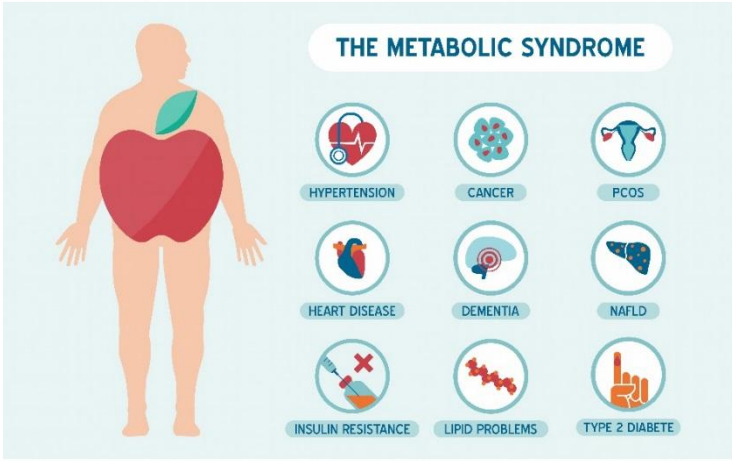


Figure 2. Metabolic Syndrome related medical conditions.

Regarding its prevalence, the international diabetes federation (IDF) estimates that one-quarter of the world's adults population suffers from MetS, and it has been considered the pandemic of the 21st century[32].

Central obesity is the centerpiece of the metabolic alterations associated to the MetS. Concretely, the appearance of the MetS phonotype is manly triggered by weight gain, concretely an increase in intra-abdominal fat accumulation [33,34]. Obesity is a direct consequence of current lifestyle trends which are characterized by abundant caloric consumption and reduced physical

activity[35]. This nutritional excess together with a reduced energy expenditure results in a disruption in the systemic metabolic regulation pathways. As we will latter discuss, inflammation is entirely associated to obesity, and insulin resistance seems to be intimately related to MetS.

MetS is associated with a three to five fold increased risk for the development of type 2 diabetes mellitus, and a two to four fold increased risk of suffering stroke. Moreover MetS doubles the risk of developing cardiovascular disease(CVD)[29].

If we come back to the previous section which described the worldwide leading causes of death we can remark that stroke and T2DM are two of the ten diseases included into the list.

Thus, bearing in mind this alarming data and the high prevalence of MetS it is easy to understand why MetS has become a significant public health problem and a clinical challenge worldwide.

1.4.2 Neurodegenerative diseases

Neurodegeneration refers to a progressive dysfunction and loss of neurons and axons in the central nervous system. Most neurodegenerative diseases share a common pathogenic mechanisms involving aggregation and deposition of misfolded proteins, which leads to progressive central nervous system disease[36].

The molecular bases underlying the pathogenesis of the neurodegenerative diseases are gradually being elucidated. Increasing evidences reveal that mitochondrial alterations, oxidative and nitrative processes, accumulation of defective and misfolded proteins, excitotoxicity events and inflammatory response play a central role in the onset of many neurodenerative disorders [37–40].

Since brain cells are rarely replaced, neurodegenerative diseases are incurable and debilitating conditions that results in a progressive loss of brain and body function for the rest of the patient's life.

Age is the largest risk factor for the development and progression of most of these neurodegenerative diseases. Consequently, the epidemic of these diseases will continue rising as life expectancy also increases. It can be predicted that, over next generations, the proportion of elderly people will almost double, that means that the number of people suffering this kind of diseases will also increase. In that sense, a better understanding of these illnesses is crucial in order to develop more effective therapies to combat the personal, social and economic costs of these diseases [41].

Among all the existent neurodegenerative diseases attention has largely focused on a handful, such as Alzheimer and Parkinson disease.

- **Alzheimer's Disease**

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder worldwide and the most common cause of dementia [42,43]. According to the World Alzheimer Report of 2016, over 46 million people live with dementia and it is estimated to increase to 131.5 million by 2050 [44]. Moreover, as previously commented, AD is one of the leading causes of death worldwide. Thus, AD represents an increasing challenge to public health.

The onset of this diseases is mainly defined by impaired ability to recall recent events, but as the condition progresses other intellectual and physical skills decline and a loss of control over body functions occur [45].

In AD, neurons of the cerebral cortex and hippocampus are selectively lost. The hallmark of this disease is, the progressive accumulation of the protein fragment beta-amyloid outside neurons and twisted strands of protein Tau inside neurons. Thus triggering the neuronal death above commented [46].

- **Parkinson's Disease**

Parkinson's disease (PD) is the second most common progressive neurodegenerative disorder. Approximately 1% of the population older than 65 years suffers from this condition [47].

Parkinson disease is associated with both, motor and non-motor symptoms. Patiens with PD manifest rest tremor, rigidity, bradykinesia and stooping posture. In addition they can also display neurobehavioral disorders such as depression, anxiety, dementia, autonomic dysfunction and visual hallucination [48].

The symptoms of PD are caused by selective and progressive degeneration of pigmented dopaminergic neurons in the substantia nigra pars compacta. Another important feature of PD is the presence of intracellular inclusions containing aggregates of misfolded proteins termed Lewy bodies[49].

Although the causes of PD remains unclear, several reports suggest that this disease may arise from an interaction between genetic and environmental factors that leads to progressive degeneration of neurons in specific regions of the brain[50]

Moreover, in the same way that in AD, inflammation and excitotoxicity play also a central role in PD [51].

In the last few decades, several studies have focus on the development of medical pharmacologic therapies aimed to improve the symptoms caused by this disorder, however, definitive disease-modifying therapy is still lacking. In that sense, further scientific knowledge is crucial to promote better quality of life for PD patients.

1.4.3 Cancer

Cancer is a leading cause of death worldwide, and it was responsible for 8.8 million deaths in 2015. Globally, nearly 1 in 6 deaths is due to cancer [28]. It is estimated that by 2030 there will be 23.6 million new cases of cancer [44].

Cancer is a complex group of diseases mainly characterized by the uncontrolled growth and spread of abnormal cells [52]. This disease arises from the transformation of normal cells into tumor cells as a result of the interaction between a person's genetic factors and several external agents. This external agents include physical carcinogens (such as ionizing and UV radiation), chemical carcinogens (such as tobacco smoke) and biological carcinogens (such as certain viral or bacterial infections). A substantial proportion of cancer cases can be prevented: changes in lifestyle by quitting smoking or better eating habits can help to reduce the risk of developing many types of cancer. Some types of cancer cells often acquire the capacity to travel to other parts of the body and spread to other organs, this process is known as metastasis. Metastases are a major cause of death from cancer.

As normal cells evolve progressively to a neoplastic state they acquire a succession of specifically hallmark capabilities that enable them to become tumorigenic and ultimately malignant. This hallmarks characterization came from Hanahan and Weinberg who proposed six essential alterations in cancer cell physiology as a manifestation of the multiple genetic mutations that collectively determine the malignant cell phenotype. These six capabilities include: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis [53]

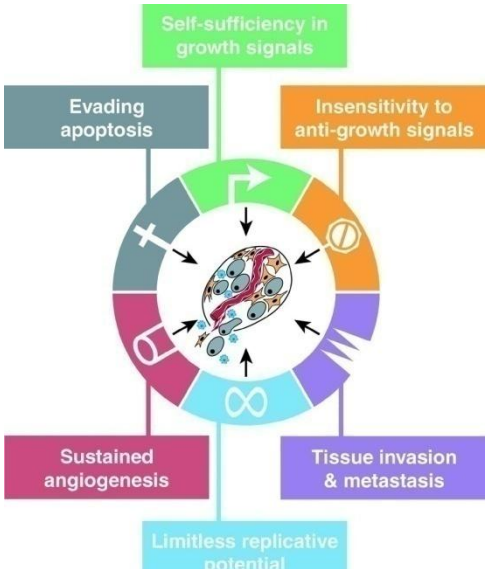


Figure 3. The hallmarks of cancer. Extracted from [53]

Regarding cancer therapy, most of the existing treatments are not effective enough to provide full protection from this disease. Cancer cells can be removed surgically or destroyed with toxic chemicals or radiation but it is very hard to eradicate all the cancer cell population. Moreover, treatments aimed to kill cancer cells are generally toxic to normal cells as well and even when those kind of treatments seems to be effective a few cancerous cells can remain and can produce a resurgence of the disease. Besides, as mentioned, metastasis events are a major added problem [52].

Hence, there is an urgent need to identify novel alternative therapies to combat this dreaded disease.

1.5. Natural products and Metabolic syndrome, neurodegenerative disease and cancer

As previously mentioned, natural products have played a significant role in promote human health and have served as a valuable source for the discovery of

new drugs. Within the past few years, scientific knowledge of biochemical pathways related to the onset of these significant diseases has expanded, thus helping research on the finding of bioactive new molecules from several sources including natural products databases.

In the following section we are going to briefly mention some of the natural products strategies that have been used for the prevention or treatment of the three major diseases covered in this thesis: metabolic syndrome, neurodegenerative diseases and cancer.

1.5.1 Natural products and Metabolic syndrome

The potential of natural products for treating MetS-related disorders is under constant scientific exploration. The use of natural compounds may be an excellent alternative strategy for developing future effective and safer anti-obesity drugs [54]. Wide ranging of NP, including crude extracts and isolated pure natural compounds, have been show to positively affect metabolic disorders through different mechanisms, such as anti-inflammatory activity, improvement of insulin sensitivity, normalization of glucose levels or improvement of blood lipid profiles [55–57]. Therefore, they have been widely used for the treatment of this medical disorders [58,59]. In most cases, the underlying molecular targets mediating this beneficial effects are not completely understood, however, there are several pathways and molecular targets that are already well established to mediate this healthy outcomes and serve as starting point for the development of this natural product based drugs. In the following table selected examples of this important targets are listed, together with their major physiological consequences and some examples of natural compounds that interact with these targets (Table 1) [60].

Molecular Target	Major Physiological Consequence	Natural Products interacting with these targets
AMPK	Activation leads among others to inhibition of fat and cholesterol synthesis, promotion of fat oxidation, enhancement of mitochondrial biogenesis, and promotion of glucose uptake in skeletal muscle and fat cells	Alkaloids, chalcones, flavonoids and other polyphenols, galegine, salicylate, terpenoids
COX-1/-2	Inhibition leads to reduced biosynthesis of pro-inflammatory prostaglandins	Alkaloids, stilbenes, flavonoids and other polyphenols, terpenoids
DPP-4	Inhibition leads to decreased incretin degradation (and thus increased insulin secretion)	Alkaloids, flavonoids and other polyphenols, polypeptides, terpenoids
eNOS	Activation leads to increased availability of anti-inflammatory nitric oxide (NO), a major antiatherogenic factor in the vasculature	Anthocyanidins, fatty acids, flavonoids and other polyphenols, ginsenosides, triterpenoic acids
NF-Kbpathway	Inhibition leads to impaired expression of pro-inflammatory mediators	Alkaloids, curcuminoids, chalcones, diterpenes, flavonoids, iridoids, naphtoquinones, salicylates, sesquiterpenelactones, stilbenes, triterpenes
PPARγ	Activation leads to insulin sensitization and normalization of blood glucose levels	Amorfrutins, diterpenequinones, flavonoids, neolignans, polyacetylenes, sesquiterpenelactones, stilbenes
PTP1B	Inhibition leads to prolonged and enhanced insulin and leptin signaling (increased insulin sensitivity and reduced food intake)	Alkaloids, bromophenols, chalcones, coumarins, diterpenes, flavonoids, lignans, *N*- or *S*-containing compounds, sesquiterpenes, sesterterpenes, steroids, triterpenes
5-LO	Inhibition leads to reduced biosynthesis of pro-inflammatory leukotrienes	Alkaloids, coumarins, depsides, quinones, flavonoids and other polyphenols, polyacetylenes, sesquiterpenes, triterpenes

Table 1.Natural Products to Counteract the Epidemic of Cardiovascular and Metabolic Disorders. Adapted from [60].

1.5.2 Natural products and neurodegenerative diseases

Although numerous studies have made important discoveries regarding the mechanism involved of these neuropathologies, there is still no definitive cure for this kind of diseases. For instance, despite intense research, there are just few synthetic drugs used for the management of AD, PD, autism and other chronic illnesses, and they possess strong side effects [61–63]. In that sense, there is an urgent need to identify and develop more effective drugs for the treatment of these disorders.

Interestingly, many NP have been used alone or in combination with other neuroprotective compounds to fight against the neurodegeneration involved in these pathologies. Several natural compounds are successfully used to improve memory and cognition in AD patients, besides, many NP have been demonstrated to be efficacious anti-Parkinson agents [64–67]. These products possess neuroprotective properties as a result of not only their well-recognized anti-oxidative and anti-inflammatory activities but also their inhibitory roles regarding iron accumulation, protein misfolding and the maintenance of proteasomal degradation, as well as mitochondrial homeostasis[62,68]. In that sense, NPs have attracted researcher's attention to study them as a potentially source for drug discovery for the treatment of several diseases that lead with neurodegeneration.

As an interesting illustrative example, figure 4 show current NP targeting different pathogenic pathways of PD [68].

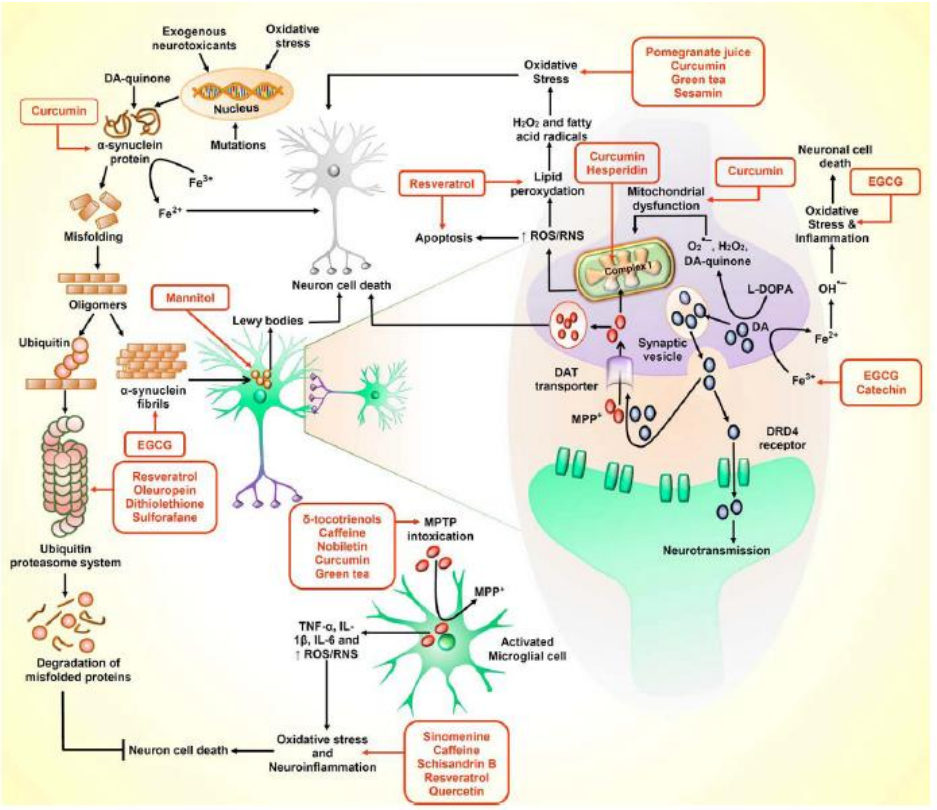


Figure 4. Natural products targeting different pathogenic pathways of Parkinson's disease. Extracted from[68].

1.5.3 Natural products and cancer

As previously mentioned, cancer is considered a major public health problem since it is one of the leading cause of death worldwide. Although there are a number of medicines in the market addressed to treat various types of cancer any drug has been found to be fully effective and safe. The major problem in cancer chemotherapy is the toxicity of the established drugs. Nevertheless, NPs have proved effectiveness and safety in the management and treatment of several cancer types. In fact, over 60 % of currently used anti-cancer drugs have been developed from natural sources [69]. As an example, herbal medicines, such as Chinese herbal products, have become very popular for cancer treatment do to

its efficacy and low toxicity [70]. In that sense, anticancer drugs derived from natural compounds or NP combined with conventional therapy may enhance anticancer therapeutic efficacy and reduce the side effects of this aggressive treatments.

The following table shows a list of many natural food items that have been found to poses some compound on its composition with anti-cancer properties. Thus, we aimed to remark the numerous cancer preventive phytochemicals present in the nature (Table 2)[71].

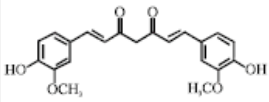
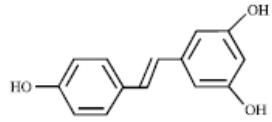
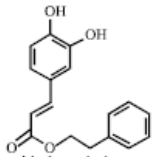
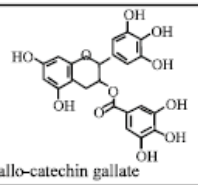
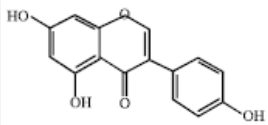
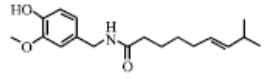
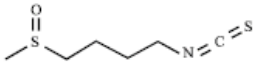
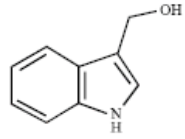
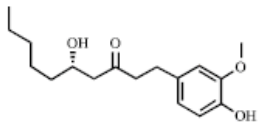
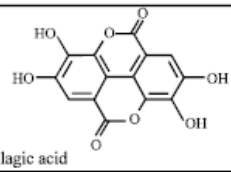
Natural source	Chemopreventive phytochemicals	Natural source	Chemopreventive phytochemicals
Turmeric 	 Curcumin	Grapes 	 Resveratrol
Honey 	 Caffeic acid phenethyl ester	Green tea 	 Epigallo-catechin gallate
Soybean 	 Genistein	Chilli pepper 	 Capsaicin
Broccoli 	 Sulphoraphane	Cabbage 	 Indole-3-carbinol
Ginger 	 [6]-Gingerol	Strawberry 	 Ellagic acid

Table 2. Cancer preventive phytochemicals. Extracted from [71].

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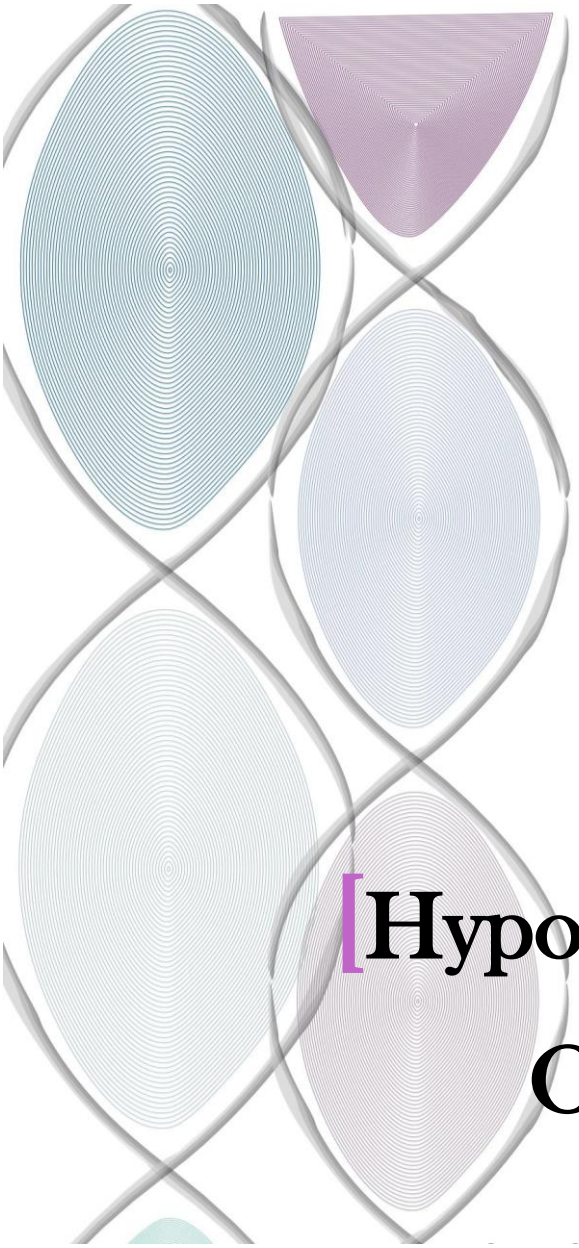


[Hypothesis and Objectives]

UNIVERSITAT ROVIRA I VIRGILI
CHARACTERIZATION OF THE BIOLOGICAL EFFECTS OF NATURAL COMPOUNDS AGAINST INFLAMMATION, METABOLIC SYNDROME AND CANCER
Sara Tomás Hernández

Natural products (NPs) have been used throughout the ages for the treatment and prevention of many diseases and illnesses. Humans have relied on nature for the cure and prevention of a wide spectrum of diseases using traditional medicines, remedies, potions and oils with bioactive natural compounds. Many of these natural molecules have gone on to become current drugs

The initial hypothesis of this PhD thesis was that, the use of natural products for modulating specific targets involved in inflammation, insulin sensitivity and autophagy processes could be a great strategy for preventing or reversing the onset of some of the most significant chronic diseases worldwide such as metabolic syndrome, neurodegenerative diseases and cancer.

Thus, the aim of this PhD thesis was to characterize the biological effects of selected natural compounds that could influence on the above mentioned molecular processes. To achieve this goal, the following specific objectives were proposed:

1. **To investigate whether the natural compound *o*-oresllinaldehyde might modulate the inflammatory and apoptotic response by acting as IKK-2 inhibitor. (Manuscript 1 and Manuscript 2)**

This main goal was divided in the following objectives:

1.1. To validate the anti-inflammatory properties that *o*-orsellinaldehyde exerts through IKK-2 inhibition in RAW246.7 cells and Balb/c mice.

1.2. To study the pro-apototic effect of *o*-orsellinaldehyde in HepG2 cancer cells.

1.3. To identify and quantify the amount of *o*-orsellinaldehyde in *Griofola frondosa* extracts in order to evaluate the feasibility of preparing useful extracts from this natural source that were enriched in *o*-orsellinaldehyde.

1.4. To investigate the neuroprotective effects of the natural compound *o*-orsellinaldehyde in murine primary glial cells.

2. To examine the anti-diabetic effects that the natural compound 2,4,6-Trimethoxybenzophenone could exert by acting as PPARγ ligand. (Manuscript 3 and Manuscript 4)

In order to asses this objective two goals were proposed:

2.1. To develop an optimal kinase assay for studying the phosphorylation of PPAR*γ*at Ser 273 and its inhibition.

2.2. To investigate the inhibitory effect of this novel PPAR*γ* ligand on the Cdk5-mediated phosphorylation of PPAR*γ*at Ser 273.

2.3. To determine the effect of 2,4,6-Trimethoxybenzophenone on adipogenesis.

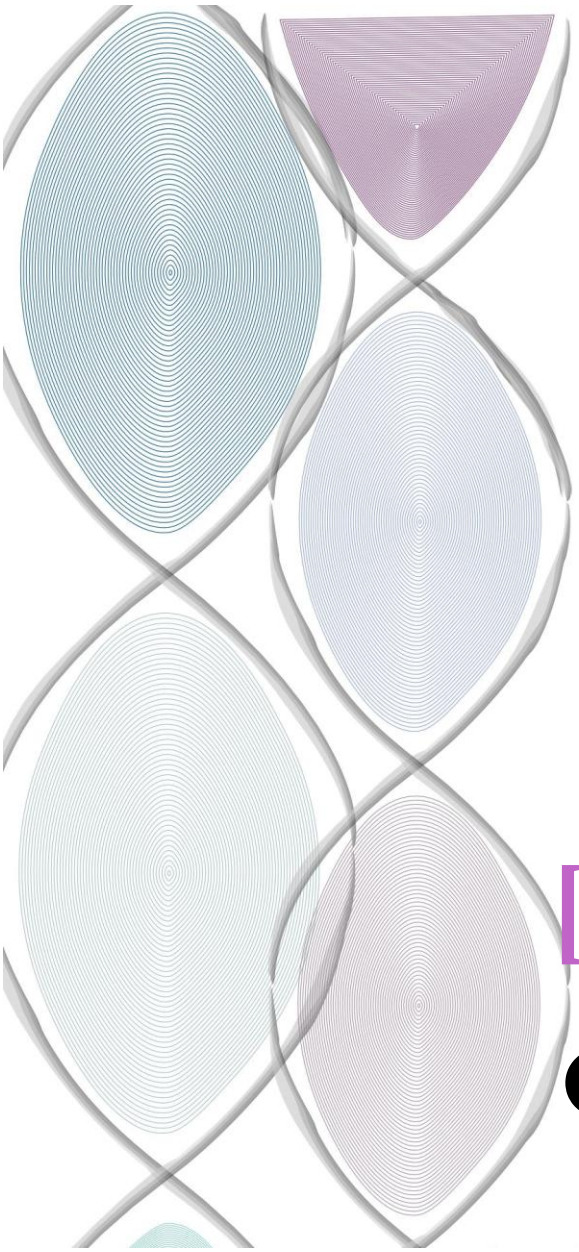
3. To study the combined effect of Quercetin and Resveratrol on the autophagic process in HepG2 cancer cells.(Manuscript 5)

This main goal was divided in the following objectives:

3.1. To establish a methodological approach to study the autophagic process

3.2. To validate the hypothetical synergistic effect of Quercetin and Resveratrol on the autophagic process.

3.3. To identify the molecular targets related to the effects exerted by these natural compounds.



[Hipòtesi i Objectius]

UNIVERSITAT ROVIRA I VIRGILI
CHARACTERIZATION OF THE BIOLOGICAL EFFECTS OF NATURAL COMPOUNDS AGAINST INFLAMMATION, METABOLIC SYNDROME AND CANCER
Sara Tomás Hernández

Els productes naturals han sigut utilitzat al llarg del temps per al tractament i prevenció de moltes malalties. L'home ha confiant en la natura per a la cura i la prevenció d'un ampli espectre de malalties utilitzant medicines tradicionals, remeis, pocions i olis que contenien compostos naturals bioactius. Moltes d'aquestes molècules naturals s'han convertit avui en dia en fàrmacs.

La hipòtesi inicial d'aquesta tesi doctoral era que l'ús de compostos naturals pera a la modulació de dianes especifiques implicades en la inflamació, la sensibilitat a insulina i els processos autofàgics podria ser una gran estratègia per prevenir o revertir l'aparició d'algunes de les malalties cròniques més significatives del món com són la síndrome metabòlica, les malalties neurodegeneratives i el càncer.

En aquest sentit, l'objectiu d'aquesta tesi ha estat caracteritzar els efectes biològics de certs compostos naturals prèviament seleccionats que podrien influir en els processos moleculars esmentats anteriorment. Per tal d'assolir aquesta fita es van proposar els següents objectius específics:

1- **Investigar si el compost natural *o*-orsellinaldehid podria modular la resposta inflamatòria i apoptòtica actuant com a inhibidor d'IKK-2. (Manuscrit 1 i Manuscrit 2).**

Aquest objectiu principal es va dividir en les següents tasques:

1.1. Validar les propietats antiinflamatòries que l'*o*-orsellinaldehid posseeix al actuar com a inhibidor d'IKK-2 en cèl·lules RAW264.7 i ratolins Balb/c.

1.2. Estudiar l'efecte pro-apoptòtic de l'*o*-orselinaldehid en la línea cel·lular cancerosa HepG2.

1.3. Identificar i quantificar la concentració d'*o*-orsellinaldehid que hi ha en extractes de *Grifola frondosa* per tal d'avaluar la viabilitat de preparar extractes útils enriquits en *o*-orsellinaldehyde a partir d'aquesta font natural.

1.4. Investigar els efectes neuroprotectors del compost natural *o*-orsellinaldeid in cultius primaris de cèl·lules glials murines.

2. Examinar els efectes antidiabètics que pot posseir el compost natural 2,4,6-trimethoxibenzofenona com a lligand de PPARγ (Manuscrit 3 and Manuscrit 4)

Per tal d'assolir aquesta fita es van proposar els següents objectius:

2.1. Desenvolupar un assaig que determine l'activitat quinasa per a estudiar la fosforilació de la Ser 273 de PPARγ així com la seua inhibició.

2.2. Investigar l'efecte inhibitori d'aquest nou lligand en la fosforilació de la Ser273 de PPARγ duta a terme per l'enzim Cdk5.

2.3. Determinar l'efecte de la 2,4,6-trimethoxibenzofenona en l'adipogènesi.

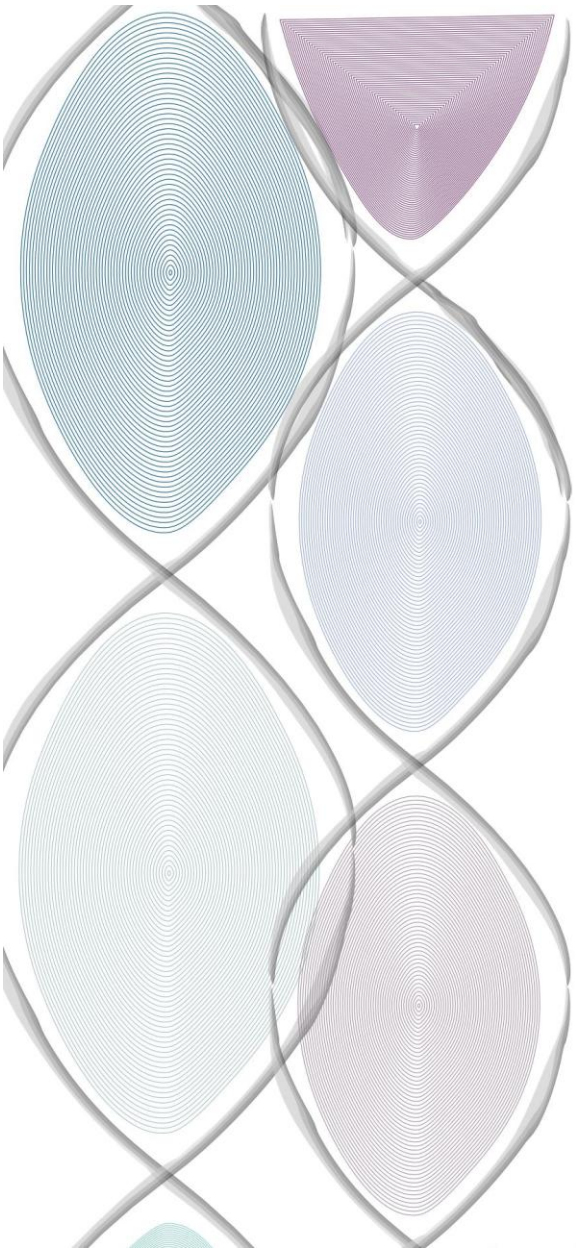
3. Estudiar l'efecte combinat de la uercetina i el resveratrol sobre els processos autofàgics en la línea cel·lular cancerosa HepG2. (Manuscrit 5)

Aquest objectiu principal es va dividir en les següents tasques:

3.1. Establir una estratègia metodològica per a estudiar els processos autofàgics.

3.2. Validar l'hipotètic efecte sinèrgic de la quercetina i el resveratrol en els processos d'autofàgia.

3.3. Identificar les dianes metabòliques involucrades en els efectes produïts per aquestos compostos naturals.



[Results]

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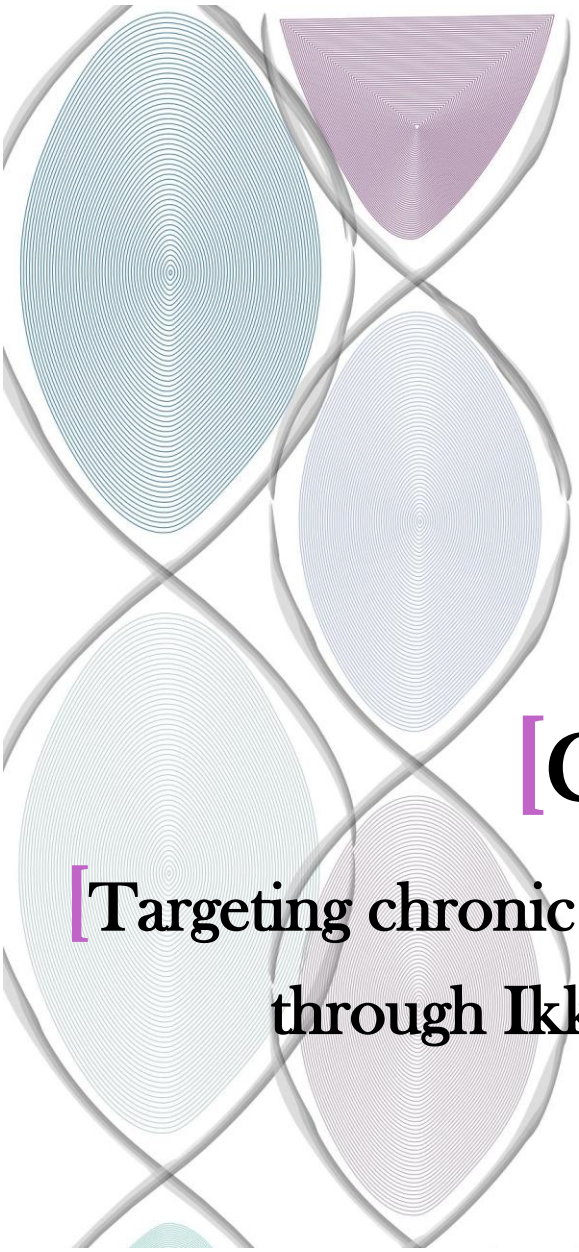
CHARACTERIZATION OF THE BIOLOGICAL EFFECTS OF NATURAL COMPOUNDS AGAINST INFLAMMATION, METABOLIC SYNDROME AND CANCER
Sara Tomás Hernández



[Chapter 1]

[Targeting chronic inflammation through Ikk-2 inhibition]

UNIVERSITAT ROVIRA I VIRGILI
CHARACTERIZATION OF THE BIOLOGICAL EFFECTS OF NATURAL COMPOUNDS AGAINST INFLAMMATION, METABOLIC SYNDROME AND CANCER
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INFLAMMATION IN METABOLIC SYNDROME AND NEURODEGENERATIVE DISEASES

Inflammation is a protective body's response intended to fight against a variety of injuries or insults such as pathogens and other harmful agents which have potential to cause damage into the organism. This short-term reaction is known as acute inflammatory response and it is very beneficial to the body due to its healing properties [1,2]. However, if this response is unnecessarily persistent or improperly focused it has harmful consequences for the body and lead to the apparition of several disorders including: atherosclerosis, asthma, diabetes, neurodegenerative diseases, cancer etc. This last type of inflammation response is known as chronic inflammation, sometimes called persistent or low-term inflammation [3–5].

In the following sections we are going to talk about the existent relation between chronic inflammations and two major medical conditions that we have previously introduced: metabolic syndrome (MetS) and neurodegenerative diseases.

1.1 Metabolic syndrome and chronic inflammation.

The connection between chronic inflammation and obesity was first proposed in 1993, with the discovery that the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) was overexpressed in the adipose tissue of obese mice [6]. Subsequent studies revealed that other inflammatory mediators such as interleukin-6 (IL-6) and C-reactive protein (CRP) were also increased in this conditions [7]. Nowadays, it is widely accepted that chronic inflammation is directly involved in the onset of MetS [8,9]. Under conditions of adiposity and nutritional excess, which are obesity hallmarks, there is a disturb in energy balance that can result in a metabolically driven, low-grade, chronic inflammatory state, known as "meta-inflammation" [10].

Changes in the inflammatory signaling by adipocytes and infiltration of adipose tissue by immune cells lead to obesity induced insulin resistance and associated metabolic disease[11,12].

It has been demonstrated that excessive levels of pro-inflammatory cytokines disrupt insulin signaling by direct serine phosphorylation of insulin receptor substrate-1 (IRS-1), and promotion of IRS degradation [13–15]. High levels of these cytokines also activate inflammatory pathways manly through via c-Jun N-terminal kinase (JNK) and NF-kB. This leads to an increase in the inflammation state that directly inhibit insulin action [16] as well as other metabolic pathways.

Figure 1 shows the inflammatory pathways involved in the development of insulin resistance [17]. Briefly, tissue macrophages activation leads to the release of cytokines which can induce insulin resistance through different pathways. Among these inflammatory molecules, TNF-α is the most studied cytokine and has been shown to promote insulin resistance. TNF-α can stimulate serine kinases, such as IKK, JNK and S6 kinase and other targets including mammalian target of rapamycin (mTOR) [16,18,19], which causes serine phosphorylation of IRS-1, reducing its ability to propagate insulin signaling.

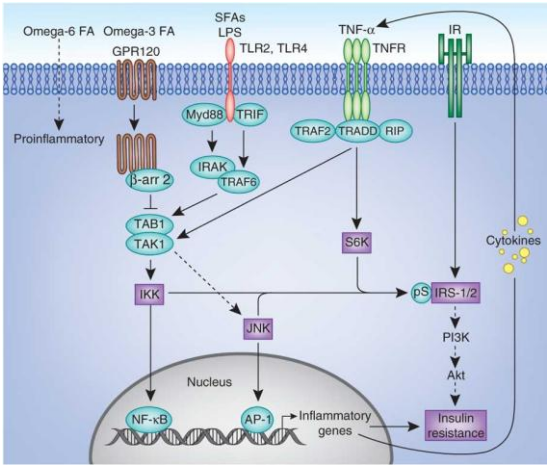

Figure 1. Inflammatory signaling pathways involved in the development of insulin resistance. Stimulation of proinflammatory signaling pathways negatively regulates insulin signaling. Extracted from [17].

Interestingly IL-1α, a cytokine highly expressed in α-cells and infiltrating macrophages, has been shown to play an important role in the atrophy of pancreatic islet function. Treatment with an IL-1 receptor antagonist significantly improved α-cells function and glycemic control in type 2 diabetes mellitus (T2DM) patients[20].

1.2 Neurodegenerative diseases and chronic inflammation

Microglia cells are the resident macrophages of the central nervous system and have pivotal roles in innate immune regulation and neuronal homeostasis. [21]. When not responding to specific major insults, microglia displays a ramified morphology and performs a very active and continuous surveillance function [22,23] (Figure 2). Microglia activation is a protective mechanism contributing to tissue homeostasis and neuronal function observed in the early phase of neurodegeneration [24]. However, excessive or long-term activation of microglia contributes to chronic neuro-inflammatory responses in the brain. The continuous release of inflammatory mediators by activated microglia induces increased oxidative and nitrosative stress leading to neurotoxic consequences [25]. This alarmed microglia have been identified in a wide spectrum of neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis, amyotrophic lateral sclerosis and inherited photoreceptor dystrophies [26–30].

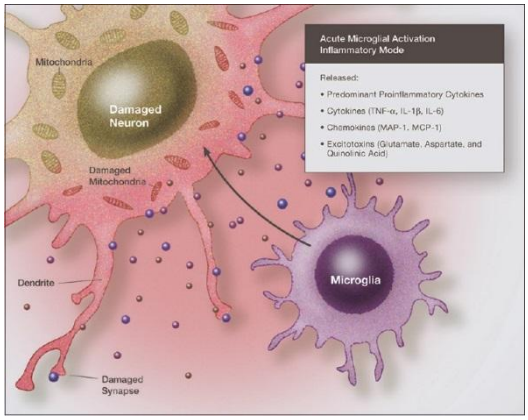

Figure2 . Neurotoxic effects of long-term microglia activation. Extracted from [23].

The presence of increased microglial activation in the brains of PD was first described by McGeer *et al* almost 30 years ago [31]. Nowadays it is widely

accepted that inflammation is a key feature in the initiation and progress of a number of neurodegenerative disorders.

In this activation process microglial cells adopt diverse functional phenotypes that range from the classical pro-inflammatory and neurotoxic phenotype, known as M1, to the alternative anti-inflammatory M2 phenotype [32,33]. "M1 like" activation of microglia is associated with the expression of inducible nitric oxide synthase (iNOS), the production of reactive oxygen species (ROS) and pro-inflammatory mediators (such as IL-1β) and the decreased secretion of neurotrophic factors. By contrast, "M2-like" phenotype is associated with the increased secretion of neurotrophic factors and proteases, the production of interleukin 4 (IL-4), the expression of the enzyme arginase 1 (Arg-1) and insulin-degrading enzyme (IDE), and enhanced phagocytic activity. These divergent responses may determine whether microglial cell activity leads to the clearance of tissue debris and the resolution of the inflammatory response or leads to chronic neuroinflammation (Figure 3) [34]

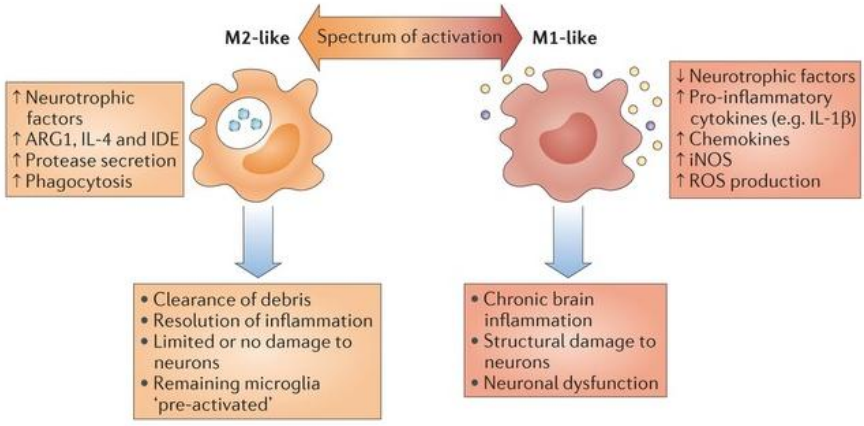

Figure 3. M1/M2 polarization of microglia. Adapted from [34].

Most of the expression and phenotype changes observed in alarmed microglia are a result of activation of the transcription factor NF-κB, a master regulator of inflammation pathways. Thus, in the recent years, agents ameliorating this

inflammation process are attracting attention as candidate drugs for neuroprotection in some neurodegenerative diseases.

1.3 Reverting chronic inflammation as strategy to combat metabolic syndrome and neurodegenerative diseases.

As previously explained, in the recent years numerous studies have shown that the inflammation process is directly involved in the onset of MetS and some neurodegenerative diseases [35–38].

Among the transcription factors that regulate chronic inflammation across multiple diseases NF-kB is one of the most significant and has become a very attractive target for de development of anti-inflammatory drugs.

NF-kB has been put forward as a major intracellular mediator of inflammation in the development of MetS and neuroinflammation processes. Interestingly, recent studies have demonstrated that high fat diet intake is associated with the activation of NF-kB. Furthermore, it has also widely been reported that activation of this transcription factor plays an important role in inflammatory mediated neurodegenerative disorders.

That would explain why targeting NF-kB has become a new challenge for preventing or decreasing the metabolic syndrome as well as the progression of a number of neurodegenerative disorders. Accordingly with this, numerous studies have been published reporting the neuroprotective effect of inhibiting NF-κB activation as well as the positive effect of this strategy for the prevention of MetS.

1.3.1 NF-κB pathway

NF-kB is an important family of transcription factors involved in propagating the cellular response to inflammation processes.NF-kB comprises various dimeric complexes of members of the Rel protein family, which include RelA (p65), RelB, c-Rel, NF-kB1 (p50 and its precursor p105) and NF-kB2 (p52 and

its precursor p100) [39]. Each possesses a homologous DNA-binding domain (the Rel homology-domain) responsible for the dimerization, nuclear translocation and DNA binding. Besides, p65, RelB and c-Rel, also contain a C-terminal transactivation domain. Of the various heterodimmers combinations, p50-p65 is the most common [39].

In resting cells, NF-kB resides in the cytoplasm trapped by the inhibitory Ikbα which prevents its entrance to the nuclei[40]. Upon activation by external stimuli the inflammatory signal converges on and activates a cluster of kinases kwon as IkB kinases (IKK) complex. Once activated, specific IKK phosphorylates two conserved N-terminal residues of IkBα, causing its ubiquitinitation and subsequent proteosomal degradation [41]. Free NF-kB then translocates into the nucleus, interacts with the promoter region of a variety of inflammatory response genes and activates their transcription [42,43] (Figure 4).

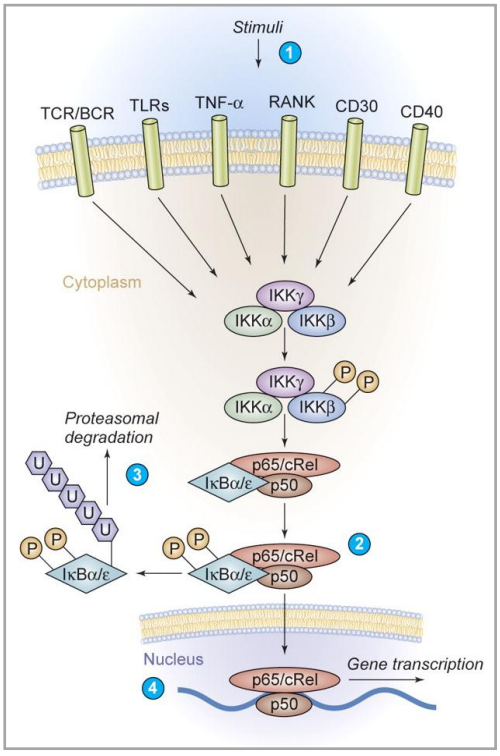

Figure 4. NF-kB activation pathway. (1) a serie of stimuly actívate the NF-kB pathway, including pro-inflammatory cytokines, such as IL-1, or TNF-α, or pathogen-associated molecular patterns that bind to TLRs like lipopolysaccharide (LPS). (2) Activated IKK phosphorylates two conserved N-terminal residues of IKBα and induces IKBα polyubiquitinylation (3) which in turns induces their recognition by the proteosome and subsequent degradation. (4) free NF-kB dimers are then released and translocate into the nucleus, where gene transcription is activated. Adapted from [43].

Once understood that the activation of NF-kB leads to the induction of multiples genes that regulates the inflammatory response, the potential for inhibiting NF-kB has received attention.

More concretely, since the phosphorylation of the IκB proteins is a key step involved in the regulation of NF-kB mechanism of action, agents that suppress the kinase activity of IKKs have become likely to be effective drugs against chronic inflammation related disorders.

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Manuscript 1

Anti-inflammatory and pro-apoptotic properties of the natural compound, o-orsellinaldehye through IKK-2 inhibition

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[Abstract]

Metabolic syndrome is a cluster of medical conditions that increases the risk of developing cardiovascular disease and type 2 diabetes. Numerous studies have shown that inflammation is directly involved in the onset of metabolic syndrome.

In this study *in silico* techniques were applied to a natural products database containing molecules isolated from mushrooms from the Catalan forests to predict molecules that can act as human nuclear-factor kB kinase 2 (IKK-2) inhibitors. IKK-2 is the main component responsible for activating the nuclear-factor kB transcription factor (NF-kB). One of these predicted molecules was *o*-orsellinaldehyde, a molecule present in the mushroom *Grifola frondosa*.

This study shows that o-orsellinaldehyde presents anti-inflammatory and pro-apoptotic properties by acting as IKK-2 inhibitor. Additionally, we suggest that the anti-inflammatory properties of *Grifola frondosa* mushroom could partially be explained by the presence of *o*-orsellinaldehye on its composition.

Keywords: Inflammation, apoptosis, IKK-2, NF-kB, o-Orsellinaldehye, *Grifola frondosa.*

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1. Introduction

Metabolic syndrome is a cluster of medical conditions that increase the risk of developing cardiovascular disease and type 2 diabetes. Considerable attention has been paid to this syndrome since it affects one in five people and it is a leading cause of death in developed countries. [1]

In the last few years, numerous studies have shown that the inflammation process is directly involved in the onset of metabolic syndrome [2,3]. Furthermore, nowadays it is accepted that chronic subclinical inflammation is a part of the insulin resistance syndrome [4,5]. That would explain why targeting inflammation mediators has become a new challenge for preventing or decreasing metabolic syndrome [6].

In recent years, natural products and their active metabolites have become increasingly important on pharmaceutical research [7]. Unfortunately, bioactivity screening in extracts exclusively by *in vitro* or *in vivo* approaches is a complex and expensive process that is difficult to afford [8]. However, virtual screening (VS) workflows have been successfully used to screen large natural products databases in order to identify new bioactive molecules for specific targets [9,10]. In particular, our group has wide experience on VS and has published several research projects which aimed to identify new natural compounds with undescribed bioactivity and with great interest in medicinal chemistry [11,12].

Concretely, one interesting target that has become very attractive for anti-inflammatory drugs development is the human inhibitor nuclear-factor kB kinase 2 (IKK-2) [7,13]. IKK-2 is a serine-threonine protein kinase belonging to the IKK complex and is the main component responsible for activating the nuclear-factor kB transcription factor (NF-kB) in response to inflammatory stimuli. NF-kB is an important transcription factor involved in propagating the cellular response to inflammation [14]. This universal transcription factor

regulates the expression of several components of the immune system including pro-inflammatory cytokines, chemokines, adhesion molecules and inducible enzymes such as cycloxygenase-2 and inducible nitric oxide synthase [15].

Inflammatory mediators stimulate the inflammation pathway by activating the IKK-2 that phosphorylates IKβα and leads to its degradation; As a consequence, free NF-kB translocates to the nucleus and induces the transcription of all the elements that controls the inflammatory response. [16].

In this study, firstly, we applied previously validated VS workflow developed by our group [17] to a natural products database containing a total of 5134 molecules isolated from mushrooms from the Catalan forests in order to identify potential IKK-2 inhibitors. One of this predicted IKK-2 inhibitors was 2,4-dihydroxy-6-methylbenzaldehyde, most commonly known as *o*-orsellinaldehyde. It has been described that this molecule is present in some natural sources such as *Agrocybe praecox*, *Aspergillus rugulosus*, and *Grifola frondosa* [18–20]. Interestingly the last one, *Grifola frondosa*, has been traditionally used as a medicinal mushroom for treating pain and some inflammatory states in Asia [21], where it is commonly known as Maitake.

Secondly, we aimed to investigate and validate the anti-inflammatory properties of *o*-orsellinaldehyde by applying *in vitro* and *in vivo* techniques. Finally, Maitake mushrooms were analyzed by liquid chromatography/quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) in order to identify and quantify the amount of this compound and evaluate the feasibility of preparing extracts from this natural source that were enriched on *o*-orsellinaldehyde.

The results show that *o*-orsellinaldehyde is able to reduce NF-kB dependent inflammatory responses by inhibiting IKβα phosphorylation through IKK-2 inactivation. Concretely, this molecule decreased the expression of pro-inflammatory mediators in LPS-stimulated RAW264.7 macrophage cells;

furthermore, in an *in vivo* context, *o*-orsellinaldehyde significantly decreased the IL-6 plasma concentration in LPS-stimulated Balb/c mice.

Additionally, we could also confirm the apoptotic effects of *o*-orsellinaldehyde in HepG2 cancer cells. Bearing in mind that NF-kB is also involved in propagating the cellular response to apoptosis and carcinogenesis we suggest that the apoptotic effects observed in *o*-orsellinaldehyde could be partially due to its IKK-2 inhibitor activity.

This study presents the anti-inflammatory and apoptotic activity of *o*-orsellinaldehyde, suggesting that it may be a potential preventive or therapeutic candidate drug for the treatment of inflammatory and cancerous diseases. Additionally, the described anti-inflammatory and pro-apoptotic properties of *Grifola frondosa* [21] could partially be explained by the presence of *o*-orsellinaldehyde on its composition.

2. Materials and Methods

2.1 *In silico* experiments

2.1.1 Building the initial database of molecules to be screened and virtual screening

The scientific name of all the mushrooms that are present at the Catalan forest were obtained from the webpage of the Catalan Society of Micology (http://www.micocat.org/). We chose the mushrooms present in the Catalan forest as source to find bioactive molecules mainly due to the easy obtention of the samples in this region. A total of 5134 molecules isolated from any mushroom that share the same genus than those found at that webpage where downloaded from the Reaxys Medicinal Chemistry database (http://www.reaxys.com). These molecules were then filtered with FaF-Drug2 [22] to keep only those that show good ADMET properties (*i.e.*, 3582

molecules) and submitted to a previously published VS workflow that has been shown to be effective to predict new IKK-2 inhibitors [11]. Details about the filters of this VS can be also found at the original publication [11].

2.2 In vitro experiments with RAW 264.7 cells

2.2.1 Cell culture and viability

RAW264.7 macrophages obtained from ECACC (Sigma-Aldrich Chemical, Madrid, Spain) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), L-Glutamine (2 mM), HEPES (25 mM) and Penicillin-Streptomycin (P/S) (100 U/mL of) at 37°C in a 5% CO_2 humidified atmosphere. DMEM, FBS, P/S and L-Glutamine were bought from Lonza, Cultek, (Barcelona, Spain). HEPES buffer solution was obtained from Gibco by Fisher (Madrid, Spain). For the analysis of cell viability, an MTT assay was performed. RAW264.7 cells (250.000 cells/well) were treated with different concentrations of *o*-orsellinaldehyde ranging from 1 to 50 µg/mL (ref: 657603 Sigma-Aldrich Chemical, Madrid, Spain) for 8 hours. After this incubation period, medium was replaced by 200 µl of fresh DMEM with 50 µl of 5mg/mL of MTT solution and the plate was incubated at 37°C for 4 hours in darkness. The colored formazan product was then dissolved in DMSO and quantified using a scanning multi-well spectrophotometer (BioTek EON, Izasa, Barcelona, Spain) at a wavelength of 570 nm.

2.2.2 Cell treatment

RAW264.7 cells were seeded into a 12-well plate at a density of 250.000 cells/well. 24 hours after platting, cells were pre-incubated with *o*-orsellinaldehyde at the selected concentrations for 30 minutes and then the inflammatory stimuli was then added (LPS 1 µg/mL). As a positive control of anti-inflammatory agent we used a commercial inhibitor (ref: 401481 IKK-2 Inhibitor IV Calbiochem, USA) which target is IKK-2. As a negative control,

cells were left untreated. 8 hours after the LPS addition the culture medium was collected for IL-6 and nitrite determination. Cells were then washed with PBS and lysed in RIPA buffer (25mM Tris–HCl; pH 7.4, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing a protease and phosphatase inhibitor mixture (Phenymethanesulfonyl fluoride solution (ref: 93482), Protease inhibitor cocktail (ref: P8340), Phosphatase inhibitor cocktail-2 (ref: P5726), and Phosphatase inhibitor cocktail-3 (ref: P0044), all from Sigma-Aldrich Chemical, Madrid, Spain). The resulting solution was collected into microcentrifuge tubes for future protein determination and Western Blot assay.

2.2.3 Nitrite and IL-6 Determination

Nitrite concentration was measured by use of Griess reaction [23]. Briefly, 50 μL of medium was added in a 96-well plate and mixed with 100 μL of 1% sulfanilamide in 0.5 M HCl. The plate was incubated at 4ºC for 10 minutes and 50 μL of NED (N-1-naftiletilendiamine) solution was then added to each well. After 30 minutes at 4°C the optical density at 540 nm was measured. The values were interpolated with a standard curve with known concentrations of nitrite oxide (NO).

Il-6 concentration in the supernatants and serum was determined by a commercial ELISA assay (IL-6 ELISA-MAX^{TM} from Biolegend) according to the manufacturer's instructions.

2.2.4 Western Blot analysis

After protein quantification using BCA assay (ref: 23225, Thermo/Pierce, Rockford,IL, USA) -proteins were separated on sodium dodecyl sulfate (SDS)–polyacrylamide 10% gels and transferred to polyvinylidene difluoride (PVDF) membranes using a Trans-Blot Turbo Blotting system (Bio-Rad, Barcelona, Spain). Membranes were blocked with 5% nonfat milk in PBS–Tween (0.1%) for one hour. Then, membranes were incubated overnight at 4ºC with the

following antibodies (dilution 1.1000): polyclonal antibody for iNOS (sc-651, Santa Cruz Biochemicals), polyclonal antibody for Phospho-Ikβ-α (Ser32) (2859; Cell Signaling Technology) or polyclonal antibody for β-actin (A 2066; Sigma-Aldrich Chemical, Madrid, Spain). After washing three times with PBS–Tween, membranes were incubated with the secondary antibody anti-rabbit (NA934, Merck, Barcelona, Spain) (dilution 1.10000) conjugated with horseradish peroxidase for 1 h and then washed three more times with PBS-Tween.

The immunoreactive proteins were visualized using an enhanced chemiluminescence substrate kit (ECL plus; Amersham Biosciences, GE Healthcare) according to the manufacturer's instructions. Digital images were obtained with a GBOX Chemi XL 1.4 system (Syngene, UK), which allows quantification of the band intensity. The protein load was monitored via the immuno-detection of β-actin.

2.2.5 IKK-2 kinase assay

The effect of *o*-orsellinaldehyde on IKK-2 kinase activity was determined using the ELISA assay Cyclex IKKα and IKKβ Inhibitor Screening Kit (MBL International, Woburn, MA, USA; Cat# CY-1178). Briefly, plates were pre-coated with a substrate corresponding to recombinant IKβα, which contains two serines (Ser32 and Ser36) that are phosphorylated by IKKα and IKKβ. In order to develop the assay, both test compounds and the enzyme was diluted in assay buffer and pipetted into the wells allowing substrate phosphorylation. The amount of phosphorylated substrate was detected using a specific anti-phospho-Ikβα (Ser32) antibody HRP-conjugate and blue color development with TMB substrate. The ELISA stop solution was then used to stop the color development, and the absorbance was read at 450 nm (with a reference wavelength of 540–600 nm) using an ELISA reader (BioTek EON, Izasa, Barcelona, Spain). The absorbance is directly related to the IKK-2 activity level. In addition, one

positive and one negative control were included on the test. As a positive control we used the commercial inhibitor (1µM), mentioned before (IKK-2 Inhibitor IV, 401481 Millipore, Madrid), as a negative control, DMSO (vehicle) was added to the wells. Three repetitions were made for the entire activity assay.

2.2.6 *In vitro* IKK-2 phosphorylation Assay

RAW264.7 cells were seeded into a 12-well plate at a density of 250.000 cells/well. After 24 hours of platting, cells were pre-incubated with *o*-orsellinaldehyde at the selected concentrations for 30 minutes and then the inflammatory stimuli was added (LPS 1µg/mL) for 45 additional minutes. As a positive control of IKK-2 inhibitor agent we used the same commercial drug mentioned before. Some cells were also treated just with the vehicle used to dissolve the test compound. Once the experiment finished, the medium was discarded and cells were then washed with PBS and lysed in RIPA buffer containing a protease and phosphatase inhibitor mixture. The mix result was recollected into microcentrifuge tubes for future protein determination and Western Blot assay.

2.3 *In vivo* experiments

2.3.1 Animals

Male Balb/c mice (5–6 weeks of age) were obtained from Charles River (Barcelona, Spain). Animals were housed under standard conditions with a 12-h light/dark cycle. The animals were acclimatized to the environment for ten days before starting the experiments. All procedures were performed in accordance with the European Communities Council Directive regarding the protection of experimental animals (86/609/EEC) and the procedure established by the Departament d'Agricultura, Ramaderia i Pesca of the Generalitat de Catalunya.

2.3.2 Intraperitoneal administration of *o*-orsellinaldehyde

For the *in vivo* assays, an LPS endotoxic shock was induced in order to evaluate the anti-inflammatory activity of *o*-orsellinaldehyde. Mice were divided into 8 groups (n=6): groups 1 to 4 received an intraperitoneal injection of *o*-orsellinaldehyde at different concentrations (10, 40, 60 100 mg/kg body weight (b.w.)). Group 5 received dexamethasone (12 mg/kg b.w.) as an anti-inflammatory agent control. 1 hour after the molecule administration, an intraperitoneal injection of LPS at a concentration of 1 mg/kg b.w. was administered. The positive and negative groups (named group 6 and group 7) received just LPS or PBS, respectively. Finally group 8 received only *o*-orsellinaldehyde at the maximum concentration (100 mg/kg b.w) in order to discard any side effects produced by this molecule. 2 h after LPS injection, mice were anesthetized and 1ml of blood was obtained by cardiac puncture in order to measure IL-6 concentration in plasma.

2.3.3 Oral administration of o-orsellinaldehyde

Mice were divided into 6 groups (n=6) depending on the treatment. Thus, o-orsellinaldehyde (100 mg/kg b.w) was orally administrated 30 minutes (group 1), one hour (group 2), or two hours (group 3), before LPS stimulation.

As anti-inflammatory control, one group (group 4) received dexamethasone. The positive and negative groups (group 5 and 6 respectively) received just LPS or PBS, respectively. Finally, 2 h after LPS injection, mice were anesthetized and 1 ml of blood was obtained by cardiac puncture in order to measure IL-6 concentration in plasma.

2.4 Cytotoxic Effect against HepG2

2.4.1 Cell culture and viability

The human hepatoblastoma HepG2 cells (obtained from ECACC, Sigma-Aldrich Chemical, Madrid, Spain) were cultured in DMEM supplemented with 10% FBS, 2% P/S, 1% L-glutamine, and 1% NEAA (non-essential amino acids) at 37°C in a 5% CO2 humidified atmosphere. In order to measure the reduction of cell viability an MTT assay was performed. For this assay cells were seeded at a density of 5×10^4 cells/well on a 96-well culture plate and were incubated overnight. Once confluence was reached, cells were treated with increasing concentrations of *o*-orsellinaldehyde and incubated for 48 hours. After this incubation period, the MTT assay was performed following the protocol mentioned above.

2.4.2 Cell treatment and analysis for cleaved caspase-3 detection

Caspases, a group of cysteine proteases, are the key mediators of apoptosis [24]. Therefore, in order to elucidate if *o*-orsellinaldehyde shows a cytotoxic effect on HepG2 cells through an apoptotic process, we treated these cells with *o*-orsellinaldehyde at increasing concentrations and we then quantified the cleaved caspase-3 protein expression.

For this purpose, HepG2 cells were seeded at a density of 3×10^5 cells/well on a 12-well culture plate and incubated overnight. Once cells reached confluence, they were treated with *o*-orsellinaldehyde at increasing concentrations for 48 hours. After this incubation period the medium was discarded and cells were washed with PBS and lysed in RIPA buffer containing a protease and phosphatase inhibitor mixture. The resulting solution was recollected into microcentrifuge tubes for future protein determination and Western Blot assay as previously explained.

After cell treatment and protein extraction and quantification, the expression of cleaved caspase-3 was determined by immunoblot following the same protocol mentioned above and using the polyclonal antibody for cleaved caspase-3 (9662; Cell Signaling Technology. dilution 1.1000).

2.5. Extraction and quantification of *o*-orsellinaldehyde into Maitake mushrooms

Finally, once evaluated the bioactivity of the pure molecule, we decided to quantify the amount of *o*-orsellinaldehyde in Maitake mushrooms in order to evaluate its potential as natural source of this compound. To do this, firstly we extracted the compound from the Maitake mushroom and after that we analyzed it by LC-QTOF-MS.

To perform the extractions, acetonitrile (HPLC grade), acetone, ethanol, ethyl acetate and acetic acid (all provided by ScharlauChemie; Barcelona, Spain) were used. Ortho-phosphoric acid (85%) was purchased from Panreac (Barcelona, Spain) and water was of Milli-Q quality (Millipore Corp, Bedford, MA, USA). A stock solution of *o*-orsellinaldehyde at 1000 mg/L was dissolved in Milli-Q water and stored in a dark flask at -18 ºC.

2.5.1 Preparation of the extract

To prepare an extract containing *o*-orsellinaldehyde from Maitake mushroom we followed the method described by Lee *et al.* (2012) with some modifications. Briefly, 3 grams of lyophilized Maitake mushrooms were mixed with 80 mL of pure ethanol and were continuously agitated during 5 hours at 65 ºC. Then, it was centrifuged at 3,000 rpm for 10 min at 4ºC. The supernatant was rotavaporated to dryness, setting the temperature at 30 ºC and was further dissolved in 80 mL of Milli-Qwater. Then, it was purified using 50 mL of ethyl acetate. To allow complete separation of the 2 phases it was kept overnight in darkness into the hood. Aqueous phase was separated, rotavaporated at 30 ºC to

remove organic solvent and finally lyophilized. The lyophilized extract was dissolved in 10 mL of Milli-Qwater and stored at -18 ºC until analysis.

The day of analysis, extracts were further purified in order to remove components that could interfere with the quantification by using microelution plates (Waters, Milford, USA) packed with 2 mg of OASIS HLB sorbent (Waters, Milford, USA) following the method previously described with some modifications [25]. Firstly, the wells were sequentially conditioned by using 250 µL of methanol and 250 µL of Milli-Qwater:acetic acid (99.2:0.2, v/v). Then, 300 µL of extract mixed with 300 µL of phosphoric acid 4% were loaded onto the plate. After that, the clean-up of the plates was sequentially done with 200 µL of Milli-Qwater and 200 µL of Milli-Qwater:acetic acid (99.8:0.2, v/v) to eliminate any interference that the sample might contain. Finally the elution of the retained compounds was done with 2x50 µL of acetone:Milli-Qwater:acetic acid (70:29.5:0.5, v/v/v). 5 µL of the eluted was directly injected into the LC-QTOF-MS.

2.5.2 LC-QTOF-MS analysis of extracted samples

The LC-QTOF-MS system consisted of an Agilent 1260 Series (Agilent Technologies, Palo Alto, U.S.A.) coupled to a 6540 ESI-QTOF (Agilent Technologies) operated in positive electrospray ionization mode (ESI+). Separation was carried out in a using a XBridge™ Shield RP18 column (3.5 µm, 150 mm x 2.1 mm i.d.) from Waters equipped with a Pre-Column Zorbax SB-C18 (3.5 µm, 15 mm x 2.1 mm i.d.) from Agilent. The software used was Masshunter. Drying gas temperature was 350 ºC and its flow rate was held at 12 L/min. On the other hand pressure of the gas nebulizer was 45 psi and the capillary voltage was set at 4000 V. Fragmentor was set at 120V, skimmer at 65V and OCT 1RF Vpp was set at 750V.

During the analysis, the column was kept at 25º C and the flow rate was 0.4 mL/min. The solvent composition was solvent A: Milli-Qwater/acetic acid

(99.8:0.2 v/v) and solvent B: acetonitrile. Solvent B was initially 10% and was gradually increased reaching 50% at 20 minutes and 100% at 22 min. Then it was maintained isocratically 5 min and after that it was reduced to 10% in 1 minute and was held at initial conditions during 7 minutes

3. Results

3.1 *In silico* virtual screening

The VS identified 20 molecules as possible IKK-2 inhibitors; one of them was *o*-orsellinaldehyde (see Figure 1A). Figure 1B shows how the docked pose of *o*-orsellinaldehyde fits at the structure-based pharmacophore that is located at the ATP binding site. This pharmacophore is formed by two hydrogen-bond donors (in blue), one hydrogen-bond acceptor (in red) and one hydrophobic region (in green), with tolerances (*i.e.*, radii) of 1.5, 1.5 and 3.0 Å, respectively. *o*-Orsellinaldehyde matches three out of the four sites (*i.e.*, **A1**, **D3** and **H4**) with one of the two hydroxyls simultaneously matching **A1** and **D3** by accepting the proton from the nitrogen main chain of Cys99 and acting as a hydrogen bond donor with the main chain carbonyl oxygen of Glu97.

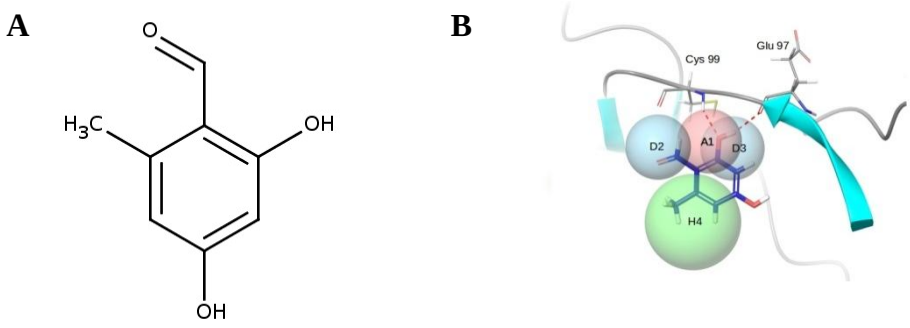


Figure 1. Virtual Screening of *o*-orsellinaldehyde. (A) Chemical structure of *o*-orsellinaldehyde. (B) Predicted binding pose for o-orsellinaldehyde at the binding site of human IKK-2

3.2 Anti-inflammatory effects of *o*-orsellinaldehyde in *in vitro* experiments with RAW 264.7 cells

3.2.1 Effect *o*-orsellinaldehyde on cell viability of RAW264.7 macrophage cells

To determine whether *o*-orsellinaldehyde influences the viability of RAW264.7 cells an MTT assay was performed. As shown in figure 2, after 8 hours of treatment, *o*-orsellinaldehyde did not affect cell viability at concentrations ranging from 1-50 µg/mL. These data indicates that *o*-orsellinaldehyde is not cytotoxic to RAW 264.7 cells at the tested concentrations.

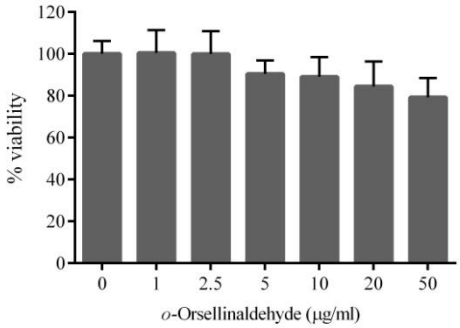


Figure 2. Effect of *o*-orsellinaldehyde on the viability of RAW264.7 macrophage cells. Cells were incubated with the indicated concentrations of *o*-orsellinaldehyde for 8 hours. Cell viability was determined by the MTT assay. Results are shown as the mean ± SD of three independent experiments.

3.2.2 *o*-Orsellinaldehyde inhibits nitrite production in LPS-activated macrophages

To evaluate the effect of *o*-orsellinaldehyde on NO production in RAW 264.7 cells we pretreated-macrophages with or without *o*-orsellinaldehyde (20, 30, 40 or 50 µg/mL) for 30 minutes before stimulation with 1 µg/mL of LPS for 8 hours. The nitrite concentration in the medium was measured by use of Griess reaction. As it can be elucidated from figure 3A and 3B when RAW264.7 cells were stimulated with LPS (1 µg/mL) the concentration of nitrite increased in the

culture medium compared to the basal levels. However 1 hour pre-treatment of the cells with *o*-orsellinaldehyde at the assayed concentrations caused a reduction of NO production in a dose-dependent manner (see figure 3A and table 3B).

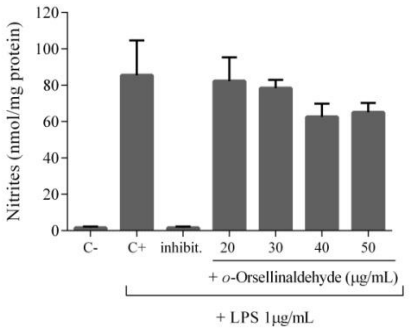
3.2.3 *o*-Orsellinaldehyde decreases iNOS protein expression in LPS-activated macrophages

In order to elucidate if the cause of NO reduction was due to decreased iNOS protein levels, the effect on iNOS protein expression was determined by immunoblot. As reflected in figure 3C, treatment with LPS increased iNOS protein expression in RAW264.7 cells, however the preincubation with *o*-orsellinaldehyde caused a significantly dose-dependent reduction of LPS-stimulated iNOS protein expression.

3.2.4 *o*-Orsellinaldehyde inhibits de release of IL-6 proinflammatory cytokine in murine macrophages

RAW 264.7 cells were pre-treated with or without *o*-orsellinaldehyde (20, 30, 40 or 50 μg/mL) for 30 minutes and then stimulated with LPS (1 μg/mL) for 8 h. In order to validate whether *o*-orsellinaldehyde was able to reduce IL-6 production an ELISA assay was performed. As figure 3D shows, the release of IL-6 was increased in the LPS-treated group and this effect was markedly decreased by *o*-orsellinaldehyde in a dose-dependent manner, reaching an 87% of inhibition with the higher concentration tested (50 μg/mL) which suggest that *o*-orsellinaldehyde seems to be even more effective than the commercial inhibitor.

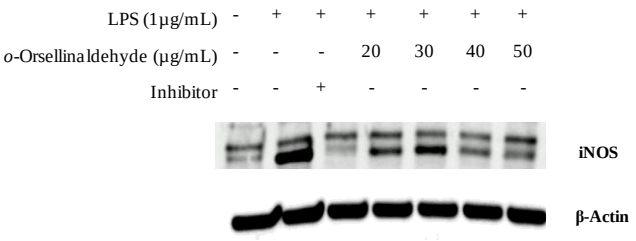
A



B

	% Inhibition
LPS + Comercial Inhibitor IV	73 %
LPS + *o*-orsellinaldehyde 20 µg/mL	3.7 %
LPS + *o*-orsellinaldehyde 30 µg/mL	8.3 %
LPS + *o*-orsellinaldehyde 40 µg/mL	15.9 %
LPS + *o*-orsellinaldehyde 50 µg/mL	19 %

C



D

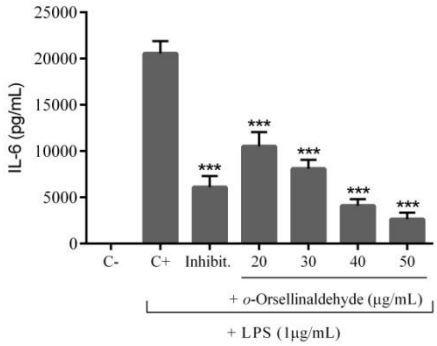


Figure 3. Effect of *o*-orsellinaldehyde on LPS-induced NO and IL-6 production and iNOS expression in RAW264.7 macrophage cells. Cells were incubated with the indicated concentrations of o-orsellinaldehyde for 30 minutes before treatment with LPS (1µg/ml) for 8 hours. **(A)** Concentration of NO was determined using Griess reaction. Results are shown as the mean of the nitrite production ± SD of four independent experiments. **(B)** Percentage of inhibition of the nitrite production. **(C)** Whole protein was extracted and then analyzed for iNOS by western blotting. One of the three experiments is shown. **(D)** Concentration of IL-6 in the media were detected using a specific enzyme immunoassay. Results are expressed as the mean ± SD of four independent experiments. Significant differences relative to the control (vehicle) were analyzed by one-way ANOVA followed by the Bonferroni post hoc test: ***p<0.001.

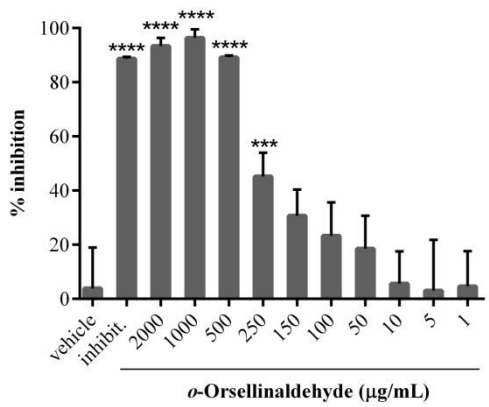
3.2.5 *o*-Orsellinaldehyde binds IKK-2 and inhibits its kinase activity

To determine whether *o*-orsellinaldehyde directly targets IKK, the *Cyclex IKKα and IKKβ Inhibitor Screening Kit* was performed. With this assay we aimed to study the effects of *o*-orsellinaldehyde on IKK-2 kinase activity by directly applying this molecule to recombinant IKKβ protein in a cell-free system. As figure 4A shows, *o*-orsellinaldehyde was able to reduce IKK kinase activity in a dose-response manner.

3.2.6 *o*-Orsellinaldehyde inhibits IKβα phosphorylation in LPS-activated macrophages

In order to validate that *o*-orsellinaldehyde was capable of inhibiting IKβα phosphorylation in an *in vitro* system, RAW264.7 cells were pretreated with *o*-orsellinaldehyde for 30 minutes and then, the inflammatory stimuli (LPS 1 μg/mL) was added for 45 additional minutes. As depicted in figures 4B and 4C, LPS drastically increased the phosphorylation of IKβα by IKK-2, however this phosphorylation is significantly decreased when cells are previously treated with *o*-orsellinaldehyde. Thus confirming that this molecule is interacting with IKK-2 enzyme preventing its kinase activity

A



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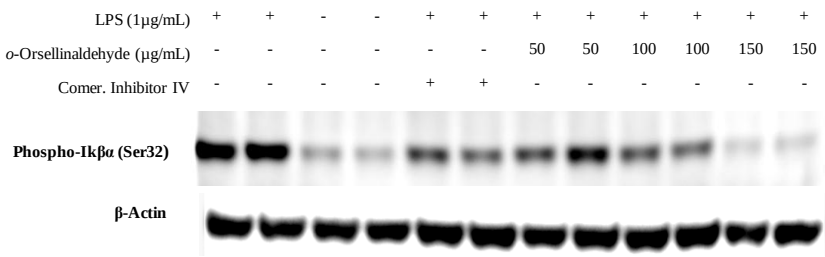

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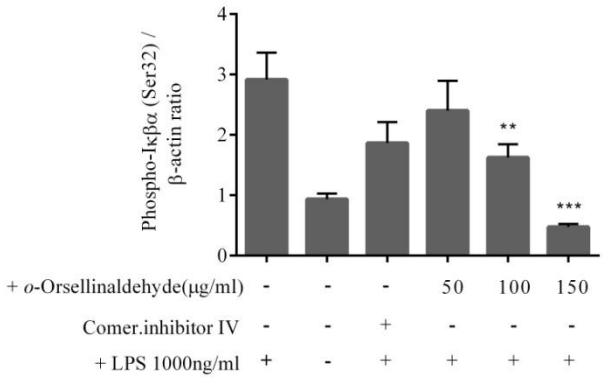


Figure 4. Effect of *o*-orsellinaldehyde on IKK-2 kinase activity. Inhibition of recombinant human IKK-2 was assayed in the presence of 10 different concentrations of *o*-orsellinaldehyde by an *in vitro* kinase assay. (A) The percentage of inhibition of IKK-2 activity is shown where each column represents the mean ± SD from three independent experiments. Significant differences relative to the control (vehicle) were analyzed by one-way ANOVA followed by the Bonferroni post hoc test: ****p<0.0001, ***p<0.001. **(B)** RAW264.7 Cells were pretreated with the indicated concentrations of *o*-orsellinaldehyde for 30 minutes, and then stimulated with LPS (1µg/ml) for 30 minutes. Whole protein was extracted and then analyzed for Phospho-Ikβα (Ser32) by western blotting. One of the three experiments is shown. **(C)** Plots representing the protein levels of Phospho-Ikβα normalized to the level of β-Actin. Results are expressed as the mean ± SD of three independent experiments. Significant differences relative to the control (vehicle) were analyzed by one-way ANOVA followed by the Bonferroni post hoc test: *** p<0.001, ** p<0.01.

3.3 *In vivo* experiments

3.3.1 *o*-orsellinaldehyde reduced the serum IL-6 concentration in LPS-stimulated animals

To assay whether anti-inflammatory effects of *o*-orsellinaldehyde occurred *in vivo*, mice received and intraperitoneal injection of the compound at different concentrations one hour prior LPS stimulation. As depicted in figure 5A, injecting mice with LPS highly increased the concentration of IL-6 in serum compared to untreated mice. Interestingly, *o*-orsellinaldehyde significantly reduced the serum IL-6 production by 21% in the LPS-stimulated animals at the higher concentration tested. This figure also shows that pretreatment with the anti-inflammatory steroid dexamethasone caused a 33% reduction in serum IL-6 in LPS-stimulated mice. It was also evidenced that *o*-orsellinaldehyde did not produced any pro-inflammatory effect by itself.

3.3.2 Oral administration of *o*-orsellinaldehyde reduced the serum IL-6 concentration in LPS-stimulated animals.

We next examined if this reduction of the inflammation was also achieved when the *o*- orsellinaldehyde was orally administrated. In this case, 300 µl of o-orsellinaldehyde at 100 mg/kg b.w. was orally administrated 30 minutes, one hour or two hours depending on the treated group and then the LPS stimuli was injected intraperitoneally. As can be seen in figure 5B serum IL-6 production markedly increased when mice were treated with LPS. However the IL-6 decreased when animals were also orally treated with the compound (100 mg/kg b.w), reaching a reduction of 20.5% when the tested molecule was administrated one hour previous to the LPS injection.

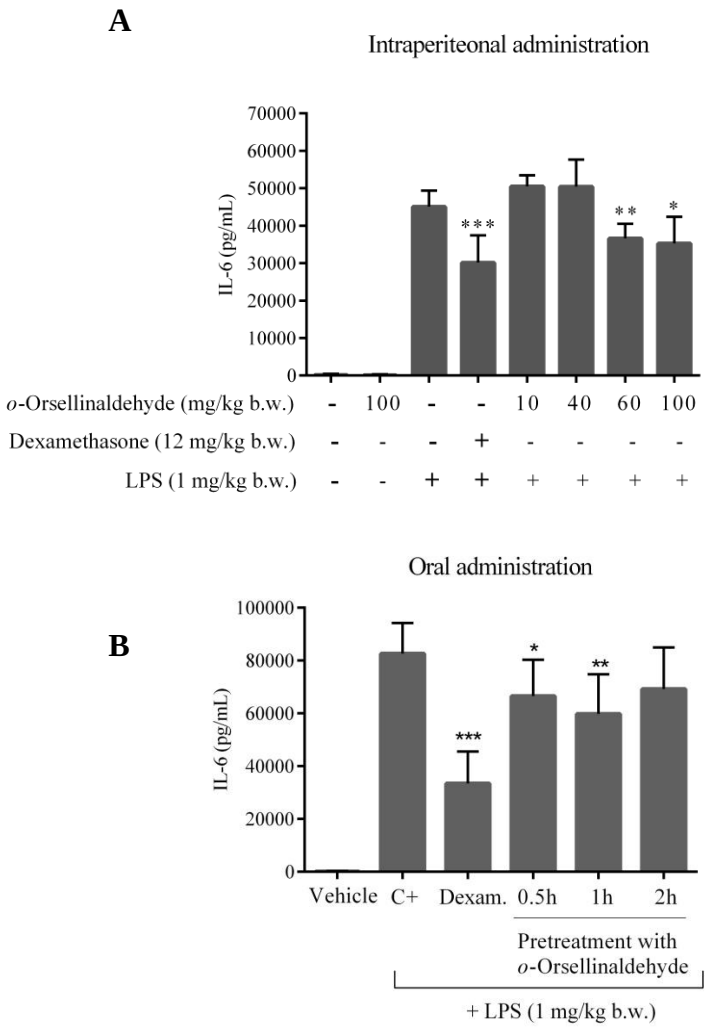


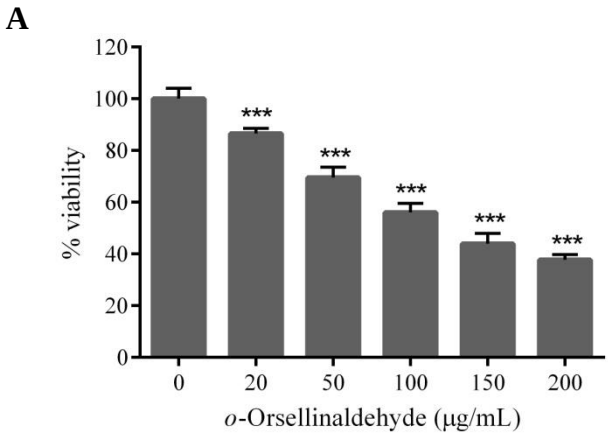
Figure 5. Effect of intraperitoneal and oral administration of *o*-orsellinaldehyde on IL-6 concentration in the serum of LPS injected mice. (A) Mice received and intraperitoneal injection of the compound at different concentrations one hour prior LPS stimulation (1 mg/kg b.w.). Serum was collected 2 hours after LPS injection and the level of IL-6 was determined. **(B)** 100 mg/kg b.w. of *o*-orsellinaldehyde was orally administrated for half, one or two hours before LPS intraperitoneal injection. Serum was collected at the indicated times and the production of IL-6 was determined .The data are represented as the mean±SD (n=6) Significant differences relative to the control (vehicle) were analyzed by one-way ANOVA followed by the Bonferroni post hoc test: *** $p<0.001$, ** $p<0.01$, * $p<0.1$.

3.4.1 Cell culture and viability

As previously reported [20] *o*-orsellinaldehyde has been isolated from different strains of *Aspergillus rugulosus* as a cytotoxic compound toward Hep3B human hepatoma cells through apoptosis. In our assays, HepG2 cells were used to evaluate the tumoricidal activity of the compound. In order to evaluate this, cells were treated with *o*-orsellinaldehyde for 48 hours and then an MTT assay was carried out. As depicted in figure 6A, *o*-orsellinaldehyde exhibits a cytotoxic effect on HepG2 cells in a dose-response manner.

3.4.2 *o*-Orsellinaldehyde increased cleaved caspase-3 expression in HepG2 cells

After 48 hours treatment with *o*-orsellinaldehyde, cleaved caspase-3 expression was quantified with a western blot assay in order to elucidate if the cytotoxic effect of *o*-orsellinaldehyde shown on HepG2 cells were mediated through an apoptotic process. As shown in figures 6C and 6D, *o*-orsellinaldehyde markedly increased cleaved caspase-3 expression in a dose response manner, confirming the apoptotic process.



B

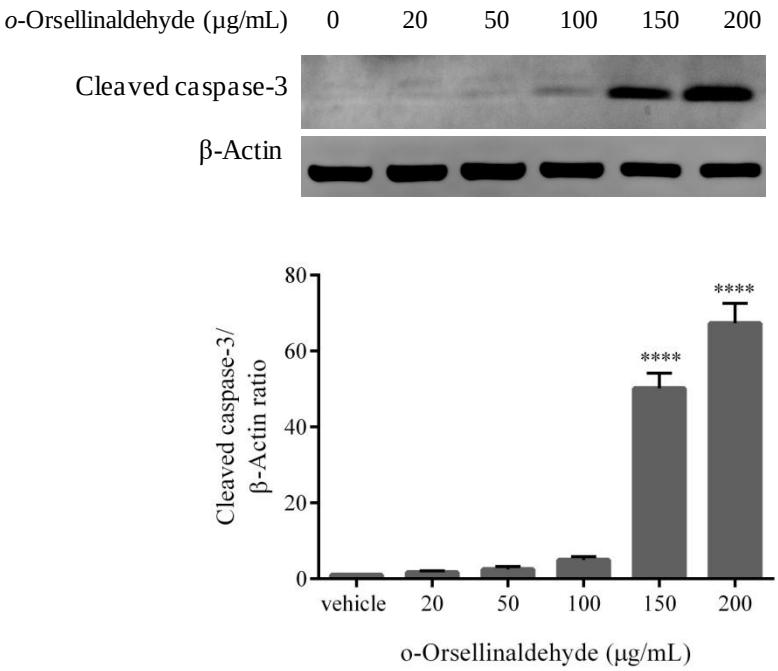


Figure 6. Cytotoxic Effect of o-orsellinaldehyde on HepG2 cells. (A) HepG2 cells were incubated with the indicated concentrations of *o*-orsellinaldehyde for 48 hours. Cell viability was determine by the MTT assay. Results are shown as the mean ± SD of three independent experiments. Significant differences relative to the control (vehicle) were analyzed by one-way ANOVA followed by the Bonferroni post hoc test: ***p<0.001. **(B)** Cells were pretreated with the indicated concentrations of o-orsellinaldehyde for 48 hours. Whole protein was extracted and then analyzed for cleaved caspase-3 by western blotting. One of the three experiments is shown. **(C)** Plots representing the protein levels of cleaved caspase-3 normalized to the level of β-Actin. Results are expressed as the mean ± SD of three independent experiments. Significant differences relative to the control (vehicle) were analyzed by one-way ANOVA followed by the Bonferroni post hoc test: **** p<0.0001.

3.5 Quantification and detection of *o*-orsellinaldehyde in extracted samples by LC-QTOF-MS.

Quantification of *o*-orsellinaldehyde in the Maitake extract obtained following the procedure above described was carried out by using a 6-points calibration curve of the standard compound. Figure 7 (supplementary material) confirms that *o*-orsellinaldehyde can be isolated from Maitake extracts. However the amount of this molecule contained in Maitake mushroom is very low (3.41 µg *o*-orsellinaldehyde /g lyophilized Maitake). In that sense, larger infrastructures would be necessary in order to isolate enough amount of the anti-inflammatory molecule for treating cells directly with the enriched Maitake extracts.

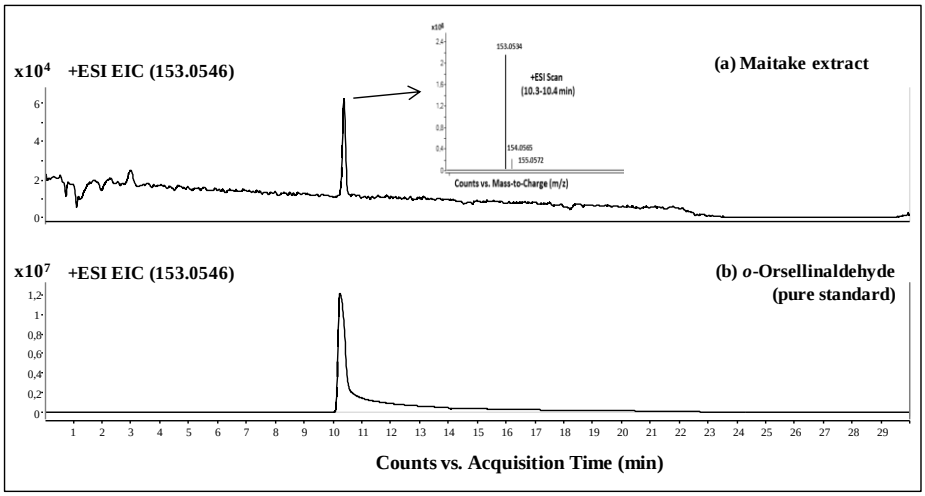


Figure S1. (Supplementary material) Extraction and quantification of o-orsellinaldehyde into Maitake mushrooms.ESI extracted ion chromatograms acquired by LC-QTOF-MS of **(A)** the Maitake extract and **(B)** a pure standard solution of *o*-orsellinaldehyde. The mass spectra scan obtained in the 10.3-10.4 interval is also shown

4. Discussion

Natural products have attracted considerable attention on pharmaceutical research field since they are a very important source when looking for novel molecules that could be used as lead compounds during the development of new drugs. More concretely, natural products and their principles have played a very important role on the discovery of new molecules with anti-inflammatory properties [26]. However, the identification process by using exclusively *in vitro* and *in vivo* approaches is very long and expensive. In that sense, using VS workflows help to improve this identification process as it can be used to easily screen large natural product databases [9–11].

The present research project aimed to identify and validate novel molecules from natural origin that can act as IKK-2 inhibitors by using *in silico* techniques. In order to achieve this goal we first applied virtual screening workflows to a natural molecules database in order to look for natural extracts that contain IKK-2 inhibitors and then we validated the anti-inflammatory properties of the selected compound in an *in vitro* and *in vivo* context.

The VS experiments successfully identified a molecule, *o*-orsellinaldehyde, with high chance of acting as IKK-2 inhibitor. It has been described that this molecule is present in the *Grifola frondosa* mushroom specie [20]. In that sense, in order to validate the *in silico* predictions we performed a kinase assay that confirmed that *o*-orsellinaldehyde directly targets IKK-2 and reduces its IKK-2 kinase activity in a dose-response manner (Figure 4A). Additionally, we validate that *o*-orsellinaldehyde significantly inhibited IKβα phosphorylation in LPS-stimulated RAW 264.7 macrophage cells (Figures 4C and 4D). These results confirmed that *o*-orsellinaldehyde exerts its anti-inflammatory effects by modulating NF-kB activity.

Moreover, the anti-inflammatory properties of the studied molecule have also been demonstrated by the obtained results referred to the reduction of nitrites, IL-6 and iNOS expression in the *in vitro* model used as it can be shown in figure 3.

To study if this compound is also effective *in vivo* we induced an LPS endotoxic shock model in Balb/c mice. This model can greatly increase the production of serum inflammatory mediators [27]. In both accomplished studies (intraperitoneal administration and oral administration of *o*-orsellinaldehyde) the molecule significantly reduced the serum IL-6 concentration (Figure 5)

All this findings provide strong evidences that *o*-orsellinaldehyde possesses anti-inflammatory properties and that it exerts its activity by influencing NF-kB activity, more concretely, acting as IKK-2 inhibitor.

Furthermore, previous studies have shown that *o*-orsellinaldehyde is also able to inhibit growth as well as induce apoptosis in various types of cancer cell lines cases (Hep3B human hepatocellular carcinoma cells and MRC-5 human lung fibroblast cells). It is also been described that in some particular cases this molecule has cytotoxic effect [19,20]. We have also observed this apoptotic activity from *o*-orsellinaldehyde in our experiments with HepG2 cells. Figure 6 confirmed that the *o*-orsellinaldehyde exhibits a cytotoxic effect on these cells and that this effect was mediated through an apoptotic process.

At this point, it is interesting to mention that NF-kB is not only related with inflammation process, this enzyme complex is also involved in propagating the cellular response to apoptosis and carcinogenesis. The functions of NF-kB gene targets span diverse cellular processes, including adhesion, immune regulation, apoptosis, proliferation and angiogenesis [28,29]. Consequently, the NF-kB transcription factor plays an important role in cancer and related diseases.

In that sense, numerous studies have been published reporting that inhibition of NF-kB -regulated genes potentiates apoptosis and has anti-proliferative effects [30–33].

So, it is feasible that some of the cytotoxic and apoptotic effects of *o*-orsellinaldehyde observed against the cancer cells could be due to NF-kB inhibition. In agreement with our results, previous interesting studies have also described this induction of apoptosis in cancer cell lines when using IKK-2 inhibitors [34–36].

Related to the natural source *Grifola frondosa*, this mushroom is known by its Japanese name Maitake. The fungus is native to the northeastern part of Japan and North America, and it has been traditionally used as a medicinal mushroom. This mushroom has been used for treating pain and some inflammation states in Asia; however, in the past few years some groups have reported that this specie has also other properties. Briefly, in the same way that *o*-orsellinaldehyde, *Grifola frondosa* is also able to induce apoptosis in cancer cell lines and also inhibit the growth of various types of cancer cells [37–39]. Other studies revealed that Maitake have anti-cancer and anti-metastatic properties [40,41]. *Grifola frondosa* has also hypoglycemic effect, and so it is beneficial for the management and treatment of diabetes [42,43]. Finally, *Grifola frondosa* extracts also contain antioxidants and cyclooxygenase 2 inhibitors [44]. As *o*-orsellinaldehyde is one of the compounds of Maitake, it is feasible to state that at least, a part of the beneficial effects observed in the *Grifola frondosa* extracts could be explained by the IKK-2 inhibition triggered by *o*-orsellinaldehyde.

The results with the LC-QTOF-MS (figure S1 in supplementary material) show that *o*-orsellinaldehyde can be isolated from Maitake extracts, however the amount of this molecule contained in Maitake mushroom is very low, suggesting that further studies would be necessary in order to isolate enough amount of the anti-inflammatory molecule from the mushroom specie.

5. Conclusions

Taken together, our findings show that the molecule *o*-orsellinaldehyde was effective in suppressing the production of inflammatory mediators *in in vitro* and *in vivo* studies. The anti-inflammatory and pro-apoptotic activity of this molecules is mediated by its interaction with NF-kB complex, more concretely, we demonstrate that *o*-orsellinaldehyde is able to act as IKK-2 inhibitor, confirming the worth and importance of using bioinformatics tools when searching for new active principles. Moreover, the anti-inflammatory properties of *Grifola frondosa* would partially be explained by the presence of *o*-orsellinaldehyde on its composition.

In summary this study presents the potential anti-inflammatory activity of *o*-orsellinaldehyde, suggesting that it may be a potential preventive or therapeutic candidate for the treatment of inflammatory disorders such as metabolic syndrome.

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Sara Tomás Hernández

Manuscript 2

Neuroprotective effects of the natural compound o-orsellinaldehyde in murine primary glial cells

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[Abstract]

Activation of microglia is a protective mechanism aimed to repair damaged tissues in early stages of neurodegeneration processes, however it has been widely reported that chronic neuroinflammation caused by sustained microglial activation is one of the main contributor of neurological disorders.

In a previous study we reported that *o*-orsellinaldehyde, a molecule contained in *Grifola frondosa* mushroom, has potent anti-inflammatory effects in RAW2647 cells as well as in Balb/c mice. On the basis of these previous results, we aimed to elucidate if *o*-orsellinaldehyde could also exert these anti-inflammatory effect in a neuroinflammation context.

This study presents the neuroprotective properties of *o*-orsellinaldehyde in mixed glia and microglia cells. Concretely, this molecule inhibits the LPS-induced inflammatory response by acting as IKK-2 inhibitor and modulating NF-kB activity. Moreover, *o*-orsellinaldehyde treatment in LPS-stimulated microglia promotes cell polarization towards anti-inflammatory M2 phenotype thus reinforcing the potential therapeutic value of this natural compound in the treatment or prevention of some neurodegenerative diseases.

Keywords: Microglia, neuroinflammation, NF-kB, *o*-Orsellinaldehyde, *Grifola frondosa*, M1/M2 polarization

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1. Introduction

Microglial cells are the resident macrophages of the nervous system and have very important roles in immune regulation and neuronal homeostasis. Under normal conditions, microglia shows a ramified morphology and displays a very active and continuous surveillance function [1,2]. Activation of microglia is a protective mechanism aimed to repair damaged tissues in early stages of neurodegeneration processes [3], however long-term or excessive activation of this cells is directly correlated with chronic neuroinflammation[4]. In this activation process microglial cells adopt diverse functional phenotypes that range from the classical pro-inflammatory and neurotoxic phenotype, known as M1, to the alternative anti-inflammatory M2 phenotype [5,6].

It the recent years, it has been widely reported that chronic neuroinflammation caused by sustained microglial activation is one of the main contributor of neurological disorders including Alzheimer's disease, Parkinson's disease, ischemia and neural death [7,8]. In that sense, regulation of microglial activation by compounds that promote the switch of microglia from inflammatory M1 type to anti-inflammatory M2 state could be a promising therapeutic option for treatment of several neurodegenerative diseases [9,10].

There is a growing interest in the identification of natural compounds with anti-inflammatory properties [10,11], in particular our group has wide experience on using virtual screening workflow to screen natural products databases in order to identify new bioactive molecules for specific targets [12,13]. In a previous study of our group, *in silico* techniques predicted that *o*-orsellinaldehyde, a molecule contained in *Grifola frondosa* mushroom, can act as IKK-2 inhibitor. IKK-2 is protein kinase belonging to the IKK complex and is the primary component responsible for activating the nuclear-factor kB transcription factor (NF-kB) in response to inflammatory stimuli [14–16].

In this previous study we demonstrated that *o*-orsellinaldehyde inhibits the inflammatory response by acting as IKK-2 inhibitor. This molecule dimished the production of nitric oxide and reduced the secretion of pro-inflammatory cytokines in LPS-stimulated RAW2647 macrophages. *o*-Orsellinaldehyde was also able to decrease IL-6 plasma concentration in stimulated mice. Furthermore, this compound exhibited a cytotoxic effect on HepG2 cancer cells.

Based on these previous results, the aim of this study was to elucidate if this molecule could also exert this potent anti-inflammatory effect in a neuroinflammation context. In that sense, we evaluate the effects of *o*-orsellinaldehyde in LPS-activated glia.

The results obtained show that *o*-orsellinaldehyde caused an inhibition on Nitrite oxide (NO) production and iNOS expression after LPS-stimulation in both mixed glia and microglia cells. We also evaluated the effect of *o*-orsellinaldehyde in the expression of the stress protein heme oxygenase-1 (HO-1). Furthermore, we observed that cell pre-treatment with *o*-orsellinaldehyde suppressed the phosphorylation of Ikβα in LPS-activated microglia. Thus confirming that *o*-orsellinaldehyde exerts its anti-inflammatory effects by acting as IKK-2 inhibitor and modulating NF-kB activity. Finally, *o*-orsellinaldehyde treatment modulates the expression of several cytokines in LPS-stimulated microglia inducing macrophage polarization towards anti-inflammatory M2 phenotype, which might contribute to the protective effects associated to *o*-orsellinaldehyde.

This study presents the anti-inflammatory effects of *o*-orsellinaldehyde in LPS-activated microglia and provides useful information about the potential therapeutic value of this natural compound in the treatment or prevention of some neurodegenerative diseases.

2. Materials and Methods

2.1 Prediction of blood-brain barrier permeation

The SwissADME website [17] was used to compute physicochemical descriptors and predict ADME parameters for the o-orsellinaldehyde. The BOILED-Egg method was used to predict blood-brain barrier (BBB) permeation [18] This method uses a representation of the predicted *n*-octanol/water partition coefficient (WLOGP) vs topological polar surface area (tPSA) to predict the BBB permeation.

2.2 Animals and in vitro cell culture procedure

2.2.1 Mixed glia cell culture and primary microglia isolation

Three-day-old pups of Wistar rats were used in this study. All animal care and experimental protocols with post-natal pups were carried out in accordance with the Directive 86/609EEC of the Council of the European Union and the procedure established by the Departament d'Agricultura, Ramaderia i Pesca of the Generalitat de Catalunya.

Mixed glial cell cultures were prepared from the cerebral cortex of three-day-old rats following the protocol described by Tamashiro et al (2012) with some modifications. Briefly, whole brains were rapidly dissected out and placed into a Petri dish with L-15 media on ice (Leibovitz's medium supplemented with 0.1% BSA and 1% Penicillin/Streptomycin (P/S)). After meninges removal, the cortices were fragmented and mechanically dissociated. The material was then dispensed through a cell strainer (100 μm pores) and centrifuged. Dissociated cells were seeded onto 75 cm^2 flasks in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% P/S and the cultures were maintained in a humidified atmosphere of5% CO_2at 37°C. The medium was replaced on the fifth day after the initial seeding and changed every third day afterward. DMEM, FBS, P/S and L-Glutamine were bought from

Lonza, Cultek (Barcelona, Spain). L-15 media was obtained from Gibco, Fisher (Madrid, Spain).

2.2.2 Isolation and Plating of Primary Microglia

Upon reaching confluence (10-12 days), the microglial cells were harvested by mild shaking (200 rpm for 4 h at 37°C) and recollected in 50 ml conical tubes. The flasks with the remaining mixed glia were returned to the incubator after adding new fresh media.

The isolated microglial cells were centrifuged at 2,500 rcf for 5 minutes, resuspended in microglial plating media (Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal equine serum and 1% P/S) and plated in at a density of 500.000 cells/ml. Twelve hours later, the cells were ready for treatment.

2.2.3 Mixed glia platting

Remaining mixed glia were detached from the flasks by mild trypsinitzation. Cells were pelleted by centrifugation at 2,500 rcf for 5 minutes and then seeded at a density of 500.000 cells/ml in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and 1% Penicillin/Streptomycin. Cells were allowed to attach overnight and treated the day after.

2.3 *o*-Orsellinaldehyde toxicity

Microglia cells were seeded in a 96-well plate and cultured in microglia plating media as mentioned above. For the analysis of cell viability the MTT reduction assay was performed. Microglia cells were treated with different concentrations of *o*-orsellinaldehyde (ref: 657603 Sigma-Aldrich Chemical, Madrid, Spain) for 24 hours. Afterwards, medium was replaced by 200µl of fresh plating medium and MTT salt was added at a final concentration of 5mg/mL. The plate was

incubated at 37°C for 4 hours without light. The colored formazan product was then dissolved in DMSO and quantified using a scanning multi-well spectrophotometer (BioTek EON, Izasa, Barcelona, Spain) at a wavelength of 570 nm.

2.4 Cell treatments

2.4.1 Mixed glia treatment

Mixed glia cells were seeded into a 12 well plate at a density of 500.000 cells/mL. Twelve hours after platting, cells were pre-incubated with vehicle (DMSO) or increasing concentrations of *o*-orsellinaldehyde for 30 minutes and then the inflammatory stimuli was added (LPS 10 ng/mL). As a negative control, some cells were left untreated. 24 hours after the LPS addition the culture medium was recollected for nitrite determination and cells were harvested for further protein analysis by western blotting.

2.4.2 Microglia treatment

In order to better characterize the influence of *o*-orsellinaldehyde in a neuroinflammation context primary microglia cells were isolated from mixed glial cell cultures as mentioned above. In that sense, microglial cells were treated with vehicle or with the highest concentration tested of *o*-orsellinaldehyde (50 µg/mL) 30 minutes prior LPS stimulation (LPS 10 ng/mL). Following the 24-h incubation, the medium was collected for NO measurement. Cells were then harvested and total RNA and protein were isolated for further analysis.

2.5 Nitrite Determination

Concentration of NO in culture supernatants were measured by use of the Griess reaction (ref). Briefly, 50 µL of sample were added in a 96-well microplate and mixed with 100 µL 1% sulfanilamide in 0.5 M HCl. The plate was incubated at 4ºC during 10 minutes and 50 µL of NED (N-1-naftiletilendiamina)/well were

then added. After 30 minutes at 4°C the optical density at 540 nm was measured with an ELISA plate reader (BioTek EON, Izasa, Barcelona, Spain). The values were interpolated with a standard curve with known concentrations of nitrite oxide (NO).

2.6 Western Blot analysis

Cells were washed with PBS and lysed in RIPA buffer (25mM Tris–HCl; pH 7.4, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing a protease and phosphatase inhibitor mixture (Phenymethanesulfonyl fluoride solution (ref: 93482), Protease inhibitor cocktail (ref: P8340), Phosphatase inhibitor cocktail-2 (ref: P5726), and Phosphatase inhibitor cocktail-3 (ref: P0044), all from Sigma-Aldrich Chemical, Madrid, Spain). Lysates were then centrifuged at 10.000 rpm for 10 min at 4ºC. The protein concentration was measured using BCA protein assay (ref: 23225, Thermo/Pierce, Rockford,IL, USA). Equal amounts of protein (30-50 µg) were separated electrophoretically on SDS–polyacrylamide gels and transferred to PVDF membranes (Trans-Blot Turbo system, Bio-Rad, Barcelona, Spain). The membranes were blocked with 5% nonfat milk in PBS–Tween (0.1%) for one hour. Then, membranes were blotted overnight at 4ºC with the proper rabbit antibody (dilution 1.1000): polyclonal antibody for iNOS (sc-651, Santa Cruz Biochemicals), polyclonal antibody for HO-1 (70081; Cell Signaling Technology), polyclonal antibody for Phospho-Ikβα (Ser32) (2859; Cell Signaling Technology), polyclonal antibody for Phospho-SAPK/JNK (Thr183/Tyr185) (9251; Cell Signaling Technology), polyclonal antibody for Phospho-p38 MAP Kinase (Thr180/Tyr182) (9211; Cell Signaling Technology) or polyclonal antibody for β-actin (A 2066; Sigma-Aldrich Chemical). After washing three times with PBS–Tween, the membranes were hybridized with secondary antibody anti-rabbit (NA934, Merck, Barcelona, Spain) (dilution 1.10000) conjugated with horseradish peroxidase for 1 h and then washed three more times with PBS-Tween. The immunoreactive proteins

were visualized using an enhanced chemiluminescence substrate kit (ECL plus; Amersham Biosciences, GE Healthcare) according to the manufacturer's instructions. Digital images were obtained with a GBOX Chemi XL 1.4 system (Syngene, UK), which allows quantification of the band intensity. The protein load was monitored via the immuno-detection of β-actin.

2.7 *In vitro* IKK-2 phosphorylation and NF-kB activity analysis

Microglial cells were seeded into a 12-well plate at a density of 500.000 cells/well. 24 hours after platting cells were pre-incubated for 30 minutes with *o*-orsellinaldehyde at 50 µg/mL or with the vehicle used to dissolve the test compound and then the inflammatory stimuli was added (LPS 10 ng/mL) for 45 more minutes. Some cells were left untreated as a negative control. Once the experiment finished, the medium was discarded and cells were washed with PBS and lysed in RIPA buffer containing a protease and phosphatase inhibitor mixture. The mix result was recollected into microcentrifuge tubes for future protein determination and Western Blot assay. Concretely Phospho-Ikβα, Phospho-SAPK/JNK and Phospho-p38 MAP Kinase levels were analyzed in this experiment.

2.8 RNA isolation and cDNA synthesis

Total RNA was obtained from the microglia treated cells using an RNeasy Mini Kit (QIAgen, Valencia, CA) according to the manufacturer's protocol. The RNA was resuspended in RNasefree water. The DNase I RNAase free kit (Fermentas, Thermo Scientific) was used to remove the genomic DNA from the RNA preparations. The RNA was quantified and tested for purity by measuring the absorbance at 260 and 280 nm with a spectophotometre (Nanodrop 1000 Spectrophotometer, Thermo Scientific). Then, 1 µg of total RNA from each sample was reverse transcribed using a First Strand cDNA Synthesis Kit (Fermentas, Thermo Scientific) according to the suppliers' protocol.

2.9 Real-time RT-PCR

The expression of various genes (iNOS, IL-1β, IL-10, Arg-1 and MRC-1) were evaluated by Q-PCR using SYBR Premix Ex Taq (Takara) according to the manufacturer's protocol and were analyzed on a CFX96 Real-Time PCR Detection System (BIORAD, Spain.) Each sample was assessed in triplicate.

The thermal cycling was composed of an initial step at 50°C for 2 min followed by a polymerase activation step at 95°C for 10 min and a cycling step with the following conditions: 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min. Therefore, at the end of the PCR cycles, the PCR products were analyzed using a heat dissociation protocol to confirm that a single PCR product was detected by the SYBR Green dye. The fluorescence data were acquired at the 72°C step. The threshold cycle (Ct) was calculated using the CFX Manager Software to indicate significant fluorescence signals above the noise during the early cycles of amplification. The software calculated copy numbers for the target samples from the Ct using interpolation from the standard curve. The relative expression levels of the target genes were calculated relative to GADPH (internal control) according to the $2^{-\Delta\Delta Ct}$ method. The primer sequences for the tested gens are shown in table 1.

Gene	Forward Primer 5´-3´	Revers Primer 3´-5´
iNOS	GCCACCTCGGATATCTCTTG	TCTGGGTCCTCTGGTCAAAC
IL-1β	CACCTCTCAAGCAGAGCACAG	GGGTTCCATGGTGAAGTCAAC
IL-10	GCCAAGCCTTGTCAGAAATGA	TTTCTGGGGCCATGGTTCTCT
Arg-1	ATATCTGCCAAGGACATCGTG	AGGTCTCTTCCATCACTTTGC
MRC-1	TGGACTAAGCCAAGGGGCAA	CAGGAGCAGGGGGAGTCTCA
HPRT	CTCATGGACTGATTATGGACAGGAC	GCAGGTCAGCAAAGAACTTATAGCC

Table 1. Primers sequences for the specific genes analyzed.

3. Results

3.1 Prediction of blood-brain barrier permeation

To be effective as neuroinflammatory modulator the molecule tested should be able to pass the hematoencephalic barrier in order to exert its activity on the brain cells. In that sense, the SwissADME Web tool was used to predict the blood-brain barrier permeation of the molecule o-orsellinaldehyde [17]. The BOILED-Egg method was used to predict blood-brain barrier (BBB) permeation. This tool predicted that this molecule was indeed capable to pass through the blood-brain barrier.

3.2 Effect *o*-orsellinaldehyde on cell viability of mixed glia and isolated microglia cells

To determine whether o-orsellinaldehyde influences the viability of glial cells an MTT assay was performed in both, mixed glia and isolated microglia cells. The results (figure 1), demonstrate that o-orsellinaldehyde treatment did not affect cell viability after 24 hours of incubation. Thus, the concentrations of 20 to 50 µg/mL were selected to further investigation.

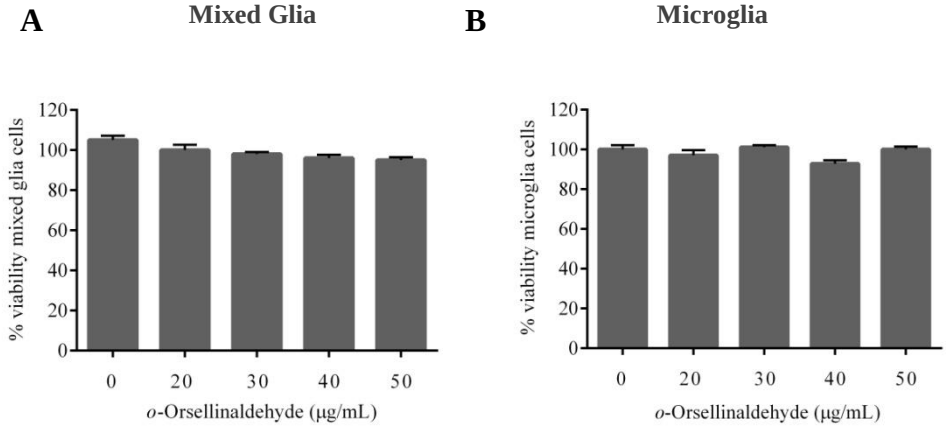

Figure 1. Effect of *o*-orsellinaldehyde on the viability of mixed glia and microglia cells. Cells were treated with increasing concentrations of o-orsellinaldehyde (20-50 µg/mL) for 24 hours. Cell viability was assessed by the MTT assay. Results are shown as the mean ± SD of three independent experiments.

3.3 *o*-Orsellinaldehyde inhibits nitrite production in LPS-activated mixed glia and microglia cells.

To evaluate the inhibitory effect of o-orsellinaldehyde on NO production, mixed glia cells were pre-incubated with vehicle (DMSO) or increasing concentrations of *o*-orsellinaldehyde 30 minutes before stimulation with LPS (10 ng/mL). As a negative control, some cells were left untreated. 24 hours after the LPS addition the culture medium was recollected and NO levels were measured by use of Griess reaction. As shown in figure 2A, LPS markedly induced nitrite production compared to that of the control, however cell pre-treatment with *o*-orsellinaldehyde significantly reduced the levels of NO in the medium in a dose-dependent manner, reaching a reduction of 25-30% with the highest doses of the compound.

In order to better characterize the influence of *o*-orsellinaldehyde, primary microglia cells were isolated from mixed glial cell cultures. Microglial cells were treated with vehicle or with the higher concentration tested of *o*-orsellinaldehyde (50 µg/mL) 30 minutes prior stimulation with LPS (10 ng/mL). Following the 24-h incubation, the medium was collected for NO measurement. As figure 2B depicted, in the same way as in mixed glia cells, *o*-orsellinaldehyde was able to significantly reduce the NO production in LPS-stimulated microglia cells. Concretely, the molecule decreases the concentration of NO by a 50%.

3.4 *o*-Orsellinaldehyde decreases iNOS protein expression in LPS-activated mixed glia and microglia cells

To confirm whether the cause of NO reduction observed in mixed and in microglial cells was due to a decrease in iNOS protein level, iNOS protein expression was determined by immunoblot. As figures 2C to 2F depicted LPS increased the expression of this protein in mixed glia and microglia cells, while a significantly suppression in iNOS protein levels was observed due to the presence of *o*-orsellinaldehyde.

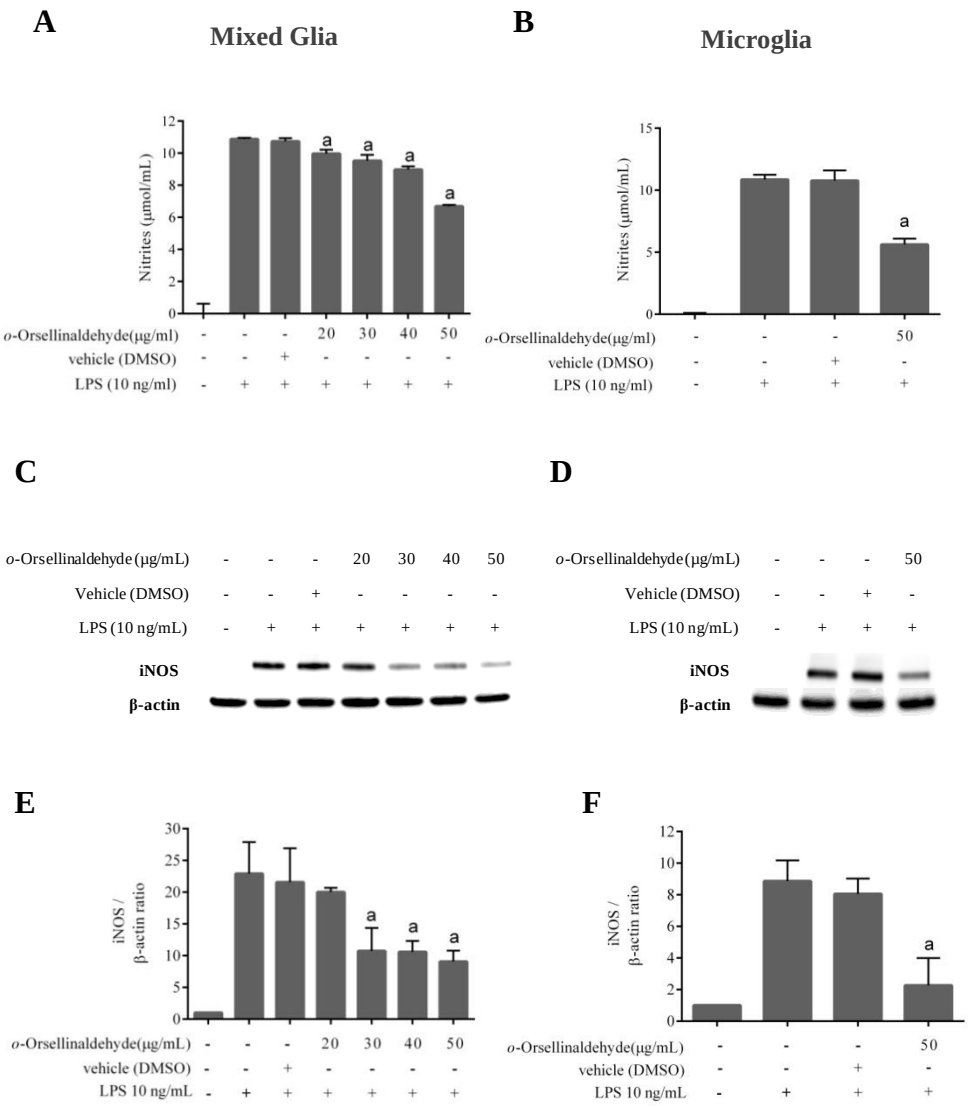

Figure 2. Effect of *o*-orsellinaldehyde on LPS-induced NO and iNOS expression in mixed glia and microglia cells. Cells were incubated with the indicated concentrations of o-orsellinaldehyde for 30 minutes before treatment with LPS (10 ng/mL) for 24 hours. The medium was recollected and cells were lysed for protein extraction. Concentration of NO in mixed glia (**Fig. 2A**) and in microglia cells (**Fig. 2D**) were determined using Griess reaction. Results are shown as the mean of the nitrite production ± SD of four independent experiments. iNOS expression was measured by Western blotting analysis in mixed glia(**Fig. 2B**) and in microglia cells (**Fig. 2E**).

The ratio of densitometry values of iNOS and β-actin was normalized to each respective control group in mixed glia (**Fig. 2C**) and in microglia cells (**Fig. 2F**). Results are expressed as the mean ± SD of three independent experiments. Significant differences relative to the control were analyzed by one-way ANOVA followed by the Bonferroni post hoc test: a=p,0.001.

3.5 *o*-Orsellinaldehyde decreases HO-1 protein expression in LPS-activated mixed glia and microglia cells

HO-1 expression was also determined by immunoblot. As shown in figure 3, LPS-activation of mixed glia and microglia cells remarkably increased the expression of HO-1 protein. However, mixed glia and microglia pretreatment with *o*-orsellinaldehyde reduced its over-expression of HO-1 caused by the LPS stimulation that could be detrimental for the cells.

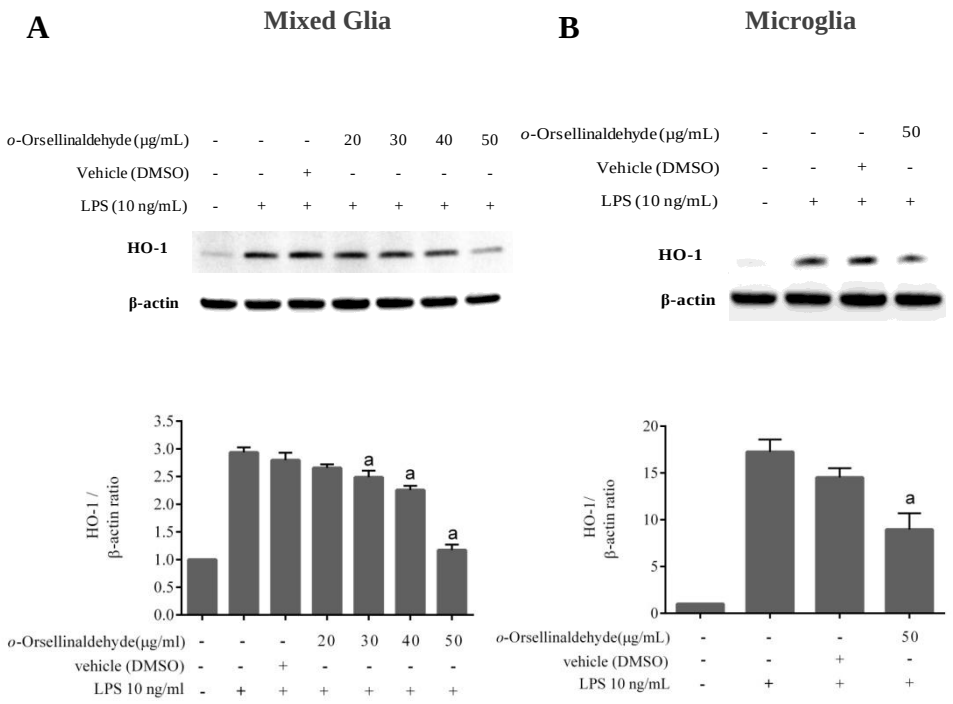

Figure 3. Effect of *o*-orsellinaldehyde on HO-1 protein expression in LPS-activated mixed glia and microglia cells. Cells were incubated with the indicated concentrations

of o-orsellinaldehyde for 30 minutes before treatment with LPS (10 ng/mL) for 24 hours. Whole protein was then extracted and analyzed for HO-1 expression by western blotting in mixed glia (**Fig. 3A**) and isolated microglia (**Fig. 3C**). One of the three experiments is shown. The ratio of densitometry values of HO-1 and β-actin was normalized to each respective control group in mixed glia (**Fig. 3B**) and in microglia cells (**Fig. 3D**). Results are expressed as the mean ± SD of three independent experiments. Significant differences relative to the control were analyzed by one-way ANOVA followed by the Bonferroni post hoc test: a=p,0.001.

3.6 *o*-Orsellinaldehyde suppressed LPS-induced microglia activation through NF-kB signaling pathway.

Our previous studies indicated that *o*-orsellinaldehyde is able to influence on NF-kB activation by inhibiting IKK-2 activity. In order to validate that *o*-orsellinaldehyde was able to inhibit IKβα phosphorylation *in vitro*, we examined the NF-kB signaling pathway in response to LPS in primary microglial cells. Microglial cells were pre-incubated with *o*-orsellinaldehyde (50 µg/mL) for 30 minutes and then, the LPS (10 ng/mL) was added for 45 more minutes. Cells lysates were subjected to western blotting for phospho-IKβα, phospho-p38, phospho-SAPK/JNK.

As figure 4 depicts, LPS stimulation markedly increased the IKβα phosphorylation, however o-orsellinaldehyde pre-treatment resulted in a strong blockade of LPS-induced IKβα phosphorylation.

Regarding the activation levels of p38 and SAPK/JNK MAPK, we could also observe that stimulation of microglial cells with LPS leads the cells to a rapid activation of this MAPK, whereas, pre-incubation of microglia with the tested compound inhibit the phosphorylation of these MAPKs, showing a total kinase inhibition in the case of SAPK/JNK MAPK.

These results confirm that the inhibition of pro-inflammatory mediators expression by *o*-orsellinaldehyde in LPS-stimulated glia cells is associated with a down-regulation of IKβα phosphorylation due to the IKK-2 inhibitory capacity of the molecule. Furthermore, the phosphorylation reduction of p38 and

SAPK/JNK suggests that the anti-inflammatory effect of *o*-orsellinaldehyde could also be caused by the inhibition of the MAPKs signaling pathway.

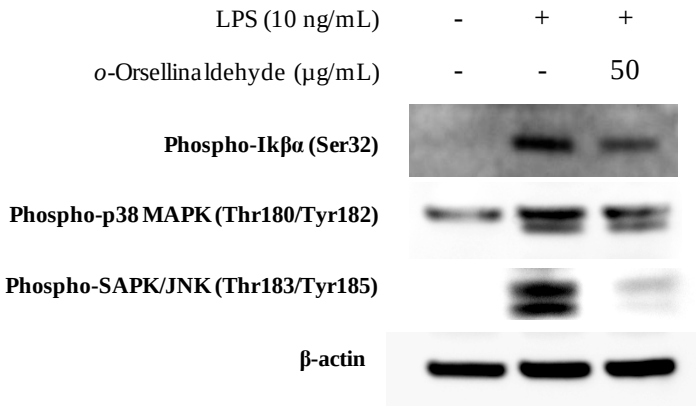

Figure 4. Effect of *o*-orsellinaldehyde on LPS-stimulated NF-KB activity in microclia cells. Microglial cells were pre-incubated for 30 minutes with *o*-orsellinaldehyde at 50 µg/mL and then stimulated with LPS (10 ng/mL) for 45 more minutes. Whole protein was then extracted and analysed for Phospho- Ikβ-α, Phospho-SAPK/JNK, Phospho-p38 MAP Kinase and β-actin expression by western blotting. One of the three experiments is shown.

3.7 *o*-Orsellinaldehyde favored M2 polarization in LPS-activated microglia.

In order to elucidate whether *o*-orsellinaldehyde could promote anti-inflammatory M2 polarization in microglial cells, M1/M2 microglia gene markers were analyzed by Q-PCR after LPS-activation of cells. M1 macrophage phenotype was induced by LPS addition (10 ng/mL) for 6 hours. As shown in figure 5, treatment with *o*-orsellinaldehyde induced a decrease in iNOS and IL-1β gene expression (M1 markers) and an increase in mRNA expression ofIL-10 and MRC-1 (M2 markers) as compared to control cultures, suggesting that *o*-orsellinaldehyde redirected M1 activation to M2 activation. Surprisingly Arg-1

expression displayed the opposite result expected, this result is further examine in the discussion.

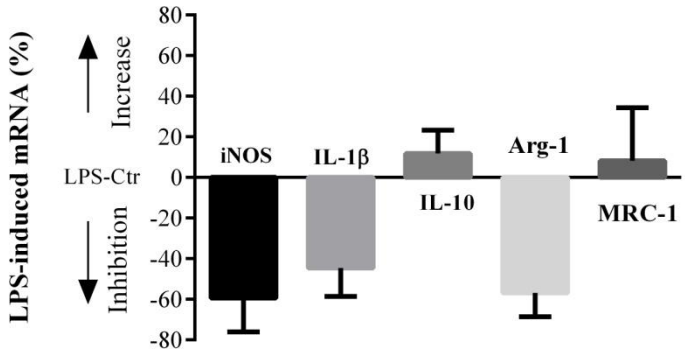

Figure 5. Effect of *o*-orsellinaldehyde on LPS-induced microglia M1/M2-related genes. Microglial cells were pre-incubated for 30 minutes with *o*-orsellinaldehyde at 50 µg/mL and then stimulated with LPS (10 ng/mL) for 6 hours. Cells were lysed for RNA extraction and analysis by real time RT-PCR. Data are given as % of increase or decrease over the corresponding gene in LPS-control medium cultures. Results are expressed as the mean ± SD of three independent experiments, run in triplicate. Significant differences relative to the LPS-control medium were analyzed by one-way ANOVA followed by the Bonferroni post hoc test: a=p,0.001.

4. Discussion

There is accumulating evidences indicating that neuroinflammation plays an essential role in the progression of neurodegenerative disorders pointing microglial activation as a hallmark of this inflammation process [19,20]. Microglia cells, the resident macrophages of the central nervous system, perform a very active and continuous surveillance function. However long-term activation of microglia results in an excessive release of cytokines and inflammatory mediators that will ultimately lead to neurotoxic consequences[4].

In that sense, the inhibition of this activated microglia and thus, reducing the inflammatory response, has been proposed as an effective therapeutic approach against many neurodegenerative disease [21,22]. In order to counteract this detrimental process, there is growing interest in the identification of natural compounds with anti-inflammatory properties [10,11].

Natural products have been isolated and characterized from a wide range of organisms. Microorganisms, mainly bacteria and fungi, are a potent source of bioactive substances and had been widely used in traditional medicine as well as for the isolation of natural compounds with beneficial properties

Among all the natural sources that could be explored for discovery of compounds with neuroprotective properties, culinary-medicinal mushroom have attracted important attention in the recent years. The potential use of this mushroom for the treatment or prevention of some neurodegenerative disease have been well studied [23,24]. For example, *Hericium erinaceus, Ganoderma lucidum, and Sarcodon scabrosus* are three mushroom species that have been reported to have important neuroprotective properties [25–27].

We were particularly interested in *Grifola frondosa* medicinal mushroom since it contains the molecule we are working with on its composition. *Grifola frondosa*, native to the northeastern part of Japan and North America, has been traditionally used as a medicinal mushroom [28]. This mushroom has been widely used for treating pain and some inflammatory states in Asia, and has also several other beneficial health properties [29–31]. Interestingly, Grifola frondosa have been also reported to have neuronal health benefits, concretely, Atsuyoshi et al identified a lysophosphatidylethanolamine in Grifola Frondosa that act as neurotrophic activator [32] .

In a previous research done by our group we demonstrated that the molecule *o*-orsellinaldehyde, also contained in *Grifola frondosa* mushroom, was effective in suppressing the production of inflammatory mediators in RAW2467

macrophages and *in vivo* studies. We were able to state that the anti-inflammatory activity of this molecules was mediated by modulating NF-kB activity, more concretely, acting as IKK-2 inhibitor.

On the basis of this previous results, the aimed of the present study, was to elucidate if the molecule *o*-orsellinaldehyde contained in *Grifola frondosa* specie had also neuroprotective effects.

To be effective as neuroinflammatory modulator the molecule tested should be able to pass the hematoencephalic barrier in order to exert its activity on the brain cells. In that sense, the SwissADME Web tool was used to predict the blood-brain barrier permeation of the molecule o-orsellinaldehyde [17]. This tool predicted that this molecule was indeed capable to pass through the blood-brain barrier.

One of the most well-known inflammatory markers is NO production. Overproduction of NO induces tissue damage associated with chronic inflammation and plays an important role on glial activation induced neuronal death. [33]. In that sense we wanted to elucidate if the molecule tested had inhibitory activity on the NO production. Our findings show that *o*-orsellinaldehyde decreased iNOS protein expression and subsequent NO release in LPS-activated mixed glia and microglia cells.

Regarding the effect of *o*-orsellinaldehyde in HO-1 protein expression, figure 3 shows that LPS-activation of mixed glia and microglia cells remarkably increased the expression of HO-1 protein. Whereas, *o*-orsellinaldehyde pretreatment decreases HO-1 protein expression in LPS-activated mixed glia and microglia cells.

Although most reports hold that up-regulation of HO-1 expression is thought to be protective given the beneficial effects of HO-1 [34], it has also been stated that an excessive expression of this protein can aggravate injury. Thus , it is been

proposed that reducing HO-1 expression could be a good strategy for certain neurodegenerative disorders [35,36]. In that sense, *o*-orsellinaldehyde could be a potential compound to reach this last objective since we stated that this molecule is able to reduce the over-expression of HO-1 caused by the LPS stimulation.

The nuclear factor NF-kB is an important mediator involved in propagating the cellular response to inflammation[37] . Under normal conditions, NF-kB is suppressed in the cytoplasm, upon stimuli, phosphorylation of IKβα by specific kinases such us IKK-2 leads to degradation of NF-kB complex. NF-kB dimmers are then able to translocate to the nucleus and active pro-inflammatory genes [14]. Activation of this transcription factor plays an important role in the inflammatory mediated neurodegenerative disorders in that sense many current anti-inflammatory therapies are focused on blocking NF-kB activity [38,39].

Here (figure 4), we demonstrate that this molecule significantly inhibit IKβα phosphorylation in LPS-stimulated microglia cells, indicating that *o*-orsellinaldehyde exerts its anti-inflammatory effects by blocking NF-kB activation.

In agreement with our results, numerous studies have been published reporting the neuroprotective effect of inhibiting NF-kB activation by natural compounds [40].

Furthermore, MAPKs, including p38 and JNK play an important role in the inflammatory response since they are involved in the microglia activation [41,42]. Several studies have been published suggesting that an effective inhibition of the MAPK pathway decreases the excessive inflammatory response observed in long-term activated glia and thus be beneficial for the treatment of some neuroinflammation states [43,44]

In that sense, we investigated the effect of *o*-orsellinaldehyde in the phosphorylation state of these MAPKs in LPS-stimulated microglia. Our results

indicate that *o*-orsellinaldehyde markedly inhibit the activation of both, p38 and JNK, after LPS stimulation in microglial cells. Thus suggesting that the anti-inflammatory effects of this molecule could also be mediated by the inhibition of the MAPKs signaling pathway.

Regarding the potential of *o*-orsellinaldehyde on promoting M2 polarization in LPS-activated microglia we studied the expression of several gens associated with either M1 or M2 phenotypes. As figure 5 shows, treatment with *o*-orsellinaldehyde induced a decrease in iNOS and IL-1β expression (M1 markers) and an increase in mRNA expression ofIL-10 and MRC-1 (M2 markers) as compared to control cultures in LPS-activated microglia. Unexpectedly, Arg-1, an M2-like marker, decreases its expression when activated microglia was treated with the tested compound. This last observation does not necessarily means that *o*-orsellinaldehyde was not able to induce a glial polarization thought an anti-inflammatory phenotype because the expression of arg-1 was not increased; classical M1/M2 classification is a simplification of matters, and further intermediate phenotypes have been described [45], furthermore it has also been reported that microglia expresses distinct M1 and M2phenotypic markers in the postnatal and adult central nervous system, and arginase-1 is one of this variable markers [46]. Having all this into account and on the basis of our results we can conclude that the molecule tested, *o*-orsellinaldehyde, is able to promote the macrophage switch from inflammatory M1 type to the anti-inflammatory M2 type.

5. Conclusions

In summary, the present study demonstrates that *o*-orsellinaldehyde is a potent modulator of microglia activation and possesses strong neuroprotective effects. This molecule significantly inhibited LPS-induced pro-inflammatory mediators by modulating NF-kB activity, concretely, acting as IKK-2 inhibitor.

Additionally, the anti-inflammatory effect of *o*-orsellinaldehyde was also evidenced in the reduction of the phosphorylation state of p38 and JNK MAPKs in LPS-activated microglia.

Furthermore, this molecule leads microglia cells to a polarized M2 phenotype with anti-inflammatory and neuroprotective characteristics. Therefore, our results show that *o*-orsellinaldehyde could improve some neuroinflammatory states and could be used as a hypothetical candidate for the treatment of several pathologies that deal with neuroinflammation.

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The authors have declared no conflicts of interest.

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CHARACTERIZATION OF THE BIOLOGICAL EFFECTS OF NATURAL COMPOUNDS AGAINST INFLAMMATION, METABOLIC SYNDROME AND CANCER
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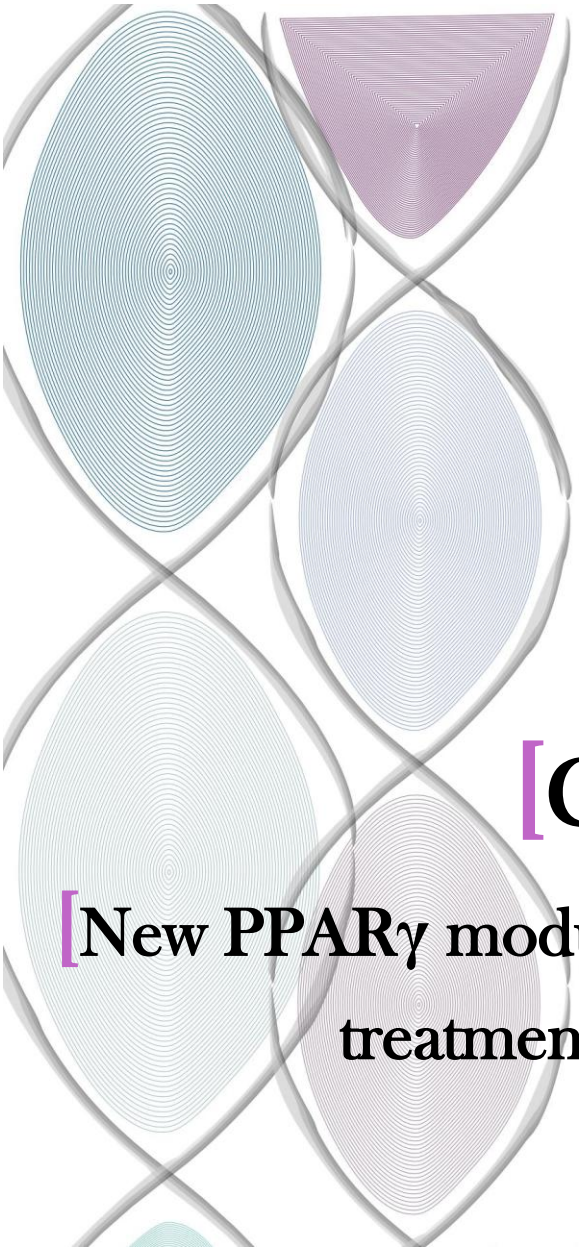

[Chapter 2]

[New PPARγ modulators for the treatment of Diabetes]

UNIVERSITAT ROVIRA I VIRGILI
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TYPE 2 DIABETES MELLITUS: OVERVIEW AND TREATMENT

1.1 Diabetes Mellitus

Diabetes mellitus (DM) is a group of physiological dysfunctions that represents a major health problem. According to the world health organization, 422 million adults worldwide have diabetes, compared to 108 million in 1980. Concretely, the global prevalence (age-standardized) of this pathology has nearly doubled since 1980 from 4.7% to 8.5% in the adult population [1]. Worse still, DM is a leading cause of death worldwide, having killed 1.6 million people in 2015 [2].

This pathology is characterized by hyperglycemia resulting directly from insulin resistance, inadequate insulin secretion, or excessive glucagon secretion [3]. There are two major types of DM, type 1 diabetes (T1DM) and type 2 diabetes (T2DM) being this last one the most prevalent. T1DM, is an autoimmune disorder leading to an absolute deficiency of insulin due to the destruction pancreatic β-cells. Whereas, TD2M is primarily a problem of progressively impaired glucose regulation due to a combination of dysfunctional pancreatic β-cells and insulin resistance [4].

Since T2DM accounts for 90-95% of the diagnosed cases of DM we are going to focus on this type of diabetes. There are many medical conditions which can potentially increase the risk of suffering T2DM such us obesity, hyperlipidemia and several other metabolic syndrome (MetS)-related pathologies [5].

As mentioned above, early T2DM is characterized by insulin resistance in several tissues such as liver, adipocytes and skeletal muscle. This event is associated with an excessive glucose production by the liver and impaired glucose utilization by peripheral tissues resulting in a hyperglycemic state. Pancreatic β-cells increase insulin secretion in order to compensate insulin resistance, however chronic over-stimulation of this cells gradually mitigate them and eventually exhaust the islet β-cell reserve. This leads to a state of

absolute insulin deficiency [4,6]. The transition of early stages of the disease characterized just by impaired glucose management to type 2 diabetes can be influenced by ethnicity, obesity, body fat distribution, sedentary lifestyle aging and other conditions [7].

The general consensus on treatment of T2DM is that this disease can be prevented by lifestyle modification. There are several studies showing that lowering the body mass index by changes on the diet and physical activity significantly reduces the incidence of T2DM [8,9]. However, when lifestyle management measures seems to be insufficient for preventing the onset of the disease, oral glucose-lowering drugs and injections of insulin are the conventional therapies [10].

Among all the existing antidiabetic drugs, thiazolidinediones (TZD) have been widely used in the treatment of T2DM. This molecules are able of reduce insulin resistance by acting as full agonists of the peroxisome proliferator-activated nuclear receptor (PPAR). Despite the demonstrated beneficial effects of this drugs they are under intense scrutiny due to the several undesired side effects that they induce, thus the use of some TZDs has been restricted or even suspended in US and Europe [11].

In that sense, new approaches are needed to prevent and treat T2TD. An interesting strategy aimed to achieve this goal could be the identification of new molecules that present anti-diabetic properties without owning the side effects associated with the existing drugs.

1.2 PPARs: therapeutic targets for metabolic disorder

Peroxisome proliferator activated receptors (PPARs) are transcriptional factors belonging to the ligand-activated nuclear receptor superfamily [12]. Upon activation mainly by dietary fatty acids and their metabolic derivates, they modulate the gene expression of numerous proteins involved in nutrient metabolism and energy homeostasis [13]. Three major types have been

identified (PPAR-α, PPAR-β/δ and PPAR-γ) which exhibit distinct tissue distribution and regulate diverse biological functions. PPAR-α and PPAR-γ are principally expressed in liver and adipose tissue respectively, whereas PPAR-β is predominantly present throughout the body but at low levels in liver [14].

All PPARs function through the formation of obligate heterodimers with the retinoid X receptor (RXR), which bind to the peroxisome proliferator response elements (PPREs) located in the promoter regions of genes. This agonist binding to PPARs triggers a conformational change in the receptor that allows specific recruitment of co-activators and co-repressors and activates the transcriptional machinery [15,16].

The importance of PPARs in physiology and disease is evidenced by the fact that this receptors are targets of effective drugs in the treatment of metabolic disorders. PPAR-γ agonists, such as TZD, are anti-diabetic agents with high insulin-sensitizing actions mediated by large pleiotropic effects in adipose tissue. Several PPAR-α agonists also revealed anti-atherosclerotic and hypolipidemic effects. Furthermore, recent studies proved that PPAR-γ and PPAR-α ligands exert also important anti-inflammatory actions. Although little is known about PPAR-δ and its relation with fat metabolism, recent investigations state that PPAR-δ ligands might have beneficial effects on circulating lipids and obesity[17–20].

1.3 New PPARγ agonists for the treatment of T2DM

An agonist is a chemical that binds to a receptor of the cell and triggers a response on that cell. Thus, when talking about a full agonist we refer to a molecule that binds to a receptor and displays full efficacy at that receptor, activating the transcription of all the genes regulated by this receptor. On the other hand, a partial agonist is a ligand that binds and activates a receptor but have only partial efficacy at this receptor. The ability of PPAR to promote or suppress the transcription of responsive genes depends on interaction of its

ligand binding domain (LBD) with ligands that stabilize receptor conformation [21,22].

The mechanism of PPARγ activation by full agonists is mediated by a molecular switch of the H12 a-helix of this receptor. H12 forms part of the ligand-dependent activation domain, AF-2, that closes on the ligand-binding site in response to ligand binding. The resulting active form can bind to several co-activator proteins that activate the cellular transcriptional machinery [23,24].

In contrast, partial agonists activate PPARγ by an H12-independent mechanism, and consequently, the key interactions between partial agonists and the LBD of PPARγ are different than those of the full agonists. This causes a lower degree of H12 stabilization, which affects the recruitment of coactivators and, in turn, decreases the transcriptional activity of PPARγ [25,26].

The (TZD) anti-diabetic agents are defined as PPARγ full agonists. This insulin sensitizing drugs act by increasing insulin action and inhibiting hepatic glucose output in several tissues [17,27]. However, as previously mentioned, despite the beneficial effects of this compounds they have also been related to significant side effects including weight gain, fluid retention, bone fractures, and cardiovascular disease [11,28]. In that sense, identification of selective PPARγ modulators (SPPARγM) that maintain the insulin sensitizing properties without inducing the undesired side effects represent the major challenge for the development of novel PPARγ-based antidiabetic compounds. Thus, SPPARγM act as partial agonist of PPARγ and display different binding properties when compared with full agonists[26,29,30].

Recent studies reported new SPPARγM that prevented Cdk5-mediated phosphorylation of PPARγ at Ser 273. These compounds demonstrated potent insulin sensitizing activity without displaying PPARγ ligand-related side effects. Thus, suggesting that the anti-diabetic effects of PPARγ ligands occurs through

the inhibition of Cdk5-mediated phosphorylation of PPARγ, whereas the several associated side effects could be related to classical agonisitic actions [31,32]

Bearing this in mind, compounds that do not promote full transcriptional activity of PPARγ but inhibit the Cdk5-mediated phosphorylation at Ser273 could represent a new class of antidiabetic agents that maintain the beneficial effects of the existent drugs without the associated adverse side effects.

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Manuscript 3

Peroxisome Proliferator-Activated Receptor γ (PPARγ) and Ligand Choreography: Newcomers Take the Stage

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Peroxisome Proliferator-Activated Receptor γ (PPARγ) and Ligand Choreography: Newcomers Take the Stage

Miniperspective

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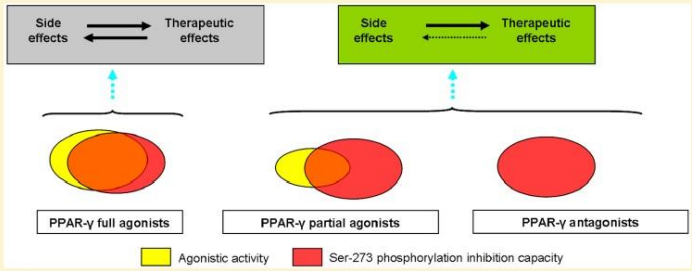
Supporting Information



ABSTRACT: Thiazolidinediones (TZDs), such as rosiglitazone and pioglitazone, are peroxisome proliferator-activated receptor γ (PPARγ) full agonists that have been widely used in the treatment of type 2 diabetes mellitus. Despite the demonstrated beneficial effect of reducing glucose levels in the plasma, TZDs also induce several adverse effects. Consequently, the search for new compounds with potent antidiabetic effects but fewer undesired effects is an active field of research. Interestingly, the novel proposed mechanisms for the antidiabetic activity of PPARγ agonists, consisting of PPARγ Ser273 phosphorylation inhibition, ligand and receptor mutual dynamics, and the presence of an alternate binding site, have recently changed the view regarding the optimal characteristics for the screening of novel PPARγ ligands. Furthermore, transcriptional genomics could bring essential information about the genome-wide effects of PPARγ ligands. Consequently, facing the new mechanistic scenario proposed for these compounds is essential for resolving the paradoxes among their agonistic function, antidiabetic activities, and side effects and should allow the rational development of better and safer PPARγ-mediated antidiabetic drugs.

INTRODUCTION

Peroxisome proliferator-activated receptor γ (PPARγ) is a ligand-activated transcription factor (TF) with a key role in adipogenesis activation.[1] It is also the molecular target of the thiazolidinediones (TZDs), such as pioglitazone and rosiglitazone, which are PPARγ full agonists and are able to promote adipocyte differentiation.[2] TZDs improve glucose metabolism and insulin sensitivity, which justifies their role as antidiabetic compounds, but they have also been linked to important side effects, including weight gain, fluid retention, bone fractures, and cardiovascular disease.[3−5] Consequently, retaining the beneficial glucose-lowering effects of PPARγ agonists while avoiding their undesired side effects represents the major challenge for the development of novel and safer PPARγ-based antidiabetic compounds. Although the objective is well-defined,

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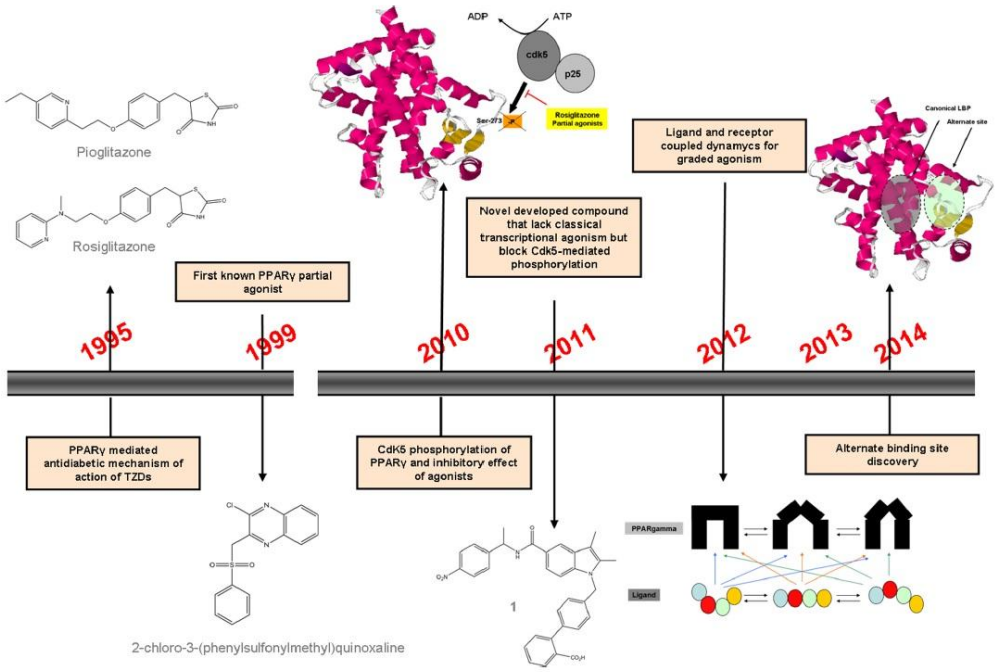

Figure 1. Milestone discoveries regarding the role of PPARγ in diabetes. The image focuses on several important events that have greatly helped to understand the mechanistic basis that governs the effects of different PPARγ ligands.

the lack of understanding of how PPARγ activation by small molecules correlates with the antidiabetic effect complicates the development of such compounds.

Nuclear receptor ligands can induce a spectrum of graded responses, including a full agonism, a partial agonism, antagonism, and an inverse agonism.[6] Full agonist compounds position helix H12 in a conformation favoring interaction with coactivator proteins.[7] Conversely, antagonist compounds block receptor activation either by promoting corepressor interaction or by positioning H12 to preclude coactivator binding.[7]

Despite the large amount of existing knowledge regarding full agonists and antagonists, the molecular mechanisms describing partial agonism remain poorly understood. For example, crystal structures of the apo ligand-binding domain (LBD) of PPARγ, as well as cocrystal structures of PPARγ LBD bound to ligands with partial agonist activity, all present H12 in the canonical position,[8] providing little insight into the mechanism of action of the full versus partial agonists. Additionally, although crystal structures of PPARγ reveal atomic differences in the ligand contacts within the receptor ligand-binding pocket (LBP), they provide no insight into the graded activity of ligands.[9] Thus, further "active" information outside of what was obtained by crystal structures has paramount value because of the dynamism among such nuclear receptors (NRs) and their different types of ligand (i.e., full vs partial agonists).

Besides the above, the application of methodologies such as genome-wide profiling of PPARγ:RXR and RNA polymerase II occupancy is bringing essential information to achieve a better understanding of the molecular mechanistic processes related to these TFs in response to different stimuli (3T3-L1 adipogenesis induction, rosiglitazone, etc.).[10−12] This could complement the knowledge acquired by studying PPARγ post-translational modifications, alternative binding, TF activation, etc. and will significantly help to obtain better and safer PPARγ targeting compounds.

■ PPARγ-TARGETED ANTIDIABETIC COMPOUNDS

The milestone discoveries regarding the role of PPARγ in diabetes are depicted in Figure 1. TZDs were developed in the late 1990s and have been widely used for the treatment of type 2 diabetes mellitus. TZDs function as insulin sensitizers; i.e., they lower serum glucose without increasing pancreatic insulin secretion by binding to PPARγ and inducing its transactivation activity. However, because of their adverse effects,[13] the use of these drugs has been restricted or they have been withdrawn from the market in the U.S., Europe, and other countries. Recently the U.S Food and Drug Administration has removed certain restrictions on prescribing and use of the drug Avandia (rosiglitazone) to reflect new information regarding the cardiovascular risk of the medicine. However, some scientific uncertainty about the cardiovascular safety of rosiglitazone still remains. Nevertheless, to overcome the adverse effects of TZDs, a new class of compounds called PPARγ partial agonists or selective modulators of PPARγ has been developed.[14,15] These compounds bind in a distinct manner to the LBP of PPARγ and cause a differential cofactor recruitment or

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Table 1. Development Status of Some Antidiabetic Drugs Whose Action Is Mediated by PPARγ

generic name	company	compound class	type of PPARγ agonist	development status
aleglitazar	Hoffmann-La Roche	glitazar	PPARα/γ dual agonist	Withdrawn in phase III in July 2013 because of safety concerns (bone fractures, heart failure, and gastrointestinal bleeding).
balaglitazone	Dr. Reddy's Laboratories Ltd.	TZD	partial agonist	Completed phase III in January 2010. Not in development.
farglitazar		glitazar	PPARα/γ dual agonist	Discontinued in 2003.
imiglitazar	Takeda Pharmaceutical Co	glitazar		Suspended during phase III in December 2004 because of abnormalities in liver enzyme tests.
indeglitazar	Wyeth	glitazar	pan agonist	Terminated in phase II in October 2007.
metaglidasen	Metabolex Inc.	non-TZD	SPPARM-gamma	Discontinued after phase II because the glycemic efficacy did not reach the commercial threshold to enter into phase III. It was repurposed for treatment of gout.
(2*R*)-2-[3-[3-(4-methoxybenzoyl)-2-methyl-6-(trifluoromethoxy)indol-1-yl]phenoxy]butanoic acid	Merck	indole derivative	partial agonist	Terminated in phase II in September 2007 based on data suggesting that none of the doses tested demonstrated benefits vs pioglitazone.
muraglitazar	Bristol-Myers Squibb, Merck	glitazar	PPARα/γ dual agonist	Terminated in phase III in May 2006 because it increased the risk of cardiovascular events.
pioglitazone (Actos)	Takeda Pharmaceutical Co., Eli Lilly and Co., Pfizer Inc.	TZD	full agonist	In the market. Withdrawn in France and Germany in June 2011 because it could raise the risk of bladder cancer.
ragaglitazar	Dr. Reddy's Laboratories Ltd., Novo Nordisk	glitazar	PPARα/γ dual agonist	Suspended during phase III in July 2002 because it caused the development of urinary bladder tumors in mice.
rivoglitazone	Daiichi Sankyo Inc.	TZD	SPPARM-γ	Terminated in phase III in May 2009. Currently in phase II for xerophthalmia.
rosiglitazone (Avandia)	GlaxoSmithKline	TZD	full agonist	In the market. Withdrawn in September 2010 in Europe and restricted since November 2011 in U.S. because it increased cardiovascular events.
saroglitazar	Zydus Group	glitazar	PPARα/γ dual agonist	Approved for use in India in June 2013.
tesaglitazar	AstraZeneca	glitazar	PPARα/γ dual agonist	Terminated in phase II and phase III in May 2006 because it was associated with renal problems.
troglitazone	Daiichi Sankyo Co.	TZD	full agonist	Withdrawn in December 1997 in England and in 2000 in U.S. because it increased the incidence of drug-induced hepatitis.

displacement.[16,17] This reduces the transactivation activity of PPARγ and, in some cases, maintains the antidiabetic effect and reduces the adverse effects. However, although several PPARγ partial agonists have reached phase II and phase III clinical trials, none have reached the marketplace because of their side effects or insufficient efficacy. Table 1 shows the development status of some antidiabetic drugs whose action is mediated by PPARγ (the structures of Table 1 compounds are shown in Supporting Information Figure 1).

■ BINDING DIFFERENCES BETWEEN PPARγ AGONISTS

The LBP of PPARγ has a Y-shaped form, consisting of a hydrophobic entrance (arm III) that branches off into two subsites: the polar arm I, which is extended toward H12, and the hydrophobic arm II, which is located between helix H3 and a β-sheet.[18] Full agonists occupy arms I and II and establish a net of hydrogen bonds with the side chains of Ser289, His323, His449, and Tyr473. These interactions stabilize H12 and are responsible for the transactivation activity of PPARγ.[16,19] However, partial agonists interact mainly with arm III through a hydrogen bond with Ser342 and with arm II through several hydrophobic interactions (Figure 2 and Supporting Information Figure 2). This binding mode has two simultaneous effects: a destabilization of H12 and a stabilization of H3 that affect the recruitment of coactivators and decrease the transactivation activity (potential) of PPARγ.[8,14]

Additionally, as shown in Figure 3, the structural studies have also provided essential information to define the "mousetrap" model of NR activation.[20] In this classical model, the ligand is attracted to the trap by the electrostatic potential of the receptor, and a conformational change takes place, preventing ligand exit.[21] The ligand forms an integral part of the hydrophobic core of the LBP and stabilizes the structure. The structural change is different for full agonists compared with partial agonists. In the latter case, the binding of the ligand induces a conformational change in the LBP, whereby the activation-function 2 (AF-2) sequences fold back against the binding pocket, obstructing the opening and causing rearrangements of the adjacent helices. In the process, a new surface is revealed and recruits specific coactivators (containing LXXLL motifs) required for transcriptional activation.

■ PPARγ ANTAGONISTS THAT INHIBIT THE Cdk5-MEDIATED PHOSPHORYLATION OF PPARγ

The different PPARγ binding modes of the full and the partial agonists do not fully explain their respective antidiabetic properties. In 2010, a new antidiabetic mechanism of PPARγ-targeted drugs was described[22] (Figures 1 and 3). This novel mechanism depends upon the inhibition of PPARγ Ser273 phosphorylation by cyclin dependent kinase 5 (Cdk5). This

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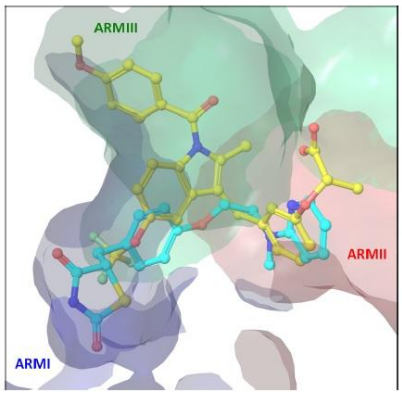

Figure 2. Binding differences between PPARγ partial and full agonists. The structure of the LBP of PPARγ forming a complex with 4 (a partial agonist, in yellow) from the PDB entry 2Q5P is superimposed with the structure of rosiglitazone (a full agonist, in blue) from the PDB entry 1FM6. 4 only shows a 21% PPARγ transactivation activity with respect to the maximum activation of rosiglitazone, but it effectively reduces hyperglycemia in the db/db model. The partial agonists occupy mainly arm II and arm III of the LBP of PPARγ, but the full agonists occupy mainly arm I and arm II. Both structures were validated by VHELIBS software[79] and then were aligned by Maestro (Schrodinger).

inhibition maintains the transcription of several insulin-response genes, such as adiponectin and adipsin.[22] Interestingly, obesity and other proinflammatory signals induce the phosphorylation of PPARγ at Ser273, explaining why most obese people also develop insulin resistance.[22] Furthermore, the Cdk5-mediated phosphorylation of PPARγ does not affect its transactivation activity and its role in adipocyte differentiation.[22] This new mechanistic scenario is in agreement with the fact that the extent to which a compound activates PPARγ is not correlated with the extent of its antidiabetic effect and may explain how PPARγ partial agonists can exhibit similar or higher antidiabetic effects than those of TZDs and other full agonists. Additionally, it might also be the reason for the differing side-effect profiles of PPARγ partial and full agonists and might explain why some predicted PPARγ antagonists exhibit antidiabetic effects.[23−25] It is therefore possible that the therapeutic benefit of PPARγ ligands as insulin sensitizers occurs through the inhibitory effect on Cdk5-mediated phosphorylation of PPARγ, whereas the various "agonistic potency" could be related to differences in side effects.[22] Thus, compounds that do not promote the transactivation activity of PPARγ but block the Cdk5-mediated phosphorylation at Ser273 could represent a new class of antidiabetic drugs that would have the antidiabetic benefits of TZDs without the associated adverse side effects. Again in 2011, Spiegelman, Griffin, and co-workers developed the novel synthetic compound **1** (SR1664),[26] which lacks the classical PPARγ transcriptional agonism activity but blocks the Cdk5-mediated phosphorylation of PPARγ at Ser273 in cultured adipocytes and in insulin-resistant mice[26] (Figure 1). In addition, **1** does not cause fluid retention and weight gain and does not interfere with bone formation,[26] two serious side effects described for TZDs and other PPARγ ligands. Because **1** antagonizes the transcriptional activity of rosiglitazone,[26] this type of compound has been referred to as a PPARγ antagonist capable of blocking phosphorylation of Ser273.[27] The structural requirements for the nonagonist actions of **1** and related compounds, such as **2** (SR1824),[26] suggest that antagonist and nonagonist activities are closely related. Ligands that do not affect the dynamic conformational switch of H12 cannot be considered as nonagonists; rather, they appear to function as partial agonists.[26] Although **1** and **2** do not interact with H12 in any detectable way, both ligands cause an increase in the conformational mobility of H11 that distorts the H12 enough to block partial agonism.[26] Whether there are other alternative modes of ligand binding that would lead to a complete lack of classical agonism remains to be determined.[26] Other PPARγ antagonists, such as the ginsenosides Rh2 and Rg3 from *Panax ginseng* and Tanshinone IIA from *Salvia miltiorrhiza*, are able to improve glucose tolerance in vivo.[28,29] PPARγ antagonists that block adipocyte differentiation have clinical potential for the treatment of type 2 diabetes and obesity.[23,24] The underlying mechanisms linking the inhibition of phosphorylation at Ser273 and the antidiabetic effects of PPARγ agonists are not fully understood. It is likely that PPARγ ligands reduce the dynamic nature of the region containing Ser273, "freezing" this region in a configuration less favorable to Cdk5 phosphorylation.[22] In addition, specific coregulator modulation of PPARγ by modifications at Ser273 is highly plausible.[22] Recently, it has been shown that thyroid hormone receptor-associated protein 3 (Thrap3) can directly interact with PPARγ when it is phosphorylated at Ser273, and this interaction controls the diabetic gene programming mediated by the phosphorylation of PPARγ.[30]

Despite the initial interest caused by the new antidiabetic mechanism of PPARγ agonists,[5,31−36] the analysis of the inhibition of PPARγ phosphorylation at Ser273 is not yet a routine way to search for new PPARγ-targeted compounds or to validate the antidiabetic effect of known compounds. Although several ligands that stimulate the insulin-induced glucose uptake of adipocytes and do not stimulate the transactivation activity of PPARγ have been identified, none of them, with the exceptions of **1**[26] and **3** (UHC1),[37] have been tested for the inhibition of the Cdk5-mediated phosphorylation of PPARγ. In some cases, although relatively potent binders that do not induce PPARγ transcriptional activity have been found, these interesting compounds were discarded.[38] This is also evidence for other related PPAR-targeted compounds, such as PPARα/γ dual agonists or PPAR pan agonists. These compounds, such as glitazars, are expected to lower high blood triglycerides (the agonist action on PPARα), improve insulin resistance, and consequently lower blood sugar (the agonist action on PPARγ).[4] However, inhibitory activity of PPARα/γ dual agonists on the Cdk5-mediated phosphorylation of PPARγ has not yet been reported.

Table 2 and Figure 4 show the PPARγ-mediated compounds that have been analyzed for their inhibitory capacity on Cdk5-mediated Ser273 phosphorylation. Rosiglitazone blocks the phosphorylation of PPARγ at Ser273 in vitro, with a half-maximal effective dose of approximately 30 nM.[22] **4** (MRL24) is more effective at blocking Cdk5-mediated phosphorylation than rosiglitazone.[9,22] 30 nM **4** inhibits the phosphorylation of PPARγ as effectively as 300 nM rosiglitazone.[22] Other PPARγ agonists, including **1**,[26] **3**,[37] **5** (MBX-102),[22] **6** (BVT.13),[22] **7** (nTZDpa),[22] **8** (compound 7b),[26] **9** (GQ-16),[39] **10** (MRL20),[9] **11** (AZ6106),[40] **12** (R35),[41] **13** (S35),[41] nonanoic acid,[42]

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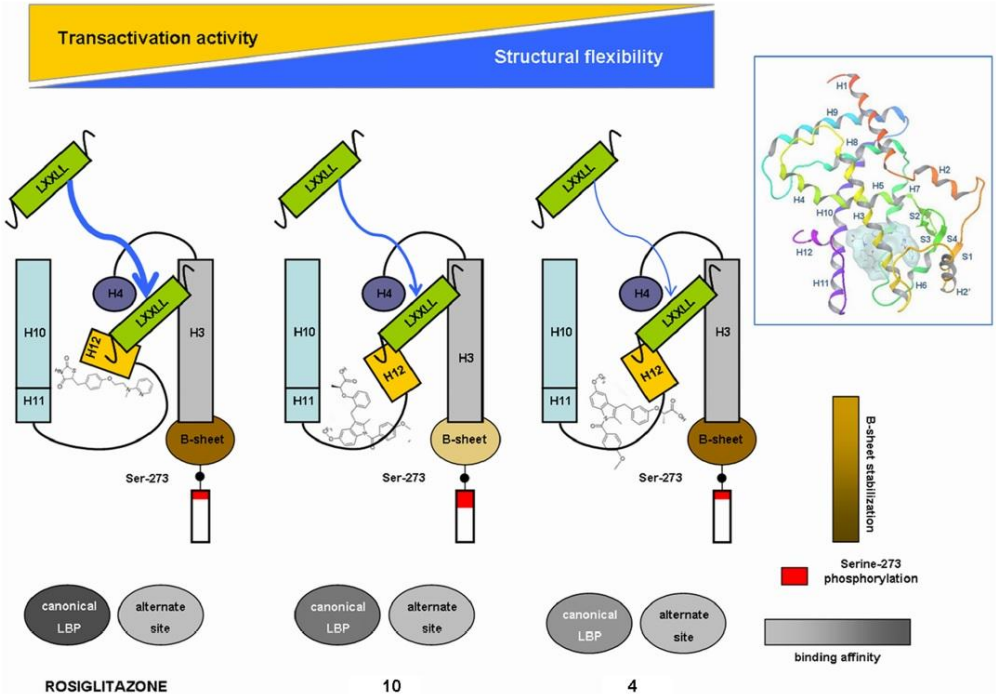

Figure 3. Mechanistic insights of the PPARγ graded agonism. The ligand forms an integral part of the hydrophobic core of the LBD, promoting rearrangements in adjacent helices that stabilize the structure (i.e., H3, H10, H11, and H12). This structural change is different for ligands that are full agonists (rosiglitazone and **10**) versus those that are partial agonists (**4**), differentially affecting the recruitment of transcriptional coactivators (containing LXXLL motifs; see blue arrows). PPARγ agonists show different binding modes with variable stabilization degrees of helix H12 promoting different levels of agonism activity (transactivation capacity vs structural flexibility; the width of the blue arrow is inversely correlated with structural flexibility). The ligand-induced degree of stabilization of the β-sheet near Ser273 (brown intensity) plays an essential role in Ser273 phosphorylation (see red bars size). The presence of an alternative binding site also modulates the final effects of PPARγ ligands. Figure is adapted from John P. Vanden Heuvel, nrresource.org.

ionomycin,[43] telmisartan,[44] and pseudoginsenoside F11,[45] have been shown to inhibit PPARγ phosphorylation in vitro. Rosiglitazone, **1**, **3**, and **4**, amorfrutin 1, and ionomycin are in addition able to inhibit the PPARγ phosphorylation in vivo in white adipose tissue (WAT).[22,26,43,46] Zhang and co-workers[47] found that **14** (C333H)[47] had no impact on PPARγ phosphorylation in WAT but observed a reduction in brown adipose tissue. However, these authors found that rosiglitazone does not significantly reduce PPARγ phosphorylation at Ser273 in WAT.[47]

Amorfrutin B and decanoic acid are unable to block the Cdk5-mediated phosphorylation of PPARγ.[48,49] Surprisingly, in the case of amorfrutin B, the authors did not observe any significant change in vivo of Ser273 phosphorylation even after the rosiglitazone treatment.[48] Both compounds, amorfrutin B and decanoid acid, are able to reduce fasting blood glucose in mice.[48,49] More evidence is therefore needed to rule out that the antidiabetic properties of these compounds are independent of the Ser273 phosphorylation inhibition of PPARγ.

■ ROLE OF STRUCTURAL DYNAMICS IN PPARγ GRADED AGONISM

Despite the discovery of the important role of the Ser273 phosphorylation inhibitory capacity played by the PPARγ agonists, experimental work and related knowledge have been rapidly evolving. As a consequence, other interesting mechanistic features about the interrelation among PPARγ receptor and ligands have recently emerged. In this sense, several important mechanistic details that could be responsible for the degree of ligand agonism as well as the therapeutic and side effects of PPARγ targeting ligands have been depicted[9] (Figures 1 and 3). Interestingly, they suggest that the level of partial agonism could be related to (a) the presence of two PPARγ receptor populations in slow intermediate conformational states, (b) the degree of receptor intermediate conformational exchange within the LBP and AF-2 region, (c) the degree of specific PPARγ stabilization brought by its interaction with different types of agonists (i.e., rosiglitazone, compounds **4** and **10**), and (d) the ligand's capacity to "assay" multiple binding modes and the relative binding mode occupancy.[9]

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Table 2. PPARγ-Mediated Compounds That Have Been Analyzed for Their Capacity To Block Cdk5-Mediated PPARγ Phosphorylation at Ser273

compd	transactivation activity, EC_{50} (μM)[a]	effects on glucose metabolism	PDB	block the phosphorylation at Ser273	ref
amorfrutin 1	0.458 (39%)	Reduces plasma insulin and glucose concentrations in leptin receptor-deficient db/db mice.	2YFE	Yes in vivo	46
amorfrutin B	0.073 (25%)	Reduces fasting blood glucose, plasma insulin, and insulin resistance in HFD-fer C57BL/6 mice.	4A4W	No in vivo	48
11 (AZ6106)				Yes in vitro	40
6 (BVT.13)	1.3 (80%)	Reduces fasting plasma glucose and plasma insulin in ob/ob mice.	3DZU	Yes in vitro	22, 74
14 (C333H)	0.155 (100%)	Ameliorates insulin resistance in MSG rats and significantly decreases fasting blood glucose levels in db/db mice.		Yes in vivo	47, 75
8 (compound **7b** and derivatives)	0.54 (15%)	Compound **7b** efficiently decreases plasma glucose levels by 25% in Zucker fa/fa rats.	3KMG	Yes in vitro	26, 76
decanoic acid	(60% approx)	The triglyceride form (glyceryl tridecanoate) decreases the fasted blood glucose levels in diabetic mice but had no significant effect on weight gain.	3U9Q	No in vivo	49
9 (GQ-16)	(33% approx)	Improves insulin sensitivity without promoting weight gain in a mouse model of diet-induced obesity and insulin resistance.	3T03	Yes in vitro	39
ionomycin	(35% approx)	Significantly reduces the levels of serum glucose and insulin in db/db mice.	4FGY	Yes in vivo, in vitro	43
5 (MBX-102)	12 (10%)	Reduces fasting plasma glucose levels in several rodent models of type 2 diabetes, including ob/ob, db/db mice, and Zucker fatty diabetic (ZDF) rats.		Yes in vitro	22, 74
10 (MRL20)	0.002 (60% approx)		2Q59	Yes in vitro	9, 77
4 (MRL24)	0.002 (21%)	Effectively reduces hyperglycemia in the db/db model.	2Q5P	Yes in vitro, in vivo	9, 22, 77
nonanoic acid and other C8−C10 MCFAs			4EM9	Yes in vitro	42
7 (nTZDpa)	0.057 (25%)	Ameliorates hyperglycemia and hyperinsulinemia in animal models.	2Q5S	Yes in vitro	22
pseudoginsenoside F11	(30% approx)			Yes in vitro	45
12 (R35), **13** (S35)	0.00038 (70% approx), 0.183 (60% approx)			Yes in vitro	41
rosiglitazone	(100%)	Reduces glucose, fatty acid, and insulin blood concentrations.	1FM6	Yes in vitro, in vivo	22
1 (SR1664)	not active (0%)	Causes a trend toward lowered (and normalized) glucose levels and a significant reduction in the fasting insulin levels in obese and insulin-resistant mice. In addition, intravenous treatment resulted in a robust improvement in insulin sensitivity in leptin-deficient ob/ob mice.		Yes in vitro, in vivo	26
telmisartan	4.5 (30%)	Reduces significantly serum glucose levels after 5 weeks of treatment in Sprague−Dawley rats on a high-fat and high-carbohydrate diet	3VN2	Yes in vitro	44, 78
3 (UHC1)	not active (0%)	UHC1 treatment dramatically improved insulin sensitivity in high-fat diet-fed mice without causing fluid retention and weight gain		Yes in vitro, in vivo	37

[a]In parentheses: % of maximal activation relative to rosiglitazone.

These new data complement the "classical knowledge" of NR structure−function relationships that have been essentially derived from information from many X-ray crystallography structures, which have been collected from "fixed" conformations of ligand−receptor complexes.[9] In this sense, it also pinpoints the necessity of using other methodologies such as solution nuclear magnetic resonance (NMR) and hydrogen/deuterium exchange to better assess the dynamic behavior of NR−ligand structures and to describe the pharmacological phenotype of the ligand that could not be inferred from the crystal structures.

Interestingly, it has been shown that by sampling of more than one binding mode to the PPARγ's LBP, two agonists (**4** and **10**) less potent than rosiglitazone were able to allosterically propagate a conformational exchange that altered the surfaces of the PPARγ receptor, which are important for function.[9] Notably, such structural changes induced by **4** and **10** were associated with a reduced degree of agonism but were not related to the ability of Ser273 phosphorylation surface stabilization.[9] In this sense, such knowledge helps to profile the molecular action of the PPARγ ligands and to understand and predict the relationship between the specific therapeutic effect (insulin sensitizing activity) and some of the undesired side effects (adipogenicity, edema, etc.). It became obvious that this type of information must not be neglected in the development of new and safer PPARγ agonists.

■ AN ALTERNATIVE BINDING SITE COULD ALSO BE RESPONSIBLE FOR PPARγ ACTIVATION

It has recently been shown that PPARγ ligands can also bind to an alternative binding site, leading to unique receptor conformational changes that impact coregulator binding, transactivation, and target gene expression.[50] Interestingly, such alternative binding is possible even if the canonical LBP is covalently bound to endogenous ligands or to irreversible antagonists[50] (Figures 1 and 3). In a series of elegant experiments Prof. Kojetin's group showed that (a) the stoichiometric addition of **4** and **10** to PPARγ induced a sequential saturation of two ^{19}F NMR resonances, which implicate a second PPARγ−ligand binding event; (b) by

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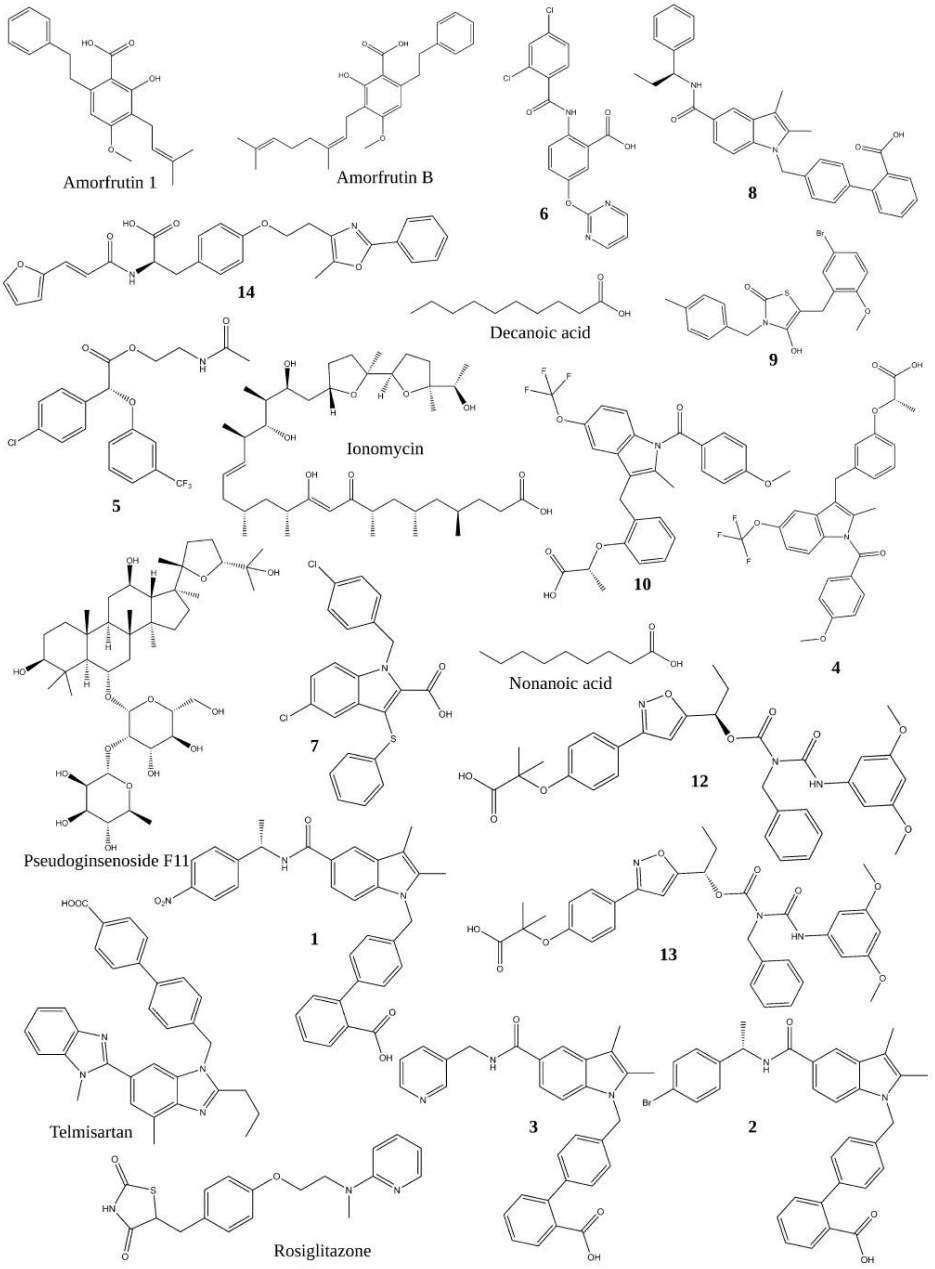

Figure 4. 2D representation of the PPARγ ligands from Table 2. The structure of 11 has not been disclosed.

blocking the canonical LBP with the irreversible agonist 2-chloro-5-nitro-*N*-phenylbenzamide with or without the PPARγ agonists (4 and 10), they were able to induce the ^{19}F NMR chemical shift, which indicates that covalent agonists do not

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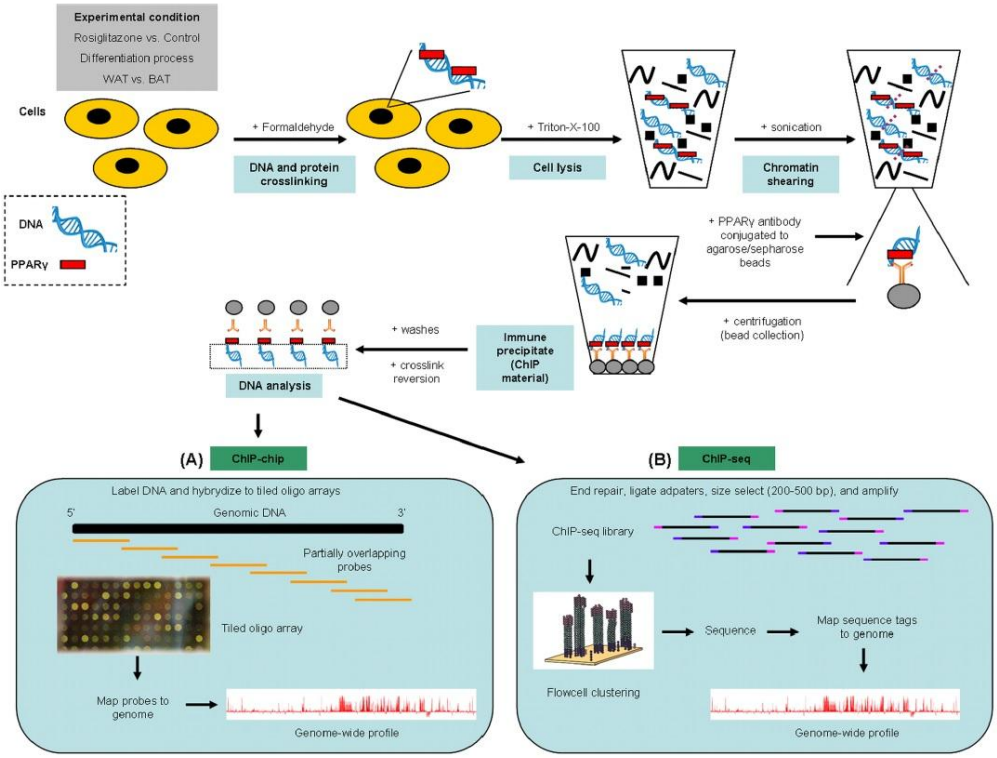

Figure 5. The chromatin immunoprecipitation (ChIP) assay is a powerful method for analyzing epigenetic modifications and genomic DNA sequences bound to specific regulatory proteins. In ChIP assay protein−DNA complexes are cross-linked, immunoprecipitated, purified, and amplified for gene- and promoter-specific analysis of known targets using real time PCR or sequencing or for the identification of new target sequences. (A) ChIP-chip. Microarray technology allows the generation of high resolution genome-wide maps of protein/protein modifications. DNA purified from the immunoprecipitated chromatin and input are labeled with fluorescent dyes using ligation mediated PCR. The fluorescently labeled DNA is applied to the microarray and after subsequent image analysis; the enrichment of the regulatory protein relative to the input is recorded in each genome locus. (B) ChIP-seq. Direct sequencing of the DNA isolated generates genome wide profiles. ChIp-seq combines ChIP and direct sequencing technology for genome-wide analysis of antigen distribution. Immunoprecipitated DNA is sequenced and mapped to the genome. Based on a figure from ref 80.

block the alternative binding site; (c) by the same strategy, but using time-resolved fluorescence resonance energy transfer and a gene reporter assay, they were able to study the coregulator interaction, as well as PPARγ transactivation, and showed that the binding to the alternative site affects coregulator interaction (thryroid hormone receptor-associated protein subunit 220 (TRAP220) and nuclear receptor corepressor (NCOR)) and transactivation (increased transactivation and aP2 expression).[50] Altogether, these results strongly support the presence of a second alternative binding site that, despite having low affinity, can play an important role in the functional final output of PPARγ agonists. Despite the efforts, a cocrystal structure of a PPARγ ligand bound in the alternative site has yet to be obtained.[50]

The conclusions raised by this study shed light on the reasons why antidiabetic partial agonists have not progressed to the clinic. Notably, the development of compounds that block PPARγ Ser273 phosphorylation with little transactivation activity can be complicated by an alternative binding site that could contribute to PPARγ hyperactivation.[50] Additionally, further research is needed to clarify the extent of the so-called "PPARγ-independent" effects of its ligands that could be mediated by the alternative binding site.

■ GENOME-WIDE ACTIONS OF PPARγ AND ITS LIGANDS

Novel strategies based on targeting post-translational modifications of PPARγ may improve the effectiveness of novel treatments against T2D. Additionally, new methods are also needed to evaluate and limit off-target PPARγ activation in non adipose tissue. Knowing the gene- and tissue-selectivity of this novel class of compounds would be an important step in selecting the more efficient ones while reducing their side effects. The integration of these data with gene expression profiling under conditions that affect PPARγ levels of activity

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may reveal cell type-specific PPARγ transcription networks that potentially could be targeted in tissue-selective ways.

Recently, chromatin immunoprecipitation (ChIP) has been combined with high throughput techniques such as tiled oligo arrays (ChIP-chip) and deep sequencing (ChIP-seq) to get an unbiased global map of TF binding and distribution of histone marks. ChIP-seq is currently the method of choice in transcriptional genomics, as this technique is cost-efficient, is very sensitive, and has a resolution that is much higher (up to 1 bp) than ChIP-chip. These techniques have contributed to the deciphering of the genome-wide actions of PPARγ full agonists (Figure 5).

Genome-wide profiling of PPARγ and retinoic X receptor (RXR) binding in 3T3L-1 adipocytes demonstrated that PPARγ:RXR was bound to thousands of sites in mature adipocytes.[10,51,52] Furthermore, it has been shown that the time course of PPARγ binding during adipogenesis followed the induction of PPARγ protein levels, whereas RXR was already bound to many sites in the undifferentiated state, probably as a heterodimer with PPARδ or other NRs.[10] Interestingly, by using ChIP-seq, it has also been demonstrated that only a small fraction (<10%) of the PPARγ binding sites were located close to genic regions whereas most of binding sites were found in distal intergenic regions or in intronic regions.[10] Additionally, ChIP-seq was also used to determine the genome-wide occupancy of RNA polymerase II during adipogenesis as a measure of transcriptional activity.

Data from genome-wide analyses also indicated that members of the CCAAT/enhancer binding protein (C/EBP) family may directly promote or modulate PPARγ activity at the majority of its binding sites.[53] Interestingly, the co-occupancy of C/EBPs with PPARγ in adipocytes was not restricted to C/EBP α, since C/EBP γ was also bound to many target sites even in the mature adipocytes. This indicated that C/EBP β in addition to being an early inducer of PPARγ expression may also cooperate with PPARγ in mature adipocytes.[53]

Importantly, it was found that the genome wide binding pattern of PPARγ in adipocytes did not change dramatically in response to synthetic agonists.[11] However, binding of PPARγ to many preexisting binding sites in 3T3-L1 was enhanced in response to acute treatment with the PPARγ full agonist rosiglitazone. This enhanced binding of PPARγ correlated with increased recruitment of mediator subunit 1 and expression of nearby genes, indicating that enhanced PPARγ recruitment plays a role in the activation of PPARγ targets in response to rosiglitazone in adipocytes.[11]

Comparison of PPARγ binding profiles in adipocytes and macrophages revealed that PPARγ binding is largely cell type-specific, with macrophage unique binding occurring near genes with functions in immune defense as well as in cytokine/chemokine-mediated signaling.[54] By contrast, the small amount of overlap that exists in binding locations between the cell types occurred near metabolic genes.[54] These findings provided a molecular mechanism for the roles of PPARγ in the function of alternatively activated macrophages, in addition to its well-established role in lipid metabolism.

Attempts to determine what drives cell type-specific PPARγ recruitment led to the identification of tissue-specific TFs that could colocalize and cooperate with PPARγ on a genome-wide scale. For example, it has been shown that whereas adipocyte PPARγ sites are located in the proximity to C/EBP α/β binding, in macrophages PPARγ tends to colocalize with the hematopoietic factor PU.1 in addition to C/EBPs.[55]

Altogether, these discoveries emphasize the potential of these genome wide-profiling techniques on knowledge acquisition of PPARγ functions on several cell types and pinpoint the need for considering such experimental approaches in drug discovery. A comparison of genome-wide effects of full vs partial PPARγ agonists could be a very interesting possibility for the discovery and understanding of particular mechanisms involved in heterodimerization, sequence targeting, binding-site genome distribution, tissue specificity, and PPARγ cooperation with other TF.

■ EXPERIMENTAL CHARACTERIZATION OF PPARγ-MEDIATED ANTIDIABETIC COMPOUNDS

A new PPARγ-mediated antidiabetic compound could be characterized in vitro by analyzing the binding affinity to the LBD of PPARγ, checking the capacity to promote the transactivation activity of PPARγ, the differentiation of preadipocytes into adipocytes, the insulin-induced glucose uptake of adipocytes, and the inhibition of the Cdk5-mediated Ser273 phosphorylation. The ideal PPARγ-targeted antidiabetic compound would be a compound that binds potently to PPARγ, does not stimulate the transactivation activity of PPARγ, does not induce adipogenesis, stimulates the insulin-induced glucose uptake, and blocks the Cdk5-mediated phosphorylation of PPARγ. In addition, it may be interesting to check the capacity of a compound to antagonize the action of rosiglitazone or other PPARγ full agonists. Further information about experimental characterization of PPARγ-mediated antidiabetic compounds can be found in the Supporting Information.

■ FUTURE DIRECTIONS

The major challenge of PPARγ-based drug discovery remains how to retain the beneficial glucose-lowering effects of PPARγ ligands and at the same time avoid the undesired side effects.[56,57] The distinction between the transcriptional activity and the Cdk5-mediated phosphorylation of PPARγ might represent a cardinal point for the discrimination among the beneficial vs unbeneficial outcomes of such drugs. Because some PPARγ activating ligands, such as amorfrutin B and decanoic acid, show a powerful glucose-lowering effects in vivo, but they do not inhibit Cdk5-mediated Ser273 phosphorylation of PPARγ, other complex mechanisms of PPARγ-mediated antidiabetic effects could be possible. Further evidence that links the block of the Cdk5-mediated phosphorylation of PPARγ and the antidiabetic effects is needed. Nevertheless, PPARγ antagonists that inhibit the Cdk5-mediated phosphorylation of PPARγ could lead to the development of a new generation of safer antidiabetic drugs.[27] To reach this goal, testing the Cdk5-mediated phosphorylation inhibition of PPARγ must become a routine analysis. Comparison of the ability of several compounds to inhibit Cdk5-mediated phosphorylation of PPARγ would improve understanding of the molecular interactions responsible for this activity. Despite the fact that the densitometry analysis has suggested an efficacy rank order of **4** > rosiglitazone > **10**,[9] **3** > **1**,[37] and **12** > **13**,[41] we lack of this type of information for other PPARγ-mediated antidiabetic compounds.

Although several ligand receptor cocrystal structures of PPARγ have been reported,[8,58,59] the publication of the cocrystal structure of the LBD of PPARγ with an antagonist such as **1**, which is capable of blocking the phosphorylation of

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Ser273, is still lacking and would be of great interest. This structure could be used to better characterize the functional differences between TZDs and diverse PPARγ agonists or antagonists and help to elucidate the mechanism of action of different ligands.

Although several effective Cdk5 inhibitors exist, Cdk5 activity is essential for proper development of the mammalian nervous system, and dysregulation of this enzyme has been implicated in several neurogenerative diseases.[33] For this reason, complete inhibition of Cdk5 would also interfere with its other functions, and drugs that specifically affect PPARγ are preferable.[31] In addition, recent results in a CdK5 adipose tissue knockout mice show that these animals present both a paradoxical increase in PPARγ phosphorylation at Ser273 and worsened insulin resistance.[60]

The last finding about the role of Thrap3 in the diabetic gene programming caused by the phosphorylayion of PPARγ at Ser273[30] opens up further possibilities for antidiabetic drug development. Thus, compounds that avoid the docking of Thrap3 on PPARγ, through the interaction with either PPARγ or Thrap3, may provide new opportunities for a new type of antidiabetic.[30] Recently it has been found that extracellular signal-regulated kinase (ERK) kinases directly phosphorylates PPARγ at Ser273.[60] Increasing concentrations of pioglitazobe block this phosphorylation.[60] In addition, MEK/ERK inhibitors improve insulin resistance in both obese wild-type and ob/ob mice.[60] These data suggest that MEK/ERK inhibitors, alone or in combination with a nonagonist ligand that blocks kinase accesibility to PPARγ Ser273, may hold promise for the treatment of type 2 diabetes.[60]

There is increasing evidence supporting novel therapeutical perspectives for PPARγ agonists. The potential beneficial role of TZDs and other PPARγ agonists in asthma,[61] psoriasis,[62] cancer,[63] diabetic nephropathy,[64] drug addiction,[65] non-alcoholic fatty liver disease,[66] Alzheimer's disease,[67] Parkinson's disease,[68] and other conditions is being investigated. This means that the development of new full or partial agonists of PPARγ is still relevant and necessary. However, the antidiabetic mechanism of TZDs has shown us that other post-transcriptional modifications of PPARγ or additional mechanisms could also be related to these novel beneficial effects of the PPARγ agonists. For example, the anti-inflammatory effect of PPARγ agonists could be mediated by the transrepression of NFkβ inflammatory genes through SUMOylation of PPARγ NR at lysine 365,[69] and the acetylation of PPARγ is related to the brown remodeling of WAT tissue, which will be an interesting therapeutic approach to staunch the current obesity epidemic.[70,71]

Recently, a PPARγ-independent mechanism has been proposed for the effect of TZDs on insulin resistance and a novel TZD analog class with very low affinity for binding and activation of PPARγ.[72,73] The beneficial effects of these compounds were preserved in hepatocytes from liver-specific PPARγ$^{-/-}$ mice, suggesting that PPARγ is not required for the action of these compounds.[72,73] However, more evidence is needed to confirm or reject the hypothesis that these compounds use one of the already known mechanisms, such as the inhibition of the Cdk5-mediated phosphorylation of PPARγ in the WAT, to induce such beneficial effects.

Finally, the concept of structural dynamics in the NR field has been derived primarily from the structural plasticity of H12 in the LBD switching between an "on" (agonist) and "off" (antagonist) conformation in ligand receptor cocrystal structures. This simplistic view must be refined by considering the novel mechanistic insights, as well as the "NR-ligand's behavior" from a static to a dynamic point of view. In this regard, although Cdk5-mediated phosphorylation of PPARγ is of paramount importance for a better understanding and profiling of the structural characteristics of novel PPARγ agonists, we must not forget the recent findings about the contribution of ligand and receptor to the mechanism of graded PPARγ agonism, as well as the possible influence of an alternative ligand binding site for PPARγ activation. Altogether this information will contribute to the understanding of the fine-tuned regulation of PPARγ activity, allowing for the design of a new generation of agonists with better and safer characteristics.

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental characterization of PPARγ-mediated antidiabetic compounds, the 2D representation of the PPARγ ligands from Table 1, and the crystal structure complex interactions of ligand-binding pocket of PPARγ with some of the compounds from Table 2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Laura Guasch received her Ph.D. in Biochemistry in 2011 at Universitat Rovira i Virgili (URV, Tarragona, Spain). There, she worked on the identification of natural products as antidiabetic agents using computer-aided drug design methods. Since 2012, she joined Marc C. Nicklaus' group at the National Institutes of Health (NIH, NA, U.S.) as a Postdoctoral Fellow. Her research interests cover several aspects of molecular modeling and chemoinformatics, such as drug design, virtual screening, and tautomerism.

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two important mediators of inflammation pathways and energy balance.

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■ ABBREVIATIONS USED

AF-2, activation function 2; C/EBP, CCAAT/enhancer binding protein; Cdk5, cyclin dependent kinase 5; ChIP, chromatin immunoprecipitation; ERK, extracellular signal-regulated kinase; LBD, ligand binding domain; LBP, ligand binding pocket; NFkβ, nuclear factor κ light chain enhancer of activated B cells; NR, nuclear receptor; Thrap3, thyroid hormone receptor-associated protein 3; TF, transcription factor; TZD, thiazolidinedione; PPAR-γ, peroxisome proliferator-activated receptor γ; RXR, retinoic X receptor; WAT, white adipose tissue

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Manuscript 4

2,4,6-Trimethoxybenzophenone as a novel PPARγ ligand with anti-diabetic properties

[Preliminary results]

UNIVERSITAT ROVIRA I VIRGILI
CHARACTERIZATION OF THE BIOLOGICAL EFFECTS OF NATURAL COMPOUNDS AGAINST INFLAMMATION, METABOLIC SYNDROME AND CANCER
Sara Tomás Hernández

2,4,6-TRIMETHOXYBENZOPHENONE AS A NOVEL PPARγ LIGAND WITH ANTI-DIABETIC PROPERTIES

1. Introduction

PPARγ is a member of the nuclear receptor superfamily and regulates the gene expression of a subset of proteins involved in energy homeostasis, adipogenesis and insulin sensitivity [1,2]. This nuclear receptor is a well validated pharmaceutical target of the treatment of several metabolic diseases [3]. In fact, the PPARγ full agonists rosiglitazone and pioglitazone (belonging to the TZDs drug class) have been widely used for the treatment of type 2 diabetes as they enhance human sensitivity to insulin [4,5]. However, despite the beneficial effects that they display, this TZDs drugs are also associated with various undesired side effects [6,7]. Nevertheless other compounds with low agonist activity for PPARγ, (PPARγ partial agonists or PPARγ modulators (SPPARγMs) retain very good anti-diabetic effect without displaying those side effects. Therefore, several partial agonists of PPARγ are being developed as new antidiabetic drugs and have revealed a new mechanism of action for the anti-diabetic effect of some PPARγ agonist. This mechanism is completely independent of the classical PPARγ transactivation and consists on the inhibition of the phosphorylation of PPARγ on Ser 273, thereby preventing the deregulation of a several PPARγ beneficial related genes involved in metabolism homeostasis [8–12]. It has been demonstrated that PPARγ full agonists are also able to inhibit this phosphorylation. Thus, given that both (partial and full agonists) have potent anti-diabetic effects it is feasible that a substantial portion of the therapeutic benefits of PPARγ ligands in metabolic disease is through the inhibition of the phosphorylation of PPARγ at Ser 273, whereas at least some of the undesirable side effects of this full agonist drugs may occur due to classical agonist actions [12,13].

These compounds act as partial agonist of PPARγ and display different binding and transcriptional activity than full agonists. Therefore the identification and characterization of new SPPARγMs have become an important clinical challenge for developing improved and safer anti-diabetic drugs. Several plant extracts have been found to possess anti-diabetic effects trough modulation of PPARγ activity with no or little effect on adipogenesis [14,15]. In that sense, PPARγ partial agonists from natural extracts are promising candidates for the treatment of some metabolic disorders, such as T2DM.

With this purpose our group, in a previous study, designed and applied a virtual screening workflow to identify novel PPARγ partial agonists among natural products databases [16,17]. This *in silico* experiment successfully identified several molecules with high chance of being effective PPARγ partial agonists. One of this predicted molecules was2,4,6-Trimethoxybenzophenone (from now referred as ZINC00394686) (Figure 1) a molecule contained in the C. sumatranum ssp. Neriifolium plant [18]. Interestingly this plant specie has been traditionally used for the treatment of some medical disorders [19].

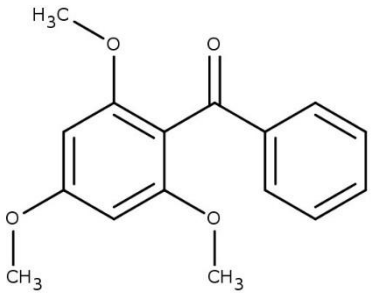

Figure 1. Chemical structure of 2,4,6-Trimethoxybenzophenone (ZINC00394686)

The aim of this study was to examine the effect of this novel SPPARγMs on the Cdk5-mediated phosphorylation of PPAR*γ* as well as its influence on adipogenesis.

In order address if ZINC00394686 was effective at blocking cdk5-mediated phosphorylation of PPARγ a kinase assay and a western blot analysis were

performed. However, previous to testing the compound we needed to validate the kinase assay and see if it was an adequate method for studying the phosphorylation reaction and its inhibition. In that sense we first developed a kinase assay method based on the protocol previously described by Choi et al but with some modifications [12]. We optimized this protocol, performed the reaction and analyzed the result by western blot. Once established the optimal conditions and got the protocol validated we investigated if the molecule ZINC00394686 was able to inhibit PPARγ Ser273 phosphorylation.

As previously mentioned, we further investigate the anti-diabetic activity of ZINC00394686 by studying the effects of this molecule in the adipogenic differentiation process of 3T3-L1 cells.

The results show that ZINC00394686 is able to block thePPARγ phosphorylation at Ser 273, moreover this molecule markedly inhibit adipogenesis in 3T3-L1 cells. Hence, this study presents a novel PPARγ ligand with low agonistic activity and potent inhibition of Cdk5-mediated phosphorylation.

2. Materials and methods

2.1. Validation and development of the kinase assay

0.5 µg of purified PPARγ LBD was incubated with active Cdk5/p35 in a 50 µl reaction containing the proper assay buffer and 200 µM ATP. The reaction was carried out at 30 °C for 15 minutes and then stopped by the addition of 6x SDS-loading buffer. As a negative control of the kinase reaction one condition did not contained ATP. Samples were resolved in SDS–polyacrylamide gels and transferred to PVDF membranes with a Trans-Blot Turbo system. The membranes were blocked with 5% nonfat milk in PBS–Tween (0.1%) for one hour. Then, they were blotted overnight at 4ºC with a phospho-specific antibody against PPARγ Ser 273 produced by New England Peptides with a synthetic

phosphopeptide corresponding to residues surrounding Ser 273 of PPARγ (Ac-KTTDKpSPFVIYDC-amide). After washing three times with PBS–Tween, the membrane was hybridized with secondary anti-rabbit antibody conjugated with horseradish peroxidase for 1 h and then washed three more times with PBS-Tween. The immune reactive proteins were visualized using an enhanced chemiluminescence substrate kit according to the manufacturer's instructions. Digital images were obtained with a GBOX Chemi XL 1.4 system, which allows quantification of the band intensity. The relative amounts of PPARγ protein loaded onto the gel were monitored by use of a mouse monoclonal anti-total PPARγ antibody.

2.2. Inhibition of PPARγ Ser273 phosphorylation by ZINC00394686

The selected compound was tested for their ability to inhibit Cdk5/p35-mediated phosphorylation of PPARγ in vitro. Different concentrations of the ligand were pre-incubated with PPARγ LBD (0.5 µg) at room temperature for 30 minutes prior to the addition of Cdk5/p35 and ATP. The reaction was then performed at 30 °C for 15 minutes and then stopped by the addition of 6x SDS-loading buffer. The Western blot analysis was made according to the procedure described in the previous section.

2.3.3T3-L1 cell culture and differentiation

3T3-L1 preadipocytes were culture in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum, 2mM glutamine, 100 U/mL penicillin and 1% penicillin-streptomycin (P/S). After reaching 80-90% confluence, cell differentiation was induced by addition of D1 medium (5 µg/mL insulin, 0.5 mM 3-isobutyl-
1-methylxanthine, 0.25 mM dexamethasone in DMEM supplemented with10% fetal bovine serum (FBS), 2mM glutamine and 1% P/S). 48 hours later, on day 3 of differentiation, cells were treated with D2 medium (5 µg/mL insulin in DMEM supplemented with10% fetal bovine serum (FBS), 2mM glutamine and

1% P/S). On day 5 of differentiation cells were re-fed with D3 medium (DMEM supplemented with10% fetal bovine serum (FBS), 2mM glutamine and 1% P/S). After a further 72 h cells were re-fed with fresh D3 medium. To study the effects of ZINC00394686 on adipocyte differentiation, different concentrations of the compounds (1 to 50 µM) were added at each of the three stages of adipocyte differentiation. As a negative control cells were left untreated.

2.4. Oil red O staining

In order to investigate the effects of ZINC00394686 on adipocyte differentiation, the intracellular lipid accumulation was measured using the Oil Red O staining method. Briefly, Oil red O stock solution was prepared dissolving Oil Red O powder in isopropanol (5mg/mL), and then the Oil Red O working solution was prepared by mixing 60% Oil Red O stock solution with 40% distilled water. After the differentiation, cells were washed twice with phosphate-buffered saline (PBS) and fixed in 3.7 % formaldehyde for 15 min at room temperature. Cells were stained with Oil Red O working solution for 30 min at 25 °C and then washed three times with PBS to remove unbound dye. Staining was visualized using bright-field microscopy.

3. Results and Discussion

3.1 The kinase assay is an optimal method for studying the phosphorylation reaction and its inhibition

In order to assess if the studied compound was able to block Cdk5-mediated phosphorylation of PPARγ we need to develop a kinase assay protocol and validate that it was an appropriate method for studying this kinase reaction and its inhibition. After protocol optimization we analyzed the result by western blot. The positive controls conditions contained all the reagents needed for the phosphorylation reaction, the negative ones did not contain ATP. As shown in Figure 2, phosphorylation of Ser 273 PPARγ was detected in the positive

conditions, whereas, as expected, in the negative control conditions (where no ATP was added) we did not observe any phosphorylation or Ser 273 PPARγ. Thus validating that the kinase reaction was working out properly and that we could use this protocol for testing if the selected molecule is able to block Cdk5-mediated phosphorylation of PPARγ.

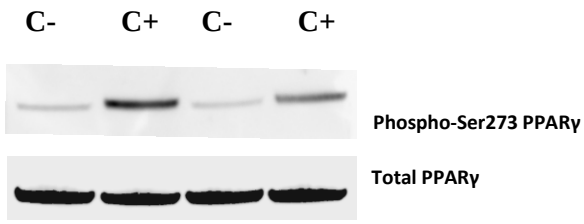

Figure 2. Validation of the kinase assay for the detection of Cdk5-mediated phosphorylation of PPARγ at Ser 273. 0.5 µg of PPARγ-LBD was incubated in vitro with Cdk5 in the presence (C+) or absence (C-) of ATP as described in materials and methods. After the reaction, samples were analyzed by western blotting One representative image of three independent experiments is shown.

3.2 ZINC00394686 inhibits obesity-linked phosphorylation of PPARγ at Ser 273.

Because the insulin-sensitizing effects of PPARγ agonists seem to be more closely correlated with their ability to inhibit theSer-273PPARγ phosphorylation than with classical receptor activation, we investigate the ability of ZINC00394686 to inhibit Cdk5 phosphorylation of PPARγ *in vitro*. Thus, PPARγ LBD was pre-incubated with the compound or rosiglitazone prior to the addition of Cdk5/p35 and ATP and then the kinase reaction was carried out. Different concentrations of the agonist were assayed. As depicted in Figure 3AZINC00394686 decreased the Ser-273 phosphorylation of PPARγ in a dose response- manner reaching a markedly inhibition of the reaction with the highest concentrations of the molecule. Furthermore, when compared with the effect of Rosiglitazone, ZINC00394686 at the highest concentrations was as efficacious as the known TZD at blocking Cdk5-mediated phosphorylation (Fig.3B).

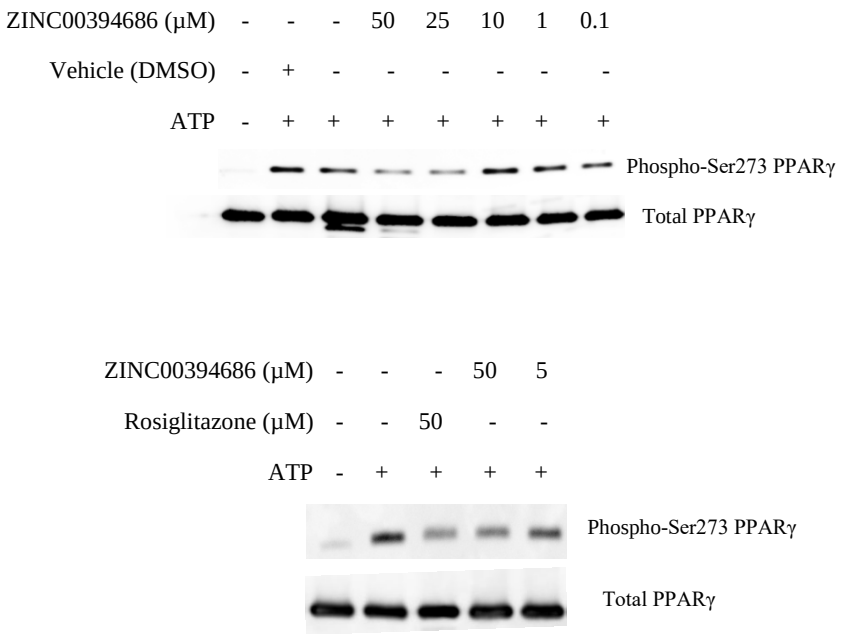

Figure 3.Inhibitory effect of ZINC00394686 on Cdk5-mediated PPARγ-Ser 273 phosphorylation. (A) 0.5 µg of PPARγ-LBD was incubated with increasing concentrations of ZINC00394686prior to the addition of Cdk5/35 and ATP. **(B)** 0.5 µg of PPARγ-LBD was incubated with ZINC00394686 or with rosiglitazone prior to the addition of Cdk5/35 and ATP. In both cases (A) and (B) Negative control was performed in the absence of ATP and positive control was performed in the presence of ATP and no inhibitory compound. After the reaction, samples were analyzed by western blotting as described in materials and methods. One representative image of three independent experiments is shown.

3.3 ZINC00394686 inhibits adipogenesis in 3T3-L1 cells

Full agonists of PPARγ have been shown to potently stimulate the differentiation of pre-adipose cell lines. A desirable sPPARγM should bound to PPARγ-LBD in a distinct manner than full agonist do and may lead a conformational change that potentiates less affinity to coactivators participated in the PPARγ mediated classical agonism, such as adipogenesis.

To determine the effect of ZINC00394686on lipid accumulation in 3T3-L1 preadipocytes, cells were treated with increasing concentrations of the compound at each of the three stages of adipocyte differentiation. After 10 days, intracellular lipid accumulation was examined with Oil red O staining as an

indicator of the degree of adipogenesis. Concretely, the differentiation of preadipocytes into adipocytes is associated with an increased number of Oil-red O stained due to lipid accumulation. As expected, most untreated control cells were Oil red O stained, however the number of detectable stained droplets decreased as the concentration of ZINC00394686 increased (Figure 4). Interestingly, cells treated with the highest doses of ZINC00394686 showed a markedly reduction in lipid accumulation compared to the control. These results indicate that ZINC00394686 effectively is a PPARγ ligand with low agonistic activity that blocks adipocyte differentiation in 3T3-L1 preadiocytes.

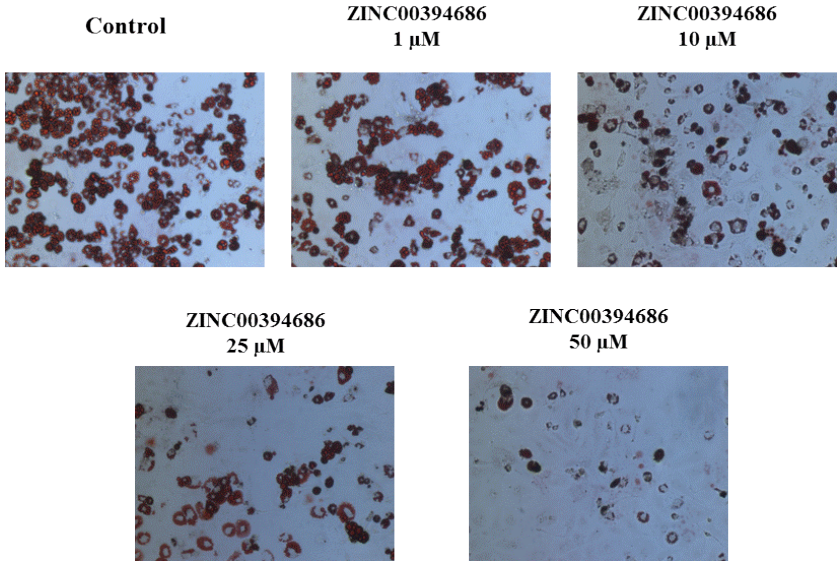

Fig 4. Effect of ZINC00394686 on adipogenesis in 3T3-L1 cells. Postconfluent 3T3-L1 cells were subjected to differentiation in the absence (control) or presence of ZINC00394686 (1, 10, 25 or 50 µM) for 10 days. At the end, 3T3-L1 cells were stained with Oil Red O and microscopically analyzed. Images shown are representative of at least three independent experiments.

4. Conclusions

Collectively, our data demonstrated that ZINC00394686 is a novel partial PPARγ agonist that retains the benefits of improving insulin resistance by inhibiting CDK5-mediated PPARγ phosphorylation at ser273, but minimizes the common side effects of TZDs, such as adipogenesis, by alleviating PPARγ agonistic activity. Although further characterization of the ant-diabetic properties of this compound is needed, these preliminary results suggest that ZINC00394686 could be a promising candidate drug for the treatment of some metabolic diseases, such as T2DM.

5. References

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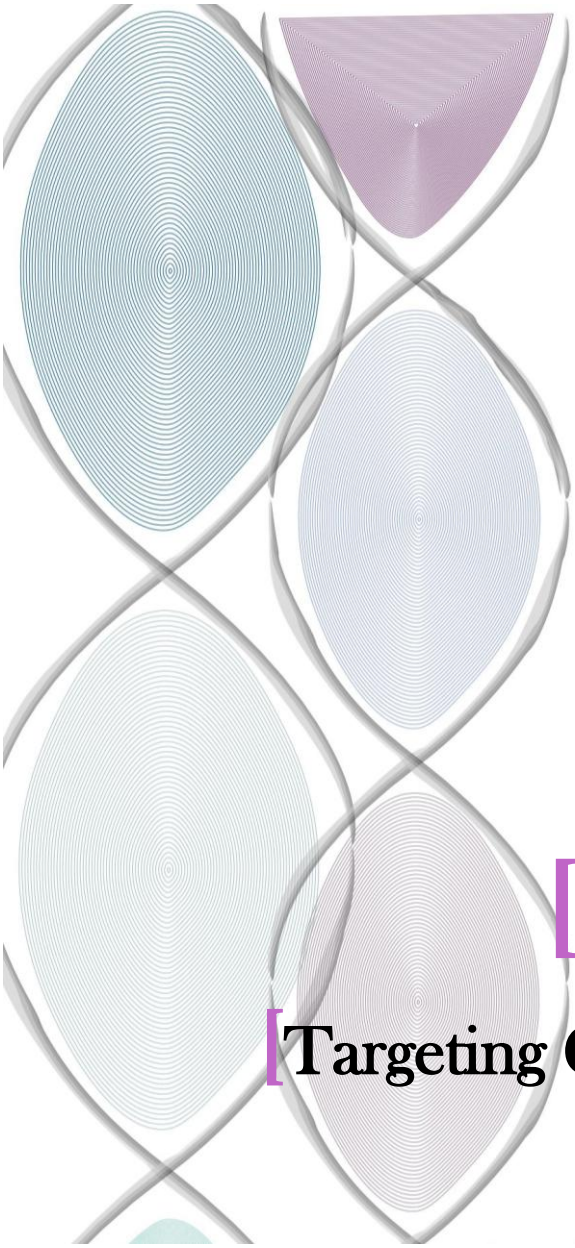

[Chapter 3]

[Targeting Cancer through Autophagy]

UNIVERSITAT ROVIRA I VIRGILI
CHARACTERIZATION OF THE BIOLOGICAL EFFECTS OF NATURAL COMPOUNDS AGAINST INFLAMMATION, METABOLIC SYNDROME AND CANCER
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CANCER AND AUTOPHAGY

Caloric restriction prolongs lifespan in various organisms and can delay the onset of several important pathologies. One of the processes that are favored by CR is autophagy (the world autophagy is Greek for self-eating). Autophagy is a survival-promoting pathway that captures, degrades, and recycles intracellular proteins and organells in lysosomes. Autophagy preserves organelles function, prevents the toxic build up of cellular waste products, and provides substrates to sustain metabolism in starvation.

There are various types of autophagy that differ in how the "cargo" is delivered into the lysosomes. (Figure 1)

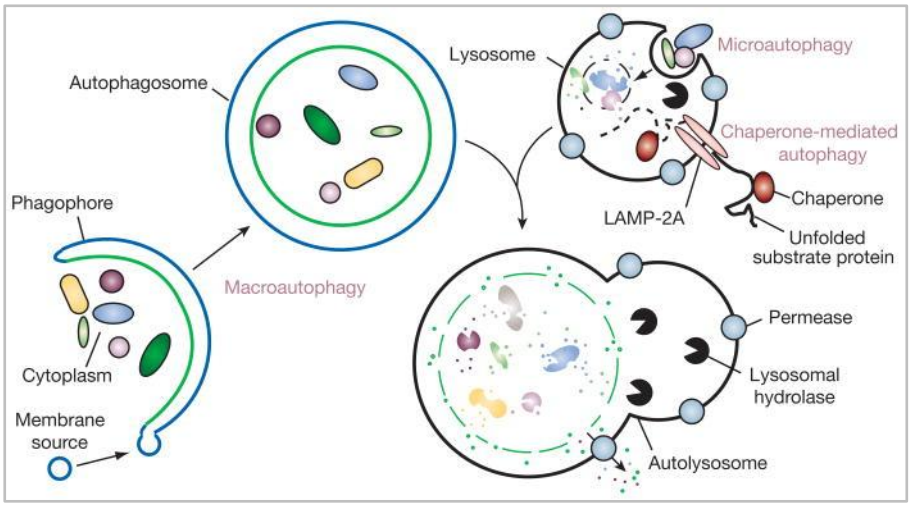

Figure 1. Different types of autophagy. Extracted from [1]

Macroautophagy is defined as the sequestration of cytosolic contents such a volume of cytoplasm, organelles or invading microbes by unique double-membrane which expands from the phaghophore and closes to became a vesicle called autophagosome. This autophagosome eventually fuses with the lysosome, thereby acquiring hydrolases and forming an autolysosome in which the inner membrane of the autophagosome and its contents are degraded.

Interestingly, the sequestration of cytosolic components may be unselective, as for example in the case of mitophagy or lipophagy. Macroautophagy regulation depends on amino acid and energy levels, growth factors and nutrient supplies. Accordingly, the classic macroautophagy pathway is regulated by a central kinase involved in sensing all these parameters, the mammalian target of rapamycin (m-TOR). This Ser/Thr kinase suppresses the autophagic machinery. Additionally, 35 autophagy genes (Atgs) have been discovered in yeast and many of them have mammals homologues. These Atgs are mostly located downstream of mTOR and they play an important role in autophagosome formation and growth. Upstream of mTOR there are several important kinases that can regulate macroutophagy function. For example, the AMP-activated protein kinase (AMPK) which is induced upon caloric restriction can induce macroautophagy via the tuberous sclerosis complex 1/2 (TSC 1/2) which is a negative regulator of mTOR. Another pathway that negatively regulates macroautophagy (activating mTOR) is induced via the class I PI3K and AKT by insulin and growth factors. Although the pathway related to mTOR inhibition is the best kwon inductor of macroautophagy, there are several other signaling cascades and transcriptional events that trigger this type of lysosomal degradation. For example, AMPK can also induce autophagy through direct phosphorylation of Ulk1 (the mammalian homologue of Atg1) or lead to SIRT1-dependent

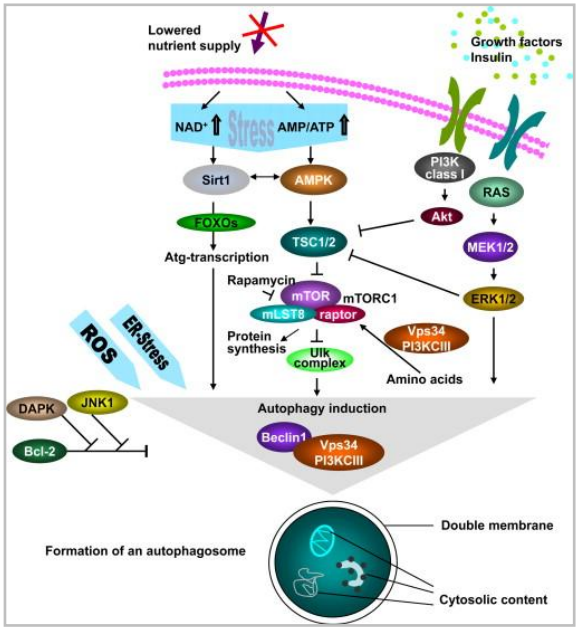

Figure 2. Main signaling pathways that lead to macroautophagy induction. Extracted from [13].

deacetylation of the trasncriptional regulators forkhead box O (FoxO). The main signaling pathways that are related to macroautophagy regulation are summarized in Figure 2.

While in macroautophagy the content is sequestered inside the characteristic double membranes, in the lesser studied microautophagy, the directly sequesters small portions of the cytoplasm through the invagination of the lysosomal membrane (see Figure 1).

In contrast to micro- and macroautophagy, which are better known for bulk degradation, chaperone-mediated autophagy (CMA) is a highly selective process for the degradation of cytosolic proteins. CMA plays a role in housekeeping, as a stress response and it is increased during long-term starvation. During this CMA process, proteins are selectively unfolded and translocated through the lysosome membrane by the cytosolic and lysosomal chaperone hsc70, and the lysosomal membrane receptor LAMP-2A (lysosome-associated membrane protein type 2A).

Growing evidence suggests that autophagy plays an important role in maintaining human health. Autophagic deficiencies have been identified in the pathophysiology of neurodegenerative disorders, pulmonary and cardiovascular disease, aging, obesity and cancer [2–5]. Thus, there has been a growing awareness of autophagy deficiencies and the resulting accumulation of defective proteins or organelles that contribute to, or result in, a variety of pathologies.

1.1 Autophagy and cancer

Cancer was the first human pathology connected to autophagy through the discovery that Beclin1 (Atg6 homologue), a core component of the autophagosome nucleation complex, was monoallelically deleted in40–75% of sporadic human breast, ovarian and prostate cancers [99]. Independent studies verified that heterozygous Beclin1+/− mice develop spontaneous tumors,

including lymphomas, lung carcinomas, hepatocellular carcinomas and mammary precancerous lesions [6,7].

Analogous generation of spontaneous hyperproliferating tumors was also reported later upon deletion of other autophagy-related genes, such as the ultraviolet resistance-associated gene (*UVRAG*), the Bax interacting factor-1 (*Bif-1*) and the LC3 (Atg8 homologue) processing protease Atg4c [8–10]. Furthermore, the products of common oncogenes, such as class I PI3K, PKB, TOR and Bcl-2, have been shown to act as autophagy repressors, whereas tumor suppressor gene products such as p53, PTEN, DAPk and TSC1/TSC2 exert a stimulatory effect on autophagy (reviewed in [4,11,12]).

Although, in light of this opposite effect of oncogenes and tumor suppressors on autophagy, this process was initially classified as an anti-oncogenic mechanism, this conclusion has been challenged by experimental evidence supporting that under certain conditions autophagy can also be pro-oncogenic. Accordingly, as it can be observed in Figure 3, autophagy can have an anti-tumoural effect, for example the active maintenance of cellular quality control for cytosolic pro-oncogenic proteins such as p62 prevents malignant transformation of nontumoural cells. In addition, the supply of energy provided through macroautophagy activation reduces the dependence on glycolysis, while assuring the energy required for maintenance of a stable genome, further preventing oncogenesis. On the other hand, autophagy can also have pro-oncogenic effect. For example, the reduction in macroautophagic activity in early stages of the oncogenic process favours malignant transformation, as the accumulation of molecules such as p62 activates signalling mechanisms that promote necrosis and inflammation. Poor quality control as a result of diminished macroautophagy can also result in accumulation of defective mitochondria, with the subsequent release of harmful molecules (cytochrome c and reactive oxygen species) that contribute to further altering genome maintenance. However, as the tumour progresses, activation of macroautophagy

is observed in many oncogenic processes, in part to compensate the poor nutritional supply associated with rapidly growing tumors and to defend cancer cells against damage induced by anti-oncogenic therapies. In addition, enhanced mitochondrial degradation in this stage may contribute to the up- regulation of glycolysis to maintain the energetic balance (Warburg effect) characteristic of malignant cells.

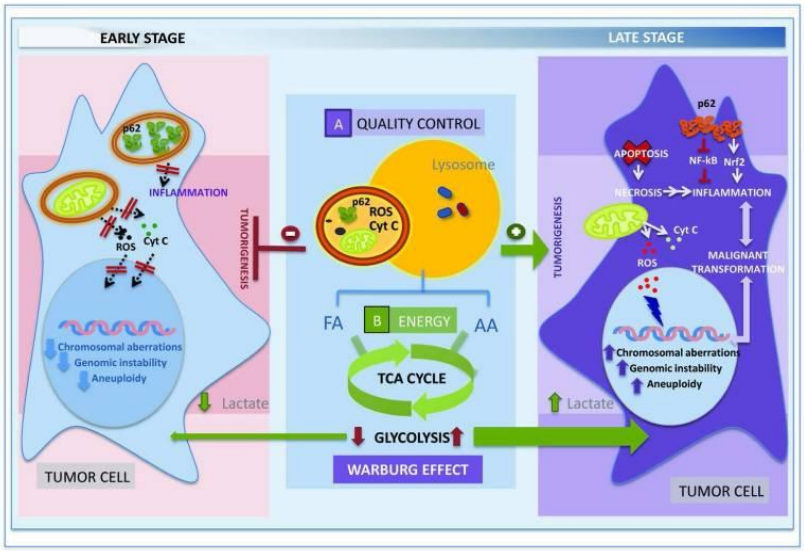

Figure 3. Autophagy may play opposite roles in the oncogenic process. Extracted from [1]

So, although in some contexts autophagy suppresses tumorigenesis, in most contexts autophagy facilitates this malignant process. Cancers can upregulate autophagy to survive microenvironmental stress and to increase growth and aggressiveness. Mechanisms by which autophagy promotes cancer include inhibiting the p53 tumor suppressor protein activity and maintaining metabolic function of mitochondria (see figure 4). In consequence, efforts to inhibit autophagy to improve cancer therapy have thereby attracted great interest.

Another important point is that autophagy is affected by and also affects diverse cancer therapies; however, these effects are not always the same. In fact, they are often diametrically opposed. Sometimes cancer therapeutics induce

autophagy, and sometimes they inhibit it. In other words, sometimes autophagy protects tumor cells against cancer therapy, and sometimes it is required for the therapy to kill the cancer cell. However, researchers are starting to detect patterns and are making rapid progress in understanding the underlying molecular mechanisms that govern these effects. This will allow them to at least begin to develop rational approaches to manipulate autophagy for clinical benefit. It seems that the most important priorities just now for autophagy research related to cancer therapy are to find ways to identify which tumors will be most effectively treated by autophagy manipulation (usually this will mean autophagy inhibition).

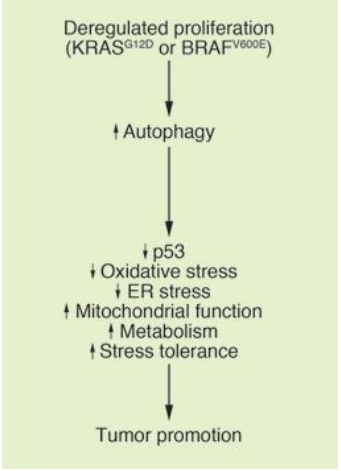

Figure4. Proposed tumor-promoting roles for autophagy in cancerExtractedfrom[14]

1.2 Natural products, autophagy and cancer

Natural products (NP) are receiving increasing attention for the prevention and/or treatment of cancer because of their promising efficacy and low toxicity to normal tissue. Therefore there is a great interest in identifying new natural products active against cancer and in understanding the mechanisms of action of these compounds to exploit their properties in the development of new therapeutic or preventive treatments.

As we have previoulsy mentioned, autophagy can act as a tumor suppressor during the early stages of carcinogenesis, but it can also be used by transformed cells as a survival mechanism to overcome the stresses imposed during tumor growth. The prosurvival role of autophagy is responsible, at least in part, for the adaptive response of cancer cells to various anticancer therapies, including radiation therapy and conventional DNA damaging chemotherapy. The pro-

death function of autophagy, at the moment poorly characterized, could be attributed to two separate functions: the pro-apoptosis function of autophagy and the induction of autophagic cell death, without the involvement of apoptosis machinery.

Interestingly, the synergism between autophagy inhibitors and conventional chemotherapeutic drugs is attracting more and more attention since this strategy could overcome resistance, indeed increasing and maximizing the clinical effectiveness of cancer therapy.

Several studies suggest that many natural compounds induced autophagy by specifically downregulating the Akt/mTOR pathway, thus indicating that autophagy may induce cell death through a specific molecular commitment. It is noteworthy that the Akt/mTOR pathway, frequently upregulated in cancer, contributes to the disease development and progression also through an extensive crosstalk with many other signaling pathways involved in cell survival, apoptosis, growth and differentiation. Since mTOR activity can be directly or indirectly modulated by a number of upstream signaling pathways, it is mandatory to uncover the mechanisms through which these natural compounds inhibit the Akt/mTOR pathway and impact on the cell fate.

In addition, a better understanding of the molecular effectors that interconnect autophagy to programmed cell death is urgently required to look at many natural compounds as a “sustainable” hope for therapeutic anticancer strategy.

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Manuscript 5

Resveratrol potently counteracts quercetin starvation-induced autophagy and sensitizes HepG2 cancer cells to apoptosis.

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[Submitted]

UNIVERSITAT ROVIRA I VIRGILI
CHARACTERIZATION OF THE BIOLOGICAL EFFECTS OF NATURAL COMPOUNDS AGAINST INFLAMMATION, METABOLIC SYNDROME AND CANCER
Sara Tomás Hernández

[Abstract]

Resveratrol (RSV) has been described as a potent antioxidant, anti-steatotic, antitumor compound, and it has also been identified as a potent autophagic inducer. On the other hand, quercetin (QCT) is a dietary flavonoid with known antitumor, anti-inflammatory and antidiabetic effects. Additionally, QCT is able to increase autophagy. In order to study the hypothetical synergistic effect of both compounds we have tested the combined effect of QCT and RSV on the autophagic process in HepG2 cells. Autophagy was studied by western-blotting, real-time RT-PCR, and cellular staining. Our results clearly indicate a bifunctional molecular effect of RSV. Both polyphenols were individually able to promote autophagy. Strikingly, when RSV was combined with QCT it promoted a potent reduction on the QCT-induced autophagic process and stressed the pro-apoptotic signaling. In conclusion, RSV will act differentially on the autophagic process depending on the cellular energetic state. We have further characterized the molecular mechanisms that are related to this effect and we have observed that the AMP-activated protein kinase (AMPK) phosphorylation and heme oxygenase 1 (HO-1) downregulation could be important modulators of such RSV related effect, and could globally represent a promising strategy to sensitize cancer cells to QCT treatment.

Keywords: Autophagy, AMPK, HO-1, Quercetin, Resveratrol.

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1. Introduction

Autophagy is a critical intracellular pathway that targets damaged organelles to the lysosome for degradation, and this process is highly preserved in eukaryotic cells [1]. As an initial protective mechanism, autophagy is usually triggered by starvation or damage to maintain cell homeostasis. Degradation of cellular components can entail the uptake of small amounts of cytoplasm at the vacuole or lysosome surface (microautophagy) or, in response to a strong stimulus such as starvation, the formation of specific double membraned-organelles termed autophagosomes, which engulf larger portions of cytoplasm or organelles before fusing with a vacuole or lysosome (macro-autophagy) [2,3]. Although this process is an important part of the normal balance between anabolism and catabolism and can prolong survival during nutrient deprivation, autophagy is also an alternate death pathway that facilitates type II programmed cell death [4–6]. For this reason, imbalances in this pathway can contribute to diverse pathologies.

Interestingly, the role of autophagy in cancer is quite complicated and controversial. Autophagy is supposed to be tumor suppressive during cancer development but to contribute to tumor cell survival during cancer progression [7,8]. Alternatively, autophagy prevents tumor cells from dying by inhibiting apoptosis during nutritional deprivation, and the cells undergo apoptosis when autophagy is prevented [9–11]. Regardless of whether they promote cell survival or cell death, the two processes engage in complex and poorly understood molecular cross-talk [12] and inducing apoptosis and inhibiting protective autophagy have become an effective means of cancer therapy.

Natural compounds are an important source of molecules with interesting healthy properties and have been widely used for prevention and treatment of metabolic, degenerative and cancer pathologies [13].

Resveratrol (RSV), a phytoalexin found in grape skins, peanuts, and red wine, has been reported to exhibit a wide range of biological and pharmacological properties in several pathological conditions such as fatty-liver [14], inflammation [15] and diabetes [16], among others. Aside from these protective effects, RSV is able to inhibit tumor initiation, promotion and progression in a variety of cell culture systems and animal models by mechanisms that include cell cycle arrest, kinase pathways inhibition and apoptosis activation [17–19]. In addition, RSV-induced autophagy has been suggested to be a key process in mediating many beneficial effects of RSV, such as reduction of hepatic steatosis, endoplasmic-reticulum stress (ER-stress) and inflammation [20,21] and induction of cancer cell death [7,22].

On the other hand, quercetin (QCT) is an antioxidative flavonoid ubiquitously distributed in plants with multiple health benefits (i.e. diabetes, inflammation, atherosclerosis and cancer) [23–26]. The anticancer effects of QCT have been attributed to antioxidative activity, inhibition of enzymes activating carcinogens, modification of signal transduction pathways, and interactions with receptors and other proteins [27]. Moreover, it has also been shown that QCT is able to induce “protective” autophagy in several cancer cell lines [28,29].

Importantly, the capacity of these compounds to empower cancer cell death that enhances the effects of standard therapies should be taken into consideration for designing novel therapeutic strategies [30]. In this sense, the synergism between autophagy-inducing plant metabolites contained in the diet should not be neglected. This will allow, by selecting natural compounds combination, a better chemosensitization and consequent cell death of cancer cells through autophagic-related mechanisms.

In this sense, the aim of our study was to identify and characterize hypothetical synergistic effects of two well stablished natural autophagic inducers, RSV and QCT, on autophagic-related mechanisms in HepG2 cancer cells.

2. Material and Methods

2.1 Chemicals

Resveratrol (RSV) ref. R5010, Quercetin (QCT) ref. Q4951, Thiazolyl Blue Tetra-zolium Bromide (MTT) ref. M5655 as well as, the protease inhibitor cocktail ref. P8340, the phosphatase inhibitor cocktail 2 ref. P5726 and the phosphatase inhibitor cocktail 3 ref. P0044 were obtained from Sigma-Aldrich (Sr.Louis, USA). Acridine orange ref. A3568 was obtained from Invitrogen. Dulbecco's modified Eagle's medium (DMEM) was purchased from Lonza (Basel, Switzerland). The human hepatoblastoma HepG2 cell line was obtained from the European Collection of Cell Cultures (Wiltshire, UK).

2.2 Cell culture and general experimental treatment

The human hepatoblastoma HepG2 cells were cultured in 75 cm^2 flasks (Orange Scientific) with DMEM supplemented with 10% fetal bovine serum, 2% PS (penicillin-streptomycin), 1% L-glutamine, and 1% NEAA (non-essential amino acids) in a humidified atmosphere with 5% CO_2 at 37°C. For the mRNA and protein extraction, cells were seeded at a density of 5×10^5 cells/well in 12-well plates (Orange Scientific). RSV and/or QCT treatments were done by incubating HepG2 cells with increasing concentrations of polyphenols (0, 25, 50 or 100 μM) or a vehicle (0.05% DMSO) and harvested at specific time points (0.75, 8 and 24 h).

2.3 MTT assay

Cells were seeded at a density of 5×10^4 cells/well on a 96-well culture plate and incubated overnight. After the treatments, medium was removed and was replaced with 200 μL fresh medium. Cells were loaded with 50 μl freshly prepared MTT (5 mg/mL in phosphate buffered saline (PBS)) and incubated for 4 h at 37°C. The blue formazan crystals formed following the reduction of the MTT dye were solubilized in 200 μl dimethyl sulfoxide (DMSO) and 25 μl

glycine buffer and quantified using the Helios Zeta UV-VIS ELISA reader (Thermo Scientific). The dye absorbance was measured at a wavelength of 570 nm (680 nm was used as the reference wavelength). The vehicle-treated cells were established as 100% viability. The relative percentage of viability was calculated as follows: Viability (%) = [A570 (compound) / A570 (control)] × 100. Cell survival or viability (%) was determined by averaging three repeated experiments.

2.4 RNA isolation and cDNA synthesis

Total RNA was obtained from HepG2 cells using a RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA was resuspended in 100 µL RNase free water. The DNase I RNAase free kit (Fermentas, Thermo Scientific) was used to remove the genomic DNA from the RNA preparations. RNA was quantified with a spectrophotometer (Nanodrop 1000 Spectrophotometer, Thermo Scientific) at an absorbance of 260 nm and tested for purity (by the A260/280 ratio) and integrity (by denaturing gel electrophoresis). The first strand of cDNA was reverse transcribed from 1 µg total RNA from each sample using a First Strand cDNA Synthesis Kit (Fermentas, Thermo Scientific) according to the manufacturer's protocol. An identical reaction without the reverse transcription was performed to verify the absence of genomic DNA.

2.5 Real-time Q-PCR

Quantitative PCR for CHOP, ATF6, ATF4 and cyclophilin was performed using SYBR Premix Ex Taq (Takara) according to the manufacturer's protocol and was analyzed on a CFX96 Real-Time PCR Detection System (Bio-Rad, Spain.). The cDNA was amplified using human-specific primers for CHOP (forward: 5'-AGG GAG AAC CAG GAA ACG GAA ACA-3'; reverse: 5'-TCC TGC TTG AGC CGT TCA TTC TCT-3'), ATF6 (forward: 5'-ATG TCT CCC CTT TCC TTA TAT GGT; reverse: 5'-AAG GCT TGG GCT GAA TTG AA-3'), ATF4

(forward: 5'-GGG TTC TCC AGC GAC AAG GCT AAG-3'; reverse: 5'-AAC AGG GCA TCC AAG TCG AAC TC-3'), and cyclophilin (forward: 5'-TTC ATC TGC ACT GCC AAG AC-3'; reverse: 5'-TCG AGT TGT CCA CAG TAG C-3'). The thermal cycling was composed of an initial step at 50°C for 2 min followed by a polymerase activation step at 95°C for 10 min and a cycling step with the following conditions: 40 cycles of denaturation at 95 °C for 15 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min. Oligonucleotides of varying lengths produce dissociation peaks at different melting temperatures. Therefore, at the end of the PCR cycles, the PCR products were analyzed using a heat dissociation protocol to confirm that a single PCR product was detected by the SYBR Green dye. The fluorescence data were acquired at the 72°C step. The threshold cycle (Ct) was calculated using the CFX Manager Software to indicate significant fluorescence signals above the noise during the early cycles of amplification. The relative levels of expression of the target genes were measured using cyclophilin mRNA as an internal control according to the $2^{-\Delta\Delta Ct}$ method.

2.6 Analysis of XBP1 mRNA splicing

Spliced XBP1 mRNA induced by activated IRE1 is translated to the protein, a potent transcription factor that induces BiP/GRP78 expression. XBP1 splicing is also induced by activated ATF6; thus, it is believed to be an important marker reflecting IRE1 and ATF6 signaling in response to ER stress. For this assay, the XBP1 cDNAs were amplified by PCR using human-specific primers for the XBP1 transcript (forward: 5'-GCT GAA GAG GAG GCG GAA G-3'; reverse: 5'-GTC CAG AAT GCC CAA CAG G-3'). These primers are useful for capturing the XBP1 spliced forms (XBP1s-172 bp amplicon) and the XBP1 unspliced form (XBP1u-197 bp amplicon). The PCR conditions were composed of an initial step at 50 °C for 2 min followed by a polymerase activation step at 95°C for 10 min and a cycling step with the following conditions: 40 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 sec, and extension at

72 °C for 30 sec. A final extension at 72°C for 10 min was also developed. The PCR products were separated by 4% agarose gel electrophoresis for 280 min and were stained with ethidium bromide.

2.7 Detection of autophagic cells by acridine orange staining.

As a marker of autophagy, the volume of the cellular acidic compartment was visualized by acridine orange staining. Cells were seeded in six well tissue culture ($5x10^5$ cells/well). 24 h following polyphenol treatments, cells were incubated with medium containing 1 μg/mL acridine orange for 15 minutes; the staining solution was then removed, cells were washed once with PBS, fresh media was added, and fluorescent micrographs were taken using an Olympus inverted fluorescence microscope. All images presented are at the same magnification.

2.8 Western blotting analysis

HepG2 cells were harvested and homogenized in RIPA lysis buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1% NP40, and 0.25% Na-deoxycholate, containing protease and phosphatase inhibitors). Aliquots of the cell lysate containing 30 μg of protein per sample were analyzed by Western blotting. Briefly, the samples were placed in sample buffer (0.5 M Tris–HCl pH 6.8, 10% glycerol, 2% (w/v) SDS, 5% (v/v) 2-β-mercaptoethanol, and 0.05% bromophenol blue) and denatured by boiling at 95-100°C for 5 min. The samples were then separated by electrophoresis on 15% polyacrylamide gels. The proteins were subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, GE Healthcare) using a transblot apparatus (Bio-Rad). The membranes were blocked for 1 h with 5% non-fat milk dissolved in TBS-T buffer (50 mM Tris, 1.5% NaCl, and 0.2% Tween 20, pH 7.5). The membranes were then incubated overnight with primary monoclonal antibodies against: cleaved caspase-3 (5A1E; Cell Signaling), eIF2α (9722; Cell Signaling), phospho-eIF2α Ser 51 (9721; Cell Signaling), LC3A/B (4108; Cell Signaling),

Bcl-2 (2876; Cell signaling), phospho-mTOR Ser 2448 (sc-293133; Santa Cruz), phospho-AMPK Thr-172 (2535; Cell Signaling), HO-1 (70081; Cell signaling), phospho-p70S6 Kinase Thr 389 (9234; Cell Signalling), phospho-S6 Ribosomal Protein Ser 240/244 (2251; Cell Signaling) and β-actin (A 2066; Sigma). The blots were washed thoroughly in TBS-T buffer and incubated for 1 h with a peroxidase-conjugated IgG antibody. The immunoreactive proteins were visualized using an enhanced chemiluminescence substrate kit (ECL plus; Amersham Biosciences, GE Healthcare) according to the manufacturer's instructions. Digital images were obtained with a GBOX Chemi XL 1.4 system (Syngene, UK), which allows quantification of the band intensity. The protein load was monitored via the immuno-detection of actin.

2.8 Docking assay

A docking assay was performed between p70S6 kinase (p70S6K) and two potential inhibitors, RSV and QCT. A subunit of 3A60 protein structure from PDB was selected for the assay. Protein was prepared with Protein Preparation Wizard and grid was further generated. In protein preparation original hydrogen were removed and then added, and missing sidechains and loops were filled in using prime. Ligands were obtained from ChemSpider database and were prepared by ligprep in order to perform the docking. Protein preparation, grid generation and ligprep were carried out using Schrödinger Suite software.

2.9 Statistical analyses

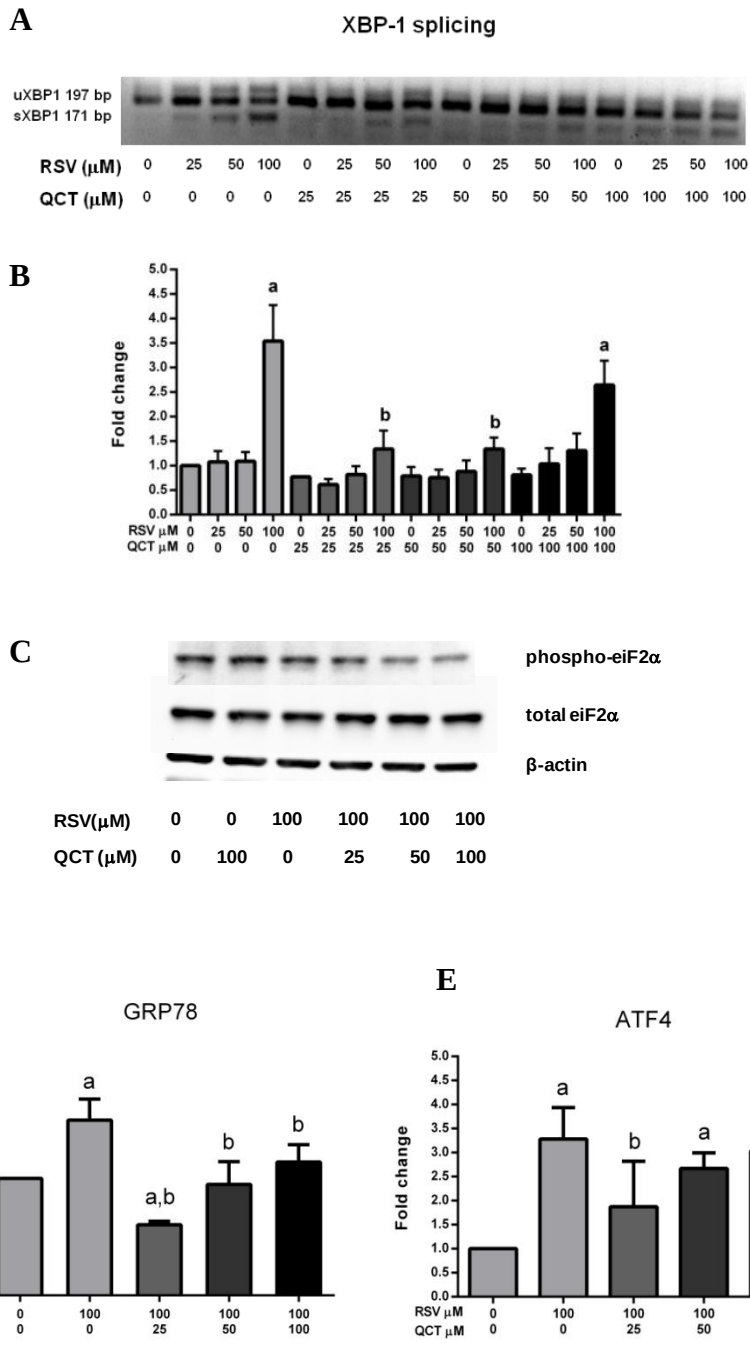
The data were evaluated by Student's T-test or one-way ANOVA, followed by the Bonferroni post hoc tests to identify significant differences between controls and treatments. GraphPad Prism version 6 software was used. The differences were considered significant when the *p* values were less than 0.05. Results are displayed as the mean±SD of at least three independent assays for each experiment.

3. Results

3.1 Quercetin abrogates Resveratrol-induced ER stress

In our attempt to discover novel synergistic effect of polyphenols we treated HepG2 cells with several combinations of QCT and RSV for 8 h. Due to our expertise in the field and our previous published [19] and unpublished results we started to study the consequences of such polyphenol combinations on ER-stress mechanisms. Despite the fact that, we have previously described that RSV is able to induce a potent ER-stress in HepG2 cells [19], the combination of RSV with QCT significantly decreased such ER-stress. As it could be seen in the figure **1** increasing doses of RSV are able to: (a) promote the splicing of X-box binding protein 1 (XBP-1), a well established marker of ER-stress activation (figure **1A**), and (b) induce C/EBP homologous protein (CHOP) overexpression (figure **1B**). In this sense, we observed that although increasing doses QCT alone are not able to induce ER-stress, in a combined treatment with RSV, it is capable to significantly reduce the RSV-mediated ER-stress. This effect is also evident when other ER-stress markers were studied, such as eiF2α phosphorylation, and GRP78 and ATF4 expression (figures **1C**, **D** and **E**, respectively). Interestingly, when we studied the apoptotic pathway, by using the caspase-3 cleavage, we observed the same profile than the observed with ER-stress, so RSV treatment at 8 h induced apoptosis activation, and the combination with QCT decreased such RSV pro-apoptotic effect (figure **1C**). It is noteworthy to mention that the lowest doses of QCT (25 and 50 μM) induce a strongest effect than the highest one (100 μM).

Figure 1


A
XBP-1 splicing
uXBP1 197 bp
sXBP1 171 bp
RSV (μM) 0 25 50 100 0 25 50 100 0 25 50 100 0 25 50 100
QCT (μM) 0 0 0 0 25 25 25 25 50 50 50 50 100 100 100 100
B
Fold change
a
b
b
a
RSV μM 0 25 50 100 0 25 50 100 0 25 50 100 0 25 50 100
QCT μM 0 0 0 0 25 25 25 25 50 50 50 50 100 100 100 100
C
phospho-eiF2α
total eiF2α
β-actin
RSV(μM) 0 0 100 100 100 100
QCT (μM) 0 100 0 25 50 100
D
GRP78
Fold change
a
a,b
b
b
RSV μM 0 100 100 100 100
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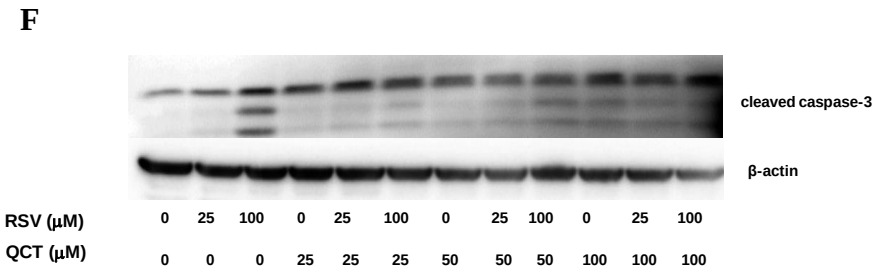


Figure 1. QCT inhibited RSV-induced ER-stress in HepG2 cells. After HepG2 cells were treated with several concentrations of RSV and/or QCT for 8 h, cell lysates were collected and proteins or mRNA were obtained. **(A)** Spliced form of XBP-1 was analyzed by PCR and gel electrophoresis. **(B)** CHOP mRNA expression was analyzed by real-time RT-PCR. **(C)** Phosphorylation level of eiF2α was analyzed by Western blotting using total eiF2α or β-actin as reference protein. **(D)** GRP78 mRNA expression was analyzed by real-time RT-PCR. **(E)** ATF4 mRNA expression was analyzed by real-time RT-PCR. **(F)** Cleaved-caspase 3 level was analized by Western blotting using β-actin as reference protein. For Western blotting and XBP-1 splicing results, one representative image of three independent experiments is shown. For real-time RT-PCR results, data are expressed as mean±SD of three independent experiments. Significant differences relative to the control (a) or to the 100 μM RSV condition (b) were analyzed when considered by one-way ANOVA followed by the Bonferroni post hoc test. Results were considered significant when $p < 0.05$.

3.2 Resveratrol silences the autophagic activation mediated by Quercetin

As QCT have been previously described to induce a "protective" autophagy [28,29] we wanted to study the viability of HepG2 cells at 24 h in the presence of high doses of both polyphenols, alone or in combination. As can be seen in figure **2B**, the MTT assay showed that QCT induced a "burst" in the HepG2 viability that was counteracted by RSV presence (RSV 100 μM + QCT 100 μM). This viability results were in agreement with the results obtained when the apoptotic process (cleaved caspase-3 and Bcl-2) was studied (figure **2B** and **2C**, respectively). In this sense, we observed a complementary profile to the one obtained with MTT when we studied the caspase-3 cleavage (caspase activation increased when the viability decreased).

Due to the previously observed effect on ER-stress at 8 h and because the autophagic pathway is intimately associated with the ER-stress process [31] we wondered if the QCT-induced effects on RSV-mediated ER-stress could be related to some modulation of the autophagic flux induced by both polyphenols. As can be seen in the figure **2C** both QCT and RSV were able to activate the autophagic process at 24 h, reflected by the 1A/1B-light chain 3 (LC3) lipidation (LC3-II), being QCT a more potent activator. This is in agreement with previous publications that describe these two polyphenols as autophagic-inducers [30,32,33].

Surprisingly, despite both compounds have been established as autophagic activators the combination of such compounds did not promote any synergistic effect. On the contrary, RSV seemed to act as an autophagic-inhibitor when QCT was present. This was in accordance with the previously observed effects on the ER-stress process (figure **1A**-**E**). In consequence, it seemed that QCT, by inducing the autophagic process, was able to relief HepG2 cells from the RSV induced ER-stress. But, on the other hand, RSV was able to silence this QCT-mediated autophagic effect, above all at the highest dose (100 µM), and would likely be responsible for the ER-stress re-activation.

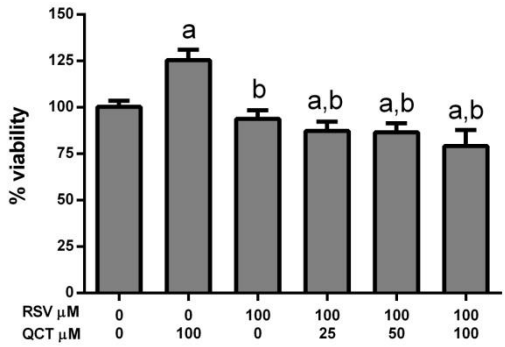
Consequently, it became clear that the QCT molecular "behaviour" on the apoptotic and autophagic processes was different depending on RSV presence. In consequence, we focused our efforts to further characterize this interesting RSV-induced modulation in HepG2 cells. To do so, instead of fixing one polyphenol concentration we combined three RSV concentrations with three QCT concentrations. Again, as it can be seen in figure **3A** and **3B**, the autophagic process was decreased when RSV was combined with the QCT treatment. More concisely, in figure **3A** we can observe an increased acridine orange staining, indicative of a high autophagic activity, due to QCT treatment (100 µM/24 h). Additionally, there was a drecrease on the staining (red colour) in HepG2 cells (acidic vacuoles) induced by RSV co-treatment (100 µM/ 24 h).

On the other hand, figure **3B** depictes the decrease of the autophagic marker LC3-II due to RSV increasing concentrations at 24 h treatment. Importantly this image also points out the fact that the autophagic process is decreased by RSV co-treatment (LC3-II decrease) despite the inactivation by the polyphenol combination of the mammalian target of rapamycin (mTOR) which is the main ihibitory kinase of the autophagic pathway (figure **3C**). This fact suggests that other alternative molecular targets are modulated by RSV and have impact on this autophagic process inactivation.

Finally, figure **3D** shows how this decrease on the autophagic process induced by RSV co-tretament triggers a potent pro-apoptotic signaling (cleaved caspase-3). At this point, it is interesting to mention that, as can be seen in figure **1B**, the increase on the ER-stress derived pro-apototic marker CHOP promotes a caspase-3 cleavage (figure **1C**). In consequence, it is likely that the decrease in the autophagic process induced by RSV co-treatment is promoting a potent pro-apoptotic signaling that could, at least partially, be mediated by an increased ER-stress. Nevertheless, we cannot rule out the fact that QCT by itself in the presence of RSV could be also inducing a more potent apoptotic signaling that could be not related to ER-stress.

Figure 2

A



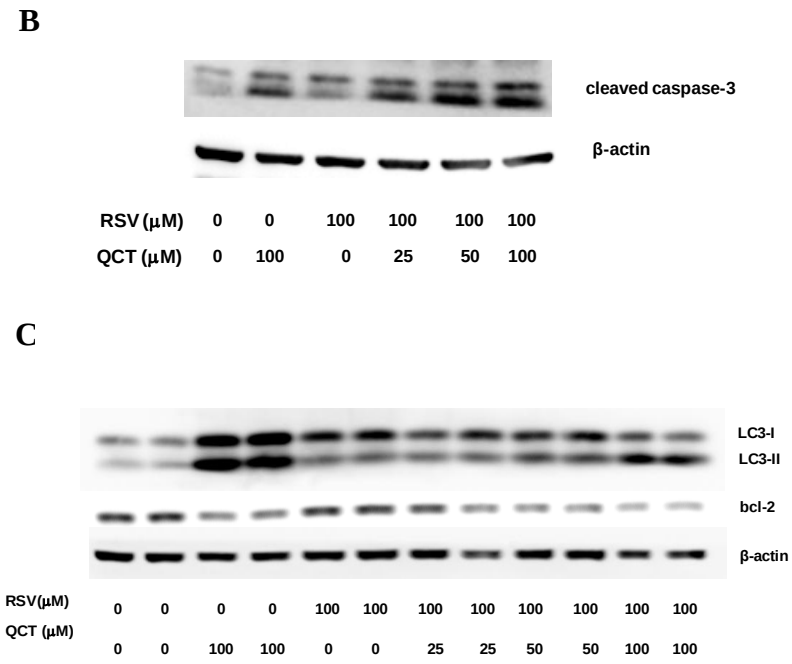
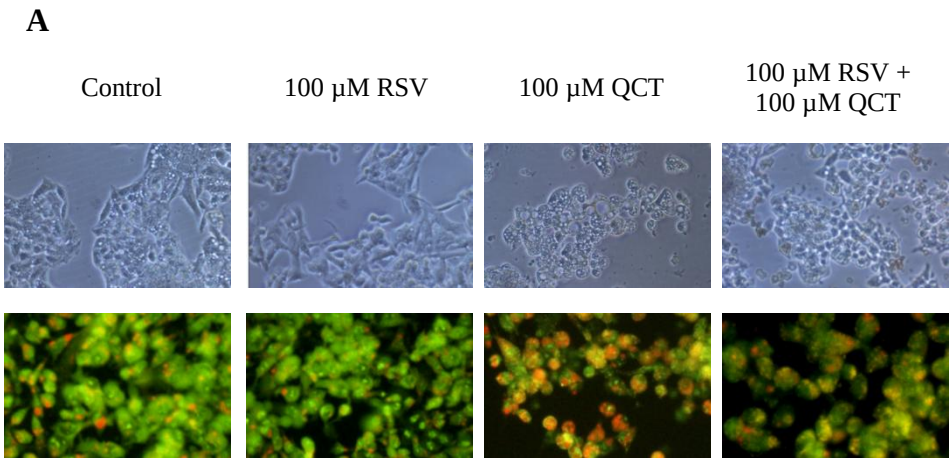


Figure 2. QCT induced a protective autophagy that was decreased by RSV. After HepG2 cells were treated with several concentrations of RSV and/or QCT for 24 h, cell viability was assessed by MTT or cell lysates were collected for Western blotting. (**A**) HepG2 viability was evaluated using a MTT assay. Data are shown as the mean ± SD of three independent experiments. (**B**) Cleaved caspase-3 analysis by Western blotting. The blot shown is a representative image of three independent experiments. (**C**) LC3-II and bcl-2 levels were analyzed by Western blotting using β-actin as reference protein. One representative image of three independent experiments is shown. For all densitometry tests, significant differences relative to the control (a) or to the 100 μM QCT condition (b) were analyzed when considered by one-way ANOVA followed by the Bonferroni post hoc test. Results were considered significant when $p < 0.05$.

Figure 3


A
Control
100 µM RSV
100 µM QCT
100 µM RSV + 100 µM QCT
B
RSV 0 µM
RSV 50 µM
RSV 100 µM
QCT 0 µM
QCT 50 µM
QCT 100 µM
LC3-I
LC3-II
β-actin
LC3-II/ β-actin ratio
RSV µM
QCT µM
C
p-mTOR (Ser 2448)
β-actin
phospho-mTOR (Ser 2448) / β-actin ratio

D

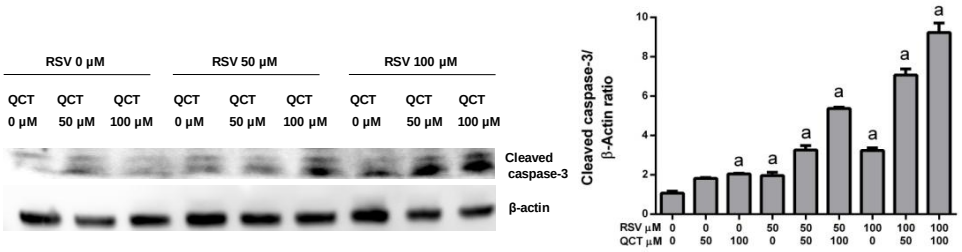


Figure 3. RSV abrogated QCT-induced autophagy despite mTOR inhibition. After HepG2 cells were treated with several concentrations of RSV and/or QCT for 24 h, fluorescent images were acquired or cell lysates were collected for Western blotting. (**A**) Acridine orange staining. One representative image of 10 images per condition is shown. All images presented are at the same magnification. (**B**) LC3-II and bcl-2 levels were analyzed by Western blotting using β-actin as reference protein. One representative image of three independent experiments is shown. Band densitometry is shown on the right part. (**C**) phospho-mTOR (Ser 2448) levels were analyzed by Western blotting using β-actin as reference protein. One representative image of three independent experiments is shown. Band densitometry is shown on the right part (**D**) Cleaved caspase-3 analysis by Western blotting. The blot shown is a representative image of three independent experiments. Band densitometry is shown on the right part. For all densitometry tests, significant differences relative to the control (a), to the 100 µM QCT condition (b) or to the 100 µM RSV condition (c) were analyzed when considered by one-way ANOVA followed by the Bonferroni post hoc test. Results were considered significant when $p < 0.05$.

3.3 Quercetin promotes a starvation-like state which is decreased by Resveratrol

In order to deep into the possible molecular mechanisms that are leading the modulation of the autophagic process we treated HepG2 cells for 45 min with QCT and/or RSV in order to study several important phosphorylated proteins. Starvation or energy deficiency is one of the hallmarks of autophagy activation. And, in fact, one of the adaptative pathways induced by the starvation process is the AMPK-mediated activation of autophagy in order to obtain energy from this catabolic process [34]. Accordingly, we focused on the study of the AMPK phosphorylation level. In this sense, figure **4A** shows that the phosphorylated AMPK state increased due to QCT treatment. This kinase is activated by a low energetic state of the cell (high 5'-adenosine monophosphate level). This

suggests that QCT is inducing a starvation-like state in HepG2 cells that is, at least partially, responsible for the autophagic process. Interestingly, RSV co-treatment decreased the phosphorylated form of AMPK. In consequence, it is likely that RSV co-treatment, by counteracting the starvation-like state mediated by QCT, could be inhibiting the autophagic process.

3.4 Resveratrol strongly downregulates hemeoxigenase 1 expression

HO-1 catalyzes the first and rate-limiting step in heme degradation. Interestingly, evidence accumulated over the past 25 years demonstrates that HO-1 is induced not only by the substrate heme but also by a variety of non-heme inducers such as heavy metals, endotoxin, heat shock, inflammatory cytokines, and prostaglandins. The chemical diversity of HO-1 inducers led to the speculation that HO-1, besides its role in heme degradation, may also play a vital function in maintaining cellular homeostasis. Interestingly, HO-1 is also highly induced by a variety of agents causing oxidative stress; and increasing HO-1 expression seems to be protective in animal and in vitro models [35]. Additionally, recent studies have highlighted the critical role of HO-1 in regulating autophagy [36,37]. Consequently, we focused on the influence of QCT and/or RSV treatments on HO-1 expression. As shown in figure **4B,** RSV strongly downregulated HO-1 expression in a dose dependent-manner. Furthermore, the highest doses of QCT for each combination with RSV promoted a slight recovery of this downregulation

Figure 4

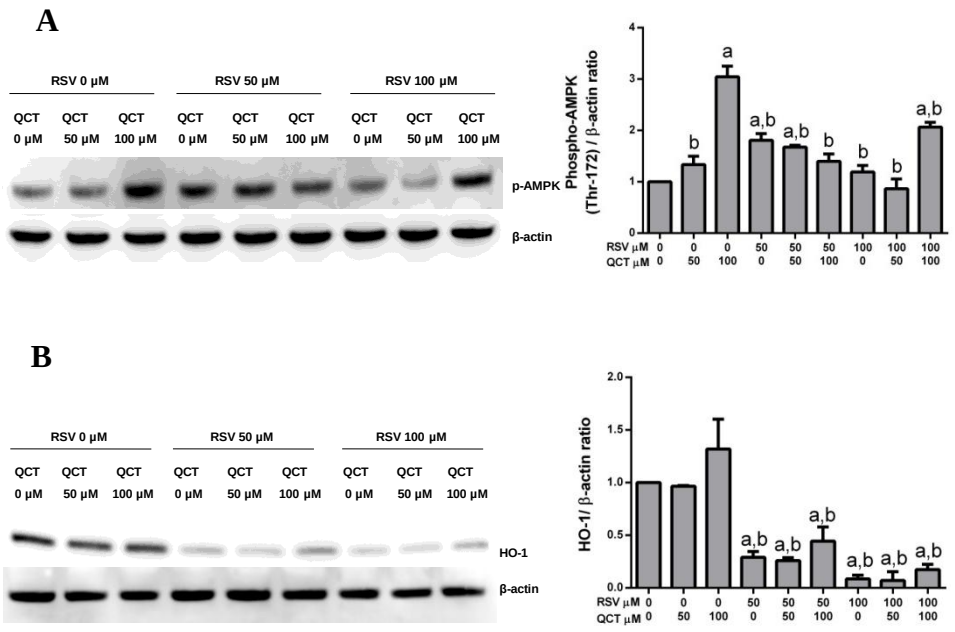


Figure 4. AMPK phosphorylation and HO-1 expression were related to the RSV autophagic inhibitory effect. After HepG2 cells were treated with several concentrations of RSV and/or QCT for 45 min, cell lysates were collected for Western blotting. (**A**) phospho-AMPK (Thr 172) levels were analyzed by Western blotting using β-actin as reference protein. One representative image of three independent experiments is shown. Band densitometry is shown on the right part (**B**) HO-1 analysis by Western blotting. The blot shown is a representative image of three independent experiments. Band densitometry is shown on the right part. For all densitometry tests, significant differences relative to the control (a) or to the 100 µM QCT condition (b) were analyzed when considered by one-way ANOVA followed by the Bonferroni post hoc test. Results were considered significant when $p < 0.05$.

3.5 Resveratrol and quercetin inhibit p70S6 Kinase

It has previously been shown that RSV can exert the same "anti-autophagic" and "pro-apoptotic" effect in cells that are under caloric restriction state (serum deprivation), and in this case the postulated mechanism of the polyphenol was

the p70S6K inhibition, the well known target of mTOR [38]. As a consequence we were also interested in elucidating if some of the observed effect, mediated by RSV, was also due to this kinase inhibition. For this purpose, we studied the phosphorylated state (threonin 389) of p70S6K. We observed that QCT and/or RSV were able to decrease such phosphorylation state. As previously seen with mTOR, the combination of such polyphenols was the treatment that induced the most potent inhibitory effect of such phosphorylation (figure **5A**). Interestingly, when we studied the phosphorylated level of S6 ribosomal protein, a well-characterized downstream target of p70S6 kinase, we observed that QCT and/or RSV were also able to strongly reduce the phosphorylated level of S6 ribosomal protein (figure **5B**). Again, the combination of such polyphenols was the treatment that induced the most potent inhibitory effect of such phosphorylation. This could indicate that both polyphenols are affecting p70S6K function. In order to further evaluate the effect of these polyphenols on this kinase we carried out a docking assay between p70S6K and both compounds. Interactions in the binding site are showed for RSV and QCT (green carbon molecules) with residues (orange carbon molecules) (figure **5C**). It is observed that three H bonds (red dashed lines) are predicted for RSV, with Glu143, Leu175 and Asp236. QCT presents the same interactions as RSV and one more, with Lys123 residue. The glide score was -10,076 for QCT, and -6,084 for RSV. This docking assay steps up the fact that those polyphenols could be affecting p70S6K function by interacting hard in its binding site.

Figure 5

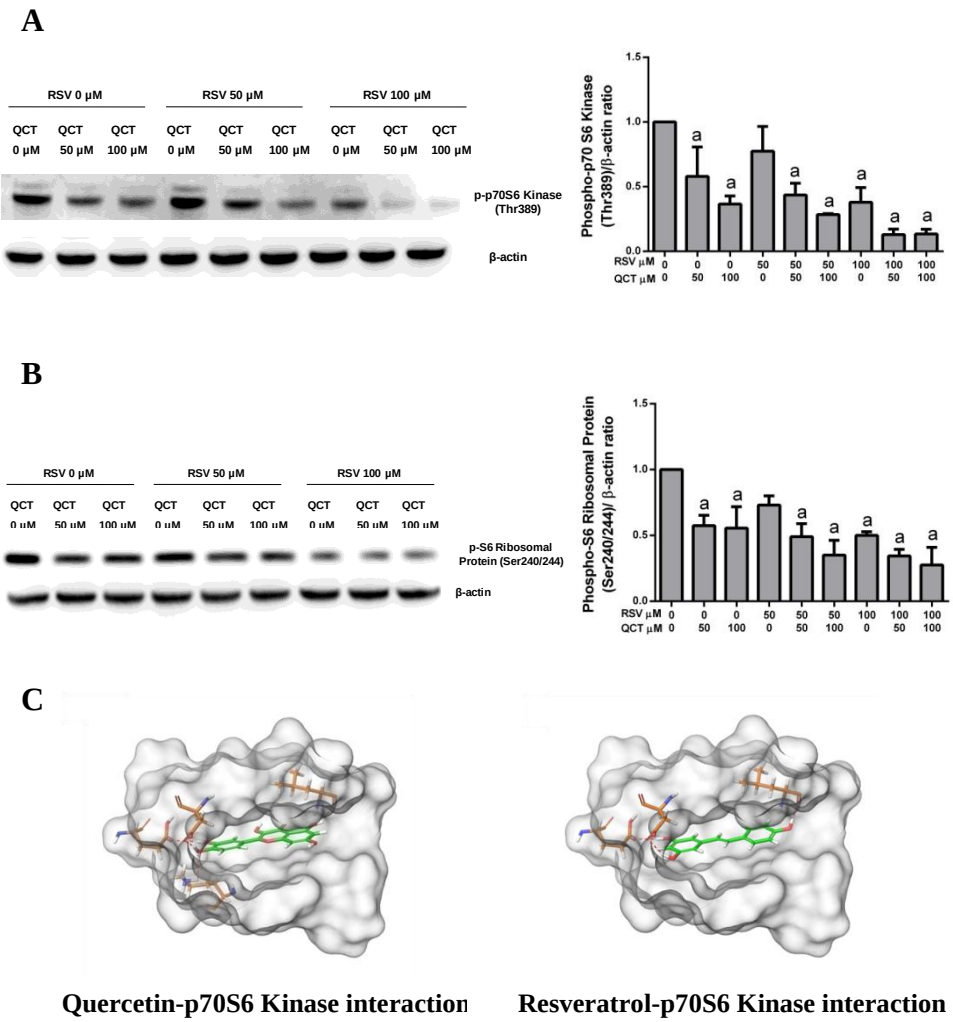


Figure 5. p70S6 kinase was inhibited by QCT and RSV. After HepG2 cells were treated with several concentrations of RSV and/or QCT for 45 min, cell lysates were collected for Western blotting. (**A**) phospho-p70S6 kinase (Thr 389) levels were analyzed by Western blotting using β-actin as reference protein. One representative image of three independent experiments is shown. Band densitometry is shown on the right part (**B**) phospho-S6 ribosomal protein (Ser 240/244) analysis by Western blotting. The blot shown is a representative image of three independent experiments. Band densitometry is shown on the right part. For all densitometry tests, significant differences relative to the control (a) were analyzed when considered by one-way ANOVA followed by the Bonferroni post hoc test. Results were considered significant when $p < 0.05$. (**C**) Docking assay of QCT and RSV with p70S6 kinase.

4 Discussion

In this study we aimed to clarify the interaction of two natural compounds such as QCT and RSV, which have been previously described as potent autophagic inducers, on this important catabolic process in HepG2 cells. Unexpectedly we discovered that the combination of both polyphenols has an opposite effect on autophagy as well as on ER-stress. For example, QCT induced a potent autophagic activation in HepG2 cells that was confirmed through acridine orange staining and conversion of LC3- II (figures **2** and **3**). This QCT-mediated activation of autophagy decreased ER-stress induced by RSV (figure **1**). On the other hand, RSV was also able to promote a significant but weaker (compared to QCT) autophagic process (figures **2** and **3**). Nevertheless, it seemed that the autophagic induction elicited by both polyphenols was slightly different.

On the one hand, QCT promoted a "starvation-like" state characterized by a potent signaling through AMPK which could be, at least partially, responsible for this autophagic process. In this sense, it has been clearly identified by other authors that QCT is able to induce a protective autophagy in cancer cells by modulating pathways such as AKT [28] , JNK [29] and AMPK [39], among others.

On the other hand, RSV have been described to induce autophagy by AMPK signaling [40], by SIRT1 induction [41] or even by directly inhibition of mTOR through ATP competition [32], among others.

Interestingly, although both compounds were not equally able to inhibit mTOR phosphorylation (figure **4C**), the combination of QCT and RSV was the treatment that induced the most potent inhibition on the phosphorylation of mTOR. This is an important aspect because theoretically under this molecular scenario (synergistic mTOR inhibition) one would expect that the autophagic process would have been exacerbated. In fact, related to this, it has been previously described that QCT in combination with other substances, can

potentiate their autophagy- and cell death inducing qualities [42]; and synergisms with other autophagy-inducing natural substances found in plants have been detected [43–45]. This is especially relevant in a nutritional context as QCT is found in numerous foods and is likely to act synergistically with other autophagy-inducing plant metabolites contained in the diet.

Nevertheless, in a cancerous context, such as our HepG2 *in vitro* model or others, QCT in a combined treatment with an autophagy inhibitor, may be an excellent therapeutic approach to reduce cancer cell proliferation and could be a promising strategy to sensitize cells to QCT treatment. In this sense, several synthetic autophagic inhibitors such as chloroquine [28,29]

or 3-methyladenine [46] have been proved successful for this purpose; reducing the "protective" autophagy and maximizing the pro-apoptotic cell death.

Furthermore, as far as we know, this is the first report that in fact describes an antagonistic effect on the autophagic process between two natural autophagic inducers such as QCT and RSV in HepG2 cancer cells. Additionally, this effect seems to be related to the energetic cellular state, due to the differences observed in AMPK phosphorylation between QCT and RSV (figure **4A**).

Interestingly, Armour et. al [38] in a series of elegant experiments showed that RSV was able to suppress autophagy on a caloric restricted cellular environment, or by direct mTOR inhibition through rapamycin treatment. So it is likely that AMPK modulation could be playing an important role on this RSV mediated effects. Nevertheless, Armour and collaborators postulated that the inhibitory effect of RSV on p70S6K was an important factor of the dual behavior of RSV on autophagy depending on the nutrional state; and hypothesized, that insulin signaling could be the key difference. In consequence, the authors postulated that under nutrient withdrawal, where insulin signaling was minimal, the inhibition of p70S6K, that promoted a reduction in autophagy, could be the dominant effect of RSV. Additionally, they also hypothesized that

when autophagy was held in check by robust signaling through insulin-PI3K-Akt-mTOR (fed conditions), the disruption of this pathway could lead to the induction of autophagy over time.

Despite we completely agree with Armour and collaborators that RSV attenuates autophagy and this could be related to starvation and mTOR inhibition; we are also convinced that, in our case, other factors rather than p70S6K inhibition are responsible for such effect. This is based on the fact that, as can be seen in figure **5**, we observed that RSV as well as QCT seems to be able to importantly inhibit this kinase. In consequence, there must be some differential effect/s of these polyphenols that are responsible for this interesting "phenotype" in HepG2 cells, and one of these effects could be related to AMPK differential modulation.

On the other hand, it has been recently described that hepatocyte death induced by experimental sepsis *in vivo* or lipopolysaccharide (LPS) *in vitro* is exacerbated by either inhibition or siRNA knockdown of HO-1, suggesting a pro-survival induction of autophagy via HO-1 action [47]. The same group also demonstrated that in macrophages, LPS induces the release of proinflammatory cytokines together with a concomitant increase in autophagy via a toll-like receptor-4 (TLR4)/HO-1-dependent pathway [48]. In an acute kidney injury model induced by cisplatin, HO-1 and LC3-II were up-regulated in proximal tubular epithelial cells [49]. By contrast, proximal tubular epithelial cells of HO-1-knockout mice showed significant apoptosis and impaired induction in autophagy [49]. These reports demonstrated the critical role of HO-1 in regulating autophagy. As a consequence, in our attempt to identify possible factors that could be characteristic of this RSV suppressive effect on autophagy we focused in HO-1 modulation due to QCT and/or RSV treatment.

HO-1, known as an anti-oxidative molecule, is the first and rate-limiting enzyme in the catabolism of heme to produce equimolar amounts of biliverdin, CO, and free iron. In addition, HO-1 is highly inducible under stress conditions. Human

HO-1 deficiency increases sensitivity to oxidative stress, resulting in severe endothelial damage. The influence of polyphenols on HO-1 have been also widely studied [50–52] but little is known about its relation on autophagy regulation. In this sense, our results (figures **4B**) clearly show that RSV is strongly downregulating HO-1 protein content and this correlates with the anti-autophagic effect of RSV. In agreement with our results, it has been shown that the HO-1 inhibitor zinc protoporphyrin (ZnPP) strongly inhibits autophagy and induces apoptosis [53], and that HO-1 induces protective autophagy [37].

In conclusion, QCT can induce protective autophagy in HepG2 cells. And it is feasible to think that under mTOR inhibition or caloric restriction, RSV could act as an autophagic silencer instead of behave as an autophagic inductor, sensitizing in consequence cells to QCT treatment. Additionally, as autophagy induced by QCT or RSV is negatively regulated by mTOR, and as both polyphenols are equally able to inhibit p70S6K, we hypothesize that other factors are responsible for this characteristic and differential effect. In view of our results, we propose that in this context (polyphenol co-treatment) RSV acts as an autophagic inhibitor by, at least, modulating AMPK phosphorylation levels and downregulating HO-1 expression. We cannot rule out the fact that other important molecular mechanisms related to autophagy could also be modulated by such polyphenol combination. For example, it could be feasible that in this context (polyphenol co-treatment) RSV is sensitizing HepG2 cancer cells to apoptosis by inhibiting the QCT- induced mitophagy of organelles that are signaling an apoptotic cell death. Further experiments are required to validate such hypothesis.

Therefore, the application of combinations of QCT and RSV in the treatment of other cancer cells or tumor models could be a worthy possibility to be further studied. And, additionally, it will be interesting to test the effects of resveratrol on autophagy in animals, especially under starvation, mTOR inhibition or tumor models, where we could observe a similar duality of function induced by RSV.

Author contributions: M.M designed the research and wrote the manuscript, S.TH conducted the research and wrote the manuscript. J.RM conducted the docking assay. All authors read and approved the final manuscript.

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The authors have declared no conflicts of interest.

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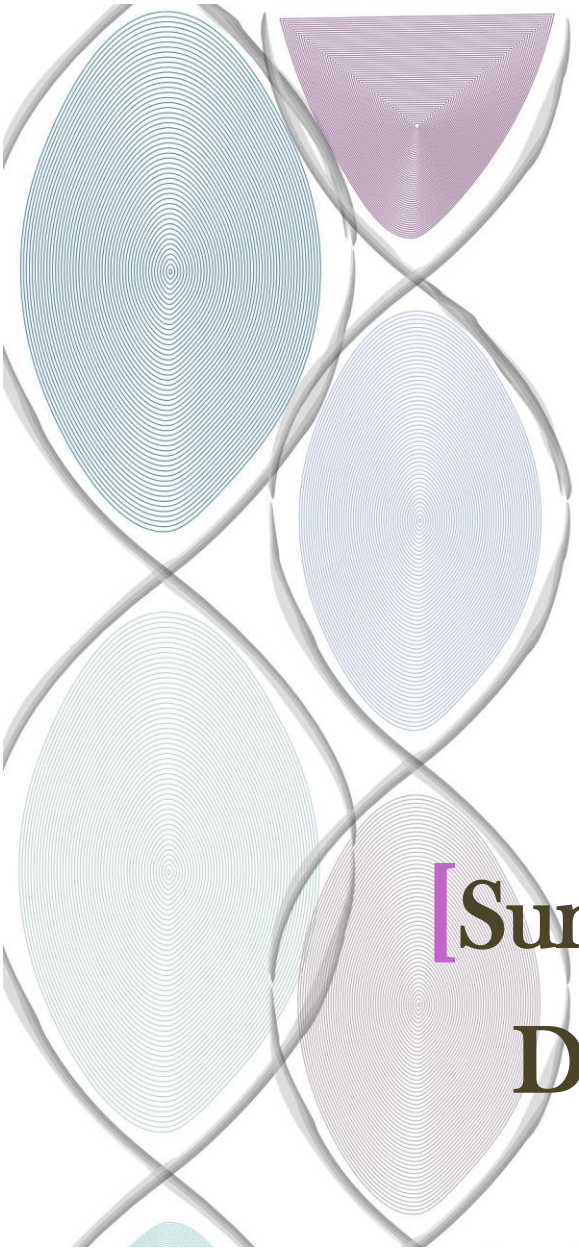


[Summarizing Discussion]

UNIVERSITAT ROVIRA I VIRGILI
CHARACTERIZATION OF THE BIOLOGICAL EFFECTS OF NATURAL COMPOUNDS AGAINST INFLAMMATION, METABOLIC SYNDROME AND CANCER
Sara Tomás Hernández

Natural products (NPs) have been used since ancient times for the prevention and treatment of many diseases and illnesses. Humans have relied on nature for the cure and prevention of a wide spectrum of diseases using traditional medicines, remedies, potions and oils with bioactive natural compounds. Many of these natural molecules have gone on to become current drugs.

The main goal of this doctoral thesis was to characterize selected bioactive natural compounds that could be used for the prevention or treatment of some major significant diseases, such as metabolic syndrome, neurodegenerative diseases and cancer. These medical conditions constitute an important health problem worldwide since they are included in the world leading causes of death.

In recent years, natural products and their active metabolites have become increasingly important on pharmaceutical research since they are a very important source when looking for novel molecules that could be used as lead compounds during the development of new drugs. Due to the enormous existent variety of biocompounds, there are literally millions of natural chemical structures that could be candidates for pharmaceutical research. Thousands of chemicals must be evaluated and tested to find a proper hit against one specific target. Unfortunately, bioactivity screening in extracts exclusively by *in vitro* or *in vivo* approaches is a complex and expensive process which is difficult to afford. In that sense, computer-aided drug design (CADD) methodologies like Virtual Screening (VS) have been successfully used to screen large NPs databases in order to identify new bioactive molecules for specific targets.

The first part of this thesis was focused on the identification and characterization of natural compounds with anti-inflammatory properties. Since numerous studies have shown that the chronic inflammation process is directly involved in the onset of metabolic syndrome and neurodegenerative disease we aimed to find a natural compound that could revert this harmful persistent inflammation state.

In these initial studies, firstly, we applied previously validated VS workflow developed by our group to a NPs database in order to identify potential IKK-2 inhibitors. One of this predicted IKK-2 inhibitors was 2,4-dihydroxy-6-methylbenzaldehyde, most commonly known as *o*-orsellinaldehyde, a molecule contained in *Grifola frondosa* mushroom specie.

In that sense, in order to validate the *in silico* predictions we performed a kinase assay that confirmed that *o*-orsellinaldehyde directly targets IKK-2 and reduces its IKK-2 kinase activity in a dose-response manner. Additionally, we validate that *o*-orsellinaldehyde significantly inhibited IKβα phosphorylation in LPS-stimulated RAW 264.7 macrophage cells. Moreover, the anti-inflammatory properties of the studied molecule have also been demonstrated by the obtained results referred to the reduction of nitrites, IL-6 and iNOS expression in the *in vitro* model used.

To study if this compound was also effective *in vivo* we induced an LPS endotoxic shock model in Balb/c mice. In both accomplished studies (intraperitoneal administration and oral administration of *o*-orsellinaldehyde) the molecule significantly reduced the serum IL-6 concentration. All this findings provide strong evidences that *o*-orsellinaldehyde possesses anti-inflammatory properties and that it exerts its activity by influencing NF-kB activity, more concretely, acting as IKK-2 inhibitor.

Other research groups working with this molecule had previously reported that *o*-orsellinaldehyde was able to inhibit growth as well as induce apoptosis in various types of cancer cell lines. Other studies stated that in some particular cases this molecule exerts cytotoxic effect. We were also able to observe these characteristics in the experiments carried out in our lab. Our results also showthat *o*-orsellinaldehyde exhibits a cytotoxic effect on HepG2 cells and that this effect was mediated through an apoptotic process. Bearing in mind that NF-kB complex is also involved in propagating the cellular response to apoptosis

and carcinogenesis we suggest that some of the cytotoxic and apoptotic effects of *o*-orsellinaldehyde observed against the cancer cells could be due to NF-kB inhibition.

Finally, *Grifola frondosa* mushrooms extracts were analyzed by liquid chromatography/quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) in order to identify and quantify the amount of this compound and evaluate the feasibility of preparing extracts from this natural source that were enriched on *o*-orsellinaldehyde. The results of this experiments show that, although we were able to isolate the molecule, the amount of this compound in the extracts prepared was very low, suggesting that further studies would be necessary in order to isolate enough amount of the anti-inflammatory molecule from the mushroom specie.

In summary this first study presented the potential anti-inflammatory activity of *o*-orsellinaldehyde, suggesting that it may be a potential preventive or therapeutic candidate for the treatment of inflammatory disorders such as metabolic syndrome. *(Manuscript 1)*

Once examined the beneficial properties of the *o*-orsellinaldehyde in RAW 264.7 cells and in Balb/c mice we wanted to elucidate if this molecule could also exert this potent anti-inflammatory effect in a neuroinflammation context. In that sense, we evaluate the effects of *o*-orsellinaldehyde in LPS-activated glia. *(Manuscript 2)*

The results obtained show that *o*-orsellinaldehyde caused an inhibition on Nitrite oxide (NO) production and iNOS expression after LPS-stimulation in both mixed glia and microglia cells. We also evaluated the effect of *o*-orsellinaldehyde in the expression of the stress protein heme oxygenase-1 (HO-1), observing that *o*-orsellinaldehyde cell pretreatment decreases HO-1 protein expression in LPS-activated mixed glia and microglia cells.

Although most reports hold that up-regulation of HO-1 expression is thought to be protective given the beneficial effects of HO-1, it is also stated that an excessive expression of this protein can aggravate injury. Thus, it has been proposed that reducing HO-1 expression could be a good strategy for certain neurodegenerative disorders. In that sense, *o*-orsellinaldehyde could be a potential compound to reach this last objective since we stated that this molecule is able to reduce the over-expression of HO-1 caused by the LPS stimulation.

Furthermore, we observed that cell pre-treatment with *o*-orsellinaldehyde suppressed the phosphorylation of Ikβα in LPS-activated microglia. Thus confirming that *o*-orsellinaldehyde exerts its anti-inflammatory effects by acting as IKK-2 inhibitor and modulating NF-kB activity.

Furthermore, MAPKs, including p38 and JNK play an important role in the inflammatory response since they are involved in the microglia activation. Several studies have been published suggesting that an effective inhibition of the MAPK pathway decreases the excessive inflammatory response observed in long-term activated glia and thus be beneficial for the treatment of some neuroinflammation states.

In that sense, we investigated the effect of *o*-orsellinaldehyde in the phosphorylation state of these MAPKs in LPS-stimulated microglia. Our results indicate that *o*-orsellinaldehyde markedly inhibit the activation of both, p38 and JNK, after LPS stimulation in microglial cells. Thus suggesting that the anti-inflammatory effect of this molecule could also be mediated by the inhibition of the MAPKs signaling pathway.

Regarding the potential of *o*-orsellinaldehyde on promoting M2 polarization in LPS-activated microglia we studied the expression of several genes associated with either M1 or M2 phenotypes. On the basis of our results we can state that the molecule tested, *o*-orsellinaldehyde, is able to promote the macrophage switch from inflammatory M1 type to the anti-inflammatory M2 phenotype.

In summary, with this study we demonstrated that *o*-orsellinaldehyde is a potent modulator of microglia activation and possesses strong neuroprotective effects. Thus suggesting that this molecule could improve some neuroinflammatory states and could be used as a hypothetical candidate for the treatment of several pathologies that deal with neuroinflammation.

The second part of this doctoral thesis was focused on the identification and characterization of novel PPARγ partial agonists from natural origin that present anti-diabetic properties without owning the side effects associated with the current existing drugs. *(Manuscript 3 and Manuscript 4)*

PPARγ is a member of the nuclear receptor superfamily and regulates the gene expression of a subset of proteins involved in energy homeostasis, adipogenesis and insulin sensitivity. This nuclear receptor is a well validated pharmaceutical target of the treatment of several metabolic diseases. In fact, the PPARγ full agonists rosiglitazone and pioglitazone (belonging to the TZDs drug class) have been widely used for the treatment of type 2 diabetes as they enhance human sensitivity to insulin. However, despite the beneficial effects that they display, this TZDs drugs are also associated with various undesired side effects. Therefore, several partial agonists of PPARγ are being developed as new antidiabetic drugs and have revealed a new mechanism of action for the anti-diabetic effect of some PPARγ agonist. This mechanism is completely independent of the classical PPARγ transactivation and consists on the inhibition of the phosphorylation of PPARγ on Ser 273, thereby preventing the deregulation of a several PPARγ beneficial related genes involved in metabolism homeostasis. It has been demonstrated that PPARγ full agonists are also able to inhibit this phosphorylation. Thus, given that both (partial and full agonists) have potent anti-diabetic effects it is feasible that a substantial portion of the therapeutic benefits of PPARγ ligands in metabolic disease is through the inhibition of the phosphorylation of PPARγ at Ser 273, whereas at least some of the undesirable side effects of this full agonist drugs may occur due to classical

agonist actions. Several plant extracts have been found to possess anti-diabetic effects trough modulation of PPARγ activity with no or little effect on adipogenesis. In that sense, PPARγ partial agonists from natural extracts are promising candidates for the treatment of some metabolic disorders, such as T2DM.

With this purpose our group, in a previous study, designed and applied a virtual screening workflow to identify novel PPARγ partial agonists among natural products databases. This *in silico* experiment successfully identified several molecules with high chance of being effective PPARγ partial agonists. One of this predicted molecules was 2,4,6-Trimethoxybenzophenone a molecule contained in the C. sumatranum ssp. Neriifoliumplant. Interestingly this plant specie has been traditionally used for the treatment of some medical disorders.

On the basis of this *in silico* results, we wanted to examine the effect of this novel partial agonist on the Cdk5-mediated phosphorylation of PPAR*γ* as well as its influence on adipogenesis.

Our results demostrated that the natural compound 2,4,6-Trimethoxybenzophenone could be a novel PPARγ ligand that would retain the benefits of improving insulin resistance since its able to inhibit Cdk5-mediated PPARγ phosphorylation at ser273, but minimizes the common side effects of TZDs, such as adipogenesis, by alleviating PPARγ agonistic activity. Although further characterization of the ant-diabetic properties or this compound is needed, these preliminary results suggest that the molecule studied could be a promising candidate drug for the treatment of some metabolic diseases, such as T2DM.

The last part of this thesis aimed to clarify the combined effect of the two natural compounds Quercetin (QCT) and Resveratrol (RSV), which have been previously described as potent autophagic inducers, on this important catabolic process in HepG2 cells. *(Manuscript 5)*

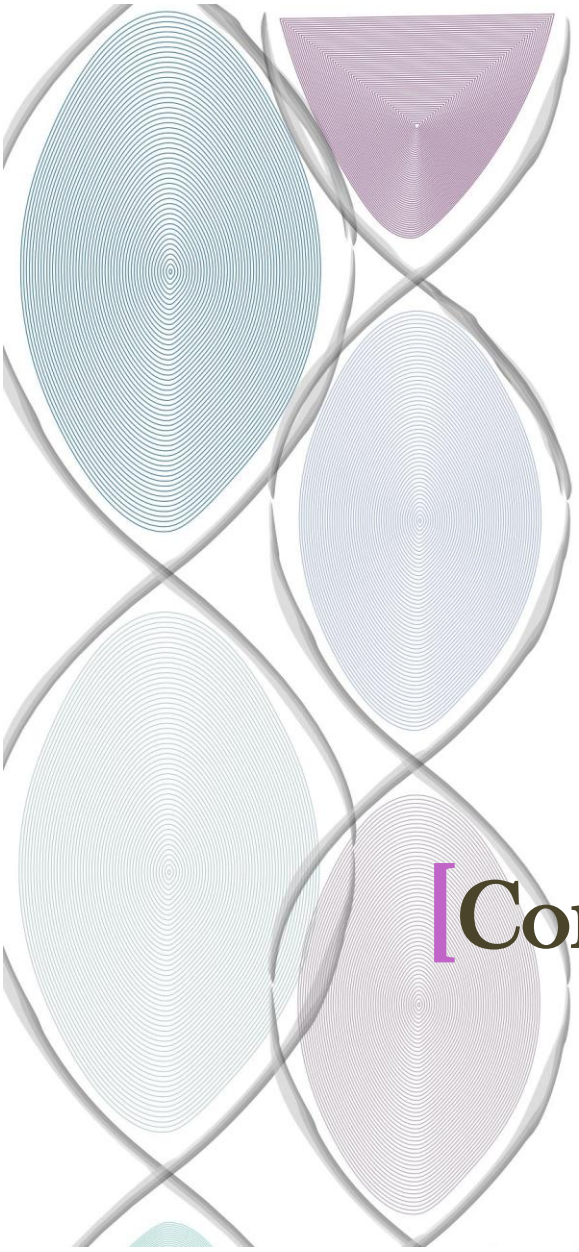
Unexpectedly we discovered that the combination of both polyphenols has an opposite effect on autophagy as well as on ER-stress. QCT induced a potent autophagic activation in HepG2 cells that was confirmed through acridine orange staining and conversion of LC3- II . This QCT-mediated activation of autophagy decreased ER-stress induced by RSV. On the other hand, RSV was also able to promote a significant but weaker (compared to QCT) autophagic process.

On the one hand, QCT promoted a "starvation-like" state characterized by a potent signaling through AMPK which could be, at least partially, responsible for this autophagic process. In this sense, it has been clearly identified by other authors that QCT is able to induce a protective autophagy in cancer cells by modulating pathways such as AKT, JNK and AMPK, among others.

Interestingly, although both compounds were not equally able to inhibit mTOR phosphorylation, the combination of QCT and RSV was the treatment that induced the most potent inhibition on the phosphorylation of mTOR. This is an important aspect because theoretically under this molecular scenario (synergistic mTOR inhibition) one would expect that the autophagic process would have been exacerbated.This is especially relevant in a nutritional context as QCT is found in numerous foods and is likely to act synergistically with other autophagy-inducing plant metabolites contained in the diet.

Nevertheless, in a cancerous context, such as our HepG2 in vitro model or others, QCT in a combined treatment with an autophagy inhibitor, may be an excellent therapeutic approach to reduce cancer cell proliferation and could be a promising strategy to sensitize cells to QCT treatment. In this sense, several synthetic autophagic inhibitors such as chloroquine or 3-methyladenine have been proved successful for this purpose; reducing the "protective" autophagy and maximizing the pro-apoptotic cell death.

In conclusion, RSV will act differentially on the autophagic process depending on the cellular energetic state. We have further characterized the molecular mechanisms that are related to this effect and we have observed that the AMP-activated protein kinase (AMPK) phosphorylation and heme oxygenase 1 (HO-1) downregulation could be important modulators of such RSV related effect, and could globally represent a promising strategy to sensitize cancer cells to QCT treatment.



[Conclusions]

UNIVERSITAT ROVIRA I VIRGILI
CHARACTERIZATION OF THE BIOLOGICAL EFFECTS OF NATURAL COMPOUNDS AGAINST INFLAMMATION, METABOLIC SYNDROME AND CANCER
Sara Tomás Hernández

Conclusions related to the objective 1→ To investigate whether the natural compound *o*-oresllinaldehyde might modulate the inflammatory and apoptotic response by acting as IKK-2 inhibitor (Manuscript 1 and Manuscript 2)

1- The natural compound *o*-oresllinaldehyde is effective in suppressing the production of inflammatory mediators in RAW246.7 cells as well as in Balb/c mice. Moreover, this molecule also exerts pro-apoptotic activity HepG2 cancer cells.
2- The anti-inflammatory properties of *Grifola frondosa* mushroom could partially be explained by the presence of *o*-orsellinaldehye on its composition.
3- The results with the LC-QTOF-MS show that, also we were able to isolate *o*-orsellinaldehyde from *Grifola frondosa* extracts, the amount of this molecule contained in *Grifola frondosa* mushroom is very low, suggesting that further studies would be necessary in order to isolate enough amount of the anti-inflammatory molecule from this mushroom specie.
4- *o*-Orsellinaldehyde is a potent modulator of microglia activation and possesses strong neuroprotective effects. This molecule leads microglia cells to a polarized M2 phenotype with anti-inflammatory and neuroprotective characteristics.
5- The anti-inflammatory and pro-apoptotic activity of *o*-oresllinaldehyde is mediated by its interaction with NF-kB complex, concretely acting as IKK-2 inhibitor

In summary, the potent anti-inflammatory activity of *o*-orsellinaldehyde suggests that it may be a potential preventive or therapeutic candidate for the treatment of several pathologies that deal with chronic inflammation such as metabolic syndrome and neurodegenerative diseases.

Conclusions related to the objective 2→ To examine the anti-diabetic effect that the natural compound 2,4,6-Trimethoxybenzophenone exerts by acting as PPARγ ligand. (Manuscript 3 and Manuscript 4)

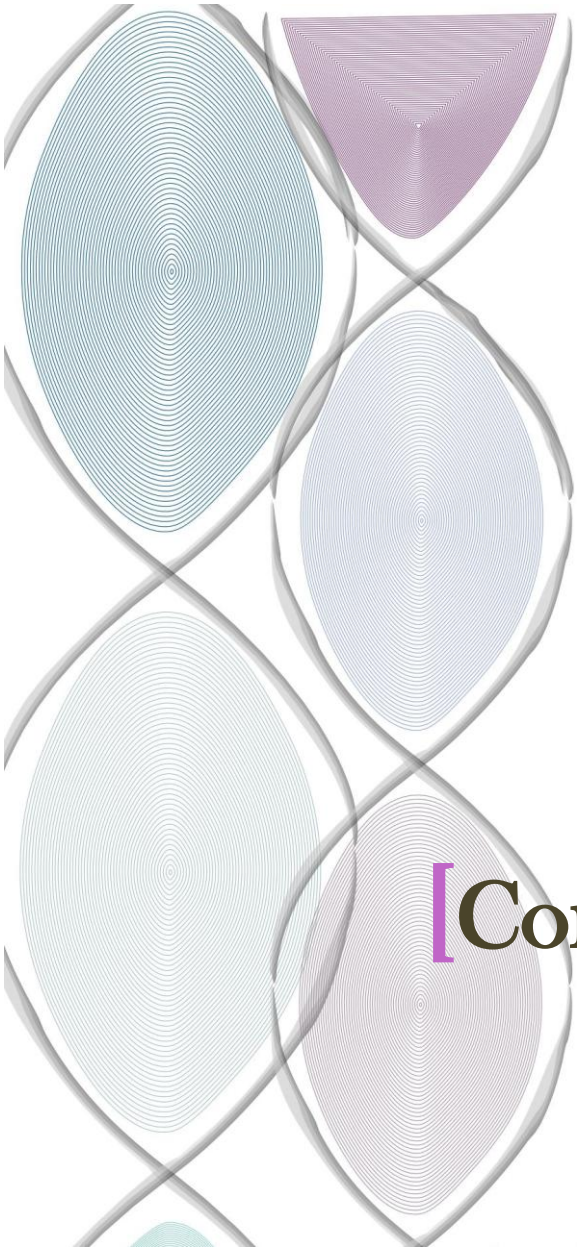
6- The kinase assay developed is an optimal method for studying the Cdk5-mediated phosphorylation of PPARγ at Ser 273 and its inhibition.
7- 2,4,6-Trimethoxybenzophenone could be a novel SPPARγM which is able to block CDK5-mediated PPARγ phosphorylation at ser273.
8- 2,4,6-Trimethoxybenzophenone inhibits adipogenesis in 3T3-L1 cells.

To sum up, our data demonstrated that the natural compound 2,4,6-Trimethoxybenzophenone could be a novel PPARγ ligand that would retain the benefits of improving insulin resistance since its able to inhibit Cdk5-mediated PPARγ phosphorylation at ser273, but minimizes the common side effects of TZDs, such as adipogenesis, by alleviating PPARγ agonistic activity. Although further characterization of the ant-diabetic properties or this compound is needed, these preliminary results suggest that the molecule studied could be a promising candidate drug for the treatment of some metabolic diseases, such as T2DM.

Conclusions related to the objective 3 →To study the combined effect of Quercetin (QCT) and Resveratrol (RSV) on the autophagic process in HepG2 cancer cells.

9- RSV could act as an autophagic silencer instead of behave as an autophagic inductor, sensitizing cells to QCT apoptotic effect.
10- Both, RSV and QCT, are able to inhibit p70S6K.
11- Some of the RSV effects regarding its autophagy inhibition could be mediated by modulation of AMPK phosphorylation and down-regulation of HO-1 expression.

In short, RSV potently counteracts QCT starvation-induced autophagy and sensitizes HepG2 cancer cells to apoptosis.



[Conclusions]

UNIVERSITAT ROVIRA I VIRGILI
CHARACTERIZATION OF THE BIOLOGICAL EFFECTS OF NATURAL COMPOUNDS AGAINST INFLAMMATION, METABOLIC SYNDROME AND CANCER
Sara Tomás Hernández

Conclusions relacionades amb l'objectiu 1→ Investigar si el compost natural *o*-orsellinaldehid podria modular la resposta inflamatòria i apoptòtica actuant com a inhibidor d'IKK-2. (Manuscrit 1 i Manuscrit 2).

1- El compost natural *o*-oresllinaldehid redueix la producció de molècules mediadores de la inflamatoris en cèl·lules RAW246.7, així com en ratolins Balb/c. A més, aquesta molècula també exerceix una activitat pro-apoptòtica en les cèl·lules canceroses HepG2.
2- Les propietats antiinflamatòries del bolet *Grifola frondosa* podrien explicar-se, en part, per la presència de la molècula *o*-orsellinaldehid en la seua composició.
3- Els resultats amb el LC-QTOF-MS mostren que tot i que hem pogut aïllar el compost *o*-orsellinaldehid a partir d'extractes de *Grifola frondosa*, la quantitat d'aquest compost continguda al bolet és molt baixa, la qual cosa suggereix que es necessiten estudis addicionals per tal d'aïllar més quantitat de la molècula antiinflamatòria a partir d'aquesta espècie de bolet.
4- L'*o*-orsellinaldehid és un potent modulador de l'activació de la micròglia i posseeix forts efectes neuroprotectors. Aquesta molècula promou la polarització de les cèl·lules de la micròglia cap al fenotip antiinflamatori M2.
5- L'activitat antiinflamatòria i pro-apoptòtica de l'*o*-oresllinaldehid és deguda a la seva interacció amb el complex NF-kB, més concretament, actuant com a inhibidor d'IKK-2.

En conclusió, la potent activitat antiinflamatòria que posseeix l'*o*-orsellinaldehid suggereix que aquest compost podria ser un possible candidat preventiu o terapèutic per al tractament de patologies relacionades amb processos d'inflamació crònica com són la síndrome metabòlica i les malalties neurodegeneratives.

Conclusions relacionades amb l'objectiu 2→ Examinar els efectes antidiabètics que pot posseir el compost natural 2,4,6-Trimethoxibenzofenona a l'actuar com a lligand de PPARγ (Manuscrit 3 and Manuscrit 4).

6- L'assaig quinasa desenvolupat es un mètode òptim per a estudiar la fosforilació de la Ser 273 de PPARγ així com la seua inhibició.
7- La 2,4,6-Trimethoxibenzofenona podria tractar-se d'un nou modulador selectiu de PPARγ capaç de bloquejar la fosforilació de la Ser273 de PPARγ duta a terme per l'enzim Cdk5.
8- La 2,4,6-Trimethoxibenzofenona inhibeix l'adipogènesi en cèl·lules 3T3-L1.

En resum, les nostres dades demostraren que el compost natural 2,4,6-trimetoxibenzofenona podria tractar-se d' un nou lligand de PPARγ que retindria la capacitat de millorar la resistència a la insulina (ja que és capaç d'inhibir la fosforilació de la Ser273 de PPARγ duta a terme per l'enzim Cdk5) però que degut a la seua baixa activitat agonística minimitza els efectes secundaris habituals dels fàrmacs existents, com ara l'adipogènesi.

Conclusions relacionades amb l'objectiu 3 → Estudiar l'efecte combinat de la Quercetina (QCT) i el Resveratrol (RSV) sobre els processos autofàgics en la línea cel·lular cancerosa HepG2. (Manuscrit 5).

9- El RSV podria actuar com un silenciador autofàgics en lloc de com un inductor d'aquest procés, sensibilitzant les cèl·lules a l'apoptòsi.
10- Tots dos, RSV i QCT, són capaços d'inhibir p70S6K.
11- Alguns dels efectes del RSV relacionats amb la seua capacitat inhibitòria de l'autofàgia podrien ser deguts a la modulació de la fosforilació de AMPK i la regulació de l'expressió de HO-1.

En resum, el RSV contraresta potentment l'autofàgia induïda per la QCT sota una situació de restricció calòrica i sensibilitza les cèl·lules canceroses a l'apoptòsi.

[Agraïments]

En aquests cinc anys que he passat realitzant la tesi he tingut moments bons i no tant bons, he fet amistats que conservaré tota la vida i he viscut anècdotes i històries que recordaré per sempre. De fet, amb totes aquestes vivències se'n podria fer una pel·lícula. No se si seria el film més taquiller de la història, però per mi ha sigut aquesta pel·lícula i els seus protagonistes el que ha fet possible que realitzés aquesta tesi doctoral. M'agradaria per tant mostrar el meu agraïment a totes aquelles persones que d'una manera o altra han estat al meu costat durant aquesta etapa i que han fet possible la realització d'aquest treball.

En primer lloc, el més sincer agraïment al Dr. Miquel Mulero, per haver sigut el Steven Spielberg d'aquesta tesi doctoral i la pel·lícula derivada d'ella. Per tota l'ajuda rebuda en aquests anys, pels consells, pels cafès i per tot el temps que m'has dedicat. Moltes gràcies per haver confiat en mi des del primer moment fins l'últim. Agrair també al Dr. Santi García el seu suport rebut durant aquesta etapa. Moltes gràcies als dos directors, he aprés molt al vostre costat. Voldria donar les gràcies també a tot els membres del grup de Quimioinformàtica i Nutrició, espere haver estat a "l'altura" de tots vosaltres. Ha sigut un plaer treballar al vostre costat.

A tots els membres del departament que d'alguna manera o altra m'han ajudat en aquesta tesi, de vegades amb aportacions científiques i de vegades amb paraules de motivació que han fet aquest camí molt més planer.

Voldria dedicar una menció especial a Niurka i Rosa. En la meua suposada pel·lícula serieu les tècnics en "sonido, iluminación, efectos especiales, resolución de catástrofes i sabiduría varia". Moltes gràcies per la vostra ajuda, sense vosaltres ni la meua tesi (ni la de ningú en aquesta universitat) hagués estat possible. Moltes gràcies a tots els companys de laboratori, els altres

protagonistes d'aquesta historia. Als que encara hi son i als que ja han marxat. Per totes els moments viscuts i rises compartides. En especial a Pan, esto no es lo mismo sin ti!

Grazie mille al Dr. Luisa Minghetti e il suo team. Grazie per avermi dato l'opportunità di lavorare con voi. Per il vostro coinvolgimento e gentilezza. Grazie per haver reso il mio soggiorno a Roma indimenticabile.

A Clàudia, Zaira, Miryam i Yorx per totes les vostres paraules d'ànim, per la vostra empatia i en alguns moments telepatia. Diuen que trobar amics amb el teu mateix desordre mental, no té preu. Amb vosaltres ho confirme.

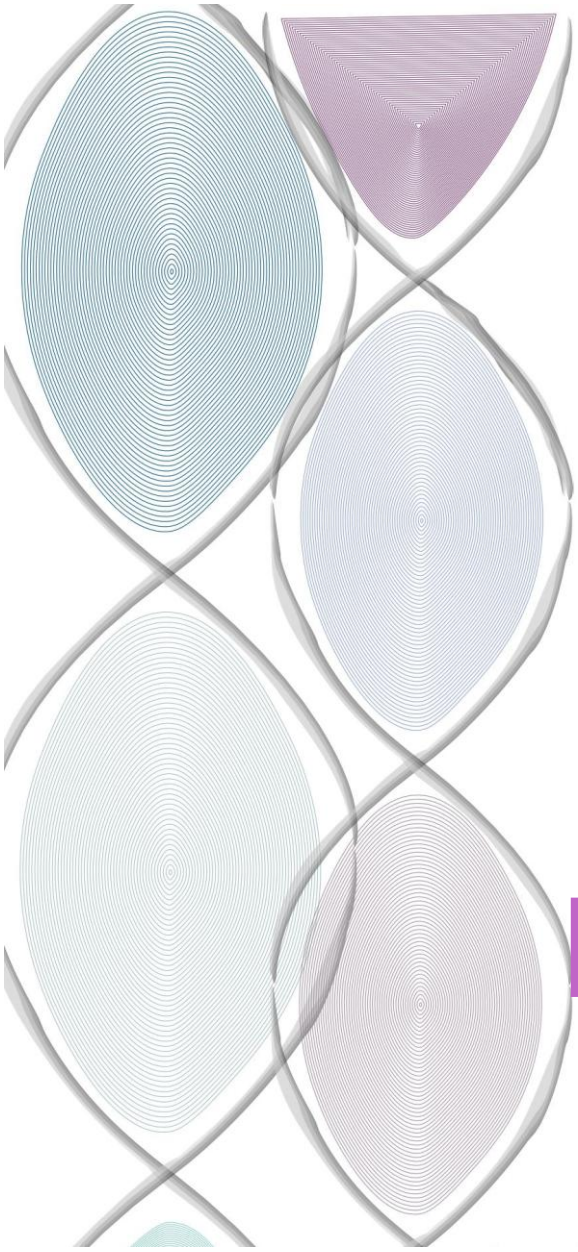
A les meues amigues, per haver estat al meu costat sempre, sense importar la distància que ens separa. Per haver-me animat en tot moment, per les vostres paraules de suport i carinyo. Per haver estat pendent de mi cada dia. Moltes gràcies.

En tota bona pel·lícula, la protagonista té sempre dos amics que l'acompanyen en tot moment. Sense aquests personatges la pel·lícula no tindria sentit. En aquest cas són María José i Manuel, moltes gràcies per tot. Per que el jeroglífic de cada matí no ha sigut l'únic problema que meu ajudat a resoldre. Per animar-me, guiar-me i comprendre'm en tot moment. Gràcies, l'Óscar és per a vosaltres.

Als meus pares, per haver confiat en mi en tot moment. Com ja he dit moltes voltes, gràcies per haver-me ajudat a arribar fins a ací i convertir-me en la persona que sóc.

I finalment a la meua germana Alba, ni esta tesi ni res del que he aconseguit mai ho haguera pogut fer sense tu. Gràcies per ser tu.

Moltes gràcies a tots



[Annexes]

UNIVERSITAT ROVIRA I VIRGILI
CHARACTERIZATION OF THE BIOLOGICAL EFFECTS OF NATURAL COMPOUNDS AGAINST INFLAMMATION, METABOLIC SYNDROME AND CANCER
Sara Tomás Hernández

[List of Publications]

1- Garcia-Vallvé S, Guasch L, **Tomas-Hernández S**, del Bas JM, Ollendorff V, Arola L, Pujadas G, Mulero M, *Peroxisome Proliferator-Activated Receptor γ (PPARγ) and Ligand Choreography: Newcomers Take the Stage*. J Med Chem. 2015 Jul 23;58 (14):5381-94. doi: 10.1021/jm501155f. Review. Impact factor: 5.5589 . Category: Chemistry medicinal. Position 3/59 (Q1)

2- **S. Tomás-Hernández**, S. Garcia-Vallve, G. Pujadas, C. Valls, M.J. Ojeda-Montes, A. Gimeno, A. Cereto-Massagué, J. Roca-Martinez, M. Suárez, L. Arola, M. Mulero, R. Beltrán-Debón. *Anti-inflammatory and pro-apoptotic properties of the natural compound o-orsellinaldehyde through IKK-2 inhibition*. Submitted.

3- **S. Tomás-Hernández**, S. Garcia-Vallve, G. Pujadas, M.J. Ojeda-Montes, A. Gimeno, A. Cereto-Massagué, L. Arola, L. Minghetti, M. Mulero, R. Beltrán-Debón. *Neuroprotective effects of the natural compound o-orsellinaldehyde in murine primary glial cells*. Submitted.

4- **S. Tomás-Hernández**, J. Blanco, C. Rojas, J. Roca-Martinez, M.J. Ojeda-Montes, R. Beltrán-Debón., S. Garcia-Vallve, G. Pujadas, L. Arola, M. Mulero. *Resveratrol potently counteracts quercetin starvation-induced autophagy and sensitizes HepG2 cancer cells to apoptosis*. Submitted.

5- T. García, **S. Tomás-Hernández**, D. J. Sánchez, M. Mulero, M. Gómez, J.L. Domingo, J. Blanco. *Effect of oral subchronic exposure to silver nanoparticles on insulin signaling in rat livers*. Submitted.

6- M.J. Ojeda-Montes, À. Casanova-Martí, A. Gimeno, **S. Tomás-Hernández**, A. Cereto-Massagué, G. Wolber, R. Beltrán-Debón, C. Valls, M. Mulero, M. Pinent, G. Pujadas, S. Garcia-Vallve, *A virtual screening strategy to mine large molecular databases to find new leads with low similarity to known actives: application to find new DPP-IV inhibitors*, Submitted to European Journal of Medicinal Chemistry.

7- M.J. Ojeda-Montes, A. Gimeno, **S. Tomás-Hernández**, A. Cereto-Massagué, R. Beltrán-Debón, C. Valls, M. Mulero, G. Pujadas, S. Garcia-Vallve, *Activity and selectivity cliffs for DPP-IV inhibitors: lessons we can learn from SAR studies and their application to virtual screening*, Submitted to Medicinal Research Reviews.

8- M.J. Ojeda-Montes, A. Ardid-Ruiz, **S. Tomás-Hernández**, A. Gimeno, A. Cereto-Massagué, R. Beltrán-Debón, M. Mulero, S. Garcia-Vallve,G. Pujadas, C. Valls, *Ephedrine as a lead compound for the development of new DPP-IV inhibitors*, Submitted to Future Medicinal Chemistry.

9- A. Cereto-Massagué, M.J. Ojeda, A. Gimeno, **S. Tomás-Hernández**, R. Beltrán-Debón, J.M. Mateo-Sanz, C. Valls, M. Mulero, G. Pujadas, S. Garcia-Vallve, *Molecular weight-based decoys: A simple decoy set finding alternative for fingerprint similarity approaches* [Submitted]

10- A. Cereto-Massagué, M.J. Ojeda, A. Gimeno, **S. Tomás-Hernández**, R. Beltrán-Debón, J.M. Mateo-Sanz, C. Valls, M. Mulero, S. Garcia-Vallve, G. Pujadas, *Anglerfish: A web server for quantitative epredictionof ligand bioactivity* [Submitted]

[Oral Communications]

1- Oral communication in the 1st National Congress of Young Researchers in Biomedicine. (Valencia 28th and 29th of November 2016)
Title: *in vitro and in vivo anti-inflammatory effects of the natural compound o-orsellinaldehyde.*
Oral Communication Accésit Prize.

I NATIONAL CONGRESS OF YOUNG RESEARCHERS IN BIOMEDICINE

III CONGRESS OF BIOMEDICINE OF PHD STUDENTS IN VALENCIA

I CONGRESO NACIONAL DE JÓVENES INVESTIGADORES EN BIOMEDICINA

III CONGRESO DE BIOMEDICINA PREDOCS VALENCIA

ORAL COMMUNICATION ACCÉSIT PRIZE

awarded to

Ms. Sarah Tomas Hernández

for the work presented in CIPF, Valencia (Spain) on the 28th to 29th November 2016 with the title *"In vitro and in vivo anti-inflammatory effects of the natural compound o-orsellinaldehyde"*.

Organizing Committee
Valencia, 2016

Centro de investigación Príncipe Felipe (CIPF). C/ Eduardo primo Yúfera, 3, Valencia 46012, Spain

Congress organized by members of:

UNIVERSITAT ROVIRA I VIRGILI
CHARACTERIZATION OF THE BIOLOGICAL EFFECTS OF NATURAL COMPOUNDS AGAINST INFLAMMATION, METABOLIC SYNDROME AND CANCER
Sara Tomás Hernández

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Roma, 18/12/2015

TO WHOM IT MAY CONCERN

This is to certify that Sarah Tomas, PhD student from Quimioinformatics and Nutrition Research Group of the Biochemistry and Biotechnology Department (Rovira i Virgily University, Tarragona), has attended our laboratories at Istituto Superiore di Sanità from the 14th of September until the 18th of December 2015.

During this period, Sarah has learn several techniques related to microglial activation and performed independent experiments that will part of her PhD thesis and that set the stage for future collaborations between the two lab in Rome and in Terragona.

During the stage, Sarah interacted very well with the personnel of the lab and we all appreciated her personal and scientific qualities.

Best Regards

Luisa Minghetti

Luisa Minghetti, PhD
Head, Experimental Neurology Unit
Department of Cell Biology and Neuroscience

Istituto Superiore di Sanità

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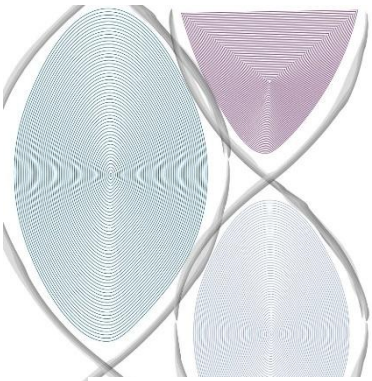
Sara Tomás Hernández



As Aristoteles said “Nature does nothing without purpose or uselessly”, this quote would explain why natural products have been used for the treatment and prevention of many diseases and illnesses since early human history. Many of these natural molecules have gone on to become current drugs.

On this basis, the initial hypothesis of this PhD thesis was that, the use of natural products for modulating specific targets involved in inflammation, insulin sensitivity and autophagy processes could be a great strategy for preventing or reversing the onset of some of the most significant chronic diseases worldwide such as metabolic syndrome, neurodegenerative diseases and cancer. Thus, the aim of this PhD thesis was to characterize the biological effects of selected natural compounds that could influence on the above mentioned molecular processes.

This book summarizes all the research conducted in order to accomplish this goal. As a result of this PhD work we have been able to identify several natural compounds with beneficial effects for human health that would represent promising candidate drugs for the prevention or treatment of several significant medical conditions.