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DISSERTATION

Titel der Dissertation

„Natural products and their analogues as nuclear receptor modulators and inhibitors of NF-κB activation“

Verfasser

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‘Any sufficiently advanced technology is indistinguishable from magic.’

Clarke's third law

Sagt denn der Ton zu dem Töpfer: "Was machst du mit mir?"

Jesaiah 45,9

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Abstract

This doctoral thesis describes the identification and partial characterization of natural products and their (semi)synthetic analogues in human embryonic kidney (HEK)-based *in vitro* luciferase reporter gene test systems as modulators of several targets. On one hand samples were tested for their transactivational activity towards the three subtypes of PPAR (peroxisome proliferator-activated receptors) and RXRα (retinoid X receptor alpha). On the other hand a luciferase reporter gene system was also employed to identify inhibitors of the TNFα (tumour necrosis factor alpha)-induced NF-κB (nuclear factor 'kappa-light-chain-enhancer' of activated B-cells) activation cascade. All of these targets have relevance in modern drug discovery.

In interdisciplinary cooperation with multiple teams of project partners several active natural compounds were identified either by a computer-aided or by a bioactivity-guided approach. Using the two strategies was successful to identify PPAR agonists from *Notopterygium incisum*, *Silybum marianum*, *Lycium chinense*, as well as from *Magnolia officinalis,* while inhibitors of the NF-κB signaling cascade could be identified from *Himatanthus sucuuba*, *Oroxylum indicum*, *Chromolaena odorata*, *Cetrelia monachorum*, *Lycium chinense* and *Eurycoma longifolia*.

Additional studies were dedicated to approach the molecular mechanism for identified inhibitors of NF-κB activation. In line this doctoral thesis describes some experiments that were performed to further characterize plumericin from *Himatanthus sucuuba*, a spirolactone iridoid that was already identified in a previous research project as a potent inhibitor of NF-κB activation.

This work also reports several characterization experiments to approach the molecular mechanism of the NF-κB inhibitory effect for the C-19 quassinoid eurycomalactone from *Eurycoma longifolia*. For this compound the inhibitory effect of submicromolar concentrations on TNFα-induced NF-κB activation was also observed in immortalized human umbilical vein endothelial cells (HUVECtert). Although the molecular target of eurycomalactone remains elusive, the obtained results from the performed mechanistical studies suggest that the molecular target of eurycomalactone is downstream of nuclear translocation and DNA binding of p65.

The last part of this work describes the characterization of a set of 57 mainly (semi)synthetic neolignan derivatives in view of their capacity to transactivate PPARγ and RXRα in the cell based luciferase reporter gene assay.

In conclusion this thesis describes the identification and partial characterization of several active natural compounds (and their analogues) in *in vitro* test systems.

Kurzzusammenfassung

Die vorliegende Doktorarbeit beschreibt die Identifizierung und partielle Charakterisierung von Naturstoffen und (semi)synthetischen Naturstoffanalogen mittels *in vitro* Luciferase-Reportergen Experimenten in humanen embryonalen Nierenzellen (HEK-Zellen). Einerseits wurden Verbindungen hinsichtlich ihres Vermögens, die drei Subtypen von PPAR (Peroxisom Proliferator-aktivierte Rezeptoren) zu transaktivieren, untersucht. Anderseits wurden auch zahlreiche Verbindungen als RXRα (Retinoid-X-Rezeptor alpha)-Agonisten erforscht. Des Weiteren wurde in Luciferase-Reportergen Experimenten Naturstoffe hinsichtlich deren Eignung als Inhibitoren des TNFα (Tumor Nekrose Faktor alpha) induzierten NF-κB Signalwegs getestet. Alle genannten Targets sind in Bezug auf die Entwicklung neuer Wirkstoffe von pharmazeutischem Interesse.

So konnten in interdisziplinärer Zusammenarbeit mit verschiedenen anderen Universitätsgruppen mittels computergestützter Aktivitätsvorhersage und aktivitätsgeleiteter Isolierung zahlreiche aktive Naturstoffe identifiziert und anschließend teilweise charakterisiert werden. In diesem Zusammenhang wurden PPAR Agonisten in *Notopterygium incisum*, *Silybum marianum*, *Lycium chinense*, sowie in *Magnolia officinalis* identifiziert, während Inhibitoren der NF-κB Aktivierung in *Himatanthus sucuuba*, *Oroxylium indicum*, *Chromolaena odorata*, *Cetrelia monachorum*, *Lycium chinense* und *Eurycoma longifolia* gefunden werden konnten.

In weiterführenden Experimenten wurde versucht, molekulare Wirkmechanismen von identifizierten Inhibitoren des NF-κB Signalwegs zu eruieren. Dementsprechend beschreibt diese Doktorarbeit auch einige Charakterisierungsexperimente für Plumericin, einem Spirolacton-Iridoid, das bereits in einem vorhergehenden Projekt als potenter Inhibitor des NF-κB Signalwegs identifiziert wurde.

Ebenfalls beschreibt diese Arbeit Charakterisierungsexperimente für das im Rahmen dieses Projekts als NF-kB Inhibitor identifizierte Eurycomalacton aus *Eurycoma longifolia*. Für dieses C-19 Quassinoid konnte eine inhibitorische Wirkung in sub-mikromolaren Konzentrationen in immortalisierten Nabelschnur-Endothelialzellen (HUVECtert) beobachtet werden. Obwohl der molekulare Wirkmechanismus von Eurycomalacton im Rahmen dieser Arbeit nicht vollständig aufgeklärt werden konnte, lassen die erhaltenen Ergebnisse weiterführender Experimente vermuten, dass das Molekül in jenen Teil der NF-κB-Aktivierung eingreift, der Translokation und DNA-Bindung von p65 nachfolgt.

Der letzte Teil dieser Arbeit beschreibt die Charakterisierung eines Sets aus 57 hauptsächlich (semi)synthetischen Neolignanderivaten hinsichtlich deren Eigenschaft in

dem HEK-zellbasierten Luciferase-Reportergen Assay PPARγ und RXRα zu transaktivieren.

Zusammenfassend beschreibt diese Arbeit die Identifizierung und teilweise Charakterisierung mehrerer aktiver Naturstoffe und naturstoffanalogen Verbindungen in *in vitro* Testsystemen.

Preface

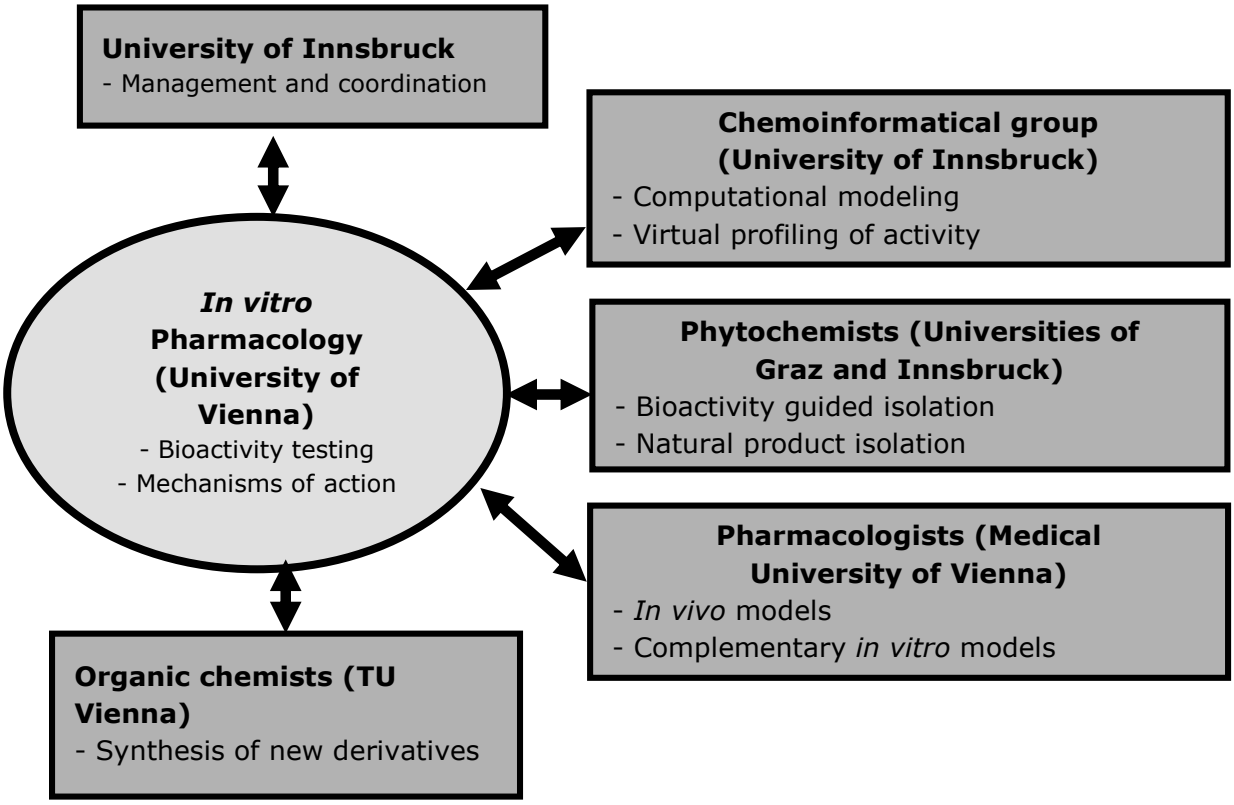
This PhD thesis was accomplished at the University of Vienna, in the Department of Pharmacognosy, in the framework of a national research network termed DNTI ("Drugs from nature targeting inflammation"). In this consortium several different scientific groups were engaged to identify and characterize natural products capable of either preventing or combating inflammatory processes. The DNTI project was started in 2008 and financed for three years by the FWF (Fonds zur Förderung der wissenschaftlichen Forschung), but eventually got prolonged for an additional period of three years. Within the first period of the project from 2008 to 2011 luciferase reporter gene assays and other *in vitro* experimental setups for the chosen molecular targets of the three subtypes of PPAR (α, β/δ and γ) and inhibition of NF-κB activation were successfully established. Overall more than 1000 isolated samples (extracts and compounds) were received and tested in the first funding period of the DNTI-project. The combined use of computational techniques and traditional knowledge about the effect of plants, high-tech chemical analysis and a broad range of *in vitro* and *in vivo* pharmacological models delivered a series of promising compounds with interesting anti-inflammatory effects, which were presented in corresponding scientific journals.

The author of this thesis started in 2011 at the beginning of the second period of the DNTI-project and this work aims to report the results of *in vitro* experiments conducted in the time period between 2011 and 2014 in a continuation of the efforts and mainly with the tools from the first project period. In addition to the already established luciferase reporter gene assays a novel experimental setup was developed to identify natural products and their (semi)synthetic analogues as RXRα agonists in luciferase reporter gene assays.

Outstanding importance for the DNTI-project had the two phytochemical groups in Graz and Innsbruck as suppliers of natural products for the testings. Their task was the isolation of active constituents from selected plants with an ethnopharmacological background and also proper pharmacognostic identification of collected plant material as well as phytochemical characterization studies. Isolated plant material was then used for *in vitro* biological testings conducted by the author's group as well as by another DNTI collaboration group with complementary *in vitro* models also located in Vienna. The chemoinformatical collaboration group in Innsbruck had on the one hand the role of developing/improving pharmacophore models to provide suggestions for the molecular mode of action of characterized compounds. On the other hand the chemoinformatical group also used their pharmacophore models to proactively predict activities and suggest

promising natural products for isolation and pharmacological investigation. Organic chemists from the Technische Universität (TU) Vienna were also engaged in the DNTI-consortium. Their task was to synthesize series of natural products analogues in order to develop lead compounds with improved potency. Finally sufficiently characterized natural compounds were forwarded to a collaboration group from the Medical University of Vienna and tested in *in vivo* models with the main focus on cardiovascular inflammatory models and novel mechanisms of anti-inflammatory action. The project team the author of this thesis was assigned to consisted of Univ. Prof. Dr. Verena M. Dirsch, the ambitious post-doc scientist Dr. Atanas G. Atanasov, laboratory technician Mia Gössinger and the author as a PhD student. All DNTI operations were coordinated by the head of the DNTI-consortium Univ. Prof. Dr. Hermann Stuppner, located at the University of Innsbruck. In the second period of the DNTI project again more than 1000 natural products (extracts and compounds) were forwarded to the author's group, collected and tested in various *in vitro* assays.

An overview of the involved groups and their part in the consortium is presented in the following scheme:



Additional information about the DNTI project can be obtained at the DNTI-homepage (http://www.uibk.ac.at/pharmazie/pharmakognosie/dnti/) and also in a recent publication about the consortium (Stuppner, 2014).

1. Literature Review

1.1 Natural products and their analogues in drug discovery

1.1.1 A short history of drug discovery

Mankind has been using natural products as medicine for millennia. Self-medication is also a common feature in non-human animals, both vertebrates and invertebrates (Rodriguez and Wrangham, 1993), so it is likely that even early ancestry forms of *Homo* used nature as a rich and exploitable source for remedies. Notably it has been hypothesized by some authors that not even the ability of associative learning is a prerequisite for self-medication (Singer et al., 2009). At the moment the earliest proven written record of natural products being systematically used as drugs by humans dates back to around 2600 BC and describes the use of approx. 1000 plant-derived substances in Mesopotamia (Cragg and Newman, 2013).

Historically, modern drug discovery began in 1805 when the pharmacist's apprentice Friedrich Sertürner isolated the first pharmacologically active pure compound from a plant: Morphine from opium produced by cut seed pods of *Papaver somniferum* (Hamilton and Baskett, 2000). Always along with technical innovations drug discovery has been developing more and more quickly since then. Many natural compounds were isolated and the overall understanding of the principles of chemistry and biology was ever improving. Not only morphine was the first natural product to be isolated, it was also the first natural product for which derivatization is reported in biomedical literature (Sneader, 2005). Along with the progress of the Industrial revolution in the 19th century also drug discovery flourished with too many milestones to be presented here, therefore the following only two isolated events of symbolic character for drug discovery: The first synthetic drug, chloral hydrate, was discovered in 1869 and is still available today in some countries (Jones, 2011). The first drug to be marketed in a modern way (mass scale production with standardized formulation and packaging) was salicylic acid, which was introduced to the market in 1875 as a progenitor drug of Aspirin, which entered the markets in 1899 to become the first blockbuster drug (Mahdi et al., 2006).

In the 20th century hallmarks of drug discovery are among others the introduction of barbiturates as sleeping aids (López-Muñoz et al., 2005), the discovery of antibacterial sulfonamides, as well as the discovery of penicillin as antibiotic. Especially the latter has

spurred drug discovery and the period between 1940 and 1960 is commonly referred to as "the golden age of drug discovery", with roughly one half of all antibiotics that are used today being discovered around this period. (Aminov, 2010)* Other relevant discoveries of the 20th century to optimize treatment interventions include the class of corticosteroids, the diuretics and beta blockers for hypertension, oral contraceptives, as well as new classes of antidepressants and tranquilizers (Grabowski, 2011).

In summary, all those discoveries and innovations have significantly contributed to doubling the average estimated lifespan of Western societies during the 20th century (Demain, 2002) and also to triplicate world population in the period between 1900 and 2000.

Natural product discovery reached its peak in the pharmaceutical industry in the period between the 70s and 80s of the last century, which resulted in a pharmaceutical landscape that was heavily relying on non-synthetic molecules. However, one of the major bottlenecks in natural product research is the isolation and purification of the active principle from an extraordinary complex matrix (Koehn and Carter, 2005). Another problem in natural product research is a supply issue: While for early testings milligram amounts are usually sufficient, to complete all clinical testings often an amount in the excess of 100 gram is needed. For therapeutic purposes is then a steady supply required, what can create serious problems for the usage of some natural compounds as drugs. A good example for such a case is the cytostatic drug paclitaxel (trade name: Taxol) isolated from the pacific yew *Taxus brevifolia*, which was rediscovered around 1980 as a cytostatic drug. When paclitaxel got approval in the beginning of the 1990s, it was already clear that sustained supply of compound will be difficult, since six pacific yews of 100 years age each were to be sacrificed for the treatment of every single cancer patient. This supply bottleneck was believed to skyrocket prices due to the disequilibrium of demand and offer and possibly also extinct the species in the long term. In turn extensive efforts to solve the supply issue were invested and yielded several semisynthetic strategies for production as well as several biosynthetic cell culture-based methods. (Malik et al., 2011)

In the 1980s natural product research became more and more unpopular for the pharmaceutical industry, since it became more and more expensive and difficult to market new drugs from natural products. It was believed that the traditional extract-based screening would increasingly result in the continuous re-discovery of already

*In this work references are set after the punctuation mark if the information from more than the previous sentence is used from the referenced source.

known active compounds, the few novel isolated bioactive natural compounds would be too expensive to re-synthesize and to derivatize, the time frame to develop a drug would become too long and supply with natural products would also become increasingly difficult. As a consequence many pharmaceutical companies disbanded their natural product research programs in the 1990s and focused on high-throughput screening (HTS) systems, a new technology that became feasible by using combinatorial chemistry. (Dias et al., 2012)

Unfortunately these early HTS programs proved to be unsuccessful, an impressive example for the early disappointments is a screening program from SmithKline Beecham (now part of GlaxoSmithKline (GSK)) to develop novel broad-spectrum antibiotics: Between 1995 and 2002 67 high-throughput screens with at least. 3×10^5 compounds each were conducted against 300 different possible targets - an unprecedented undertaking at that time. Sixteen of those 67 screens led to hits, of those hits only five compounds could be translated into lead compounds. For three of the five compounds it was found out in subsequent experiments that the target they showed activity against was not appropriate to develop a broad-spectrum antibiotic, while the two remaining compounds could not be successfully developed into molecules with drug-like properties. In conclusion the project had an unsustainable low level of success in relation to the large efforts invested. (Payne et al., 2007)

This fail is prototypical for the early, widely futile efforts to develop drugs with combinatorial chemistry as the only drug that could be marketed from early HTS programs is sorafenib (an orphan drug to treat advanced kidney, liver and thyroid cancer) (Dias et al., 2012).

The low success of high-throughput screening was accompanied by the disappointment of the so called "genomic revolution": Elucidation of the human genome was expected to yield a plethora of new targets and treatment opportunities, a prognosis that did not yet apply (Dias et al., 2012).

As a consequence natural product discovery has been receiving revived scientific interest. Contemporary approaches aim to avoid the classical tedious isolation, screening and dereplication strategy and attempt to be more fast-forward by using a combination of *in silico* based bioprospecting approaches to directly transform genomic information into biosynthetic outputs. (Schmidt-Dannert, 2015)

1.1.2 Current situation of rational drug discovery

The number of approved drugs per billion US dollars spent on R&D has been decreasing over the last decades and is believed to further decrease in future, while the overall investments for drug discovery will increase or remain constant. In literature, several reasons for this development are discussed, including among others the "better than the Beatles" theory and the "cautious regulator"-theory. The "better than the Beatles" theory uses the analogy of drugs as Beatles' songs available almost for free (comparable to generic drugs) and new drugs/songs are only consumed if they are significantly better than those available ones. In turn an ever-improving stock of drugs increases the hurdles in the development process of new drugs, which poses a progressive and intractable problem. The "cautious regulator" problem states that progressive lowering of risk tolerance by legal authorities increases development costs, is highly bureaucratic and thereby limits competition to a few big players that have a strong financial background. Both theories are suitable to explain the low amount of new approvals and the relatively high amount of newly approved orphan and anti-cancer drugs among them. In general the approved drugs per billion US dollars spent on research and development have halved roughly every 9 years since 1950, which equals an 80-fold decline in inflation-adjusted terms. This phenomenon is referred to as "Eroom's Law" (the backward spelling variant of "Moore's Law" which states an exponential growth of the number of transistors in a dense integrated circuit). (Scannell et al., 2012)

Currently discovery and marketing of a new drugs takes 10 – 15 years and needs a budget of one (to three) billion USD (Scannell et al., 2012), with only about 50.000 USD and three months to be spend on isolation and identification of an active compound from its natural source (Dias et al., 2012). However, the non-scientific business magazine "Forbes" has written a read-worthy article on this topic, claiming that the one (to three) billion number is an underestimation on purpose to attract investors and that the true cost to develop and market a new drug are at least 4 billion USD[*]. On the other hand there is an article from the non-scientific daily newspaper "The New York Times" that claimes the one (to three) billion number as highly over-exaggerated to justify bold

[*] "The Truly Staggering Cost Of Inventing New Drugs" – written by Matthew Herper, 2/10/2012 http://www.forbes.com/sites/matthewherper/2012/02/10/the-truly-staggering-cost-of-inventing-new-drugs/ retrieved on May, 11th 2015

retail prices and argues that the true costs are manifold lower[**]. But all numbers are speculative as long as the pharmaceutical companies do not have to explicitly declare all their expenses, what is unlikely to happen.

1.1.3 Nature as an unexplored lore

The trend of declining numbers of approved drugs is definitely not because knowledge of natural products has reached its ceiling with only little more to discover in future and the difficult synthetic approach as the only alternative. Regarding mankind's knowledge about natural products, rather the opposite is true: Currently the structures of about 1.6 x 10^5 natural products are known, 25 % of which are reported to show activity (including toxicity) with an annual increase of 1 x 10^4 elucidated structures of natural compounds. (Demain, 2014) This relatively small number opposes the huge number of 3.7 x 10^{30} different microorganisms that are estimated to live alone in marine environments and are almost completely unknown (Kennedy et al., 2008). Even if only one new natural product is to be found in every billionth of these species of marine microorganisms the resulting number of new natural product would yield an enormously vast number (3.7 x 10^{21}) of yet undiscovered natural products.

Extrapolating from the number of investigated plants (~6 x 10^4) and the number of drugs that originate from those (~140), it can be suggested that the so far uninvestigated plants (~3 x 10^5) could be source of another approx. 600 drugs that are yet to be discovered (David et al., 2014). Interesting in regard to drug discovery from plant sources is that it is very common to not find single products from natural sources, but rather suites or complexes of structurally closely related analogues. The biological significance of this phenomenon is unclear, especially since frequently members of those complexes are present in major quantities, but seem to lack any biological activity or function. (Koehn and Carter, 2005) However, it has been hypothesized that the strategy of generation and retention of chemical diversity at low cost is an evolutionary adaption to cope with changing environmental conditions and thereby increases evolutionary fitness (Firn and Jones, 2003).

[**] "$2.6 Billion to Develop a Drug? New Estimate Makes Questionable Assumptions" – written by Aaron E. Carrol, 18/11/2014 http://www.nytimes.com/2014/11/19/upshot/calculating-the-real-costs-of-developing-a-new-drug.html, retrieved on May, 11th 2015

Similar to plants and marine microorganisms also fungi are an attractive source for the discovery of active natural products and also similar to those our understanding of fungi is in its infancy: It is estimated that there are approx. 1.5×10^6 different species of fungi with only as little as $\sim 7 \times 10^4$ species currently described in literature. Unfortunately and in addition the majority of described fungi is currently not cultivable and therefore not suitable as a source for natural products (Jiang and An, 2000).

1.1.4 Differences between synthesized and biosynthesized compounds

Apart from the vast numbers of undescribed species in all three domains of life that render natural products to an interesting source for the development of new drugs, there are also fundamental differences in structural aspects of synthesized and biosynthesized compounds. This leads to a superior sample-to-hit ratio in the average library of natural products in comparison to synthetic compound libraries. (Koehn and Carter, 2005)

The latter are usually prepared with regard to the ease of chemical accessibility which leads to significant deficiencies in the types of generated chemical structures in view of diversity. In turn chemical properties of synthesized and biosynthesized are fundamentally different. While the median for the number of chiral centers among natural compounds is four, it is zero in synthesized compounds. This is a very relevant difference, because the presence of chiral centers contributes to the selectivity of molecules for their predominantly stereospecific binding sites. Synthesized compounds also often lack rigidity in comparison to their biosynthesized counterparts, which is a result of combinatorial chemistry generally introducing new rotatable bonds that join the basic building blocks of synthesized molecules. Another main difference is the composition of molecules: While synthesized compounds on average comprise of three times as many nitrogen atoms per molecule compared to biosynthesized compounds, the number of oxygen atoms is on average only half the number compared to biosynthesized compounds. These differences can be explained by the different building blocks used in biosynthesis and combinatorial chemistry and is relevant because the usually observed higher specificity of natural products derives from the correct matching of complementary polar and non-polar surfaces of the interaction partners. Other differences include a different ratio of hydrogen donors and acceptors, differences in the average number of different bond types and also differences in lipophilicity. (Feher and Schmidt, 2003)

Another disparity between synthesized and biosynthesized compounds that is possibly most important is the discrepancy in their purpose of creation. While synthesized compounds are usually prepared without knowledge if they have any activity, biosynthetic compounds are typically created by an organism in order to fulfill an inherent function, often by binding to a protein. Although the number of possible protein sequences is theoretically indefinite, the number of basic shapes in which proteins fold seems to be restricted to no more than 10.000 folds in existence. Interestingly, the majority of protein families belong to only approximately 1.000 common folds. (Koonin et al., 2002) This occurrence of common folds explains why organisms can yield ligands for biological targets that are completely out of their biological context (e.g. lovastatin from *Aspergillus terreus* as inhibitor of the human 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase to lower cholesterol levels). In conclusion natural products are generally structurally optimized to bind enzymes or protein receptors (Li and Vederas, 2009) and are therefore a fascinating source for the development of new lead compounds and drugs.

1.2 Nuclear receptors

1.2.1 Discovery and general aspects of nuclear receptors

Nuclear receptors are a superfamily of transcription factors that regulate a vast array of physiological functions. Speculations that such receptors may exist can be traced back in the history of biomedical research at least several decades prior to their discovery in the mid-1980s, but most of the techniques required for their identification and classification were simply beyond the limits of classic biochemical analysis. (Evans and Mangelsdorf, 2014)

Advances in biochemistry, especially the discovery of the PCR reaction that triggered a new era of biological sciences, led in turn to the molecular and genetic characterization of genes encoding the first steroid receptors, which were the glucocorticoid and the estrogen receptor. Comparison of their cDNA sequence suggested the presence of structural highly conserved domains of those receptors, which eventually led to the discovery of several more evolutionary related proteins. Because for some of them associated ligands could not be identified, those newly discovered proteins were termed "orphan receptors". (Evans and Mangelsdorf, 2014)

Especially one remarkable and innovative technological achievement, the cotransfection assay, has become one of the most widely used tools to discover new ligands for nuclear receptors and to gain structural insights about receptors. In this assay the receptor's cDNA is cotransfected with a hormone-responsive reporter gene, thereby creating a highly sensitive regulatory switch that is sensitive to the binding of exogenous compounds. In variations of the assay also elucidation of receptor's DNA and ligand binding domains, as well as determination of ligand and target gene specificity are feasible. (Evans and Mangelsdorf, 2014)

One of the main advantages of this assay is the extreme sensitivity towards small ligands that enabled screening programs with milligram amounts of isolated compounds.[*] Identification of endogenous ligands could often also give hints about the physiological role of the receptor: For example, the insight that PPARs are responsive towards fatty

[*] In comparison, the classic endocrinology methods that led to the isolation of thyroid hormone in 1914 required 3.5 tons of bovine thyroid gland (Evans and Mangelsdorf, 2014).

acids led to the discovery that they are important in fatty acid metabolism. (Latruffe and Vamecq, 1997)

In a combinatorial use of the cotransfection strategy and electrophoretic mobility shift assays it was discovered that nuclear receptors always bind as dimers to their target gene sequences. In this dimerization process another nuclear receptor termed RXR has the outstanding role of being the obligate heterodimerization partner for many other nuclear receptors. Thus the superfamily of nuclear receptors can be divided into two groups: One group of nonsteroid receptors that require RXR as a mandatory dimerization partner and the other group of steroid receptors that bind as homodimers to their target gene promoters without involvement of RXR. The group that necessarily requires RXR for dimerization can be further split into two subgroups: One subgroup of dimers that can only be formed by ligand binding of RXR's heterodimerization partner (the so called "non-permissive dimers") and the other subgroup of dimers that can be created by ligand binding to one or the other dimerization partner (the so called "permissive dimers"). (Pérez et al., 2012)

By genome sequence analysis a total of 48 different nuclear receptors have been identified, 24 of which are classified as orphan nuclear receptors that do not require ligand binding to fulfill their designated role in the cellular machinery (Benoit et al., 2006). Interestingly, also some orphan receptors are considered in literature as viable drug targets (Blumberg and Evans, 1998).

Ligand binding to responsive nuclear receptors initiates transcriptional induction. In this highly orchestrated and dynamic process activated nuclear receptor dimers target specific DNA sequences in the promoter regions of target genes to modulate their expression levels. Ligand binding is usually followed by dissociation of corepressors and their replacement with coactivator complexes. (Sever and Glass, 2013) Coactivator complexes typically contain proteins with enzymatic functions to mediate enzymatic reactions, remodel chromatin and facilitate the association of the RNA polymerase II complex with the general transcriptional machinery (Burris et al., 2013). Overall there have been 400 different coactivator and corepressors identified. This enormous diversity offers the organism another layer to fine tune transcriptional regulation, because cofactor abundance is tissue specific and their displacement/recruitment is ligand specific. (Lemon et al., 2001)

In basic nuclear receptor research the evolutionary origin as well as the possible ancestral function of the primordial nuclear receptor have been a matter of scientific debate basically since their discovery, especially after the first orphan nuclear receptors

were discovered. Without extensive phylogenetic analysis feasible in the early 1990s it was unclear whether the primordial receptor was orphan and has diversified via gene duplication to gain the ability of ligand binding or *vice versa*. But even the extensive phylogenetic data available today is inapt to completely rationalize one theory over the other. Possibly nuclear receptors have lost and gained their ability of ligand binding several times during evolution and some authors speculate that the primordial receptor might have had been a nutritional sensor. (Markov and Laudet, 2011)

1.2.2 Structural aspects of PPARs and RXR

All nuclear receptors share a common organization of six homology regions (designated as A -F) which also overlap with functional domains. The evolutionary most conserved region of nuclear receptors is the DNA binding domain, which is composed of two C4 type zinc fingers. (Cotnoir-White et al., 2011) Interestingly, the commonly suggested "mousetrap mechanism" for ligand activation of nuclear receptors, where ligand binding induces a conformational change of helix 12 to entrap the ligand, was recently reported to be a misinterpretation of experimental data. Rather than that there is now evidence that the H12 region is highly mobile and lacks a strong structural organization such as a helix. Instead, ligand binding is suggested to yield a stable but dynamic helical conformation in H12 residues and thereby adds global stability to the LBD fold. This proposed new mechanism is commonly referred to as "H12 dynamic stabilization". (Rastinejad et al., 2015) The H12 region is important for recruitment of a diverse set of coactivators and for displacement of corepressors, which is necessary to initiate target gene modulation (Burris et al., 2013).

PPARs

The A/B domain at the N-terminus harbors a ligand-independent transcriptional activating function (AF-1) motif. The C domain has a typical two-zinc finger structure to interact with the DNA of peroxisome proliferator response elements (PPREs). The D domain or hinge region is responsible for receptor dimerization and also facilitates DNA binding. The E/F domain is also required for receptor dimerization and in addition also

contains the ligand-binding domain (LBD) and the ligand-dependent transcriptional-activating function (AF2) motif. (Markt et al., 2007)

The LBDs of the three different PPAR subtypes are 60 – 70 % homologous and this difference explains also the different ligand binding affinities of agonists towards the three subtypes. Generally all three PPAR subtypes have a large Y-shaped ligand binding pocket with a volume of approx. 130 nm^*, which is typically not completely filled by ligands. (Batista et al., 2012)

PPARα has the largest and most hydrophobic binding pocket among the subtypes, PPARβ/δ the smallest. PPARγ's more polar binding pocket shows more affinity towards hydrophilic ligands than PPARα's and the ligand binding pocket of PPARβ/δ cannot accommodate large hydrophobic residues and is generally most diverse from the two other subtypes. (Markt et al., 2007)

RXR

There are three different subtypes of RXR (α, β and γ) and as a result of alternative splicing there are several isoforms for each subtype, all of which have different expression levels in different tissues. While RXRα and RXRβ can be found in almost every type of tissue, RXRγ is mainly restricted to muscle and brain cells. The three RXR subtypes have a high degree of homology, suggesting that they have similar roles and respond to common ligands. (Thomas et al., 2012) RXR is reported to be present in physiologically relevant concentrations as transcriptionally silent tetrameric complexes that dissociate into dimers upon ligand binding (Boerma et al., 2014).

Like other NRs, RXR proteins have six major functional/structural domains that follow the same pattern as for the PPARs, apart for the F domain, the function of which in RXR remains yet to be established (Dawson and Xia, 2012). Special structural importance has the ligand binding domain that was in turn extensively studied: RXRα has an L-shaped ligand binding pocket (Zhang et al., 2011) and upon ligand binding the coactivator peptide GRIP-1 is recruited in 1:1 stoichiometry. Recruitment of GRIP-1 results in four structural changes of amino acid interaction, thereby stabilizing H3, H11

* The referenced research paper reports a "volume" of 1300 Å, possibly here volume is inverted with diameter and the true volume is a multitude of the 130 nm^3, but the key information is an untypically large binding pocket for PPARs, irrespective of a precise measure.

and H12. Stabilization of H12 is necessary for recruitment of coactivator proteins and activation of transcription. (Boerma et al., 2014)

1.2.3 Physiological roles of PPARs and RXRα

Although modulation of target gene expression is the prime role for nuclear receptors, PPARs and RXR supposedly also have a role beyond modulating target gene expression, implied by the abundance of some nuclear receptors in cells that lack a nucleus (Dawson and Xia, 2012).

PPARs

Generally, PPARs are shown to have an anti-inflammatory role with partly non-redundant mechanisms which can be both direct and indirect (Wahli and Michalik, 2012). In addition members of the PPAR family are important in cell differentiation and embryonic development, where they again have distinct, but also complementary functions. (Michalik et al., 2002)

PPARα

PPARα is especially abundant in metabolically active tissue with a high capacity for fatty acid oxidation, such as liver, heart, kidney and muscle (Montagner et al., 2011). Typically ligands primarily control the expression of genes involved in lipid metabolism and activation of PPARα results in increased energy consumption and reduced fat storage. Without an endogenous high-affinity ligand so far identified, the role of PPARα is supposedly to sense the total influx of fatty acids as a nutritional sensor. (Georgiadi and Kersten, 2012) In turn expression levels of PPARα follow a diurnal rhythm and contribute to the connection between the circadian clock and energy metabolism (Montagner et al., 2011). PPARα furthermore shows a pronounced sexual dimorphism in hepatic gene

expression, with a more repressive function in females that is hypothesized to be needed to cope with elevated estrogen levels in females (Leuenberger et al., 2009).

PPARβ/δ

The PPARβ/δ subtype is sometimes considered as the black sheep of the PPAR family and is therefore the least understood. This is because of the ubiquitous expression of PPARβ/δ in all kinds of tissues which is a clear disadvantage in view of rational drug discovery. (Willson et al., 2000) Also the physiological roles of PPARβ/δ are most diverse, but since PPARβ/δ is particularly abundant in liver, intestine, kidney, abdominal adipose tissue and skeletal muscle, one of the main roles is probably participation in the lipid metabolism (Grygiel-Górniak, 2014). Together with PPARγ, PPARβ/δ is also known to have several important functions in skin biology (Montagner et al., 2011). Additionally PPARβ/δ has also been found to play an especially important role in skeletal muscle, since overexpression of PPARβ/δ and receptor stimulation lead to a remarkable change of gene expression patterns that lead to an increase of oxidative muscle fibers and in turn to improved endurance under certain conditions (Narkar et al., 2008).

PPARγ

The PPARγ gene encodes two different PPARγ isoforms which are produced via alternative splicing. Both isoforms share key functions but have different expression patterns: While the PPARγ1 isoform is expressed in almost every tissue, PPARγ2 is mainly limited to adipose tissue. (Janani and Ranjitha Kumari, 2015)

The most prominent physiological role for PPARγ is its involvement in glucose and lipid metabolism. Stimulation of the receptor enhances storage of triglycerides and thereby lowers the concentration of free fatty acids in blood. This shifts catabolism from free fatty acid utilization to glucose utilization and thereby also lowers blood glucose levels. (Spiegelman, 1998) In addition to regulating storage of triglycerides, PPARγ also controls genes that are directly responsible for release and transport of fatty acids (Janani and Ranjitha Kumari, 2015). PPARγ is furthermore reported to have a crucial role in the maintenance of skeletal muscle insulin action (Hevener et al., 2003).

In adipocytes PPARγ controls the secretion of adipocytokines thereby modulating the insulin action in peripheral tissues and also protects non-adipose tissues against excessive lipid overload (Kintscher and Law, 2005). PPARγ furthermore also participates in the regulation of cancer progression and modulation can context-dependently either promote or inhibit tumor growth (Panigrahy et al., 2005).

RXRα

The prime and most important physiological role of RXRα is to be an auxiliary receptor for many other nuclear receptors that require RXRα for heterodimerization (Zhang et al., 1992). Thereby RXRα regulates a vast array of signaling pathways, actively those that rely on permissive heterodimers and passively the non-permissive ones*. Beyond this indispensable function required for signaling through the pathways mediated by those receptors evidence of specific physiological roles for RXRα is rather scarce (Pérez et al., 2012). However, the insight that fatty acids are RXRα ligands implies a role of RXRα that is beyond that of a mandatory heterodimerization partner and suggests importance as intracellular sensor of the cellular metabolic status (Rőszer et al., 2013). Also the RXRα/RXRα homodimer supposedly has a distinct physiological role, but generally RXR has rather been studied as a subordinate partner of other nuclear receptors than as a regulator itself (Menéndez-Gutiérrez et al., 2015). However, some researchers speculate that there are even RXRα- homodimer specific pathways, while others question an *in vivo* relevance of RXRα- homodimers at all (Röszer et al., 2013).

1.2.4 PPARs and RXRα as drug targets

PPARα

PPARα agonists have a long tradition as drugs since the antidiabetic effects of fibrates are explained mainly via their agonistic effect on PPARα. Interestingly, the fibrate class

* For some non-permissive receptors an additive effect upon ligand-binding to both heterodimerization partners is is reported, referred to as "RXR subordination" (Perez et al., 2012).

of drugs was discovered prior to the discovery of the receptor and introduced in the end of the 1960s, while PPARα was discovered in 1992. Some fibrate class drugs are still in clinical use to combat different aspects of the metabolic syndrome (hyperlipidemia and hypertriglyceridemia) presenting a moderately effective alternative to statin medication. Interestingly, in addition to their supposed main effect of inhibiting the HMG-CoA-reductase statins also modulate PPARα. Recent research activities indicate a potential beneficial effect upon co-treatment with fibrates and statins. (Choi et al., 2015)

In literature PPARα is also considered as a viable target to treat certain forms of cancer (Pozzi and Capdevila, 2008), as well as a target to treat diseases that are associated with inflammation (e.g. atherosclerosis (Willson et al., 2000) or neurodegenerative disorders (Bordet et al., 2006).

PPARβ/δ

Unlike the other subtypes there are no clinically used drugs known that exert their effects via modulation of PPARβ/δ. However, as PPARβ/δ is co-responsible for lipid metabolism, it is clearly considered as target to combat metabolic disorders. Interestingly, PPARβ/δ agonists are also suggested as potential exercise mimetics (Narkar et al., 2008) and their use in professional sport is prohibited by the world anti-doping agency (WADA) (Earl et al., 2014). Other suggested targets are similar to the other two PPAR subtypes and include inflammatory disorders (Kilgore and Billin, 2008) and certain types of cancer (Zaveri et al., 2009).

PPARγ

PPARγ is the best researched and therefore also the best understood PPAR-subtype. This is because PPARγ is the molecular target of a class of highly effective antidiabetic agents known as thiazolidinediones (or "glitazones"). Similar to the fibrate class of drugs also the glitazones were discovered through empirical compound screening in rodents prior to the discovery of their appendent target. Subsequently glitazones were identified as high-affinity subtype-selective agonists of PPARγ. (Willson et al., 2000)

However, although highly effective in lowering blood glucose levels, all hitherto been marketed glitazone drugs are under scrutiny of an unfavorable risk profile and some

glitazone drugs were subsequently banned from the markets by authorities. In many countries (including Austria*) glitazones are only recommended to be used for second-line-therapy (Yau et al., 2013). Nonetheless novel PPARγ agonists remain sought after in biomedical research, because some of the side-effects that led to withdrawal of glitazones seem to be compound-specific, while PPARγ dependent side effects can be overcome by selective coactivator recruitment and sub-maximal receptor activation. Sub-maximal receptor activation induced by partial agonists has been shown to retain effectivity with reduced side effects in animal experiments. Consequently several partial PPAR agonists have been in clinical trials. (Agrawal et al., 2012; Chigurupati et al., 2015)

Similar to the other two PPAR subtypes PPARγ was furthermore identified as a target in inflammatory disorders (Martin, 2010)**,** as well as in cancer (Woo et al., 2011), but beyond that also as a target for a variety of diseases including for example various skin pathologies (Toffoli and Desvergne, 2015) or neurological disorders (Zhao and Aronowski, 2014), as well as kidney diseases (Yang et al., 2012).

Apart from modulation of PPARγ with antagonists or agonists, the receptor was also shown to serve as a drug target independent of receptor modulation: Blocking of phosphorylation of Ser-273 was reported to exhibit anti-diabetic effects *in vivo* independent of receptor agonism (Choi et al., 2011).

RXRα

In general, modulation of RXRα is expected to yield pleiotropic effects, considering RXRα's outstanding role as an obligate dimerization partner for many other nuclear receptors. In turn unspecific RXRα modulation can modulate expression for those genes that are under control of permissive RXRα-containing-heterodimers (Germain et al., 2006). Therefore RXRα is considered as a difficult target and the enthusiasm of pharmaceutical companies to develop drugs targeting RXRα is rather dampened and research is in turn mainly restricted to academia (Dawson and Xia, 2012).

Nevertheless there are two drugs marketed that target the RXRα receptor: Bexaroten (trade name: Targretin), an anti-neoplastic agent used in second-line therapy to treat cutaneous T-cell lymphoma and 9-cis retinoic acid for topical treatment of Kaposi's

* Source: "therapie tipps" – Information für Vertragsparter Sammelband 02/2014 issued by the WGKK

sarcoma (trade name: Panretin) and systematic treatment of refractory chronic hand eczema (trade name: Toctino). Unfortunately both drugs have significant adverse effects. (Dawson and Xia, 2012) In biomedical literature RXRα agonists are reported to possibly have pharmaceutical potential as cancer preventive agents (Tanaka et al., 2004), as neuroprotective agents (Friling et al., 2009), to combat different aspects of the metabolic syndrome (Shulman and Mangelsdorf, 2005) and as anti-inflammatory agents (Xu and Drew, 2006). However, the main challenge to develop RXR agonists as drugs is to identify compounds that can selectively induce the formation of desired heterodimers over undesired ones. It has already been shown that compounds can exhibit some degree of selectivity (Pérez et al., 2012), but it remains yet elusive if sufficient selectivity is achievable at all.

1.3 NF-κB

1.3.1 NF-κB - General aspects

The transcription factor family NF-κB (nuclear factor 'kappa-light-chain-enhancer' of activated B-cells) was discovered in 1986 and was initially suggested to be a B-cell specific DNA-binding activity regulator. Within a few years of subsequent research, however, it became clear that NF-κB is by far not B-cell specific, but instead a stimulus-responsive pleiotropic regulator of gene control. (Hinz et al., 2012) In fact, while the name NF-κB remained, none of the information as implied by the name is correct, since NF-κB is neither a specific regulator of the kappa-light-chain-gene, nor it is B-cell specific, nor a nuclear factor (Hoffmann and Baltimore, 2006).

In the following years various members of the NF-κB family were identified and some of their functions were deciphered as well as additional aspects of the pathway were uncovered. In the early to mid-1990s, NF-κB signaling was established as a transcription factor having pivotal importance in inflammation, immune response and cancer. This insight has boosted NF-κB research and the number of publications that mention NF-κB in any way has skyrocketed ever since. In 2012 nearly 40.000 hits could be obtained in a PubMed search with the term "NF-kB" (in 2015 almost 50.000 hits compared to 1995 with less than 1000 hits) with an annual increase of around 3500 studies. As a result NF-κB-related studies account approximately for 0.5 % of all new publications in biomedical research. (Hayden and Ghosh, 2012) The high complexity and importance of NF-κB regulation can be underlined by a study that reports more than 15.000 putative binding regions for NF-κB in the human genome (Kasowski et al., 2010).

Interestingly, it was believed for a long time that members of the NF-κB signaling cascades cannot be found in organisms simpler than *Drosophila melanogaster* and that NF-κB did not exist prior to the appearance of arthropods (approx. 550 million years ago). This speculation was based on the absence of NF-κB in model organisms like *Caenorhabditis elegans* or yeast. However, in recent studies it has been shown that several morphologically primitive organism have genes for single members of the NF-κB cascades, placing the evolutionary origin of NF-κB at the base of opisthokonts (an eukaryotic supergroup that includes metazoa and fungi), which are believed to have arisen approximately 1000 million years ago. (Gilmore and Wolenski, 2012) By comparison of the genomic data of different NF-κB and NF-κB-like proteins in various

organisms, it was also possible to suggest an evolutionary timeline for the development of NF-κB: It is speculated that the first NF-κB protein was created by a fusion of a primordial RHD-only gene with the gene encoding an ANK repeat sequence, possibly to ensure the interaction of the two proteins by virtue of the proximity of their encoding sequences. The gene for the inhibitory protein of the NF-κB pathway IκB seems to have emerged later as a result for the need to cope with more genes being regulated by the NF-κB protein. The gene for the ancestral IKK seems to be the youngest in the NF-κB family and was originally more similar to IKK-ε than to IKK-α/IKK-β. The latter were derived by gene duplication and divergence from the ancestral IKK-ε variant. In mammalians many of the ancient genes for NF-κB members were duplicated during evolution in order to fine-tune the NF-κB pathway with different partner affinities, different biological functions and different tissue-specific expression patterns. (Basith et al., 2013)

Although much knowledge about NF-κB signaling has accumulated since its discovery in 1986, there is still much inadequately understood about the NF-κB pathway, including three major mechanistic aspects: The specific regulation of IKK activity by receptor/adapter complexes, the regulation of NF-κB transcriptional activity by the IκB family and the mechanisms of how transcriptional activation is initiated and terminated (Hayden and Ghosh, 2012). Also computational biology has had and will have an important part in complementing wet-lab based techniques to yield information about the mechanistic behavior of the various components of the signaling pathway, and also to compute and rationalize quantitative data (Williams et al., 2014).

1.3.2 NF-κB signaling cascades

As it simplest, the NF-κB signaling cascade consists of three members: A dimeric transcription factor that resides in the cytoplasm, an interacting inhibitor and an upstream kinase. The kinase is activated by a physiological signal, in turn phosphorylates the inhibitor, which leads to proteolysis of the inhibitor. The now freed dimeric transcription factor translocates to the nucleus to modulate the transcription of a specific set of genes. In mammals there are multiple proteins and genes for all of the three basic members of the cascade, namely five different proteins that pair up to a multitude of different dimeric transcription factors, seven different interacting inhibitors and four different upstream kinases. (Gilmore and Wolenski, 2012)

The physiological signals leading to activation of the cascade can be manifold and are usually conducted via ligand binding to receptors on the cell surface. These include, but are not limited to, the TNF receptor (TNFR), the Toll-like receptor (TLR) and the IL-1 receptor (IL-1R). (Hayden and Ghosh, 2014)

Since none of the receptors has an intrinsic enzymatic activity, the physical act of binding as a signal inducer must be transmitted across the lipid bilayer and translated by adapter proteins into kinase activation. These adapter proteins typically possess well described protein-protein interaction domains, some of which are at example a "death fold domain" (DD), a "receptor interacting protein" domain (RIP), "homotypic interaction motifs" (RHIM) and the TNF receptor specific "TNF receptor associated factor" (TRAF) domain. The variety of adapter proteins adds additional levels of complexity to the regulation of NF-κB. (Hayden and Ghosh, 2014)

There are several different NF-κB pathways described in literature, with the so called canonical (or classical) being best understood and described, especially in regard to stimulation with the cytokine TNFα:

In this pathway a complex variety of transcription factors (with p50-p65 (RelA)[*] heterodimers being most relevant) bound to the inhibitory protein IκB is activated by an IKK complex containing the components IKK-α, IKK-β and NEMO, with IKK-β being the primary kinase in the canonical pathway. It is reported that binding of TNFα to its attendant receptor leads to the assembly of an NF-κB initial complex consisting of the adaptor molecules TRADD, TRAF2/5 and RIP1. Subsequent K63-linked polyubiquitination of RIP1 leads then to the recruitment of TAK1 and TAB2 to phosphorylate the IκB kinase in its activation loop at Ser177 and Ser181. (Li et al., 2014) Activated IκB kinase in turn phosphorylates IκB at the two serine residues (at position 32 and 36) within the so-called destruction box motif. The phosphorylated destruction box is recognized by the ubiquitin ligase subunit βTrCP leading to K48-linked polyubiquitination by the SCF/UbcH5 ubiquitin ligase complex that is followed by degradation via the 26S proteasom. (Huang et al., 2010) Once IκB is degraded the NF-κB dimer translocates to the nucleus to bind accessible NF-κB sites with 5′ GGGRNWYYCC[**] 3′ sequence motifs. The degenerate nature of the NF-κB sequence and the ability of NF-κB to form complexes with other transcription factors, allows NF-κB to regulate the expression of a vast array of target genes. In addition NF-κB dimers can also bind to enhancer elements and have been

[*] The following the alternative name RelA for p65 is omitted

[**] G – guanin; R – purine; N – any base; W – adenine or thymine; Y – pyrimidine and C - cytosin

shown to also regulate chromatin remodeling. The activity of nuclear NF-κB dimers is further regulated by a variety of post-translational modifications that alter DNA and co-factor binding, but those many possible post translational modifications and their implications are currently not very well understood. (Huang et al., 2010)

In addition to the canonical pathway that has received most scientific attention so far the so called "non-canonical pathway" is also relatively well understood. This pathway was discovered approximately ten years after the canonical pathway around the year 2000. In this activation cascade signal transduction is not facilitated via degradation of IκB, but via inducible processing of the so called "NIK-responsive domain" of p100. In particular NIK stimulates phosphorylation of p100, which is then further processed via ubiquitinylation and sumoylation to undergo proteolysis. Interestingly, p100 is not only the precursor of p50, but also has an inhibitory IκB-alike role for certain NF-κB dimers. Similar to the canonical pathway where IκB needs to be degraded for downstream translocation of the NF-κB dimer, also p100 has to undergo proteolysis in order to proceed signals through the cascade. (Sun, 2012) The underlying mechanisms for this prerequisite are still elusive (Vatsyayan et al., 2008). The predominantly activated NF-κB dimer for nuclear translocation in the non-canonical pathway is the p52/RelB heterodimer (Sun and Ley, 2008).

The differences between the two pathways are manifold, the most striking difference (apart from the differing signal transduction mechanisms) is how quickly the onset of activation is: While in the canonical pathway the activation of NF-κB is rapid and independent of protein synthesis, in the non-canonical pathway activation of NF-κB is comparatively slow, because it is dependent on synthesis of NIK, which is only present in extremely low concentrations under basal conditions. (Sun, 2012) Also the signals leading to activation of the two cascades are fundamentally different: While the canonical pathway is responsive to signals elicited by diverse receptors, the non-canonical pathway seems to be restricted to a small specific set of receptors (Sun and Harhaj, 2006). Consequently also the function of the non-canonical pathway is far more selective than the canonical pathway, but mostly the two pathways appear to cooperate in their biological functions (Sun, 2011). Interestingly, NIK-activated IKK is reported to possibly also induce IκBα degradation and can thereby activate the canonical NF-κB pathway (Xiao and Fu, 2011).

Although the canonical and the non-canonical pathway seem to have most importance in eukaryotic cells, there is evidence that NF-κB can also be activated with stimuli that work independently of those two pathways. The most prominent example for such a

situation is genotoxic stress inducing a so called "atypical NF-κB pathway" (Hoesel and Schmid, 2013).

1.3.3 NF-κB transcription factor family

The NF-κB transcription factor family consists of five proteins that are related through a highly conserved N-terminal Rel homology domain (RHD). This domain has a sequence of approx. 300 amino acids and is responsible for nuclear localization, dimerization with other NF-κB transcription factor family members and also for DNA binding. (Gilmore and Wolenski, 2012)

Based on the sequence C-terminal to the RHD the family can further be divided into two subfamilies: On one side the two so called "NF-κB proteins" p100 and p105 and on the other side the "Rel proteins" with c-Rel, p65 and RelB in mammals. NF-κB proteins contain C-terminal IκB-like inhibitory domains consisting of ankyrin (ANK) repeats that need to be removed for maturation into the transcription factors p50 and p52, while Rel proteins share a C-terminal transactivation domain allowing them to initiate transcription through co-activator recruitment. (Hayden and Ghosh, 2014)

In general all NF-κB and Rel proteins form both homodimers and heterodimers *in vivo* and the composition of the dimers is one of the main variables to specify target gene regulation. Target gene regulation is often achieved by transcriptional activation, but also repression can be reached e.g. by direct competition with dimers that promote transcriptional activation. (Gilmore and Wolensky, 2012)

The abundance of the different dimers is cell-type specific, but in most cells a p50/p65 heterodimer is prevailing (Haddad and Abdel-Karim, 2011). Accordingly the p50/p65 dimer is best studied and understood at the present time. Especially for the p65 subunit there are many interactions and posttranslational modifications reported, but the logic how these interactions and modifications influence transcription has not yet been sufficiently elucidated. (Hayden and Ghosh, 2014)

1.3.4 IκB family

The IκB family of proteins can be divided into two subfamilies: Typical IκBs that undergo stimulus-dependent phosphorylation, degradation and resynthesis and the atypical IκBs that do not follow this scheme.

The group of typical IκBs consists of three members, IκBα, IκBβ and IκBε, all of them share six ankyrin repeats as a common feature. They also share the redundant role in the cytoplasm to inhibit translocation of the NF-κB dimer with distinctly different binding affinities to different NF-κB dimers. The use of knock-out models to define non-redundant functions was partly successful, but hampered by the observation that depletion of one member of the family often led to compensation by other members. (Hinz et al., 2012) In general IκBs bound to transcription factors to inhibit their translocation are fairly stable (half life time of at least several hours). Upon stimulation IκBs get degraded rapidly, but are in turn also replenished quickly (Ferreiro and Komives, 2010).

The IκB family member IκBα has been discovered first and is also best researched. The quantitative regulation of IκBα is highly dependent on NF-κB, thereby generating a negative feedback-loop (Moss et al., 2012). IκBα is reported to remove NF-κB transcription factors from the promoter regions of their target genes to modulate the transcriptional activity of NF-κB transcription factors. In addition, several studies have demonstrated shuttling between nucleus and cytoplasm by NF-κB:IκBα complexes under basal conditions. (Gordon et al., 2011)

The role of IκBβ is very similar to that of IκBα, the main difference is their binding affinity to NF-κB dimers. Another difference between the two is the absence of shuttling events between cytosol and nucleus for IκBβ bound NF-κB dimers. (Malek et al., 2001)

IκBε was discovered the latest and is currently also least understood. It is suggested that IκBε has a very similar role to IκBα, but with significantly delayed kinetics to fine tune distinct expression dynamics of NF-κB target genes together with IκBα. (Hinz et al., 2012)

Of note is that also the NF-κB precursor proteins p100 and p105 have an IκB-like function and inhibit translocation of certain NF-κB dimers, which is important in the non-canonical NF-κB pathway (Heissmeyer et al., 1999).

The atypical IκBs (Bcl-3, IκBξ/MAIL, IκBNS and IκBη) do not predominantly reside in the cytoplasm, but are located in the nucleus. This implicates that their main role is to

modulate gene expression and not to prevent the dimeric NF-κB transcription factors from translocation. Atypical IκBs (except IκBη) have seven or eight ankyrin repeats and are only expressed in low levels in basal conditions, but their expression levels increase significantly upon stimulation with NF-κB inducing agents. Most attention of all atypical IκBs has so far received Bcl-3, a protein that was initially classified as a proto-oncogene. (Schuster et al., 2013)

1.3.5 IκB kinase complexes

IκB kinase (IKK) complexes are essential to phosphorylate IκB (in the canonical pathway) and p105 (in the non-canonical pathway) at conserved destruction boxes to tag it for proteolysis by the 26S proteasome. The kinase complex contains 3 subunits, which are the two catalytic kinases IKK-α and IKK-β and the non-enzymatic scaffold protein NEMO (an acronym for "NF- κB essential modifier"). (Hinz and Scheidereit, 2014) Similar to other kinases IKK-α and IKK-β require phosphorylation of two serine residues located in an activation loop domain for activation. While IKK-α activation is achieved by NIK that does not rely on an upstream kinase for activation (de Leon-Boenig et al., 2012), it is currently not understood how in detail the activation of IKK-β is mechanistically induced. (Hinz and Scheidereit, 2014)

IKK-α and IKK-β have approx. 50 % sequence similarity and the two enzymes share several motifs: An N-terminal kinase domain (KD) and extended regions C-terminal to the KD, which contain conserved leucine zipper (LZ) and helix-loop-helix (HLH) motifs. For kinase activity dimerization of the subunit is required, which is mediated by the LZ motif. (Scheidereit, 2006)

The third member of the IKK complex is NEMO, which is bound to IKK-α and IKK-β via their N-terminal NEMO-binding domain. Although the crystal structure of NEMO is still to elucidate, the most important structural features of NEMO have been resolved in biochemical assays: NEMO is predicted to be a helical protein containing two coiled-coil-domains (CC), a LZ and a C-terminal zinc-finger (ZF) region. In addition two more domains were identified that probably enable the formation of NEMO-oligomers. (Tegethoff et al., 2003)

The whole IKK complex has been determined in gel filtration experiments to have a molecular weight between 700 to 900 kDa, while none of the single complex members exceeds a molecular weight of 100 kDa. This indicates oligomerization of the complex

members, so a composition of IKK-α_2IKK-β_2NEMO$_4$ has been proposed, but experimental proof for this stoichiometric composition is still elusive. (Hinz and Scheidereit, 2014)

Intrinsic attenuation of IKK has been proposed to involve IKK-β-mediated autophosphorylation and also of note are several ubiquitinylation events for activation and termination of IKK activity, but again those mechanisms are currently not entirely understood (Hinz and Scheidereit, 2014).

Furthermore it is reported in literature that the activity of the IKK complex is not restricted to NF-κB dependent pathways, but can also mediate cross talk with other signaling cascades, such as the MAPK or the mTOR pathways (Dan et al., 2008).

1.3.6 Physiological roles of NF-κB

NF-κB has many physiological roles by modulation of at least 300 genes and 15.000 putative binding regions in the human genome (due to the highly variable sequence of the consensus motifs) (Kasowski et al., 2010). The most important role of NF-κB is that as a mediator of immune and inflammatory response (Hinz and Scheidereit, 2014). The importance of NF-κB in immunity covers both innate and the adapted immunity and also the development of the immune system itself. In particular, NF-κB has been shown to play an important part both in initial and dynamic spleen architecture as well as in the development of B cell and T lymphocytes, natural killer (NK) cells, dentric cells (DCs), monocytes, granulocytes, and other cellular components of the immune system. In response to acute inflammation NF-κB plays an outstanding role as mediator for a diverse range of different stimuli as different receptors particularly rely on NF-κB signaling cascades. In addition NF-κB is also responsible for the transcription of genes encoding many pro-inflammatory cytokines and chemokines and also for macrophage differentiation. Furthermore NF-κB is important for the production of enzymes that generate prostaglandins and reactive oxygen species and NF-κB is also speculated to be involved in signaling induced by prostaglandins. (Hayden and Ghosh, 2011)

Also in bone formation and maintenance NF-κB is important since a disequilibrium in activity of osteoplasts and osteoclasts can often be linked to activation of NF-κB. In line for disease where the equilibrium between osteoplast- and osteoclast-activity is impaired (e.g. arthritis or osteoporosis) modulation of NF-κB activity has been proposed as a possible treatment strategy (Novack, 2011).

In addition there is also a regulatory function of NF-κB for both the central and the peripheral nervous system described in literature. These functions are not yet entirely characterized, but include modulation of mechanistic aspects (e.g. there is a complex interplay between Ca^{2+} responsive neuronal activation and NF-κB activation (Lilienbaum and Israël, 2003)), modulation of structural plasticity (Gutierrez and Davies, 2011), but also long term behavioral modulations (Lehmann et al., 2010).

NF-κB has also an interesting physiological role in the fate of a cell choosing to undergo apoptosis. The evidence that NF-κB activation can have an anti-apoptotic effect is overwhelming and well reported in literature. This anti-apoptotic effect is mediated mostly by directly upregulating the expression of genes whose products can inhibit apoptosis (e.g. TRAF1 and TRAF2). (Karin and Lin, 2002) However, there is also growing evidence that NF-κB activation is not only anti-apoptotic, but can also mediate cell death in certain conditions. The most obvious mechanisms by which NF-κB acts pro-apoptotic is by up-regulation of pro-apoptotic genes (e.g. DR5 or p53) or down-regulation of anti-apoptotic genes. (Radhakrishnan and Kamalakaran, 2006) Apart from that there is also a p100-mediated, transcription-independent apoptosis mechanism reported in literature that was observed in cell culture experiments (Wang et al., 2002), but it is yet unclear if this mechanism has any *in vivo* relevance (Häcker and Karin, 2002). In conclusion, NF-κB cannot be easily classified as either pro- or anti-apoptotic, since the outcome of NF-κB activation is heavily context-dependent.

In summary NF-κB has a vital role in diverse biological processes, some of which are relatively well understood, for others there is only some evidence or hypothesis. In turn aberrant activation of NF-κB has been linked to a plethora of human pathologies, most importantly cancer (Xiao and Fu, 2011) and diseases associated with chronic inflammation (Killeen et al., 2014). Especially in cancer persistent activation of NF-κB has been linked to the disease, but the particular molecular mechanisms by which the NF-κB signaling pathways become constitutively activated during cancer pathogenesis still remain mainly obscure. However, as the old Chinese proverb stresses, everything has both yin and yang aspects and this is reported to be also true for NF-κB in the development and progression of cancer. On one hand there is ample of evidence that NF-κB activation contributes to tumorigenesis in general, which is one of the main reasons in the vivid interest of the scientific community in discovery of novel NF-κB inhibitors. On the other hand, however, NF-κB activation may also play a negative role in certain stages of tumorigenesis and can possibly also hamper tumorigenesis under certain circumstances. Especially in cancer models of skin and liver there is evidence reported that activation of NF-κB has a tumor suppressive function. (Xiao and Fu, 2011)

1.3.7 NF-κB signaling pathways as drug targets

Because of the multitude of cellular processes regulated by NF-κB and the fact that in several pathological conditions NF-κB signaling is aberrant, there has been a great interest in modulators of this pathway. Consequently the number of discovered NF-κB inhibitors has been staggering: While in 1999 125 inhibitors of the NF-κB signaling cascade were reported (Epinat and Gilmore, 1999), the same authors counted 765 inhibitors in 2006 (Gilmore and Herscovitch, 2006). Considering the ongoing scientific interest in NF-κB signaling it is likely that the number of known inhibitors has in the meanwhile exceeded the one thousand mark.

Generally inhibition of NF-κB can be achieved by tackling the pathway with three different approaches: (1) blocking of the incoming stimulus signal at a very early stage of signaling (e.g. by inhibiting binding of ligand to its receptor), (2) interference with one of the cytoplasmatic activation steps of NF-κB by blockage of a specific component of the cascade (e.g. inhibition of the phosphorylation of IκB by IKK) and (3) blockage of NF-κB nuclear activity (e.g. inhibition of DNA-binding). (Gilmore and Herscovitch, 2006)

In general for all three approaches a multitude of drug candidates have been identified, but of note is that for many reported NF-κB inhibitors the molecular target is often only vaguely known, because many performed assays show activity, but do not suggest a specific molecular target (Gilmore and Herscovitch, 2006).

Development of inhibitors of IKK were considered as especially promising, partly because IKK is the earliest target that funnels all different upstream signal that can activate NF-κB, but also because the development of kinase inhibitors for some other therapeutic applications has been extraordinary successful[*] (Epinat and Gilmore, 1999).

Since the NF-κB cascade is interlinked at several levels with many other pathways it is generally challenging to develop NF-κB specific drugs. Another obstacle is that blockade of the NF-κB cascade over extended periods of time is very likely to cause serious adverse effects, especially immunosuppressive effects (Papa et al., 2006).

[*] An impressive example for the success of kinase inhibitors as drugs is imatinib (trade name: Imatinib) to treat chronic myelogenous leukemia (CML). Prior to the introduction of this tyrosine kinase inhibitor the median survival rate for patients suffering from CML was 3-5 years. Strikingly, of patients that receive the drug and respond to treatment, less than 1 % die from leukemia progression. In fact, CML has become the first cancer in which standard medical treatment may provide normal life expectancy. (Gambacorti-Passerini et al., 2011)

As a possible alternative, the combined low dose use of inhibitors tackling different NF-κB signaling members has been proposed to reduce side effects. In near future systemic application of NF-κB seems not as a fruitful approach, but topical, local or cell-type specific (to treat cancer) application could hold some promise. (Gilmore and Herscovitch, 2006)

Interestingly, many commonly used drugs, especially anti-inflammatory drugs, are reported to also target the NF-κB signaling pathway, although it remains often unclear if or how much this activity contributes to the overall anti-inflammatory effect *in vivo*. Possibly also the promoted anti-inflammatory, antiaging, and anti-cancer effects of prolonged intake of some natural compounds (e.g. green tea or curcumin) can at least in part also be due their described inhibitory effect on NF-κB signaling. (Gilmore and Herscovitch, 2006)

The role of NF-κB in the nervous system suggests NF-κB modulation as a possible strategy to combat a number of neurodegenerative disease (e.g. Alzheimer's disease), although it is not always entirely clear if the up- or down-regulation in a neuron under pathological conditions is the origin of the disease or the attempt of a neuroprotective reaction by the organism itself (Mincheva-Tasheva and Soler, 2013).

At the current time there is no drug marketed that specifically exerts its effects via inhibition of NF-κB activation. The nicotine related alkaloid anatabine is sold as a food supplement and claims to exert its anti-inflammatory effects due to the inhibition of NF-κB activation. However, since food supplements in contrast to drugs only need to prove safety, but not efficacy, it remains unclear if this claim is substantiated. Very recently the distributor of anatabine announced that the United Kingdom's Medicines Healthcare Products Regulatory Agency (MHRA) has approved a clinical trial application to commence a Phase I study of anatabine citrate as an anti-inflammatory drug.[*]

Inhibitors of NF-κB activation might possibly also be useful in defense of a diverse number of lentivirus (such as HIV-1) that rely for their replication not exclusively on the host cell machinery, but also on viral proteins they encode themselves (Herbein et al., 2010). In case of HIV-1 it is reported that the viral protein Nef plays a critical role in "hijacking" the NF-κB signaling pathway to promote viral replication and to destroy uninfected bystander cells (Swingler et al., 2003).

[*] Source: http://investors.rockcreekpharmaceuticals.com/2015-01-30-Rock-Creek-Pharmaceuticals-Receives-Clinical-Trial-Application-Approval, retrieved on May, 11th 2015

Very interesting is also the possible application of NF-κB modulators for preventing insect based-transmission of microbial pathogens to humans. This approach is based on the observation that also in insects NF-κB plays a pivotal role in innate immunity. In line it has been shown that depletion of NF-κB-IκB family member gene products can promote immunity against parasite invasion**.** (Frolet et al., 2006)

2. Aim of the study

Aim of this study was to participate in DNTI-research activities that intend to identify hit and lead compounds for the development of new drugs. More specific aims of this study can be worded the following:

- To screen incoming samples for their biological activity as inhibitors of the TNFα-induced NF-κB signaling cascade and for their transactivational activity towards RXRα and the three subtypes of PPAR in luciferase reporter gene assays

- The gained data was used by cooperation partner's for the following purposes:
    - ❖ Data forwarded to the phytochemical groups was used for bioactivity guided isolation of active compounds
    - ❖ Data forwarded to cheminformatical groups was used to support them in their work to create and improve computational models and also to challenge their *in silico* predictions.
    - ❖ Data forwarded to the organic chemistry groups was used as a decision guidance for subsequent derivatization steps
    - ❖ Data forwarded to the pharmacological groups was used to indicate which compounds are most interesting for subsequent *in vivo* studies

- To further characterize a limited number of active samples with a diverse variety of experimental setups in regard of their suitability as hit or lead structures for further research activities

3. Material and methods

3.1 Cell culture

All cells lines were cultured in a cell culture incubator at 37 °C in humidified atmosphere with 5 % CO_2. All cell handling (except lysis for Western blotting) was done under aseptic conditions in a laminar flow cabinet and prior to disposal cells were decontaminated with an aqueous 0.1 % Hexaquart™ solution. Unless otherwise stated all liquids used on cells were prewarmed to 37 °C in a water bath. Testings for eventual contamination of cell lines with mycoplasma were routinely done by chief technician Ing. Daniel Schachner in sporadic intervals without ever detecting contamination in any of the used cell lines. All cultured cell lines were regularly investigated by phase contrast microscopy to detect early signs of eventual contamination.

All centrifugation steps in cell culture were done with a Heraeus Multifuge 1 S-R centrifuge (Thermo Fisher Scientific Biosciences GmbH, Oberhausen, Germany) using their Swinging Bucket Rotor TTH 400. Centrifugation speed is the following given in rpm values, the graph for g-force conversion for the used rotor is provided in the Appendix chapter.

Cell culture solutions:

The following two solutions were commonly used for all cell lines:

Phosphate buffered saline (PBS) pH 7.4

NaCl	36 g
Na_2HPO	7.4 g
KH_2PO_4	2.15 g

per 5000 ml aqua dest.,

autoclaved and stored at +4 °C.

trypsin solution

Trypsin	0.5 g
EDTA	0.2 g

per 1000 ml PBS, sterile filtered,

aliquoted and stored at -20 °C

3.1.1 HEK293 / HEK293/NF-κB-luc cells

Background information

The HEK293 cell line was created in 1973 by transfection of cells with sheared adenovirus 5 DNA (Graham et al., 1977). The name "HEK" is derived from the organ used as donor for the cells, which was a human embryonic kidney (HEK), while the number 293 refers to the chronological number of the transformation experiment. Although kidney tissue was used for transformation, HEK293 cells do not have the typical shape and behavior of kidney cells, but rather appear as cells related to neuronal cells. This was suggested to be the result of transformation of adrenal cells being present in kidney tissue which are, although only present in minor quantities, much more prone to infection with adenovirus (Shaw et al., 2002). The transformation resulted in the incorporation of approx. 4.5 kb from the viral genome into human chromosome 19 and the transformed aneuploid cell line is described as hypotriploid (Louis et al., 1997). The use of HEK293 cells is widespread because the cell line is relatively easy to handle and easily transfectable, but HEK293 cells should not be used as a model for kidney cells (Hamid et al., 2005). Overall, HEK293 cells are second most widely used cell line in cell biology research (after HeLa cells) and also second most widely used in biopharmaceutical production (after CHO cells) (Lin et al., 2014).

Over the years of extensive use in research and biopharmaceutical production several HEK293 daughter cell lines with added features were introduced. Among them are the HEK293T-cell line that carries mutations to express the SV40 large T-antigen leading to improved protein expression (Thomas and Smart, 2005) or the HEK293.2sus cell line that grows in suspension (Aydin et al., 2012). The HEK293/NF-κB-luc cells used in this project were provided by Panomics (Fremont, California, USA) and were obtained by co-transfection of HEK293 cells with pNFκB-luc and pHyg, followed by hygromycin selection. These cells maintain a chromosomal integration of a luciferase reporter construct regulated by six copies of the NF-κB response element. Upon stimulation with TNFα HEK293/NF-κB-luc cells produce the enzyme luciferase. In this study HEK293 and HEK293/NF-κB-luc cells were mainly used for screening of natural products in luciferase reporter gene assays, but also for characterization experiments of identified PPAR agonists and inhibitors of NF-κB activation.

Culture media and supplements

HEK culture medium

DMEM (4.5 g/l glucose)	
without phenol red	500 ml
fetal bovine serum (FBS)	10 % (v/v)
benzylpenicillin	100 U/ml
streptomycin	100 µg/ml
glutamine	2 mM

Cell handling of HEK 293 and HEK293/NF-κB-luc cells

Thawing of cells

For long term storage HEK293 and HEK293/NF-κB-luc cells were kept in liquid nitrogen at –196 °C. For cultivation frozen cell aliquots were retrieved from the nitrogen tank and pre-thawed by gentle agitation in a water bath at 37 °C until the frozen pellet was detachable, which was then poured into a cell culture flask containing 20 ml *HEK culture medium*. Medium was exchanged daily until the first subcultivation.

Cultivation

HEK 293 and HEK 293/luc cells were grown in 175 cm^2 vessels in 20 ml *HEK culture medium.* Cells were cultured and used for experiments until passage number 50.

Sub-cultivation

Confluent cells were usually split twice per week. For this procedure all liquid from the cell culture flask was drained and the cells were washed with 10 ml prewarmed PBS. Then cells were detached by treatment with 3 ml *trypsin solution* for 3 minutes at room temperature. The trypsinization was stopped with 12 ml *HEK culture medium*, the cell suspension was transferred to a falcon and centrifuged for 4 minutes at 1200 rpm. The supernatant got discarded and the cell pellet resuspended in 10 ml *HEK culture medium*. 1 ml of the suspension was used to approximate cell density in a cell counter. Cell density numbers were used to calculate the required volume of cell suspension for the desired quantity of HEK293 cells, which was transferred to the cell culture flask and filled up with *HEK culture medium* to an overall volume of 20 ml. Usually 6 x 10^6 cells were seeded to reach confluence within 3 days.

3.1.2 HUVECtert

Background information

All blood vessels, including the umbilical vein, have a monolayer of endothelial cells lining the interior surface of the vessel to form an interface between the circulating blood and the rest of the vessel wall (Tan et al., 2004). Since the umbilical cord has no function in the postnatal period it is an attractive source for human endothelial cells in research. HUVEC (human umbilical vein endothelial cells) have therefore become a classical model to study many aspects of function and disease of the human endothelium. This can be explained by their relative ease of isolation and availability (Ganguly et al., 2012), while the isolation of sufficient amount of endothelial cells from most other blood vessel would be a highly invasive procedure. HUVECtert are HUVEC immortalized by infection with lentiviral particles encoding the human telomerase reverse transcriptase (hTERT) (Schiller et al., 2009). Immortalized cells were a kind gift from Univ. Prof. Dr. Stockinger (University of Vienna) and were used in this study to overcome batch-to-batch variability of HUVEC and also because of economic reasons.

HUVEC are reported in literature as primary cells that mimic many processes that occur also *in vivo* and their use has been providing better understanding of many human diseases (Ganguly et al., 2012; Park et al., 2006). However, the increase in data quality compared to the data gained from less physiological cell lines (e.g. HEK293 cells) comes at the price of much more difficult cell handling. In addition HUVEC and HUVECtert require expensive culture media with many additives, grow rather slowly and can only be cultured until low passage numbers.

In this study the HUVECtert model was used to verify the observed NF-κB inhibitory effects from HEK293-luciferase reporter gene screening. This was done by quantification of pro-inflammatory cell adhesion proteins, whose expression levels are regulated by NF-κB activation. In addition HUVECtert were used to study the molecular mechanism of action of verified inhibitors of NF-κB activation.

Culture media and supplements

HUVEC culture medium

Endothelial growth medium EBM™	500 ml
FBS	10 % (v/v)
benzylpenicillin	100 U/ml
streptomycin	100 µg/ml
amphotericin B	1 % (w/v)
EBM™ Single Quots™:	human endothelial growth factor (hEGF),
	Hydrocortisone,
	Gentamicin/amphotericin
	Bovine brain extract
	Ascorbic acid

sterile filtered and stored at +4 °C

stop solution

10 % FBS in PBS, sterile filtered, stored at +4 °C

Cell handling of HUVECtert

Thawing of HUVECtert

Aliquots of HUVECtert were retrieved from the liquid nitrogen tank and were pre-thawed by gentle agitation in a water bath at 37 °C until the frozen pellet was detachable, which was then poured into a falcon containing 10 ml prewarmed HUVEC *culture medium*. The falcon was centrifuged for 10 minutes at 1000 rpm, the supernatant discarded and the cell pellet resuspended in 10 ml prewarmed HUVEC *culture medium.* The cell suspension was then transferred into a precoated (0.1 % gelatin in PBS incubated for 10 minutes at 37 °C) 75 cm^2 cell culture flask, already containing 10 ml *HUVEC culture medium*. Medium was then exchanged daily until the first subcultivation.

Cultivation of HUVECtert

HUVECtert were grown in 20 ml *HUVEC culture medium* in precoated (0.1 % gelatin in PBS) 175 cm^2 cell culture flasks with exchange of medium every second day. Cells were cultured and used for experiments until passage number 15.

Sub-cultivation of HUVECtert

For sub-cultivation all liquid from the cell culture flask was drained and cells were washed with 10 ml prewarmed PBS. Then HUVECtert were detached by incubation with 3 ml *trypsin solution* for 5 minutes at 37°C. In the meanwhile cell culture flasks were coated with 0.1 % gelatin in PBS for 10-15 minutes. Trypsinization was stopped by addition of 3 ml *stop solution* to the cell culture flask and the cell suspension was collected in a falcon. The emptied cell culture flask was washed once more with 5 ml *stop solution*, which was also collected in the same falcon and all of the suspension was centrifuged for 10 minutes at 1000 rpm. The supernatant was discarded and the cell pellet resuspended in 10 ml *HUVEC culture medium*. 1 ml of the suspension was used to approximate cell density in a cell counter. Cell density numbers were used to calculate the required volume of cell suspension for the desired quantity of HUVECtert, which was

transferred to the (precoated) 75 cm^2 cell culture flask and filled up with *HUVEC culture medium* to an overall volume of 20 ml. Usually 2 x 10^6 cells were seeded to reach confluence within 3 days.

3.1.3 3T3-L1 cells

Background information

In 1962 American scientist used disaggregated Swiss Albino mouse embryonic tissue to create a cell line of fibroblasts they named 3T3 (an abbreviation of the applied protocol "3-day transfer, inoculum 3 x 10^5 cells"). In subsequent experiments two sister cell lines 3T3-L1 and 3T3-F422A were prepared by clonal isolation and are now the most frequently used preadipocyte lines used in research (Moreno-Navarrete and Fernández-Real, 2012). The two cell lines are different in their maturation state to adipocytes (Gregoire et al., 1998). Immortalized fibroblast cultures are primarily used over primary cultures of isolated fat tissue in medical research. On one hand it is difficult to isolate preadipocytes, since they only constitute a minor percentage of total fat tissue. Also isolated preadipocytes are at different stages of maturation what could impair a definite response to treatments. (Ntambi and Young-Cheul, 2000)

The 3T3-L1 cell line has become an important model for diabetes research. The long term complications associated with diabetes are mostly the result of chronically elevated blood glucose levels resulting from an impairment of muscle and fat cells to maintain glucose homeostasis. Although muscle cells are far more relevant for glucose uptake than adipose tissue most of the available muscle cell lines are not insulin sensitive in terms of glucose transport and can therefore not be used for *in vitro* experiments where the effect of insulin is to be studied. (Lakshmanan et al., 2003)

In this project 3T3-L1 preadipocytes were used to differentiate them into adipocyte-like fibroblasts and then test their glucose uptake efficacy upon treatment with PPARγ agonists.

Culture media and supplements

3T3 culture medium

DMEM (4.5 g/l glucose)	
without phenol red	500 ml
NBS	10 % (v/v)
glutamine	2 mM

Cell handling

Thawing of 3T3-L1 cells

Thawing of 3T3-L1 cells was done as described for the HUVEC cell line, except that *HUVEC culture medium* was replaced by *3T3 culture medium* and instead of precoated 75 cm^2 vessels 175 cm^2 cell culture flasks were used. Accordingly the volume of prewarmed media was increased to 20 ml.

Cultivation of 3T3-L1 cells

3T3-L1 cells were grown in 30 ml *3T3 culture medium* in 175 cm^2 cell culture flasks until passage number 12 and cells were never allowed to become confluent.

Subcultivation of 3T3-L1 cells

Subcultivation of the 3T3-L1 cell line was done every third day. The procedure was the same as described for the HEK293/HEK293luc cells, except that *3T3 culture medium* was used instead of *HEK culture medium* and the overall amount of volume was 30 ml instead of 20 ml. Usually 4 x 10^6 3T3-L1 preadipocytes were seeded in a 175 cm^2 cell culture flask to reach 80 % confluence within 3 days.

3.2 Plasmid propagation

Background information

Since the major breakthroughs in biotechnology triggered by the discovery of the polymerase chain reaction that allows rapid amplification of DNA, plasmid DNA has become an increasingly important tool in bioscience. The propagation of plasmids can be divided into a three step procedure, consisting of transformation, propagation and isolation.

The first step is to insert the DNA into the bacteria, a process termed transformation. For transformation of bacterial cells there are two methods commonly applied, electroporation and chemical transformation. While electroporation is at least ten times more efficient in terms of transformation efficiency, the disadvantage of this method is the requirement of a specialized apparatus (a so called "electroporator") that has a very limited field of application and is therefore not very widespread. (Fregel et al., 2008) The comparatively low transfection efficiency of chemical transformation methods is not considered as a problem, since the yield of only one colony forming unit per transformation would in theory be sufficient to produce any desired amount of plasmid DNA. Most laboratories that use a chemical transformation method apply $CaCl_2$ transformation, either in a variation of the original protocol from 1970 (as in this project), or in one of the subsequently improved protocols (Chen et al., 2001). Although mechanistically not entirely understood it is hypothesized that chemical transformation works via transiently opening gated membrane channels that allow rapid influx of extracellular medium into the bacterium after temperature gradient and ionic shock (Casali and Preston, 2003). Since all plasmids used for transfection carry in addition to the genetic sequence to be multiplied (the so called "insert") also a selectable marker, only successfully transformed bacteria will grow in medium supplemented with the antibiotic the selectable marker conveys resistance against. In the commonly applied strategy first bacterial cultivation and isolation of the plasmids are only done in small scale for analytical restriction with restriction enzymes fitting to the used vector. As soon as the identity of the propagated plasmid is verified, cultivation is repeated in a bigger scale. In the project described here both small scale isolation as well as bigger scale isolation of plasmid DNA were done using commercially available kits that provide all equipment and reagents necessary for isolation. The separation between plasmid DNA and chromosomal DNA relies on differences in their physicochemical parameters due to

their vastly different size and the circular structure of plasmid DNA: During bacterial lysis with the alkaline lysis buffer both chromosomal and plasmid DNA become denaturated, but in the subsequent neutralization step with acetate only the covalently closed plasmid DNA reanneals and stays in solution, while chromosomal DNA precipitates along with protein and can be separated by centrifugation (Ehrt and Schnappinger, 2003). Further purification is then done in both small and bigger scale propagation employing a column-based purification strategy, where the ability of DNA to bind to a silica membrane is exploited.

Plasmids are usually produced in bacterial suspension cultures and some special bacterial strains were developed that carry certain mutations to facilitate efficient plasmid propagation. The *E.coli* DH5α cell line used in the project described here is one of those common strains for plasmid propagation and is reported in literature to provide a good yield of high-quality DNA (Taylor et al., 1993). The plasmids amplified in this project were used for luciferase reporter gene assays.

Concentrations of harvested plasmid dilutions were determined by photometry and were usually between 200 – 500 ng DNA/µl depending on the specific propagated plasmid. Usually six plasmids were produced at the same time, each usually yielding a sufficient amount of plasmid DNA to conduct at least 100 transfection experiments.

Solutions and buffers

LB-medium

25 g powdery LB-medium

per 1000 ml aqua dest.

Autoclaved and stored at +4 °C

bacterial freezing solution

glycerol	50 % (v/v)

aqueous

electrophoresis buffer

$CaCl_2$ solution

PIPES	10 mM
$CaCl_2$	60 mM
glycerol	15 % (v/v)

aqueous, autoclaved

SOC medium

yeast extract	0,5 % (w/v)
trypton	2 % (w/v)
NaCl	10 mM
KCl	2,5 mM

(0.5x TBE diluted from 10x TBE)

Tris-base	45 mM
Boric acid	45 mM
EDTA pH 8.0	0.5 mM

aqueous

$MgCl_2$	10 mM
$MgSO_4$	10 mM
glucose	20 mM

aqueous, stored at -20 °C

Experimental procedure

Preparation of competent E.coli

An aliquot of *E.coli* DH5-alpha cells was thawed and cells were streaked on an agar plate and grown overnight at 37 °C. Next day a single colony was inoculated overnight in 50 ml *LB medium* and on the day after 4 ml of the culture were transferred to 400 ml *LB medium* and grown at 37 °C to an OD_{590} of 0.37. The culture was then split into aliquots of 50 ml and those were left for 10 minutes on ice. Then the cells were ultracentrifuged for 7 minutes at 3000 rpm (equivalent to ~1600 *g*), the supernatant was discarded and the cells resuspended in 10 ml ice-cold $CaCl_2$ solution. The suspension was ultracentrifuged at 4 °C for 5 minutes at 2500 rpm (equivalent to ~1100 *g*) and the supernatant was again discarded and the cells resuspended in another 10 ml ice-cold *$CaCl_2$ solution*. The cells were kept on ice for 30 minutes and were then ultracentrifuged at 4 °C for 5 minutes at 2500 rpm (equivalent to ~1100 *g).* The supernatant was discarded and the cell pellet first resuspended in 2 ml ice-cold *$CaCl_2$ solution* and then divided into smaller aliquots of 250 µl each. Competent bacteria were stored at – 80 °C.

Bacterial transformation

The plasmid of interest and one aliquot of competent *E.coli* DH5α cells were thawed on ice and 40 µl of the cell suspension were pipetted into two microcentrifuge vials, one vial

for the plasmid of interest, as well as one vial as negative control. The volume containing 100 ng of plasmid of interest was added to one of the microcentrifuge vials and both vials were incubated on ice for 30 minutes. Then both cell suspensions were heated for 45 seconds at 42 °C and were then incubated for 60 seconds on ice, before 450 µl prewarmed *SOC medium* was added. Both vials were incubated at 37 °C for one hour. After that the vials were centrifuged in a table top centrifuge at top speed for 1 minute, the supernatant got discarded and the cell pellets resuspended in 50 µl LB-medium. The cells were streaked on agar plates containing (depending on the plasmid of interest) either 50 µg/ml kanamycin or 100 µg/ml ampicillin and were incubated overnight at 37 °C.

Plasmid isolation and verification

Next day several single colonies from the agar plate were grown overnight in 5 ml LB-medium supplemented with antibiotics. 500 µl *bacterial freezing solution* were added to 1 ml of the suspension culture and frozen at – 80 °C until further use. Then the plasmid of interest was isolated from 1.5 ml of the bacterial suspension using a *PureYield™ Plasmid Miniprep System* (Promega, California; USA) according to the manufacturer's instructions.

Yield and purity of the gained plasmid dilutions were determined photometrically using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific Biosciences GmbH, Oberhausen, Germany).

For plasmid verification 0.5 µg DNA were digested with appropriate restriction enzymes (see table below) by incubation for 2 hours at 37 °C. SYBR™ Safe DNA gel stain was then added and the digested DNA was separated via gel electrophoresis (0.1 µg DNA in a 1 % agarose gel, 100 V for one hour in *electrophoresis buffer*) and visualized. Plasmid identities were verified by usage of reference plasmids or size estimation of gained DNA fragments.

Plasmid propagation

Verified plasmids were propagated from the frozen cultures by first growing them overnight in 5 ml LB medium with antibiotics at 37 °C and then using this culture to inoculate 400 ml LB medium with antibiotics and incubate again overnight. Plasmids were then isolated using a *PureYield™ Plasmid Midiprep System* (Promega, California, USA) according to the manufacturer's instructions and isolated plasmids were characterized again by analysis of prepared restriction fragments.

List of used plasmids

Name of plasmid	Source	Antibiotic resistance	Used restriction enzymes
EGFP (pEGFP-N1)	Clontech (Mountain View, CA, USA)	Kanamycin	NheII, EcoRI
PPARα (pSG5-PL-hPPARalpha)	Univ. Prof. Walter Wahli and Univ. Prof. Beatrice Desvergne (Center for Integrative Genomics, University of Lausanne, Switzerland)	Ampicillin	EcoRI, HindIII
PPARβ/δ (pSG5-hPPAR-beta)	Univ. Prof. Walter Wahli and Univ. Prof. Beatrice Desvergne	Ampicillin	EcoRI, HindIII
PPARγ (pSG5-PL-hPPAR gamma1)	Univ. Prof. Walter Wahli and Univ. Prof. Beatrice Desvergne	Ampicillin	EcoRI, HindIII
PPAR_RE (tk-PPREx3-luc	Univ. Prof. Ronald M. Evans (Howard Hughes Medical Institute, La Jolla, CA, USA)	Ampicillin	EcoRI, HindIII
RXRα	Missouri S&T cDNA Res (Rolla, Missouri, USA)	Ampicillin	EcoRI, XhoI

RXR_RE (RXR(2) Luciferase Reporter Vector)	Panomics S.r.l. (Milan, Italy)	Ampicillin	HindIII, BamHI
IKK-β CA	Addgene (Cambridge, Massachussets, USA)	Ampicillin	EcoRI, Xhol
IKK-β WT	Addgene (Cambridge, Massachussets, USA)	Ampicillin	EcoRI, Xhol

3.3 Sample preparation

The author's group was one of the two main recipients for samples from the collaborating groups of organic chemists and phytochemists. Samples were usually obtained dry and were delivered in 1.5 ml microcentrifuge tubes along with information specifying identity and mass of the sample. Received extracts were usually dissolved under aseptic conditions in sterile DMSO in a concentration range between 10 to 30 mg/ml, while compounds were usually dissolved in a concentration of 30 mM. Upon necessity extracts were often put in an ultrasonic bath for 20 minutes at room temperature to improve solubility. Dissolved samples were split into aliquots and stored at -20°C to create a small library of natural products.

3.4 Luciferase reporter gene assays in HEK293 and HEK293/NF-κB-luc cells

Background information

The usage of reporter genes in academic science dates back to 1980, when the first publication describing the usage of a *lacZ* fusion reporter gene was made available (Ghim et al., 2010). In biomedical science the term reporter gene is used to define a gene with a readily measurable phenotype that is easily distinguishable over the background of

endogenous proteins (Naylor, 1999). In their early years reporter gene assays were usually performed using enzymes that catalyze the conversion of chromogenic substrates which can be measured in colorimetric assays. While for qualitative answers (e.g. a blue white screen with *lacZ*) colorimetric assays are still widely used, for quantitative answers they have in the meanwhile been widely replaced with fluorescent or bioluminescent detection strategies due to their superior dynamic range and sensitivity. In general fluorescence and bioluminescence detection strategies are currently the main tools in science with approx. ¾ of all employed assays utilizing either a fluorescent or bioluminescent detection approach.* (Thorne et al., 2010)

Historically one of the initial disadvantages of luciferase transporter systems was the localization of the enzyme inside the cell which requires lysis of cells prior to measurement. This was inconvenient since the lysis step is difficult to automatize in large scale testings. However, this problem can in the meanwhile be overcome by usage of secreted luciferases (Wu et al., 2007). Recent improvements of the luciferase-reporter-system include the introduction of engineered luciferases with improved biochemical and physical characteristics (Branchini et al., 2014), also assays using two different luciferases for measuring two independent parameters have been developed some time ago (Grentzmann et al., 1998). In addition the development of multicolor luciferase assays using even several luciferases simultaneously has also been successful (Giger et al., 2011).

The strategy for luciferase assays employed here in this project relies on the classical luciferase reporter gene assay approach. In the first step HEK293 cells get transfected with the plasmids of interest via the calcium phosphate transfection method. Luciferase reporter gene experiments require the transfection with a plasmid encoding the target receptor (to increase endogenous receptor levels for a better signal-to-noise ratio) and a plasmid encoding the luciferase enzyme under control of a response element that initiates target gene transcription via binding of the stimulated target receptor. To account for differences in cell number also an EGFP-plasmid was used and the derived fluorescent signal served as a normalization signal. The used mass ratios of response element plasmids to receptor plasmids and normalization plasmids for the experiments with nuclear receptor modulators were already established in the first project period for PPARs and NF-κB activation, while the mass ratios for transfection experiments with RXRα were derived in preliminary experiments with the RXR agonist 9-cis retinoic acid

* Since this number is derived from the approx. 2000 assays listed at PubChem database there is possibly a bias against simpler assays that might not be entirely included in this list.

conducted by diploma student Amina Cocic in co-supervision from the author of this thesis.

The calcium phosphate transfection method is an excellent method for transfection, since it is very cost-effective and can reach transfection efficiencies close to 100 % in the used HEK293 cell lines. The main disadvantage of this method is that is not applicable for most cell lines, so the susceptibility of HEK cells towards this method is one of its biggest assets. Interestingly, although the calcium phosphate transfection method was described already in 1973, they exact underlying mechanisms for transfection are still not entirely understood (Giger et al., 2011).

After transfection the cells are incubated for 6 hours in which the receptor and the protein used for normalization of cell numbers become biosynthesized. Then the transfected cells are seeded into 96 well plates and get treated with samples of interest, usually in quadruplicates (every treatment condition in four wells). Upon activation of the receptor (typically by ligand binding), it targets the introduced response element, which leads to synthesis of luciferase.

In the last step of the experiment the medium is drained and the 96-well plates are frozen until measurements of luciferase and EGFP levels in the TECAN multiplate reader (Tecan, Grödig, Austria)

Luciferase reporter gene assays are a well applicable method to screen a considerable amount of samples, since 20 different samples can be measured on every 96-well plate in quadruplicate (four quadruplicate conditions are used for the necessary control treatments on every plate) and an experienced experimenter can handle several plates simultaneously. One experimental round takes three days, so several (overlapping) rounds of experiments can be performed per week, if desired. Usually samples were tested in luciferase reporter gene assay in at least three independent experiments.

The assay as described here has also proofed to be suitable for experimenters that do not have very much experience in cell culture or with pipetting of small volumes (such as undergraduate students).

For luciferase reporter gene experiments aiming to identify inhibitors of NF-κB activation in HEK293/NF-κB-luc cells transfection with EGFP was abandoned during the second phase of the DNTI project and HEK293/NF-κB-luc cells were instead treated with 5-chloromethylfluorescein diacetate (CMFDA), commercialized as CellTracker Green (Thermo Fisher Scientific Biosciences GmbH, Oberhausen, Germany) for the same purpose. CMFDA is a fluorescent dye that can freely pass through cell membranes into cells, where it is transformed into a cell-impermeant fluorescent product. This change in

methodology increased sample throughput, but has the disadvantage in comparison to EGFP transfection that an eventual total block of protein synthesis caused by treatments might be misinterpreted as inhibition of NF-κB activation. However, on the other hand one advantage of CMFDA staining is that it provides direct information about cell viability and not about the capacity of the cells to produce a certain protein such as EGFP.

Solutions and buffers

HEK culture medium

No serum medium

DMEM (4.5 g/l glucose)

without phenol red	500 ml
benzylpenicillin	100 U/ml
streptomycin	100 µg/ml
glutamine	2 mM

stored at +4°C

Transfection mixture

HEPES	10.5 mM
glucose	2.75 mM
KCl	25 mM
Na_2HPO_4	3.53.mM
$CaCl_2$	0.12 mM

+ Plasmids /transfection

for nuclear receptor activation:

PPARβ/δ/PPARγ	: PPAR_RE	: GFP	6µg: 6µg: 3 µg
PPARα	: PPAR_RE	: GFP	1µg: 6µg: 3 µg
RXRα	: RXR_RE	: GFP	6µg: 6µg: 1µg

for inhibition of NF-κB activation

GFP	6µg

for transfections with IKK-β

IKK-β (CA/WT)	: GFP	6µg: 6 µg

Plasmids were added to the transfection mixture immediately prior to transfection

Luciferase lysis buffer

Tris-HCl pH 7.8	125 mM
DTT	10.1 mM
CDTA	10.1 mM
glycerol	50 % (v/v)
Triton X-100	5 % (v/v)
Coenzyme A	270 µM

sterile filtered

and stored at +4 °C, 0.1 mM DTT and

270 µM coenzyme A added immediately prior to usage

For TECAN measurements:

ATP-buffer (Injector A)

ATP	3.8 mM
Tricine pH 7.8	20 mM
$MgCl_2$	21.5 mM

sterile filtered and aliquoted, stored at -80°C

Luciferin-buffer (Injector B)

Luciferin	32 mg/ml
Tricine pH 7.8	21 mM

sterile filtered and aliquoted, stored at – 80 °C

Experimental procedures

Experiments for nuclear receptor activation

6 x 10^6 cells were seeded in 20 ml *HEK culture medium* in 15 cm cell culture dishes and grown overnight. Next morning cells were transfected by dropwise addition of 1.5 ml *transfection mix* and incubated for 6 hours. In the meanwhile test samples were diluted in *HEK culture medium* in four-fold concentration. 50 µl of each of sample were pipetted in four wells of the 96-well plate and the plate was then stored at 37 °C until the transfected cells were added.

All medium was aspired from the transfected cells and they were washed with PBS and then detached by treatment with 3 ml *trypsin solution* for 5 minutes at 37 °C. The *trypsin solution* was neutralized by addition of 12 ml HEK culture medium and the cell suspension was transferred to a falcon which was centrifuged for 4 minutes at 1200 rpm. The cell pellet was then resuspended in 10 ml *HEK culture medium* and 1 ml of the suspension was used to approximate cell density in a cell counter. Cell density numbers were used to prepare the required volume of cell suspension to seed 4 x 10^4 transfected cells in 150 µl/well, which were already containing 4-fold concentrated treatment conditions.

Four wells of the 96-well plate were used for untransfected cells, which were seeded in parallel to the other cells on the day before the experiment (1 x10^6 cells in 5 ml *HEK culture medium* in a 5 cm dish). On the day of the experiment these cells were detached as described for HEK cells and seeded also at a density of 4 x 10^6 cells, but an overall volume of 200 µl.

The 96-well plate was then incubated for 18 hours at 37 °C, then all liquid was aspired and the plate wrapped in aluminum foil and frozen at – 80 °C until measurement.

Experiments for inhibition of NF-κB activation

Experiments for inhibition of NF-κB activation were following a similar protocol, but here the detached EGFP-transfected HEK293/NF-κB-luc cells were resuspended in *no serum medium* instead of *HEK culture medium* and 4 x 10^4 cells/well were seeded in a volume of 100µl instead of 150 µl. Cells were then incubated overnight, before the cells were preincubated with samples diluted in *no serum medium* (instead of *HEK culture medium*) for 30 minutes in a volume of 50 µl/well in four-fold concentration. Then the cells were stimulated with 50 µl TNFα (8 ng/ml) dissolved in *no serum medium* and incubated for 6 hours, before all liquid was aspired and the plate wrapped in aluminum foil and frozen at –80 °C until measurement.

Accordingly also the untransfected cells were resuspended in in *no serum medium* instead of *HEK culture medium.* In addition to the untransfected cells four wells of the plate were used as control containing cells that were not stimulated with TNFα.

Staining of HEK293/NF-κB-luc cells with CMFDA

For staining HEK293/NF-κB-luc cells with CMFDA 10 x 10^6 cells were seeded in 20 ml *HEK culture medium* in a 175 cm^2 cell culture flask and incubated overnight. Next day the medium was replaced with 20 ml *no serum medium* supplemented with 2 µM CMFDA and incubated for one hour. Reseeding was done identically as for HEK293/NF-κB-luc cells that were transfected with EGFP. Also for experiments with CMFDA four wells of the 96-well plate were used for unstained cells, which were prepared as described for the untransfected cells in NF-κB experiments.

Measurement

50 µl *luciferase lysis buffer* were pipetted in each well of the 96-well plate and the plate was agitated on a plate shaker for 10 minutes. 40 µl from each well were then pipetted into a non-transparent 96-well plate and the TECAN Genios pro multiplate reader was used to measure fluorescence and luminescence. The measurement parameters were as follows:

Luminescence measurements

Measurement mode : Luminescence

Integration time (manual) : 2000 ms

Attenuation : None

Plate definition file : GRE96fb

Part of the plate : A1 – H12

Time between move and integration : 50 ms

Well kinetic number : 1

Well kinetic interval (minimal) : 2020 ms

Injector A volume : 50 µl

Injector A speed : 200 µl/s

Injector B volume : 50 µl

Injector B speed : 200 µl/s

Injection mode : Standard

Fluorescence measurement

Measurement mode : Fluorescence

Excitation wavelength : 485 nm

Emission wavelength : 520 nm

Gain : Optimal

Number of reads : 1

Integration time : 1000 µs

Lag time : 0 µs

Mirror selection : 40 ms

Important note: Although the author of this thesis performed an exhaustive number of luciferase assays in all kinds of variations himself, the team technician Mia Gössinger dedicated her work time almost exclusively to luciferase reporter gene experiments. In turn Ms. Gössinger has also harvested a significant amount of the data presented in this work. Because she was formally working under guidance and supervision of the author there is no designation which luciferase data was gained by Ms. Gössinger, especially since some of the data were gained by both the author of this thesis and Ms. Gössinger by combined efforts.

3.5 Glucose uptake experiments

Background information

Since the brain of vertebrates can only metabolize glucose as energy source, it is very important for the organism to always provide sufficiently high levels of glucose in the bloodstream in order to avoid impairment of the central nervous system. On the other hand sustained elevated high levels of glucose in the bloodstream (hyperglycemia) lead to vascular injury (Xu and Zou, 2009). Therefore physiological glucose levels in humans are kept within a range between 4 to 7 mM and normally do not exceed 10 mM after ingestion of food. The organism can control glucose levels via several regulatory mechanisms, with hormonal secretion being the prime mechanism for glucose homeostasis (Polakof et al., 2011). Recurrent or persistent hyperglycemia is the key feature of a prominent disease termed diabetes mellitus (the suffix "mellitus" is commonly omitted). There are several different types of diabetes known with diabetes type II being the most prominent with a share of 90 % of all diagnosed cases. Diabetes type II is characterized by elevated levels of blood glucose in the context of insulin resistance and a subsequently developing insulin deficiency. In Western societies diabetes has become an increasingly relevant health burden, global prevalence is expected to grow from 2.8 % (equals 171 million people) in 2000 to 4.4 % (equals 366 million people) in 2030 (Wild et al., 2004). Also economically diabetes increasingly wears out societies: in 2012 the total economic burden of diabetes in USA was estimated to be 245 billion USD, an increase of 41 % compared to 2007 (Giquel et al., 2014)[*].

There are several strategies available to treat hyperglycemia, all of which intend to lower blood glucose levels. A prominent treatment approach is to sensitize tissue towards insulin so that more glucose is taken up from the bloodstream. The first marketed insulin sensitizing drug was troglitazone, a PPARγ agonistic glitazone that got FDA approval in 1997. Troglitazone has later been at withdrawn from the markets due to severe side effects and also its successor drugs are under scrutiny of an unfavourable risk profile. Nevertheless PPARγ agonists are referred to as prototypical insulin sensitizers and

* In Austria health officials report that the 600.000 diagnosed diabetes patients were responsible for 8.4 % of all public health expenses, so on average each diabetes patient costs around 2.5 times more than the average insurant (source: Österreichischer Diabetesbericht 2013).

especially partial agonists are considered as promising candidates for new insulin sensitizing drugs (Mudaliar and Henry, 2015).

A widely used *in vitro* cell culture model to mimic insulin sensitization are differentiated 3T3-L1 preadipocytes. This seems at first glance as a paradox, because for glucose uptake the adipose tissue is quantitatively far less important than the skeletal muscle, but since most of the available muscle cell lines are not insulin sensitive in terms of glucose transport, they are not suitable for this kind of experimental setup (Lakshmanan et al., 2003).

However, testing glucose uptake in matured 3T3-L1 preadipocytes proofed to be a difficult task: Testing considerable numbers of samples is denied due to the low-number-well format to be used, the considerably long maturation time of preadipocytes of 2 weeks with many exchanges of culture media, matured cells very susceptible to detachment and in the last steps of the experiment many time dependent handling that require a well experienced and adept experimenter.

The experimental setup relies on usage of a 2-deoxy-D-glucose solution spiked with 2-deoxy-D-(1H^3)-glucose. Unstable tritium can be measured with a liquid scintillation counter. In liquid scintillation part of the kinetic energy of ionizing particles, produced by the decay of tritium, is transferred to the 'scintillator' which converts this energy into light photons and these light photons are directed towards photomultiplier tubes that convert photons into measurable electrical pulses. The data accumulated in the multichannel analyzer is used to determine the rate of radioactivity or counts per minute (cpm). The cpm are the total number of pulses in the channels of the multichannel analyzer divided by the total time in minutes for the obtained counts. Since all 2-deoxy-D-(1H^3)-glucose that was not uptaken by the cells is washed out prior to scintillation counting, glucose uptake can be determined using this approach.

Buffers and solution

Differentiation mix 1

DMEM, (4.5 g/l glucose)	
without phenol red	50 ml
FBS	10 %

Differentiation mix 2

DMEM (4.5 g/l glucose)	
without phenol red	50 ml
FBS	10 % (v/v)

glutamine	2 mM
insulin	1 µg/ml
dexamethasone	500 nM
IBMX	50 µM

glutamine	2 mM
insulin	1 µg/ml

Both sterile filtered and prepared immediately prior to usage

Adipocyte culture medium

DMEM, (4.5 g/l glucose)

without phenol red	50 ml
FBS	10 % (v/v)
glutamine	2 mM

sterile filtered, stored at +4°C

Adipocyte experimental medium

DMEM (4.5 g/l glucose),

without phenol red	50 ml
BSA	0.1 % (w/v)

sterile filtered, stored at +4°C

Adipocyte starvation medium

HEPES	50 mM
NaCl	136 mM
$CaCl_2$	1.25 mM
KCl	23.5 mM
$MgSO_4$	1.25 mM

BSA	0.1 % (w/v)

sterile filtered, stored at +4 °C

Glucose uptake solution

2-deoxy-D- glucose	0.1 mM

spiked with 2-deoxy-D-($1H^3$)-glucose (0.45 µCi/ml)

prepared immediately prior to usage

Adipocyte lysis buffer

HEPES	20 mM
Igepal	1 % (v/v)

sterile filtered, stored at +4 °C

Experimental procedure

1.5 x 10^5 3T3-L1 preadipocytes suspended in 3 ml *differentiation mix 1* were seeded in 12-well-format and after 2 days medium was renewed. After 4 days overall incubation time in *differentiation mix 1* the cells were incubated for another 2 days in *differentiation mix 2* and then 6 days in *adipocyte culture medium* with exchange of medium every second day.

The matured adipocytes were then treated for 48 hours with 3 ml compound or solvent vehicle as indicated, dissolved in *adipocyte culture medium.* Each treatment was done in duplicate. Then cells were incubated for 4 hours in *adipocyte experimental medium* and for one additional hour in 3 ml *adipocyte starvation medium* before 5 µl *insulin stimulus* was added to each of the duplicates (to obtain values for "basal uptake" and "insulin-stimulated uptake"). After 15 minutes glucose uptake was initiated by addition of 100 µl glucose *uptake solution* to each well. After 10 min the reaction was stopped by three rapid washes with ice-cold PBS and cells were lysed overnight by treatment at +4 °C with 500 µl *adipocyte lysis buffer.* Next day the plate was thawed, cells were scraped together and transferred to microcentrifuge vial. 200 µl from each sample was used for the scintillation counter, 10 µl were used for protein quantification and the rest was stored as backup.

To obtain a readout of incorporated mol glucose per mg protein and minute results were normalized to protein content and uptake time.

Important note: Glucose uptake experiments in this project were generally guided by former PhD student Matthias Kramer, whose PhD thesis focused on this topic. As far as the data shown in this project is concerned, the author of this thesis had an assisting role in some experiments with honokiol, while glucose uptake experiments with falcarindiol were performed in parallel. This creates a partial overlap and undesired redundancy with the doctoral thesis of Dr. Kramer, because the experiments and results for both falcarindiol and honokiol are already thoroughly discussed in his work. Therefore a graph of results and detailed discussion for the experiments with honokiol are omitted here, while the results for falcarindiol are provided, but only briefly discussed. The interested reader is referred to Dr. Kramer's worth reading dissertation for further information and a full scope of gained insights.

3.6 Flow cytometric measurement of pro-inflammatory surface proteins

Background information

Flow cytometry was developed in the 1950s as an alternative to automated microscopy and has become a standard method in biomedical science and diagnostic medicine. The method relies on the principle of hydrodynamic focusing to align single cells, so that they can individually pass a series of detectors to become characterized. The characterization usually relies on one hand on the usage of fluorescent antibodies targeting specific structures of interest and on the other hand on the scattering of light the cell produces upon passing a laser beam. To detect this scattering usually one detector is placed in a position so that it can measure light refracted in the forward direction (forward scattering) and another one that can detect light scattering orthogonal to the incident laser beam (side scattering). Since the observed scattering is characteristic for a certain cell shape, appropriate conclusions can thereby be made.

Flow cytometry has grown from a few custom-made instruments in the 1950s into a benchmark tool in contemporary biomedical cell research and currently allows measurements of various parameters from >1 x 10^4 cells/sec (Chattopadhyay and Roederer, 2012).

In this work flow cytometry was used to detect the pro-inflammatory cell adhesion proteins VCAM-1 (vascular cell adhesion molecule 1), ICAM-1 (intercellular adhesion molecule 1) and E-selectin (endothelial adhesion molecule 1) in HUVECtert with the respective antibodies from BD Biosciences (New Jersey, USA). Endothelial cells upregulate the expression of these three proteins for the recruitment of leukocytes as one of the major events in the development of an inflammation. The induced expression of VCAM-1, ICAM-1 and E-selectin requires activation of NF-κB signaling. (Zhang et al, 2002)

In the hands of the author of this thesis flow cytometric measurements of ICAM-1, VCAM-1 and E-selectin proofed to be a robust, uncomplicated and also fast method to determine the parameters of interest. Due to the uncomplicated experimental handling and the stability of the used FITC-labelled antibodies several 12-well-plates can be handled simultaneously, the measurement of one sample is usually done within one minute and the experiments generally showed a satisfyingly high degree of

reproducibility. In conclusion this method is an excellent choice to confirm anti-inflammatory effects of compounds from the luciferase reporter gene assay for inhibition of NF-κB activation in the HEK293 cell line in the physiologically more relevant HUVECtert. However, although definitely more convenient, the experimental setup using HUVECtert is not competitive with luciferase reporter gene assays in HEK293 cells in terms of sample throughput and costs.

Solutions and buffers

HUVEC culture medium

Washing solution

BSA 2 % (w/v)

in PBS, sterile filtered, stored at +4 °C

Experimental procedure

2 x 10^5 HUVECtert were seeded into precoated (0.1 % gelatin in PBS incubated 10-15 minutes at 37 °C) 12-well-plates in 2 ml *HUVEC culture medium* and grown overnight. Next day cells were pre-incubated in duplicate for 30 minutes with 2 ml treatment condition or solvent vehicle as indicated and then 2 µl TNFα (10 µg/ml dissolved in 0.1 % BSA in PBS) was added to each well. For measurement of the endothelial surface protein E-selectin workup of cells was done 4 hours after addition of TNFα, while measurements for ICAM-1 and VCAM-1 were performed after 18 hours incubation time.

To workup samples, all liquid from the well was aspired, cells were washed once with PBS and were then detached by treatment with 1 ml *trypsin solution* for 5 minutes at 37 °C. Neutralization of the reaction was done by addition of 1 ml *HUVEC culture medium* and the cell suspensions were then transferred to FACS-vials, which were centrifuged for 4 minutes at 1200 rpm. Supernatant was discarded and the cell pellets were washed with *washing solution*. After 3 washing steps 10 µl of the respective FITC-labelled

antibody were added to each sample, for measurements of ICAM-1 and VCAM-1 the cell suspension was split into halves prior to addition of the antibody to measure both ICAM-1 and VCAM-1 from the same population of cells. After 1 h incubation with the antibody at room temperature in the dark, cells were pelleted once more (4 minutes at 1200 rpm) washed again twice with *washing solution* before the samples were analyzed in the flow cytometer. 1 x 10^4 cells per sample were measured and the average of detected fluorescence is shown relative to the readout of TNFα stimulated cells treated with solvent vehicle.

To not only access the anti-inflammatory effect of treatments upon stimulation with TNFα, but also an eventual pro-inflammatory effect the endothelial surface markers ICAM-1, VCAM-1 and E-selectin were also measured using the same protocol without addition of TNFα to the treatment conditions.

3.7 Protein quantification

Background information

Having its historical origins in food science, where protein content can be used as quality parameter, protein quantification has become an integral part in modern science of every scientific laboratory whose workflow involves protein extraction, purification, labeling or analysis. The importance of protein quantification in modern science is probably best observable by studying the most frequently cited scientific papers of all times: In fact, two of the three most frequently cited publications describe a protein quantification method (Van Noorden et al., 2014).

Protein quantification methods are available in many different levels of accurateness and sophistication, with its simplest by solely measuring the UV-absorbance of proteinogenic aromatic amino acids (only applicable in pure protein solutions), the most commonly used different colorimetric methods up to highly complex coupled mass spectrometric approaches (González-Antuña et al., 2015)**.**

In this work protein quantification was done to ensure using equal amounts of protein from each sample for PAGE (polyacrylamide gel electrophoresis) and also for quantification of p65 in nuclear extracts with the TransAM™ NF-κB p65 kit. In this project two different colorimetric approaches were utilized: The Bradford method that relies on

Coomassie G-250 binding to certain amino acids yielding a shift in absorbance and the BCA (bicinchoninic acid) method that relies on the protein-mediated transition of $Cu^{2+}$to Cu^{+} that can be detected by BCA. Although the Bradford method is generally excellent and well applicable, in the author's hands it sometimes tended to give single off-values. In the opposite the BCA method yielded constantly excellent results and was therefore his preferred method for protein quantification.

Solutions and buffers

BCA master mix

50 parts of reagent A mixed with one part of reagent B immediately prior to the experiment

Reagent A		Reagent B	
BCA	10 g	$CuSO_4.H_2O$	4 g
$NaCO_3$	23 g	per 100 ml aqua dest	
$Na_2C_4H_4O_6$	1.6 g		
per 1000 ml aqua dest.,			
pH adjusted to 11.25 with NaOH			

Bradford master solution

1 part Roti™ -Quant solution diluted with 4 parts H_20

Experimental procedure

Bradford method

190 µl Bradford master solution were added in triplicates of 10 µl of 1:10 diluted sample in 96-well format and the absorbance was measured at 595 nm. Protein quantification was done using a BSA standard curve on every plate.

Pierce BCA method

190 µl *BCA master mix* were added to triplicates of 10µl of 1:10 diluted sample in 96-well format and the plate was incubated for 30 minutes at 37 °C. Absorbance was measured at 560 nm and protein quantification was done using a BSA standard curve on every plate.

3.8 SDS-PAGE and Western blotting

Background information

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and Western blotting are a combinatorial technique to isolate, detect and (semi)quantify specific proteins of interest. The combinatorial technique is in colloquial language often abbreviated to "Western blotting" and was developed in the late 1970s almost simultaneously by three independent research groups. The name was chosen based on a method to detect DNA fragments named Southern blot, which was developed by the British molecular biologist Edwin Southern in 1975. Also today, 35 years after its introduction Western blotting remains a crucial technique for investigators who need to identify and semi-quantify specific proteins (Aebersold et al., 2013). Improvements of the technique to increase reproducibility, sensitivity, quantifiability, and also the speed of Western blot experiments have been developed, but the principles of the method still remain unchanged (Eaton et al., 2013).

To detect a protein using the SDS-PAGE and Western blotting approach, the first step of the procedure is to lyse cells. Here the lysis buffer to be used is highly dependent on which proteins are to be detected and is often chosen by an iterative approach. DNA of the sample is removed by centrifugation and the proteins become denaturated by heat and reducing chemicals. Denaturation is done to eliminate the effect of the three-dimensional protein on electrophoretic mobility, while denaturated proteins usually all have linear structures. In the next step the samples become incubated with SDS. This is done to eliminate the effects of different amino acid compositions of proteins on electrophoresis, since SDS is so highly negatively charged that the differing negatively charged amino acids of proteins have a negligible effect on the overall ionic mobility in the electric field. The positively charged amino acids of the proteins are irrelevant for the separation because they carry no charge in the alkaline electrophoresis buffer. The samples are then loaded on a gel, where the proteins first become stacked in a stacking gel by isotachophoresis and then separated by electrophoresis according to their size: The bigger the protein, the slower it can migrate through the gel pores towards the anode.

In the next step the separated proteins become electrotransferred from the susceptible gel to a durable membrane, most often made of nitrocellulose, PVDF or nylon. To prevent unspecific binding of antibodies to unoccupied binding sites of the membrane, the membrane is then treated with blocking buffer that occupies all unoccupied binding sites of the membrane.

Protein-specific primary antibodies that can bind to their epitope on the protein of interest are then applied. After removing unbound primary antibody the membrane is treated with a secondary antibody that is linked to a reporter enzyme and can bind to an epitope of the primary antibody. After elution of unbound secondary antibody the substrate of the reporter enzyme is added and the gained signal from the reporter enzyme is relative to the amount of protein of interest being present in the sample.

Although in theory a persuasive and elegant method to detect and quantify proteins, in practice Western blotting is plagued by poor reproducibility, lack of accurate quantitation, extensive time to result and reliability issues and none of the improvements has fully overcome the challenges and bottlenecks still experienced by researchers today (Nguyen et al., 2011). In particular (semi)quantification of proteins is often impaired by an insufficiently quantitative transfer of proteins from gel to membrane, a challenge that was already anticipated by the inventors of the method (Towbin et al., 1979). Further reproducibility problems may arise from the many necessary steps from cell lysis to readout, where single minor deviations of the applied procedure can add up to major

differences in the obtained readouts. However, despite the many associated problems the combinatorial technique of SDS-PAGE and Western blotting is still widespread in biomedical research and has been facilitating major scientific breakthroughs.

Also the author of this thesis has extensively used the Western blotting method, but the obtained quantitative results were unfortunately often poorly inconsistent and disappointing.

Results for Western blotting are generally always shown in comparison to a normalization protein, in this project the normalization protein was usually actin (for detection of nuclear proteins lamin was used). Results for the proteins of interest are shown relative to the result of control treatments.

In the foreseeable future possibly Western blot might be outdated for the generation of (semi)quantitative data in favour of mass spectrometry (MS) methods, particularly such as the so called "Selected Reaction Monitoring" (SRM) approach (Aebersold et al., 2013).

Solutions and buffers

Western lysis buffer

Tris Hcl pH 6.8	50 mM
NaCl	500 mM
NP40 (Igepal)	1 % (v/v)
Natrium-desoxycholat	0.5 % (w/v)
SDS	0.1 % (w/v)
NaN_3	0.05 (w/v)

stored at – 20°C and completed by addition of

Complete™	4 % (v/v)
PMSF	1 mM
NaF	1mM
Na_3VO_4 (activated)	1 mM

immediately prior to use

Washing buffer

Tris base pH 8.0	25 mM
NaCl	0.19 M
Tween-20	0.1 % (v/v)

aqueous

PAGE sample buffer

Tris HCl pH 6.8	25 mM
SDS	0.2 M
bromphenol blue	0.2 mM
β-mercaptoethanol	15 % (v/v)
glycerol	30 % (v/v)

aqueous, stored at -20 °C

For the experiments described in this study the following antibodies were used: Anti-IκB-α, anti-phospho-IKK-β, anti-phospho-IκBα and anti-p65 (Cell Signaling Technology, Danvers, Massachusetts, USA), anti-actin (MP Biomedicals, Illkirch, France), anti-tubulin (Santa Cruz Biotechnology, Santa Cruz, California, USA) and anti-lamin (Abcam, Cambridge, UK). All antibodies were used in a dilution of 1:1000

Blotting buffer

Tris base pH 8.5	25 mM
glycine	194 mM
methanol	20 % (v/v)
aqueous	

Blocking buffer

skimmed milk powder	5 %
Tris base pH 8.0	25 mM
NaCl	0.19 M
Tween-20	0.1 % (v/v)
aqueous, stored at -20°C	

Development buffer

Tris-base pH 8.5	100mM
luminol	1.24 mM
p-coumaric acid	0.02 mM
H_2O_2	0.018 %
aqueous, prepared immediately prior to use	

Experimental procedure

Harvesting of cells

All liquid was aspired from the cells and the cells were washed once with cold PBS, before *Western lysis buffer* was added and incubated on ice for 15 minutes. All subsequent steps until freezing the samples were done on ice. Cells were scraped off vigorously with a cell scraper and transferred to a microcentrifuge vial. The suspension was sonicated for 15 seconds and then centrifuged for 20 minutes in a table top centrifuge at top speed

for 20 minutes at +4 °C. The supernatant was collected and stored at -20 °C. Prior to continuation of the protocol cell lysates were subjected to protein quantification.

SDS-PAGE and protein blotting

For SDS-PAGE volumes of samples containing 20 µg protein were filled up with H_2O to a total of 20 µl and 10 µl *PAGE sample buffer* were added. Samples were then heated for 5 minutes at 95 °C and loaded on a pre-casted 10 % PAA-gel together with a protein standard.

Proteins were separated for 90 minutes with 25 mA per gel and then blotted on an activated PVDF membrane for 2 hours in *blotting buffer* with 100 V. After blotting the membrane was incubated for 1 hour in *blocking buffer* and then washed three times for 5 minutes in *washing buffer*.

Protein detection

For protein detection the membrane was incubated overnight in diluted *primary antibody solutions* and then washed 3 times for 5 minutes in *washing buffer*. The membrane was then incubated for 1 hour in *secondary antibody solution* and again washed for 5 minutes each in *washing buffer.* For signal development the membrane was submerged in *development buffer* and the chemiluminescence was detected with a LAS-3000 luminescent image analyzer (Fujifilm Life Science, Düsseldorf, Germany). Quantification of proteins was done by densitometry using AIDA™ software from raytest (Straubenhardt. Germany)

Important note: Western blotting experiments with eurycomalactone were performed in kind cooperation with guest PhD student Enrico Sangiovanni (University of Milan, Italy).

3.9 Resazurin assay in HUVECtert cells

Background information

Resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) is a blue dye widely applied in biochemistry as an agent to test metabolic activity of cells. Inside living cells the non-fluorescent resazurin is converted via reduction to the strongly fluorescent pink resorufin. The reaction of resazurin to resorufin was shown to be proportional to aerobic respiration. (González-Pinzón et al., 2012) The conversion can be assessed by both colourimetry or fluorimetry, with greater sensitivity achieved using the fluorescent property of resorufin. Further incubation of resorufin will result in the formation of the non-coloured and non-fluorescent hydroresorufin, so the incubation time is a vital parameter in the proper application of this assay. (O'Brien et al., 2000)

The use of resazurin as a metabolic indicator dates back to 1929, when it was first used to quantify bacterial load in milk (Rampersad, 2012). Resazurin assays (sometimes commercialized as Alamar blue assays) are simple, rapid, reliable, safe and cost efficient and therefore offer an attractive tool to measure metabolic activity. Metabolic activity can be used as an inverse correlation parameter to determine the cytotoxicity of test compounds. Linking metabolic activity and cytotoxicity so closely, however, bears the risk that toxicity of certain compounds might become misinterpreted: Compounds that slow down the metabolism of cells will be misclassified as toxic, while the toxicity of compounds that are toxic while accelerating the metabolism will be underestimated.

In this study the resazurin assay was used to assess the toxicity/metabolic impairment of inhibitors of NF-κB activation with and without TNFα stimulation. Resazurin assays proofed to be an uncomplicated method that can easily be applied to a considerable amount of samples due to the uncomplicated low-cost experimental setup.

Solutions and buffers

HUVEC culture medium

Experimental procedure

HUVECtert were seeded in *HUVEC culture medium* into precoated (0.1 % gelatin in PBS for 10-15 minutes at room temperature) 48-well-plates at a density of 2 x 10^4 cells/well and grown for 48 hrs. Then the cells were incubated with 400 µl/well either medium alone, or medium supplemented with solvent vehicle, the indicated treatments or digitonin (100 µg/ml) as positive control. Each condition was tested in four wells. After 18 hour supernatant were aspired and the cells washed once with PBS and then incubated for 2 hours with 10 µg/ml resazurin in PBS. Metabolic activity through conversion of resazurin was measured as an increase in fluorescence at a wavelength of 580 nm (excitation wavelength: 535 nm) using the TECAN multiplate reader. Background values derived from resazurin solution in empty wells were subtracted and results are shown relative to the conversion rate of the solvent vehicle treatment.

3.10 Extraction of nuclear proteins from HUVECtert

Background information

NF-κB dimers translocate from the cytoplasm to the nucleus upon activation of the NF-κB cascade. To approach the mechanism of action for an identified inhibitor of NF-κB signaling it is therefore a reasonable approach to separate nuclear from cytosolic proteins in TNFα-stimulated cells: Eventually the abundance of the dimers in both nuclear and cytoxolic fractions prior to and after activation of the cascade can be determined. If an inhibitor of the NF-κB pathway blocks signaling upstream of the important translocation event of the NF-κB dimers, there will be no increase in their abundance in the nuclear fraction after activation of the cascade. If the inhibitor blocks NF-κB signaling downstream of the translocation event the opposite is true.

The separation relies on first bursting the cells by treatment with a hypotonic solution to release cytosolic proteins, followed by separation of nuclei by centrifugation and then treatment with a high salt extraction buffer. In this step the nuclei incubated in the high-

salt extraction buffer shrinks and nucleic proteins become extracted through the nuclear pores and solubilized in the extraction buffer. (Luo et al., 2014) In the last step a buffer with lower salt concentration is added to dilute the high-concentration and high-purity nuclear proteins. The successful separation can be verified by immunoblotting for proteins specific for cytosol or nucleus. In this project lamin was chosen as a reference protein for nucleic proteins and tubulin as reference for cytosolic proteins. The separated fractions were used to detect p65 in ELISA experiments. The extraction protocol described here is a standard method in our laboratory to separate cytosolic from nucleic proteins and is applicable for many different cell lines. One main advantage of this method is that all necessary reagents can be prepared by the experimenter to evade the necessity to purchase a costly commercial kit for this application.

Solutions and buffers

HUVEC culture medium

Separation buffer 1:

HEPES pH 7.5 10 mM	
EDTA	0.2 mM
KCl	10 mM,
NP40 (Igepal)	1 % (v/v)
DTT	1 mM,
PMSF	0.5 mM
Complete™	4 % (v/v)

Separation buffer 2:

HEPES pH 7.5	20 mM
EDTA	1.1 mM
NaCl	420 mM
DTT	1 mM,
PMSF	0.5 mM
Complete™	4 (v/v)

Separation buffer 3:

HEPES pH 7.5	20 mM
EDTA	1.1 mM
KCl	100 mM
glycerol	20 % (v/v)

NP40 (Igepal)	1 % (v/v)
DTT	1 mM,
PMSF	0.5 mM
Complete™	4 % (v/v)

Experimental procedure

0.8 x 10^6 HUVECtert cells were grown in *HUVEC culture medium* in four pre-coated 10 cm dishes for 48 h and then preincubated for 30 min with treatments as indicated. One dish with cells treated with compound as well as one dish with cells treated with solvent vehicle were worked up before addition of TNFα (10 ng/ml in PBS) and the other two dishes were worked up 1 h after stimulation with TNFα.

To separate nuclear from cytosolic proteins the dishes were first washed with cold PBS and then treated with 200 µl of *separation buffer 1*. Cells were scraped together and transferred into a microcentrifuge tube and incubated for 15 minutes on ice, with vigorous vortexing every 2-3 minutes. Then the cell lysates were centrifuged in a table-top centrifuge for 5 minutes at top speed. The supernatant was collected as cytosolic fraction.

The pellets were then washed once with *separation buffer 1* and were then resuspended in 100 µl *separation buffer 2* and incubated on ice for 15 minutes with vigorous vortexing every 2-3 minutes, followed by centrifugation for 5 minutes and 13 000 rpm in a table centrifuge. After that the supernatant were combined with 100 µl of *separation buffer 3* representing nuclear proteins. Isolated cytosolic and nucleic proteins were both stored at -80°C. To verify separation all samples were probed for the nuclear protein lamin and the cytosolic protein tubulin in immunoblot experiments.

Important note: The nuclear/cytosolic extraction experiments yielding the data presented in this thesis were performed in kind cooperation with the guest PhD student Enrico Sangiovanni in parallel experiments.

3.11 Detection of DNA binding ability of activated p65 in nuclear extracts (TransAM™ NF-κB p65 kit)

Background information

After translocation of activated NF-κB dimers from the cytosol to the nucleus in the next step of the NF-κB activation cascade the dimers have to bind accessible DNA-sequences to modulate target gene expression. Since inhibition of this binding step is a known inhibitory mechanism for some NF-kB inhibitors (e.g. Lyß et al.,1998), it is a compelling strategy to test the ability of NF-kB inhibitors to inhibit this step of the cascade, if the inhibitory effect of a compound can not be attributed to an earlier step in the activation cascade. In line this experimental approach expands the information gained from the immunoblotting experiment where the abundance, but not the binding ability of NF-κB dimers is measured from nuclear extracts.

The kit follows a classical ELISA-approach that is utilized in many commercially available assay kits: Multiple copies of the NF-κB consensus sequence oligonucleotide are immobilized in each well of the 96-well plate. The nuclear cell lysate is added and NF-κB dimers bind to the immobilized oligonucleotide if they are not inhibited by compound treatment to do so. In the next step added NF-κB antibody binds the bound NF-κB. The antibody is then conjugated with an anti-IgG-HRP-secondary antibody and upon addition of one of the many substrates for HRP (in this kit it is likely to be 3,3´,5,5´tetramethylbenzidin (TMB) according to the gained colour reaction) and stopping the reaction with acid, the amount of converted substrate is proportional to the bound NF-kB dimers and can be determined photometrically.

In conclusion this experimental setup expands the information gained from the immunoblotting method that detects the presence of p65 in nuclear extracts, with the additive information if the activated NF-κB dimers (in particular the most prevailing p65-subunit) can also bind to an NF-kB consensus sequence.

Buffers and solutions

All necessary reagents except H_2O were supplied by the manufacturer.

Experimental procedure

For detection of p65 in nuclear extracts of HUVECtert the experiments were done according to the manufacturer's protocol:

Briefly, all reagents provided by the manufacturer were completed and the required wells of the 96-well plate activated as described. 20 µg nuclear extract with and without addition of 2 µM eurycomalactone and the provided control conditions were pipetted in a volume of 20 µl in duplicate into the activated wells. Binding steps of primary and secondary antibody were done as recommended by the manufacturer. After addition of 100 µl/well development solution the plate was incubated for 5 minutes, then 100 µl stop solution were added and absorbance was measured with the TECAN multiplate reader at a wavelength of 450 nm (reference wavelength: 650 nm).

To further investigate a possible effect of eurycomalactone on the DNA binding ability of p65, eurycomalactone was also directly added to the nuclear extracts of TNFα stimulated cells to a final concentration of 2 µM in separate experiments.

Important note: Experiments for detection of p65 in nuclear extracts levels were performed in kind cooperation with guest PhD student Enrico Sangiovanni in parallel experiments.

3.12 Quantification of cAMP levels in HUVECtert (Direct cAMP ELISA kit)

Background information

Cyclic adenosine monophosphate (cAMP) is an important second messenger molecule in eukaryotic cells and is biochemically derived from ATP. Second messenger molecules are responsible for intracellular signal transduction and intracellularly propagate signals from first messengers (hormones and neurotransmitters) that cannot pass cell membranes due to their hydrophilicity. The importance of cAMP as a second messenger was realized in the 1960s and cAMP research is closely linked to two Nobel Prizes in Physiology or Medicine: In 1971 an American scientist was awarded for his discovery of second messenger molecules and in 1991 two American scientists were awarded for their discovery of G protein-coupled receptors. The latter is important in context with cAMP because cAMP-dependent signaling pathways rely on G protein-coupled receptor triggering. Physiologically cAMP plays a key role in many processes, including the role as a modulator of energy metabolism and of both the innate and the adaptive immune system (Serezani et al., 2008). There is an extensive crosstalk of cAMP or cAMP dependent pathways with NF-κB activation described in literature, but the experimental results are often contradictory. However, the majority of publications notes a negative effect of cAMP on NF-κB activation, but the outcome of the crosstalk appears to be highly cell line and context specific (Gerlo et al., 2011).

Experiments for determining the effect of inhibitors of NF-κB activation on cAMP-levels were not planned initially, but for the publication of plumericin as a new inhibitor of the NF-κB pathway one of the reviewers of the manuscript submitted to the *British Journal of Pharmacology* requested to determine an eventual effect of plumericin treatment on cAMP levels. To yield this information a commercially available cAMP determination kit from Enzo (Lausen, Switzerland) was used. The kit relies on a standard competitive ELISA strategy: cAMP from sample and cAMP conjugated to alkaline phosphatase that is supplied with the kit compete for binding to the same antibody the reaction plate is covered with. Unbound cAMP is washed away and the amount of bound conjugated cAMP can be quantified in a colorimetric reaction using a pnp (p-nitrophenyl phosphate)-substrate that is converted by the alkaline phosphatase. The conversion can be measured photometrically and is indirect proportional to the amount of cAMP in the

sample. Standard solutions of cAMP with known concentrations were used to generate a standard curve to allow quantification of the results.

Solutions and buffers

HUVEC culture medium

All other reagents of the experiments are included in the kit.

Experimental procedure

3 x 10^5 HUVECtert were cultured overnight in *HUVEC culture medium* in 12-well plate format. Half an hour before stimulation with TNFα (10 ng/ml) cells were pre-incubated in quadruplicates with either solvent vehicle, 5 µM plumericin or 20 µM forskolin as positive control. Two wells of each quadruplicates were stimulated with TNFα. Ten minutes after stimulation of half the plate with TNFα all liquid from the wells was drained and the cells lysed by treatment with 300 µl 0.1 M HCl for 10 minutes. The suspensions were transferred into microcentrifuge tubes and centrifuged in a table top centrifuge for 10 minutes at top speed. The supernatant was collected and frozen at -80 °C until detection of cAMP levels following the manufacturer's protocol for the optional acetylated assay format:

Briefly, after all reagents were completed, cAMP-standards were prepared by serial dilution, samples and standards were acetylated and all reagents combined as instructed by the manufacturer. The samples were incubated for 2 hours, then worked up according to the manufacturer's protocol and absorbance was measured at 450 nm. Results are shown as pmol cAMP per 1 x 10^6 cells.

Important note: The data of experiments for quantification of cAMP levels shown in this thesis were performed in kind cooperation with team member Dr. Atanas Atanasov.

3.13 Lanthascreening (LanthaScreen® TR-FRET PPAR gamma Competitive Binding Assay Kit)

Background information

Lanthascreening relies on FRET (fluorescence resonance energy transfer), a term used to describe the observable exchange of energy between an activated and an inactivated chromophore with overlapping emission/excitation wavelengths in close proximity. The energy transfer does not rely on photons, but on dipol-dipol interactions and can therefore not be measured directly, but the effect is easily observable on the spectral levels of both donor and acceptor. FRET is dependent on the inverse of the sixth power of the distance separating the two chromophores and observable effects are therefore limited to distances between 1-10 nm. This is reported to yield a far better resolution than light microscopy where resolution is limited to approx. 200 nm (Sun et al., 2011). As in biomedical research such close proximity of two molecules is usually interpreted as interaction, FRET is ideally suitable to measure interactions of labeled molecules (Padilla-Parra and Tramier, 2012).

FRET was discovered in the 1940s by the German physical chemist Theodor Förster and has become an increasingly attractive tool in biomedical science over the decades. The number of publications using a FRET approach has grown exponentially over the past 40 years (Sun et al., 2011). This is on the one hand because of the developments in synthetic chemistry that increasingly allow to produce and label molecules at more and more competitive prices, as well as due to instrumentation improvements and innovations that make FRET measurements much more sensitive and convenient (Clegg, 2006). For a long time one of the main drawbacks using FRET were the many auto-fluorescent biomolecules being present in most biological samples that interfered with measurement. This problem has in the meantime been overcome by the introduction of TR (time resolved)-FRET, where chromophore-donors with an increased half-life are used. Their usage creates a delay between excitation of the donor and data acquisition to eliminate short-lived auto-fluorescent noise. (Thibon and Pierre, 2009) Over the years different commercial systems have been established, the one used in during this project (from Invitrogen, Vienna, Austria) relies on the interaction between the lanthanide terbium and a proprietary fluorescent small-molecule pan-PPAR ligand called Fluormone™ Pan-PPAR Green. The terbium is linked to an anti-GST antibody that can bind human PPARγ LBD tagged with GST. Test-compounds binding to the human PPARγ

LBD are competing with the fluorescently labeled ligand for binding, which results in a decrease of the FRET signal that is proportional to the binding of the test substance. The signal obtained at 520 nm is normalized to the signal obtained from the terbium emission at 495 nm, in line the decrease in the 520 nm/495 nm ratio can used as a measure for the ability of the tested compounds to bind to the human PPARγ LBD.

Routine application of this assay in an academic setup to screen larger quantities of compounds is hampered by the comparatively high price of the kit, the inconvenient handling of sub-microliter volumes in the 384-well plate as well as the impaired stability of reagents. However, in combination with the cell-based *in vitro* luciferase reporter gene assay this non-cell based assay is valuable to establish a direct correlation between the transactivational activity observed in the luciferase reporter gene assay and the ability to bind PPAR.

Solutions and buffers

All reagents except DMSO were included it in the kit.

Experimental procedure

Lanthascreen time-resolved fluorescence resonance energy transfer (TR-FRET) competitive binding assays were done following the manufacturer's protocol:

Briefly, first all necessary reagents were completed and test compounds as well as a positive control were diluted in assay buffer in a logarithmic range of concentrations. DMSO levels were kept constant for all samples and also a negative control (only DMSO) was tested. All samples and reagents were added in duplets (one treatment condition for two wells) to the 384-well plate according to the manufacturer's instructions and the plate was then incubated at room temperature for 4 hours covered in aluminum foil. The TECAN multiplate reader was used to determine the emission wavelengths of 495 nm and 520 nm (excitation wavelength 340 nm). Results are shown in fold induction values relative to the negative control (DMSO vehicle treatment).

3.14 Statistical evaluation

All statistical evaluations presented in the graphs of this thesis were done using Graph Pad Prism software (GraphPad Software Inc., La Jolla, California, USA). Employed type of statistical comparison is indicated in the figure legends. Generally P-values below 0.05 were considered as significant. Non-linear regression (sigmoidal dose response) was used to calculate EC_{50}, IC_{50} and E_{max} values. EC_{50} and IC_{50} values are used to describe the concentrations at which 50 % of maximal activation or inhibition is achieved, while E_{max} values are used to describe maximal inducible luciferase activity in fold induction over solvent vehicle treatment.

The error bars in the bar graphs always either represent SD (standard deviation) or SEM (standard error of the mean), as it is indicated in the respective figure legends. Both SD and SEM are commonly used statistical parameters in bioscience to describe sample distribution. While SD is calculated as the square root of variance (variance is a statistical term to describe the averaged squared deviation of the single results from the arithmetic mean), SEM is calculated by further dividing SD through the square root of sample size. In line error bars showing SEM are smaller than those showing SD.

4. Results and discussion

Important notes: Since academic publications are one of the few powerful methods at a scholar's disposal to demonstrate academic talent to peers (Rawat and Meena, 2014), the following presentation and discussion of results is focusing on data that have been published or will be published in foreseeable future. This is primarily done to keep this doctoral thesis concise and to avoid an unnecessary and undesired lengthiness. The author of this thesis was also involved in some projects that do not meet the title of this doctoral thesis or did not culminate into publication in a scientific journal, data of which are therefore omitted. Unfortunately, also a full scope of all published experiments and results gained within the DNTI-consortium and their appropriate discussion would rather go beyond the scope of a doctoral thesis. Therefore presentation of results is limited to those where the author actively contributed and is generally kept concise. As far as the results of experiments where the author of this thesis did not contribute are necessary to provide context, they are also described, but not shown. The interested reader is advised to study the original publications for a full scope of conducted experiments and conclusions made that are generally more detailed than the fragments of those presented here.

All presented graphs (except those for the mechanistical studies with eurycomalactone which have not yet been published) are directly adopted from the respective publications, only the figure legends were modified. This adoption and the following magnification of the figures has sometimes resulted in sub-optimal quality, a shortcoming that the reader is asked to kindly excuse.

Since most of the publications are unconnected to each other and stand for their own, a continuous numbering system of the individual compounds has not been used. Instead compound numbering starts with "1" for every publication.

The provided discussion of results does not always follow the narrative of the respective publications and tries to avoid conclusions that might sometimes appear as overly optimistic as a result of a "bias by pressure"-situation in contemporary (academic) science (Mullane et al., 2014)*.

The structures of all relevant compounds are provided in the Appendix chapter of this work.

* This reference is uttermost excellent and highly recommended for a read.

4.1 Polyacetylenes from *Notopterygium incisum* as partial PPARγ agonists – published in *PLoS One* (Atanasov et al., 2013a)

and

Polyyne hybrid compounds from *Notopterygium incisum* with peroxisome proliferator-activated receptor gamma agonistic effects - published in *Journal of Natural Products* (Liu et al., 2014)

Introduction

Notopterygium incisum is a perennial plant belonging to the family of Apiaceae and the roots and rhizomes of this plant are among the most popular and widely applied traditional Tibetan folk herbal medicines. The plant is listed in the Chinese Pharmacopoeia together with the closely related *Notopterygium forbesii* under the same name Qianghuo (羌活). (Jiang et al., 2007)

In Chinese traditional medicine the herb is used to *"release exterior and disperse cold, expel wind-damp and alleviate pain"* and sustained commercialization is endangering the plant in its natural habitats (Sun et al., 2009). Pharmacognostical studies report main bioactive constituents as coumarins, phenoloids and essential oils (Liu et al., 2009). *Notopterygium incisum* is a rich source of bioactive molecules and with approx. 1000 scientific documents to be found using the plant's name as keyword in Google Scholar also relatively well investigated.

Experimental (Polyacetylenes)

Crude extracts of the rhizomes and roots of the plant were prepared by the phytochemical collaboration group in Graz and sent to the author's group where modest transactivational activity towards PPARγ was determined in luciferase reporter gene experiments at a concentration of 10 µg/ml (Fig. 1).

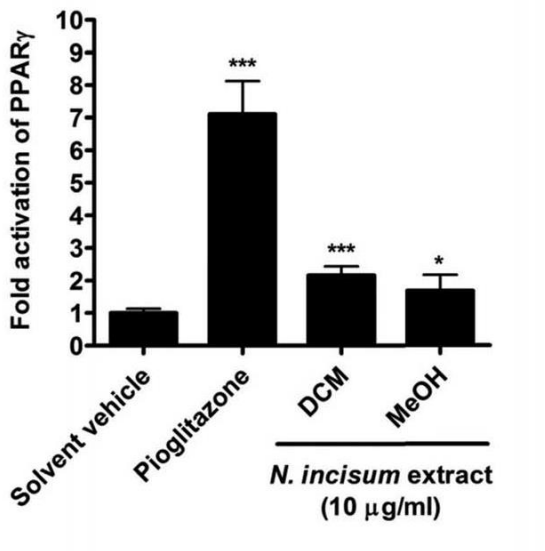
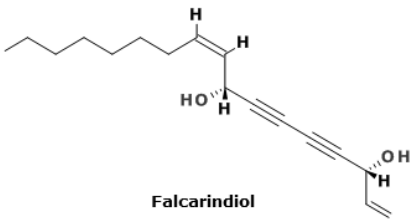


Fig. 1: PPARγ activation by N. incisum extracts *– A DCM (dichloromethane) and a methanolic extract of rhizomes and roots were tested in the luciferase reporter gene assay with PPARγ for transactivational activity as described in the "Material and Methods" section of this work. Results are expressed as fold induction compared to the solvent vehicle control. The data shown are means + SD of three independent experiments each performed in quadruplet. ***p <0.001, *p <0.05 (ANOVA/Bonferroni)*

In turn the DCM extract was used by the phytochemical collaboration group for activity guided fractionation to yield six active polyacetylenes, which were again sent to the author's group and tested in luciferase reporter gene experiments for their affinity towards all three subtypes of PPAR in dose-response experiments (six concentrations between 0.1 – 30 µm) to yield EC_{50} and E_{max} values for the individual polyacetylenes (Table 1).

From the six identified bioactive compounds falcarindiol was chosen for further characterization. Additional experiments performed with falcarindiol included luciferase reporter gene experiments where cells were co-treated with 10 µM falcarindiol and 1 µM of the PPARγ antagonist T0070907 to verify the PPARγ dependency of the observed effect (Fig. 2A). Also Lanthascreen experiments with logarithmic concentrations of falcarindiol in comparison to pioglitazone were conducted to study the binding affinity of falcarindiol to PPARγ in cell-free environment (Fig. 2B). In addition luciferase reporter gene experiments where cells were co-treated with varying concentrations of falcarindiol and



Falcarindiol

1 µM pioglitazone were performed. This was done to show that the two compounds compete for the same binding site (Fig. 2C).

	PPARγ activation		PPARα activation		PPARβ/δ activation	
Compound	EC_{50} (µM)	E_{max}*	EC_{50} (µM)	E_{max}*	EC_{50} (µM)	E_{max}*
Pioglitazone	*0.41*	*9.28*	-	-	-	-
GW7647	-	-	*0.0021*	*3.08*	-	-
GW0742	-	-	-	-	*0.0017*	*20.2*
8-Acetoxyfalcarindiol	3.59	2.36	n.d.	n.d.	n.d.	n.d.
Falcarindiol	**3.29**	**3.26**	**n.d.**	**n.d.**	**n.d.**	**n.d.**
9-Epoxy-falcarindiol	2.03	1.88	n.d.	n.d.	n.d.	n.d.
Crithmumdiol	4.58	2.29	n.d.	n.d.	n.d.	n.d.
9-Heptadecene-4,6-diyne-1-ol	11.31	1.92	n.d.	n.d.	n.d.	n.d.
2*Z*,9*Z*-Heptadecadiene-4,6-diyne-1-ol	4.18	1.73	n.d.	n.d.	n.d.	n.d.

Table 1: Transactivational activity of the isolated polyacetylenes towards the three subtypes of human PPAR (α, β/δ and γ) in the luciferase reporter gene assay - *n.d. not detected up to 30 µM; Luciferase activity is expressed as fold induction of the vehicle control after normalization to the EGFP-derived fluorescence. To verify the specificity of the performed assays, GW7647, GW0742 and pioglitazone were used as selective agonist for PPARα, β/δ and γ, respectively. The data shown are means of three independent experiments performed in triplicate.*

* E_{max} values were only determined up to a test concentration of 30 µM

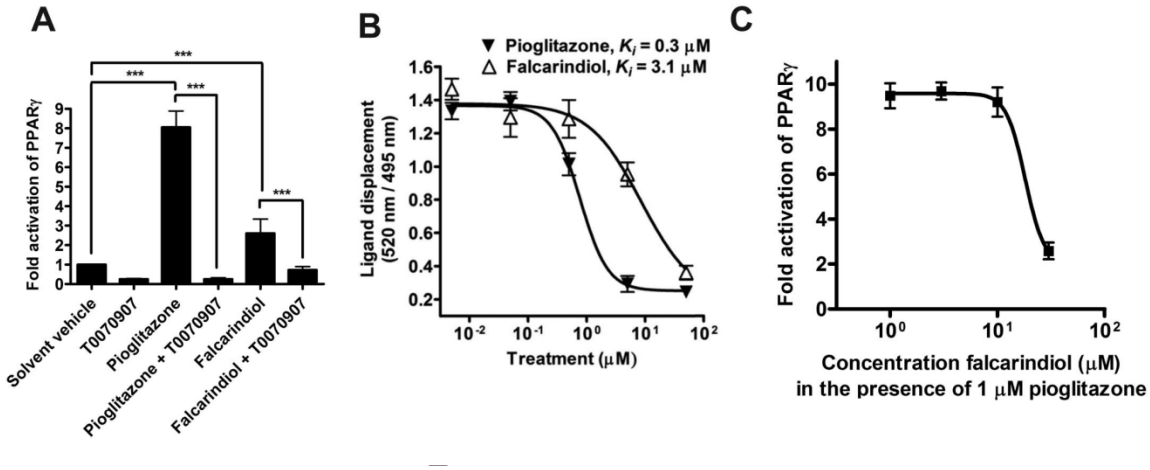


Fig. 2: Characterization experiments of falcarindiol as a partial PPARγ agonist – *(A) Co-treatment of transfected HEK293-cells with falcarindiol and the PPARγ antagonist T00770907 blunts falcarindiol-induced luciferase activity in the luciferase reporter gene assay. Experiments were performed as described in the "Material and Methods" section of this work. The data shown are means + SD of six independent experiments each performed in quadruplet. ***p <0.001 (ANOVA/Bonferroni) (B) Ligand displacement of falcarindiol and pioglitazone in Lanthascreen experiments. Experiments were performed as described in the "Material and Methods" section of this work. Displacement ability of falcarindiol is several folds lower than that of pioglitazone. Each data point represents the mean ± SEM from four independent experiments performed in duplicate (C) Co-treatment experiments with four different concentrations of falcarindiol in presence of 1 μM pioglitazone in the luciferase reporter gene assay in HEK293 cells. Experiments were performed as described in the "Material and Methods" section of this work The data shown are means ± SD of three independent experiments each performed in quadruplet.*

To further study the effect of falcarindiol in a functional cell model with endogenous PPARγ levels, glucose uptake experiments with 10 μM falcarindiol in comparison to the

prototypical PPARγ agonist pioglitazone (5 µM) were performed in differentiated 3T3-L1-cells (Fig. 3).

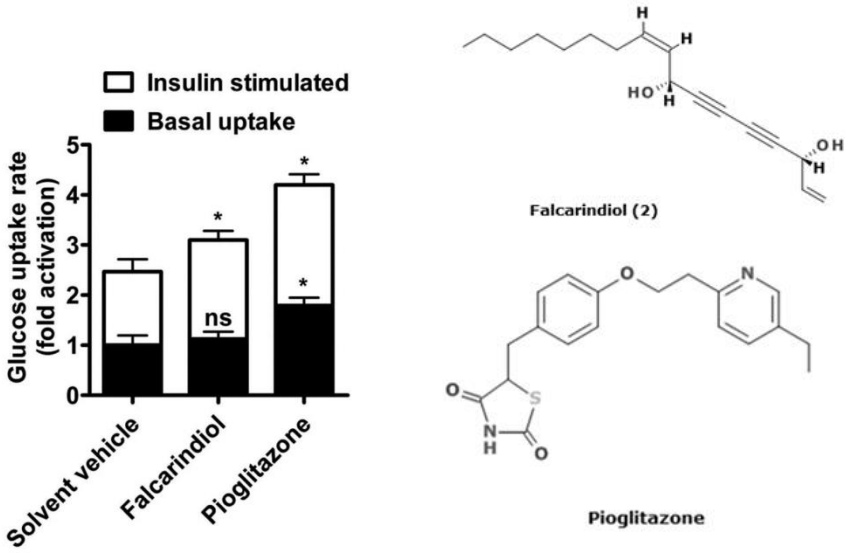


Fig. 3: Assessment of glucose uptake enhancing properties of falcarindiol – *Glucose uptake with or without insulin stimulus of differentiated 3T3-L1 adipocytes treated with solvent vehicle, falcarindiol (10µM) or pioglitazone (5 µM) as described in the "Material and Methods" section of this work. Results are shown relative to glucose uptake of cells treated with vehicle control without insulin stimulation. The data shown are means + SEM of five independent experiments. *p <0.05 (two-tailed paired t-test). Glucose uptake experiments were done in kind cooperation with former PhD student and collague Dr. Matthias Kramer.*

Discussion (Polyacetylenes)

The multitude of performed experiments clearly identify falcarindiol as partial PPARγ agonist with a maximal activation potential several folds lower than the activation induced by the full PPARγ agonist pioglitazone. The suggested partial agonism was verified by the outcome of the co-treatment experiments with varying concentrations of falcarindiol and 1 µM pioglitazone (Fig. 2C), since the partial agonist falcarindiol can be considered as an antagonist in presence of pioglitazone. A direct correlation between treatment with falcarindiol and the observed PPAR agonism could further be supported by the outcome of the Lanthascreen experiments, where falcarindiol was shown to be able to bind PPARγ in an experimental setup that does not rely on a cellular system (Fig. 2B). This result is able to exclude the possibility that metabolites of falcarindiol or indirect effects are solely responsible for the observed PPARγ-agonistic effects in the used cell

models, although such effects might generally contribute to the test results yielded in cellular systems. Partial agonists of PPARγ are considered in literature as interesting compounds, since they possibly retain the needed pharmaceutical effectiveness as antidiabetic drugs, while showing reduced side effects (Agrawal et al., 2012). Falcarindiol was chosen for further characterization, because it was able to induce strongest PPARγ activation among the six compounds, but also because it was among the most abundant polyacetylenes to be found in *Notopterygium incisum* by our collaboration partners. Apart from that falcarindiol is one of the best investigated polyacetylenes in general, also found in many other members of the Apiaceae-family (e.g. carrots, dill or parsley) (Christensen and Brandt, 2006). However, prior to this work the ability of polyacetylenes or in particular falcarindiol to induce subtype-selective activation of PPARγ was not reported in literature. Interestingly, falcarindiol was also able to increase glucose uptake in insulin-stimulated differentiated 3T3-L1-cells.

In conclusion the identification of PPARγ agonists in *Notopterygium incisum* could contribute to the rational explanation why the plant extract is used as a remedy in traditional Tibetan and Chinese medicine, although it is always difficult to conclude from the effects of isolated compounds to the effect of a complex matrix such as plant material. Accordingly it is even more difficult to conclude from the effects of isolated compounds in relatively simple *in vitro* experiments to the effects of formulations used in highly complex organisms.

In future with possibly more promising *in vitro* data it would also be necessary to consider animal testings as part of the drug development process. Unfortunately reproducibility of this study is impaired, because it was omitted to report which mobile phase was used in the HPLC-experiments for isolation.

Experimental (Polyyne hybrid compounds)

The phytochemical collaboration group in Graz used the DCM extract to yield another 11 poyyne derivatives, namely 8 notoethers (1 - 8) and three notoincisols (9 -11) (the former consist of four pairs of isomeric ethers, each composed of a falcarindiol unit and a sesquiterpene unit while the latter are adducts of a polyacetylene and a

phenylpropanoid unit). The isolated compounds were sent to the author's group, dissolved in DMSO, aliquoted and eventually tested for their ability to induce PPARγ activation in the luciferase reporter gene assay at a concentration of 10 µM (Fig. 4).

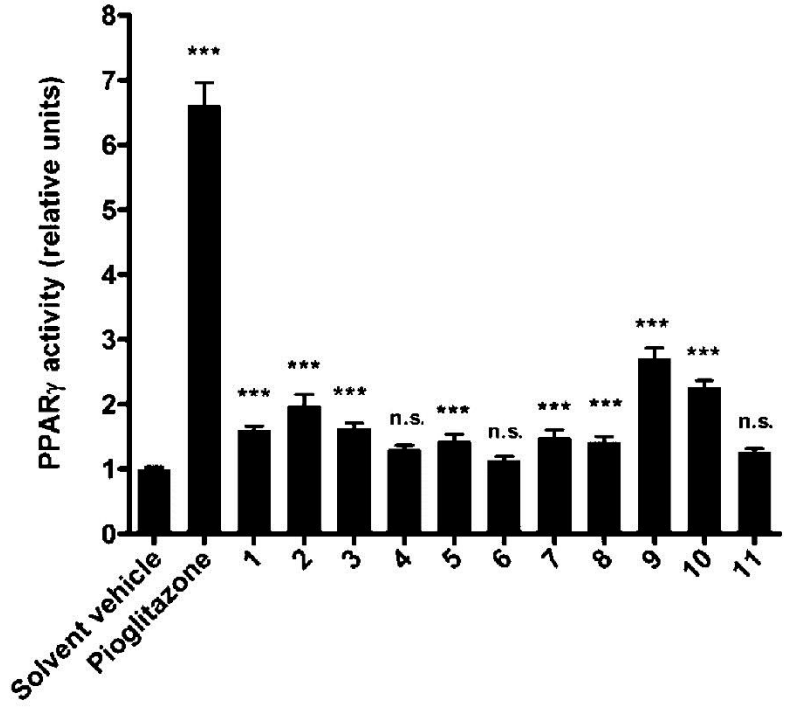


Fig. 4: PPARγ agonistic effects of polyacetylene adducts from N. incisum *– Several of the polyyne hybrid compounds could induce PPARγ activation in the luciferase reporter gene assay. Experiments were performed as described in the "Material and Methods" section of this work .The data shown are the means + SEM of three independent experiments, each performed in quadruplet. ***p ≤ 0.001, n.s.: not significant (ANOVA/Dunnett)*

Several of the compounds could significantly activate PPARγ and for the five most active compounds EC_{50} and E_{max} values were determined in dose-response experiments (Table 2).

Compound	EC_{50} (µM)	E_{max} (maximum fold luciferase induction over solvent vehicle)
Pioglitazone	0.2	8.8
1	1.9	1.6
2	1.7	2.0
3	2.0	1.6

9	2.3	2.8
10	1.7	2.3

Table 2: Derived EC_{50} and E_{max} values for the five most active isolated polyacetylene adducts from N. incisum. The structures of the compounds can be found in the Appendix chapter of this work.

Discussion (Polyyne hybrid compounds)

Eight out of the eleven test compounds showed a statistically significant activation of PPARγ applied in a concentration of 30 µM. The subsequent dose-response experiments for the five most active compounds yielded EC_{50} values between 1.7 and 2.3 µM and maximal fold activation of PPARγ below 3-fold compared to the activity of the solvent vehicle. Comparing these values to the effect of the full agonist that was always tested in comparison (in these experiments pioglitazone showed and EC_{50} value of 0.2 µM and an E_{max} value of 8.8) suggests partial agonism for the compounds. As already mentioned partial PPARγ agonism is supposedly a beneficial feature for a compound, since the clinically applied PPARγ full agonists have serious side effects that could possibly be alleviated by submaximal PPARγ activation. Further experiments to characterize the observed PPARγ agonism for those compounds could include experiments in more physiological relevant cell systems and/or experiments with a setup that does not require the usage of cells. Further interesting parameters which could also be interesting to obtain in an early phase of drug discovery include assessment of hERG activity and inhibition of P450 cytochrome (Ritchie and Macdonald, 2009).

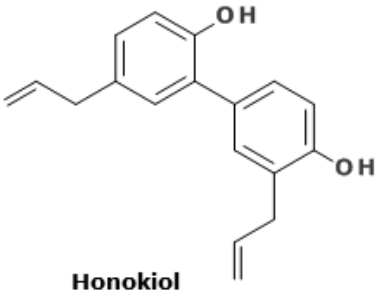
4.2 Honokiol: A non-adipogenic PPARγ agonist from nature –

published in *Biochimica et Biophysica Acta* (Atanasov et al., 2013b)

Introduction

Honokiol is a neolignan that can be isolated from a variety of plants belonging to the genus Magnolia (Fried and Arbiser, 2009). Neolignans and lignans are a large group of natural products derived from a coupling reaction of two C_6C_3 (propylbenzol) molecules. Biochemically most (neo)lignans are products of cinnamic acid derivatives and related to phenylalanine metabolism. The difference between lignans and neolignans is the type of coupling between the two C_6C_3 molecules: While a linkage of the two molecules via a β,β'-bond* yields lignans, any other linkage forms neolignans (Moss, 2000).

In the first funding period of the DNTI-consortium several neolignans were identified with a computer-aided approach as novel partial agonists occupying the PPARγ ligand-binding domain as dimers (Fakhrudin et al., 2010). In this study the generated and further optimized *in silico* model enabled us to identify and further characterize the neolignan honokiol, a major bioactive constituent of the traditional Chinese herbal drug Magnolia bark, as a novel non-adipogenic partial PPARγ activator.



Honokiol

The biphenyl-type neolignan honokiol is highly bioactive with numerous effects described in biomedical literature, including antineoplastic activity (Arora et al., 2012), inhibition of NF-κB activation (Zhang et al., 2013) as well as impressive effects on the central nervous system (Sulakhiya et al., 2014) and pronounced antioxidant activity (Ogata et al., 1997).

Possible limitations of honokiol's usage for therapeutic purposes are a poor solubility in aqueous solutions, as well as a predicted low oral bioavailability observed in *in vitro* experiments (Böhmdorfer et al., 2011). The impaired bioavailability is also suggested by the biphenylic structure of the compound as (bi)phenyls usually undergo first-pass metabolism resulting from glucuronidation of the hydroxyl groups. However, biphenyl

* β and β’ refer to the propyl(en) side chains with the terminal carbons being α and α’

neolignans are nevertheless regarded to possibly provide new scaffolds for pharmaceutical drug design, considering their pronounced binding affinity to proteins and the fact that around 4 % of all known drugs have this substructure present* (Taferner et al., 2011).

Interesting and in support of the (predicted) low bioavailability is that in many animal studies when honokiol is administered through a parenteral route a dosage as little as 0.2 mg/kg shows effects (Kuribara et al., 1998), while for oral administration dosages up to 100 mg/kg are often used (e.g. Singh et al., 2013).

Experimental

The virtually predicted PPARγ agonist honokiol was isolated from the powdered bark of *Magnolia officinalis* with a complex array of chromatographic techniques by the collaborating phytochemical group from the University of Innsbruck and forwarded to the author's group for testing in the luciferase reporter gene assay for PPARγ. The observed activity of honokiol towards PPARγ was verified in cell-free environment in Lanthascreen experiments in a logarithmic range of concentrations in comparison to the full PPARγ agonist (Fig. 5A) and honokiol was also tested in dose-response experiments to determine EC_{50} and E_{max} values in comparison to the full agonist pioglitazone (Fig. 5B).

* Although the source omits to report whether those 4 % of all drugs were designed for peroral application

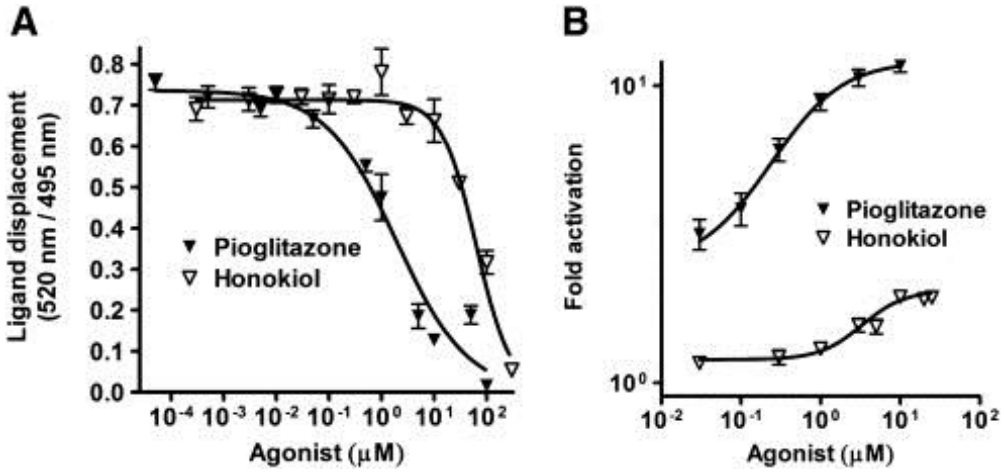


Fig. 5: Honokiol is a PPARγ agonist – *(A) Binding affinity of honokiol to PPARγ was tested in Lanthascreen experiments as described in the "Material and Methods" section of this work. Each data point represents the mean ± SEM from three independent experiments performed in duplicate. (B) Induction of luciferase activity determined in luciferase reporter gene assays for PPARγ over a range of concentrations for honokiol in comparison to pioglitazone. Experiments were performed as described in the "Material and Methods" section of this work. The data shown are means ± SEM of three independent experiments each performed in quadruplicate.*

In additional characterization experiments low micromolar concentrations of honokiol were found out to stimulate basal glucose uptake in 3T3-L1 adipocytes in glucose uptake experiments. To test the adipogenic potential of honokiol MEF cells and matured 3T3-L1 adipocytes were treated with several concentrations in low micromolar range of honokiol in comparison to pioglitazone. Since treatment with honokiol did not lead to a markedly enhanced formation of intracellular lipid droplets above basal levels these promising findings were also challenged in an *in vivo* model. In these experiments with diabetic KKAy mice conducted by an external collaboration group from the China Academy of Chinese Medical Sciences (Beijing, PRC) honokiol (100 mg/kg) was administered with a gavage for 35 days in comparison to pioglitazone (10 mg/kg). Evaluated parameters for the animal experiments were blood glucose levels, body weight of diabetic KKAy mice, as well as glucose tolerance and blood insulin levels.

Discussion

In this study we report for the first time the biphenyl type neolignan honokiol as a subtype-selective partial PPARγ agonist. The classification of honokiol as a partial PPARγ

agonist is based on the results of the cell-based luciferase reporter gene assay and the subsequent Lanthascreen experiments in cell free environment, where honokiol could significantly induce luciferase expression (EC_{50} value of 3.9 µM and luciferase activity below 2-fold for all tested concentrations) and displacement of fluorescently labeled ligand, respectively, but several fold lower than the full agonist pioglitazone (EC_{50} value of 0.3 µM and maximal fold induction above 10-fold over solvent vehicle at 5 µM).

Furthermore honokiol was found out not do trigger adipogenesis in two *in vitro* cell systems and to prevent weight gain in the murine KKAy *in vivo* diabetes model, while retaining anti-hyperglycemic activity *in vitro* and *in vivo*.

In the *in vivo* experiments a dosage of 100 mg/kg/day was used. This dosage is equivalent to a theoretical dosage of 2.6 gram honokiol/day for a human with 70 kg[*]. Although this quantity exceeds the dosage for any currently clinically applied drug it can still be considered as reasonable for a hit/lead compound, as among other parameters improvement of pharmacokinetics is one of the goals to achieve in the lead optimization stage of drug discovery. A rather obvious approach to improve bioavailability could be allosteric replacement of the biphenyl-moieties or masking of the hydroxyl groups. However, the predicted low bioavailability of honokiol in particular (or of compounds with phenolic structures in general (Thomsen and Bundgaard, 1993)) was also observed in the present study, considering the discrepancy between the observed effects of similar concentrations of honokiol and pioglitazone in the applied *in vitro* assays and the necessity to vastly increase honokiol dosage in comparison to pioglitazone in the *in vivo* situation.

In conclusion we show here the natural product honokiol for the first time as subtype-selective partial PPARγ agonist that has also been effective in suppression of hyperglycemia and weight gain in *in vivo* experiments. In continuative *in vivo* experiments it might be advantageous to tackle the low bioavailability of honokiol.

[*]70 kg and 100 mg/kg/day yields 7000 mg, which is divided by three for dose translation from mouse to human (Reagan-Shaw et al., 2008)

4.3 Imbricaric Acid and Perlatolic Acid: Multi-targeting Anti-Inflammatory Depsides from *Cetrelia monachorum* (Zahlbr.) - published in *PloS One* (Oettl et al., 2013)

Introduction

Cetrelia monachorum is a lichen, which are not separate life forms but symbiosis-alike communities between fungi and alga or cyanobacteria. The fungi typically belong to the phylum of ascomycetes, while the symbiotic partners show a high diversity and cannot easily be classified, but are generally phototropic. It is believed that lichenisation occurred several times independently during evolution, rather than an initial lichenisation event that was inherited and has diversified (Gargas et al., 1995).

Lichens are believed to have been the first colonizers of terrestrial habitats on this planet, where they could have contributed to the formation of soil which is a prerequisite for morphologically more complex organisms to persist (Taylor et al., 1995). Some lichen are able to adapt to extremely hostile environments, possibly enabling them to survive in outer space or even on Mars' surface (de Vera et al., 2004).

Lichens have a long tradition to be used by humans, application includes the usage as nutritional source (Dayan and Romagni, 2001), the usage as remedy (Boustie et al., 2010) as well as psychotropic drug (Schmull et al., 2014) or poison (Stephenson and Rundel, 1979), the usage as dye (Taylor, 1986) as well as an ingredient for the cosmetic industry (Huneck, 1999).

From a pharmacological point of view lichen are as important as the organisms they are composed of: Ascomycetes are *inter alia* highly appreciated as source for novel antimicrobials (Korzybski et al., 2013), and algae and cyanobacteria comprise a huge number of species that have been found out to contain a multitude of pharmaceutically interesting (secondary) metabolites (Tan, 2007; Patterson et al. 1994).

Opposite to many other lichen species that have specialized to live in certain ecological niches and have declined in anthropogenically influenced habitats (Singh et al., 2012), *Cetrelia monachorum* seems to be rather common and is therefore not listed on any of Austria's red lists of endangered lichen. However, research of *Cetrelia monachorum* is still at its infancy with less than 100 scientific documents obtainable in a Google Scholar search using the plant's name as keyword (both with and without quotation marks).

This study reports the *in vitro* screening of 17 Alpine lichen species for their inhibitory activity against 5-lipoxygenase, microsomal prostaglandin E2 synthase-1 and NF-κB.

Experimental

Ethanolic crude extracts of air-dried ground thalli were prepared by the collaborating phytochemical group in Innsbruck and sent to the authors group. There the extracts were dissolved in DMSO and tested at a concentration of 10 µg/ml in the luciferase reporter gene assay for inhibition of NF-κB activation with GFP (Fig. 6).

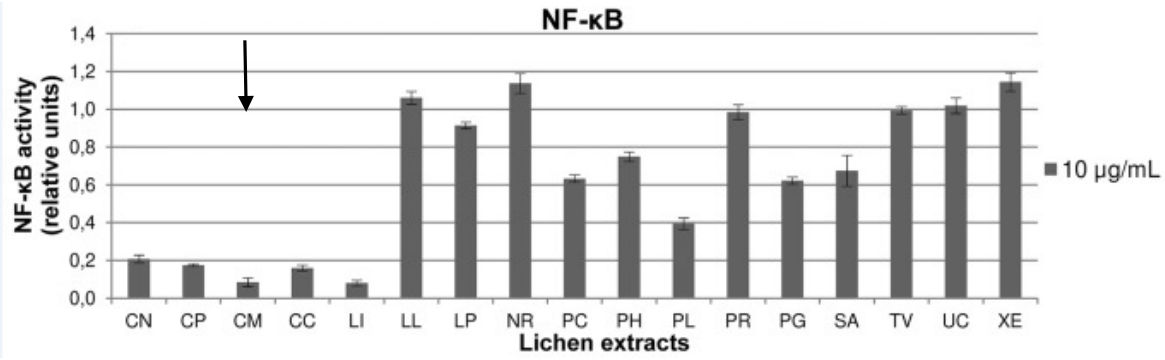


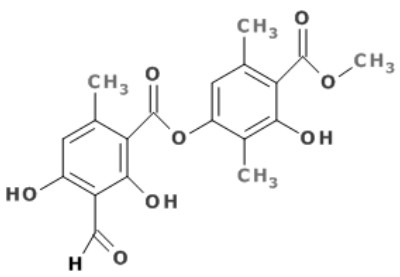
Fig. 6: Effect of ethanolic crude extracts from various lichen species on inhibition of NF-κB activation in the luciferase reporter gene assay – *Samples are abbreviated with the first letters of genus and epithet, full names of the species can be found in the corresponding publications and are omitted here due to a lack of space. The extract of interest for the following studies is indicated by an arrow and originates from Cetrelia monachorum. Results shown are the mean from three independent experiments each performed in quadruplicate. No statistical evaluation was performed. Experiments were performed as described in the "Material and Methods" section of this work. Parthenolide was always tested in comparison as positive control along with other control-conditions to verify functionality of the performed experiments (data not shown).*

Since the EtOH extract from *Cetrelia monachorum* showed best activity not only in the NF-κB assay (on this target IC_{50} concentration was determined as 2.6 µg/ml in subsequent dose-response experiments in our test system – data not shown), but also in the other *in vitro* screening systems, it was chosen for further analysis. The subsequent phytochemical isolation conducted by our collaboration partners in Innsbruck yielded three depsides and eight monoaromatic derivatives, some of which were reported for the first time to be isolated from this lichen species. The three depsides were

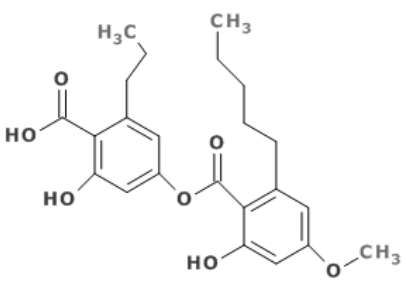
subsequently tested in dose-response experiments to determine IC_{50} values in the luciferase reporter gene assay for NF-κB with EGFP (Table 3). Finally the second most active compound in the NF-κB assay among the three, perlatonic acid, was also challenged by one of the DNTI-collaboration partners in an *in vivo* situation in a thioglycollate-induced peritonitis mouse model, where the anti-inflammatory activity could be verified.

Compound	Determined IC_{50} values (μM)
Imbricaric acid	2.0
Perlatolic acid	7.0
Atranorin	20.0

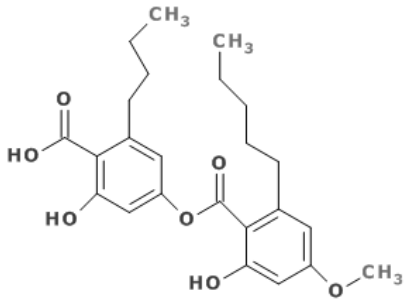
Table 3: The IC_{50} values for inhibition of NF-κB activation of three depsides isolated from the lichen species Cetrelia monachorum determined in the luciferase reporter gene assay



Atranorin



Imbricaric acid



Perlatonic acid

Discussion

The three depsides yielded IC_{50} values of 2, 7 and 20 μM and should therefore contribute to the observed anti-inflammatory effect of the *C. monachorum* extract and can possibly also rationalize the usage of *C. monachorum* in traditional medicine as an anti-inflammatory remedy to some degree. For the two more active compounds, imbricaric

acid and perlatolic acid, our collaboration partners also employed an assay to investigate whether those two could directly inhibit IKK-β, but the outcome of this assay was negative, suggesting that the two compounds have a different molecular target in the TNFα-induced NF-κB activation cascade.

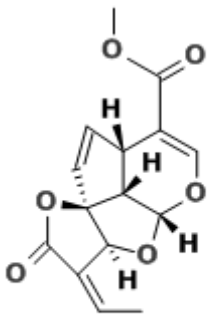
In conclusion this is the first report of ingredients of *Cetrelia monachorum* to be reported as inhibitors of the NF-κB activation cascade and also the first report of depsides interacting with this pathway. The relatively low IC_{50} value of imbricaric acid with 2.0 µM in our test system suggest further studies to continue evaluation of this compound as an interesting anti-inflammatory hit compound.

4.4 Identification of plumericin as a potent new inhibitor of the NF-κB pathway with anti-inflammatory activity *in vitro* and *in vivo*. -

published in *British Journal of Pharmacology* (Fakhrudin et al., 2014)

Introduction

Plumericin is a spirolactone iridoid which can be isolated from the bark of *Himatanthus sucuuba. Himatanthus sucuuba* is a tree belonging to the family of Apocynaceae and can be found in the Amazonian region where *H. sucuuba* is one of the few tree species that can inhabit both floodplains and non-flooded ecosystems (Ferreira et al., 2009). This ability of the tree to colonize both ecosystems is an advantage over those plants that can only colonize the floodplains and are endangered through intense deforestation and overexploitation of the Amazonian rainforest (Wittmann and Wittmann, 2010). In traditional medicine different plant parts are used as wound-healing agents on superficial and internal wounds (Villegas et al., 1997), as vermifuge, against rheumatism and as a depurative (Sanz-Biset et al. 2009), as well as anti-cancer drug and to treat cough (Af et al., 2007). The plant *H. succuba* is not very well researched with less than 700 scientific documents to be found in a Google Scholar query with the plant's name in double quotes as search term.



Plumericin

Plumericin is reported in biomedical literature to exhibit antimicrobial activity (Trost et al., 1997) and to show notably pronounced cytotoxic effects as an anti-cancer agent

(Kuete and Efferth, 2011). However, an anti-inflammatory effect of plumericin as inhibitor of NF-κB activation was not reported prior to this publication.

The phytochemical work of this study that led to the isolation of plumericin and the related compounds was performed by the collaborating research group in Innsbruck and is described in a separate publication (Waltenberger et al., 2011).

Experimental

For this publication most of the (key) experiments were already conducted by former PhD student Nanang Fakhrudin who worked in the first period on the author's position for the DNTI-consortium.

Plumericin was identified by Dr. Fakhrudin as an inhibitor of NF-κB activation via the activity guided approach in the luciferase based test system as well as in HUVECtert cells. His methods were the same (or very similar) as those described in the material and methods part of this work. He has also shown in Western blotting experiments that the molecular target of plumericin is IKK, as he could observe inhibition of IκB phosphorylation and in turn also no degradation of IκB. Our collaboration partners the phytochemists in Innsbruck also performed an assay in cell free environment to verify the inhibitory effect of plumericin on IKK.

In a LDH cytotoxicity assay plumericin was determined to be as intoxic as the solvent vehicle as determined 4h after stimulation with TNFα. To study the effect of plumericin in comparison to the other isolated, structurally similar compounds in the physiologically more relevant HUVECtert cells the induction of the adhesion molecules VCAM-1, ICAM-1 and E-selectin with and without stimulation with TNFα was measured (Fig. 7).

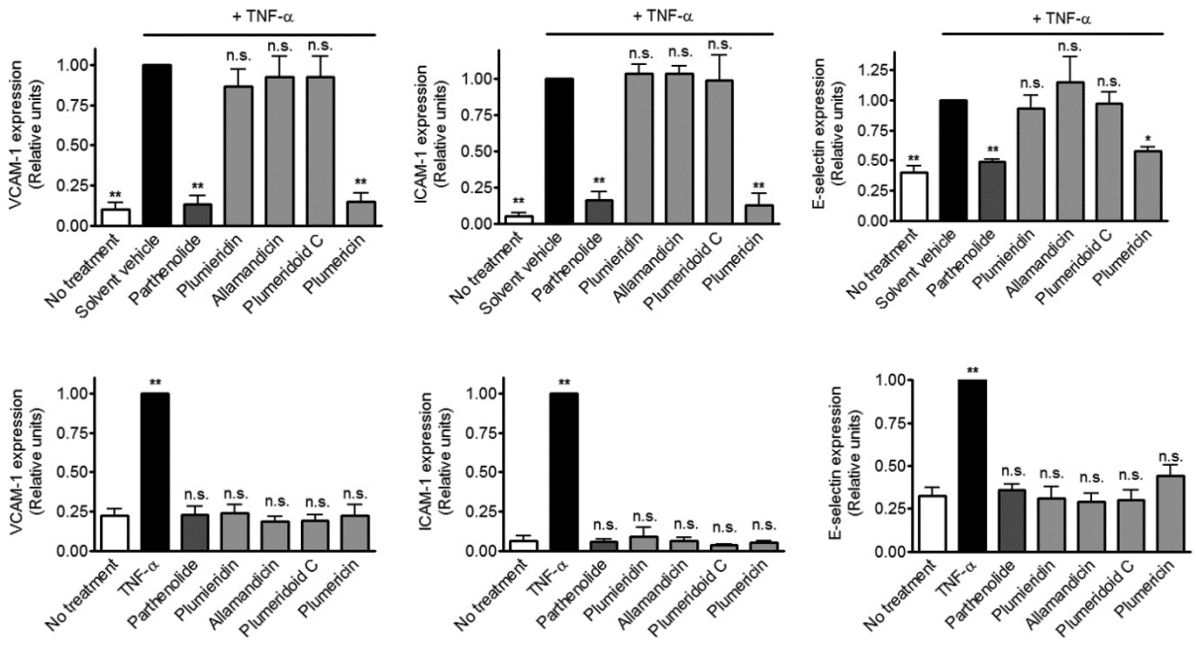


Fig. 7: Plumericin and structurally similar compounds and their ability to inhibit TNFα induced induction of adhesion molecules in HUVECtert (top panel) and their effect on basal adhesion molecule expression (lower panel) - *Experiments were performed as described in the "Material and Methods" section of this work, protein expression levels were determined by flow cytometry. Data shown are mean + SEM from three independent experiments (for measurements of E-selectin: four independent experiments). *p < 0.05; **p <0.01; n.s. not significant (ANOVA/Dunnett)*

To further support the obtained evidence that plumericin is tackling the IKK complex, luciferase reporter gene experiments with transfection of a constitutively active IKK-β (IKK-β CA) were performed (Fig. 8).

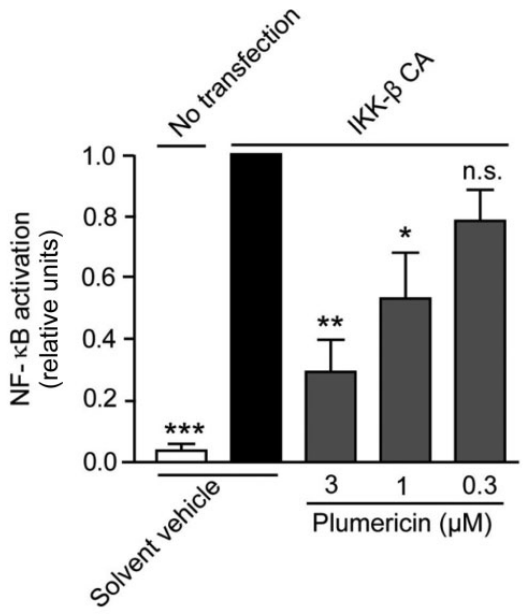


Fig. 8: Luciferase reporter gene assays with HEK293/NF-κB-luc cells cotransfected with IKK-β CA and EGFP and treated with the indicated concentrations of plumericin for 4 hours – *Data shown are means ± SEM from three independent experiments performed in quadruplicate. *p <0.05; **p <0.01; ***p <0.001; n.s. not significant (ANOVA/Dunnett)*

To gain further insights about the effect of plumericin in our HUVECtert test system cAMP levels were determined upon treatment with plumericin (5 µM) in comparison to the solvent vehicle and the positive control forskolin (20 µM) with and without TNFα-stimulation (10 ng/ml) using a commercial kit for determination of cAMP levels purchased from Enzo Life Science, Lausen (Switzerland) (Fig. 9)

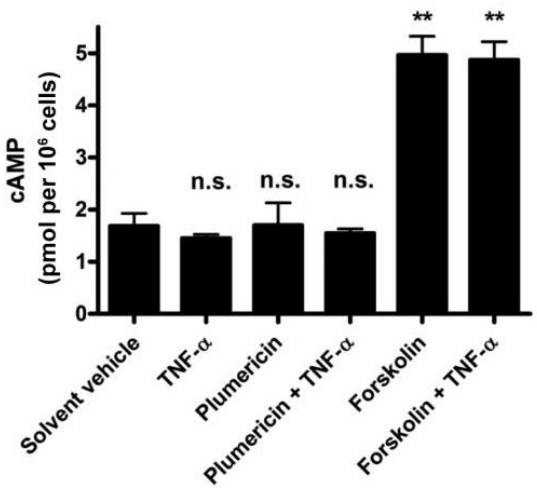
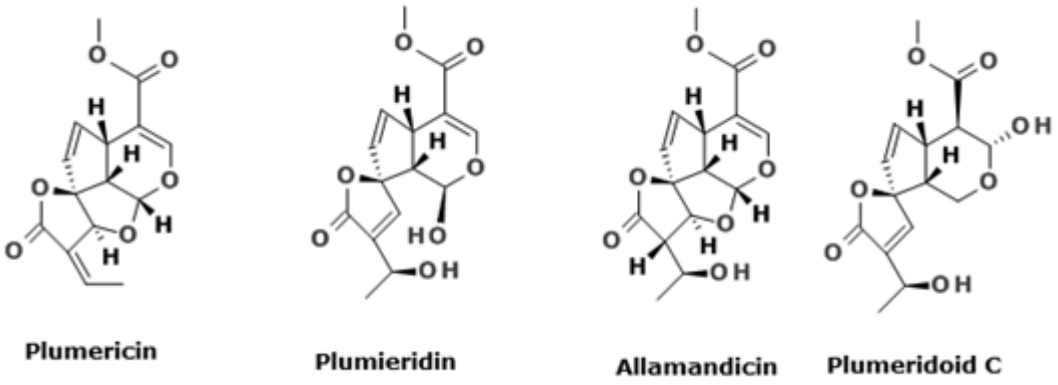


Figure 9: Effect of plumericin on cAMP levels of HUVECtert determined in ELISA assays *– Experiments performed as described in the "Material and Methods" section of this work. The data presented are derived from four independent experiments and are means ± SEM. **p <0.01; n.s. not significant (ANOVA, Dunnett) Experiments to determine cAMP levels were performed in kind cooperation with team member Dr. Atanas Atanasov.*

Finally plumericin was also tested by our pharmacological collaboration partners in Vienna in *in vivo* experiments using a thioglycollate-induced peritonitis mouse model, where the anti-inflammatory activity could be confirmed.



Discussion

Here plumericin could be identified as a potent inhibitor of NF-κB activation in difference to the structural similar compounds plumieridin, allamandicin and plumeridoid C that could not suppress TNFα-induced activation of the NF-κB cascade (Fig. 7). None of the tested compounds showed any induction of the pro-inflammatory endothelial surface proteins VCAM-1, ICAM-1 and E-selectin over basal levels without TNFα stimulation (Fig. 7). The hypothesis that the molecular target of plumericin in the NF-κB signaling cascade is not upstream of IκB-phosphorylation could further by supported by the experiments conducted with a constitutively active IKK-β (IKK-β CA). This IKK-β mutant is reported to activate the NF-κB signaling cascade independently of upstream signaling events, such as stimulation with TNFα (Mercurio et al., 1997). Also in our luciferase reporter gene assay with the HEK-293/ NF-κB-luc cell line transfection with IKK-β CA yielded a strong luciferase signal without TNFα addition. This lucifersase signal could be suppressed dose-dependently by treatment with plumericin (Fig. 8). In parallel control experiments with co-transfections of EGFP with the wild-type form of IKK-β no luciferase-induction was observed in the absence of TNFα, which validates the results obtained for the mutant form of IKK-β. Interestingly, the observed inhibitory activity of plumericin in these experiments yielded a similar potency of plumericin to that observed upon TNFα stimulation, where the IC_{50} value of plumericin was determined to be 1.07 µM in the applied experimental setup.

The experiments conducted to investigate an eventual effect of plumericin treatment on cAMP metabolism yielded no changes in intracellular cAMP levels in the presence or absence of plumericin with or without TNF-α treatment (Fig. 9). This finding suggests to exclude the possibility that the anti-inflammatory effect of plumericin can be connected to cAMP-metabolism.

The performed LDH-assay clearly shows plumericin as intoxic as the solvent vehicle under the employed experimental conditions, which suggests that the observed inhibitory effects on NF-κB activation in the initial screening were not a secondary effect due to cytotoxicity. However, due to insufficient description of experimental details it might be very difficult for third parties to exactly reproduce these experiments.

In conclusion this publication identifies plumericin as a potent inhibitor of the TNFα induced NF-κB signaling cascade with IKK-β as the suggested molecular target. The data, as it is shown in the present publication, might rank this compound among the most interesting natural products that have yet been described as NF-κB pathway blockers.

Surprisingly, the commonly reported cytotoxic effects of plumericin could not be observed in the applied experimental setup, possibly due to the limited exposure time of plumericin to the cells of less than five hours in the LDH-assay.

4.5 Activity-guided isolation of NF-κB inhibitors and PPAR agonists from the root bark of *Lycium chinense* Miller –

published in *Journal of Ethnopharmacology* (Xie et al., 2014)

Introduction

Lycium chinense Miller is a deciduous shrub with a height between one to three meters and belongs to the family of Solanaceae, commonly known as nightshades. The plant is closely related to *Lycium barbarum*, both plants comprise the species of boxthorn and are used to harvest the famous goji berry. (Potterat, 2010) Boxthorn is native all over Eurasia and several plant parts are used in traditional Chinese medicine (Mocan et al., 2014). Special importance has the root bark of *L. chinense* and *L. barbarum* (Cortex Lycii) that finds application as an antipyretic as well as for the treatment of pneumonia, night-sweats, cough, mild forms of hematemesis, inflammation and diabetes (Zhang et al., 2013). A beneficial effect of improving insulin resistance and lipid metabolism upon treatment with Cortex Lycii has also been observed *in vivo* using an obese-diabetic rat model (Ye et al., 2008). In difference to many other members of genus Lycium that are endangered from extinction due to anthropogenic causes, both *L. barbarum and L. chinense* are extensively cultivated due to their commercialization (Wang et al., 2013).

Lycium chinense is relatively well researched with around 1500 retrievable scientific documents listed in a Google Scholar search with the plant's name in double quotes as keyword. This is approx. one tenth of the retrievable documents compared to the much better investigated closely related *L. barbarum.*

Experimental

The phytochemical work of this publication was not done by DNTI-collaboration partners but by department-colleagues of the University of Vienna, who prepared root bark extracts of *L. chinense* with different solvents by a complex series of extraction steps. These extracts were subjected to luciferase assays for activation of PPARγ and inhibition of NF-κB with CMFDA to yield an inhibitory effect on NF-κB activation for the EtOAC and the MeOH extracts (Fig. 10A) and PPARγ activation for the EtOH and the DCM extract. (Fig. 10B).

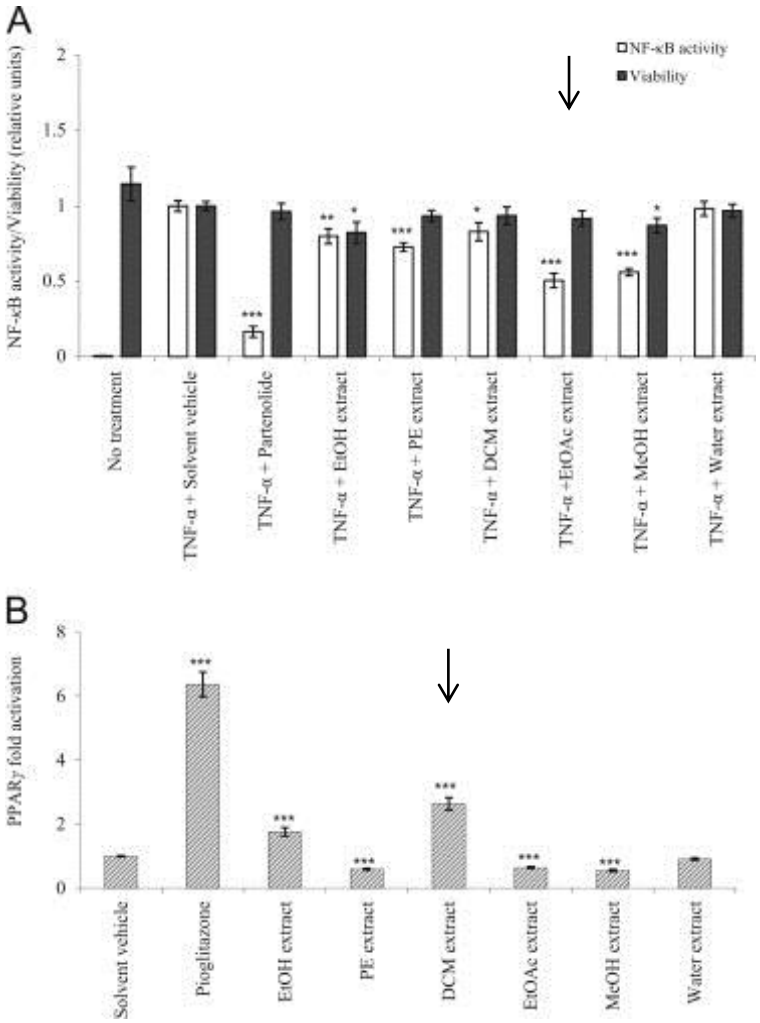


Fig. 10: Extracts from L. chinense and their effects on inhibition of NF-κB activation (A) and activation of PPARγ (B) in luciferase reporter gene assays – *Experiments were performed as described in the "Material and Methods" section of this work. Results are presented*

*as means ± SEM from three independent experiments each performed in quadruplicate. The extracts used for further purification by the phytochemists are indicated by arrows. *p <0.05; **p <0.01; ***p <0.001 (ANOVA/Bonferroni)*

In turn the department-colleagues used the EtOAc extract to isolate four phenolic amides, three neolignanamids and two lignanamides (both kinds of amids can be considered as phenolic amides) which were again tested for their ability to suppress TNFα induced activation of NF-κB (Fig. 11) at a concentration of 30 µM. Only compound 3 (*trans*-N-caffeoyltyramine) could be identified as an inhibitor of NF-κB activation in our test system and eventually the IC_{50} value for this compound was determined in luciferase reporter gene assays in dose-response experiments (data not shown).

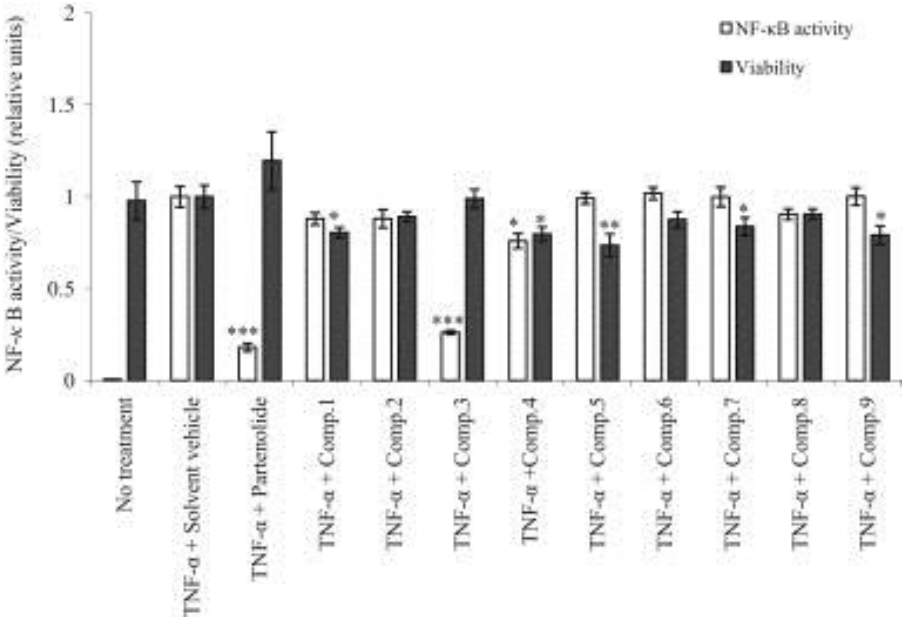


Fig. 11: Effect of isolated phenolic amides (30 µM) on TNFα-induced activation of the NF-κB cascade in HEK293-cells *– Experiments were performed as described in the "Material and Methods" section of this work. Results are presented as mean ±SEM from three independent experiments.* $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ *(ANOVA/Bonferroni). The structures of the phenolic amides can be found in the Appendix chapter and are omitted here due to a lack of space.*

Since the DCM extract showed activity towards PPARγ the bioactivity-guided fractionation approach was employed by our department-colleagues to identify the active principle. This led to one subfraction that was significantly active towards PPARγ (4.64-fold activation over solvent vehicle at a concentration of 30 µg/ml – data not shown)

which was used for further purification to yield a palmitic-acid enriched fraction. This fraction contained several saturated and unsaturated fatty acids according to LC–MS analysis. Since fatty acids are already well known as activators of PPARγ the palmitic-acid enriched fraction was not further purified to yield the isolated individual fatty acids, but tested in dose-response experiments with the luciferase reporter gene assay for PPARγ in comparison with the full agonist pioglitazone (Fig. 12A), as well as in co-treatment experiments with the PPARγ antagonist T0070907 (Fig. 12B).

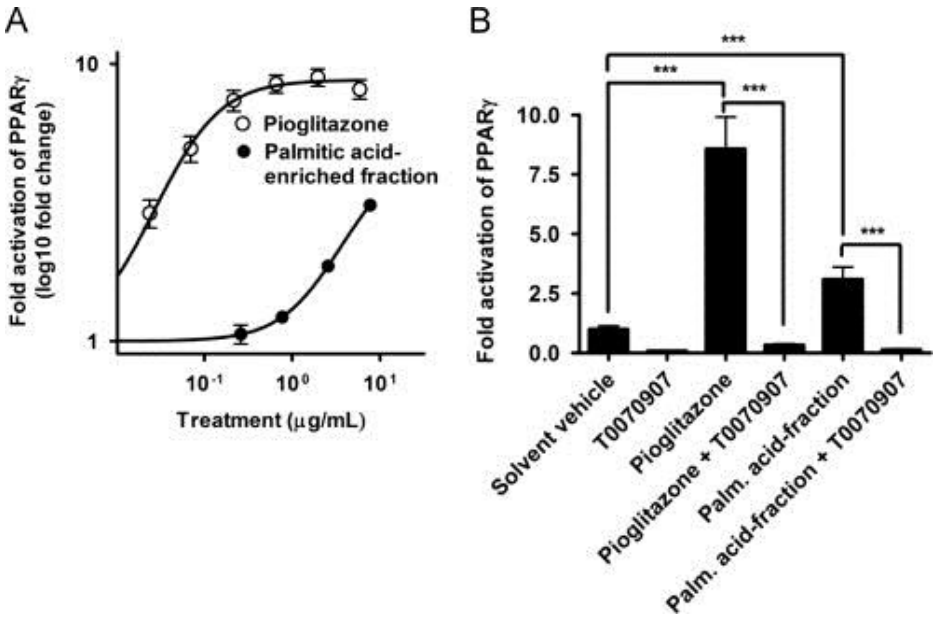
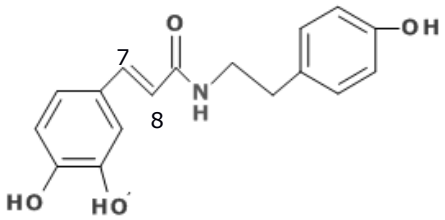


Fig. 12: Characterization experiments for a palmitic acid-enriched fraction extracted from the root bark of Lycium chinense – *(A) The palmitic acid-enriched fraction tested in four concentrations up to 8 µg/ml in the luciferase reporter gene assay for PPARγ in comparison to the full agonist pioglitazone. Experiments were performed as described in the "Material and Methods" section of this work. The data points shown are means ± SD of three independent experiments each performed in quadruplicate. (B) Co-transfected HEK293-cells treated with the palmitic acid-enriched fraction (8 µg/ml) in the luciferase reporter gene assay for PPARγ in comparison to co-transfected HEK293-cells co-treated with palmitic acid-enriched fraction (8 µg/ml) and PPARγ antagonist T0070907 (1 µM). T0070907 could blunt activation induced by both pioglitazone (5 µM) and palmitic acid-enriched fraction. Experiments were performed as described in the "Material and Methods" section of this work. Presented data + SEM are derived from three independent experiments performed in quadruplicate. ***p<0.001 (ANOVA/Bonferroni)*

Discussion

Among the isolated phenolic amides, we could only identify *trans*-N-caffeoyltyramine (Comp. 3) as an inhibitor of NF-κB activation in our luciferase reporter test system that could suppress NF-κB activation more than fifty percent at a test concentration of 30 µM. In subsequent dose-response experiments *trans*-N-caffeoyltyramine was determined to have an IC_{50} concentration of 18.4 µM under the described conditions, which we would consider as relatively modest in comparison to other inhibitors of NF-κB activation that we have identified in our laboratory. Therefore we have decided not to attempt to elucidate the mechanism of action for this compound because of too high opportunity costs. However, to the best of our knowledge this is the first time to show phenolic amides as inhibitors of NF-κB activation. The structural similarity of the isolated compounds prompted us to speculate about possible structure activity relationships (SAR) to yield the following insights: Comparing the structure of the active *trans*-N-caffeoyltyramine with the structural closely related other isolated phenolic amides suggests that the increased polarity of *trans*-N-caffeoyltyramine might play an important role for the observed activity. Apart from that also the double bond between C-7 and C-8 seems also to be crucial for the observed activity since it comprises the only difference between *trans*-N-caffeoyltyramine (active) and dihydro-N-caffeoyltyramine (inactive).



trans-N-caffeoyltyramine

The conducted experiments for the observed PPARγ agonism could finally traced back to fatty acids that are already well known as PPAR agonists. These experiments exemplify one of the major problems in past and present natural product research, which is the missing opportunity to include dereplication studies in early phases of the isolation process of natural compounds. In turn, much effort has to be invested to yield isolated compounds via the bioactivity guided isolation process to finally only end up with compounds that are already known to be active against a biological target. This severity of this problem has been exacerbating over time when more and more plants and isolated active compounds are investigated and reported in literature. A suggested possible future solution of this problem is employing a combination of *in silico* based bioprospecting approaches to directly transform genomic information into biosynthetic outputs (Schmidt-Dannert, 2015).

In conclusion we show here for first time the phenolic amid *trans*-N-caffeoyltyramine as an inhibitor of NF-κB activation, although with rather modest potency in our test system.

4.6 Identification of isosilybin a from milk thistle seeds as an agonist of peroxisome proliferator-activated receptor gamma - published in *Journal of Natural Products* (Pferschy-Wenzig et al., 2014)

Introduction

Silybum marianum (L.) Gaertn. (commonly known as milk thistle) is an annual or biennial plant belonging to the family of Asteraceae. The plant is native in the Mediterranean region but also abundant in the Middle East and Southern parts of Russia. In America and Oceania milk thistle is present as a neophyte. The fruits and seeds of milk thistle have a long tradition of application in traditional medicine to treat diseases of the biliary tract and the liver (Flora et al., 1998) and also today milk thistle preparations are still in use to treat some ailments, at example as supportive agents in hepatitis therapy (Post-White et al., 2007). However, due to a lack of high-quality studies a beneficial effect of milk thistle treatment against alcoholic and/or hepatitis B or C virus liver diseases is questioned by some authors (Rambaldi et al., 2007). The active principle of the milk thistle has been traced back to silymarin (Kvasnička et al., 2003), a phenolic mixture that largely consists of seven flavonolignans (silybin A and B, isosilybin A and B, silychristin, isosilychristin and silydianin) and the flavonoid taxifolin, which is the probably the precursor for the former seven flavonolignans. Silymarin and its constituents are reported to have a variety of biological activities *in vitro* and *in vivo*, including anti-oxidant, anti-inflammatory and anticarcinogenic effects. (Post-White et al., 2007) This present study aimed to investigate whether silymarin and its purified flavonolignan and flavonoid constituents are able to activate PPARγ.

Experimental

The phytochemical work of this study was conducted by the collaboration partners in Graz and started from silymarin, which was purchased from Sigma Aldrich (Vienna, Austria). A minor quantity of silymarin was forwarded to the author's group, dissolved in DMSO, aliquoted and tested in the luciferase reporter gene assay for PPARγ, where it was identified as a weak PPARγ agonist at a concentration of 30 µg/ml with less than 2-fold activation of PPARγ compared to the solvent vehicle (data not shown). The phytochemists in Graz applied an array of chromatographical steps to isolate six main constituents from silymarin, namely silybin A and B, isosilybin A and B, as well as silychristin and silydianin. These six isolated compounds were sent to the author's group along with taxifolin which is another main constituent of silymarin. The seven compounds were subjected to the luciferase reporter gene assay for PPARγ, where despite their high structural similarity only isosilybin A could statistically significant activate PPARγ in our *in vitro* luciferase reporter gene test system in a concentration of 30 µM (Fig. 13).

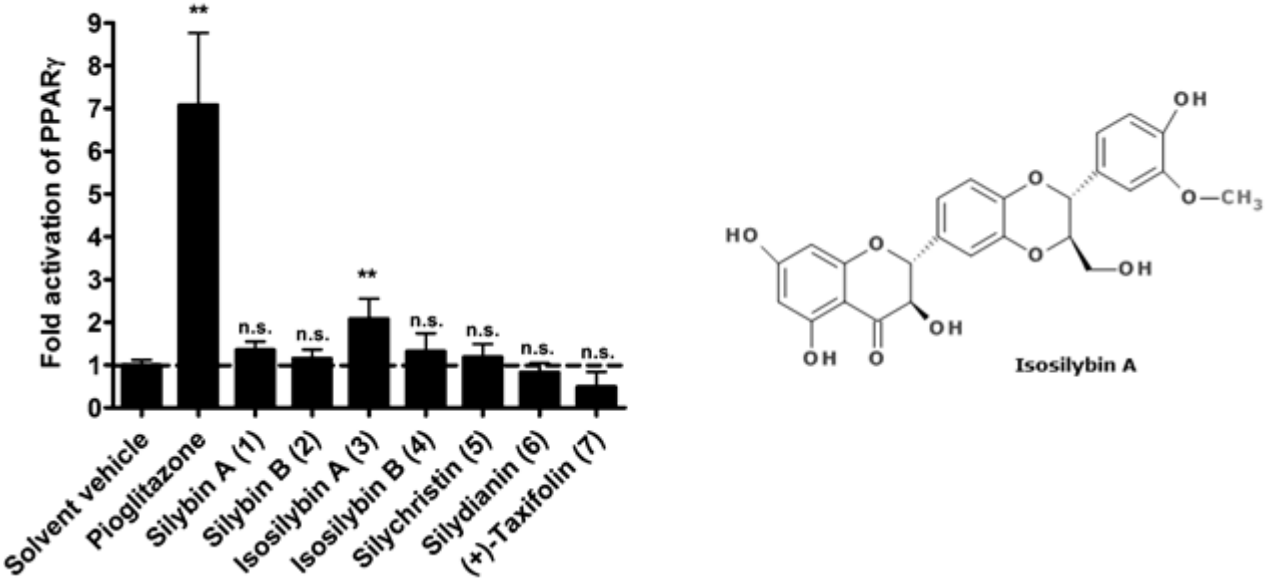


Fig. 13: PPARγ activation in luciferase reporter gene assays by silymarin constituents at a concentration of 30 µM – *Experiments were performed as described in the "Material and Methods" section of this work. The data shown are means + SD of three independent experiments each performed in quadruplicate. **p <0.01; n.s. not significant (ANOVA/Bonferroni)*

For characterization isosilybin A was tested using the luciferase reporter gene assay for PPARγ in dose-response experiments in comparison with pioglitazone (Fig. 14A). To further characterize the compound co-treatment experiments with the full PPARγ agonist pioglitazone (Fig. 14B) and co-treatment experiments with the PPARγ antagonist T0070907 (Fig. 14C) were performed.

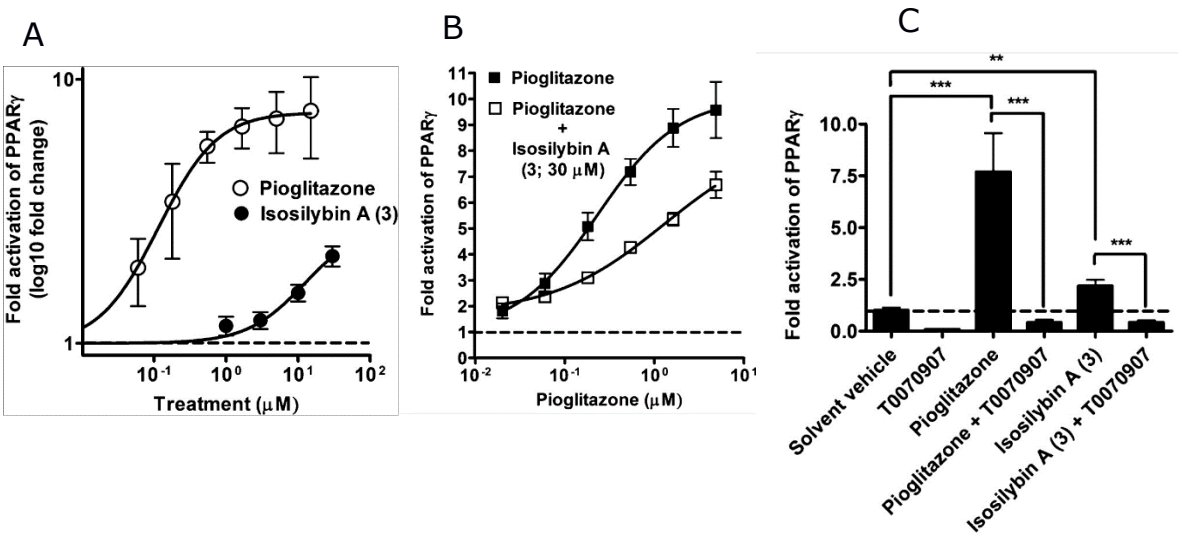


Fig. 14: Some characterization experiments for Isosilybin A - *(A) Dose-response luciferase reporter gene experiments for PPARγ with Isosilybin A in comparison to the full agonist pioglitazone. Experiments were performed as described in the "Material and Methods" section of this work. The data shown are means ± SD of three independent experiments each performed in quadruplicate. (B) Co-treatment luciferase reporter gene experiments with Isosilybin A (30 μM) and varying concentrations of pioglitazone. Luciferase assays were performed as described in the "Material and Methods" section of this work. The data shown are means ± SD of three independent experiments each performed in quadruplicate. (C) Luciferase reporter gene experiments with HEK293-cells treated with pioglitazone (5 μM), T0070907 (1 μM), isosilybin A (30 μM), or combinations as indicated on the x-axis. Experiments were performed as described in the "Material and Methods" section of this work. The data points shown are means ± SD of three independent experiments each performed in quadruplicate. **p < 0.01; ***p < 0.001; (ANOVA/Bonferroni)*

Discussion

From the seven in a concentration of 30µM tested constituents of silymarin, only isosilybin A could statistically significantly activate PPARγ in our test system with 2.08 fold activation of PPARγ in comparison to the solvent vehicle. Subsequent dose-response experiments yielded the insight that the activation of PPARγ is dose-dependent and therefore in correlation with isosilybin A-treatment, but since the activity-curve of isosilybin A did not reach plateau level at 30 µM, neither EC_{50}, nor E_{max} values were determined (Fig. 14A).

However, structural similarities of isosilybin A to the inactive other tested compounds motivated the collaborating chemoinformatical group in Innsbruck to perform docking studies of all tested compounds within the receptor binding pocket of PPARγ. Their studies yielded that only isosilybin A is able to form a hydrogen bond with Ser342 in the entrance region of PPARγ, but not the also tested regio- and stereoisomers, which was eventually also reported in the corresponding publication to provide a plausible explanation for the observed effects.

In cotreatment experiments of isosilybin with the PPARγ antagonist T0070907 the luciferase signal was depleted, which further confirms the PPARγ dependency of the observed effect (Fig. 14C). In cotreatment experiments of isosilybin A with the PPARγ full agonist pioglitazone diminished activity of pioglitazone in presence of isosilybin A was observeable. The diminished activity of pioglitazone in presence of isosilybin A could be partly overcome by increasing pioglitazone concentration (Fig. 14B). This indicates that the two compounds compete for the same binding pocket. In presence of a full agonist pioglitazone a partial agonist can be considered as an antagonist and in turn diminished the observed activity of the full agonist.

In conclusion this study reports for the first time that the flavonolignan isosilybin A from the milk thistle seed extract silymarin acts as a partial PPARγ agonist.

4.7 Identification of chromomoric acid C-I as an Nrf2 activator in *Chromolaena odorata* - published in *Journal of Natural Products* (Heiss et al., 2014)

Introduction

Chromolaena odorata is a perennial semi-lignified herbaceous plant that belongs to the family of Asteracae. The plant is native to South and Central America, but was later introduced to Asia and Africa, where it became part of traditional medicine. The leaves of the plant are used to treat a variety of medical conditions and the effect of the plant extract can broadly be summarized to be anti-inflammatory, anti-microbial and antioxidative (Thang et al., 2001). Interestingly, in context of this thesis some fatty acid derivatives isolated from a methanolic extract of aerial plant material are reported to be able to inhibit LPS induced NF-κB activation in *in vitro* experiments in micromolar concentrations (Hanh et al., 2011).

Apart from that *Chromolaena odorata* is also prominent as one of the most aggressive known neophytes that is very difficult to control because of rapid attainment of reproductive maturity, large production of wind-dispersed seeds and a (short-term) persistent seed bank (Witkowski and Wilson, 2001). Overall there are more than 5000 scientific documents to be found at Google Scholar using the plant's name in double quotes in a keyword search.

This publication focuses mainly on the observed Nrf2-effect of some isolated compounds. Since NF-κB and Nrf2 are suggested to interplay (Bellezza et al., 2010), those compounds were also tested using the luciferase reporter gene assay for inhibition of NF-κB activation.

Experimental

A crude methanol extract of *C. odorata* was identified by a department colleague in luciferase reporter gene studies as a potent Nrf2 activator. In turn this extract was further fractionated by the participating phytochemists by liquid-liquid extractions to

yield a diethyl ether fraction that elicited potent concentration-dependent activation of Nrf2. From this extract our collaboration partners, the phytochemists in Innsbruck, employed bioactivity-guided fractionation to isolate 13 flavonoids, one phenolic compound as well as five phytoprostanes all of which were again tested in a luciferase reporter gene assay for Nrf2 activation by department colleagues. While the flavonoids did not show activity up to concentrations of 30 µM, from the five phytoprostanes only one of the compounds, chromoric acid C-I, was able to significantly activate Nrf2 in the luciferase reporter gene assay. The five phytoprostanes were finally also tested in the luciferase reporter gene assay for inhibition of NF-κB activation with CMFDA (Fig. 15) and for the most active compound IC_{50} values were determined in dose-response experiments.

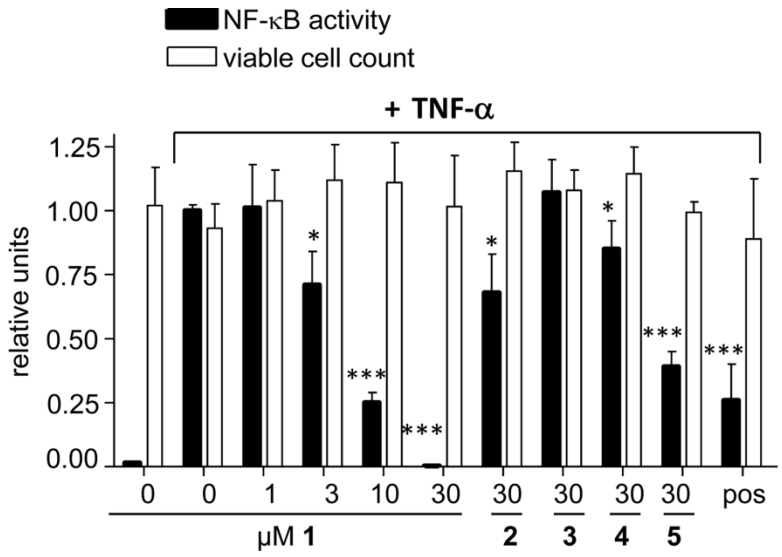


Fig.15: Inhibition of NF-κB activation by phytoprostanes isolated from C. odorata – *Experiments were performed as described in the "Material and Methods" -section of this work. The data represent means + SD of three independent experiments performed in quadruplicate. *p <0.05, ***p <0.001(ANOVA/Bonferroni). The numbers given on the x-axis refer to the used concentrations in µM (top) and the compounds (bottom), the structures of whose are to be found in the Appendix chapter of this work and are omitted here due to a lack of space.*

Discussion

The testings of the five phytoprostanes in the luciferase reporter gene assay for inhibition of NF-κB activation with CMFDA yielded three compounds that could not suppress TNFα

induced activation of NF-κB more than 50 % up to a test concentration of 30 µM (2, 3 and 4), one compound that did not completely inhibit NF-κB activation at a test concentrations of 30 µM, which equivalents an IC_{50} value above 20 µM in the applied experimental setup, as well as chromoric acid C-I (1) that was identified as a NF-κB inhibitor with an IC_{50} of 6.9 µM as determined in dose-response experiment under the described experimental conditions (Fig. 15). The identification of chromoric acid C-I as an inhibitor of NF-κB activation is interesting, because the structure of the compound shows a high structural similarity to the human 15-deoxy-Δ12,14-prostaglandin J2, a prostaglandin found under conditions of inflammation and redox stress. Interestingly, also 15-deoxy-Δ12,14-prostaglandin J2 is a known Nrf2 activator and NF-κB inhibitor (Cernuda-Morollón et al., 2001). This could suggest the existence of a common molecular anti-inflammatory language in plant and mammalian cells that relies on similar structural features.

In conclusion this study reports *Chromolaena odorata* as a source for phytoprostanes that can be possibly find future application as Nrf2 activators and/or NF-κB inhibitors.

4.8 Screening of Vietnamese medicinal plants for NF-κB signaling inhibitors: assessing the activity of flavonoids from the stem bark of *Oroxylum indicum*.

- published in *Journal of Ethnopharmacology* (Tran et al., 2015)

Introduction

Vietnam can be considered as a hot spot for plant diversity with more than 12.000 different plant species (Rhind, 2012). Some of these plants are used in traditional Vietnamese medicine but often their use is only insufficiently rationalized by insights which plant substances could be responsible for observed effects. To rationalize and assess the activity of Vietnamese plants, the DNTI-consortium conducted a screening of 17 plants which are listed in the textbook "Medicinal plants and animals in Vietnam" for their potential to inhibit TNFα induced NF-κB activation in our cellular test system.

Among those 17 plants was *Oroxylum indicum*, a species of flowering plants belonging to the family of Bignoniaceae. The tree is native from the Indian subcontinent to Southern China, also including most areas of Southeast Asia. The plant reaches a height

of approx. 12 meters and is a night bloomer that relies on bats as pollinators (Panda, 2011). All parts of the plant are used in traditional medicines, special importance has the stem bark, that finds application in treatment of gastric disorders (Hari Babu et al., 2010), skeletal diseases (Rout et al., 2010), jaundice (Hemadri and Rao, 1984) and diseases of the respiratory system (Patil et al., 2008).

Pharmacological studies of the stem bark of *O. indicum* indicate the presence of phenolic compounds, flavonoids, alkaloids (Kalaivani and Lazar, 2009), as well as saponins (Bisht et al., 2010).

Oroxylum indicum is reasonably well researched with more than 2500 scientific documents to be found in a Google Scholar search using the plant's name in double quotes. However, low seed and seedling viability in combination with indiscriminate and unsustainable utilization have put this species on the red list of endangered plants (Tiwari et al., 2007).

Experimental

The phytochemical work of this study was done by the collaborating phytochemical group in Innsbruck. They prepared a total of 40 methanolic and DCM extracts from different parts of 17 plants and sent the lyophilized extracts to the author's group where they were dissolved in DMSO, aliquoted and subjected to the luciferase reporter gene assay for inhibition of N F-κB activation with CMFDA at a concentration of 10 µg/ml (Fig. 16).

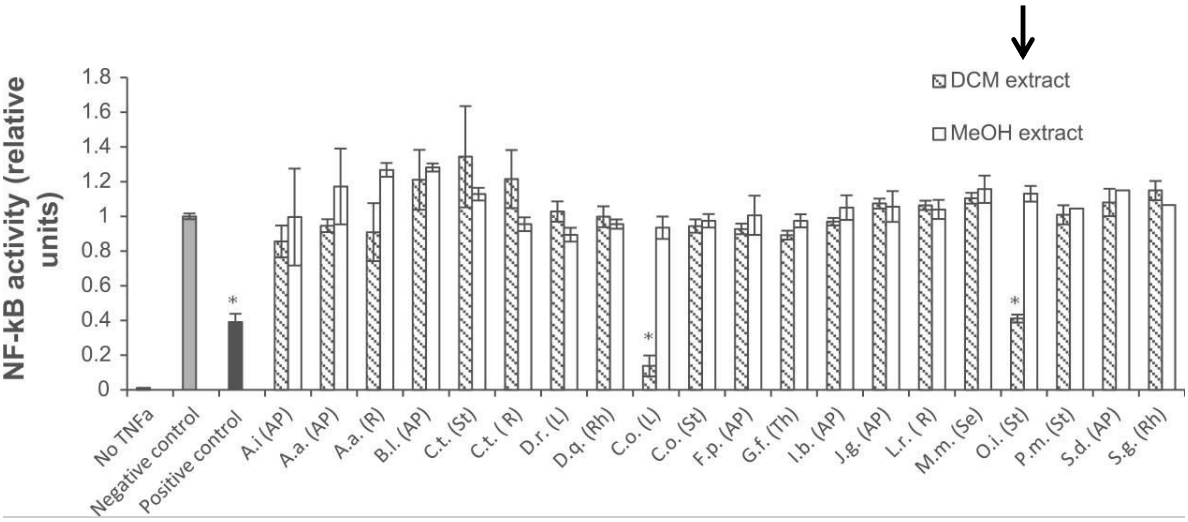


Fig. 16: Effect of crude extracts from various plants used in traditional Vietnamese medicine on inhibition of NF-κB activation in the luciferase reporter gene assay – *Samples are abbreviated with the first letters of genus and species, full names of the plants can be found in the corresponding publications and are omitted here due to a lack of space. The abbreviation in brackets for each sample refers to part of the plant that was used for the extraction process; a description is again omitted here for a lack of space and can be found in the corresponding publication. The extract of interest for the following studies is indicated by an arrow and originates from the stems of Oroxylum indicum. Experiments were performed as described in the "Material and Methods" section of this work. Results shown are the mean ±SEM from three independent experiments each performed in quadruplicate.*p <0.05 (ANOVA/Turkey)*

Best results were observed for the DCM extracts of *Chromolaena odorata* and *Oroxylum indicum* and in turn the phytochemists in Innsbruck focused on the characterization of the DCM extract of *O. indicum* and also prepared extracts with other solvents from the stem bark of *O. indicum*. Their work succeeded in isolating ten compounds (1-10) that were sent to the author's group for testing in the luciferase reporter gene assay for inhibition of NF-κB activation with CMFDA at a concentration of 30 µM (Fig. 17).

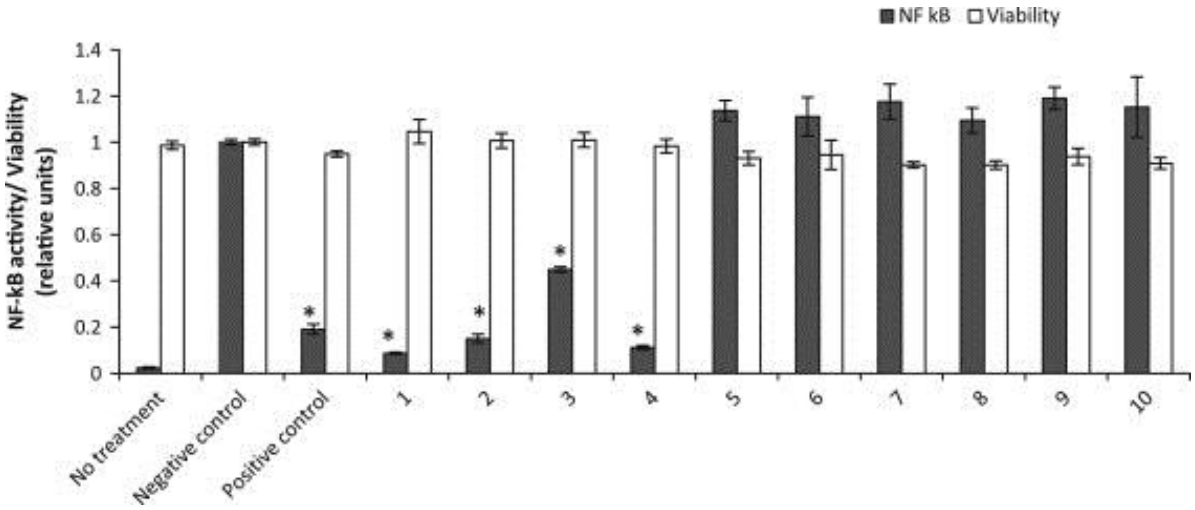


Fig.17: Effect of isolated compounds 1-10 (30 µM) on inhibition of NF-κB activation in luciferase reporter gene assays with CMFDA *- The numbers on the x-axis refer to the isolated compounds, whose structures are depicted on the next page and also in the Appendix chapter of this work.Experiments were performed as described in the "Material and Methods" section of this work. Bar graphs represent mean ±SEM of three independent experiments. *p <0.05 (ANOVA/Turkey)*

The testings resulted in the identification of four active flavonoids, for which IC_{50} values were determined in dose-response experiments in the luciferase reporter gene assay for inhibition of NF-κB activation with CMFDA. (Fig. 18) and six inactive flavonoid glycosides.

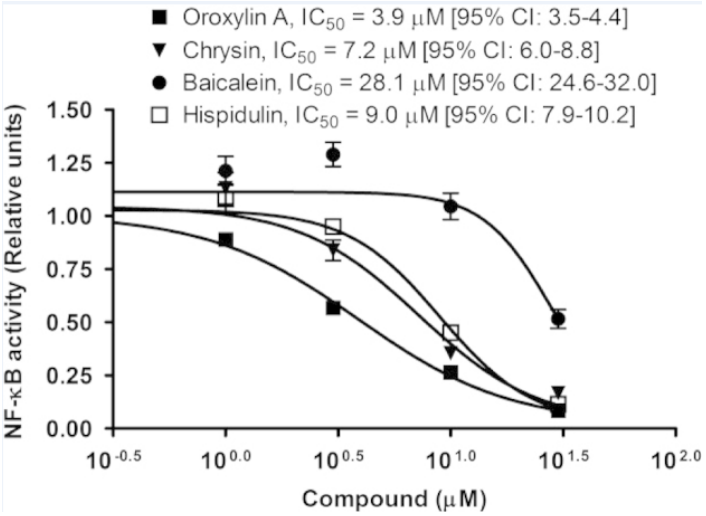
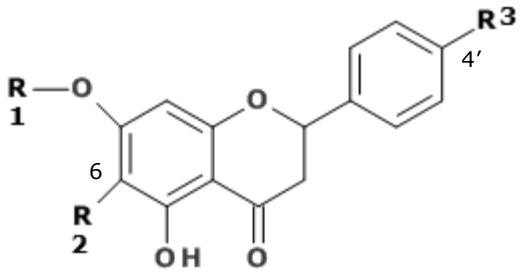


Fig. 18: Dose-response curves and IC_{50} values of four isolated flavonoids from O. indicum with respect to NF-κB activation – *Dose-response experiments for inhibition of NF-κB activation in luciferase reporter gene assays with CMFDA were performed as described in the "Material and Methods" section of this work.*



1. Oroxlin A	**R1 = H**	**R2 = OCH3**	**R3 = H**
2. Chrysin	**R1 = H**	**R2 = H**	**R3 = H**
3. Baicalein	**R1 = H**	**R2 = OH**	**R3 = H**
4. Hispidulin	**R1 = H**	**R2 = OH**	**R3 = OH**
5. Oroxylin A-7-O-beta-D-glucoronide			
	R1 = glucoronic acid	**R2 = OCH3**	**R3 = H**
6. Chrysin-7-O-beta-D-glucuronide			
	R1 = glucoronic acid	**R2 = H**	**R3 = H**
7. Baicalein-7-O-beta-D-glucoside			
	R1 = glucose	**R2 = OH**	**R3 = H**
8. Baicalin	**R1 = glucoronic acid**	**R2 = OH**	**R3 = H**
9. Oroxylin A-7-O-beta-D-methyl glucuronide			
	R1 = methyl glucuronide	**R2 = OCH3**	**R3 = H**
10. Baicalein A-7-O-beta-D-methyl glucuronide			
	R1 = methyl glucuronide	**R2 = OH**	**R3 = H**

Discussion

The four active flavonoids were determined to have IC_{50} values between 3.9 and 28.1 µM under the applied test conditions. Interestingly, only the four flavonoids showed activity in our hands, while the six flavonoid glycosides were inactive. This offers the suggestion that the activity of the flavonoid glycosides is lost due to the increase in polarity and molecular weight which could negatively influence the penetration of cell membranes in our *in vitro* test systems. The close structural relationship also prompted us to speculate about SAR of the compounds: The presence of a hydroxyl group at the position 6 of the A ring reduces the activity, while a methoxy-group or no substitution at this position leads to a better inhibitory activity in our test system. One of the compounds (hispidulin) is identical with a less active compound (baicalein), except for one additional hydroxyl group at position 4′ of ring B, which therefore should be important for the observed activity. This would be in good accordance with literature

where it is reported that the hydroxyl group at position 4’ of the B ring can be crucial for observed anti-inflammatory activity (Chen et al., 2004).

However, from the view of rational drug discovery flavonoids are difficult candidates for the development of drugs used for oral application. This is because they often get efficiently conjugated after absorption from the small intestine and in such cases no free flavonoid aglycones can be found in blood plasma or urine. Those flavonoids who escape absorption in the small intestine will often be degraded by microorganisms in the colon and are therefore hardly bioavailable. (Hollmann, 2004) However, although as a whole flavonoids are rather borderline candidates for the development of drugs used for oral application, there are some flavonoids reported in biomedical literature that show potent effects (and thus also bioavailability) in *in vivo* models after peroral application (Drees et al., 1997). Also in context of food intake flavonoids are believed to support health (Yao et al, 2004). In turn additional characterizing experiments for the here identified flavonoids could be beneficial to gain insights about the suitability of the identified compounds as candidates for rational drug development.

In conclusion the here performed experiments identify the stem bark of *Oroxylum indicum* as a valuable source of flavonoids with anti-inflammatory activity in the applied *in vitro* test system.

4.9 NF-κB inhibitors from Eurycoma longifolia. - published *in Journal of Natural Products* (Tran et al., 2014)

and

The NF-κB inhibitor Eurycomalactone acts downstream of nuclear translocation of p65 – manuscript in preparation

Introduction

Eurycoma longifolia Jack is described as a slender shrub with around 10 meters height and belongs to the plant family of Simaroubaceae. The plant is native to the tropical and

subtropical regions of South-East Asia, where many different parts of the plant are used in traditional medicine. The traditional applications include the usage as aphrodisiac tonic, as an antimalarial drug, as well as as health supplement and the plant is generally reported to exhibit anxiolytic properties, to increase muscle strength and to show antiplasmodial and cytotoxic properties (Miyake et al., 2009). Apart from that one study also reports an antihyperglycaemic activity of *E. longifolia* in *in vivo* experiments (Husen et al., 2004). The major bioactive chemical components of the plant are reported as quassinoids, alkaloids and squalene derivatives (Park et al., 2014).

Commercially most exploited is the (traditional) use as an aphrodisiac tonic and indeed studies with human test subjects claim to have found an increase in testosterone levels (Tambi et al., 2012) as well as an improvement in semen-quality (Tambi and Imran, 2000).

Due to sustained uncontrolled harvesting *E. longifolia* is considered as endangered by extinction in its natural habitats (Bhat and Karim, 2010). The plant is relatively well researched with more than 2000 scientific documents to be found in a Google Scholar search using the plant's name as search term.

Experimental (NF-κB inhibitors from *Eurycoma longifolia*):

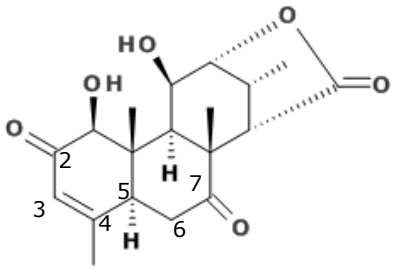
Starting from a methanolic root extract of *E. longifolia* our collaborating phytochemical partners in Innsbruck prepared liquid-liquid extractions with various solvents and the gained dried extracts were sent to the author's group. There they were redissolved, aliquoted and tested for their ability to inhibit TNFα induced NF-κB activation in the luciferase reporter gene assay with CMFDA (data not shown). The best effects were observed in the diethyl ether extract as well as in the ethyl acetate extract. In turn our collaboration partners isolated a total of 28 compounds from those two extracts, among them one quassinoid of previously unreported structure, which was termed eurycomalide C. All 28 isolated compounds were tested in the luciferase reporter gene assay at a concentration of 30 µM and 15 of them could inhibit NF-κB activation more than 50 % in our experimental setup (data not shown). For these compounds IC_{50} values were determined in the luciferase reporter gene assay (Table 4).

Compound	**IC_{50} value (µM)**
Eurycomalide C (1)	18.4
Eurycomalactone (2)	0.5
7α-hydroxyeurycomalactone (3)	1.5
5,6-dehydroeurycomalactone (4)	6.2
Eurycolactone E (5)	3.8
Longilactone (6)	4.7
14,15β-dihydroxyklaieanone (7)	1.0
11-dehydroklaieanone (8)	1.9
Eurycomanone (9)	2.4
13,21-dehydroeurycomanone (10)	0.7
1-methoxycarbonyl-*β*-carboline (13)	29.3
9-hydroxycanthin-6-one (14)	3.8
9-methoxycanthin-6-one (15)	7.4
9,10-dimethoxycanthin-6-one (16)	19.5
3,5,6,7,8,3´,4´-heptamethoxyflavone (28)	23.3
Parthenolide (positive control)	1.5

Table 4: IC_{50}-values for inhibition of NF-κB activation of compounds isolated from the roots of E. longifolia.

Discussion (NF-κB inhibitors from *Eurycoma longifolia*)

Here we have shown that a methanolic root extract of *E. longifolia* contains several compounds that can inhibit NF-κB activation in our experimental setup, which are 10 (C_{19}-type and C_{20}-type) quassinoids (1-10), four alkaloids (13-16) and the isolated flavonoid 3,5,6,7,8,3',4'-heptamethoxyflavone (28). Interestingly, in contrast to the C_{19}-type and C_{20}-type quassinoids the two isolated C_{18}-type quassinoids were both inapt to inhibit NF-κB activation in our hands. However, the structural similarity of the active quassinoids enabled us to speculate about possible structure-active relationships: The obtained data indicates that the double bond between C-3 and C-4 seems to be more relevant for inhibition of NF-κB activation than the double bond between carbons 5 and 6, because eurycomalide C (1) has a more than 30-fold higher IC_{50}-value than eurycomalactone (2). Interestingly, substitution of the carbonyl functionalities at C-2 or C-7 resulted in a significant decrease of NF-κB inhibition, observable as both eurycolactone E (5) and 7α-hydroxyeurycomalactone (3) have higher IC50-values than eurycomalactone (2).

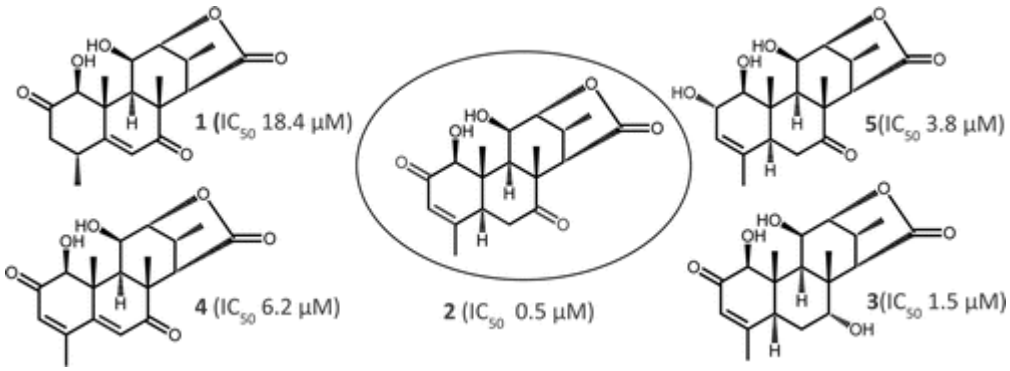


Eurycomalactone

None of the tested compounds showed any cytotoxicity as observable from the fluorescence derived signal (that is primarily used for normalization of the luciferase signal) indicating that cytotoxicity can be ruled out as an interfering factor for the active compounds in these assays. However, since the incubation time of 4.5 hours might be too short for a realistic evaluation of cytotoxic effects, it could be interesting to further challenge the cytotoxicity of the active isolated compounds, especially in experimental setups that forgo TNFα and serum-free conditions in view of that many quassinoids are reported as cytotoxic (Chakraborty and Pal, 2013). However, under the employed experimental conditions no cytotoxicity was detected.

In conclusion we have identified 15 compounds from a methanolic *E. longifolia* extract that can inhibit TNFα-induced NF-κB activation in our experimental setup, two of the compounds gave IC_{50} values in the submicromolar range (2 and 10). The most active compound among those was eurycomalactone (2), for which we determined an IC_{50} value of 0.53 µM in dose-response experiments in our experimental setup. The observed anti-inflammatory effects of the isolated compounds can possibly help to rationalize the traditional use of *E. longifolia*, although it is always difficult to conclude from the effects

of isolated compounds to the effect of a complex plant derived mixture such as an alcoholic extract or a decoction. However, the observed potency and non-toxicity under the employed experimental conditions warrant further studies of the C_{19}-quassinoid eurycomalactone that is here for the first time reported as an inhibitor of NF-κB activation.



<u>Experimental</u> (The NF-κB inhibitor Eurycomalactone acts downstream of nuclear translocation of p65 and DNA binding):

Here we try to elucidate the mechanism of action for eurycomalactone (2) as a potent inhibitor of NF-κB activation. We chose eurycomalactone for further characterization, because it was the most active compound the phytochemists in Innsbruck were able to isolate from the roots of *Eurycoma longifolia*. To first reduce the possibility that an eventual toxicity of eurycomalactone impairs the relevance of the obtained results, the effect of eurycomalactone on metabolic activity in the HUVECtert test system was evaluated with the straightforward resazurin conversion assay with and without stimulation with TNFα. (Fig. 19).

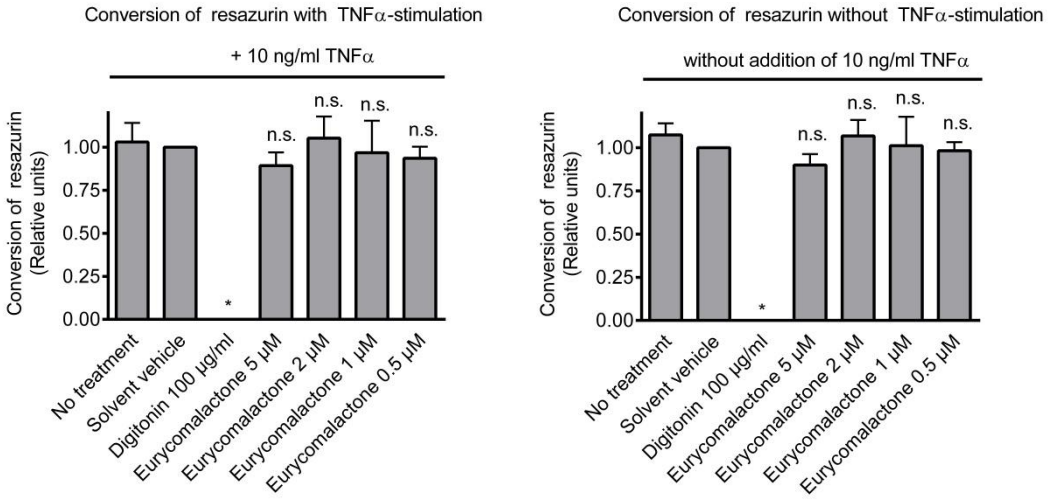


Fig. 19: Resazurin conversion assay in HUVECtert with and without stimulation with TNFα *– Experiments were performed as described in the "Material and Methods" -section of this work. The data represent means +SEM of three independent experiments performed in quadruplicate. *p <0.05, n.s. not significant (ANOVA/Dunnet)*

The subsequent flow cytometry experiments to detect the endothelial surface proteins ICAM-1, VCAM-1 and E-selection were conducted to test eurycomalactone as a potent inhibitor of NF-κB signaling in the physiologically more relevant HUVECtert system (Fig. 20).

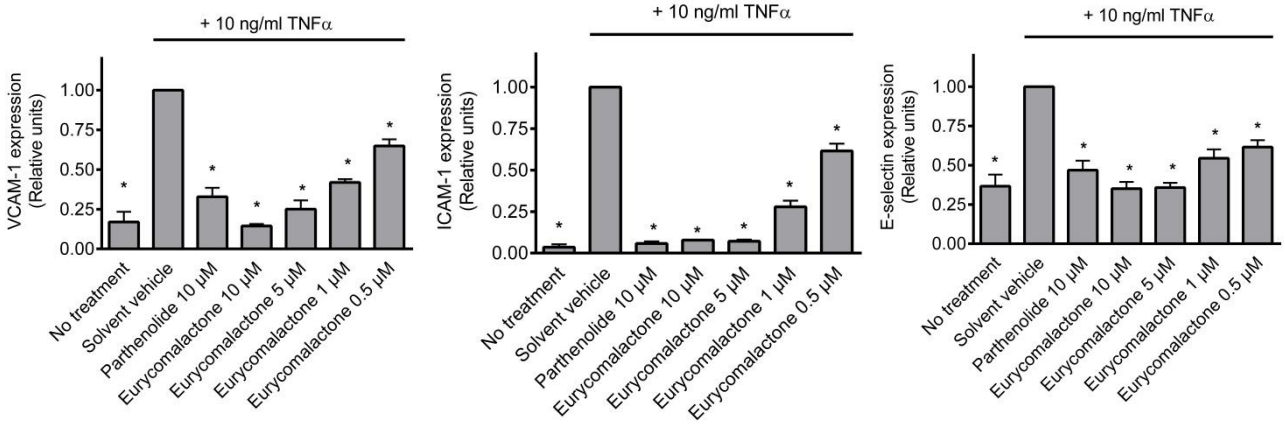


Fig. 20: Flow cytometric detection of the pro-inflammatory surface proteins ICAM-1, VCAM-1 and E-selectin in HUVECtert after stimulation with TNFα - *Experiments were performed as described in the "Material and Methods" -section of this work. The data represent means +SEM of three independent experiments performed. *p <0.05, n.s. not significant (ANOVA/Dunnet)*

To elucidate the molecular mechanism of action for eurycomalactone the effects of 2 µM eurycomalactone pretreatment prior to stimulation with TNFα (10 ng/ml) on phosphorylation of IKK-β and IκBα as well as degradation of IκBα were evaluated in Western blotting experiments (Fig. 21).

Since we could not detect statistically significant impairment of IκBα degradation upon pretreatment with eurycomalactone and stimulation with TNFα we next separated cytosolic and nuclear proteins to detect the NF-κB subunit p65 in immunoblotting experiments (Fig. 22).

To test whether p65 in the nucleic extract of eurycomalactone treated cells can bind to a NF-κB consensus sequence the TransAM™ NF-κB p65 kit was used. To test in addition whether eurycomalactone can directly inhibit binding of p65 to the consensus sequence eurycomalactone was also added to nuclear extract to a final concentration of 2 µM (Fig. 22).

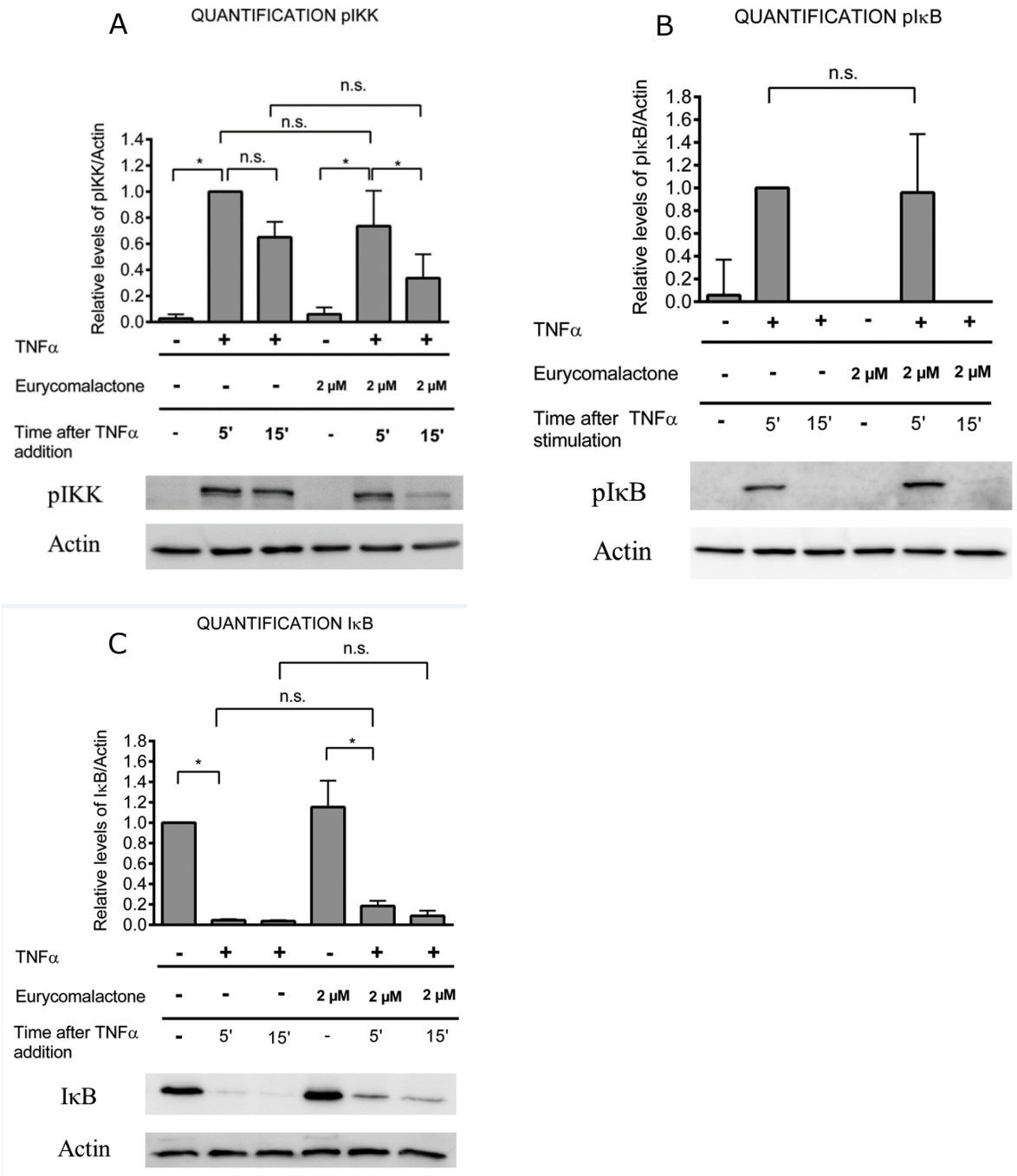


Fig. 21: Immunoblot detection of pIKK (A), pIκB (B) and IκB (C) of HUVECtert pretreated with solvent vehicle and stimulated with TNFa - *Experiments were performed as described in the "Material and Methods" -section of this work. The provided Western blots are representative results for the data shown. The data represent means +SD of three independent experiments performed in kind cooperation with guest student Enrico Sangiovanni. *p <0.05, n.s. not significant (ANOVA/Dunnet)*

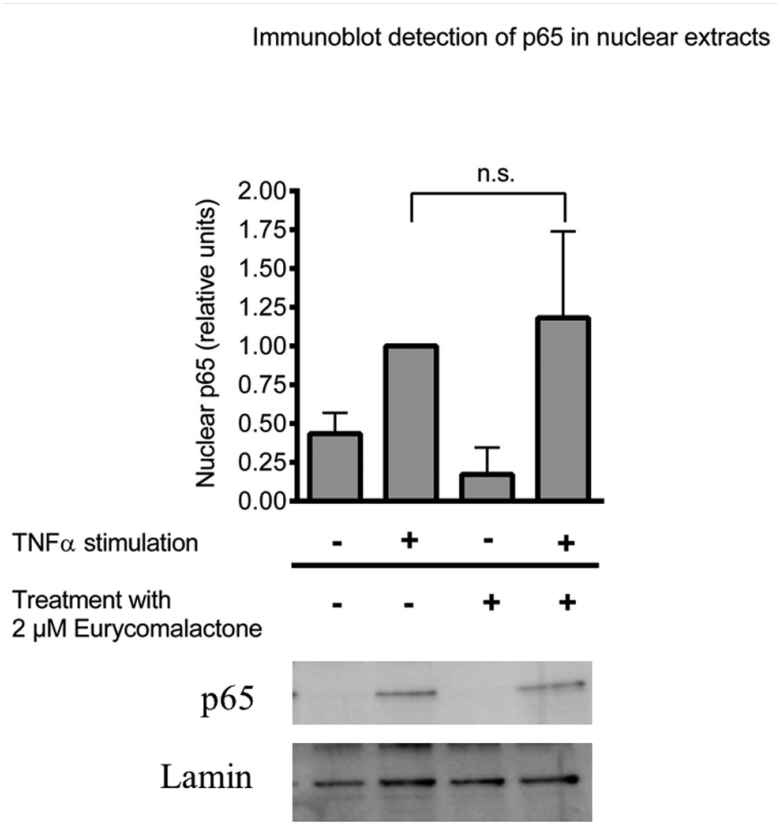


Fig. 21: Immunoblot-detection of p65 in nuclear extracts of HUVECtert – *Cells were pretreated with 2 µM eurycomalactone or solvent vehicle 30 min prior to stimulation with TNFα (10 ng/ml). Nuclear extracts were prepared 1h after stimulation as described in the "Material and Methods" section of this work. The data shown represent means +SD from three independent experiments performed in kind cooperation with guest student Enrico Sangiovanni. *p <0.05, n.s. not significant (ANOVA/Dunnet)*

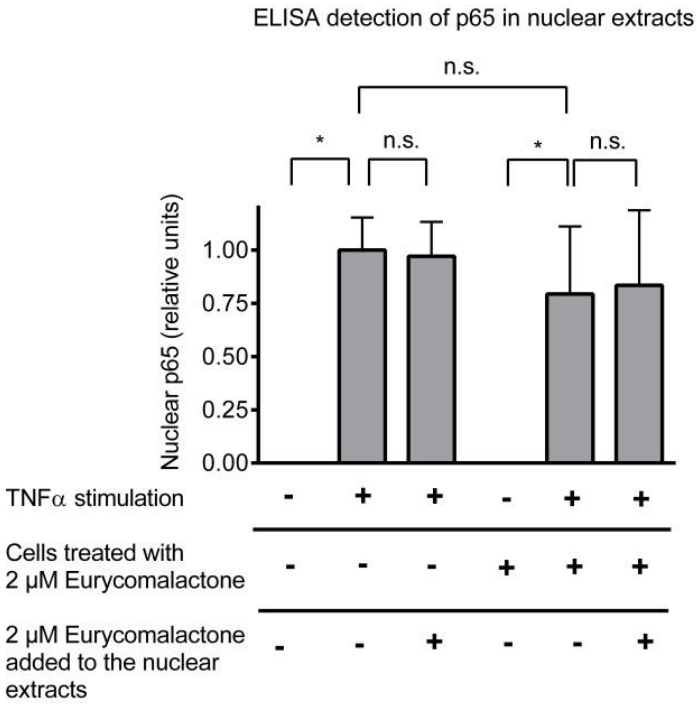


Fig. 22: ELISA-detection of DNA-bound p65 in nuclear extracts of HUVECtert – *Cells were pretreated with compound or solvent vehicle 30 min prior to stimulation with TNFα (10 ng/ml). Nuclear extracts were prepared 1h after stimulation as described in the "Material and Methods" – section of this work and binding capacity of p65 was tested in an ELISA assay as described in the "Material and Methods" –section. In separate experiments eurycomalactone was also added directly to nuclear extracts to a final concentration of 2 µM. The data represent means +SEM of three independent experiments and was yielded in kind cooperation with guest student Enrico Sangiovanni. *p <0.05, n.s. not significant (ANOVA/Dunnet)*

<u>Discussion</u> (The NF-κB inhibitor Eurycomalactone acts downstream of nuclear translocation of p65 and DNA binding)

The result of the cytotoxicity evaluation warranted further attention to eurycomalactone: While the positive control digitonin (100 µg/ml) could completely suppress metabolic activity, none of the tested concentrations between 0.5 and 5 µM of eurycomalactone showed significant impairment of resazurin conversion compared to the solvent vehicle (Fig 19a). Stimulation with TNFα had a negligible effect on metabolic activity in all tested conditions (Fig. 19b).

The subsequent flow cytometry experiments showed eurycomalactone as a potent inhibitor of TNFα induced NF-κB activation in HUVECtert. Inhibition of ICAM-1, VCAM-1 and E-selectin expression yielded IC_{50} values in the submicromolar range (IC_{50} for VCAM-1 = 0.54 µM; ICAM-1 = 0.58 µM; E-selectin = 0.56 µM). Interestingly, these obtained IC_{50} values correspond very well to the results of our initial luciferase reporter gene screening where the IC_{50} of eurycomalactone was determined to be 0.5 µM.

We therefore chose a concentration of 2 µM for the subsequent experiments to elucidate the mechanism of action for eurycomalactone as a potent inhibitor of NF-κB activation. It was also observed in flow cytometer experiments that the treatment of HUVECtert with concentrations of eurycomalactone between 0.5 and 10 µM without TNFα stimulation did not show any effect on basal expression of the pro-inflammatory endothelial surface proteins.

In the subsequent immunoblotting experiments to elucidate the molecular target of eurycomalactone, phosphorylation of IKK-β and IκBα as well as degradation of IκBα were evaluated in comparison to HUVECtert that were only pretreated with solvent vehicle prior to stimulation with TNFα. Because we could not observe a statistically significant difference for any of the three proteins tested in comparison to control cells, we conclude that the molecular target of eurycomalactone as a potent inhibitor of NF-κB activation is downstream of IκB degradation.

In the next step we were interested to determine whether pretreatment with eurycomalactone prior to stimulation with TNFα could impair nuclear translocation of the NF-κB subunit p65. In turn we prepared nuclear extracts and immunoblotted against p65, but could not observe a statistically significant difference in nuclear p65 levels upon pretreatment with 2 µM eurycomalactone without stimulation with TNFα. We therefore conclude that the molecular mechanism of eurycomalactone does not comprise impairment of nuclear p65 translocation to an extent that would explain the observed IC_{50} value in the submicromolar range in this cell model.

In the subsequent experiments to test whether pretreatment with 2 µM eurycomalactone inhibits DNA-binding of p65 after stimulation with TNFα we again prepared nuclear protein extracts of HUVECtert pretreated with either solvent vehicle or 2 µM eurycomalactone, before stimulation with TNFα and 1h after stimulation with TNFα. In these experiments we could detect DNA-bound p65 after stimulation with TNFα irrespective of eurycomalactone pretreatment, thereby excluding also impairment of DNA-binding as the molecular mechanism of action of eurycomalactone as NF-κB inhibitor. To obtain further confirmation for these finding we also directly added

eurycomalactone to a final concentration of 2 µM to the nuclear protein extracts, but this again did not show any impairment of p65 DNA-binding. In conclusion we suggest that the molecular target of eurycomalactone is also downstream of DNA binding of p65.

However, although we could provide evidence that eurycomalactone acts downstream of nuclear translocation of p65 and DNA binding the molecular target of eurycomalactone remains elusive at the present time. Many quassinoids are reported in literature as general inhibitors of protein synthesis in eukaryotic cells by targeting ribosomal peptidyl transferase (Guo et al., 2005), but most of them are C-20 quassinoids and their picrasane skeleton is distinct from the structure of the C-19 quassinoid eurycomalactone with its special structural feature of a lactone moiety connecting C-12 and C-14. We also speculate that general inhibition of protein synthesis would be reflected in a decrease of metabolic activity within our experimental setup, considering the multitude of enzymes that contribute to the overall reduction of resazurin (Rampersad, 2012) and the short half-life time of most cellular proteins (Toyama et al., 2013). In conclusion we find eurycomalactone a highly interesting natural product that warrants further research activities. However, one possible drawback of eurycomalactone or quassinoids in general in regard to their usability as lead structures is that they are known for their poor oral bioavailability resulting from extensive first-pass metabolism and a relatively low P-value. (Low et al., 2005).

4.10 (Semi)synthetic neolignans as agonists of PPARγ and RXRα – ongoing project

Important note: The data presented in this chapter is a synopsis of all data harvested during the author's engagement in the DNTI-consortium and has not yet been used in its entirety in any other academic work. Development of the assay (in view of determination of suitable mass ratios of plasmids for transfection) was done by Amina Cocic in the framework of her diploma thesis. Screening of most samples was done by Amina Cocic, guest student Gökhan Küçükkara and diploma student Reem Selim. Mrs. Selim also determined most E_{max} and EC_{50} values for the active derivatives in the framework of her diploma thesis. All of the mentioned students were co-supervised and co-guided by the author of this thesis. In summary most of the data presented the

following were harvested by guest students or diploma-students, either in parallel experiments together with the author of this thesis or independently. Only a minor quantity of samples was exclusively tested by the author of this thesis.

Introduction

We have recently identified several different neolignans as partial agonists of PPARγ) by a computer-aided approach (Atanasov et al., 2013b; Fakhrudin et al., 2010. In the present ongoing project we have been investigating a small set of 57 mainly (semi)synthetic neolignans as agonists of PPARγ and also as agonists of RXRα in HEK293-based luciferase reporter gene assays.

The set of neolignans can be divided into 36 mainly semisynthetic analogues of honokiol which were obtained from the collaborating group of phytochemists in Innsbruck as a kind gift originating from Dr. Wolfgang Schühly (Karl-Franzens-Universität- Graz, Austria). The remaining 21 samples were synthesized *de novo* with an innovative photo-coupling strategy by our collaboration partners from the TU Vienna. In addition to their reported activity as PPARγ agonists, neolignans have also been reported as modulators of RXRα (Kotani et al., 2010; Zhang et al., 2011). One of the goals of the present study is to identify derivatives with improved biological activity in our test system and to also use the harvested data to further refine the *in silico* model that has led to the identification of magnolol and honokiol as PPARγ agonists in our recent publications (Atanasov et al., 2013b; Fakhrudin et al., 2010).

Experimental

Received compounds were dissolved in DMSO, aliquoted and subjected to testings towards PPARγ and RXRα in the luciferase reporter gene assay at a concentration of 10 µM. Threshold value for activity towards PPARγ was set 2.5-fold induction over solvent vehicle, while threshold value for activity towards RXRα was set to 10-fold induction over solvent vehicle. For those derivatives yielding activities above the threshold values EC_{50} and E_{max} values were determined in dose-response experiments. The obtained results have been forwarded to the collaborating chemoinformatical group in Innsbruck to support their computational modeling and to the group of organic chemists in Vienna as

a decision support for their future synthesis of neolignans as PPARγ and RXRα modulators. Used positive controls were the prototypical insulin-sensiting full PPARγ agonist piolitazone (5 µM) and the full RXR-agonist retinoic acid (5 µM).

	PPARγ			**RXRα**		
Compound ID	**Initial activity at 10 µM**	**EC_{50} (µM)**	**E_{max}**	**Initial activity at 10 µM**	**EC_{50}(µM)**	**E_{max}**
9-cis retinoic acid (5 µM)	*n.d.*	*n.d.*	*n.d.*	*37*	*0.054*	*37.98*
Pioglitazone (5 µM)	*6.9*	*0.196*	*9.25*	*n.d.*	*n.d.*	*n.d.*
727 (Magnolol)	2.65	1.62	3.03	27.83	3.39	24
728 (Honokiol)	1.44	n.d.	n.d.	17.9	1.07	21.53
729 (Dieugenol)	3.57	0.62	3.58	1.29	n.d.	n.d.
730	3.61	0.33	3.34	1.14	n.d.	n.d.
752	1.67	n.d.	n.d.	23.46	0.69	24.32
753	2.83	3.55	2.8	19.46	3.69	18.1
754	2.99	3.38	2.77	1.47	n.d.	n.d.
755	1.44	n.d.	n.d.	1.32	n.d.	n.d.
756	1.25	n.d.	n.d.	1.48	n.d.	n.d.
757	1.25	n.d.	n.d.	1.84	n.d.	n.d.
758	1.94	n.d.	n.d.	4.61	n.d.	n.d.
759	1.71	n.d.	n.d.	1.74	n.d.	n.d.
760	1.15	n.d.	n.d.	1.06	n.d.	n.d.

761	0.98	n.d.	n.d.	1.01	n.d.	n.d.
762	1.91	n.d.	n.d.	20.36	0.73	20.93
763	2.20	n.d.	n.d.	20.78	1.98	20.89
764	1.14	n.d.	n.d.	2.32	n.d.	n.d.
765	1.43	n.d.	n.d.	18.47	9.38	33.09
766	1.82	n.d.	n.d.	0.83	n.d.	n.d.
767	1.40	n.d.	n.d.	2.42	n.d.	n.d.
768	1.60	n.d.	n.d.	3.63	n.d.	n.d.
769	1.27	n.d.	n.d.	4.45	n.d.	n.d.
770	1.06	n.d.	n.d.	0.86	n.d.	n.d.
771	1.08	n.d.	n.d.	1.36	n.d.	n.d.
772	1.73	n.d.	n.d.	2.83	n.d.	n.d.
773	1,75	n.d.	n.d.	1.13	n.d.	n.d.
774	1.72	n.d.	n.d.	8.57	n.d.	n.d.
775	2.03	n.d.	n.d.	7.15	n.d.	n.d.
776	2.65	2.45	2.7	2.16	n.d.	n.d.
777	1.99	n.d.	n.d.	21.84	3.04	21.46
778	0.77	n.d.	n.d.	1.13	n.d.	n.d.
779	1.25	n.d.	n.d.	16.97	8.19	25.67
780	2.01	n.d.	n.d.	2.51	n.d.	n.d.
781	1.58	n.d.	n.d.	18.38	0.47	21.62
782	0.96	n.d.	n.d.	25.13	3.81	25.1
783	1.19	n.d.	n.d.	2.63	n.d.	n.d.
2813	1.48	n.d.	n.d.	3.69	n.d.	n.d.
2814	1.84	n.d.	n.d.	1.49	n.d.	n.d.
2815	1.49	n.d.	n.d.	19.32	1.03	21.76
2816	3.02	3.36	2.87	22.28	5.24	23.81

2817	2.21	n.d.	n.d.	22.04	0.59	21.97
2818	1.67	n.d.	n.d.	7.71	n.d.	n.d.
2279	1.19	n.d.	n.d.	4.7	n.d.	n.d.
2280	1.09	n.d.	n.d.	6.48	n.d.	n.d.
2281	1.51	n.d.	n.d.	23.46	6.35	27.2
2282	1.27	n.d.	n.d.	2.02	n.d.	n.d.
2283	0.95	n.d.	n.d.	1.23	n.d.	n.d.
2284	1.18	n.d.	n.d.	12.1	0.41	15.2
2285	1.01	n.d.	n.d.	27.4	4.21	15.2
2286	0.68	n.d.	n.d.	1.42	n.d.	n.d.
2287	1.10	n.d.	n.d.	19.91	2.19	20.11
2288	1.28	n.d.	n.d.	1.36	n.d.	n.d.
2956	1.10	n.d.	n.d.	1.11	n.d.	n.d.
2957	2.45	n.d.	n.d.	24.82	1.08	26.77
2958	1.33	n.d.	n.d.	23.12	7.86	33.41
3097	2.65	4.27	3.02	0.96	n.d.	n.d.
3098	4.11	0.88	4.39	0.98	n.d.	n.d.

Table 5: Initial biological activities at a test concentration of 10 µM, determined EC_{50} and E_{max} values towards PPARγ and RXRα for the set of neolignans. Structures of the compounds can be found in the Appendix chapter of this work.

Discussion

From the 57 tested samples a total of 14 samples showed activity above 2-fold induction over solvent vehicle treatment towards PPARγ and 20 samples showed activity above 10-fold induction over solvent vehicle treatment towards RXRα. Six of those samples (2816, 2817, 2957, 727,752, 763) were identified as dual agonists.

For four of the tested derivatives (727-730) the biological activity towards PPARγ has already been reported in our previous studies (Atanasov et al., 2013b; Fakhrudin et al.,

2010) and could be reproduced: Honokiol (728) was verified to be active towards PPARγ, and also the known RXRα agonism (Kotani et al., 2010) was reproducible in our test system, the same is true for the dual agonist Magnolol (727) (Zhang et al., 2011). Interestingly, no activity towards RXRα was observed for the known PPARγ agonists dieugenol (729) and tetrahydrodieugenol (730).

In the vast majority of derivatives the benzyl rings are directly connected, there are only two derivatives where the connection is bridged by an ether (783) or an alkyl (770), both derivatives show no activity towards PPARγ or RXRα above the threshold values in our biological test system. This suggests the possibility that directly connected neolignans are more promising as agonists of our targets compared to their bridged counterparts, although it would be necessary to test more bridged neolignan-derivatives to substantiate this structure-activity relationship.

From the 57 samples in the present set, only six samples do not possess any hydroxyl- (or acetyloxy-) groups that will supposedly impair bioavailability in *in vivo* testing, of those samples only dimethylhonokiol (779) showed activity and was characterized as an RXRα agonist with similar potency as honokiol. Consequently in view of rational drug discovery of drugs intended for oral use dimethylhonokiol is probably the most promising RXRα agonist that has been identified from this small set of neolignans. However, RXRα is generally not regarded as a very well druggable target considering its subordinate role in the activation of many permissive nuclear receptors, particularly stimulation of the closely related retinoic acid receptor (RAR) is reported to be undesirable (De Lera et al., 2007). Interestingly, honokiol is reported to selectively activate RXRα over RARα (Kotani et al., 2010). In turn it would also be interesting to determine if the same is also true for dimethylhonokiol. However, although likely more bioavailable the solubility of dimethylhonokiol will be worse compared to honokiol that is already not very well water-soluble.

The predictable low bioavailability of most derivatives in the used set of neolignans could be overcome by advanced drug formulation or parenteral application. Possibly also masking some of the hydroxyl groups, while leaving others intact (as it was done for many of the derivatives in the present set) can significantly improve bioavailability, but generally their depletion would be most desirable. Another possible option to overcome the problem with the vulnerable hydroxyl- (or acetyloxy-)groups would be the introduction of neighboring substituents on the benzene rings to provoke steric hindrances and thus render hydroxyl-groups protected from first-pass-metabolization. Some of the samples in the present set indeed have such structural features present, but the resulting derivatives are either inactive (e.g. 755, 756 or 757) or the substituent

is too small to likely qualify for steric hindrances (e.g. 729 or 730) or is susceptible to first pass effects itself (e.g. 754).

Two of the derivatives are considerably larger than their counterparts and can be considered as sesqui*-magnolol (3098) or sesqui-trimethylmagnolol (3097), respectively. Those derivatives can be understood as intermediates towards the synthesis of dimagnolol derivatives, while sesqui-trimethylmagnolol can be regarded as the protected intermediate of sesqui-magnolol. Such dual-magnolol-like derivatives could be interesting PPARγ agonists, considering that in our computational model two magnolol molecules cooperatively accommodate into the Y-shaped ligand-binding pocket of PPARγ (Fakhrudin et al., 2010). Interestingly, while sesqui-trimethylmagnolol was inactive in our test system, sesqui-magnolol exhibited highest activity towards PPARγ among all tested derivatives in the present set. This finding supports the intention of the collaborating organic chemists to develop the sesqui-magnolol into the corresponding dimagnolol, although the resulting derivatives will probably be even more difficult to dissolve in aqueous media than honokiol. However, although none of the experimenters reported observable precipitation for any of the samples, a certain level of caution will be adequate for derivatives that are more lipophilic than the ones tested.

An interesting, but inconclusive, SAR can be observed by studying the effect of side chain variations. Many of the derivatives have either propyl or propenyl units as their only structural difference. In case of dimethylhonokiol (779) a propenyl unit is present, while in a similar derivative (780) the propenyl unit has been (formally) reduced to a propyl unit and in turn the derivative has lost its activity towards RXRα. On the other hand, the reduced honokiol derivative (782) shows approx. 1.5 fold the activity towards RXRα of honokiol (728) with its propenyl structure present. Similar observations can be made for PPARγ: While reduction of dieugenol (729) or magnolol to the corresponding derivatives (730 and 753) had no effect on biological activity, reduced dimethylhonokiol (780) is about 1.5 fold more active than the parent structure (779).

Many of the tested synthetic samples (2279-2288 and 2956-2958) have the 2-propenyl,5-phenyl** skeleton present that is also the structural feature of honokiol's and magnolol's ring A. In these derivatives substituents were introduced on different positions on ring B, the degenerated derivative without substitutions on ring B (yielding

* The Latin term "sesqui" means "one-and-one-half times"

** To avoid confusion for this set of neolignans a numbering system is used that does not follow IUPAC nomenclature (Moss, 2001), but numbers the ring-connecting carbons consequently 1 and 1´.

a benzene for ring B) did not show any activity towards PPARγ or RXRα (2279). Also almost none of the introduced substitutions could induce activity of the derivatives towards PPARγ, the only two derivatives with two rings in the present set that have a 2-propenyl,5-phenyl structure of ring A and are active towards PPARγ are the parent structure magnolol (727) that consists of two 2-propenyl,5-phenyl units and a derivative with a 2'-methyl-benzene structure of ring B (2957). Interestingly, also the saturated magnolol derivative composed of two 2-propyl,5-phenyl units (753) is a dual agonist, but with slightly reduced biological activity in our test system. Also interestingly, from the four tested substituents for ring B used for the derivatives with magnolol's ring A, substitutions in *ortho*- positions tend to show no (2286, 2283, 2956) or impaired (2280) activity towards RXRα compared to magnolol, while substitutions in *meta*-positions mainly yielded active derivatives (2815, 2281, 2284 and 2287, while 2818 is inactive) and substitutions in *para*-positions yielded two RXRα-active (2285, 2958) and two RXRα-inactive derivatives (2288 and 2282).

Finally some considerations about the potency of active compounds from the tested set of neolignans: Among all tested derivatives the most potent sample for PPARγ is still tetrahydrodieugenol (730) (EC_{50} = 0.33 µM and E_{max}= 3.34 fold induction over solvent vehicle), a compound that was already tested in our previous study (Fakhrudin et al., 2010). While sesqui-magnolol (3098) yielded a higher E_{max} value of 4.39, it shows significantly reduced potency (EC_{50} = 0.88 µM). For RXRα several compounds with increased potency (752, 762, 781, 2817, 2284) could be identified compared to the parent structure honokiol (EC_{50} = 1.07 µM and E_{max}= 21.53) and those derivatives also have similar E_{max} values. The most potent among those with approx. half the EC_{50} value of honokiol is 781 (EC_{50} = 0.47 µM and E_{max}= 21.62), while one compound (2284) shows a lower EC_{50} value of 0.41 µM, but a considerably lower E_{max} value of 15.2 fold luciferase-induction over solvent vehicle treatment. However, considering that that the average potency of approved drugs is around 0.02 µM (Nicolaou, 2014) none of the samples qualifies as an excellent lead compound. This is not very surprising since around 1×10^4 molecules are usually to be synthesized before such a molecule is identified (Nicolaou, 2014).

I. Acknowledgements

First and most importantly I want to thank the financier of the costly academic infrastructure that has also nourished me during my doctoral studies at the University of Vienna, which is the taxpayer. Although the revenue of these expenses might not always be obvious, the value of discovering the truth should be self-evident.

Thank you also to my supervisors Univ. Prof. Dr. Verena M. Dirsch and Dr. Atanasov Atanasov and also to the many DNTI-collaboration partners who contributed to this work.

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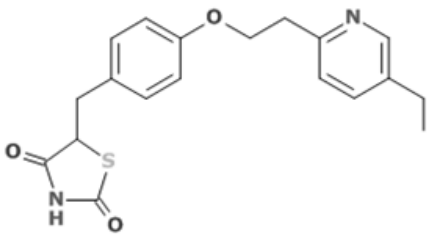
Also all colleagues in the Department of Pharmacognosy deserve appreciation for their efforts to make studying at the University of Vienna a more worthwhile endeavor, especially office colleague and team-technician Mia Gössinger and office colleague Linh Dieu Nguyen.

Please grant me the serenity to accept these things I cannot change, courage to change those things I can and the wisdom to always know the difference.

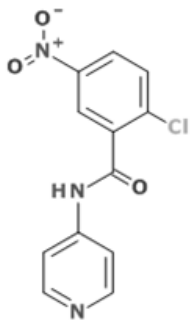
II. Structures:

all of the following structures were prepared using Accelrys Draw (Bioviva, California, USA).

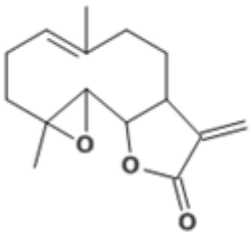
Positive controls and antagonists



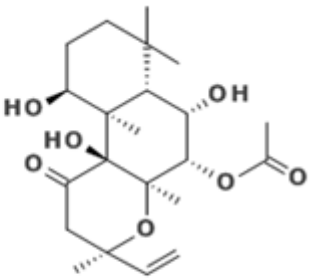
Pioglitazone



Antagonist T0070907

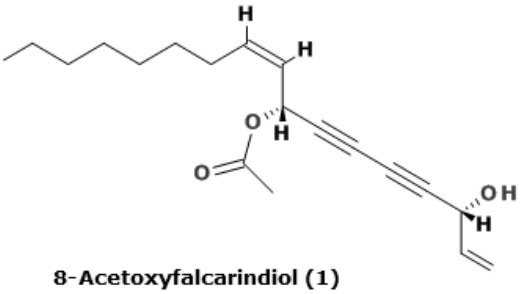


Parthenolide

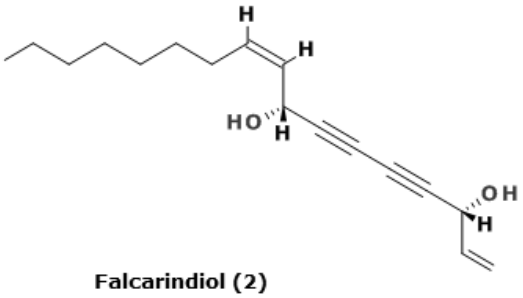


Forskolin

Polyacetylenes from *Notopterygium incisum* as partial PPARγ agonists



8-Acetoxyfalcarindiol (1)



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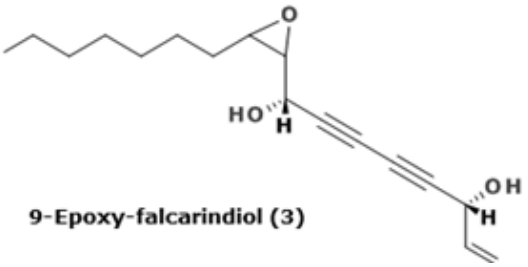
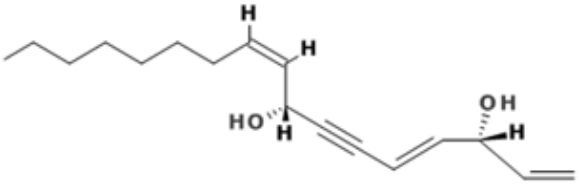
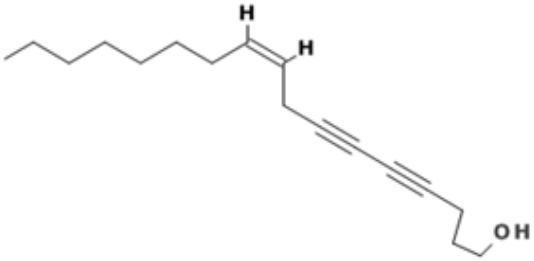


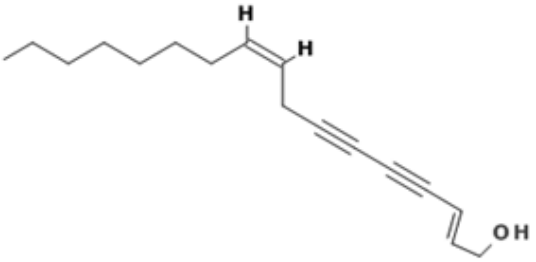
9-Epoxy-falcarindiol (3)



Crithmumdiol (4)

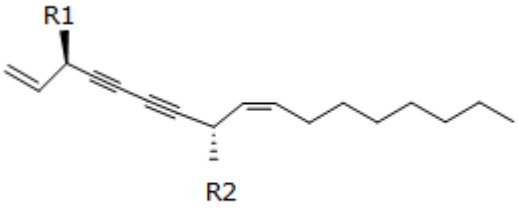


9Z-Heptadecene-4,6-diyne-1-ol (5)

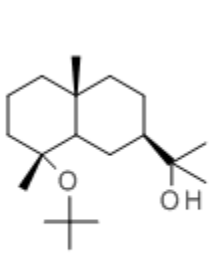


2Z, 9Z-Heptadecene-4,6-diyne-1-ol (6)

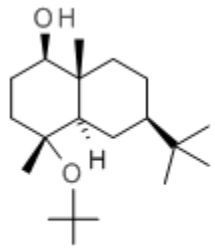
Polyyne hybrid compounds from *Notopterygium incisum* with peroxisome proliferator-activated receptor gamma agonistic effects.



R1R2
1AOH
2OHA
3BOH
4OHB
5COH
6OHC
7DOH
8OHD



A



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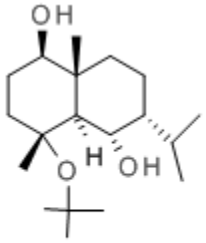

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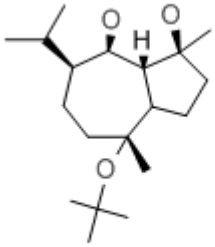
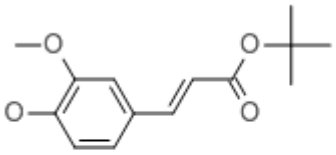


D



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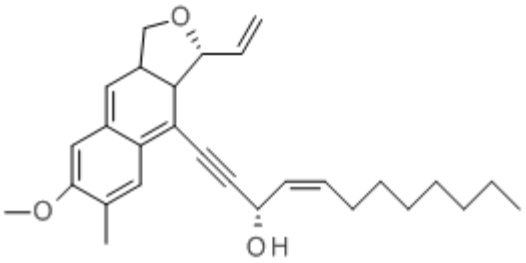

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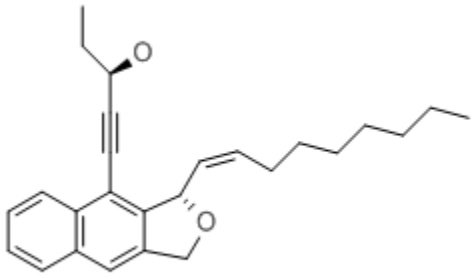
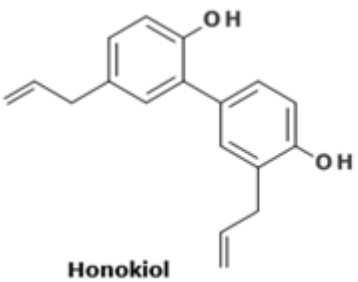
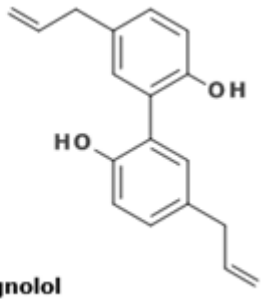


11

Honokiol: A non-adipogenic PPARγ agonist from nature

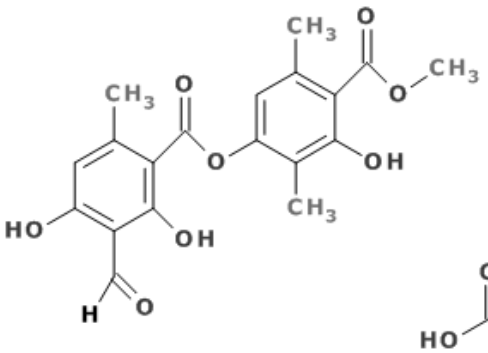


Honokiol

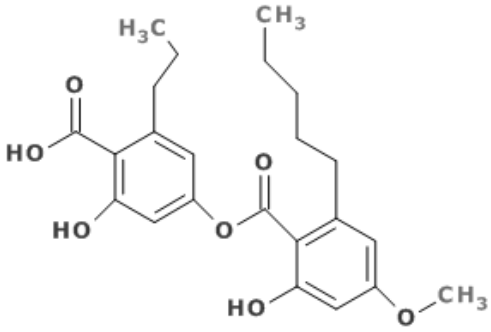


Magnolol

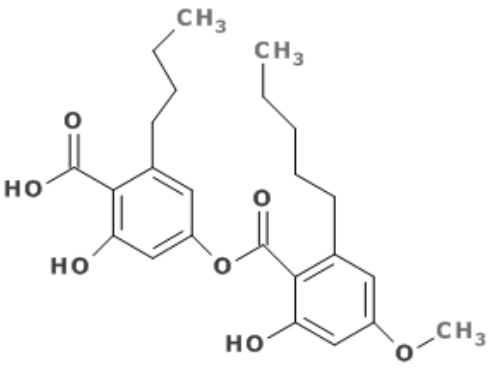
Imbricaric Acid and Perlatolic Acid: Multi-targeting Anti-Inflammatory Depsides from Cetrelia monachorum (Zahlbr.)



Atranorin

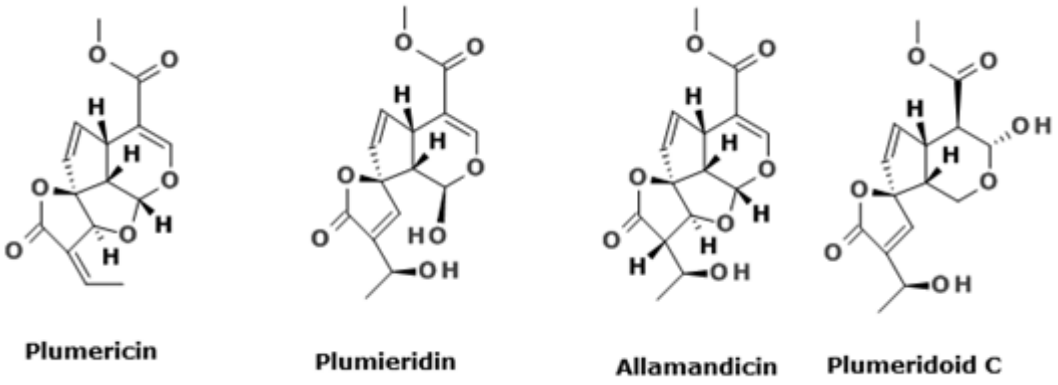


Imbricaric acid

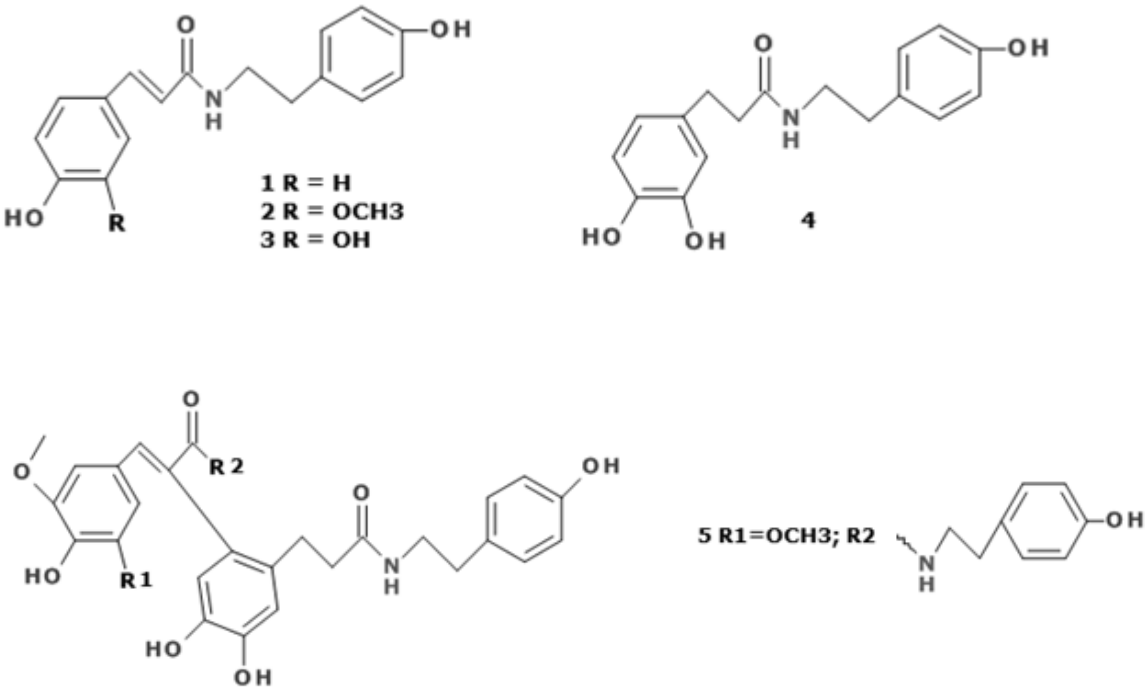


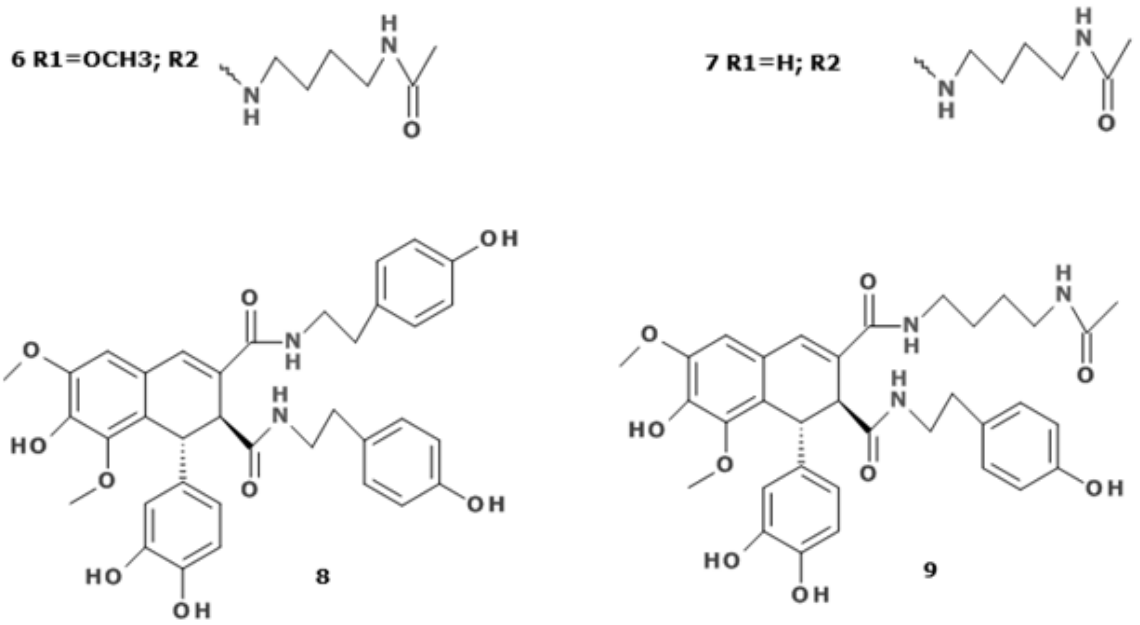
Perlatonic acid

Identification of plumericin as a potent new inhibitor of the NF-κB pathway with anti-inflammatory activity in vitro and in vivo.

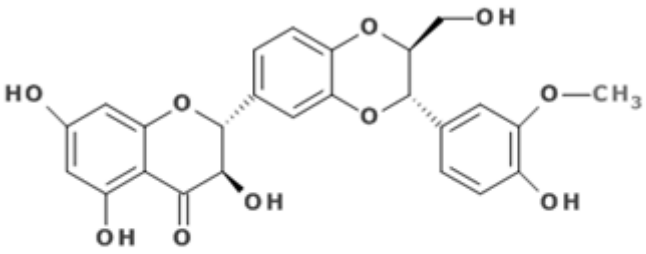


Activity-guided isolation of NF-κB inhibitors and PPAR agonists from the root bark of Lycium chinense Miller

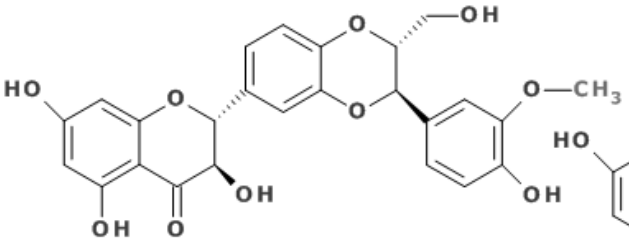




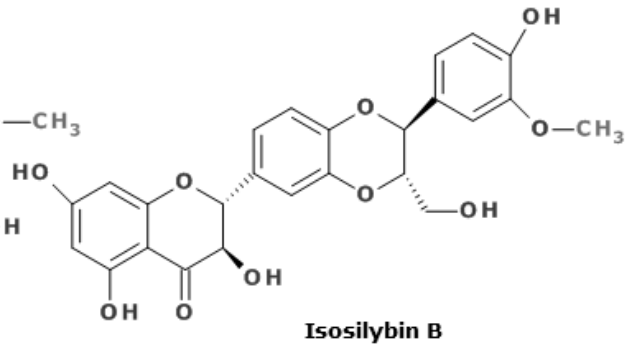
Identification of isosilybin a from milk thistle seeds as an agonist of peroxisome proliferator-activated receptor gamma.



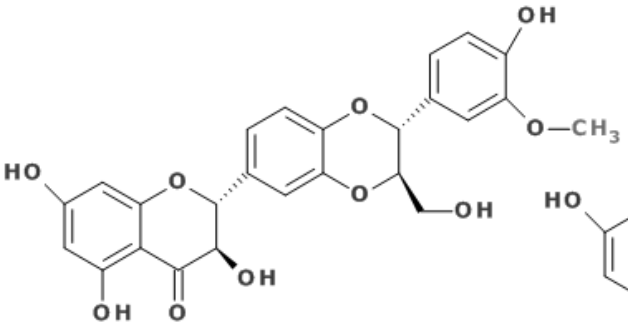
Silybin B



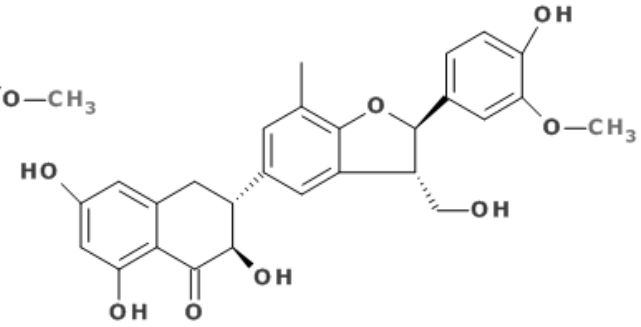
Silybin A



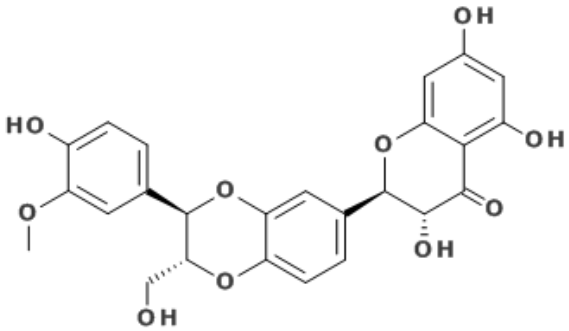
Isosilybin B



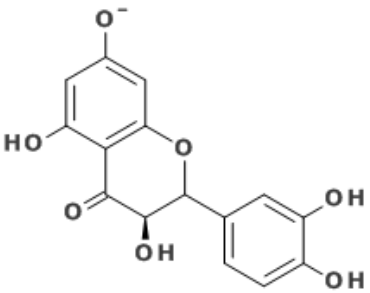
Isosilybin A



Silychristin

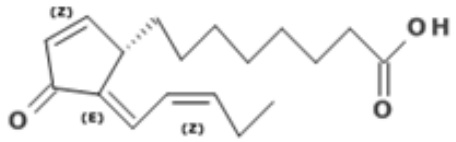


Silydianin

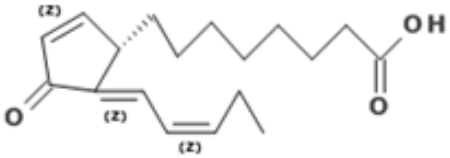


(+) - Taxifolin

Identification of chromomoric acid C-I as an Nrf2 activator in *Chromolaena odorata*.



1 - chromoric acid C-I



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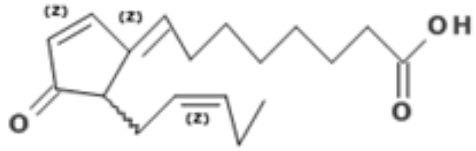

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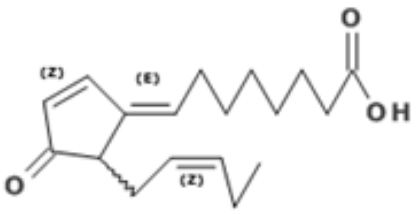

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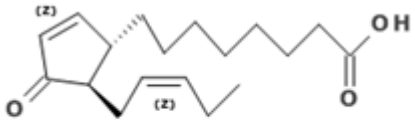
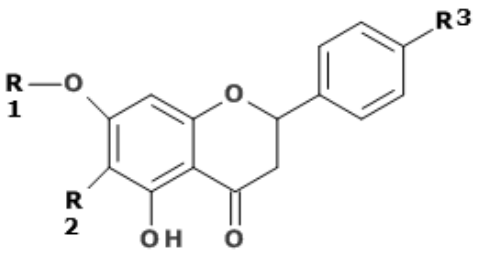


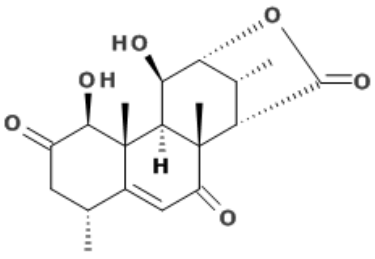
5

Screening of Vietnamese medicinal plants for NF-κB signaling inhibitors: assessing the activity of flavonoids from the stem bark of Oroxylum indicum.

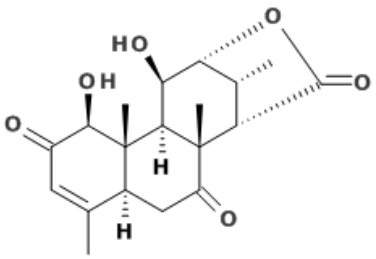


1. Oroxlin A	R1 = H	R2 = OCH3	R3 = H
2. Chrysin	R1 = H	R2 = H	R3 = H
3. Baicalein	R1 = H	R2 = OH	R3 = H
4. Hispidulin	R1 = H	R2 = OH	R3 = OH
5. Oroxylin A-7-O-beta-D-glucoronide			
	R1 = glucoronic acid	R2 = OCH3	R3 = H
6. Chrysin-7-O-beta-D-glucuronide			
	R1 = glucoronic acid	R2 = H	R3 = H
7. Baicalein-7-O-beta-D-glucoside			
	R1 = glucose	R2 = OH	R3 = H
8. Baicalin	R1 = glucoronic acid	R2 = OH	R3 = H
9. Oroxylin A-7-O-beta-D-methyl glucuronide			
	R1 = methyl glucuronide	R2 = OCH3	R3 = H
10. Baicalein A-7-O-beta-D-methyl glucuronide			
	R1 = methyl glucuronide	R2 = OH	R3 = H

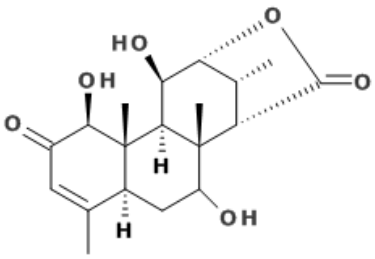
NF-κB inhibitors from *Eurycoma longifolia*.



1



2 (Eurycomalactone)



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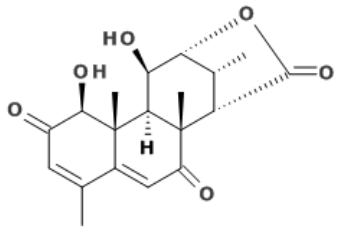

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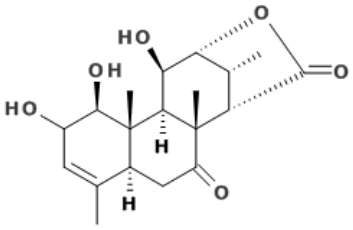

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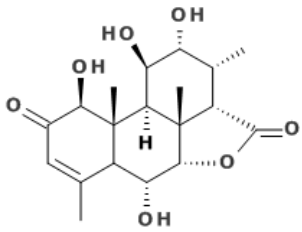

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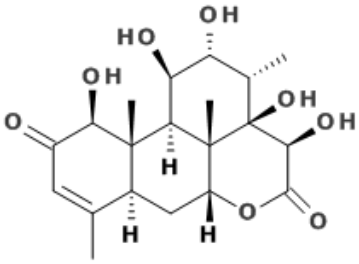

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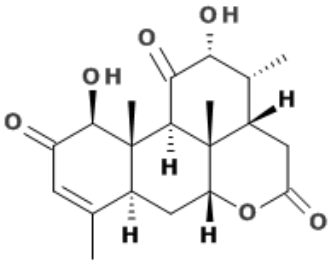

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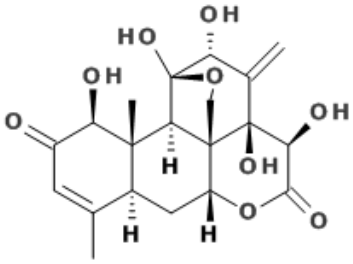

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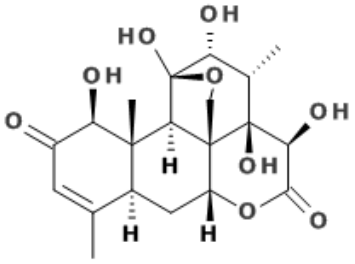

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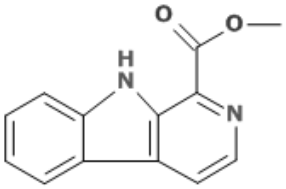

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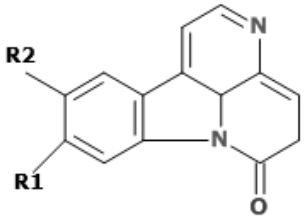
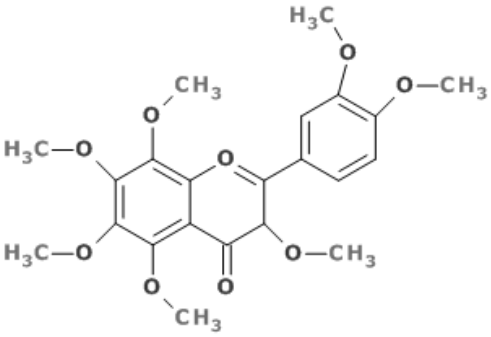
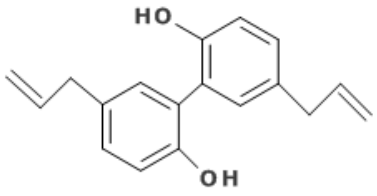


14: R1 = OH, R2 = H
15: R1 = OCH3 R2 = H
16: R1 = OCH3 R2 = OCH3

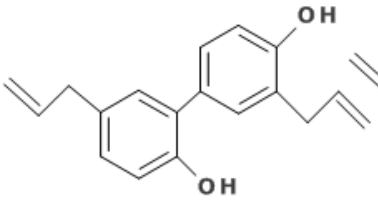


28

(Semi)synthetic neolignans as agonists of PPARγ and RXRα



727 (Magnolol)



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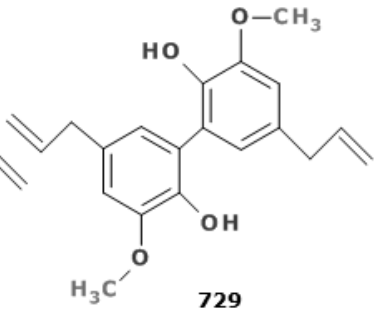

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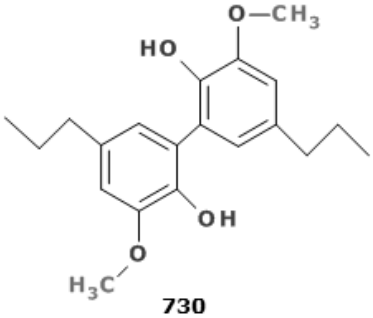

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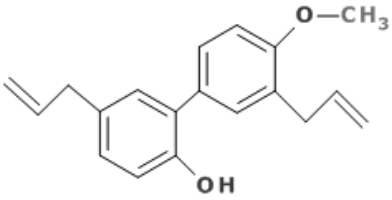

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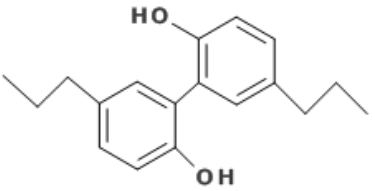

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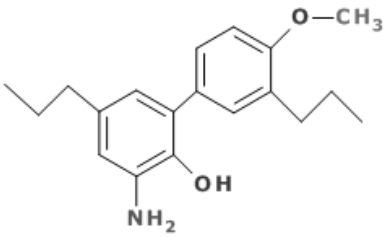

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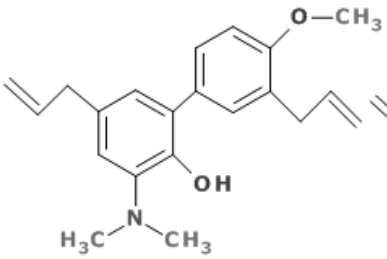

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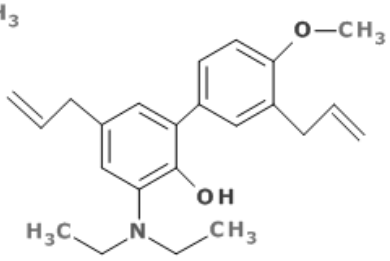

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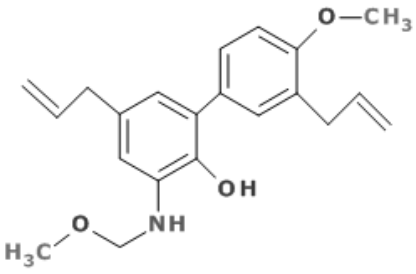

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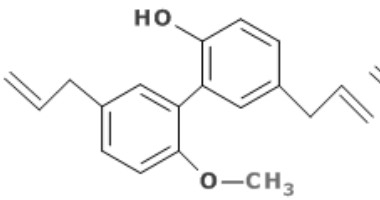

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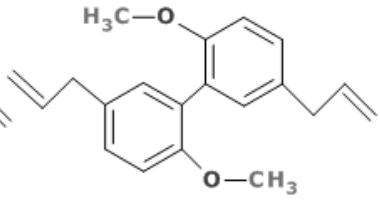

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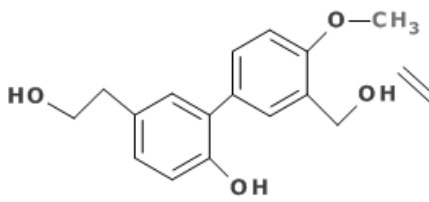

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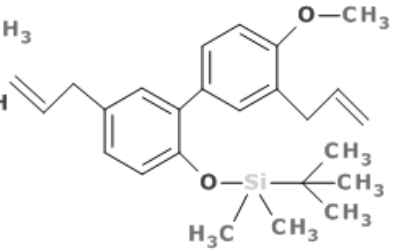

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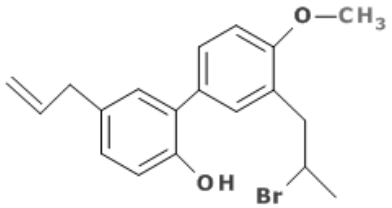

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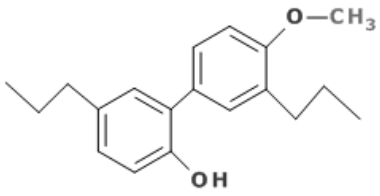

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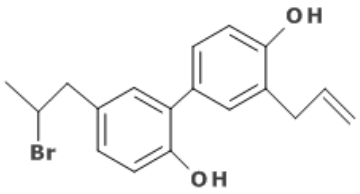

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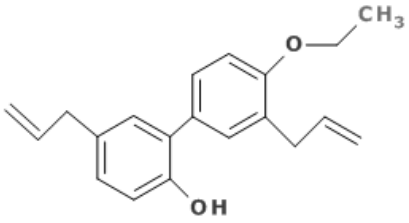

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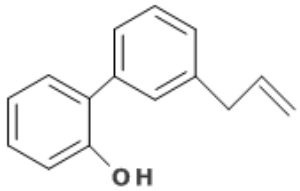

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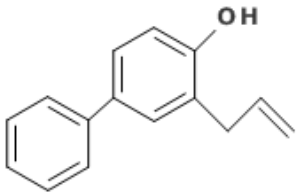

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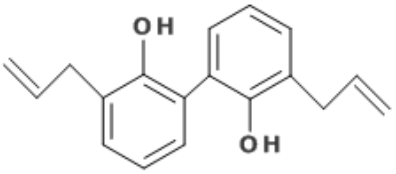

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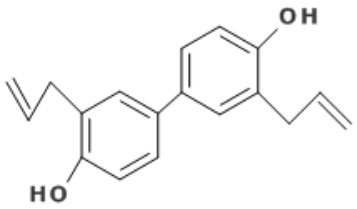

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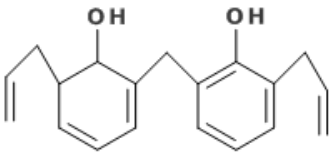

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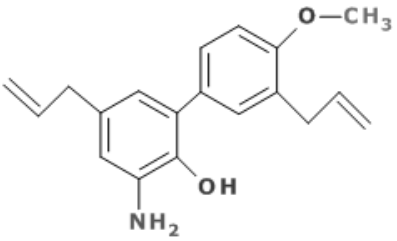

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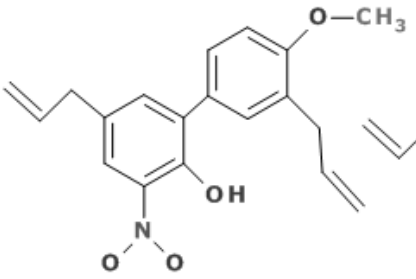

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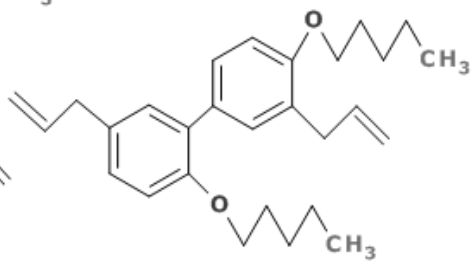

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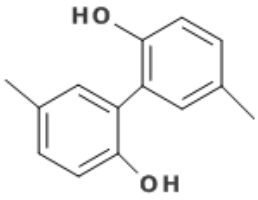

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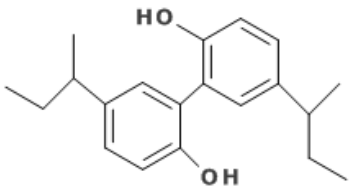

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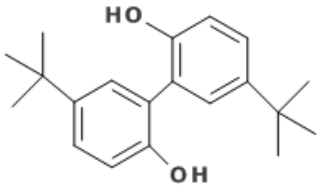

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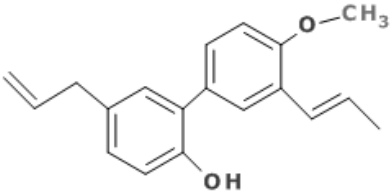

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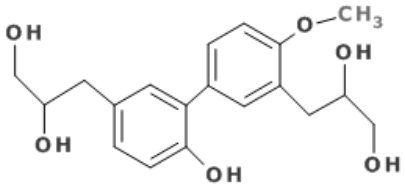

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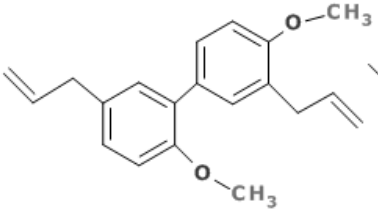

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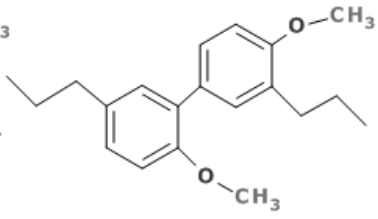

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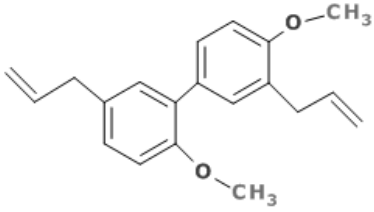

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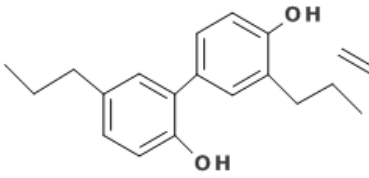

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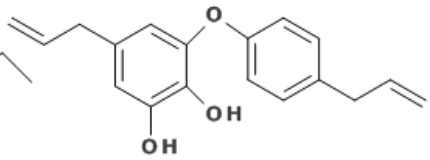

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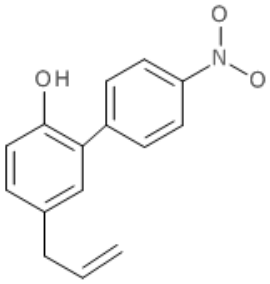

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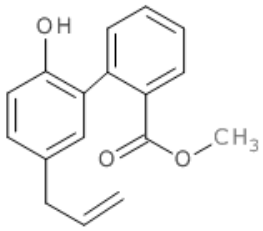

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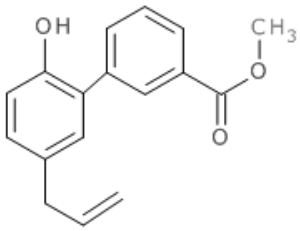

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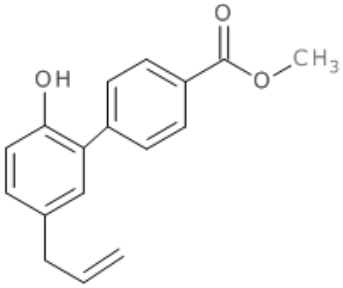

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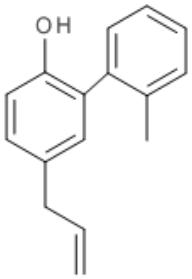

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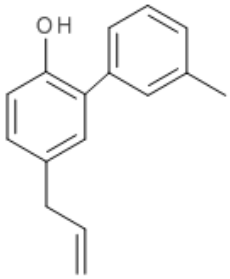

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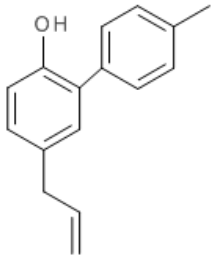

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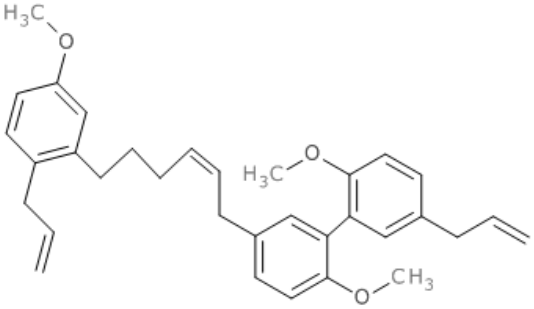

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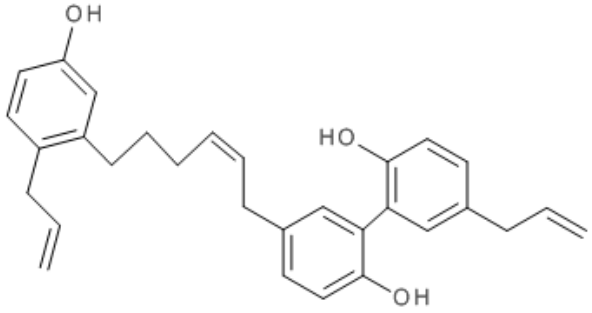

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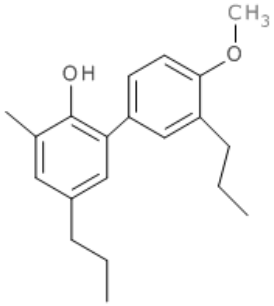

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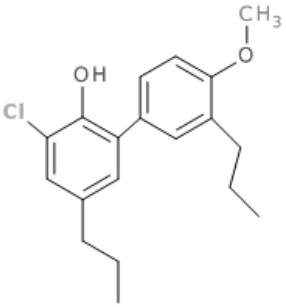

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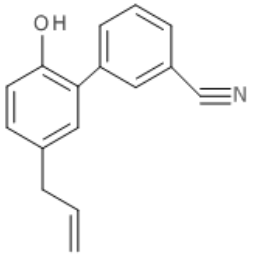

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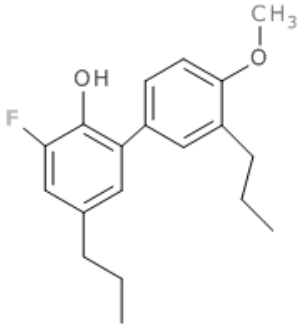

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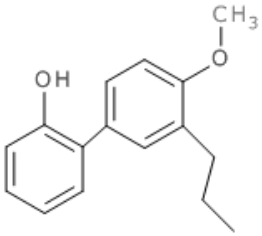

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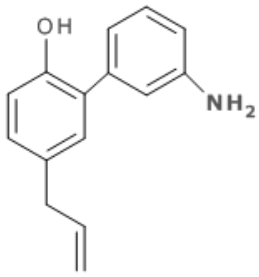

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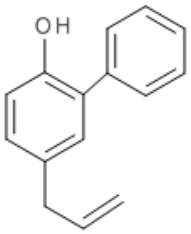

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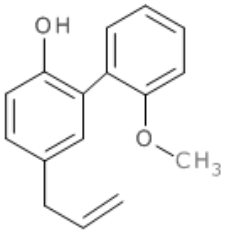

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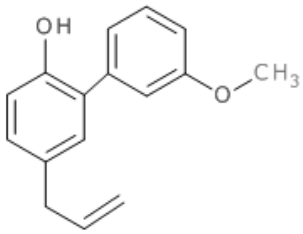

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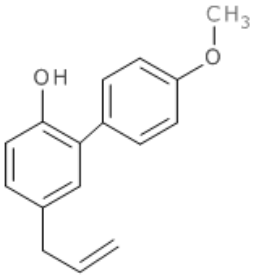

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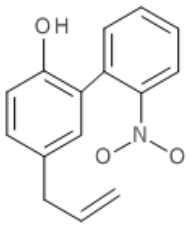

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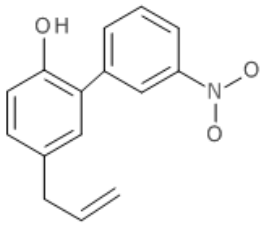

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III. List of Abbreviations (in alphabetical order, abbreviations used in the provided references are not included):

ATP.....adenosine triphosphate; AG.....Aktiengesellschaft (German for public limited company); **B**CA.....bicinchoninic acid; BSA…..bovine serum albumin; **c**AMP.....cyclic adenosine monophosphate; CC.....coiled-coil domain; cpm.....counts per minute; CMC.....chronic myoloid cancer; CMFDA....5-chloromethylfluorescein diacetate, CTA.....clinical trial application; CV.....curriculum vitae; **D**C.....dentric cells; DCM.....dichloromethane; DD....death domain; DI.....Diplomingeneur (German for graduate engineer); DMEM.....Dulbecco's Modified Eagle Medium; DMSO.....dimethyl sulfoxide; DNA.....deoxyribonucleic acid; DNTI.....Drugs from Nature Targeting Inflammation; Dr. rer. nat.....*doctor rerum naturalium* (Latin for Doctor of Natural Sciences); **e**.g.....*exempli gratia* (Latin for at example); EDTA....ethylenediaminetetraacetic acid; EGFP.....enhanced green fluorescent protein; ELISA.....enzyme linked immunosorbent Assay; E-selectin….. endothelial adhesion molecule 1; et al.....et alia (Latin for and others); EtOH.....ethanol; **F**ACS.....fluorescence assisted cell sorting; FBS.....fetal bovine serum; FH.....Fachhochschule (German for University of Applied Sciences); FITC.....fluorescein isothiocyanate;FRET.....flourescence resonance energy transfer FWF.....Fonds zur Förderung der wissenschaftlichen Forschung (German for Fund for Support of Scientific Research) **G**RIP.....glutamate receptor-interacting protein; GmbH.....Gesellschaft mit beschränkter Haftung (German for limited company); GSK.....GlaxoSmithKline; **H**EK.....human embryonic kidney; HIV....human immunodeficiency virus; HMG-CoA.....3-hydroxy-3-methylglutaryl-coenzyme A; HRP.....horseradish peroxidase; hTERT..... human telomerase reverse transcriptase; HTS.....high throughput screening; HUVEC.....human umbilical vein endothelial cells; **I**CAM-1…..intercellular adhesion molecule 1; IκB.....inhibitor of NF-κB; IKK.....IκB kinase; IL-1.....interleukin 1; **L**B.....liquid broth; LBD.....ligand binding domain; lt.....laut (German for according to); LZ.....leucin zipper; **M**eOH…..methanol; MS.....mass spectrometry; MAPK.....mitogen-activated protein kinase; MEF.....mouse embryonic fibroblast; MHRA..... United Kingdom's Medicines Healthcare Products Regulatory Agency; mTOR.....mechanistical target of rapamycin; **N**K.....natural killer; Nrf2.....Nuclear factor (erythroid-derived 2)-like 2; NF-κB.....nuclear factor kappa-light-chain-enhancer of activated B cells; **O**D_{590}.....optical density at 590 nm; **P**AGE…..polyacrylamide gel electrophoresis; PBS.....phosphate buffered saline; PMA.....phorbol-12-myristate-13-acetate; pnp.....p-nitrophenyl phosphate; PPAR.....peroxisome proliferator-activated receptor; PRC....People's Republic of China; PVDF.....polyvinylidene difluoride; **R**AR.....retinoic acid receptor; RHD.....Rel homology domain; RIP..... receptor interacting protein; RNA.....ribonucleic acid; RXR.....retinoid X receptor; **S**AR.....structure-activity-relationship; SD……standard deviation; SEM…..standard error of the mean; SDS.....sodium dodecyl sulfate;

SRM.....Selected Reaction Monitoring; **T**LR…..toll-like receptor; TMB.....3,3´,5,5´tetramethylbenzidin; TNF....tumor necrosis factor; TR.....time resolved; TRAF.....TNF receptor associated factor; TU.....Technische Universität (German for Technical university); **U**niv. Prof.....Universitätsprofessor (German for university professor); USD.....United States dollar; UV.....ultraviolet; **V**CAM-1…..vacular cell adhesion molecule 1; **W**ADA.....world anti-doping agency; WGKK.....Wiener Gebietskrankenkasse (German for Viennese Regional Health Insurance Fund); **Z**F.....zinc finger

IV. List of suppliers (alphabetical order of the items)

Apparatus

Bacterial incubator:	Incubator Hood TH 30 (Edmund Bühler, Hechingen, Germany)
Cell culture incubator:	Hera Cell 150 Incubator (Heraeus, Hanau bei Frankfurt, Germany)
Cell counter:	VI-Cell (Beckmann Coultier, Vienna, Austria)
Centrifuge:	Heraeus Multifuge 1 S-R (Heraeus, Hanau bei Frankfurt, Germany)
Flow cytometer:	BD FACS Excalibur (BD Biosciences, New Jersey, USA)
Laminar flow cabinets	Herasafe ((Thermo Fisher Scientific Biosciences GmbH, Oberhausen, Germany)
Liquid scintillation counter:	LSA 307 (Perkin Elmer, Waltham, Massachusetts, USA)
Luminescent image analyzer:	LAS 3000 (Fujifilm, Tokyo, Japan)
Ultracentrifuge:	Sorvall RC 5C Plus Ultracentrifuge (Thermo Fisher Scientific Biosciences GmbH, Oberhausen, Germany)
Photometers:	Nanodrop 2000 (Thermo Fisher Scientific Biosciences GmbH, Oberhausen, Germany)
	TECAN GENios pro (Tecan, Mannedorf, Switzerland)

Chemicals and biologicals

Chemicals used for the experiments described within this work, but not mentioned in the following list were either purchased from Carl Roth, (Karlsruhe, Germany) or from Sigma-Aldrich Handels GmbH (Vienna, Austria) in the highest purity available. All of the listed chemical were also purchased in the highest purity available.

2-Deoxy-D-glucose	Sigma Aldrich (Vienna, Austria)
2-deoxy-D-(1H^3)-glucose	Sigma Aldrich (Vienna, Austria)
9-cis-retinoic acid	Enzo (Lausen, Switzerland)
ATP	Sigma Aldrich (Vienna, Austria)
anti-IκB-α	
anti-phospho-IKK-β	Cell Signaling Technology (Danvers,
anti-phospho-IκB-α	Massachusetts, USA)
anti-p65	
anti-actin	MP Biomedicals (Illkirch, France)
anti-tubulin	Santa Cruz Biotechnology (Santa Cruz, California, USA)
anti-lamin	Abcam (Cambridge, UK)
BCA master mix	Thermo Fisher Scientific Biosciences GmbH (Oberhausen, Germany)
BSA	Sigma Aldrich (Vienna, Austria)
Coenzyme A	Sigma Aldrich (Vienna, Austria)
DMEM	Lonza (Basel, Switzerland)
Endothelial growth medium EBM™	Lonza (Basel, Switzerland)
EBM™ single quots	Lonza (Basel, Switzerland)
FBS	Thermo Fisher Scientific Biosciences GmbH(Oberhausen, Germany)
FITC antibodies	BD Biosciences, New Jersey, USA
Hexaquart™	B. Braun Melsungen AG (
LB-medium	Sigma Aldrich (Vienna, Austria)
Luciferin	Sigma Aldrich (Vienna, Austria)
NBS	Lonza (Basel, Switzerland)
Resazurin	Sigma Aldrich (Vienna, Austria)
RotiQuant	Carl Roth (Karlsruhe, Germany)
TNFα	Sigma Aldrich (Vienna, Austria)
Trypsin	Carl Roth (Karlsruhe, Germany)
Yeast-extract	Carl Roth (Karlsruhe, Germany)

Kits

LanthaScreen® TR-FRET PPAR gamma Competitive Binding Assay Kit distributed by Invitrogen (Lofen, Austria)

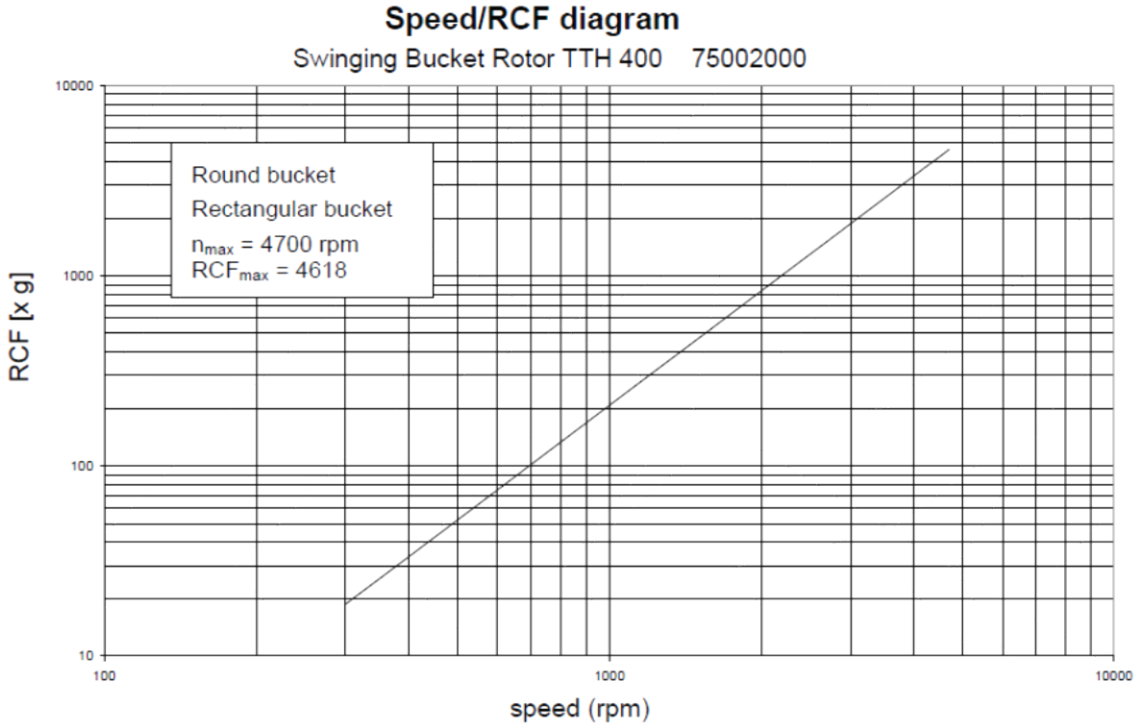
Direct cAMP ELISA kit distributed by Enzo (Lausen, Switzerland)

TransAM™ NF-κB p65 kit distributed by Active Motif (Vienna, Austria)

V. Graph for g-force conversion

Speed/RCF diagrams

* n_{max} = max. speed



VI. Curriculum vitae

Persönliche Daten

Name:	Clemens Malainer
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E-mail:	c_malainer@hotmail.com

Schulausbildung:

Handelsakademie Wiener Neustadt

08/1997- 07/2002 Matura mit "ausgezeichnetem Erfolg"

Studienverlauf:

Fachhochschulstudium "Biotechnologie" mit Schwerpunkt "Wirkstoffchemie" am FH Campus Wien

09/2003 – 10/2007 Erlangung des akadem. Titels "DI(FH)" mit "gutem Erfolg"

Titel der Diplomarbeit: Synthesis of Chitosan derivatives- Investigation of N-[1-carboxymethyl-2-quaternary ammonium] substituted chitosan

Masterstudium "Lebensmittelwissenschaften und -technologie" an der BOKU Wien

04/2008 – 01/2011 Erlangung des akadem. Titels "DI" mit "ausgezeichnetem Erfolg"

Titel der Masterarbeit: Enzymatic synthesis and investigation of novel galacto-oligosaccharides

Doktorratsstudium "Pharmazie" an der Universität Wien

seit 06/2011 zur Erlangung des akadem. Titels "Dr. rer. nat."

Titel der Dissertation: Natural products as nuclear receptor modulators and inhibitors of NF-κB activation

Auslandsaufenthalte zu Studienzwecken

08/2006 – 07/2007 Absolvierung eines halbjährigen Pflichtpraktikums an der Háskoli Islands, Reykjavik, Island, Department for Medicinal Chemistry, anschließend Verlängerung des Aufenthalts für das Diplomarbeitsprojekt

Publikationsliste (Korrespondenzautoren sind unterstrichen)

1. tert-Butyldimethylsilyl O-protected chitosan and chitooligosaccharides: useful precursors for N-modifications in common organic solvents

Ögmundur Vidar Rúnarsson, ***Clemens Malainer****, Jukka Holappa, Snorri Th. Sigurdsson and Már Másson,* published in Carbohydrate Research, Volume 343, Issue 15, 13 October **2008**, Pages 2576–2582

2. Antibacterial activity of N-quaternary chitosan derivatives: Synthesis, characterization and structure activity relationship (SAR) investigations

Ögmundur Vidar Rúnarsson, Jukka Holappa, ***Clemens Malainer****, Hákon Steinsson, Martha Hjálmarsdóttir, Tapio Nevalainen and Már Másson* published in European Polymer Journal, Volume 46, Issue 6, June **2010**, Pages 1251–1267

3. Honokiol: a non-adipogenic PPARγ agonist from nature

Atanas G. Atanasov, Jian N. Wang, Shi P. Gu, Jing Bu, Matthias P. Kramer, Lisa Baumgartner, Nanang Fakhrudin, Angela Ladurner, ***Clemens Malainer****, Anna Vuorinen, Stefan M. Noha, Stefan Schwaiger, Judith M. Rollinger, Daniela Schuster, Hermann Stuppner, Verena M. Dirsch and Elke H. Heiss* published in Biochimica et Biophysica Acta (BBA) - General Subjects, Volume 1830, Issue 10, October **2013**, Pages 4813–4819

4. Polyacetylenes from Notopterygium incisum–new selective partial agonists of peroxisome proliferator-activated receptor-gamma

Atanas G. Atanasov, Martina Blunder, Nanang Fakhrudin, Xin Liu, Stefan M. Noha, ***Clemens Malainer****, Matthias P. Kramer, Amina Cocic, Olaf Kunert, Andreas Schinkovitz, Elke H. Heiss, Daniela Schuster, Verena M. Dirsch and Rudolf Bauer* published in PLOSone, published: April 22, **2013** DOI: 10.1371/journal.pone.0061755

5. Imbricaric Acid and Perlatolic Acid: Multi-Targeting Anti-Inflammatory Depsides from Cetrelia monachorum

Sarah K. Oettl, Jana Gerstmeier, Shafaat Y. Khan, Katja Wiechmann, Julia Bauer, Atanas G. Atanasov, ***Clemens Malainer,*** *Ezzat M. Awad, Pavel Uhrin, Elke H. Heiss, Birgit Waltenberger, Daniel Remias, Johannes M. Breuss, Joel Boustie, Verena M. Dirsch, Hermann Stuppner, Oliver Werz and Judith M. Rollinger* published in PLOSone, published: October 09, **2013** DOI: 10.1371/journal.pone.0076929

6. Identification of isosilybin a from milk thistle seeds as an agonist of peroxisome proliferator-activated receptor gamma

Eva-Maria Pferschy-Wenzig, Atanas G. Atanasov, ***Clemens Malainer****, Stefan M. Noha, Olaf Kunert, Daniela Schuster, Elke H. Heiss, Nicholas H. Oberlies, Hildebert Wagner, Rudolf Bauer and Verena M. Dirsch* published in Journal of Natural Products, **2014**, 77 (4), pp 842–847

7. Activity-guided isolation of NF-κB inhibitors and PPARγ agonists from the root bark of Lycium chinense Miller

Lian-Wu Xie, Atanas G. Atanasov, De-An Guoc, ***Clemens Malainer****, Jing-Xian Zhang, Martin Zehl, Shu-Hong Guan, Elke H. Heiss, Ernst Urban, Verena M. Dirsch and Brigitte Kopp* published in Journal of Ethnopharmacology, Volume 152, Issue 3, 28 March **2014**, Pages 470–477

8. Natural product agonists of peroxisome proliferator-activated receptor gamma (PPARγ): a review

Limei Wang, Birgit Waltenberger, Eva-Maria Pferschy-Wenzig, Martina Blunder, Xin Liu, ***Clemens Malainer****, Tina Blazevic, Stefan Schwaiger, Judith M. Rollinger, Elke H. Heiss, Daniela Schuster, Brigitte Kopp, Rudolf Bauer, Hermann Stuppner, Verena M. Dirsch and Atanas G. Atanasov* published in Biochemical Pharmacology, Volume 92, Issue 1, 1 November **2014**, Pages 73–89

9. NF-κB Inhibitors from Eurycoma longifolia

Thi Van Anh Tran, ***Clemens Malainer****, Stefan Schwaiger, Atanas G. Atanasov, Elke H. Heiss, Verena M. Dirsch, and Hermann Stuppner* published in Journal of Natural Products, **2014**, 77 (3), pp 483–488

10. Identification of plumericin as a potent new inhibitor of the NF-κB pathway with anti-inflammatory activity in vitro and in vivo

Nanang Fakhrudin, Birgit Waltenberger, Muris Cabaravdic, Atanas G. Atanasov, ***Clemens Malainer****, Daniel Schachner, Elke H. Heiss, Rongxia Liu, Stefan M. Noha, Anna M. Grzywacz, Judith Mihaly-Bison, Ezzat M. Awad, Daniela Schuster, Johannes M. Breuss, Judith M. Rollinger, Valerie Bochkov, Hermann Stuppner and Verena M. Dirsch* published in British Journal of Pharmacology, Volume 171, Issue 7, pages 1676–1686, April **2014**

11. Polyyne Hybrid Compounds from Notopterygium incisum with Peroxisome Proliferator-Activated Receptor Gamma Agonistic Effects

Xin Liu, Olaf Kunert, Martina Blunder, Nanang Fakhrudin, Stefan M. Noha, ***Clemens Malainer****, Andreas Schinkovitz, Elke H. Heiss, Atanas G. Atanasov, Manfred Kollroser,*

Daniela Schuster, Verena M. Dirsch and Rudolf Bauer published in Journal of Natural Products, **2014**, 77 (11), pp 2513–2521

12. Identification of Chromomoric Acid C-I as an Nrf2 Activator in Chromolaena odorata

Elke H. Heiss, Thi Van Anh Tran, Kristin Zimmermann, Stefan Schwaiger, Corina Vouk, Barbara Mayerhofer, ***Clemens Malainer****, Atanas G. Atanasov, Hermann Stuppner and Verena M. Dirsch* published in Journal of Natural Products, **2014**, 77 (3), pp 503–508

13. Screening of Vietnamese medicinal plants for NF-κB signaling inhibitors: Assessing the activity of flavonoids from the stem bark of Oroxylum indicum

Thi Van Anh Tran, ***Clemens Malainer****, Stefan Schwaiger, Tran Hung, Atanas G. Atanasov, Elke H. Heiss, Verena M. Dirsch and Hermann Stuppner* published in Journal of Ethnopharmacology Volume 159, 15 January **2015**, Pages 36–42

14. Working title: **The NF-κB inhibitor Eurycomalactone acts downstream of nuclear translocation of p65 and DNA binding**

***Malainer et al.,* 2015** manuscript in preparation

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