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FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ

Katedra biochemických věd

UNIVERSITY OF VIENNA

FACULTY OF LIFE SCIENCES

Department of Pharmacognosy

EFFECT OF SYNTHETIC MAGNOLOL DERIVATIVES ON ACTIVITY OF NUCLEAR RECEPTORS PPARγ AND RXRα

VLIV SYNTETICKÝCH DERIVÁTŮ MAGNOLOLU NA AKTIVITU NUKLEÁRNÍCH RECEPTORŮ PPARγ A RXRα

Diploma thesis / Diplomová práce

Supervisors of the thesis: Mag. Simone Latkolik

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Hradec Králové 2016   Andrea Dymáková

Prohlašuji, že tato práce je mým původním autorským dílem. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Práce nebyla využita k získání jiného nebo stejného titulu.

I declare that this thesis is my original author piece. Literature and other sources that were used during the writing process are all listed in References and are properly cited throughout the work. The thesis was not previously used to acquire a Master's or any other degree.

V Hradci Králové   Andrea Dymáková

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Abstract

Charles University in Prague

University of Vienna

Faculty of Pharmacy in Hradec Králové

Faculty of Life Sciences

Department of Biochemical Sciences

Department of Pharmacognosy

Candidate: Andrea Dymáková

Supervisors: Mag. Simone Latkolik, doc. Ing. Barbora Szotáková, Ph. D.

Title of diploma thesis: Effect of Synthetic Magnolol Derivatives on Activity of Nuclear Receptors PPARγ and RXRα

The nuclear receptors, peroxisome proliferator-activated receptor γ (PPARγ) and its heterodimerization partner retinoid X receptor α (RXRα) are drug targets in the treatment of diseases like the metabolic syndrome and *diabetes mellitus* type 2. The effort has been made to develop new agonists for PPARγ to obtain ligands with more favourable properties than currently used drugs (Berger et al. 2002, Berger et al. 2005).

Magnolol was previously described as a dual agonist of PPARγ and RXRα (Fakhrudin et al. 2010, Zhang et al. 2011). Based on the bi- aryl structure of magnolol, the effort has been made to design and synthesize linked magnolol dimers.

The aim of this thesis was to investigate the agonistic potential of these compounds with respect of the nuclear receptors PPARγ and RXRα in comparison to magnolol. We evaluated the ligand binding properties of the compounds and their functionality as PPARγ agonists *in vitro* and in intact cells, with a purified PPARγ ligand binding domain and in a cell-based nuclear receptor transactivation model in HEK293 cells, respectively.

We found that magnolol dimer binds with much higher affinity to the purified PPARγ ligand binding domain than magnolol (K_i values of 5.03 and 64.42 nM, respectively). However, there was no significant difference of PPARγ-dependent luciferase gene expression between magnolol dimer and between magnolol in intact cells. This is likely due to the PPARγ-specific activity of magnolol dimer, and the lack of RXRα activation by this compound (as specified above magnolol is dual PPARγ and RXRα agonist). The only derivative which is able to activate both receptors in intact cells is sesqui magnolol B, its affinity to PPARγ is similar to magnolol, but RXRα is affected only slightly.

Abstrakt

Univerzita Karlova v Praze   University of Vienna

Farmaceutická fakulta v Hradci Králové   Faculty of Life Sciences

Katedra biochemických věd   Department of Pharmacognosy

Kandidát: Andrea Dymáková

Školitelé: Mag. Simone Latkolik, doc. Ing. Barbora Szotáková, Ph. D.

Název diplomové práce: Vliv syntetických derivátů magnololu na aktivitu nukleárních receptorů PPARγ and RXRα

Mezi terapeutické cíle v léčbě civilizačních onemocnění, jako jsou například metabolický syndrom nebo *diabetes mellitus* typu II, patří nukleární receptory, receptor aktivovaný peroxisomovými proliferátory γ (PPARγ) a jeho heterodimerizační partner retinoidní X receptor α (RXRα). V současnosti užívané léky však mají řadu nežádoucích účinků, proto se hledají noví PPARγ agonisté, kteří by disponovali lepšími vlastnostmi než tyto stávající léky (Berger et al. 2002, Berger et al. 2005).

Právě magnolol byl již dříve popsán jako duální agonista PPARγ a RXRα (Fakhrudin et al. 2010, Zhang et al. 2011). Na základě bi-arylové struktury jeho molekuly byly navrženy a syntetizovány tzv. spojené magnolol dimery.

Cílem této diplomové práce bylo studium agonistického potenciálu těchto derivátů na výše zmíněné nukleární receptory PPARγ a RXRα, výsledky byly porovnávány s magnololem. Aktivita těchto sloučenin byla studována v trans-aktivačním modelu v neporušených HEK293 buňkách a také byla posuzována afinita k purifikované PPARγ ligand-vázající doméně *in vitro*.

Zjistili jsme, že magnolol dimer (K_i = 5,03 nM) se váže s mnohem větší afinitou k purifikované PPARγ ligand-vázající doméně než magnolol (K_i = 64,42 nM). Nicméně v neporušených buňkách nebyl nalezen žádný signifikantní rozdíl mezi jejich PPARγ-závislou luciferázovou genovou expresí. To je pravděpodobně způsobeno tím, že magnolol dimer je schopný aktivovat specificky pouze PPARγ avšak ne RXRα (jak bylo uvedeno výše, magnolol je duální agonista PPARγ a RXRα). Jediným derivátem, který je schopen aktivovat oba receptory PPARγ a RXRα v neporušených buňkách, je sesqui magnolol B, jehož afinita k PPARγ je podobná magnololu, avšak RXRα ovlivňuje pouze slabě.

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1 Introduction

The number of people that develop lifestyle diseases is growing faster and faster. Examples of typical lifestyle diseases are metabolic diseases such as *diabetes mellitus type II* (DM II) or hyperlipidemia. A strategy for treatment of such diseases is to target nuclear receptors that play a crucial role in keeping metabolic homeostasis. Therefore, scientists are developing new drugs to target nuclear receptors, and nature is a rich source of biologically active natural products with potentials in medicine.

One of these natural compounds is magnolol. Magnolol is a dual agonist of the nuclear receptors peroxisome proliferator-activated receptor γ (PPARγ) and retinoid X receptor α (RXRα). Magnolol has been shown to be PPARγ partial agonist in comparison to thiazolidinedione pioglitazone that is clinically used as an insulin sensitizer and to treat DM II. The full agonists have the disadvantage to possess severe side effects such as weight gain or edema. Partial agonists still preserve the beneficial metabolic activity, and it is hypothesized that they do not produce such severe side effects. In the aim of finding new compounds with more selective properties, magnolol derivatives were synthesized, and four of them were chosen for this investigation to evaluate their biological function.

Synthetic magnolol derivatives were tested using different *in vitro* approaches to find out their possible ability to activate nuclear receptors PPARγ or RXRα. The luciferase assays and the mammalian one-hybrid assays were performed in HEK293 cells. A cell viability assay (resazurin conversion) was performed to determine possible cytotoxic properties of the investigated compounds. Furthermore, *in vitro* binding studies were performed in a PPARγ competitive binding assay with a purified PPARγ ligand binding domain (PPARγ-LBD).

2 Theoretical Part/ Research Background

2.1 Nuclear Receptors

The human nuclear receptors form a superfamily of 48 transcription factors (Costa et al. 2010, Polvani et al. 2014) that is divided into several families (Polvani et al. 2014). About one-half is ligand-activated (Delfosse et al. 2014), but there are still described receptors with unknown ligands, so-called orphan nuclear receptors (Zhang et al. 2015). The nuclear receptors are transcription factors, that regulate gene expression and that are involved in the initiation of transcription (Yen 2015). Each nuclear receptor has certain target genes and binds to specific sequences in the promoter regions of these genes (Kota et al. 2005, Yen 2015).

The nuclear receptors are mostly activated by lipophilic ligands like steroids and thyroid hormones (Rastinejad et al. 2013, Sever et al. 2013, Yen 2015), retinoids (Rastinejad et al. 2013) or fatty acids and sterols (Yen 2015). Alternatively, the nuclear receptors can be activated by phosphorylation or other covalent modifications and through interactions with other proteins (Smirnov 2002).

A basic structure of the nuclear receptors is common for the whole superfamily (Pawlak et al. 2012) (Fig. 1). Each nuclear receptor is composed of four (Kota et al. 2005) to five domains with certain functions (Smirnov 2002). The N-terminal A/B domain plays the main role in receptor phosphorylation and contains the ligand independent activation function 1 (AF-1) (Kota et al. 2005) responsible for the specificity of receptor activity (Smirnov 2002). The C domain, the so-called DNA binding domain (DBD), affects receptor binding to the response elements in the promoter region of target genes (Kota et al. 2005, Rastinejad et al. 2013). The D domain interacts with certain cofactors. Ligand specificity is dependent on the E/F domain, the so-called ligand binding domain (LBD) which also promotes activation of receptor binding to its response elements (Kota et al. 2005). The LBD is responsible also for receptor dimerization (Smirnov 2002). The C-terminal domain, the so-called ligand binding activation function 2 (AF-2), is a part of the E/F domain and recruits receptor coactivators (Kota et al. 2005). The LBD and the nearby located AF-2 sequence are important mediators of receptor transactivation (Smirnov 2002).

Structural Organization of Nuclear Receptors
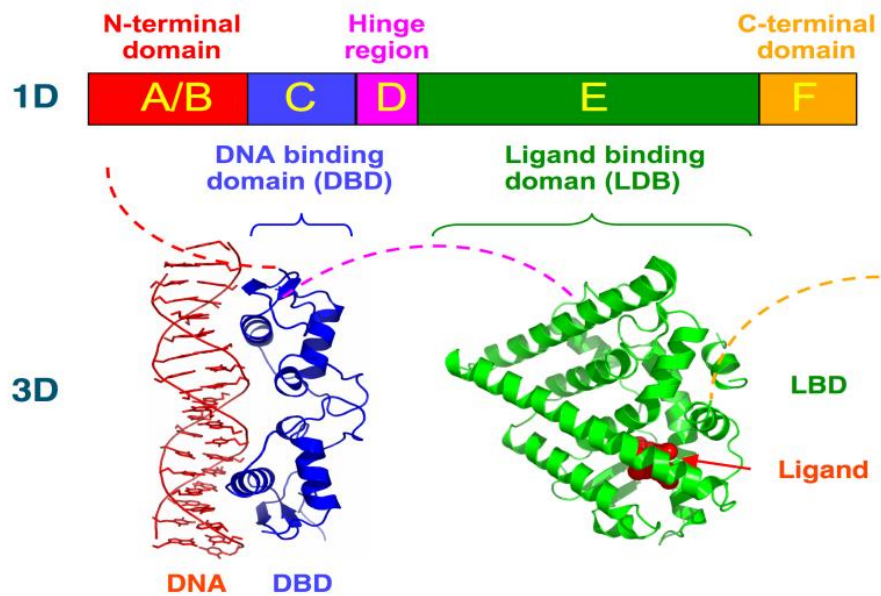
Fig. 1: Structure of nuclear receptors: One dimensional and three dimensional (3D) pictures. 3D shows the DBD associated with DNA and a ligand-bound LBD.

Nuclear_Receptor_Structure.png [online]. Latest revision 09.10.2014 [cit. 2016-04-19]. Available from: <https://upload.wikimedia.org/wikipedia/commons/3/3e/Nuclear_Receptor_Structure.png>

2.2 Peroxisome Proliferator-activated Receptor

PPARs belong to the superfamily of the ligand-activated nuclear receptors. In general, there are three types of PPARs: PPARα, PPARβ/δ and PPARγ (Zoete et al. 2007). All these types of receptors improve lipid and glucose metabolism, play important roles in inflammatory and cardiovascular diseases and have been shown to regulate various tumor growth (Berger et al. 2002, Kota et al. 2005).

2.2.1 Peroxisome Proliferator-activated Receptor α

PPARα is expressed in liver, heart, kidney, skeletal muscles (Wang et al. 2014), brown fat (Berger et al. 2005) and vessel walls (Fakhrudin et al. 2010). PPARα reduces the blood level of triglycerides and very low-density lipoprotein and elevates the level of high density lipoprotein (Grygiel-Gorniak 2014). The endogenous ligands are saturated and unsaturated fatty acids such as palmitic acid, oleic acid, linoleic acid and arachidonic acid (Berger et al. 2002). The most important synthetic agonists of PPARα are fibrates that are used in the treatment of hypertriglyceridemia, and these drugs also improve insulin sensitivity and blood glucose level (Grygiel-Gorniak 2014).

2.2.2 Peroxisome Proliferator-activated Receptor β/δ

PPARβ/δ is also widely expressed in many tissues throughout the body, especially in the brain, adipose and skin (Berger et al. 2002). The activity of this nuclear receptor subtype ameliorates lipid metabolism (Grygiel-Gorniak 2014) primarily in adipose tissue, heart and skeletal muscles (Wang et al. 2014).

Saturated and unsaturated fatty acids have the function of the endogenous ligands similarly as by the other subtypes (Berger et al. 2002).

2.2.3   Peroxisome Proliferator-activated Receptor γ

PPARγ is located particularly in adipocytes (Janani et al. 2015) and at lower concentrations in skeletal muscles or liver (Leonardini et al. 2009, Grygiel-Gorniak 2014). The subtype exists in two isoforms PPARγ1 and PPARγ2 (Medina-Gomez et al. 2007) that vary in the N-terminal domain (Leonardini et al. 2009). PPARγ1 is expressed in adipose tissue, the large intestine and hematopoietic cells (Wang et al. 2014), PPARγ2 is exclusively expressed in white and brown adipose tissue (Medina-Gomez et al. 2007).

Physiological Effects of Peroxisome Proliferator-activated Receptor γ

This PPAR subtype is mainly important for insulin sensitivity, vascular integrity (Grygiel-Gorniak 2014) and lipid homeostasis similar as PPARα (Kota et al. 2005).

Activated PPARγ improves insulin sensitivity in liver and skeletal muscles (Berger et al. 2005) and enhances gene expression of glucose transporter type 4 (Wang et al. 2014). PPARγ also provides an inhibition of expression of tumor necrosis factor α in adipose tissue in rodents and prevents tumor necrosis factor α induced insulin resistance (Berger et al. 2002). Expression of other pro-inflammatory cytokines could be suppressed by PPARγ as well; pro-inflammatory cytokines are responsible for an enhancement of insulin resistance (Berger et al. 2005).

PPARγ stimulates adipocytes differentiation (Lehrke et al. 2005). The process where pre-adipocytes differentiate into mature adipocytes is called adipogenesis. PPARγ regulates changes in gene expression during adipogenesis that lead to changes in cell morphology and hormone sensitivity (Leonardini et al. 2009).

Cholesterol efflux from macrophages can be induced by PPARγ activation to preclude the transformation into foam cells as well (Berger et al. 2005). On the other hand, PPARγ causes decrease of the leptin activity, adipocyte-selective protein, that inhibits feeding and improves catabolic lipid metabolism (Berger et al. 2002). Furthermore, some PPARγ ligands can inhibit the cyclin-dependent kinase 5 (Cdk5) mediated phosphorylation of PPARγ at serin 273 (Ser273) in adipose tissue, and the Cdk5-mediated phosphorylation is proven to play a crucial role in the development of obesity. (Wang et al. 2014).

PPARγ is highly expressed in primary colon tumors and colon cancer cell lines (Berger et al. 2002), also in breast, prostate, pancreatic or gastric tumors (*in vitro* and animal models) (Kota et al. 2005). PPARγ ligands hypothetically block cell proliferation that is based on the assumption that PPARγ improves adipocyte differentiation and thereby inhibits their proliferation. This proposed mechanism has potential in the treatment of cancer (Berger et al. 2002). The various roles of PPARγ are summarized in figure 2.
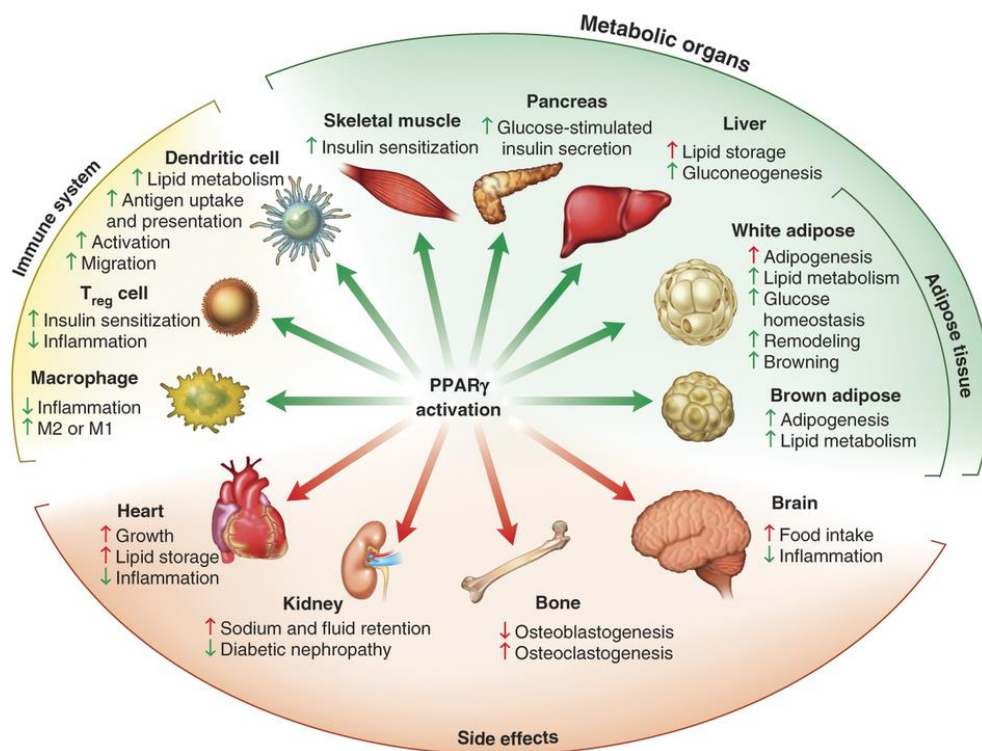

Fig. 2: Summary of the PPARγ activation effects. Green arrows show positive effects and red arrows mean adverse side effects (Ahmadian et al. 2013)

As mentioned above (2.1), the LBD is essential for binding of the receptor specific ligands, and there are differences in the LBD structure between individual receptors (Rastinejad et al. 2013). The PPARγ-LBD form is Y-shaped and is composed of a hydrophobic entrance (arm III) that forks into the polar arm I and the hydrophobic arm II (Garcia-Vallve et al. 2015). PPARγ-LBD is divided into two sub-pockets, the so-called AF-2 sub-pocket and β-sheet sub-pocket (Zhang et al. 2011).

Full agonists interact with arms I and II that leads to a stabilization of helix 12 in the conformation that enhances interactions with coactivator proteins that is followed by an increase of transactivation activity (Guasch et al. 2011, Garcia-Vallve et al. 2015). Unlike full agonists, PPARγ partial agonists bind to arms II and III that causes destabilization of helix 12 and stabilization of helix 3 (Guasch et al. 2011). Stabilization of helix 3 causes a dislocation of nuclear receptor cofactors and thus interferences with coactivator-binding and thus decreases transactivation activity of PPARγ. Partial agonists have the advantage of fewer side effects, and there are some partial agonists which still maintain antidiabetic effect (Garcia-Vallve et al. 2015)

Another key point in the regulation of PPARγ activity is Cdk5-mediated phosphorylation within the LBD at Ser273 (Ahmadian et al. 2013). PPARγ antagonists can inhibit phosphorylation at Ser273 that keeps transcription of several insulin genes (e. g. adiponectin, adipsin) active, but has no effect on the transactivation activity and differentiation of adipocytes (Garcia-Vallve et al. 2015). Currently, there are known several PPARγ ligands that have a weak agonistic activity, but still retain an anti-diabetic effect and all of them can inhibit phosphorylation of PPARγ by Cdk5. Targeted inhibition of PPARγ phosphorylation by Cdk-5 is an upcoming approach in the development of new antidiabetic drugs with a better side effect profile (Wang et al. 2014, Garcia-Vallve et al. 2015)
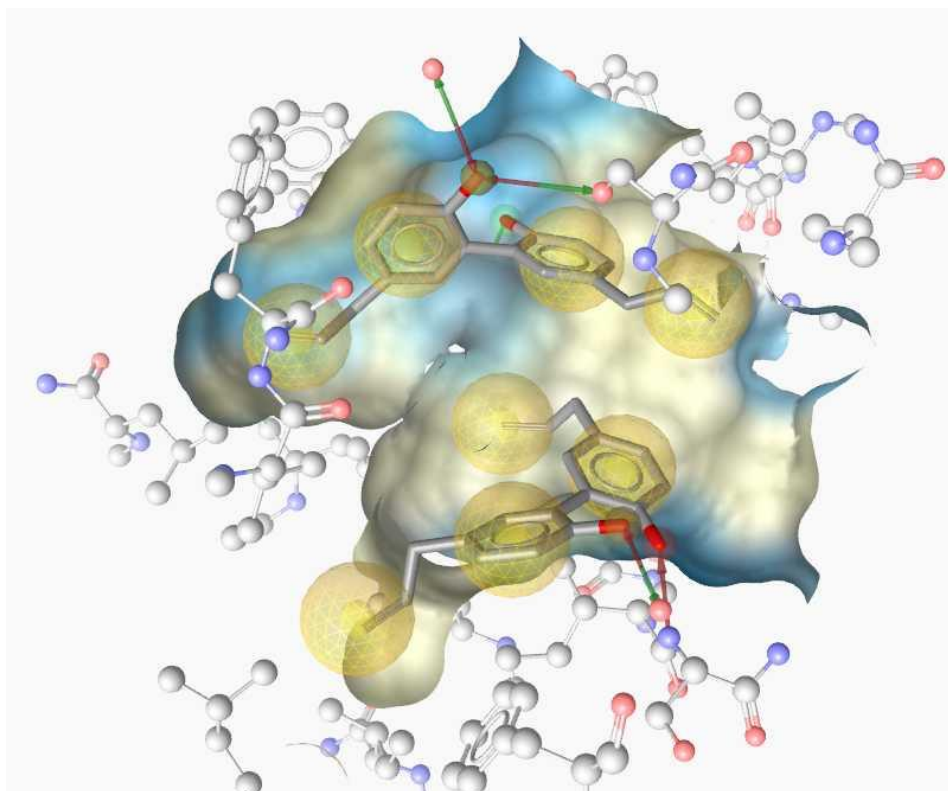
Fig. 3: Pharmacophore model of binding of two magnolol molecules to PPARγ-LBD
based on the crystal structure PDB: 3r5n of Zhang et al. (2011)

Chemical protein-ligand interactions are shown as yellow spheres (hydrophobic contacts) and arrows
(hydrogen bonds). The shape of the binding site is in colour blue (hydrophilicity) and grey (lipophilicity).

This model was made by Mag. Pharm. Dr. Daniela Schuster (Institute of Pharmacy/ Pharmaceutical
Chemistry and Center for Molecular Biosciences Innsbruck (CMBI), University of Innsbruck, Innsbruck,
Austria)

The endogenous ligands of PPARγ are polyunsaturated fatty acids such as essential linoleic acid, linolenic acid, arachidonic acid, eicosapentaenoic acid, prostaglandin-related compounds or oxidize lipids (Kota et al. 2005).

The most important synthetic agonists are thiazolidinediones (TZDs) which were developed to treat DM II (Cariou et al. 2012). TZDs can decline serum glucose level and do not enhance secretion of pancreatic insulin simultaneously (Garcia-Vallve et al. 2015). Because of their high ability to activate PPARγ (Wang et al. 2014), they have many side effects such as weight gain, edema, hemodilution, plasma-volume expansion (Berger et al. 2005), bladder cancer (Cariou et al. 2012), cardiovascular diseases and bone fractures (Garcia-Vallve et al. 2015). Despite their side effects, they are still used in the treatment of DM II (Wang et al. 2014).

Weak or partial agonists of PPARγ have less undesired side effects (Guasch et al. 2011). Some partial agonists were tested clinically, but neither showed better properties for the treatment of DM II than full agonists (Garcia-Vallve et al. 2015). Another class of compounds are selective PPARγ modulators that cause binding of selective cofactors to the receptor that modulates target gene expression differently. Furthermore, the group of inhibitors of Cdk5-mediated PPARγ phosphorylation has a potential as antidiabetic drugs (Cariou et al. 2012). The last interesting group of the potential antidiabetic drugs are dual PPARα+γ agonists that have a complementary effect on energy homeostasis. These dual agonists are more efficient than individual agonists due to their synergistic effect (Berger et al. 2005, Garcia-Vallve et al. 2015).

2.3 Retinoid X Receptor

RXRs are nuclear receptor subfamily with the specific endogenous ligand 9-*cis* retinoic acid (9-*cis*RA), vitamin A derivative (Kiss et al. 2013), but its position as natural RXR agonist is still discussed (Dawson et al. 2012, Huang et al. 2014). 9-*cis*RA is a ligand of retinoic acid receptor (RAR) as well, but is not specific for this receptor because RAR also binds all *trans*-retinoic acid isomers (Mark et al. 2003).

RXRs play a role in cell differentiation, lipid and glucose metabolism and the immune response. RXR ligands have potential in the treatment of metabolic diseases like atherosclerosis and insulin resistance, autoimmunity or neurodegeneration (Roszer et al. 2013).

Currently, it is known that except 9-*cis*RA, some fatty acids like oleic acid or docosahexaenoic acid can activate RXR too. RXRs natural ligands are retinoids, the compounds based on the structure of vitamin A. Retinoids, which bind to RXR as well as to RAR, have an important role in cell proliferation, cell differentiation or epithelial cell growth (Roszer et al. 2013). The most important synthetic retinoid is the RXR agonist bexarotene (Targretin®) that is used to treat cutaneous T-cell lymphoma (Farol et al. 2004, Perez et al. 2012).

Like the PPAR subfamily, this subfamily consists of three subtypes: RXRα, RXRβ and RXRγ. RXRα occurs in macrophages (Roszer et al. 2013), liver, lung, kidney and as the major subtype in skin (Perez et al. 2012). RXRβ is expressed throughout the body and RXRγ is especially in the brain, cardiac and skeletal muscles (Perez et al. 2012). Each subtype occurs in two major isoforms, isoform 1 and isoform 2 (Dubuquoy et al. 2002).

There are the differences in the RXRα-LBD structure compared to PPARγ-LBD. RXRα-LBD is L-shape formed only with one hydrophobic pocket. In general, it means that RXRα has only one binding site (Fig. 4) but PPARγ has two binding sites for the ligands (Fig. 3) (Zhang et al. 2011).
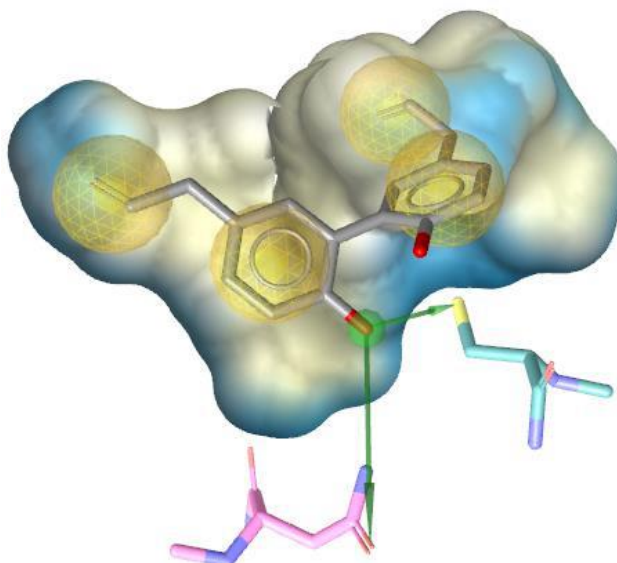
Fig. 4: Pharmacophore RXRα-LBD model with one bound molecule of magnolol based on the crystal structure PDB: 3r5n of Zhang et al. (2011)

This model was made by Mag. Pharm. Dr. Daniela Schuster (Institute of Pharmacy / Pharmaceutical Chemistry and Center for Molecular Biosciences Innsbruck (CMBI), University of Innsbruck, Innsbruck, Austria)

2.4 Peroxisome Proliferator-activated Receptor γ / Retinoic X Receptor α Heterodimers

RXR can operate as a homodimer or create a heterodimer with another nuclear receptor like PPAR, liver X receptor or farnesoid X receptor (Roszer et al. 2013). RXR is the most often occurring heterodimerization partner. RXR heterodimer partners are divided into two groups; so-called permissive nuclear receptors like PPAR or farnesoid X receptor, that can be activated by their agonists as well as by RXR agonists, and the second group are so-called non-permissive nuclear receptors like RAR, which can be affected only by their ligands (Nohara et al. 2009). PPARγ/RXRα heterodimers act in the intestinal tract, pancreas or liver (Dubuquoy et al. 2002) and have an important function in glucose and lipid metabolism (Nohara et al. 2009).

2.5 Peroxisome Proliferator-activated Receptor γ – mediated Gene Transcription

PPARγ is always activated by a ligand first. A ligand-activated PPARγ creates then a heterodimer with another ligand-activated nuclear receptor, RXRα (Wang et al. 2014). Newly-created heterodimer binds to the promoter region of PPAR response elements that are found in the promoter regions of their target genes. Heterodimer-mediated activation leads to an increase of transcription activity of various genes (Kota et al. 2005). The mechanism of PPARγ-mediated activation is shown in figure 5. Elucidation of the crystal structure of their LBD has brought new insights in the mechanism of heterodimer activation.
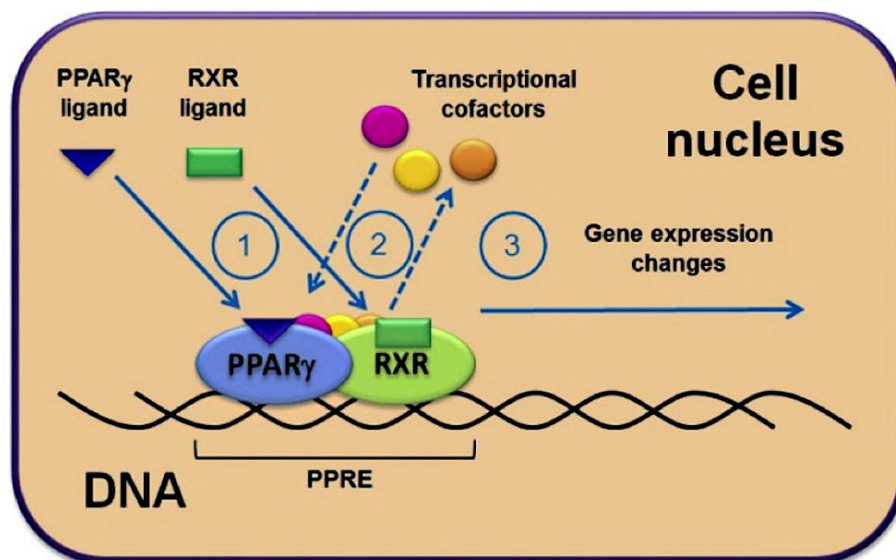
Fig. 5: Mechanism of PPARγ-mediated gene transcription. Specific ligands activate PPARγ and RXRα followed by their heterodimerization. Heterodimer binds to PPAR response element and recruitment of cofactors is necessary (Wang et al. 2014).

2.6 Natural Products

Natural products are used as medicine for thousands years. Between the most famous medicine systems belong Egyptian medicine, Indian Ayurveda or traditional Chinese medicine (Atanasov et al. 2015). People have always tried to find something to heal them and nature was and still is a good drug resource (Petrovska 2012). People have collected herbals and plants, prepared mixtures and extracts out of them, unguents, infusions or decoctions and a lot of other different forms, which are still used today (Halberstein 2005). In the last two centuries, attention has been more concentrated on chemical drugs, for example, acetylsalicylic acid also well known as aspirin (Rishton 2008) that was the first synthetic drug (History and Background of Drug Discovery [online]. [cit. 2016-04-19]. Available from: http://www.uga-cdd.org/background.php). Today, the influence of natural based drugs and development of new drugs based on natural products has become more and more important (Bohlin et al. 2010, Fakhrudin et al. 2010).

PPARγ ligands are often found in plants, especially in the plants which are used as food or herbal medicine, for example, (-)–catechin from *Camellia sinensis*, Δ9-tetrahydrocannabiol from *Cannabis sativa*, genistein from *Glycine max* or isosilybin A in silymarin from *Silybum marianum*. The most of active substances from these food plants have often only a little ability to activate PPARγ (Wang et al. 2014). Dual agonists of PPARγ and PPARα are also important compounds, for example isoprenols farnesol and geranylgeraniol or high soy isoflavone protein have been shown to be dual PPARγ/α agonists (Huang et al. 2005).

2.6.1 Magnolol

Magnolol (Fig. 6) is a bioactive neolignan found in *Magnolia officinalis*. The bark and flowers of this plant are used in traditional medicine like herbal medicine in China, Korea or Japan. The eastern doctors have used this plant to treat gastrointestinal disorders, anxiety or allergic diseases. In *Magnolia officinalis* extract, there are a lot of different biologically active compounds such as the neolignans magnolol, honokiol then syringaresinol or 4-*O*-methylhonokiol. It has been shown that magnolol is antioxidant with anticancer, antidepressant, anti-inflammatory or anti-Alzheimer properties and can also operate hepatoprotective (Lee et al. 2011).
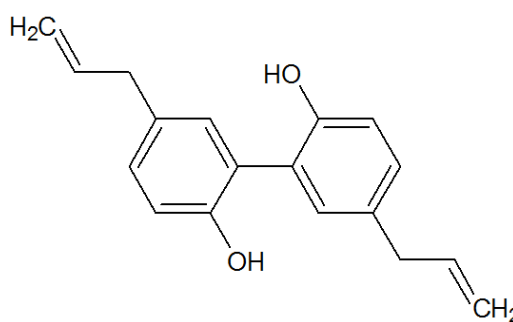
Fig. 6: Formula of magnolol (727)

Magnolol was identified as a dual agonist of the nuclear receptors PPARγ and RXRα (Fakhrudin et al. 2010, Zhang et al. 2011). In the work of Fakhrudin et al. (2010), it has been shown that magnolol is PPARγ partial agonist with a positive effect on lipid and glucose metabolism and thus has potential in the treatment of metabolic diseases like *diabetes mellitus* or to decrease the cholesterol blood level (Wang et al. 2014). The effects of magnolol and its isomer honokiol, that is another bioactive neolignan in *Magnolia officinalis* (Wang et al. 2014), are summarized in figure 7.
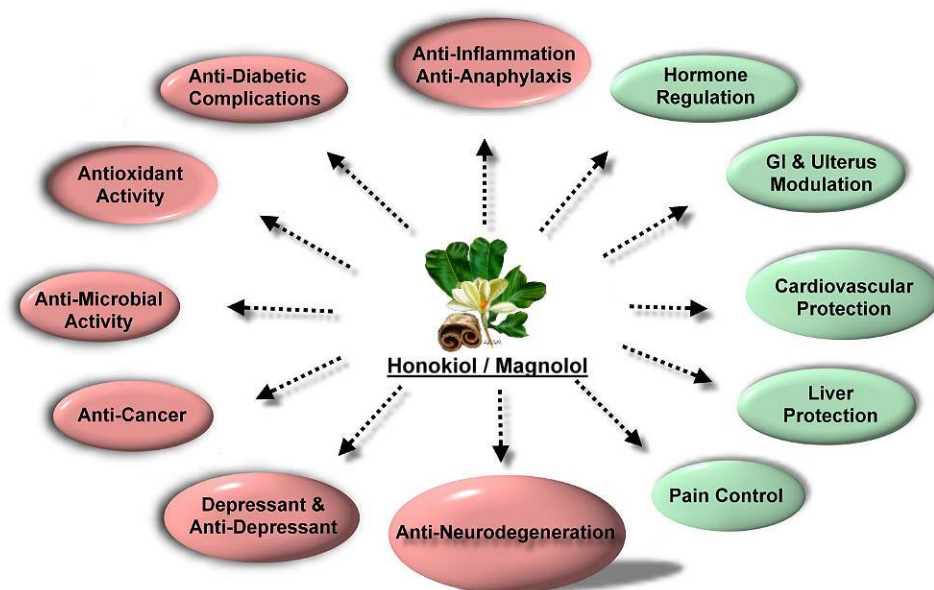
Fig. 7: The medicine targets of magnolol and honokiol (Shen et al. 2010)

Based on the bi-aryl structure of magnolol and the crystal structure of PPARγ-LBD and RXRα-LBD together with magnolol, synthetic derivatives of magnolol were synthetized by Synthesis Group of Marko D. Mihovilovic (Dominik Dreier, Lukas Rycek, Univ. Prof. Dipl.-Ing. Dr. techn. Marko D. Mihovilovic, Institute of Applied Synthetic Chemistry, Vienna University of Technology, Vienna, Austria), and four of them were chosen for testing in this thesis: truncated magnolol dimer (Fig. 8), protected sesqui magnolol B (Fig. 9), sesqui magnolol B (Fig. 10) and magnolol dimer (Fig. 11). Actually, truncated magnolol dimer, protected sesqui magnolol B and sesqui magnolol B are intermediate products of magnolol dimer synthesis.
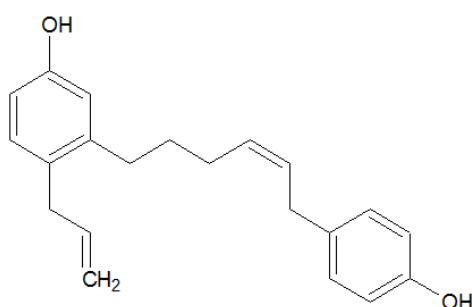
Fig. 8: Formula of truncated magnolol dimer (3280)
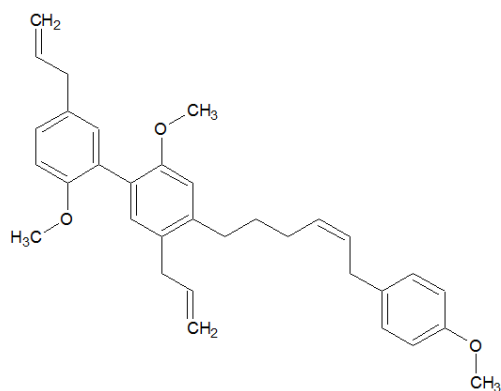
Fig. 9: Formula of protected sesqui magnolol B (3281)
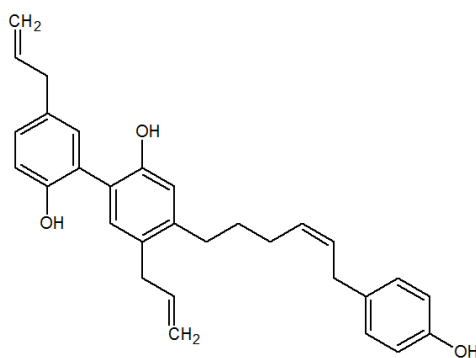
Fig. 10: Formula of sesqui magnolol B (3282)
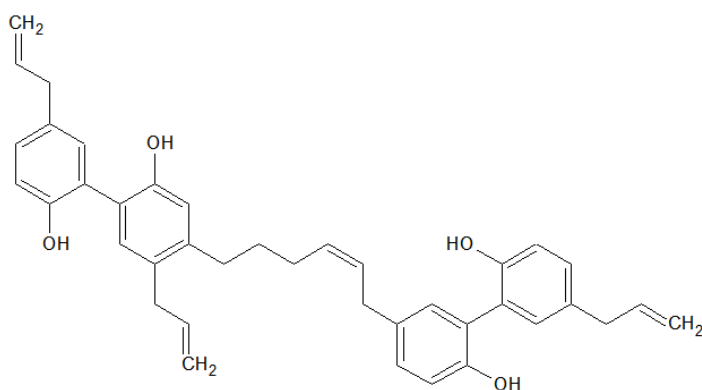
Fig. 11: Formula of magnolol dimer (3283)

3 Aim

The aim of this diploma thesis was to investigate a potential effect of selected synthetic magnolol derivatives on activation of the nuclear receptors PPARγ and RXRα and to compare the results to magnolol.

More attention was paid to magnolol dimer and its effect on PPARγ due to its interesting molecule structure. It is supposed that there are two binding pockets in PPARγ-LBD thus two magnolol molecules bind to PPARγ-LBD, but only one magnolol molecule interacts with RXRα-LBD because there is only one LBD sub-pocket. Therefore, it was proposed that magnolol dimer may have more selective properties towards PPARγ compared to magnolol.

The effects of magnolol derivatives were examined using different *in vitro* approaches:

- Luciferase reporter gene transactivation assay
- Mammalian One-Hybrid assay
- LanthaScreen[TM] TR-FRET PPARγ Competitive Binding assay

All *in vitro* approaches, except a competitive binding assay, were performed in HEK293 cells. A possible influence on cell viability of the magnolol dimers was assessed using a resazurin conversion assay.

4 Materials

4.1 Resazurin Assay

Tab. 1: Reagents used for splitting and seeding of HEK293 cells

Dulbecco´s modified eagle medium (DMEM) - complete medium	Recipe see below (Tab. 19)
Phosphate buffered saline (PBS), pH 7.4	Recipe see below (Tab. 21)
Trypsin/ EDTA	Recipe see below (Tab. 20)

Tab. 2: Reagents used for dilution of the samples

	Source
DMEM with 1% glutamine	Recipe see below (Tab. 23)
100% dimethyl sulfoxide (DMSO)	*Fluka*

Tab. 3: Reagents used in the step of addition of PBS/ Resazurin solution

	Source
PBS, pH 7.4	Recipe see below (Tab. 21)
Resazurin sodium salt	*Sigma Aldrich*

Tab. 4: Compounds used in resazurin assay

Stock solutions of the samples in DMSO	Source
30 mM 727 Magnolol	*Group of V. M. Dirsch (Department of Pharmacognosy, Faculty of Life Sciences, University of Vienna, Austria)*
30 mM 3280 Truncated magnolol dimer	*Synthesis Group of M. D. Mihovilovic (Vienna University of Technology, Vienna, Austria)*
30 mM 3281 Protected sesqui magnolol B	*Synthesis Group of M. D. Mihovilovic (Vienna University of Technology, Vienna, Austria)*
30 mM 3282 Sesqui magnolol B	*Synthesis Group of M. D. Mihovilovic (Vienna University of Technology, Vienna, Austria)*
30 mM 3283 Magnolol dimer	*Synthesis Group of M. D. Mihovilovic (Vienna University of Technology, Vienna, Austria)*
500 mM Deoxycholate	*Sigma Aldrich*
5 mg/ml Digitonin (in ethanol)	*Sigma Aldrich*

Tab. 5: Devices used in resazurin assay

	Company
Biological Safety Cabinets Herasafe™	*Thermo Fisher Scientific Inc.*
Hera cell 150 Incubator	*Thermo Fisher Scientific Inc.*
Microscope	*Olympus*
Mini Vortexer	*VWR International*
Tecan Genios Pro	*Tecan*
Vi-Cell™ (Cell viability analyzer)	*Beckmann Coulter*
Water-bath	*Julabo*

4.2 Plasmid Preparation

Tab. 6: Reagents used for bacterial transformation and obtaining of single colonies

	Source
Agar	Recipe see below (Tab. 9)
Ampicillin	*Sigma Aldrich*
Lysogeny broth (LB) medium	Recipe see below (Tab. 10)
SOC medium	
10 mM Tris HCl buffer, pH ~ 7.6	
ddH$_2$O	
Competent *Escherichia coli* bacteria	
Tk-PPAREx3-luc plasmid	*Prof. Ronald M. Evans (Howard Hughes Medical Institute, California, USA)*

Tab. 7: Reagents used for pre-culture

	Source
Ampicillin	*Sigma Aldrich*
LB medium	Recipe see below (Tab. 10)

Tab. 8: Reagents used in DNA midipreps/ DNA purification by vacuum

	Source
PureYield^{TM} Plasmid Midiprep System	*Promega*
ddH$_2$O	

Tab. 9: Recipe of agar medium

Agar medium	Quantity	Source
Bacto agar	7.5 g	*Sigma Aldrich*
LB medium	500 ml	Recipe see below (Tab. 10)

Tab. 10: Recipe of LB medium

LB medium	Quantity	Source
Bacto tryptone	5 g	*Sigma Aldrich*
Bacto yeast	2.5 g	*Sigma Aldrich*
NaCl	5 g	*Sigma Aldrich*
ddH$_2$O	up to 500 ml	
Medium had to be sterilized		

Tab. 11: Devices used in plasmid preparation

	Company
Bacterial Incubator	*Edmund Bühler*
Eluator^{TM} Vacuum Elution Device	*Promega*
Heating block	*Grant*
Heraeus^{TM} Biofuge^{TM} fresco	*Thermo Fisher Scientific Inc.*
Mini Vortexer	*VWR International*
NanoDrop 2000c	*Thermo Fisher Scientific Inc.*
PureYield^{TM} Clearing Column	*Promega*
PureYield^{TM} Binding Column	*Promega*
Sorvall RC 5C Plus Ultracentrifuge	*GMI*
Vacuum Manifold	*Promega*
Water-bath	*Julabo*

4.3   Luciferase Assay

Tab. 12: Reagents used for splitting and seeding of HEK293 cells

	Source
DMEM complete medium	Recipe see below (Tab. 19)
PBS, pH 7.4	Recipe see below (Tab. 21)
Trypsin/ EDTA	Recipe see below (Tab. 20)

Tab. 13: Reagents used for transfection of HEK293 cells

2 M CaCl$_2$	
HEPES buffered saline (HBS) 2x, pH 7.5	Recipe see below (Tab. 22)
ddH$_2$O	

Tab. 14: Reagents used for dilution of the samples

DMEM with 1% glutamine	Recipe see below (Tab. 23)
100% DMSO	*Fluka*

Tab. 15: Reagents used for reseeding of HEK293 cells

DMEM complete medium	Recipe see below (Tab. 19)
DMEM with 1% glutamine	Recipe see below (Tab. 23)
PBS, pH 7.4	Recipe see below (Tab. 21)
Trypsin/ EDTA	Recipe see below (Tab. 20)

Tab. 16: Reagents used for measurement of luciferase activity

	Source
ATP assay buffer	Recipe see below (Tab. 27)
270 mM Coenzyme A (CoA)	*Sigma*
1 M Dithiothreitol (DTT)	*Fluka*
1x Luciferase buffer	Recipe see below (Tab. 24)
Reporter lysis 5x buffer	*Promega*
ddH$_2$O	

Tab. 17: Compounds used in luciferase assay

Stock solution of the samples in DMSO	Source
5 mM 9-*cis*RA	*Enzo® Life Science*
30 mM Pioglitazone hydrochloride	*Molekula*
30 mM 727 **Magnolol**	*Group of V. M. Dirsch (Department of Pharmacognosy, Faculty of Life Sciences, University of Vienna, Austria)*
30 mM 3280 **Truncated magnolol dimer**	*Synthesis Group of M. D. Mihovilovic (Vienna University of Technology, Vienna, Austria)*
30 mM 3281 **Protected sesqui magnolol B**	*Synthesis Group of M. D. Mihovilovic (Vienna University of Technology, Vienna, Austria)*
30 mM 3282 **Sesqui magnolol B**	*Synthesis Group of M. D. Mihovilovic (Vienna University of Technology, Vienna, Austria)*
30 mM 3283 **Magnolol dimer**	*Synthesis Group of M. D. Mihovilovic (Vienna University of Technology, Vienna, Austria)*

Tab. 18: Plasmids used in luciferase assay

Plasmids	Concentration	Source
pSG5-PL-hPPARγ	680.3 µg/ml	*Genomics, University of Lausanne* *(Switzerland)*
Tk-PPAREx3-luc	30 µg/ml; 31.8 µg/ml; 38.7 µg/ml; 764 µg/ml	*Prof. Ronald M. Evans (Howard Hughes* *Medical Institute, California, USA)*
Retinoid X nuclear receptor alpha	526 µg/ml	*Missouri S&T (Missouri, USA)*
RXR Luciferase Reporter Vector	637 µg/ml	*Panomics Affymetrix (Milano, Italia)*
pEGFP-N1	409.5 µg/ml; 409.9 µg/ml	*Clontech (CA, USA)*

Tab. 19: Recipe of DMEM complete medium

DMEM complete medium	Quantity	Source
DMEM	440 ml	*Lonza Group Ltd.*
10% Fetal bovine serum	50 ml	*Lonza Group Ltd.*
2 mM L-Glutamine	5 ml	*Lonza Group Ltd.*
100 UI/ml Penicillin + 100 μg/ml Streptomycin	5 ml	*Lonza Group Ltd.*
Everything was pre-warmed in a water-bath at 37°C and sterile filtrated		

Tab. 20: Recipe of Trypsin/ EDTA

Trypsin/ EDTA	Quantity
EDTA	0.02%
Trypsin	0.05%
PBS	1 000 ml

Tab. 21: Recipe of PBS

PBS, pH 7.4	Quantity
$CaCl_2 \cdot 2\ H_2O$	0.10 g
KCl	0.20 g
KH_2PO_4	0.20 g
NaCl	8.00 g
Na_2HPO_4	1.15 g
$MgCl_2 \cdot 6\ H_2O$	0.10 g
ddH_2O	up to 1 000 ml

Tab. 22: Recipe of HBS 2x

HBS 2x, pH 7.5	Concentration
NaCl	280 mM
KCl	10 mM
Dextrose	12 mM
2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), pH 7.6	50 mM
$NaH_2PO_4 \cdot H_2O$	1.5 mM

Tab. 23: Recipe of DMEM with 1% glutamine

DMEM with 1% glutamine	Quantity	Source
2 mM L-Glutamine	5 ml	*Lonza Group Ltd.*
DMEM	495 ml	*Lonza Group Ltd.*

Tab. 24: Recipe of 1x luciferase buffer

1x Luciferase buffer	Quantity	
10x Luciferin	82.70 ml	Recipe see below (Tab. 25)
1 M Tricine, pH 7.8	16.54 ml	Recipe see below (Tab. 26)
ddH$_2$O	691.67 ml	
Stored at - 80°C		

Tab. 25: Recipe of 10x luciferin

10x Luciferin	Quantity	Source
Luciferin	25 mg	*Molecular Probes/ Invitrogen*
ddH$_2$O	8.27 ml	
The aluminum foil was used to protect luciferin from light		

Tab. 26: Recipe of 1 M tricine

1 M Tricine, pH 7.8	Quantity	Source
Tricine	17.92 g	*Sigma Aldrich*
ddH$_2$O	80 ml	
pH was adjusted to 7.8, he volume was filled up to 100 ml with ddH$_2$O and it was kept at room temperature		

Tab. 27: Recipe of ATP assay buffer

ATP assay buffer	Quantity	Source
0.1 M ATP, pH 7.0	30.599 ml	Recipe see below (Tab. 28)
0.5 M MgCl$_2$	35.561 ml	*Sigma Aldrich*
1 M Tricine, pH 7.8	16.50 ml	Recipe see above (Tab. 26)
ddH$_2$O	744.3 ml	
Stored at - 80°C		

Tab. 28: Recipe of 0.1 M ATP

0.1 M ATP, pH 7.0	Quantity	Source
ATP disodium salt	2.5 g	*Sigma Aldrich*
1M NaOH	9.9 ml	*Sigma Aldrich*
ddH$_2$O	33.5 ml	
pH was adjusted to 7.0 with 2 M HCl		

Tab. 29: Devices used in luciferase assay

	Company
- 80°C freezer	*Thermo Fisher*
Biological Safety Cabinets Herasafe™	*Thermo Fisher Scientific Inc.*
Centrifuge Heraeus™ Multifuge™ 1 S-R	*Thermo Fisher Scientific Inc.*
Fluorescent microscope	*Olympus*
Hera cell 150 Incubator	*Thermo Fisher Scientific Inc.*
Microscope	*Olympus*
Mini Vortexer	*VWR International*
Multi- MicroPlate Genie®	*Carl Roth*
Tecan Genios Pro	*Tecan*
Vi-Cell™ (Cell viability analyzer)	*Beckmann Coulter*
Water-bath	*Julabo*

4.4 Mammalian One-Hybrid Assay

All materials and devices are same as for the luciferase assay (see above 4.3) except the different plasmids.

Tab. 30: Plasmids used in mammalian one-hybrid assay

Plasmid	Concentration	Source
hPPARgamma Gal4	622 µg/ml	*Dr. Ronald Evans (Salk Institute, USA)*
hRXRalpha Gal4	808 µg/ml; 692 µg/ml	*Dr. Ronald Evans (Salk Institute, USA*
pEGFP-N1	409.5 µg/ml; 409.9 µg/ml	*Clontech (CA, USA)*
tk-LUC	752 µg/ml	*Dr. Ronald Evans (Salk Institute, USA)*

4.5 LanthaScreen™ TR-FRET PPARγ Competitive Binding Assay

Tab. 31: Reagents used in LanthaScreen™ TR-FRET PPARγ Competitive Binding Assay

	Source
100% DMSO	*Fluka*
1 M DTT	*Fluka*
LanthaScreen™ TR-FRET PPARγ Competitive Binding Assay	*Invitrogen*

Tab. 32: Compounds used in LanthaScreen™ TR-FRET PPARγ Competitive Binding Assay

Stock solution of the samples in DMSO	Source
30 mM Pioglitazone hydrochloride	*Molekula*
30 mM 727 **Magnolol**	*Group of V. M. Dirsch (Department of Pharmacognosy, Faculty of Life Sciences, University of Vienna, Austria)*
30 mM 3283 **Magnolol dimer 3283**	*Synthesis Group of M. D. Mihovilovic (Vienna University of Technology, Vienna, Austria)*

**Tab. 33:** Devices used in LanthaScreen™ TR-FRET PPARγ Competitive Binding Assay

	Company
Mini Vortexer	*VWR International*
Tecan Genios Pro	*Tecan*

5 Methods

5.1 Resazurin Assay

Resazurin is a blue dye that has no fluorescent activity, but in presence of viable cells get reduced into pink fluorescent resorufin and the fluorescent activity of resorufin can be detected at 590 nm.
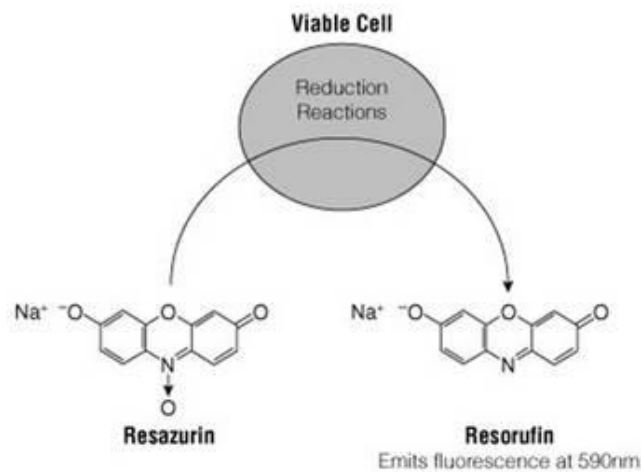
Fig. 12: Principle of resazurin transformation into resorufin

CellTiter-Blue Cell Viability Assay Technical Bulletin TB317 - celltiter-blue cell viability assay protocol.pdf [online]. [cit. 2016-04-19]. Available from: <http://at.promega.com/~/media/files/resources/protocols/technical%20bulletins/101/celltiter-blue%20cell%20viability%20assay%20protocol.pdf>

5.1.1 Seeding of HEK293 Cells (First Day)

HEK293 cells were split and seeded the same way as for the luciferase assays (see below 5.3), but there were seeded at a number of 40 000 cells per well in 100 µl DMEM complete medium. Four wells were let empty for background subtraction, 2 wells of resazurin solution only and 2 wells total empty. The plate was incubated at 37°C for 24 hours.

5.1.2 Addition of Samples (Second Day)

After 24 hours, the old medium was removed and in each well, 100 µl of DMEM with 1% glutamine with our sample was added. First, stock solutions of the 1000x concentrated samples than the final concentrations were prepared in DMSO. These stock solutions were then diluted 1000x with pre-warmed DMEM with 1% glutamine (37°C, 30 min) and 1000 µl was always prepared. Digitonin at the final concentration of 5 mg/ml and 500 µM deoxycholate were used as positive controls because of their known cytotoxicity on HEK293 cells. The plate was incubated at 37°C for another 24 hours.

5.1.3 Addition of PBS/ Resazurin Solution (Third Day)

After another 24 hours, the medium was aspirated, and the cells were washed carefully with 100 µl PBS (pre-warmed at 37°C for 30 min) that was removed again. 100 µl PBS/ resazurin solution, that contained resazurin at the concentration of 10 µg/ml, was added to the each well (except two wells for the background of plastic). PBS/ resazurin solution was always prepared fresh from the 0.1 mg/ml stock solution of resazurin in PBS, the stock solution was diluted 10x with pre-warmed PBS (37°C, 30 min), and then sterile filtrated. The plate was put back in the incubator.

5.1.4 Measurement of Fluorescence (Third Day)

An increase of fluorescence was measured at the wavelength of 590 nm by an excitation wavelength of 535 nm after 4 hours using Tecan Genius Pro.

5.1.5 Calculation of Results

First, a background subtraction was done, the background of the plate and the background of resazurin solution alone. The average calculation followed and the values were then normalized to the solvent 0.1% DMSO.

5.2 Plasmid Preparation

5.2.1 Bacterial Transformation and Obtaining of Single Colonies (First Day)

The plasmid preparation had to be done under sterile conditions in the range of the gas-fire.

Morning

The agar plates were prepared first. The agar medium was thawed in a water-bath and ampicillin was added at the final concentration of 100 µg/ml, the solution was poured in the small (5 cm) petri-dishes and left at room temperature until afternoon.

Afternoon

First, 40 µl of competent *Escherichia coli* bacteria were taken into the 1.5 ml Eppendorf tube (epp.), one epp. tube was used for each plasmid and also a negative control. Then, 2 µl of plasmid was added, mixed slightly and incubated for 30 minutes. Everything had to be done on ice. The epp. tubes were put on the 42°C heating block for exactly 45 seconds and instantly moved back on ice for 1 minute. Then, 450 µl of SOC medium (pre-warmed in a water-bath at 37°C) were pipetted in each epp. tube and the mixtures were incubated at 37°C for 1 hour.

After 1 hour, mixtures were centrifuged at maximal speed (13 000 rpm) for 1 minute. The supernatant was removed, and the bacterial pellet was re-suspended in 50 µl of LB medium. The bacterial mixtures were transferred into the agar plates, and the lines were made in three different directions. The agar plates were incubated with shaking at 37°C overnight. Any colonies were not supposed to grow up on the negative control plate.

5.2.2 Pre-culture (Second Day)

In the morning, LB medium was supplied with ampicillin at its final concentration of 100 µg/ml freshly, and 5 ml of this supplied LB medium was put in each 15 ml falcon tube. Finally, a single bacterial colony was added with an inoculation needle.

The suspensions were shaken at 37°C until the afternoon. The pre-cultures were transferred into the big vessels which contained 250 ml of LB medium. LB medium was first supplied with ampicillin at the final concentration of 100 µg/ml. Shaking followed at 37°C overnight. The main cultures were then obtained.

5.2.3 DNA Midipreps/ DNA Purification by Vacuum (Third Day – Afternoon)

The bacterial cultures were transferred into the centrifugation vessels; the volumes had to be balanced with distilled water and centrifuged (6 900 rpm; 10 min; 20°C).

The supernatant was discarded. The cell pellets were re-suspended in 6 ml of the Cell Resuspension Solution, and the cell solutions were put in the small centrifugation vessels. 6 ml of the Cell Lysis Solution was then added followed by inverting mixing (up and down) 3-5 times and incubation at room temperature for 3 minutes. Finally, 10 ml of the Neutralization Solution was pipetted followed by mixing by inverting 3-5 times and incubation at room temperature for 3 minutes again. The tubes had to be balanced with the Neutralization Solution, and the lysates were centrifuged (11 220 rpm; 15 min; 20°C).

The column system had to be properly set together; the blue PureYield™ Clearing column was located above the white PureYield™ Binding column, and the white column was placed onto the vacuum manifold (Fig. 13).

Fig. 13: Column positions on the vacuum manifold

PureYield™ Plasmid Midiprep System [online]. [cit. 2016-04-19]. Available from: <http://at.promega.com/products/dna-purification-quantitation/plasmid-purification/pureyield-plasmid-midiprep-system/?activeTab=2>

The lysate was poured into the PureYield™ Clearing column and incubated at room temperature for 2-3 minutes; the vacuum was turned on until all liquid went through both columns. The vacuum was released slowly. The PureYield™ Clearing column was taken away, and the PureYield™ Binding column stayed on the vacuum manifold. 5 ml of Endotoxin Removal Wash with added isopropanol (315 ml Endotoxin Removal Wash + 57 ml isopropanol) was pipetted into the PureYield™ Binding column, and the solution passed through the column due to the vacuum. 20 ml of Column Wash Solution with added ethanol (381 ml Column Wash Solution + 635 ml 95% ethanol) was then poured into the column and because of the vacuum again, the solution got through the column. The binding membrane was dried by the running vacuum; it was necessary to be completely dry. The opened 1.5 ml epp tubes were located on the base of the Eluator™ Vacuum Elution Device (Fig. 14).

Fig. 14: Right position of the epp. tubes on the base of the Eluator^{TM} Vacuum Elution Device

PureYield™ Plasmid Midiprep System [online]. [cit. 2016-04-19]. Available from: <http://at.promega.com/products/dna-purification-quantitation/plasmid-purification/pureyield-plasmid-midiprep-system/?activeTab=2>

The PureYield^{TM} Binding column was placed over the epp. tube, and the Eluator^{TM} Vacuum Elution Device was located on the vacuum manifold. 1 ml of Nuclease-Free Water was pipetted in the PureYield^{TM} Binding column, incubation followed at room temperature for 1 minute, and then the vacuum was turned on until all the solution got through the column. The solution was centrifuged at maximal speed (13 000 rpm) for 10 minutes, and the supernatant was transferred to a new 1, 5 ml epp. tube.

5.2.4   Determination of DNA amount (Third day – Afternoon)

The DNA amount was quantified with a spectrophotometric method. 1 µl of the DNA mixture was put into NanoDrop 2000c. The plasmid concentration was measured against the background of distilled water and defined as the concentration of ng/µl. The measurement was repeated three times for each mixture, and the average was calculated.
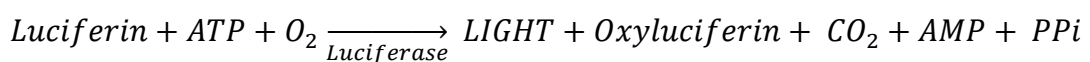
5.3 Luciferase Assay

The luciferase assay is a bioluminescent assay. HEK293 cells are transfected with three different plasmids: an expression plasmid that encodes a human nuclear receptor gene, a reporter plasmid containing the corresponding nuclear receptor response element coupled to a luciferase gene, and a third one encoding green fluorescent protein (GFP). GFP serves as an internal control and an indicator of cell viability. Luciferase gene is translated into the functional luciferase protein that mediates a reaction of luciferin with ATP (Fig. 15 and 16). The light signal is released in this reaction, and it could be detected.
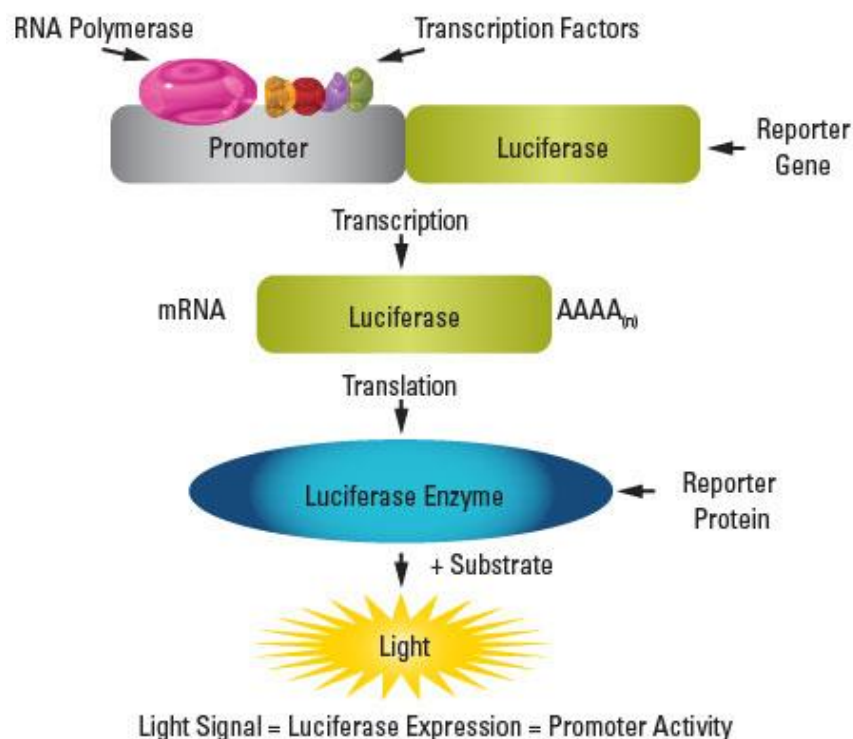
Fig. 15: Principle of luciferase assay

Luciferase Reporters | Thermo Fisher Scientific [online]. [cit. 2016-04-19]. Available from: <https://www.thermofisher.com/at/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/luciferase-reporters.html>

$$Luciferin + ATP + O_2 \xrightarrow[Luciferase]{} LIGHT + Oxyluciferin + CO_2 + AMP + PPi$$

Fig. 16: Luciferase-mediated reaction between luciferin and ATP in the presence of O_2

5.3.1 Splitting and Seeding of HEK293 Cells (First Day)

The HEK239 cell culture grew in a flask with 10 ml of DMEM complete medium in the incubator at 37 ˚C. First, DMEM complete medium had to be aspirated from the flask. Each solution had to be always pre-warmed in a water-bath at 37 ˚C for at least 30 minutes. The cells were washed with 10 ml PBS that had to be removed too. Trypsinization was started with 2 ml 0.05% trypsin followed by incubation at 37 ˚C for 1 min. Trypsinization was then stopped with 8 ml of DMEM complete medium. It was very important to get all cells from the wall. A number of HEK293 cells was counted with the Vi-Cell^{TM} (Cell viability analyzer) in 1 ml of the cell suspension. The cells were seeded in the petri-dishes, two big (20 cm) petri-dishes and one small (5 cm) petri-dish were needed for 1 experiment (one 96-well plate). For transfected cells, 6 million cells was seeded per one big dish and DMEM complete medium was added to the volume of 20 ml. For un-transfected cells, 2 million cells was seeded in a small dish with 5 ml of DMEM complete medium.

5.3.2 Transfection of HEK293 Cells (Second Day - Morning)

The second day started with the preparation of the transfection mix; it was important to respect the following pipetting order: 1. sterile water, 2. GFP, 3. PPRE/ RXRE, 4. PPARγ/ RXRα, 5. HBS and 6. CaCl$_2$ (Tab. 34). The volume of the used plasmids was calculated, there was the excel sheet for calculation on PC. Calcium chloride had to be added dropwise; vortexing and waiting about 20 min followed the mix could form. The mix was pipetted dropwise on the big dish surface. The dishes were incubated at 37 ˚C for 6 hours.

Tab. 34: Quantity of each component in transfection mix (one big petri-dish)

PPARγ/ RXRα	Quantity
pEGFP-N1	3 µg
pSG5-PL-hPPARγ/ Retinoid X nuclear receptor alpha	6 µg
Tk-PPAREx3-luc/ RXR Luciferase Reporter Vector	6 µg
2 M CaCl$_2$	94 µl
2x HBS	750 µl
ddH$_2$O	up to 1.5 ml

5.3.3 Dilution of Samples (Second Day - Between the Transfection and the Reseeding)

First, 1000x concentrated stock solutions of the samples were prepared in DMSO. These concentrated samples were then diluted 500x with DMEM with 1% glutamine; 500 µl was prepared for each sample. It was always necessary to test pure DMSO because of the compound dilution in DMSO (a negative control). The maximal DMSO concentration was less than 0.1%. 5 µM pioglitazone was used as a positive PPARγ control and 9-*cis*RA as a positive control of RXRα. 100 µl of the sample in DMEM with 1% glutamine were pipetted into four wells of a 96-well plate (quadruplet). In the last column of the plate, only 100 µl of DMEM with 1% glutamine was pipetted that served as a background of un-transfected cells. The plate was stored in the incubator at 37 °C.

5.3.4 Reseeding of HEK293 Cells (Second Day - Afternoon)

The reseeding could start 6 hours after transfection. The first step was to aspirate the medium with a pump followed by washing with 10 ml PBS. Trypsinization was started with 2 ml 0.05% trypsin for transfected cells and 1 ml 0.05% trypsin for un-transfected cells. The dishes were incubated at 37 °C, 2 minutes for transfected cells and 1 minute for un-transfected cells was enough. Trypsinization was stopped with 8 ml of DMEM complete medium. It was important to get all cells in the suspension. The cell suspension was transferred to a falcon tube (the cell suspensions of the same receptor were put in the same falcon tube) and the dish was washed with about 5 ml of DMEM complete medium again. The cell suspension was centrifuged at 1400 rpm for 4 minutes at room temperature. The supernatant was discarded and the cell pellet was re-suspended in 10 ml of DMEM with 1% glutamine for transfected cells and about 3 ml of DMEM with 1% glutamine for un-transfected cells. The number of cells per milliliter of HEK293 cell suspension was measured with the cell counter. About 50.000 cells were needed in 100 µl of the cell suspension; DMEM with 1% glutamine was used for dilution. 100 µl of cell suspension was added to each well of the 96-well plate except the last column. The un-transfected cell suspension was pipetted only in the last column (the background). The plate was incubated in the incubator at 37 °C for 18 hours.

5.3.5 Freezing of the Plate (Third Day)

The transfection efficiency was checked under the fluorescent microscope; the shining GFP could be seen in transfected cells. The medium was aspirated with a pump, and the plate was instantly frozen in the –80°C freezer. The plate could be stored in the -80°C freezer for a few weeks.

5.3.6 Measurement of Luciferase Activity (Third or Another Day)

The measurement of luciferase activity could start one hour after the freezing. The complete lysis buffer solution had to be prepared fresh (Tab. 35), and 50 µl was pipetted into each well of the plate. The plate was shaken for 10 min and 40 µl was then transferred from each well to the corresponding well of a black plate. 5.5 ml of ATP and 5.5 ml of luciferin was needed for one measurement; they had to be thawed about 1 hour before the measurement. Luciferase activity was measured at excitation wavelength 485 nm and emission wavelength 520 nm on Tecan Genios Pro.

Tab. 35: Recipe of complete lysis buffer (one 96-well plate)

Complete lysis buffer	Quantity
270 mM CoA	6 µl
1 M DTT	6 µl
Reporter lysis 5x buffer	1.2 ml
ddH$_2$O	4.8 ml

5.3.7 Analysis

Two tables were obtained after the measurement, the first table with relative fluorescence unit (RFU) values corresponding to the emission wavelength of GFP and the second table with relative luminescence unit (RLU) values corresponding to measured luciferase activity.

First, a background subtraction (the average of the values of un-transfected cells) was done in each table. After that subtraction, the ratio of RLU/ RFU was used to calculate relative luciferase activity under each condition (Fig. 17). The results were expressed relative as fold activation to the solvent 0.1% DMSO. The compounds are considered as active if fold activation is higher than 2.5 for PPARγ activation and 10 for RXRα activation. A compound seems to be toxic for the cells if the amount of GFP is less than 50% of GFP amount of DMSO, but a potential cytotoxicity had to be confirmed in resazurin cell viability assay (see above 5.1). The program GraphPad Prism 4 was used to design the dose-response curves and to calculate EC$_{50}$ and EC$_{max}$ values.

$$Normalized\ luciferase\ activity = \frac{Luminiscence}{GFP\ fluorescence}$$

Fig. 17: Calculation formula

5.4 Mammalian One-Hybrid Assay

The process of the mammalian one-hybrid assay (the so-called Gal4 assay) is generally according to the method of the luciferase assay (see above 5.3) However, the different plasmids are used. HEK293 cells are transfected with a chimeric expression plasmid encoding for a fusion protein of the DBD of the yeast transcription factor Gal4 and the LBD of the nuclear receptor and a reporter plasmid encoding a luciferase gene under the control of upstream activating sequences that can be specifically recognized by Gal4. An eGFP-plasmid serves as an internal control.

The different receptors have various response elements (usually DNA direct repeats), and the mammalian one-hybrid system is independent of any response elements of the receptor subtypes.

For example, in the transactivation luciferase gene expression assay, where the full-length human PPARγ is used, the presence of intrinsic RXR is necessary to cause a luciferase gene expression. That means if an RXR agonist is used in the PPARγ luciferase assay, luciferase activation is seen because of the permissive nature of PPARγ receptor. But if we do the same in the PPARγ-Gal4 assay, there is no visible luciferase activation because of the independence of heterodimerization.

The plasmids used for this experiments are listed in (Tab. 36).

Tab. 36: Quantity of each compound in transfection mix (one big petri-dish)

PPARγ-Gal4/ RXRα-Gal4	Quantity
pEGFP-N1	2 µg
Tk-LUC	2 µg
hPPARgamma Gal4/ hRXRalpha Gal4	10 µg
2M CaCl$_2$	94 µl
2x HBS	750 µl
ddH$_2$O	up to 1.5 ml

5.5 LanthaScreen^{TM} TR-FRET PPARγ Competitive Binding Assay

The competitive binding assay is a method based on fluorescence resonance energy transfer. A terbium-labeled anti- glutathione S-transferase (GST) antibody and a nuclear receptor with tagged with a GST is used in this assay. In the case that the antibody is binding to its GST-tag the nuclear receptor will indirectly be labeled by terbium. If a fluorescent ligand (Fluormone^{TM} Pan-PPAR Green) is binding to the nuclear receptor, the fluorescent ligand will be able to absorb energy from terbium and emit light of a different wavelength than terbium and a high TR-FRET ratio is observed. Emission at 495 nm (a terbium signal) and 520 nm (a signal of the acceptor fluor) is measured upon excitation at 340 nm, and the 495 nm/ 520 nm TR-FRET ratio is obtained. The compounds, that can activate the nuclear receptor (agonists), can displace the fluorescent ligand, and decrease in TR-FRET ratio is then observed because there is not any acceptor of terbium light energy (SELECTSCREEN™ KINASE PROFILING SUMMARY TABLE - 20130328 Custom Nuclear Receptor LanthaScreen Binding Customer Protocol and Assay Conditions.pdf [online] .
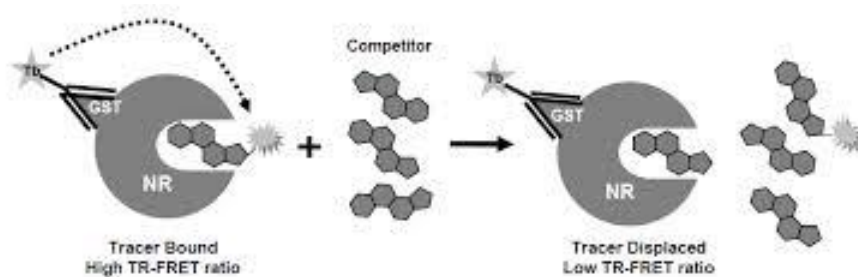
Fig. 18: Principle of competitive binding assay

SELECTSCREEN™ KINASE PROFILING SUMMARY TABLE - 20130328 Custom Nuclear Receptor LanthaScreen Binding Customer Protocol and Assay Conditions.pdf [online]. [cit. 2016-04-19]. Available from: <https://www.thermofisher.com/content/dam/LifeTech/global/life-sciences/DrugDiscoveryDevelopment/files/PDF-10-14/20130328%20Custom%20Nuclear%20Receptor%20LanthaScreen%20Binding%20Customer%20Protocol%20and%20Assay%20Conditions.pdf>

First, the 100x concentrated serial dilutions of the tested compounds were prepared in the solvent DMSO. The solutions were then diluted 50x with complete TR-FRET PPAR Assay Buffer. Complete TR-FRET PPAR Assay Buffer had to be always prepared fresh (Tab. 37). TR-FRET PPAR Assay Buffer was stored at + 4°C therefore, it was necessary to leave it before an addition of DTT at room temperature for a few minutes. 10 µl were pipetted from these 2x concentrated solutions into two wells (duplicate) of a black 384-well plate.

The rest of reagents had to be kept on ice, and everything had to be vortexed except PPARγ-LBD that is very fragile.

Tab. 37: Final reagents of competitive binding assay

Reagents (the final concentration in a well)	1. Component	2. Component
Complete TR-FRET PPAR Assay Buffer	1.6 ml TR-FRET PPAR Assay Buffer	8 µl 1 M DTT
20 nM Fluormone™ Pan-PPAR Green (5 nM)	200 µl Complete TR-FRET PPAR Assay Buffer	2 µl 2 µM Fluormone™ Pan-PPAR Green
20 nM Tb anti-GST antibody (5 nM)	200 µl Complete TR-FRET PPAR Assay Buffer	1.11 µl 3.6 µM Ab
2 nM PPARγ-LBD/ Tb-anti-GST Ab (0.5 nM)	190 µl 20 nM Tb anti-GST antibody	1.62 µl 233.9 nM PPARγ-LBD
233.9 nM PPARγ-LBD	15 µl Complete TR-FRET PPAR Assay Buffer	0.3 µl 11695 nM PPARγ-LBD

5 µl of 20 nM Fluormone™ Pan-PPAR Green (see table 38) then were added to each well. The assay had two controls; no receptor control without PPARγ-LBD and the negative control for comparison to the solvent 1% DMSO. A positive control we used pioglitazone (a full agonist of PPARγ) The last, 5 µl of 2 nM PPARγ-LBD/ Tb-anti-GST antibody (see table 38) had to be pipetted into each well except two wells for no receptor control there, 5 µl of 20 nM Tb-anti-GST antibody was added. The plate was mixed gently and incubated at room temperature for 2 hours.

Tab. 38: Final mix of reagents in each well

|		20 nM Fluormone™ Pan-PPAR Green	20 nM Tb anti-GST antibody	2 nM PPARγ-LBD/Tb-anti-GST Ab
No receptor control	10 µl 2% DMSO	5 µl	5 µl	-
Negative Control	10 µl 2% DMSO	5 µl	-	5 µl
Sample	10 µl of 2x compound	5 µl	-	5 µl

The fluorescent emission signal was measured at 495 nm and 520 nm upon excitation at 340 nm on Tecan Genios Pro. The measurement was repeated two times.

Two result tables were obtained, one for 495 nm wavelength and second for 520 nm wavelength. The first step was to subtract an average of no receptor control (a background) from the 495 nm/ 520 nm ratio that was followed by normalization to the solvent 1% DMSO. The results were then expressed relative as fold activation to the solvent 1% DMSO. The final values were put in GraphPad Prism 4 and analyzed. For calculation of K_i, the values $K_D = 2.8 \pm 0.8$ nM and the concentration of the tracer (Fluormone™ Pan-PPAR Green), which was 5 nM, had to be used.

6 Results

6.1 Effect of Synthetic Magnolol Dimers on HEK293 Cell Viability

The influence of magnolol dimers on cell viability in HEK293 cells was tested using a resazurin conversion assay and the results were compared to magnolol. The cells were treated with the compounds at different concentrations (dilution series 0.3 µM, 1 µM, 3 µM, 10 µM and 30 µM) for 24 hours. Digitonin at the concentration of 50 µg/ml and 500 µM deoxycholate were used as positive controls. The results are displayed in figure 19 and data are expressed as average of at least three independent experiments compared to the solvent control (0.1% DMSO). Error bars are shown as standard deviation (SD). The tested dimers, except protected sesqui magnolol B, reduce cell viability significantly at 30µM in HEK293 cells but do not reduce the cell viability at concentrations up to 10 µM (Fig. 19). Statistical analysis was performed using OneWay ANOVA (GraphPad Prism 7.00) with P value interpreted as not significant (n. s.) for $p > 0.1$, * for $p < 0.1$, ** for $p < 0.01$, *** for $p < 0.001$ and **** for $p < 0.0001$. The results of compounds were compared to DMSO.
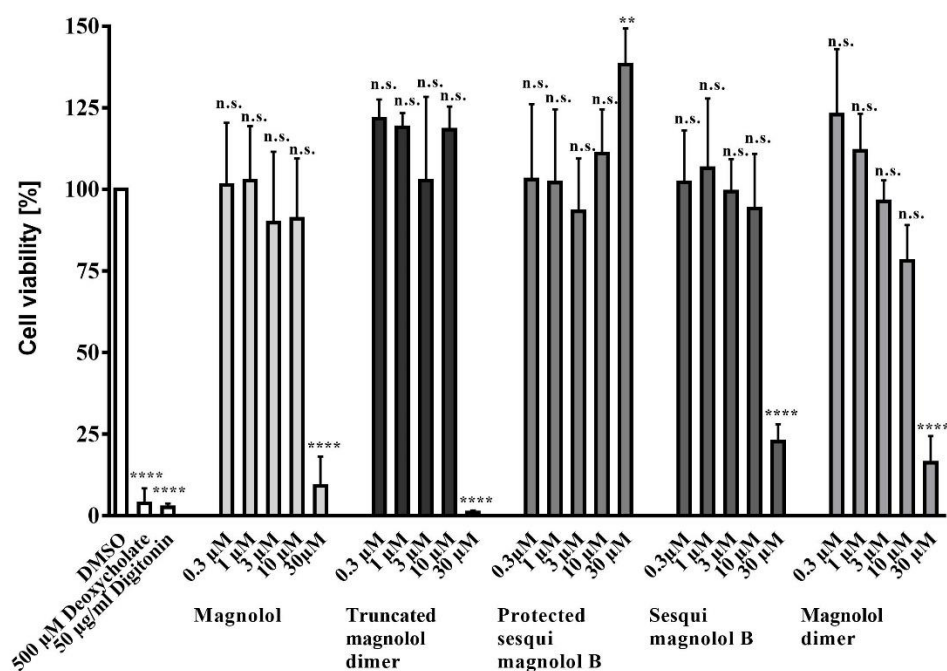
Fig. 19: Column graph of the HEK293 cells viability after the compound treatment in different concentrations for 24 hours in comparison to the solvent 0.1% DMSO and positive controls 500 µM deoxycholate and 50 µg/ml digitonin

6.2   Plasmid Preparation

The plasmids have been provided by the Department of Pharmacognosy, Faculty of Life Sciences, University of Vienna except the luciferase reporter plasmid carrying the PPAR response element (PPRE).

The PPRE reporter plasmid was isolated and functionally verified. The concentrations of the obtained plasmid solutions after plasmid preparation are listed in table 39.

Tab. 39: Concentrations of the prepared plasmid

Plasmid	Concentration
1) **Tk-PPAREx3-luc**	764 µg/ml
2) **Tk-PPAREx3-luc**	126 µg/ml

## 6.3 Effect of Synthetic Magnolol Dimers on Nuclear Receptors

6.3.1 Peroxisome Proliferator-activated Receptor γ Activation

Magnolol dimers (magnolol dimer, truncated magnolol dimer, protected sesqui magnolol B, and sesqui magnolol B) were tested first at the concentrations of 10 µM and 3 µM using a luciferase reporter gene transactivation assay.

The full agonist pioglitazone was used as a positive control and magnolol in order to compare PPARγ transactivation with the derivatives.

In general, in this assay, a compound that transactivates the luciferase gene expression higher than 2.5 times compared to the solvent 0.1% DMSO, is considered to be an agonist.

The activities of magnolol, magnolol dimer and sesqui magnolol B are very similar both at 3 µM (Fig. 20) and at 10 µM (Fig. 21). Truncated magnolol dimer and protected sesqui magnolol B had no effect on PPARγ (data not shown). All data are displayed as average of at least three independent experiments that are expressed as fold activation compared to the solvent control (DMSO 0.1 %). Error bars are presented as SD. Statistical analysis was performed using OneWay ANOVA (GraphPad Prism 7.00) with P value interpreted as not significant (n. s.) for $p > 0.1$, * for $p < 0.1$, ** for $p < 0.01$, *** for $p < 0.001$ and **** for $p < 0.0001$. The results of compounds were compared to DMSO.
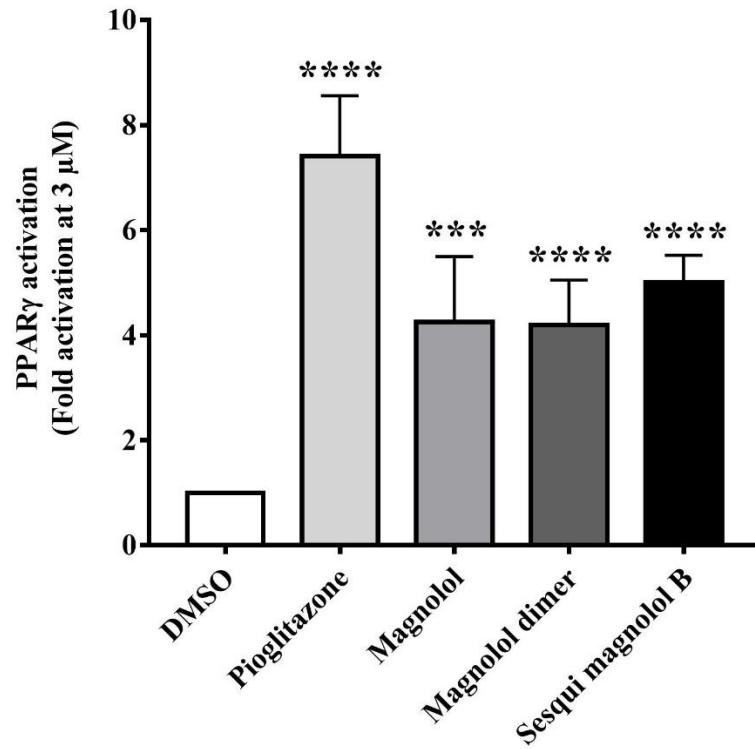
Fig. 20: PPARγ-dependent luciferase gene transactivation by magnolol dimer and sesqui magnolol B at 3 µM compared to pioglitazone and magnolol
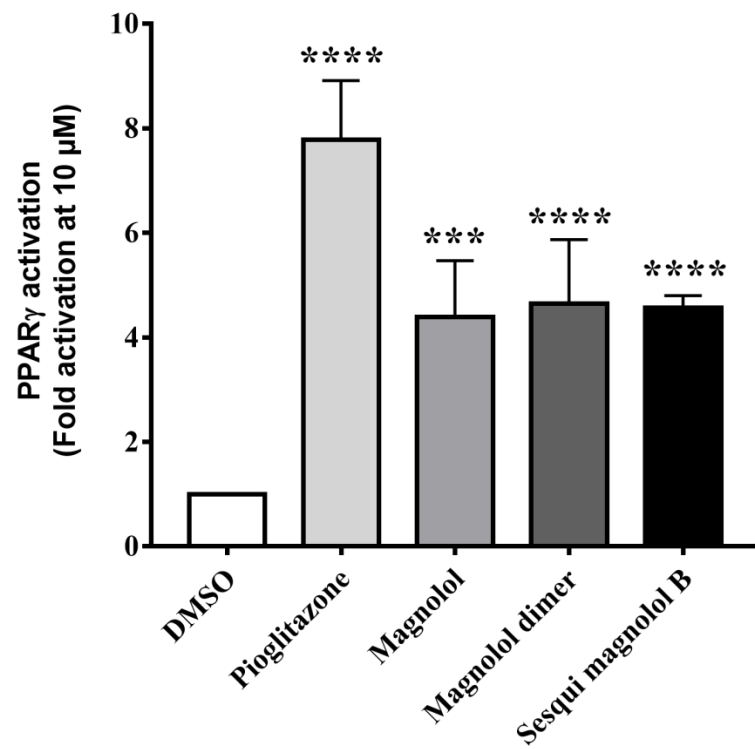
Fig. 21: PPARγ-dependent luciferase gene transactivation by magnolol dimer and sesqui magnolol B at 10 µM compared to pioglitazone and magnolol

Subsequently dose-response experiments of identified agonists were preformed (Fig. 20, 21) using concentrations ranging from 0.01 µM to 10 µM (Tab. 40) in order to gain sufficient data to calculate EC_{50} and EC_{max}. EC_{50} describes the half maximal effective concentration that is the concentration where the drug gives half-maximal response. The term is widely used in order to assess the potency of a drug. EC_{max} is the maximal effective concentration where the drug provides the maximum efficiency.

Dose-response curves of magnolol, magnolol dimer and sesqui magnolol B show that all these compounds are partial agonists of PPARγ in comparison to the full agonist pioglitazone in our assay. The potency and efficiency of magnolol and sesqui magnolol B turned out to be very similar when comparing values of fold activation at 10 µM concentration and the values of the EC_{50} (Tab. 41).

Tab. 40: Tested concentrations of the compounds in the PPARγ-luciferase assay

	0.01 µM	0.03 µM	0.1 µM	0.3 µM	1 µM	3 µM	5 µM	7 µM	10 µM
Pioglitazone	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$	×	×	$\checkmark$
Magnolol	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$	×	×	$\checkmark$
Magnolol dimer	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$	×	×	$\checkmark$
Sesqui magnolol B	×	×	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$

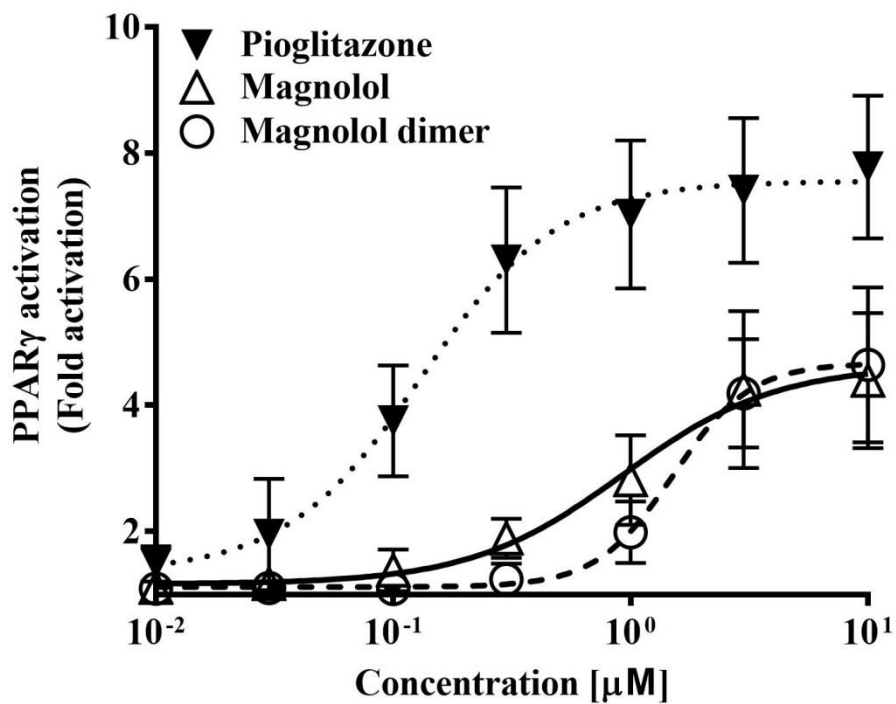($\checkmark$ tested concentration, × not tested concentration)

Fig. 22: Dose-response curve of PPARγ-dependent luciferase transactivation of magnolol dimer in comparison to magnolol and pioglitazone
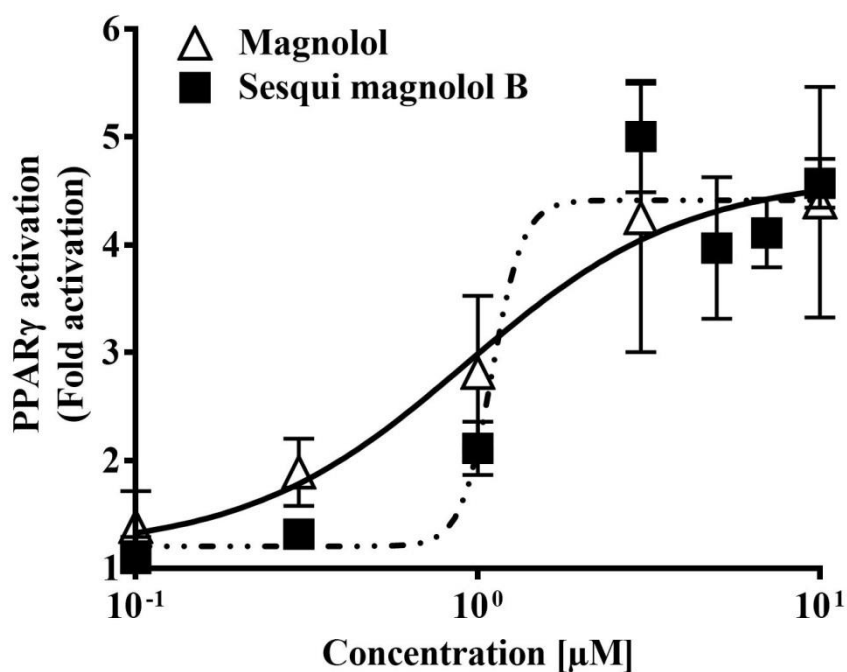

Fig. 23: Comparison of dose-response curves of PPARγ-dependent luciferase transactivation of magnolol and sesqui magnolol B

Tab. 41: Summary of the activities of pioglitazone, magnolol, magnolol dimer and sesqui magnolol B in the PPARγ-luciferase assay

	Fold activation (10 µM)	Fold activation (3 µM)	EC_{50} [µM]	EC_{max} [µM]
Pioglitazone	7.78 ± 1.13	7.41 ±1.15	0.13	7.56
Magnolol	4.39 ± 1.07	4.25 ± 1.25	0.93	4.63
Magnolol dimer	4.64 ± 1.23	4.19 ± 0.86	1.52	4.67
Sesqui magnolol B	4.57 ± 0.23	5.00 ±0.52	1.06	4.41

Magnolol dimer was also tested in a mammalian one-hybrid assay to further assess its specificity to PPARγ. In this assay a fusion protein of PPARγ-ligand-binding domain (LBD) and the DNA-binding domain (DBD) of the yeast transcription factor Gal4 (Gal4-DBD) is used. The reporter plasmid in this assay carries a recognition sequence for the Gal4-DBD coupled to a luciferase reporter gene. Pioglitazone was used as a positive control. The ability of magnolol, magnolol dimer and pioglitazone to activate PPARγ was compared directly in dose-response experiments using following serial dilutions: 0.01 µM, 0.03 µM, 0.1 µM, 0.3 µM, 1 µM, 3 µM and 10 µM.

Data are presented as average of at least three independent experiments, expressed as fold activation to the solvent 0.1% DMSO with error bars shown as SD.

Magnolol dimer is similar active to magnolol in accordance with the EC_{50} and EC_{max} values (Tab. 42) but their dose-response curves and activities at 10 µM indicate that magnolol dimer could be more active than magnolol in the Gal4 assay.

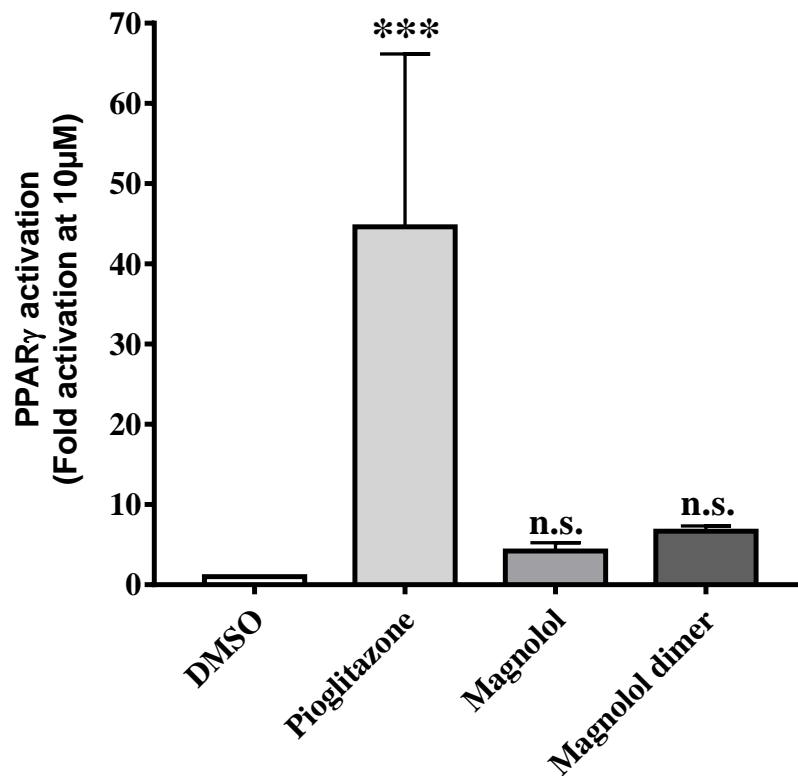**Fig. 24:** Column graph of PPARγ transactivation at 10 µM of magnolol dimer
compared to magnolol and pioglitazone in the Gal4 assay

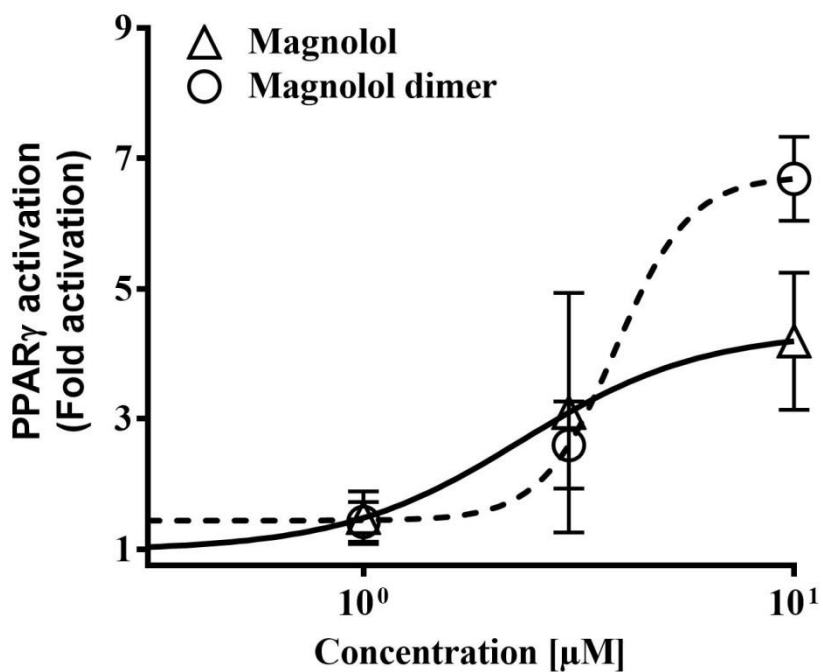**Fig. 25:** PPARγ-Gal4 transactivation dose-response curves of magnolol and
magnolol dimer

Tab. 42: Summary of compound activities in the PPARγ-Gal4 assay

|	Fold activation (10 μM)	EC$_{50}$ [μM]	EC$_{max}$ [μM]
Pioglitazone	44.62 ± 21.55	n. d.	n.d
Magnolol	4.19 ± 1.06	2.33	4.35
Magnolol dimer	6.69 ± 0.64	3.86	6.73

6.3.2 Retinoid X Receptor α Activation

The magnolol dimers were first tested at the concentration of 10 μM in a luciferase assay using the full-length RXRα receptor. 9-*cis*RA at the concentration of 5 μM was used as a positive control.

Interestingly, truncated magnolol dimer, protected sesqui magnolol B (data not shown) and magnolol dimer (Fig. 26) are not able to significantly activate RXRα. The only compound that slightly activated RXRα was sesqui magnolol B (Fig. 26). This result is one of the key outcomes showing that, magnolol dimer cannot activate RXRα and thus seems to be specific for PPARγ. In this assay, only the compounds with fold activations higher than 10 times (compared to the solvent 0.1% DMSO) can be considered as active on RXRα. Data of magnolol activity were used from a preliminary work of a diploma student of the University of Vienna, Reem Selim (2014).

Data are shown as average of three independent experiments and are expressed as fold activation compared to the solvent 0.1% DMSO with error bars presented as SD.
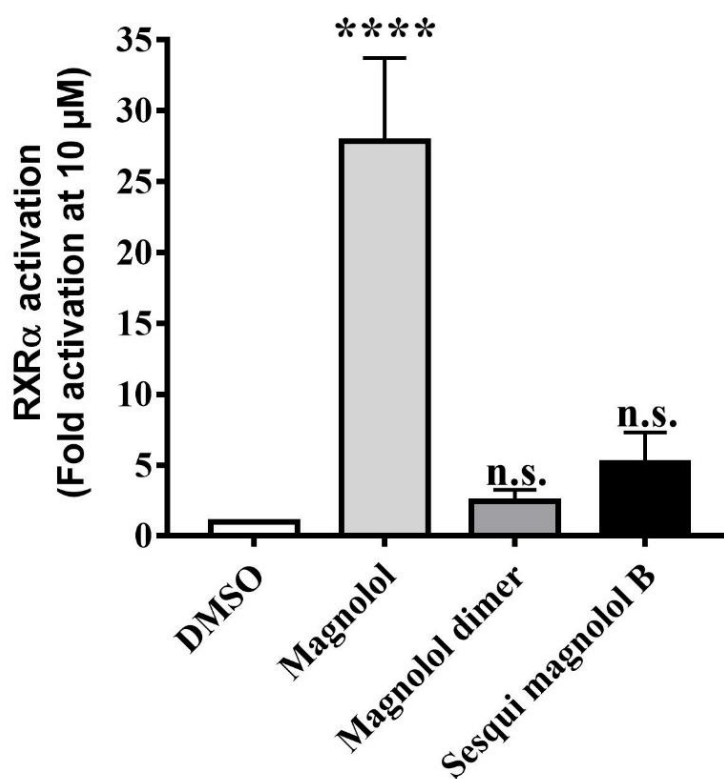
Fig. 26: Column graph of RXRα transactivation at 10 μM of magnolol dimer and sesqui magnolol B in comparison to magnolol

Statistical analysis was performed using OneWay ANOVA (GraphPad Prism 7.00) and data were compared to DMSO. P value was presented as not significant (n. s.) for p > 0.1, * for p < 0.1, ** for p < 0.01, *** for p < 0.001 and **** for p < 0.0001.

Transactivation experiments of RXRα were performed in dilution series ranging from concentration 0.01 µM to 10 µM (Tab. 43). The results are summarized in table 44. Magnolol dimer is not active on RXRα actually. Sesqui magnolol B transactivates RXRα slightly only at 10 µM.

Tab. 43: Tested concentrations of the compounds in the RXRα-luciferase assay

	0.01 µM	0.03 µM	0.1 µM	0.3 µM	1 µM	3 µM	5 µM	7 µM	10 µM
Magnolol dimer	✓	✓	✓	✓	✓	✓	✗	✗	✓
Sesqui magnolol B	✓	✓	✓	✓	✓	✓	✓	✓	✓

(✓ tested concentration, ✗ not tested concentration)

Tab. 44: Summary of tested compound activities in the RXRα-luciferase assay and comparison to magnolol data from Reem Selim (2014).

	Fold activation (10 µM)	EC_{50} [µM]	EC_{max} [µM]
Magnolol	27.83 ± 5.89	3.39	23.99
Magnolol dimer	2.45 ± 0.81	not determinated (n. d.)	n. d.
Sesqui magnolol B	5.18 ± 2.14	n. d.	n. d.

A mammalian one-hybrid assay was done as well to confirm the luciferase assay results of magnolol dimer effect on RXRα activation in comparison to magnolol. The cells were also treated with 5 µM 9-*cis*RA that served as a positive control. The same dilution series as for the PPARγ-Gal4 assay were used (0.01 µM, 0.03 µM, 0.1 µM, 0.3 µM, 1 µM, 3 µM and 10 µM). Data are presented as average of at least three independent experiments expressed as fold activation to the solvent 0.1% DMSO with error bars shown as SD. According to the RXRα-luciferase assay, compounds with fold activation higher than 10 (in comparison to the solvent 0.1% DMSO) were considered as active on RXRα. Like in the luciferase assay, magnolol dimer has no effect on RXRα activation in comparison to the solvent 0.1% DMSO (Tab. 45).
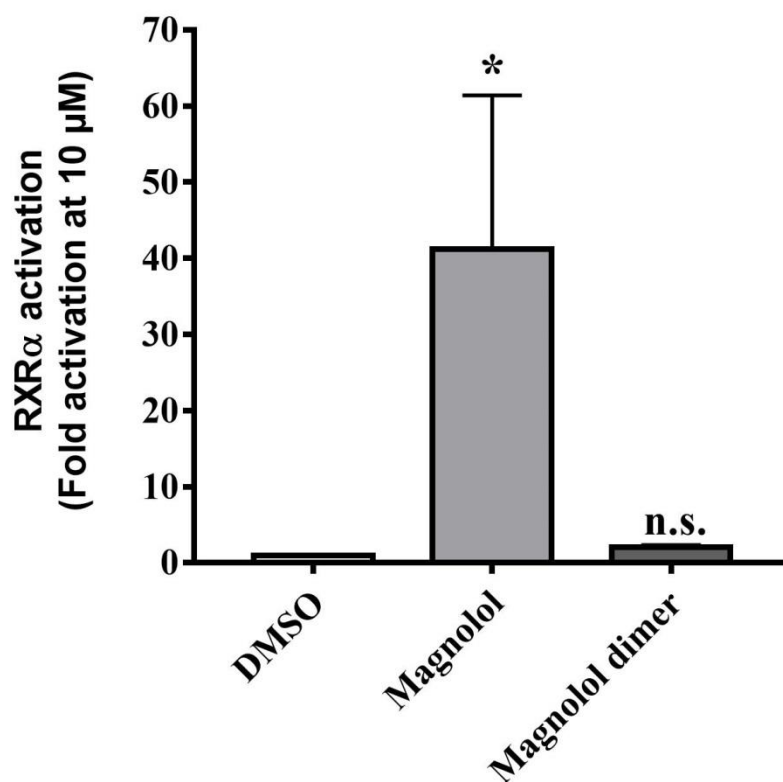
Fig. 27: Column graph of magnolol dimer RXRα-Gal4 transactivation at 10 µM in comparison to magnolol
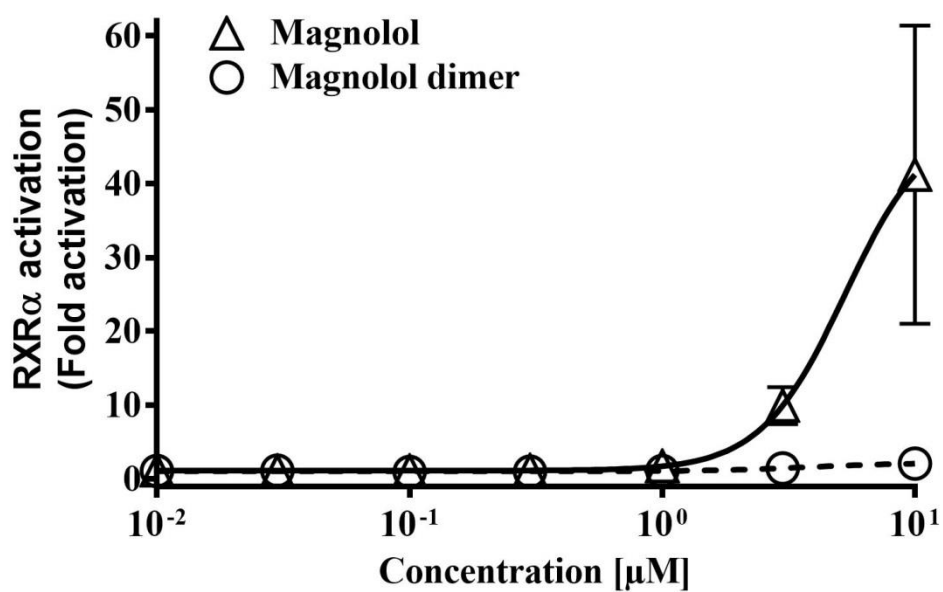
Fig. 28: Dose-response curves of magnolol dimer and magnolol in the RXRα-Gal4 assay.

**Tab. 45:** Summary of compound activities at 10 µM in the RXRα-Gal4 assay (the values of EC_{50} and EC_{max} were not determinated)

	Fold activation (10 µM)
Magnolol	41.19 ± 20.20
Magnolol dimer	2.08 ± 0.25

6.4 Magnolol Dimer Binding Properties to Peroxisome Proliferator-activated Receptor γ – Ligand Binding Domain

An *in vitro* competitive binding assay with a purified PPARγ LBD was performed to assess magnolol dimer binding properties to PPARγ. Magnolol, magnolol dimer and also pioglitazone were tested in dilution series of 0.01 µM, 0.1 µM, 1 µM, 10 µM and 100 µM. The assay was performed in a 384-well plate format. Magnolol dimer showed more than 12-fold higher affinity to the PPARγ LBD compared to magnolol, and more than 16-fold higher affinity compared to pioglitazone (Tab. 46).

Data are expressed as average from four independent experiments and presented as fold activation compared to the solvent 1% DMSO. Error bars are shown as standard error of mean (SEM). In table 46, the values of K_i and EC_{50} are presented as well. K_i characterizes the relationship between enzyme and competitor, it is the equilibrium dissociation constant and its value is the lower the more easily the complex enzyme-competitor is created. In this case, EC_{50} is the concentration that causes 50% tracer displacement.
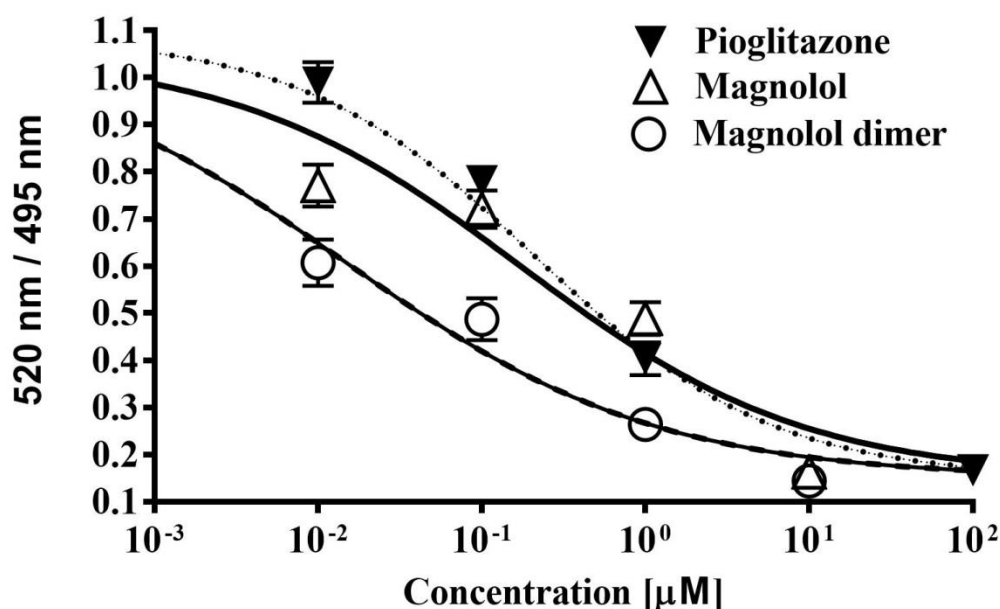
Fig. 29: Binding of magnolol dimer, magnolol and pioglitazone to the PPARγ-LBD in the competitive *in vitro* assay

Tab. 46: Summary of K_i and EC_{50} values of pioglitazone, magnolol and magnolol dimer in the competitive binding assay

	K_i [nM]	EC_{50} [nM]
Pioglitazone	85.43	238.00
Magnolol	64.42	179.50
Magnolol dimer	5.03	14.01

7 Discussion

The aim of this diploma thesis was to investigate the action of magnolol dimers on the nuclear receptors PPARγ and RXRα in comparison to magnolol. Magnolol has been shown previously to be a dual agonist of PPARγ and RXRα (Fakhrudin et al. 2010, Zhang et al. 2011). It was previously described that two magnolol molecules bind to PPARγ-LBD (one molecule in AF-2 sub-pocket and second in β-sheet sub-pocket) (Fakhrudin et al. 2010, Zhang et al. 2011) but only one magnolol molecule fits to RXRα-LBD because RXRα-LBD has a similar size like each PPARγ-LBD sub-pocket (Zhang et al. 2011). The effort has been made to synthesize the new compounds with higher specificity for PPARγ by designing a dimeric molecule that would fit only to PPARγ-LBD.

Magnolol derivatives and magnolol were first tested in the resazurin cell viability assay to assess their influence on the HEK293 cell viability. The magnolol dimers did not reduce the cell viability at concentrations up to 10μM. Truncated magnolol dimer, sesqui magnolol B and magnolol dimer reduced cell viability at the concentration of 30μM significantly. Following these results, it can be concluded that the synthetic magnolol dimers had no negative effect on the cell viability in the following cell-based luciferase reporter gene assays.

Fakhrudin et al. (2010) have shown that magnolol is a partial PPARγ agonist (Fig. 30) in comparison to the full agonist pioglitazone (EC_{50} values of 1.62 μM and 0.26 μM, respectively) in a luciferase-based cell model in HEK293 cells.

Our results of magnolol activity in the luciferase assay show that magnolol (EC_{50} = 0.93 μM) is a partial agonist of PPARγ in comparison to the full agonist pioglitazone (EC_{50} = 0.13 μM) (Fig. 22) that is in accordance with conclusions in the publication of Fakhrudin et al. (2010).
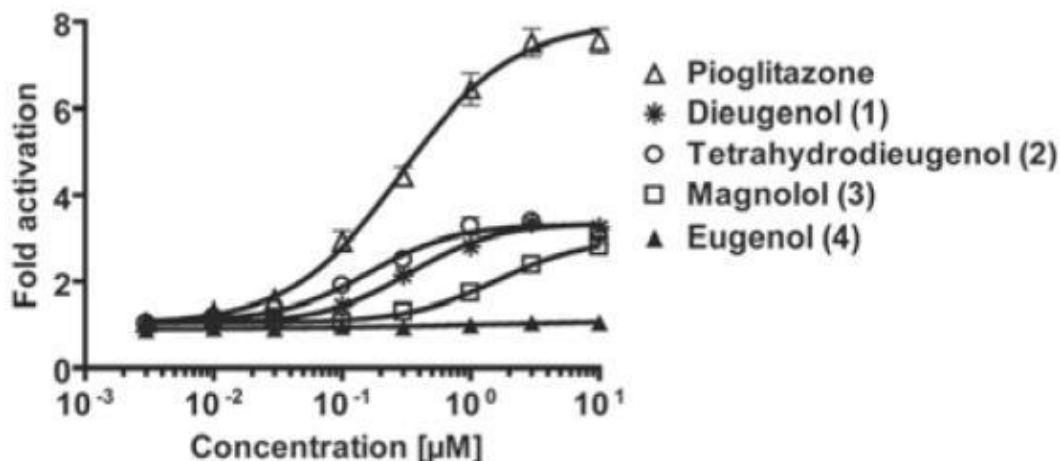
Fig. 30: Dose-response curves of PPARγ transactivation of pioglitazone and neolignans in the luciferase reporter gene transactivation assay in HEK293 cells that indicate a partial agonism of magnolol (Fakhrudin et al. 2010)

For magnolol dimer (EC_{50} = 1.52 µM) and sesqui magnolol B (EC_{50} = 1.06 µM), the dose-response curves similar to magnolol were obtained in the luciferase reporter gene transactivation assay (Fig. 22, 23). Both compounds are partial agonists of PPARγ in this assay.

The activation effect of magnolol and magnolol dimer on PPARγ (EC_{50} values of 2.33 µM and 3.86 µM, respectively) was also confirmed in the mammalian one-hybrid assay using the full-length receptor.

Binding to the PPARγ-LBD of magnolol dimer and magnolol was tested *in vitro* with the purified PPARγ-LBD in a competitive binding assay. The affinity of magnolol dimer to the purified PPARγ-LBD is more than 12-fold higher than the affinity of magnolol (K_i values of 5.03 nM and 64.42 nM, respectively) and more than 16-fold higher than the affinity of pioglitazone (K_i values of 5.03 nM and K_i = 85.43 nM, respectively).

Based on the PPARγ and RXRα crystal structure and according to previous research from Zhang et al. (2011), full agonists interact with both AF-2 sub-pocket and β-sheet sub-pocket, while partial agonists occupy only β-sheet sub-pocket in the PPARγ-LBD (Zhang et al. 2011). This binding model could explain lower affinities of magnolol compared to magnolol dimer in the competitive binding assay because magnolol dimer may dock more effectively to PPARγ-LBD due to its structure.

However, there was no significant difference of magnolol dimer compared to magnolol in the cell-based luciferase gene reporter assay and the mammalian one-hybrid assay in HEK293 cells. This may be due to the fact, that magnolol dimer does not cross the cell membrane that easily compared to magnolol dimer. One of the reasons could be the polarity or the size of magnolol dimer molecule. Lower activity of magnolol dimer in the luciferase assay could be also explained by the fact that magnolol dimer is able to specifically activate only PPARγ, not RXRα.

Nuclear receptor RXRα has only one sub-pocket in RXRα-LBD, which has the similar size as each PPARγ-LBD sub-pocket (Zhang et al. 2011). Magnolol dimer is not able to activate RXRα, and this might be because magnolol dimer does not fit into the sub-pocket of the RXRα-LBD.

The ability of sesqui magnolol B to activate PPARγ (mentioned above) and also slightly RXRα was proven in the luciferase assay. In future experiments, it would be interesting to assess the binding affinity of sesqui magnolol B to see its binding properties to the purified PPARγ-LBD. The binding properties of magnolol, magnolol dimer and sesqui magnolol B could then be compared and linked to their molecule structures.

8 Conclusion

In this study, the biological activities of synthetic magnolol dimers were evaluated. First, the influence on cell viability of different magnolol dimers was tested in HEK293 cells. Protected sesqui magnolol B, truncated magnolol dimer, sesqui magnolol B and magnolol dimer did not reduce the cell viability up to 10 µM and were therefore considered to be save for further cell-based assays.

All selected magnolol derivatives were tested in nuclear receptor–dependent luciferase reporter gene transactivation assays in HEK293 cells using the full-length human PPARγ and RXRα.

Magnolol dimer activated PPARγ comparable effective as magnolol in these assays. However, magnolol dimer did not activate RXRα-dependent luciferase gene expression in HEK293 cells, proofing the selectivity of magnolol dimer. Sesqui magnolol B activated both receptors, but RXRα only slightly.

Magnolol dimer and sesqui magnolol B have been shown to be PPARγ partial agonists in comparison to the full agonist pioglitazone in this work. Magnolol dimer activation effect on PPARγ was also confirmed in the mammalian one-hybrid assay, its efficiency was again similar to magnolol.

Magnolol dimer binding properties to PPARγ-LBD were also tested in the competitive binding assay with the purified PPARγ-LBD. Magnolol dimer affinity was more than 12-fold higher than magnolol affinity and also more than 16-fold higher in comparison to the full agonist pioglitazone.

Truncated magnolol dimer and protected sesqui magnolol B are not able to activate neither PPARγ nor RXRα.

9 Abbreviations

3D	Three dimensional
9-*cis*RA	9-*cis* retinoic acid
AF-1	Ligand independent activation function 1
AF-2	Ligand binding activation function 2
ATP	Adenosine triphosphate
Cdk5	Cyclin-dependent kinase 5
CoA	Coenzyme A
DBD	DNA binding domain
DM II	*Diabetes mellitus* type 2
DMEM	Dulbecco´s modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EC	Effective concentration
EDTA	Ethylenediaminetetraacetic acid
Epp. tube	Eppendorf tube
Gal4-DBD	DNA binding domain of the yeast transcription factor Gal4
GFP	Green fluorescent protein
GST	Glutathione S-transferase
HBS	HEPES buffered saline
HEK293 cells	Human embryonic kidney 293 cells
HEPES	2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid
K_i	Inhibition constant/ equilibrium constant
K_D	Equilibrium binding constant
LB medium	Lysogeny broth medium
LBD	Ligand binding domain
N. d.	Not determinated
N. s.	Not significant
PBS	Phosphate buffered saline
PC	Personal computer
PPAR	Peroxisome proliferator-activated receptor

PPRE	PPAR response element
RAR	Retinoic acid receptor
RFU	Relative fluorescence unit
RLU	Relative luminescence unit
Rpm	Revolutions per minute
RXR	Retinoid X receptor
RXRE	RXR response element
SD	Standard deviation
SEM	Standard error of mean
Ser273	Serin 273
SOC medium	Super optimal broth with catabolite repression medium
TR-FRET	Time-resolved fluorescence resonance energy transfer
TZD	Thiazolidinedione

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