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“Influence of piperine derivatives on ABCA1 protein expression”

verfasst von / submitted by

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2. CURRICULUM VITAE

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PUBLICATIONS

Wang, Limei, Veronika Palme, Susanne Rotter, Nicole Schilcher, **Malsor Cukaj**, Dongdong Wang, Angela Ladurner et al. "Piperine inhibits ABCA1 degradation and promotes cholesterol efflux from THP-1-derived macrophages." *Molecular Nutrition & Food Research* (2016).

ABSTARCT

Atherosclerosis is a syndrome that affects arterial blood vessels due to the accumulation of inflammatory cells in the walls of arteries. It is initiated when low-density lipoproteins (LDL) penetrate into compromised endothelium of the artery, leading to an activation of macrophages which then will be transformed into foam cells. The accumulation of foam cells leads to the formation of atheromatous plaque within the arteries. This process progresses slowly and can be asymptomatic for decades but if not treated, the so-called atheromatous plaque can rupture causing myocardial infarction or a stroke.

Removal of cholesterol from macrophages can prevent these two fatal events. This is achieved through Reverse Cholesterol Transport (RCT) with high-density lipoprotein (HDL) particles playing a key role in transporting cholesterol from peripheral tissues back to the liver and then excreted from the body in the feces. The first step in this process is cholesterol efflux where HDL particles come in contact with foam cells and take up cholesterol via cellular efflux mediated by ATP-binding cassette transporters ABCA1 and ABCG1 and by scavenger receptor class B type 1 (SR-B1).

Piperine, a major component of black pepper and long pepper, enhances macrophage cholesterol efflux by up-regulation of ABCA1. Based on this mechanism of action, 28 piperine derivatives were tested using Western blot technique with the purpose of finding a stronger compound in increasing cholesterol efflux than piperine. Also, cell viability assay was performed to determine if the tested compounds have any effect on cell proliferation or show direct cytotoxic effects that eventually lead to cell death.

Western blot analyses have revealed that one piperine derivative can increase significantly ABCA1 protein levels, making it an interesting candidate for further investigation, to determine the precise mechanism of action and to evaluate its potential in the treatment of atherosclerosis.

ZUSAMMENFASSUNG

Atherosklerose ist ein Syndrom, das die Arterien als Folge der Akkumulation von Entzündungszellen in den Wänden der Arterien auswirkt. Ausgelöst wird sie wenn die “low-density“ Lipoproteinen (LDL) in der kompromittiert arterielle Endothelium eindringen, die auf die Aktivierung von Makrophagen führt und die dann in Schaumzellen umgewandelt werden. Die Akkumulation der Schaumzellen führt dann zur Bildung von atheromatösen Plaquen in der Arterienwand. Dieser Vorgang erfolg langsam und kann über Jahrzente asymptomatisch sein, aber wenn sie nicht behandelt wird, kann ein Atheromatöseplaques Bruch geben, und kann einen Infarkt oder Schlaganfall verursachen.

Entfernung von Cholesterin aus der Makrophagen kann diese beinden tödliche Ereignisse verhindern. Dies wird erreicht durch ’’Reverse Cholesterol Transport“ (RCT), wobei die ‘‘high density“ Lipoproteinen (HDL) eine Schlüsselrolle in den Transport von Cholestrin aus peripheren Geweben zur Leber spielen, und dann aus dem Körper durch den Fäkalien ausgeschieden werden. Der erste Schritt in diesem Verfahren ist Cholesterol Efflux, wo HDL in Kontakt mit der Schaumzellen kommen und nehmen den Cholesterin durch Zellulären Efflux, die von ABC-Transporter ABCA1 und ABCG1 und von Scavenger-Receptor B1 (SR-BI) vermittelt werden.

Piperine, ein Hauptbestandteil von schwarzem Pfeffer und Langpfeffer, erhöht den Efflux von Cholesterin in Makrophagen und regulieren das Niveau der ABCA1. Auf der Grundlage dieser Wirkmechanismus, 28 Piperine Derivate wurden durch die Western blot-Technik getestet, mit dem Ziel, ein stärkeren Komponenten als Piperine zu finden, die den Efflux der Cholesterin erhöhen kann. Ebenfalls wurde der Zellviabilität Test durchgeführt, um zu bestimmen, ob die getesteten Komponenten einen Enfluss auf die Zellproliferation oder zytotoxische Wirkung zeigen, die zum Zelltod führen.

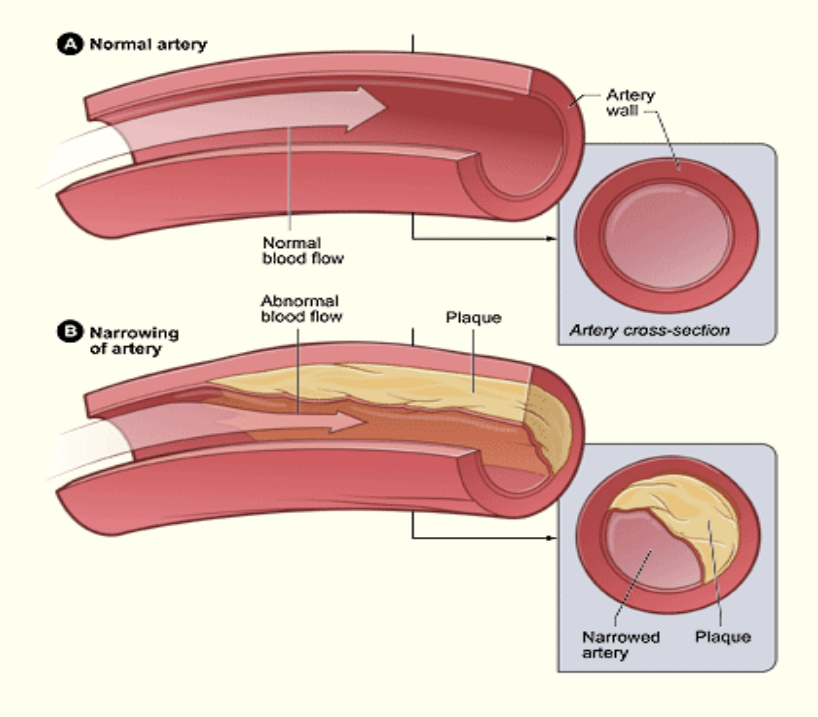
Die Western blot-Analyse hat gezeigt, das eine der Piperine Derivate deutlich dem Niveau von Protein ABCA1 erhöhen kann, und dass macht sie zum einen interessanten Kandidaten für die weitere Forschung, damit genau das Wirkungsmechanismus und ihre potentielle Verwendung bei der Behandlung von Atherosklerose bestimmt wird.

CONTENTS

A. INTRODUCTION

1. ATHEROSCLEROSIS

Atherosclerosis is a chronic disease which is caused by a continuous deposition of white blood cells in the large and medium-sized artery walls. This deposition that happens continuously for decades develops into atheromatous plaque. Plaque is a sticky composition made up of fat, cholesterol, calcium and other substances found in the blood. This plaque formation causes the arteries to lose their elasticity and as a result, arteries can no longer expand to increase blood flow when more blood is needed, for example during exercise. In small and also medium-sized arteries this atheromatous plaque can lead to a complete blockage of blood circulation, causing the death of the tissues fed by the artery. In large arteries the atheromatous plaque may rupture causing the formation of a thrombus which will rapidly block the artery leading to cell death. This is called infarction or stroke if caused by an interruption in the flow of blood to the heart or brain, respectively, and is overall the leading disease with over 80% of cardiovascular related deaths. According to World Health Organization, 17.5 million people die each year from cardiovascular diseases, an estimated 31% of all deaths worldwide.



http://www.nhlbi.nih.gov/health/health-topics/topics/atherosclerosis/

Fig. 1: Fig A shows a normal artery with normal blood flow. Fig B shows an artery with plaque buildup.

Until now, a significant number of therapies are used to treat atherosclerosis, mainly drugs that lower plasma cholesterol concentration like “statins”, which have proved to be successful in significantly reducing cardiovascular diseases (Steffens, Sabine, et al. 2005). However, still up to 50% of all deaths in Western countries are related to atherosclerosis. So the development of new therapies with the aim to stabilize the atheromatous plaque is of great interest (Leach, Colin A., et al. 2001). Still, the first line of treatment should be by dietary and lifestyle changes. Recently, agents in forms of dietary supplements have been recommended as cholesterol lowering agents, which also have a low-cost effectiveness (Duangjai, Acharaporn, et al., 2013).

One of the oldest and most used food supplements in the world is black pepper (*Piper nigrum* L.) also known as “King of Spices”, used also in traditional Indian medicine, because of its diverse pharmacological properties (Vijayakumar, Ramasamy Subramaniam, 2006). The ability of black pepper in lowering blood lipids *in vivo* and inhibition of cholesterol uptake *in vitro* appears interesting in the context of atherosclerotic treatment. As proved in a study led by Duangjai, Acharaporn, et al., (2013), this black pepper ability was due to its major active component called piperine. It is an alkaloid responsible for pungency of black pepper and long pepper.

2. LDL, HDL AND REVERSE CHOLESTEROL TRANSPORT - (RCT)

Atherosclerosis is initiated when low-density lipoproteins (LDL) also called “bad” cholesterol starts to accumulate within the artery wall (Medbury, Heather J., 2014). Cholesterol is a lipid essential to health, but too much cholesterol can be very harmful especially in the cardiovascular system. Cholesterol is produced normally in the liver or is obtained from food and circulates through blood stream usually in forms of LDL and HDL (high-density lipoprotein).

LDL circulates in the blood stream in order to supply nerve tissues, cells and other tissues with cholesterol. However, when cholesterol plasma concentration is too high and endothelium conditions compromised, LDL can get into the walls of arteries (Ross, Russell. 1999). Then, LDL can undergo an oxidation process. Oxidized LDL is toxic, and activates endothelial cells to produce chemokines that stimulate monocyte recruitment, which leads to an inflammatory process (Medbury, Heather J., 2014). As an immune response, monocytes can rapidly travel to the injured tissues and differentiate (under the influence of M-CSF factor) into macrophages which can uptake oxidized LDL and transform into so-called foam cells (Maria, Fernandez-Velasco, Gonzalez-Ramos Silvia, 2014). However, this inflammatory response is pathogenic because macrophage foam cells cannot leave the site of inflammation and as a result form a so-called fatty streak, which is a precursor of atheromatous plaque.

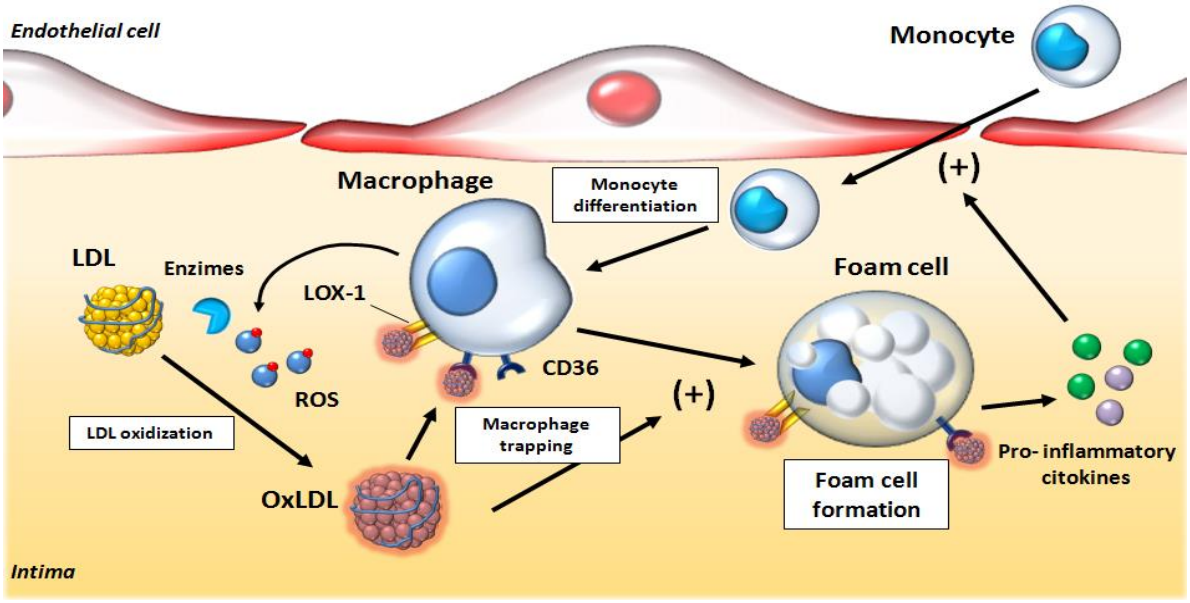


Fig. 2: The role of OxLDL in foam cell formation. Leiva, E., et al. (2015).

To achieve neutral cholesterol balance and to prevent the formation or to reduce the size of the atheromatous plaque, a process called reverse cholesterol transport (RCT) happens. This process results in cholesterol transport from peripheral tissues back to the liver and its excretion through the bile (Attie, Alan D., John P. Kastelein, 2001). The key mediator in reverse cholesterol transport is HDL. Its production starts in the liver and intestines. The circulating levels of HDL are inversely related to the risk of atherosclerosis (Fisher, Edward A., Jonathan E. 2008).

The HDL particles circulating in the blood stream will reach the vessel wall, where they come in contact with foam cells that are rich in cholesterol. They take up cholesterol via cellular efflux mediated by ATP-binding cassette transporters ABCA1 and ABCG1 and transfer it to the liver for secretion into the bile and further carriage to the intestine to be excreted from the body in the feces.

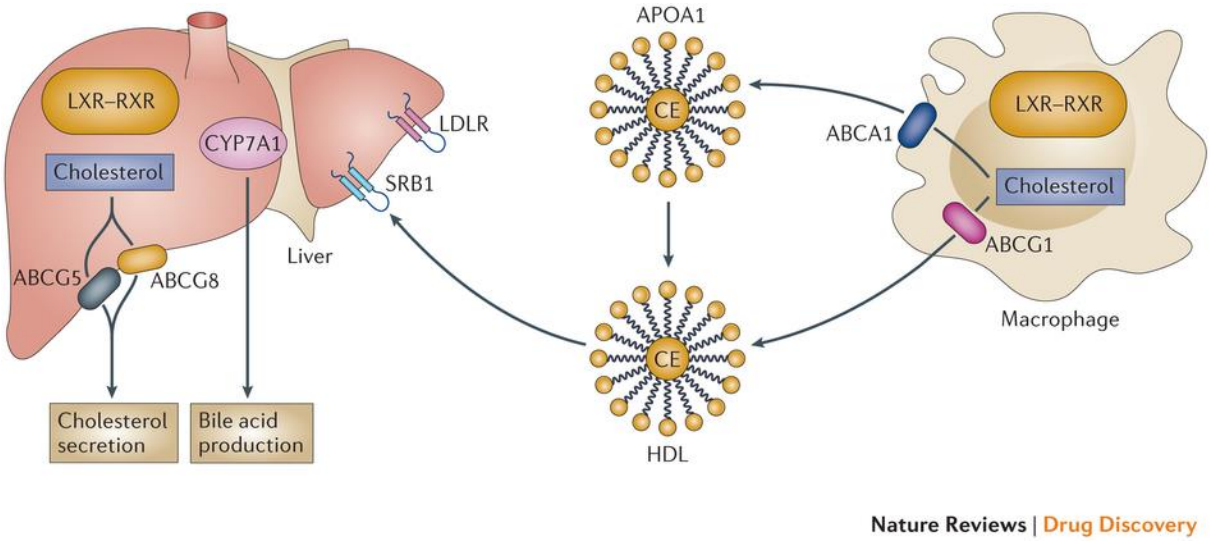


Fig. 3: HDL promotes and facilitates the process of Reverse Cholesterol Transport (RCT), whereby excess macrophage cholesterol is effluxed to HDL and ultimately returned to the liver for excretion (Duffy, Danielle, and Daniel J. Rader. 2006).

There is a wide consensus that cholesterol accumulated in macrophages is responsible for the formation of atherosclerosis. On the other hand, factors that play a key role by enhancing cholesterol efflux in macrophages should be of major importance in the treatment of atherosclerosis (Attie, Alan D., John P. Kastelein, 2001).

3. MACROPHAGE CHOLESTEROL EFFLUX

Macrophages have four pathways for removing free cholesterol to extracellular high-density lipoproteins (HDL). Two pathways are passive processes, including simple diffusion via the aqueous phase (aqueous diffusion pathway) and facilitated diffusion mediated by scavenger receptor class B type 1 (SR-B1), (SR-B1-mediated pathway). The other two pathways are active processes mediated by the ATP-binding cassette (ABC) transporters, ABCA1 and ABCG1 (Phillips, Michael C. 2014). Around two-thirds of the cholesterol efflux is mediated by active pathways with the ABCA1 transport being predominant.

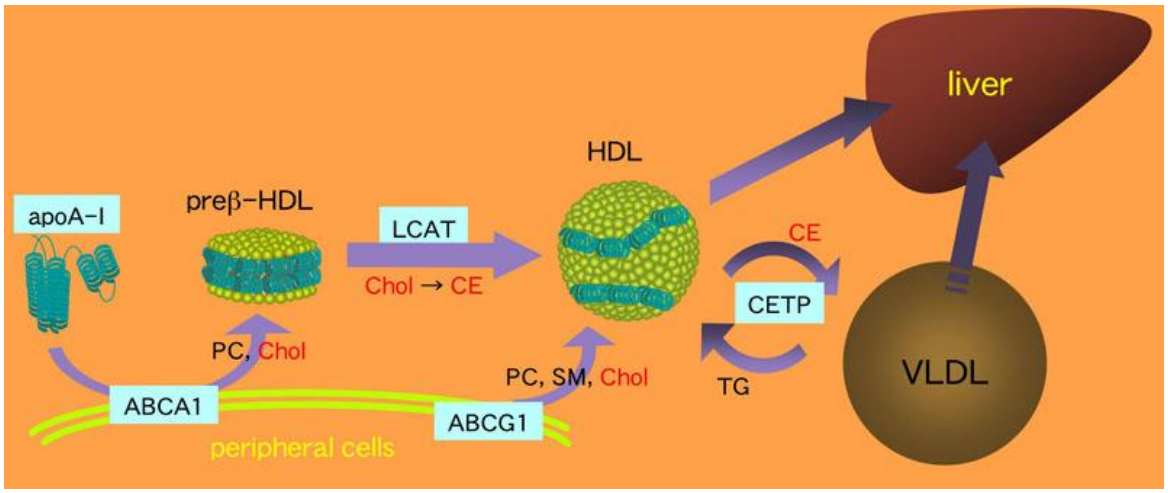
ATP-binding cassette transporters (ABC transporters) are found in all phyla including bacteria, plants, yeast, flies, fish and mammals (Tarling, Elizabeth J., and Peter A. Edwards 2011). They are localized to the plasma membrane, endoplasmic reticulum, mitochondria, peroxisomes and lamellar bodies. They bind with and hydrolyze ATP to facilitate transport of substrates across the membrane bilayer.

Most eukaryotic ABC transporters are effluxers. ABCA subfamily contains some of the largest transporters, responsible for transportation of cholesterol and lipids. ABCG subfamily transports lipids, drug substrates, bile, cholesterol and other steroids.

The ATP-binding cassette transporter A1 (ABCA1) mediates efflux of cellular phospholipids and cholesterol to lipid-poor apolipoproteins, such as apoA-1 and apoE, thereby developing the nascent HDL particle. However, ABCA1 interacts poorly with HDL-2 and HDL-3 (Wang, Nan, et al. (2004). ABCG1 and ABCG4 stimulate cholesterol efflux to both smaller HDL-3 and larger HDL-2, developing the mature HDL particle, but not to lipid-poor apoA-1. Both ABCA1 and ABCG1 have been shown to be highly induced in lipid-loaded macrophages (Klucken et al., 2000; Venkateswaran et al., 2000b).

ABCA1 is a key regulator of HDL metabolism. ABCA1 is involved in the first steps of the reverse cholesterol transport pathway and in the control of HDL levels in plasma which is the most important factor counteracting atherosclerosis. It is expressed on macrophages during cholesterol uptake and facilitates cellular cholesterol and phospholipid efflux (Van Eck,

Miranda, et al. (2002), removing excess cholesterol from peripheral cells to extracellular acceptor particles and transporting it to the liver to be metabolized and excreted.



http://www.pha.u-toyama.ac.jp/biointerface/research-e.html

Fig. 4: HDL biogenesis and the role of ABCA1 and ABCG1 as key regulators of HDL metabolism.

Incubation of apoA-1 with macrophage foam cells leads to cholesterol efflux and formation of HDL particles, a process that does not happen with fibroblasts isolated from individuals with Tangier disease. Tangier disease is an inherited disorder, associated with low plasma HDL levels, which is a consequence of mutations in the ABCA1 gene (Phillips, Michael C. 2014). This explains the key role of ABCA1 in the HDL metabolism, whereas the ability of HDL particles to mediate cholesterol efflux from cells, contributes to the anti-atherogenic properties of this lipoprotein.

The formation of nascent HDL is highly dependent on ABCA1 but not on ABCG1 or SR-B1 (Ji, Ailing, et al. (2012). However, ABCG1 is very important in cholesterol efflux from macrophages. It has a synergistic relationship with ABCA1, serving as a mediator of cholesterol efflux to newly formed nascent HDL particles generated through ABCA1 action.

Much is known about the cholesterol efflux mediated by ABCA1 and SR-B1, but little has been established about the efflux process mediated by ABCG1. There is an evidence that ABCG1 increases the availability of cholesterol in plasma membrane. So, it may not serve as direct transporter of cholesterol, but plays a role by enriching the cell membrane with cholesterol, which then can be transported through a variety of acceptor particles (Sankaranarayanan, Sandhya, et al. (2009).

The expression ABCA1 is stimulated by peroxisome proliferator-activated receptor gamma (PPARγ). PPARγ is a nuclear receptor that regulates lipid and glucose metabolism. It induces the expression of the gene for ABCA1, thus stimulating the ABCA1 pathway (Chinetti, Giulia, et al. (2001). PPARγ is activated by fatty acids and fatty acid metabolites. The activation of PPARγ leads to induction of a nuclear receptor, LXRα, which is a mediator in the expression of ABCA1. So ABCA1 gene expression is induced by both PPARγ and LXRα (Chinetti, Giulia, et al. (2001). The effect of PPARγ activation on ABCA1 mRNA levels is completely LXRα pathway dependent (Ozasa, Hideki, et al. 2011).

Piperine, a major active component of black pepper and long pepper, reduces total cholesterol, free fatty acids, phospholipids and triglycerides in plasma and tissues through several mechanisms.

Cholesterol that circulates in the blood stream comes from the synthesis of cholesterol in the liver and also from the uptake of cholesterol through intestines. Piperine has the ability to inhibit intestinal cholesterol uptake by reducing micellar cholesterol solubility, increasing micelle size or by inhibiting the function of the carrier protein, mechanisms that lead to low blood cholesterol (Duangjai, Acharaporn, et al. 2013).

Macrophages accumulate cholesterol in form of cytosolic lipid droplets and are converted to foam cells, which promote the development of atherosclerosis in the arterial wall. Piperine can inhibit the progression of atherosclerosis by inhibiting lipid droplet accumulation through blocking the activity of acyl-CoA:cholesterol acyltransferase (ACAT) in macrophages (Matsuda, Daisuke, et al. 2008).

The progression of atherosclerosis can also be inhibited by increasing cholesterol efflux from cells to HDL. The most important transporter proteins for cholesterol efflux in THP-1 macrophages are ABCA1, ABCG1 and SR-B1. Piperine significantly up-regulates ABCA1 expression, inducing apo A1-mediated cholesterol efflux (Wang, Limei, et al. 2016) and creating nascent HDL particles that facilitate cholesterol efflux through ABCG1 and turn into mature HDL (Ozasa, Hideki, et al. 2011).

So, piperine up-regulates ABCA1 but does not alter ABCA1 mRNA level (Wang, Limei, et al. (2016), meaning that piperine has the ability to regulate post-translational ABCA1 protein

levels by preventing the degradation of the protein, more specific by inhibiting calpain-mediated ABCA1 degradation (Yokoyama, Shinji, et al. 2012).

This makes black pepper a potentially important food supplement for atherosclerosis, and its main active component piperine might represent a starting point for developing synthetic compounds in order to create better and stronger drugs against the number one ranked disease in the world today.

28 piperine derivatives were tested with Western blot technique to gain further knowledge about their ability to enhance cholesterol efflux in macrophages compared to piperine.

B. AIM OF THE WORK

Preliminary research has shown that piperine, an alkaloid found in black and long pepper, has the ability to induce cholesterol efflux in macrophages by significantly up-regulating the key cholesterol transporter protein ABCA1. The aim of this study is to find between 28 piperine derivatives one or more compounds that will induce higher ABCA1 protein expression levels than piperine. For this purpose, Western blot technique is used and also cell viability assay to determine if the tested compounds have any effect on cell proliferation or show direct cytotoxic effects. The tested compounds consist of 9 natural derivatives, isolated and provided by the research team of Prof. Judith Rollinger, Department of Pharmacognosy, University of Vienna. The remaining 19 compounds are synthetized and provided by the group of Prof. Marko Mihovilovic, Institute of Applied Synthetic Chemistry, Vienna University of Technology.

C. MATERIALS AND METHODS

1. CELL LINE AND MEDIUM

1.1. HUMAN MONOCYTIC (THP-1) CELL LINE

THP-1 is a human monocytic cell line obtained from the peripheral blood of a 1 year old infant with an acute monocytic leukemia. They are one of the most widely used cell lines because of their ability to proliferate (average doubling time is 35-50 hours) and differentiate into macrophages when they are treated with phorbol 12-myristate 13-acetate (PMA). Compared with other human myeloid cell lines, macrophages derived from differentiation of THP-1 cell line behave more like macrophages derived from native monocytes (Auwerx, Johan. 1991), which is the reason that the THP-1 cell line serves as a broadly used model for studying the role of pathological macrophages in atherosclerosis. They are handled under Biosafety Level 1 containment.

1.2. GROWTH MEDIUM

For cultivation and continuous sub-cultivation of the THP-1 cell line (obtained from ATCC®), RPMI-1640 medium (obtained from Lonza Group Ltd. Basel, Switzerland) was used. 10% fetal bovine serum (FBS), which contains growth factors, important for the cultured cells to survive, grow and divide, L-glutamine, important for cell growth and function, and penicillin/streptomycin to inhibit bacterial infection, are added to the medium (concentrations and providers are given on Tab. 1). These supplements were added via a sterile filter in order to avoid contamination of the cell culture. This prepared medium was stored in 4^{o}C.

Medium	**Final concentration**	**Provider**
complete RPMI-1640 medium		
RPMI-1640 medium		Lonza Group Ltd (Basel, Switzerland)
Penicillin/streptomycin mixture (10 000 U/ml potassium penicillin, 10 000 μg/ml streptomycin sulphate)	100 U/ml penicillin, 100 μg/ml streptomycin	Lonza Group Ltd (Basel, Switzerland)
L-glutamine 200 Mm	2 mmol/L	Lonza Group Ltd (Basel, Switzerland)
Heat-inactivated Fetal Bovine Serum (FBS)	10 %	Gibco (Lofer, Austria)

Tab. 1: Cell growth medium, components and used amounts.

2. COMPOUNDS

2.1. PIPERINE

Piperine is an alkaloid and a major component of black pepper (*Piper nigrum*, Piperaceae) and long pepper (*Piper longum*, Piperaceae), and also is responsible for the pungency of the fruits. Black pepper is used since ancient times and also today is widely used as a condiment. It has also been used in some form of traditional medicine as pain reliever, against chills, flu, fever, colds, rheumatism, muscular pain, enhancing appetite etc. It has been proven also that piperine as part of black pepper and long pepper has the ability to lower blood lipids *in vivo* and inhibits cholesterol uptake *in vitro* (Duangjai, Acharaporn, et al., 2013).

Fig. 5: Fruits of *Piper nigrum* L. left and *Piper longum* L. right. http://www.sahultrading.com/black-pepper/, http://spicetrekkers.com/products-page/peppers/long-pepper-india/

Based on lipid modulating effects of piperine, a number of natural compounds are obtained and also synthetic compounds are prepared (Tab. 2) by performing small changes in the chemical structure of piperine, seen in Fig. 6.

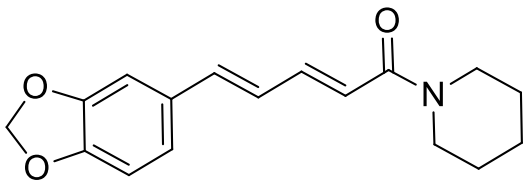


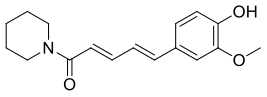
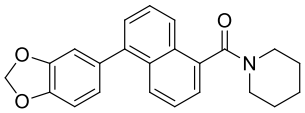
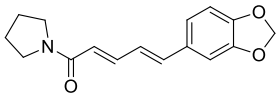
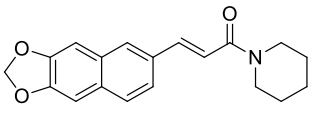
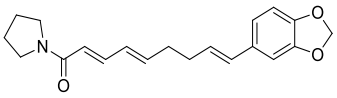
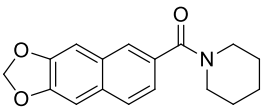
Fig. 6: Chemical structure of piperine

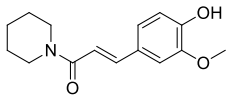
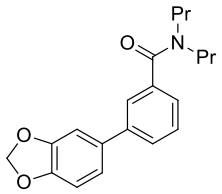
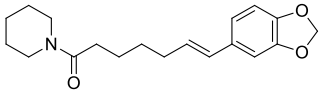
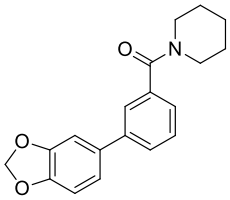
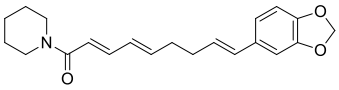
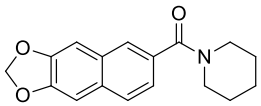
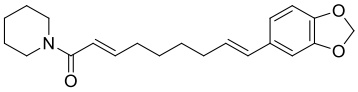
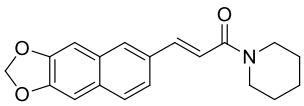
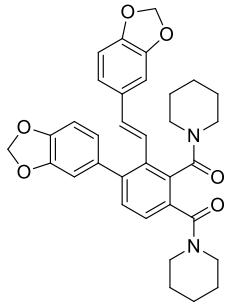
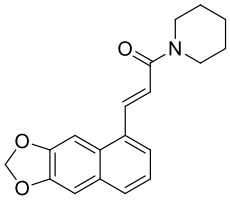
2.2. PIPERINE DERIVATIVES

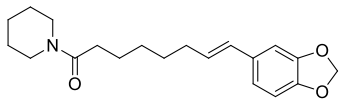
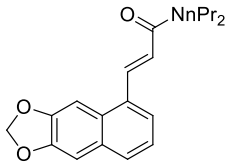
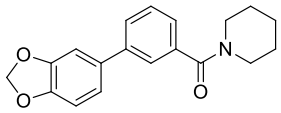
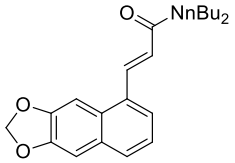
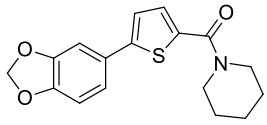
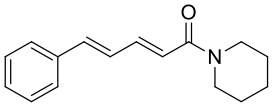
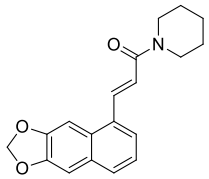
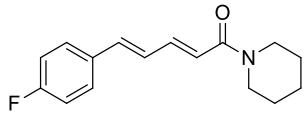
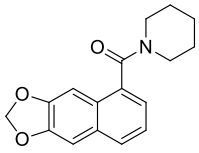
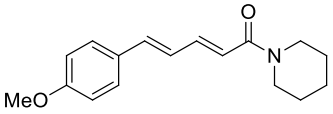
28 piperine derivatives consist of 9 natural derivatives, isolated and provided by the research team of Prof. Judith Rollinger, Department of Pharmacognosy, University of Vienna. The remaining 19 compounds are synthetized and provided by the group of Prof. Marko Mihovilovic, Institute of Applied Synthetic Chemistry, Vienna University of Technology.

For an easy approach and to avoid complications, we will refer to piperine derivatives with the numbers assigned to them in tab. 2.

	Stock solution	**Tested concentration**
Piperine	100 mM	50 µM
Piperine derivatives	30 mM	30 µM

3114  Feruperine	2964  (5-(Benzo[d][1, 3]dioxol-5-yl)naphthalen-1-yl)(piperidin-1-yl)methanone
3115  Piperylin	2965  (E)-3-(Naphtho[2,3-d][1, 3]dioxol-6-yl)-1-(piperidin-1-yl)prop-2-en-1-one
3116  (2E,4E,8E)-9-(Benzo[d][1,3]dioxol-5-yl)-1-(pyrrolidin-1-yl)nona-2,4,8-trien-1-one	2966  Naphtho[2,3-d][1, 3]dioxol-6-yl(piperi-din-1-yl)methanone

3117  N-trans Feruloylpiperidine	3079  3-(Benzo[d][1, 3]dioxol-5-yl)-N,N-dipropylbenzamide
3118  Piperoleine A	3080  (3-(Benzo[d][1, 3]dioxol-5-yl)phenyl)(pi-peridin-1-yl)methanone
3119  Dehydropipernonaline	3081  Naphtho[2,3-d][1, 3]dioxol-6-yl(piperi-din-1-yl)methanone
3120  Pipernonaline	3082  (E)-3-(Naphtho[2,3-d][1, 3]dioxol-6-yl)-1-(piperidin-1-yl)prop-2-en-1-one
3121  Chabamide	3083  (E)-3-(Naphtho[2,3-d][1, 3]dioxol-5-yl)-1-(piperidin-1-yl)prop-2-en-1-one

3122  Pipertipine	3084  (E)-3-(Naphtho[2,3-d][1, 3]dioxol-5-yl)-N, N-dipropylacrylamide
2959  (3-(Benzo[d][1, 3]dioxol-5-yl)phenyl)(piperidin-1-yl)methanone	3085  (E)-N,N-Dibutyl-3-(naphtho[2,3-d][1, 3] dioxol-5-yl)acrylamide
2960  (5-(Benzo[d][1, 3]dioxol-5-yl)thiophen-2-yl)(piperidin-1-yl)methanone	3086  (2E,4E)-5-Phenyl-1-(piperidin-1-yl)penta-2,4-dien-1-one
2961  (E)-3-(Naphtho[2,3-d][1,3]dioxol-5-yl)-1-(piperidin-1-yl)prop-2-en-1-one	3087  (2E,4E)-5-(4-Fluorophenyl)-1-(piperidin-1-yl)penta-2,4-dien-1-one
2962  Naphtho[2,3-d][1,3]dioxol-5-yl(piperidin-1-yl)methanone	3088  (2E,4E)-5-(4-Methoxyphenyl)-1-(piperidin-1-yl)penta-2,4-dien-1-one

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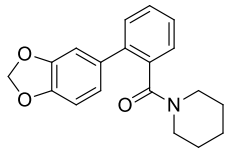	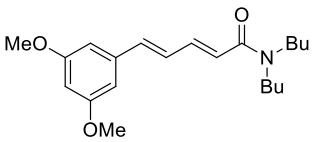
(2-(Benzo[d][1, 3]dioxol-5-yl)phenyl)(pi-peridin-1-yl)methanone	(2E,4E)-N,N-Dibutyl-5-(3,5-dimethoxy-phenyl)penta-2,4-dienamide

Tab. 2: The chemical structure of 28 piperine derivatives (3114-3122 natural derivatives, isolated and provided by the research team of Prof. Judith Rollinger, Department of Pharmacognosy, University of Vienna. The remaining compounds are synthetized and provided by the group of Prof. Marko Mihovilovic, Institute of Applied Synthetic Chemistry, University of Technology, Vienna. Stock solution and tested concentration of piperine and piperine derivatives.

3. SUBCULTURING

THP-1 cells were maintained by replacement of medium every 2 to 3 days. First, they have been seeded at $0.2x10^6$ viable cells/ml following with subcultivation every second day when cell concentration reached $0.8x10^6$ viable cells/ml. This was achieved by taking the culture that reached the later concentration and centrifuging it for 4 min at 150 x g. The supernatant was poured out of the tube which is left with precipitated cells at the bottom. After this, fresh medium was added and the suspension that originated was measured with Vi-Cell™ XR Cell Viability Analyzer from Beckman Coulter (Fullerton, CA. USA) to determine cell concentration. The calculation was made in order to get a cell culture of 20 ml with a concentration of $0.2x10^6$ viable cells/ml. This suspension was stored in the incubator (HERAcell 150 incubator, Thermo Fisher Scientific Inc., Waltham, CA, USA) at 37°C and 5% CO_2.

Sub-culturing is a measure to prevent cell culture to reach an undesirable concentration of $1x10^6$ viable cells/ml. This may cause a slowing down or a complete inhibition of cell proliferation or a change in their appearance, which would make the obtained results unreliable.

4. SEEDING

The aim of the work described above, was to maintain cells in a good form for the next step of the experiment, which was the differentiation of cells into macrophages that was triggered by addition of phorbol 12-myristate 13-acetate (PMA). The calculation was made to reach the concentration of $0.2x10^6$ viable cells/ml. The calculated amount of medium was pipetted into a 50 ml tube, followed by 6 µl of 200 nM PMA and completed by adding the calculated amount of the cell suspension. This preparation was pipetted into a 6-well plate (4 ml/well), with a concentration of $0.2x10^6$ viable cells/ml. The plate was put in the incubator at 37°C and 5% CO_2 for 72 hours to allow cell differentiation.

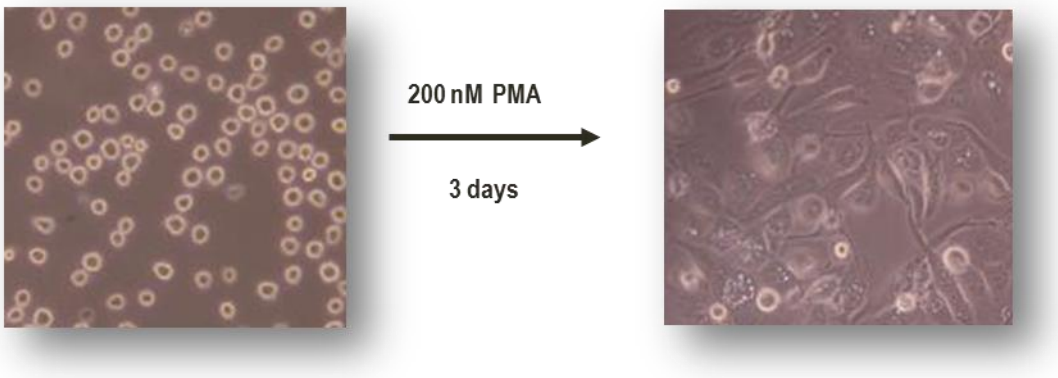

Fig. 7: Differentiation of THP-1 cells when treated with PMA (seen under microscope), provided by Limei Wang a PhD student at Vienna University.

5. TREATMENT

After 72 hours of the incubation period, the 6-well plate is observed under microscope to determine the complete differentiation of cells into macrophages.

For one 6-well plate, 4 compounds (piperine derivatives) were used, piperine (at 50 μM) as a positive control and dimethyl sulfoxide (DMSO) as a solvent vehicle (blank). Piperine derivatives (at 30 μm) are dissolved in DMSO and kept in -20°C.

Serum-free medium (shown in tab. 3) with 0.1% BSA (Bovine serum albumin) and 20 μg/ml unesterified cholesterol (details on stock solutions and providers are given in tab. 4) is prepared. Compounds dissolved in DMSO were thawed and added to the pre-warmed serum-free medium to achieve the needed concentrations for testing.

The 6-well plate, incubated for 72 h with PMA, is washed with 4 ml/well warm PBS (phosphate-buffered saline, tab. 5). After aspiration of PBS, 2 ml of the compound-containing medium is added to the wells (each well containing a different compound for testing). The plate is then incubated for 24 hours in the incubator (37°C, 5% CO_2).

For information on stock solution and final concentration of the compounds see Tab. 2.

Serum-free RPMI-1640 medium		
RPMI-1640 medium		Lonza Group Ltd (Basel, Switzerland)
Penicillin/streptomycin mixture (10 000 U/ml potassium penicillin, 10 000 μg/ml streptomycin sulphate)	100 U/ml penicillin, 100 μg/ml streptomycin	Lonza Group Ltd (Basel, Switzerland)
L-glutamine 200mM	2 mmol/L	Lonza Group Ltd (Basel, Switzerland)

Tab. 3: Composition of serum-free medium.

	Stock solution	**Final concentration**	**Provider**
PMA (phorbol 12-myristate 13-acetate)	1 mM	200 nM	Sigma-Aldrich (Vienna, Austria)
Unesterified cholesterol (delivered as a water-soluble complex with methyl-β- cyclodextrin)	1 mg/ml	20 μg/ml	Sigma-Aldrich (Vienna, Austria)
(BSA) Bovine Serum Albumin	2%	0,1%	

Tab. 4: Stock solution, final concentration and provider of PMA, unesterified cholesterol and BSA.

PBS (phosphate-buffered saline pH 7.4) components	
NaCl	36.0 g (123 mM)
Na_2HPO_4	7.4 g (10 mM)
KH_2PO_4	2.15 g (3 mM)

Tab. 5: Concentrations of PBS components.

6. CELL LYSIS AND PROTEIN EXTRACTION

After the incubation period of 24 hours, cells were lysed and protein extraction was conducted. The procedure was performed on ice.

First, the lysis buffer is prepared, containing NP-40 (Tab. 6), a surfactant that is important to extract and solubilize proteins. The 6-well plate with compound-containing medium was kept on ice and the following steps were performed: the cell culture medium (supernatant) was aspirated carefully with a Pasteur pipette without scratching the cells. After removing the supernatant the cells were washed with 4 ml/well PBS. A total volume of 280 µl of early prepared cold lysis solution (NP-40 buffer, tab. 6) was added to each well. The 6-well plate is then incubated for 30 minutes at 4 °C on a smoothly moving shaker.

Afterwards, a mechanical method was performed using cell-scratcher. The lysate was transferred then into an Eppendorf tube (0.5 ml; also kept on ice). The tubes were put in a centrifuge at 4°C, 16,060 x g for 15 min to remove cell debris. The supernatant was transferred carefully, by not touching the precipitated debris, into another corresponding tube (0.5 ml). The acquired supernatant was stored at -80°C until further use for Western blotting.

NP40 Stock solution	
NaCl	150 mM
HEPES (pH 7.4)	50 mM
NP40	1 %
NP40 Lysis buffer (1000 µl)	
NP40	940 µl
complete® 25×	40 µl
PMSF (0.1 M)	10 µl
NaF (1 M)	5 µl
Na_3VO_4 (100 mM)	5 µl

Tab. 6: NP40 solution and NP40 buffer components and concentrations

7. BRADFORD ASSAY

Bradford protein assay is an analytical method used to determine the protein concentration in a solution. In this case, to determine the protein concentration in the acquired supernatant that will facilitate loading procedure in an upcoming step, the gel electrophoresis.

First, the samples for Bradford assay were prepared: protein supernatant was diluted in double distilled water (DDW) to reach a protein dilution 1:10. Reagent solution was freshly made by diluting RotiQuant in DDW 1:3.75 and albumin solutions with known concentrations were also prepared (Tab. 7). Each sample was measured in triplicate. A 96 transparent well plate was used, in which the samples were loaded in this order: in each well was loaded 190 µl of reagent solution, followed by 5 µl of 10 times diluted lysis buffer - that was used for protein extraction - for the calibration curve, 10 µl of double distilled water (DDW) for the samples, followed by 10 µl of albumins with a known concentration for the calibration curve and finishing by adding 5 µl of diluted protein samples (illustrated in picture 7 below).

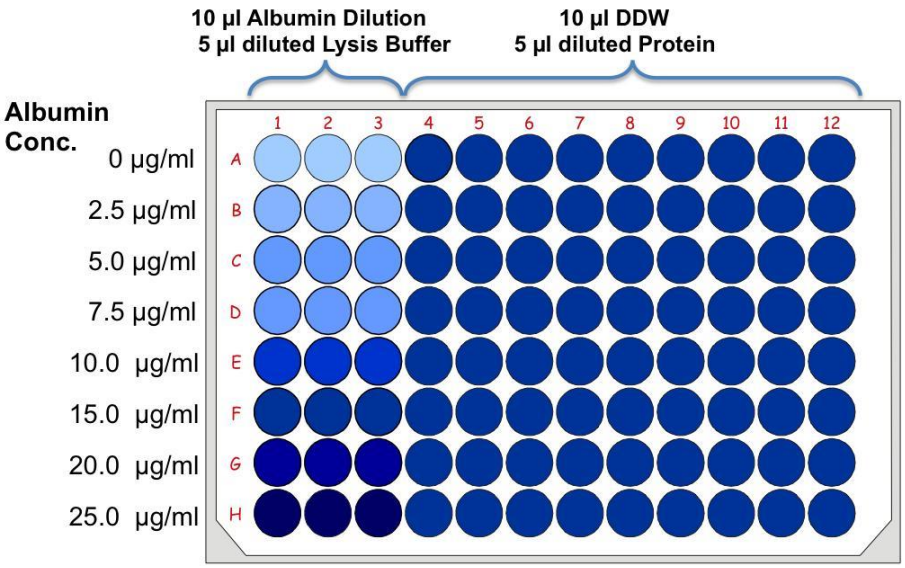

Fig. 7: Schematic pipetting plan for Bradford Assay.

At the end, the total volume in each well was 205 µl. After a slight shaking, the absorption was measured with Tecan SunriseTM (Tecan, Grödig, Austria) at 595 nm. From the values obtained from this measurement, a calibration curve was built which was used to determine the protein concentration of the samples in each well, respectively the needed amount of proteins that are going to be loaded in the next step, the gel electrophoresis.

Reagent solution		Provider
Roti®-Quant 5×Konzentrat	1	Roth (Vienna, Austria)
DDW (double distilled water)	3.75	
Albumin serial dilutions	0 µg/ml 2.5 µg/ml 5 µg/ml 7.5 µg/ml 10 µg/ml 15 µg/ml 20 µg/ml 25 µg/ml	

Tab. 7: Reagent solution and albumin serial dilutions.

8. ELECTROPHORESIS AND WESTERN BLOTTING

The acquired supernatant stored at -80^{o}C, was prepared for electrophoresis by adding Sample buffer (tab. 8.; SDS + ß mercaptoethanol) to each protein sample in a volume that corresponds to 1/3 of the sample volume, a procedure that leads to protein denaturation. 10% polyacrylamide-gel (Tab. 9) was used to separate proteins according to their electrophoretic mobility.

The samples were loaded in each lane of 10% polyacrylamide-gel in the amount that was gained during Bradford assay measurement. For protein separation, one gel required an electric current of 25 mA for 85 minutes in an electrophoresis buffer 10x (Tab. 10). After this, the proteins from polyacrylamide-gel were transferred to a polyvinylidene fluoride membrane (PVDF) using voltage at 100 V for 120 minutes in a blotting buffer (Tab. 10). The membrane was then incubated in 5% skimmed milk/TBS-T (Tab. 11) for 60 minutes to block the bindings of other proteins (antibody) at non-specific binding sites. Next, the membrane was washed 3 times with TBS-T, each time for 15 minutes and then was incubated overnight in primary anti-ABCA1 antibody (for ABCA1 detection), respectively primary ß-actin antibody (for β-actin detection). Antibodies used for incubation are displayed in tab. 12 below.

On the next day the membrane was washed again 3 times with TBS-T, each time for 15 minutes and was incubated for 90 minutes with the secondary HRP (horseradish peroxidase) antibody, respectively secondary ß-actin antibody that will bind to the primary antibody and enhance the signal. In the meantime, the chemiluminescence reagent was prepared (Tab. 13) and added to the PVDF membrane in order to detect the chemiluminescence with a LAS-3000^{TM} Luminescent Image Analyzer (Fujifilm, Tokyo, Japan).

The acquired images were then analyzed with so-called AIDA software (Raytest GmbH, Straubenhardt, Germany). The results from this analyzer were calculated by normalizing protein bands of ABCA1 with corresponding bands of actin, which led to a schematic display of the activity of the compounds that were tested.

3× SDS Sample buffer	
Tris-HCl 0.5 M pH 6,8	37.5 ml
SDS	6 g
Glycerol	30 ml
Bromphenol blue	15 mg
DDW (double distilled water)	ad 100 ml
Completed sample buffer	
3×SDS Sample buffer	85% (255 µl)
β-mercaptoethanol	15% (45 µl)

Tab. 8: Sample buffer.

10% polyacrylamide-gel	
Resolving gel 10%	
DDW (double distilled water)	3.05 ml
PAA (30%)	2.5 ml
Tris-HCl (1.5 M; pH 8.8)	1.875 ml
SDS (10%)	75 µl
TEMED	7.5 µl
APS (10%)	37.5 µl

Stacking gel	
DDW (double distilled water)	2.62 ml
PAA (30%)	640 µl
Tris-HCl (1.25 M; pH 6.8)	375 µl
SDS (10%)	37.5 µl
TEMED	7.5 µl
APS (10%)	37.5 µl

Tab. 9: Preparation of 10% polyacrylamide-gel.

Electrophoresis buffer 10×	
Tris-base	30 g
Glycine	144 g
SDS	10 g
DDW (double distilled water)	ad 1000 ml
Blotting buffer 5×	
Tris-base	15.169 g
Glycine	72.9 g
DDW (double distilled water)	ad 1000 ml

Tab. 10: Buffers and reagents for electrophoresis.

TBS-T 10× (pH 8.0)	
Tris-base	30 g
NaCl	111 g
Tween 20	10 ml
DDW (double distilled water)	ad 1000 ml

Tab. 11: TBS-T 10x preparation.

Immunogen	**Molecular weight**	**Source of antibody**	**Provider**	**Dilution**
ABCA1	220 kDa	Rabbit polyclonal	Novus Biologicals (Littleton, CO, USA)	1:500 in 3% milk in TBS-T
Actin	42 kDa	mouse, monoclonal	MP Biomedicals (Santa Ana, CA, USA)	1:1000 in TBS-T
rabbit IgG	Various	mouse, pc linked to HRP	Cell Signaling Technology (Danvers, MA, USA)	1:1000 in 3 % milk in TBS-T
mouse IgG	Various	goat, polyclonal, linked to HRP	MP Biomedicals (Santa Ana, CA, USA)	1:1000 in TBS-T

Tab. 12: Antibodies used for incubation.

Chemiluminescence reagent	
DDW (double distilled water)	4.5 ml
Tris-base (1 M pH 8.5)	500 µl
Luminol	22.5 µl
Coumaric acid	11.2 µl
H_2O_2 (30%)	1.5 µl
Luminol stock solution	
Luminol	0.44 g
DMSO	10 ml
p-Coumaric acid stock solution	
Coumaric acid	0.15 g
DMSO	10 ml

Tab. 13: Chemiluminescent reagents.

D. CELL VIABILITY – RESAZURIN ASSAY

Resazurin is a redox dye that exhibits both colorimetric and fluorometric changes which are related to cellular metabolic activity (Vega-Avila, Elisa, and Michael K. Pugsley 2011).

Resazurin assay is used to determine if the tested compounds have effects on cell proliferation or show direct cytotoxic effects that eventually lead to cell death (Riss, Terry L., et al. (2015). This assay requires the incubation of resazurin reagent with viable cells, which convert resazurin into resorufin, which is pink and fluorescent and can be detected with a plate reader.

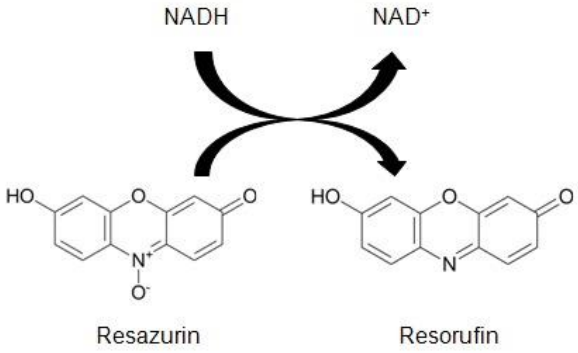

Fig. 8: The conversion of resazurin into resorufin.

The amount of resorufin produced is proportional with the number of viable cells present. If the cells are dead they can't convert resazurin to resorufin.

Resazurin assay procedure:

First THP-1 cells with a density of $0.2x10^6$ per mL are seeded in a 96-well plate. The amount of medium per well was 100 µL. Subsequently, cells are treated with 200 nM PMA in RPMI-1640 medium supplemented with 10% heat-inactivated FBS and then incubated for 72 h. This leads to differentiation of THP-1 cells into macrophages. After the differentiation, compounds are tested at different concentrations from 1, 10, 20 and 40 µM (compound concentration and stock solution is shown on tab. 2) prepared with RPMI-1640 FBS-free medium. 0.1% BSA and 10 µg/mL unesterified cholesterol are added to the medium. After THP-1 macrophages were washed once with warm PBS (37 ^oC) they are treated with

indicated compounds at given concentration and incubated for 24 h. Digitonin was used as a positive control (50 µg/mL). After the incubation period, cells were washed once, again with warmed PBS (37 ^{o}C) and then were incubated for 4 h with 10 µg/mL resazurin which is dissolved in PBS to allow the conversion to the highly fluorescent resorufin. After the incubation period, the plate was read with a Tecan GENiosPro plate reader (Männedorf, Switzerland) at a wavelength of 580 nm (emission) and 535 nm (excitation).

E. RESULTS AND DISCCUSION

Based on the chemical structure of piperine, a number of piperine derivatives have been developed.

There were 28 piperine derivatives, where the first nine of them from 3114 to 3122 (for identification of piperine derivatives numbers see tab. 2) are natural derivatives, isolated and provided by the research team of Prof. Judith Rollinger, Department of Pharmacognosy, University of Vienna and the remaining compounds synthetized and provided by the group of Professor Marko Mihovilovic, Institute of Applied Synthetic Chemistry, Vienna University of Technology. They were tested through a widely used analytical technique known as Western blot, a technique for protein detection. The aim of the work was to discover if one or more of these piperine derivatives can display a stronger effect than piperine.

Piperine derivatives were tested in series, four compounds at a time, in one 6-well plate with piperine as a positive control and DMSO as a solvent vehicle. These piperine derivatives were tested on the THP-1 cell line, a human monocytic cell line, widely used as *in vitro* model because of its long lifespan and also the ability to differentiate into macrophages when treated with PMA. All of the compounds were tested at a concentration of 30 µM, since the half maximal effective concentration (EC_{50}) of the piperine inducing cholesterol efflux is around 35 µM.

Each group was tested 3 times and every compound induced different ABCA1 protein levels compared with piperine, as shown in the tab. 14 below:

		mean±SD	
	Piperine	1,00	
np	3114	0,85±0,081, n.s.	n=3
np	3115	0,82±0,076, *	n=3
np	3116	0,89±0,046, n.s.	n=3
np	3117	0,65±0,041, ***	n=3
np	3118	1,03±0,091, n.s.	n=3
np	3119	1,12±0,120, n.s.	n=3
np	3120	0,79±0,116, n.s.	n=3
np	3121	1,04±0,309, n.s.	n=3
np	3122	0,5±0,011, **	n=3
s	2959	1,14±0,138, n.s.	n=3
s	2960	0,93±1,224, n.s.	n=3
s	2961	1,09±0,161, n.s.	n=3
s	2962	0,94±0,313, n.s.	n=3
s	2963	1,21±0,255, n.s.	n=3
s	2964	0,60±0,079, **	n=3
s	2965	0,94±0,36, n.s.	n=3
s	2966	1,08±0,301, n.s.	n=3
s	3079	0,94±0,194, n.s.	n=3
s	3080	1,12±0,179, n.s.	n=3
s	3081	1,06±0,144, n.s.	n=3
s	3082	0,99±0,154, n.s.	n=3
s	3083	1,39±0,293, n.s.	n=3
s	3084	1,02±0,131, n.s.	n=3
s	3085	0,47±0,269, *	n=3
s	3086	1,26±0,152, n.s.	n=3
s	3087	1,3±0,106, n.s.	n=3
s	3088	1,31±0,27, n.s.	n=3
s	3089	4,07±0,951, ***	n=3

Tab. 14: Results of ABCA1 protein expression levels normalized to piperine. Np=natural piperamides, isolated from black pepper fruits, s-synthesized piperine derivatives. For identification of sample numbers see chapter 2.2.

The first group of four compounds entitled with numbers 3114, 3115, 3116 and 3117 (for identification of compound numbers see chapter 2.2) provided the results shown in the fig. 9A below:

Every compound of this group provided a lower expression level (no significance) of ABCA1 protein, compared to the positive control piperine, with 3117 providing a significantly lower expression level of ABCA1 protein.

Since this could be due to a cytotoxic effect of 3117 on the THP-1 cell line, we have measured its ability to affect resazurin conversion. Digitonin, a cytotoxic natural product, was used as positive control. 3117 was tested at different concentrations from 1, 10, 20 and 40 µM. As presented in fig. 9B, 3117 did not exhibit any effect on the viability of THP-1 macrophages from neither of the concentration, whereby, digitonin at 50 µg/mL decreased THP-1 cell viability by around 90%.

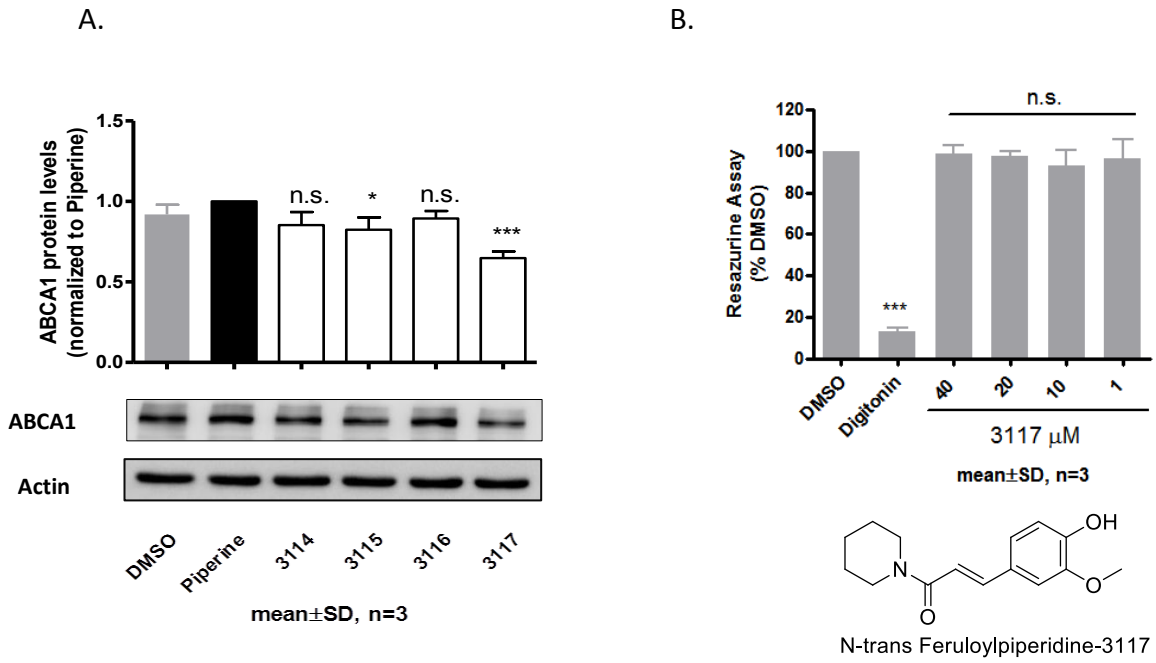

Fig. 9: A. ABCA1 protein expression levels after treatment with 3114, 3115, 3116 and 3117. THP-1 cells were differentiated with 200 nM PMA for 72 h and then treated with the compounds mentioned above. Piperine was used as positive control and DMSO as solvent vehicle. Protein bands of ABCA1 are normalized with corresponding bands of actin as a control for equal protein loading. All values are means ± S.E.M. of at least three independent experiments and statistical significance was determined by one-way ANOVA with Bonferroni post hoc test; *p<005, ***p<0.001, n.s. no significance. B. Cell viability assay for 3117.

The second group of four compounds entitled with numbers 3118, 3119, 3120 and 3121 (for identification of compound numbers see chapter 2.2) provided the results shown in fig. 10A below.

Every compound of this group provided a nonsignificant expression level of ABCA1 protein, compared to the positive control piperine, with 3120 providing a lower but also nonsignificant expression level of ABCA1 protein, compared with the positive control piperine.

Since this could be due to a cytotoxic effect of 3120 on the THP-1 cell line, we have measured its ability to affect resazurin conversion. Digitonin, a cytotoxic natural product, was used as positive control. 3120 was tested at different concentrations from 1, 10, 20 and 40 µM. As presented in fig. 10B, 3120 exhibits a small change (significant) at a concentration of 40 µM by decreasing THP-1 cell viability by around 10%. The other tested concentrations did not exhibit any effect (no significance) on the viability of THP-1 macrophages. Digitonin at 50 µg/mL decreased THP-1 cell viability by around 90%.

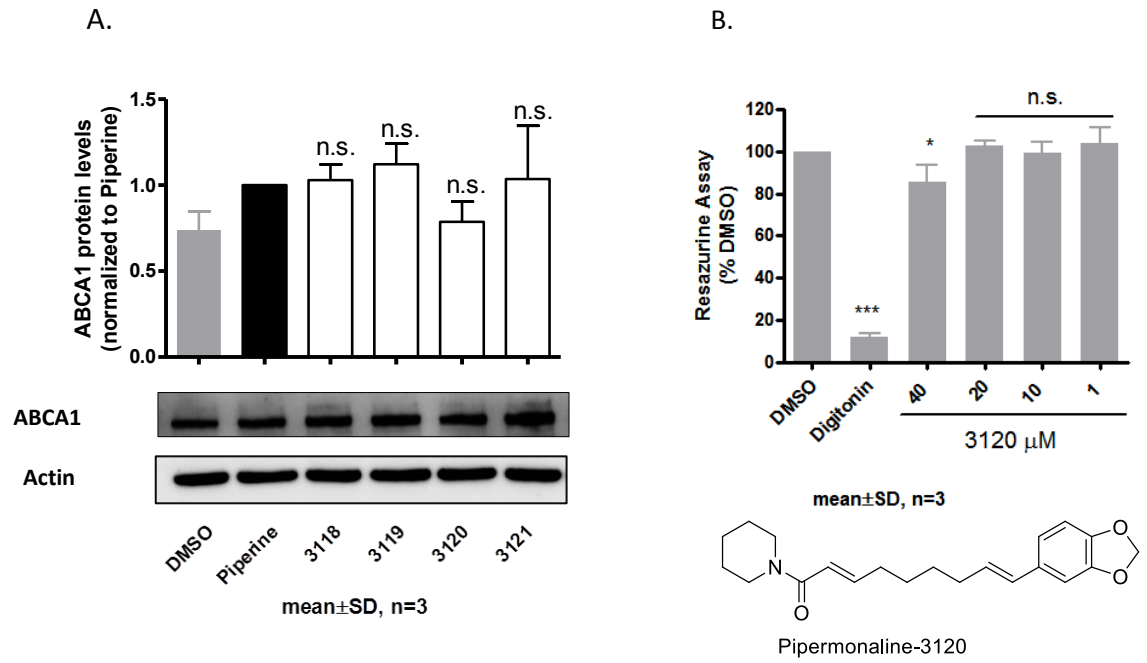

Fig. 10: A. ABCA1 protein expression levels after treatment with 3118, 3119, 3120 and 3121. THP-1 cells were differentiated with 200 nM PMA for 72 h and then treated with the compounds mentioned above. Piperine was used as positive control and DMSO as solvent vehicle. Protein bands of ABCA1 are normalized with corresponding bands of actin as a control for equal protein loading. All values are means ± S.E.M. of at least three independent experiments and statistical significance was determined by one-way ANOVA with Bonferroni post hoc test; n.s. no significance. B. Cell viability assay for 3120.

The third group of four compounds entitled with numbers 3122, 2959, 2960 and 2961 (for identification of compound numbers see chapter 2.2) provided the results shown in fig. 11A.

By this group 2959, 2960 and 2961 provided a nonsignificant expression level variation of the ABCA1 protein, compared to the positive control piperine, with 3122 providing a significantly lower expression level of the ABCA1 protein.

Since this could be due to a cytotoxic effect of 3122 on the THP-1 cell line, we have measured its ability to affect resazurin conversion. Digitonin, a cytotoxic natural product, was used as positive control. 3122 was tested at different concentrations from 1, 10, 20 and 40 µM. As presented in fig. 11B, 3122 exhibits cytotoxic effects by decreasing THP-1 cell viability by around 20% at a concentration of 10 µM, by around 40% at a concentration of 20 µM and by around 50% at a concentration of 40 µM. Only at concentration of 1 µM, 3122 did not affect the viability of THP-1 macrophages. Digitonin at 50 µg/mL decreased THP-1 cell viability by around 90%.

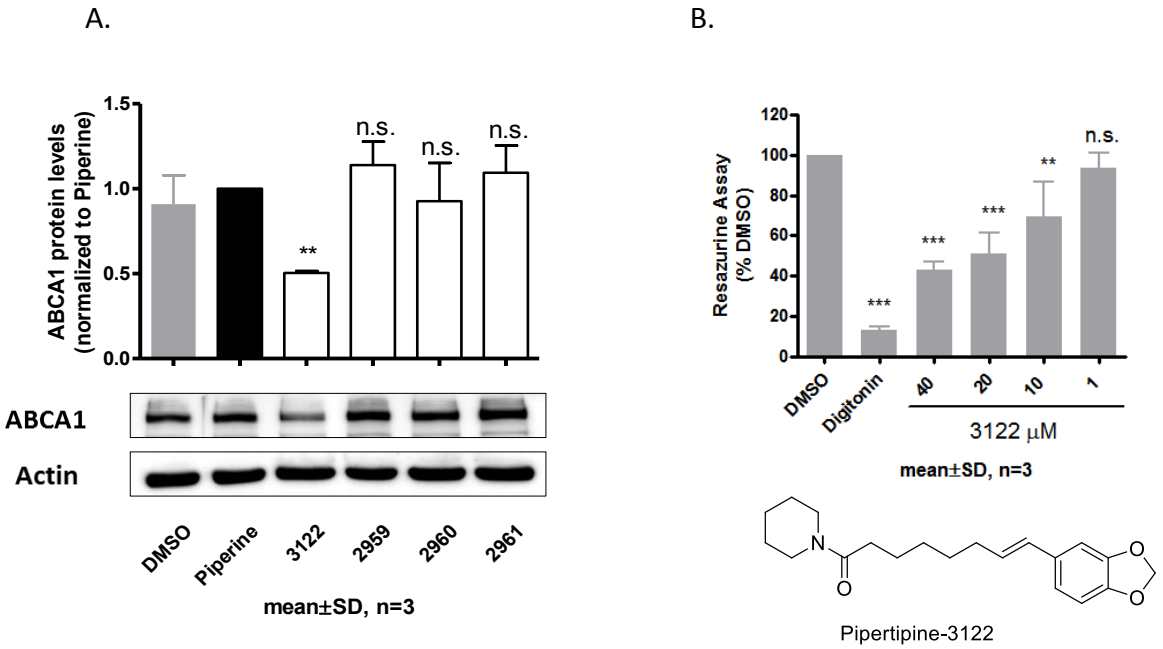

Fig. 11: A. ABCA1 protein expression levels after treatment with 3122, 2959, 2960 and 2961. THP-1 cells were differentiated with 200 nM PMA for 72 h and then treated with the compounds mentioned above. Piperine was used as positive control and DMSO as a solvent vehicle. Protein bands of ABCA1 are normalized with corresponding bands of actin as a control for equal protein loading. All values are means ± S.E.M. of at least three independent experiments and statistical significance was determined by one-way ANOVA with Bonferroni post hoc test; **p<001, n.s. no significance. B. Cell viability assay for 3122.

The fourth group of four compounds entitled with numbers 2962, 2963, 2964 and 2965 (for identification of compound numbers see chapter 2.2) provided the results shown in fig. 12A below:

2963 provided a slightly higher but nonsignificant expression level of ABCA1 protein. 2962 and 2965 provided a lower but also nonsignificant expression level of ABCA1 protein, whereas 2964 provided a significantly lower expression level of ABCA1 protein compared to positive control piperine.

Since this could be due to a cytotoxic effect of 2964 on the THP-1 cell line, we have measured its ability to affect resazurin conversion. Digitonin, a cytotoxic natural product, was used as positive control. 2964 was tested at different concentrations from 1, 10, 20 and 40 µM. As presented in fig. 12B, 2964 exhibits cytotoxic effects by decreasing THP-1 cell viability by around 5% at a concentration of 10 µM, by around 10% at a concentration of 20 µM and by around 40% at a concentration of 40 µM. Only at concentration of 1 µM, 2964 did not affect the viability of THP-1 macrophages. Digitonin at 50 µg/mL decreased THP-1 cell viability by around 90%.

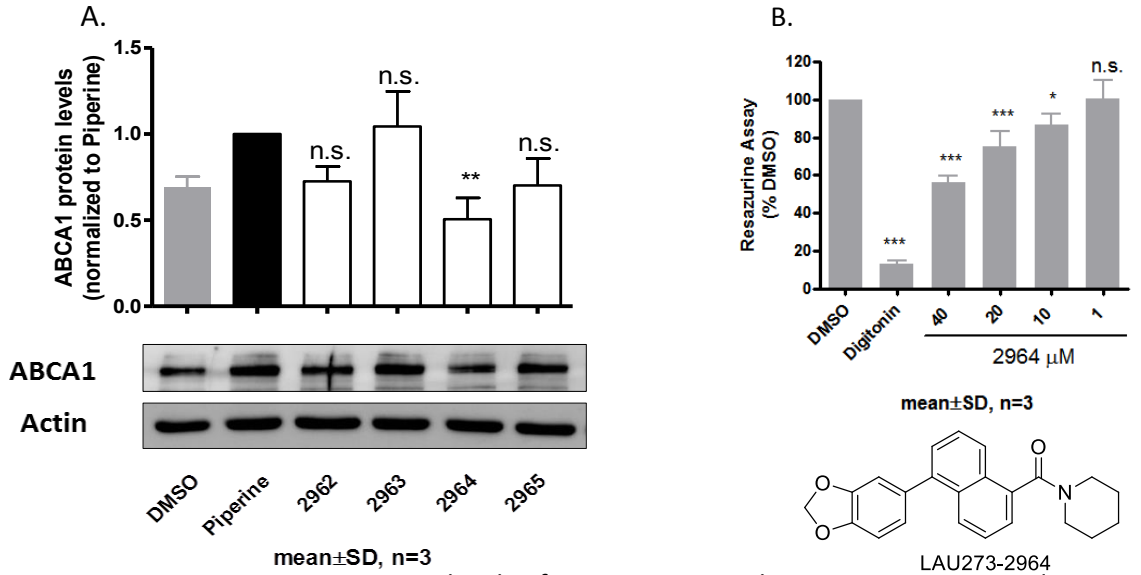

Fig. 12: A. ABCA1 protein expression levels after treatment with 2962, 2963, 2964 and 2965. THP-1 cells were differentiated with 200 nM PMA for 72 h and then treated with the compounds mentioned above. Piperine was used as positive control and DMSO as a solvent vehicle. Protein bands of ABCA1 are normalized with corresponding bands of actin as a control for equal protein loading. All values are means ± S.E.M. of at least three independent experiments and statistical significance was determined by one-way ANOVA with Bonferroni post hoc test; **p<001, n.s. no significance. B. Cell viability assay for 2964.

The fifth group of four compounds entitled with numbers 2966, 3079, 3080 and 3081 (for identification of compound numbers see chapter 2.2) provided the results shown in fig. 13 below:

Every compound of this group provided a nonsignificant expression level of ABCA1 protein, compared to the positive control piperine.

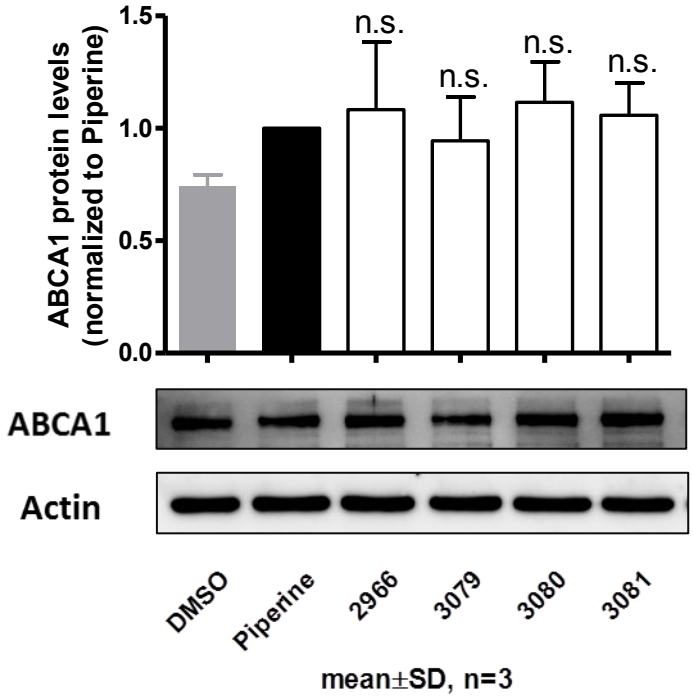

Fig. 13: ABCA1 protein expression levels after treatment with 2966, 3079, 3080 and 3081. THP-1 cells were differentiated with 200 nM PMA for 72 h and then treated with the compounds mentioned above. Piperine was used as positive control and DMSO as a solvent vehicle. Protein bands of ABCA1 are normalized with corresponding bands of actin as a control for equal protein loading. All values are means ± S.E.M. of at least three independent experiments and statistical significance was determined by one-way ANOVA with Bonferroni post hoc test; n.s. no significance.

The sixth group of four compounds entitled with numbers 3082, 3083, 3084 and 3085 (for identification of compound numbers see chapter 2.2) provided the results shown in fig. 14A below:

From this group, the first three compounds provided a nonsignificant expression level of ABCA1 protein, with 3085 providing a significantly lower expression level of ABCA1 protein compared to the positive control piperine.

The cell viability assay for 3084 and 3085 show (Fig. 14B) that both compounds at 40 µM have decreased THP-1 cell viability by up to 90% respectively 80%. 3084 at 1, 10 and 20 µM did not exhibit any effect on the viability of THP-1 macrophages, whereas 3085 decreased THP-1 cell viability also at 20 µM by around 20%, with other concentrations 1 and 10 µM not affecting cell viability. Digitonin at 50 µg/mL decreased THP-1 cell viability by around 90%.

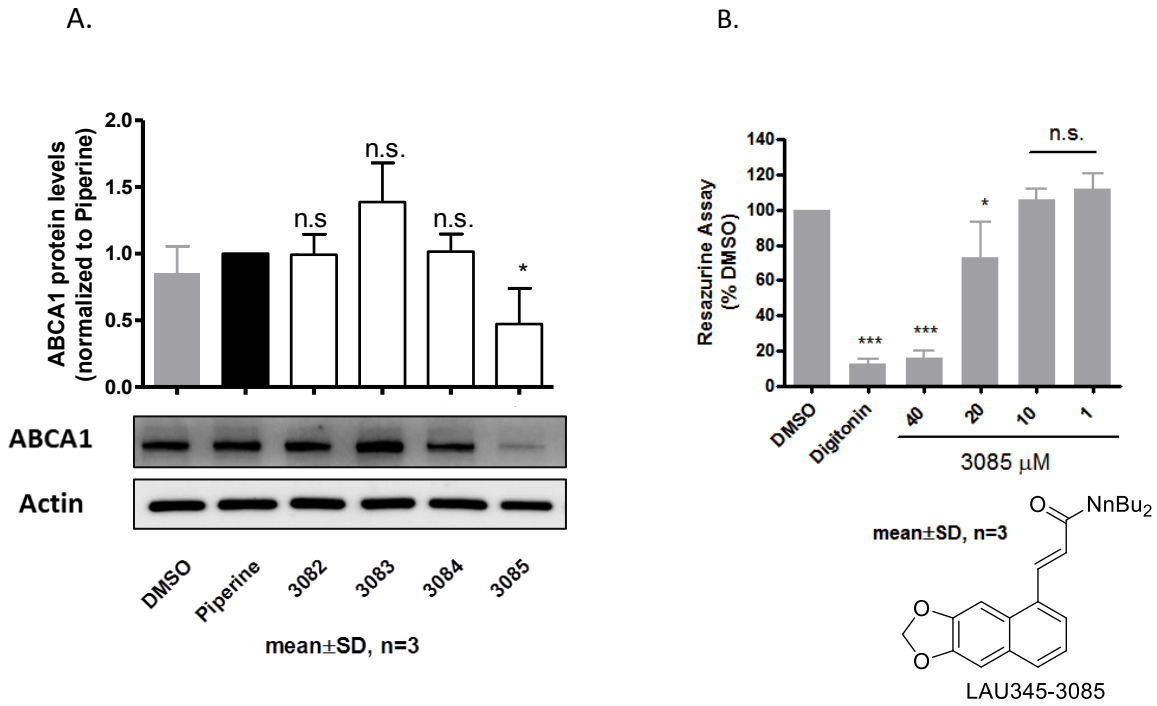

Fig. 14: A. ABCA1 protein expression levels (normalized with β-actin) after treatment with 3082, 3083, 3084 and 3085. THP-1 cells were differentiated with 200 nM PMA for 72 h and then treated with the compounds mentioned above. Piperine was used as positive control and DMSO as a solvent vehicle. Protein bands of ABCA1 are normalized with corresponding bands of actin as a control for equal protein loading. All values are means ± S.E.M. of at least three independent experiments and statistical significance was determined by one-way ANOVA with Bonferroni post hoc test; *p<005, n.s. no significance. B. Cell viability assay for 3085.

The last group of four compounds entitled with numbers 3086, 3087, 3088 and 3089 (for identification of compound numbers see chapter 2.2) provided the results shown in fig. 15A below:

The first three compounds of this group provided a nonsignificant elevated levels of ABCA1 protein, compared to the positive control piperine, whereas 3089 provides a significantly higher expression level of ABCA1 protein compared to the positive control piperine. So in the case of 3089 the expression level of ABCA1 protein normalized to piperine was threefold.

The cell viability assay for 3089 showed around 20% decrease on THP-1 cell viability at a concentration of 20 µM. The other tested compound concentrations were at 1, 5 and 10 µM and as presented in fig. 15B, they did not exhibit any effect on the viability of THP-1 macrophages. Digitonin, a cytotoxic natural product, was used as positive control, where at 50 µg/mL decreased THP-1 cell viability by around 90%.

A. B.

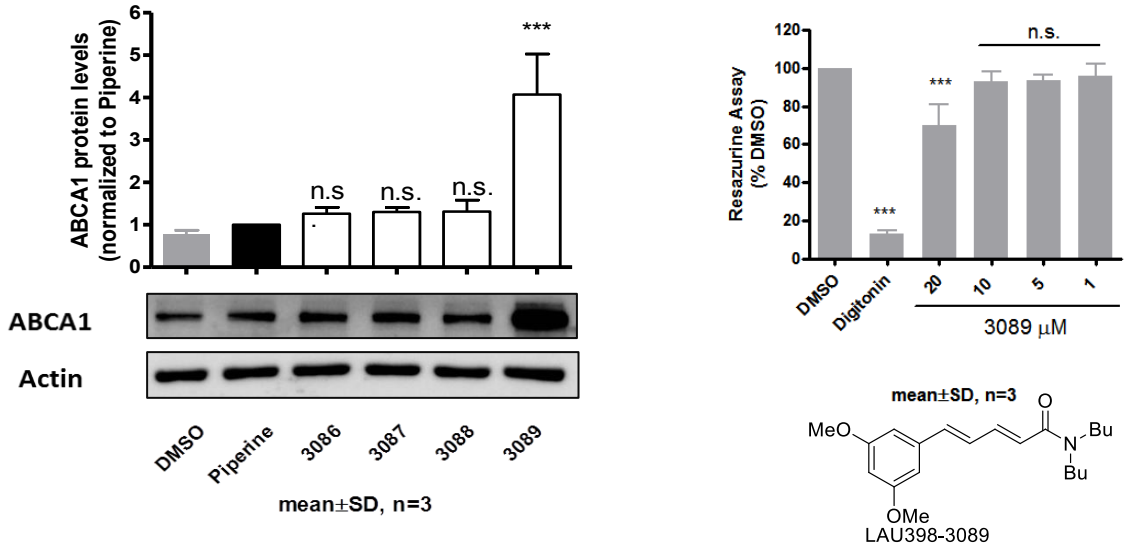

Fig. 15: A. ABCA1 protein expression levels (normalized with β-actin) after treatment with 3086, 3087, 3088 and 3089. THP-1 cells were differentiated with 200 nM PMA for 72 h and then treated with the compounds mentioned above. Piperine was used as positive control and DMSO as a solvent vehicle. Protein bands of ABCA1 are normalized with corresponding bands of actin as a control for equal protein loading. All values are means ± S.E.M. of at least three independent experiments and statistical significance was determined by one-way ANOVA with Bonferroni post hoc test; ***p<0001, n.s. no significance. B. Cell viability assay for 3089.

Since 3089 was the only compound from 28 with values that could be of great importance, further evaluations have been performed. As shown in fig. 16, 3089 was tested in different concentrations in order to get information about the concentration-dependent protein expression changes of ABCA1 in this case normalized to DMSO. The first two concentrations at 0.3 µM and 1 µM provided nonsignificant elevated levels of ABCA1 protein normalized to DMSO. Then from 3 µM there was a significantly elevated level of ABCA1 protein which reaches higher levels at 10 µM but didn't change again even at 30 µM.

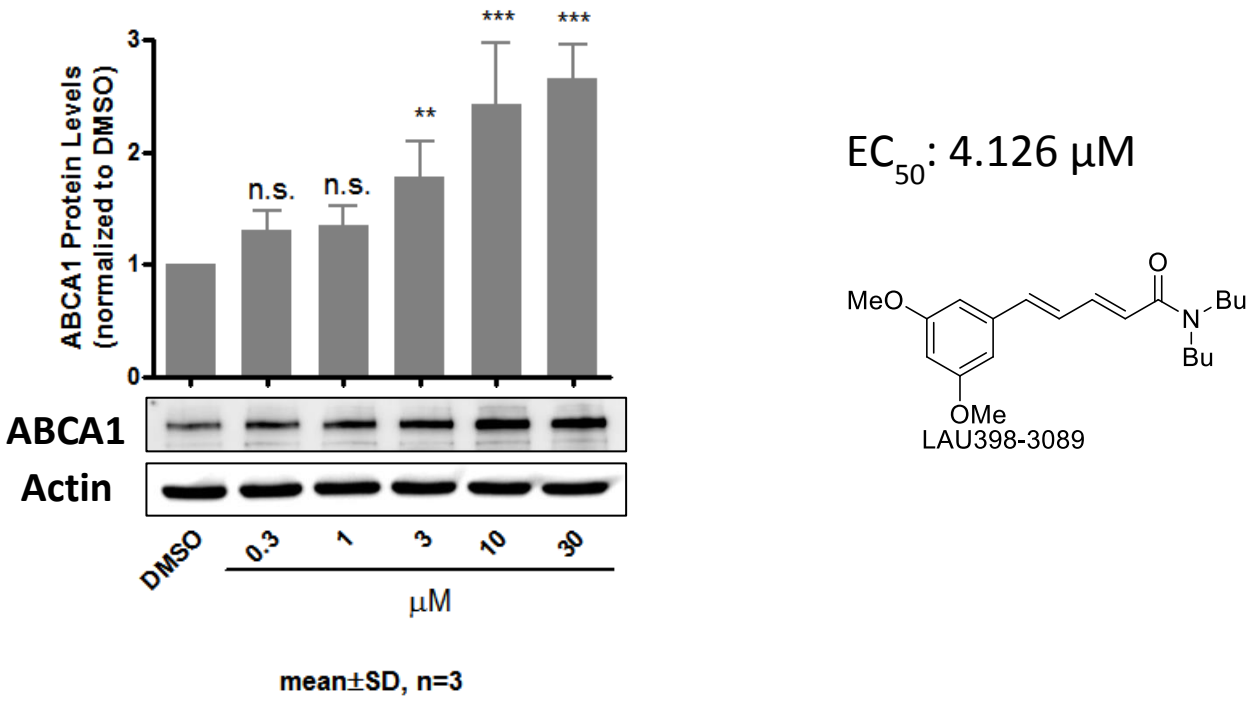

Fig. 16: ABCA1 protein expression levels after treatment with 3089 at different concentrations. THP-1 cells were differentiated with 200 nM PMA for 72 h and then treated with the compound mentioned above, normalized to DMSO as a solvent vehicle. Protein bands of ABCA1 are normalized with corresponding bands of actin as a control for equal protein loading. All values are means ± S.E.M. of at least three independent experiments and statistical significance was determined by one-way ANOVA with Bonferroni post hoc test; **p<0.01, ***p<0001, n.s. no significance.

Compared with piperine, where the half maximal effective concentration (EC_{50}) is around 37.4 µM, 3089 has a half maximal effective concentration (EC_{50}) around 4.126 µM.

The ability of 3089 to induce protein expression levels of ABCA1, makes it an interesting candidate for further investigation, to determine the precise mechanism of action and to evaluate its potential in the treatment of atherosclerosis.

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