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Novel vitamin D_3-hydroxyderivatives as candidates for the therapy against skin-aging and photo-aging: bioinformatical analysis

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ABSTRACT

Vitamin D3 acts through its most active form, calcitriol, 1α,25-dihydroxyvitamin D3 [1,25(OH)2D3] as agonist of one of the receptors involved in this ligand action, vitamin D receptor (VDR), which is also a transcription factor. Numerous modifications of calcitriol at its side-chain, C-ring, A-ring, triene system, alone or in combination, as well as nonsteroidal mimics provided new VDR agonists and some antagonists with biological activity and possible therapeutical potential. Some of the D3 metabolites, including 20,23(OH)2D3 and 20(OH)D3 are able to inhibit RORE-mediated transactivation, as well as the interaction between the RORα/γ ligand-binding domain (LBD) with an LXXLL coactivator peptide. Our analysis of recently reported microarray data on vitamin D3 (D3) induced changes in cultured human keratinocytes indicated that D3 hydroxyderivatives stimulate the expression of genes involved in anti-aging activities. Furthermore, we noted upregulation of the kallikrein gene family by 1,25(OH)2D3 after 24-hour treatment, including stimulation of KLK6, KLK13, KLK3, KLK9, KLK5, KLK7, and KLK10. Also, after 6-hour incubation with 1,25(OH)2D3, the upregulation of KLK6, KLK13, and KLK3 was seen. Interestingly, ACEIs administered to hypertensive rats doubled the lifespan of these animals. In humans, ACEIs prevent hallmarks of aging, such as organ fibrosis and cardiac hypertrophy. We noted also that vitamin D3-hydroxyderivatives act against oxidative stress through upregulation of thioredoxin reductase (TXNRD1) and heme reductase-1 (HMOX-1) gene expression in keratinocytes treated for 24h. Another mechanism of anti-aging properties of inverse agonist RORa/γ is the resolution of inflammation caused by T helper (Th17) lymphocytes and switching the immune response into T regulatory (Treg) lymphocytes activation, with silencing of the inflammation state and reducing the inflammation process. The gene connected with inflammatory response, AKR1C3 (which encodes prostaglandin F synthase) is also strongly downregulated by 20,23(OH)2D3 in keratinocytes after incubation for 24 h. We suggest that vitamin D3 analogs, such as 20,23(OH)2D3, 1,25(OH)2D3, and 20(OH)D3 may have anti-aging properties through action on different pathways connected with DNA repair.

Key words: skin aging, photo-aging, ceramide, melanosis, atopic dermatitis, vitamin D_3-hydroxyderivatives

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Introduction

An immune profile during aging was established to uderstnad better which compounds may be beneficial for promoting healthy aging. T cells from the older group of patients (60-year-old people) produced more cytokines associated with Th17 in in-vitro studies, a group of T lymphocytes, which promotes inflammation occurring in many diseases, including cancer cardiac disease, and neurodegeneration [1, 2].

Some of the vitamin D_3 analogs with anti-inflammatory properties that can modulate the immune response, including $20,23(OH)_2D_3$ and $20(OH)D_3$, are able to inhibit the interaction between an LBD of rexinoid orphan receptor a/γ (RORa/γ) with an LXXLL coactivator peptide and can act as inverse agonists of RORα/γ [3–14].

In the current paper, we describe the impact of vitamin D_3-hydroxyderivatives on the inhibition of inflammaging and a decrease of gene expression associated with the aging process.

Structure and chemical properties of vitamin D analogs

Vitamin D_3 acts through its active form, calcitriol, 1α,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] as an agonist of one of the receptors involved in this ligand action, vitamin D receptor (VDR), which is also a transcription factor [15]. Numerous modifications of calcitriol at its side-chain, C-ring, A-ring, triene system, alone or in combination, as well as nonsteroidal mimics, provided new VDR agonists and some antagonists with biological activity and a possible therapeutical potential [16]. About 150 crystal structures of VDR's ligand-binding domain were discovered with various vitamin D derivatives, which allows molecular studies of the dependences between the structure of the particular compound and their mechanism of action (Fig. 1) [15]. The VDR is a nuclear receptor and an endocrine receptor. The mechanisms of its action are comparable to those connected with receptors for estrogen and glucocorticoids. VDR's ligand-binding domain (LBD) is considered as structurally conserved and comprises 11–15 α-helices, varies solved crystal complexes. The LBD structure depends on the folding of an intrinsically disordered region included between α-helices H1 and H3 and a presence of the helix HX between α-helices H11 and H1228 [17, 18]. The lower part of the LBD contains a ligand-binding pocket (LBP), which has a structure of a cavity of ~700 Å^3 volume formed by some of the 40 mostly nonpolar amino acids [19–21]. Within the LBP, the three pairs of polar amino acids fix by hydrogen bonds each one of the three particulars OH groups (at C-1α, C-25, C-3β position) of $1,25(OH)_2D_3$. The group of 1α-OH interacts with helix H5 (S278), as well as helix H1 (Y143), and the 3β-OH group links helix H3 (S237) and helix H5 (R274). The 25-OH group interacts with H305 (the loop located between helices H7 and H6) and H397 (in helix H11) [22, 23].

The ligands of VDR have induced a conformational shift to the LBD, which allows the replacement of co-repressor molecules by coactivator proteins. This interaction is responsible for the effect connected with ligand binding that induces a different protein-protein interaction profile of the VDR receptor [24]. This finding is also connected with changes in the gene expression profile in many biological processes including aging and is connected also with the strength of biomedical activity. The agonists of VDR cause an efficient co-repressors dissociation from the LBD and are responsible for specific binding of the mediator complex and coactivators. Additionally, coactivators attract the chromatin-modifying enzymes, which erase write or read post-translational modifications of histones, such as with methyl and acetyl groups, changing histone proteins of nucleosomes within the genomic VDR binding sites [24].

Interaction of vitamin D3--hydroxyderivatives with rexinoid orphan receptor a/γ (RORa/γ)

Isoforms of RORs possess different biological functions. Human RORγ has two isoforms (RORγ1 and RORγ2), and RORα-4 isoforms (α1–4) [25, 26]. The described isoforms differ only in their N-terminus. The molecules have different patterns of expression, therefore regulating different genes, as well as biological processes. RORγ1 isoform is expressed in liver, adipose, kidney, muscle tissue, where the receptor regulates glucose and lipid metabolism and circadian rhythm [27–29]. However, RORγ2 is selectively expressed in immune cells, such as T helper Th17 cells that promote inflammation, CD4+CD8+ (DP) thymocytes, type 3 innate lymphoid (ILC3) cells, the proinflammatory immune cells, and lymphoid tissue inducer cells (LTi) [30,31]. RORγ2's physiological function is the regulation of the DP thymocytes' survival and apoptosis. The molecule is also crucial for the development of both ILC3 and Th17 cells and the production of the proinflammatory cytokine, IL-17 [32, 33].

Vitamin D_3 metabolites, such as $1,20(OH)_2D_3$, $20(OH)D_3$, and $20,23(OH)_2D_3$ possess the ability to inhibit the interaction between a RORα/γ LBD with an LXXLL coactivator peptide and RORE-mediated transactivation [34, 35]. Molecular modeling with the established crystal structures of LBDs of RORγ and RORα showed that the inverse agonists possess high docking scores revealing the interaction of the vitamin D_3- hydroxyderivatives with a ligand-binding pocket of RORα/γ [29, 34, 36, 37]. Therefore, these produced endogenously noncalcemic vitamin D_3 metabolites can act as RORα/γ inverse agonists and further modulate RORs functions and activity. We expect a decrease of the inflammaging process through the reduction of inflammation mediated by Th17 lymphocytes. This action is due to inverse the agonism of the vitamin D_3 analogs in relation to RORα/γ.

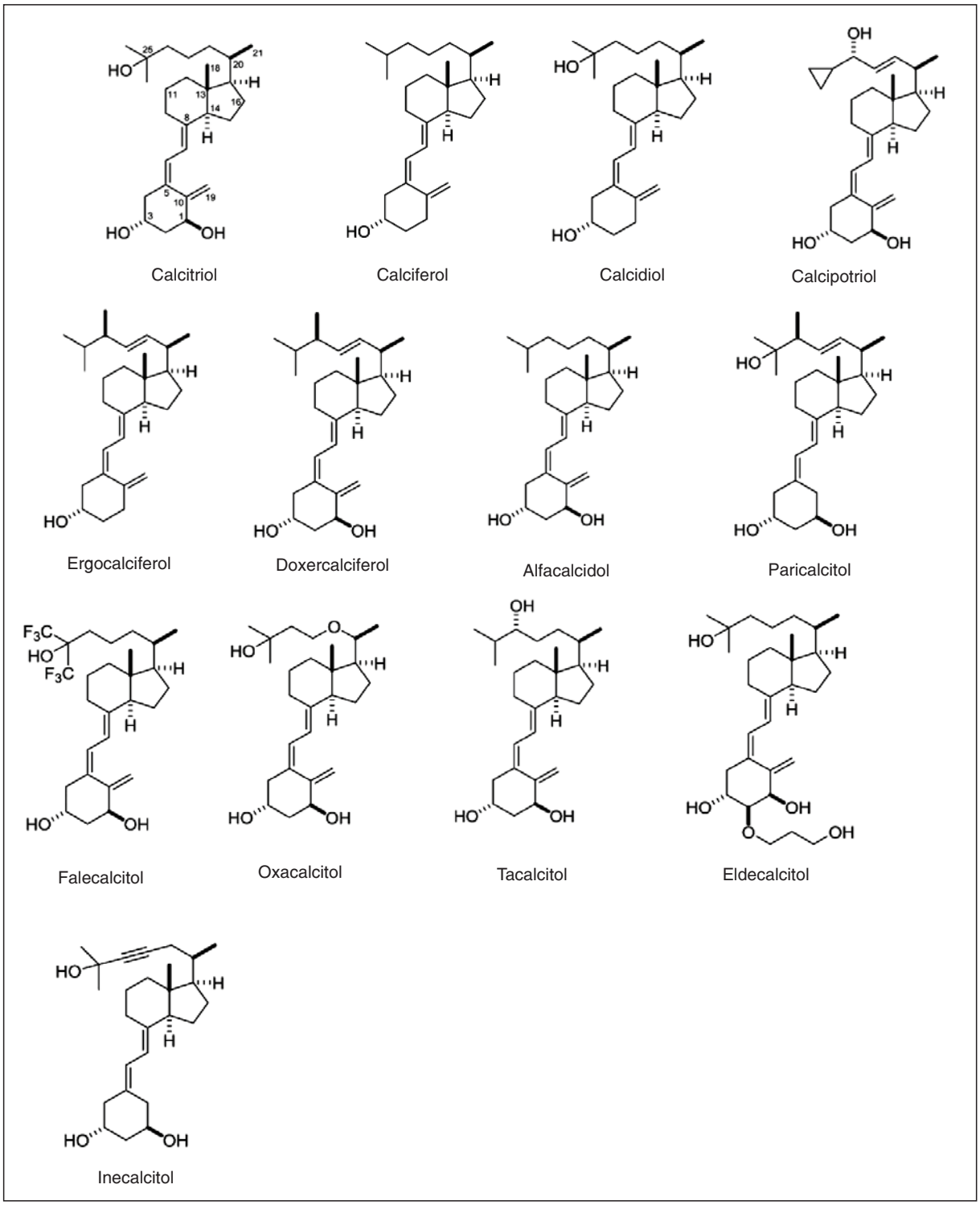

Figure 1. Examples of vitamin D compounds. Vitamin D_2 (ergocalciferol) used in the prevention of vitamin D deficiency and associated diseases, like as rickets [15]; Vitamin D_3 (calciferol) is used worldwide in the prevention of vitamin D deficiency and associated diseases, such like also rickets; Calcidiol (25(OH)D3) is used in the treatment of chronic hypocalcemia, renal osteodystrophy, rickets; Calcitriol [1,25(OH)2D3] is prescribed for renal osteodystrophy and psoriasis; Calcipotriol [22-ene-26,27-dehydro-1,25(OH)2D3] is used for psoriasis; Doxercalciferol [1α(OH)D2] prescribed for secondary hyperparathyroidism; Alfacalcidol (1α(OH)D3) is used for renal osteodystrophy, secondary hyperparathyroidism, osteoporosis as well as rickets; Tacalcitol (1α,24(OH)2D3), prescribed for psoriasis; Paricalcitol [19-nor-1,25(OH)2D2], used for secondary hyperparathyroidism; Oxacalcitriol (22-oxa-1,25(OH)2D3), used for secondary hyperparathyroidism treatment and psoriasis in Japan; Falecalcitriol [1,25(OH)2-26,27-F6-D3], prescribed for secondary hyperparathyroidism in Japan; Eldecalcitol [2α-(3-hydroxypropoxy)-1,25(OH)2D3] [15]

Physiology of vitamin D_3-hydroxyderivatives

The immediate precursor of cholesterol, 7-Dehydrocholestrol (7DHC), absorbs ultraviolet B radiation (UVB) producing pre-vitamin D_3 that isomerizes to vitamin D_3 (D_3) [56, 83–85]. Within the canonical pathway of calcitriol biosynthesis, enzymes 25-hydroxylase (CYP27A1) and 1α-hydroxylase (CYP27B1) activate D_3 for producing $1,25(OH)_2D_3$. $25(OH)D_3$, as well as $1,25(OH)_2D_3$, are degraded by sequential oxidation reactions of the side-chain catalyzed by CYP24A1 to produce calcitroic acid [56, 86, 87]. D_3 is also activated by CYP11A1 in a novel noncanonical pathway [56, 87, 88], with initial hydroxylation at C20 with the generation of $20(OH)D_3$ [56, 89, 90] or C22 [56, 91] and further subsequent reactions of hydroxylation at the positions of C20, C22, C23 and in some compounds at C17 [92]. The generated products of these reactions are selectively hydroxylated by different kinds of cytochromes: CYP24A1, CYP24A1, CYP27A1, CYP2R1, and/or CYP3A4, with additional hydroxylation at the position of C1α for all de novo synthesized compounds, with the exemptions of those which have a hydroxyl group at C17 [93–98]. Many of the described intermediates can be synthesized in the placenta, skin cells, as well as adrenal glands, and some are present in the pig adrenal gland and human serum and/or epidermis [99–102]. Native vitamin D_2 also undergoes reaction of hydroxylation through CYP11A1 with the generation of products, such as $20(OH)D_2$, $17,20,24(OH)_3D_2$, $17,20(OH)_2D_2$ [103, 104], leading to hydroxylation of $20(OH)D_2$ by CYP27B1 to $1,20(OH)_2D_2$ compound [105]. The described vitamin D_2 metabolites are also produced by pig adrenal glands, as well as in human skin cells and the placenta [106].

7-dehydrocholesterol (7-DHC), as well as lumisterol (L_3), also became substrates for CYP11A1 [56]. Finally, in the skin, the 5,7-dienal compounds with a shortened or full-length side-chain undergo a UVB-induced photoisomerization conversion to give the corresponding L_3, D_3 or T_3 products [56].

More of the UVB photons can be absorbed by the stratospheric ozone layer with an increase in the solar zenith angle [107]. While the zenith angle of the sun is so oblique that very few UVB photons penetrate to the surface of the earth, this phenomenon causes little, if any, vitamin D3 cutaneous production. This is a cause of little production (if any) of vitamin D3 in the skin during the winter at latitudes above or below 35°N and 35°S [108]. A season of the year, time of day, altitude, and latitude all markedly affect the vitamin D3 cutaneous production [109]. Cutaneous levels of 7-dehydrocholesterol decrease with the age. This markedly reduces the capacity of the aging skin to produce vitamin D_3 [110]. On the other hand, despite the up-to-fourfold reduction in the production of vitamin D_3 in the 70-year-olds compared to the 20-year-olds, the skin has such a high capacity to produce vitamin D_3 that elders with exposure to sunlight can produce an adequate amount of vitamin D to meet their vitamin-D needs [109, 111–114].

The concentration of 10^{-7} M for *in vitro* treatment of keratinocytes is an amount that refers to *in vivo* concentration of vitamin D_3 analogs, such as $20,23(OH)_2D_3$ and $1,25(OH)_2D_3$ in the physiological state [38]. Native vitamin D_3 (cholecalciferol) is widely used both as a nutritional supplement and a drug for the treatment of vitamin-D deficiency and its complications like rickets, osteoporosis, or decreased immunological resistance.

However, in the pathological conditions, the development of some types of cancer, like hepatocellular carcinoma (HCC) is associated with vitamin-D deficiency, and vitamin-D deficit occurs in hepatocellular carcinoma patients [115]. Thus, treatment of severe deficiency of serum concentration of 25(OH)D (below 10 ng/mL) by administration of vitamin D_3 analogs might prevent the development of HCC; they also should inhibit its progression.

Effect of vitamin D_3-hydroxyderivatives on the genes associated with anti--aging activities

Our retrospective analysis of recently reported microarray data on vitamin D_3 (D_3) induced changes in cultured human keratinocytes indicated that D_3-hydroxyderivatives stimulate the expression of genes involved in anti-aging activities [38]. Primary neonatal human epidermal keratinocytes were treated with 10^{-7} M of either $1,25(OH)_2D_3$ (a classical active form of D_3) or $20,23(OH)_2D_3$ (non-calcemic form of D_3) or vehicle. RNA was isolated and submitted for gene expression analysis by Illumina's HumanWG-6 chip/arrays (Tab. 1, 2).

Native vitamin D_3 acts against inflammaging and stimulates circadian clock genes

Cell-autonomous circadian clocks were identified as temporal orchestrators of many biological processes. Disruptions of the circadian clock are one of the causes of aging, as well as inflammation. Some of the types of molecules act through receptors that modulate the expression of circadian clock genes and reduce an inflammaging process. The effect of cholecalciferol (the classic form of vitamin D3) on the inflammatory process was investigated through its influence on the circadian clock and inflammation relief.

Table 1. 24h *in vitro* stimulation of HEKn by vitamin D_3-hydroxyderivatives, microarray data [38]

Gene	$20,23(OH)_2D_3$	$1,25(OH)_2D_3$	Description
S100A9	–2.80	1.10	S100 calcium binding protein A9; Marker of aging
TXNRD1	1.94	2.53	Thioredoxin reductase 1; antioxidative stress enzyme
PSMB7	1.46	1.24	Proteasome 20S subunit beta 7; proteasomes act against accumulation of the disrupted proteins in the process or aging
HMOX1	2.5	1.3	Heme oxygenase 1; an enzyme against oxidative stress
FOXO3	2.3	1.1	Forkhead box P3
KLK6	1.4	25.0	Kallikrein 6; an anti-aging protein involved in ACEI pathway (hypotensive effect)
SIRT1	1.9	–	Sirtuin 1; an anti-aging protein
AKR1C3	–12.12	–	Aldoketo reductase family 1 member C3 (prostaglandin F synthase); strong proinflammatory molecule
KLK13	–	3.1	Kallikrein 13
KLK3	–	2.1	Kallikrein 3
KLK9	–	2.0	Kallikrein 9
KLK5	–	2.0	Kallikrein 5
KLK7	–	1.8	Kallikrein 7
KLK10	–	1.8	Kallikrein 10
NR1D1	1.7	–	Nuclear receptor subfamily 1 group D member 1 (Rev-Erb-Alpha); $20,23(OH)_2D_3$ is an agonist of expression of Rev-Erba
NR1D2	3.0	1.0	Nuclear receptor subfamily 1 group D member 2 (Rev-Erb-Beta); $20,23(OH)_2D_3$ is an agonist of expression of Rev-Erbb

Table 2. 6h *in vitro* stimulation of HEKn by vitamin D3-hydroxyderivatives, microarray data [38]

Gene	20,23(OH)2D3	1,25(OH)2D3	Description
TXNRD1	1.1	3.0	Thioredoxin reductase, cytosolic form; decreases oxidative stress
SERPINB1	1.1	3.7	Inhibition of cathepsin G by SERPINB1 reduces GSDMD-driven inflammation
IL20	1.0	–1.9	Proinflammatory cytokine
IL24	1.1	–1.6	Cytokine contributing in sking inflammation
IL8	–1.4	–1.7	Proinflammatory cytokine
MMP9	–1.3	–2.0	Matrix metalloproteinase 9; proinflammatory molecule degradating extracellular matrix
MMP10	–1.3	–1.9	Matrix metalloproteinase 10; proinflammatory molecule degradating extracellular matrix
IL1F9	1.2	–1.6	Interleukin 1 family, member 9; proinflamamtory factor
CXCR7	–1.1	–1.5	C-X-C chemokine receptor type 7; Proinflammatory molecule
KLK6	1.0	3.0	Kallikrein 6; an anti-aging factor
KLK13	–	1.9	Kallikrein 13
KLK3	–	1.9	Kallikrein 3

The influence of native vitamin D_3 on the gene expression was evaluated by the analysis of the microarray data from blood cloth of the patients after oral administration of cholecalciferol at a dose of 10000 UI for 6 months with no toxic and side effects [39]. In humans, vitamin D_3 caused an increase of the

Table 3. Differentiate expressed genes under influence of native vitamin D_3 after oral administration (microarray data) [39]

Gene	Cholecalciferol change	Description
NR1D2	1.6	Nuclear receptor Subfamily 1 Group D member 2 (Rev-Erb-Beta); cholecalciferol is an agonist of expression of Rev-Erbβ
ALOX5	–1.5	Arachidonate 5-lipoxygenase; ALOX5 – rate-limiting enzyme in the synthesis of leukotrienes, that are family of lipid proinflammatory mediators
COX-2	–1.5	Cyclooxygenase 2 (prostaglandin-endoperoxide synthase 2); proinflammatory enzyme
NLRP 12	–1.6	NLRP12 inflammasome (NLR family pyrin domain containing 12); proinflammatory factor
IL6R	–1.6	Interleukin 6 receptor; (IL 6 is a proinflammatory cytokine)
IL7R	–1.5	Interleukin 7 receptor (IL7 is a proinflammatory chemokine)
IL-1RA	1.7	Interleukin 1 receptor antagonist; interleukin 1 is a proinflammatory cytokine
NR4A2	8.4	Nuclear receptor subfamily 4 group A member 2; a potential target for anti-aging therapy (improve mitochondrial function)
NR4A3	1.7	Nuclear receptor subfamily 4 group A member 3; a potential target for anti-aging therapy by improvement of mitochondrial function
PER1	4.2	Period circadian regulator 1 (genes involved in circadian clock act against aging process)
CRY1	2.0	Cryptochrome circadian regulator 1
TLR1	–1.9	Toll-like receptor 1 (toll-like receptors are proinflammatory agents and contributes to development of age-related diseases like atherosclerosis)
TLR8	–1.8	Toll-like receptor 8
TLR4	–1.8	Toll-like receptor 4
TLR5	–1.6	Toll-like receptor 5
CCR2	–1.6	C-C Motif chemokine receptor 2 (chemokines are proinflammatory agents)
CXCR1	–1.7	C-X-C motif chemokine receptor 1
CXCR2	–1.9	C-X-C motif chemokine receptor 2
CX3CR1	–1.9	C-X3-C motif chemokine receptor 1
CCR3	–1.5	C-C motif chemokine receptor 3
PSMD7	1.9	Proteasome 26S subunit, non-ATPase 7
PSMD12	1.6	Proteasome 26S subunit, non-ATPase 12

expression of NR1D2 gene (1.6 fold), reduced inflammaging by a decrease of the expression of ALOX5 gene (–1.5 fold), cyclooxygenase 2 (COX-2) (–1.5 fold), NLRP 12 inflammasome (–1.6 fold), IL6R (–1.6 fold), an expression increase of the Interleukin 1 Receptor Antagonist (1.7 fold). By upregulation of NR4A2 (8.4-fold) and NR4A3 (1.7-fold), cholecalciferol causes the stimulation of antioxidative activity, DNA repair machinery, and improvement of intrinsic mitochondrial functions. The tested substance induces also genes connected with the circadian clock, such as PER1 (4.2) fold, CRY1 (2.0-fold). A further anti-inflammatory action of vitamin D_3 is connected with influence on the genes involved in inflammatory response: TLR1 (–1.9 fold), TLR8 (–1.8), TLR4 (–1.8), TLR5 (–1.6), CCR2 (–1.6). CXCR1 (–1.7 fold), CXCR2 (–1.9 fold), CX3CR1 (–1.9), CCR3 (–1.5 fold) (Tab. 3).

Agonists of nuclear receptor Rev-Erb α/β may have anti-aging properties

SR-9009, selective REV-ERB (nuclear receptor subfamily 1 group D member 2) agonist administered intraperitoneally to Bmal1$^{flox/flox}$/MHC-Cre$^{-/-}$ mice (a control mice to circadian disruption mice model of heart) at a dose of 100 mg/kg b.w./daily for 8 days, has shown anti-aging properties by exerting influence on gene expression in heart (RNAsequence data from biventricular samples) [40]. SR-9009 acts against oxidative stress through upregulation of Heme oxygenase 1 gene expression (fold change *vs.* vehicle = 3.01) and decreasing the expression of NQO2, and decreasing inflammation by downregulation of CYP26B1 (–14.30 fold). Therefore, it has anti-aging properties and may be useful in preventing CNS dysfunction in Alzheimer's Disease

Table 4. Differentiate expressed genes after treatment intraperitoneally with SR-9009 (RNAseq. data) [40]

Gene	SR-9009 fold change	Description
HMOX1	3.0	Heme oxygenase 1; anti-oxidative enzyme
CYP26B1	–14.3	Cytochrome P450 family 26 subfamily B member 1
NQO2	–1.1	N-ribosyldihydronicotinamide quinone reductase 2
NRGN	1.2	Neurogranin; molecule involved in the prevention of CNS dysfunction in Alzheimer's disease
APBB2	–1.2	Amyloid beta (A4) precursor protein binding family B, member 2; downregulation of this gene prevents against Alazheimer's disease development
HMG-CoA synthase 2	–1.3	3-hydroxy-3-methylglutaryl-coenzyme A synthase 2; downregulation of this gene prevents against development of age-related diseases like atherosclerosis, obesity, atherosclerotic dementia
uPA	1.4	Plasminogen activator, urokinase; prevents against stroke and ischemic gangrene
FGFR1OP2	–1.0	FGFR1 oncogene partner 2
BCL7A	–1.1	B cel CLL/lymphoma 7A
LYZ2	1.3	Lysozyme 2
LYNX1	–1.1	Ly6/neurotoxin 1; Adult *Lynx1–/–* mice shew visual cortex plasticity similar to the plasticity of juveniles, what demonstrates that LYNX1 caused as a break for cortical plasticity [63]. Based on the studies in mice model, LYNX1 is involved in modulatory role in the brain under process of aging [63, 64]. SR9009 might improve visual transduction process in aged visual system by decrese of the expression of LYNX1 gene

by increasing the expression of neurogranin and decreasing the expression of amyloid beta (A4) precursor protein binding family B, member 2. The compound prevents also age-related disorders like atherosclerosis or stroke by the expression decrease of HMG-CoA synthase 2 and an increase for plasminogen activator, urokinase receptor. SR-9009 prevents oncogenesis by decreasing the expression of FGFR1 oncogene partner 2 and B cell CLL/lymphoma 7A. The expression of lysyl oxidase-like 2, a protein involved in the induction of oxidative vascular stress, was decreased after treatment with SR-9009. Some of the engineering approaches to extending lifespan are focused on the intervention into the lysozyme expression, and we noticed that SR-9009 induces the lysozyme 2 gene. The compound upregulated also the circadian clock gene, E4BP4. PPARγ, a strong anti-inflammatory molecule, which exhibits a circadian rhythm of the expression controlled by E4BP4 (Tab. 4) [40].

Free radical scavenging activity of atorvastatin, an inhibitor of HMG-CoA reductase

Atorvastatin, a popular hypolipemic drug may be considered a free radical scavenger and anti-aging therapeutic because it modulates expression of the genes connected with antioxidant function in hepatocellular carcinoma (HepG2) cell line (Tab. 5) [41]. Free Radical Scavenging gene set (-log(p value) = 1.89E-03-1.61E-02) (see some of the involved genes in this pathway in the Tab. 6) belongs to overrepresented Biological Functions in Ingenuity pathway Analysis of RNA sequence data, obtained in HepG2 cells treated with atorvastatin at the concentration of 10 μM for 24 hours. Indirect AMPK activators, metformin, resveratrol, as well as exercise, are widely tested as candidates for anti-aging drugs [42–44]. An increased level of neuregulin is associated with an increased lifespan in rodents [45, 46].

Comparison of the genetic profile of the compounds with metformin, a compound with anti-aging potential

Expression profile of the genes involved in DNA repair pathways, organ regeneration, proteasomal degradation of excessively expressed proteins, and mitochondrial metabolism after metformin treatment of HepG2 cells reveals similarity to the gene profile after incubation of keratinocytes with vitamin D_3-hydroxyderivatives, SR-9009 or atorvastatin (Fig. 2, Tab. 7) [47]. DAVID functional annotation of RNAseq. data from primary human hepatocytes was performed after treatment with metformin [2.5 mM], an anti-diabetic drug, for 8 hours or with 40 μM compound C, an inhibitor of AMPK along with 2.5 mM metformin for 8 hours (Tab. 7) [47].

The study showed a hierarchical gene clustering heatmap containing 1906 differentially expressed genes

Table 5. Canonical pathway analysis (IPA) in HepG2 under *in vitro* treatment with atorvastatin [10 μM] for 24 hours (n = 3), RNAseq. data [41]

Enriched canonical pathway	–log (P-value)	Molecules involed in pathway
AMPK signaling	1,71E+00	PIK3R3 PFKFB4 FASN ACACA HMGCR
Telomerase signaling	2,65E-01	PIK3R3
Neuregulin signaling	3,01E-01	PIK3R3
Neuroprotective role of THOP1 in Alzheimer's disease	5,56E-01	SERPINA3
Ceramide signaling	3,24E-01	PIK3R3
Melanocyte development and pigmentation signaling	3,05E-01	PIK3R3
Circadian rhythm signaling	6,28E-01	BHLHE40
RAR activation	4,00E-01	PIK3R3, RDH11
IL-4 signaling	3,53E-01	PIK3R3
IL-9 signaling	6,17E-01	PIK3R3
p38 MAPK signaling	2,23E-01	PLA2G3
HIF1α signaling	2,54E-01	PIK3R3
VDR/RXR activation	3,28E-01	SULT2A1
Oxidative ethanol degradation III	3,75E+00	ACSL3 ACSS2 ACSL1
Mitochondrial L-carnitine shuttle pathway	3,49E+00	ACSL3 ACSL4 ACSL1
NAD biosynthesis III	1,50E+00	NAMPT
IL-6 signaling	2,13E-01	PIK3R3
IL-15 signaling	4,07E-01	PIK3R3

Table 6. Genes connected with response to oxidative stress in HepG2 cells treated with atorvastatin [10 μM] for 24h (Differentiate expressed genes, RNAseq. data) [41]

Gene	Atorvastatin fold change	Description
ACSS2	2.2	acyl-CoA synthetase short-chain family member 2
MT1E	1.4	Metallothionein 1E; metallothionein scavenges reactive oxygen species [44]
UCP2	1.5	Uncoupling protein 2 (mitochondrial, proton carrier)
DDIT4	1.2	DNA-damage-inducible transcript 4
DHCR24	1.2	24-dehydrocholesterol reductase
SREBF2	1.2	Sterol regulatory element binding transcription factor 2
ACOT1	1.3	Hepatic acyl-CoA thioesterase 1; acot1 knockdown caused increased FA oxidation, reduced PPARα activity, and further increased inflammation and hepatic oxidative stress
ACSL1	1.3	acyl-CoA synthetase long-chain family member 1

after metformin treatment (adjusted $p \leq 0.05$) with segregation into 10 groups [56]. Cluster 1 included 194 genes with an increase of the expression in response to treatment with metformin that remained elevated also after incubation with the compound C (metformin increased, AMPK-independent). Cluster 3 contained 575 genes with an increase of the expression in response to incubation with metformin, which decreased the expression while also under treatment with the compound C (metformin increased, AMPK-dependent).

In the study, the further generation of AMPK-independent and AMPK-dependent clusters was presented by comparing the tested conditions [47]. Since compound C has also off-target effects, only the genes whose expression changed due to metformin response were considered for this experiment. Clusters 2 (containing 134 genes), 3 (with 575 genes), 7 (83 genes), 8 (168 genes) contain molecules whose expression increased after metformin incubation but was reduced under a simultaneous treatment with metformin and

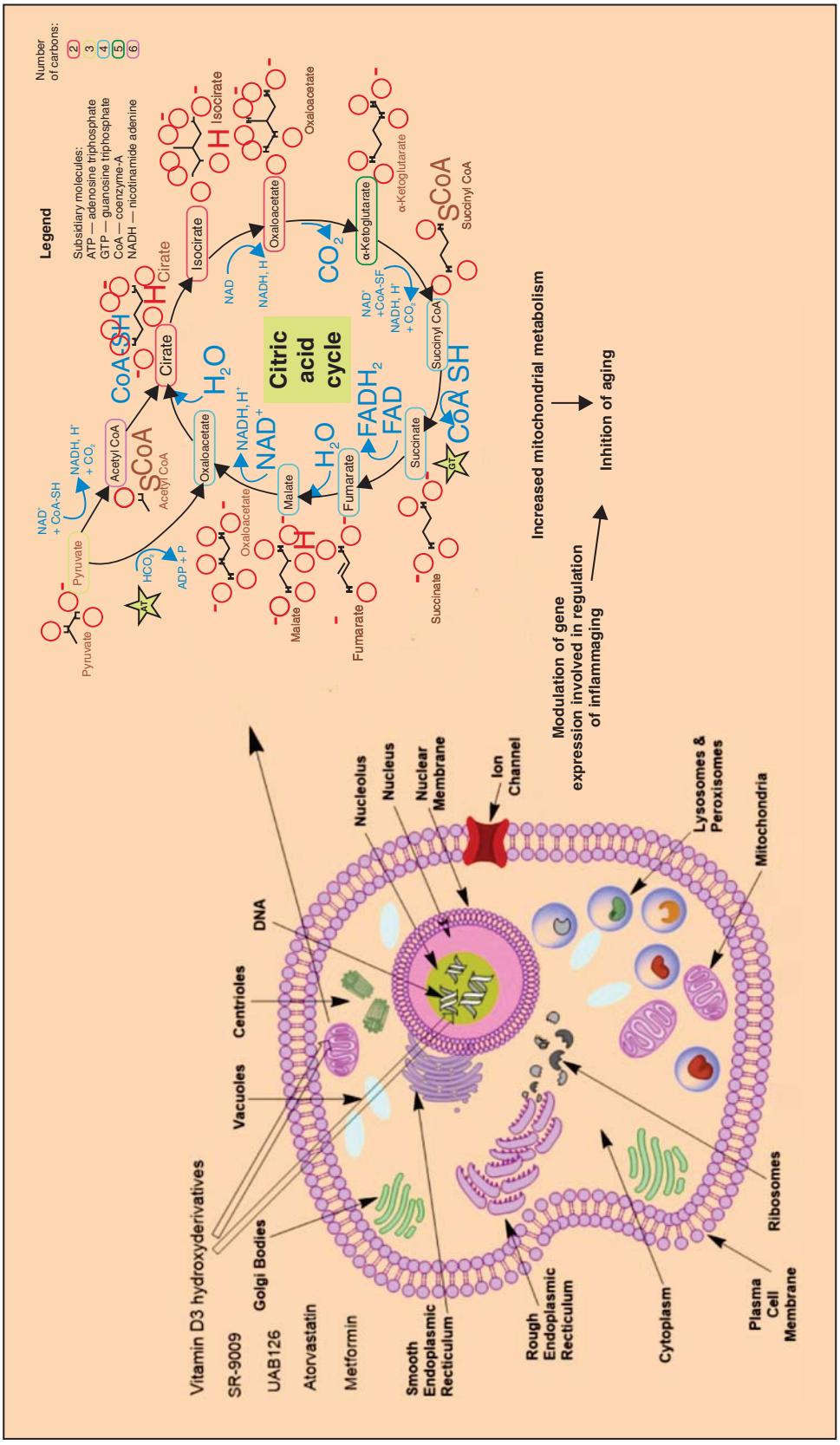

Figure 2. Comparative analysis of the compounds with antiaging properties according their similarity in mechanism of action

Table 7. Results of DAVID functional annotation of RNAseq. Data from HepG2 treated *in vitro* with metformin [2.5 mM] for 8h [47]

David results from RNA-seq cluster 1 (metformin-dependent increased genes, AMPK independent)				
Annotation cluster 8	**Enrichment score: 1.3114286631285683**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_BP_FAT	GO:0031099~regeneration	5	3.184713376	0.004
GOTERM_BP_FAT	GO:0042246~tissue regeneration	3	1.910828025	0.034
Annotation cluster 17	**Enrichment score: 0.6264708391271191**			
GOTERM_BP_FAT	GO:0048534~hemopoietic or lymphoid organ development	4	2.547770701	0.427
Annotation cluster 20	**Enrichment score: 0.42660630022201657**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_BP_FAT	GO:0006508~proteolysis	17	10.82802548	0.031
Annotation cluster 23	**Enrichment score: 0.36641602542817736**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_BP_FAT	GO:0006974~response to DNA damage stimulus	6	3.821656051	0.257
GOTERM_BP_FAT	GO:0033554~cellular response to stress	8	5.095541401	0.262
GOTERM_BP_FAT	GO:0006259~DNA metabolic process	5	3.184713376	0.687
GOTERM_BP_FAT	GO:0006281~DNA repair	3	1.910828025	0.738
David results from RNA-seq cluster 2 (met increased, AMPK dependent)				
Annotation cluster 10	**Enrichment score: 0.8868509634642516**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_BP_FAT	GO:0043161~proteasomal ubiquitin-dependent protein catabolic process	3	2.4	0.158
GOTERM_BP_FAT	GO:0010498~proteasomal protein catabolic process	3	2.4	0.158
GOTERM_BP_FAT	GO:0006508~proteolysis	7	5.6	0.750
Annotation cluster 17	**Enrichment score: 0.5947274210343121**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_BP_FAT	GO:0033554~cellular response to stress	8	6.4	0.098
GOTERM_BP_FAT	GO:0006974~response to DNA damage stimulus	5	4	0.261
GOTERM_BP_FAT	GO:0006259~DNA metabolic process	6	4.8	0.275
GOTERM_BP_FAT	GO:0006281~DNA repair	3	2.4	0.590
David results from RNA-seq cluster 3 (met increased, AMPK dependent)				
Annotation cluster 10	**Enrichment score: 1.9018121386666416**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_BP_FAT	GO:0032436~positive regulation of proteasomal ubiquitin-dependent protein catabolic process	3	0.53	0.0188
GOTERM_BP_FAT	GO:0045862~positive regulation of proteolysis	4	0.71	0.043
GOTERM_BP_FAT	GO:0032434~regulation of proteasomal ubiquitin-dependent protein catabolic process	3	0.53	0.045
Annotation cluster 13	**Enrichment score: 1.6825557240354716**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_BP_FAT	GO:0046513~ceramide biosynthetic process	4	0.71	0.011
Annotation cluster 33	**Enrichment score: 0.8581528728649036**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_BP_FAT	GO:0042787~protein ubiquitination during ubiquitin-dependent protein catabolic process	4	0.71	0.015
GOTERM_BP_FAT	GO:0006508~proteolysis	31	5.53	0.751

Table 7 cont. Results of DAVID functional annotation of RNAseq. Data from HepG2 treated *in vitro* with metformin [2.5 mM] for 8h [47]

Annotation cluster 77	**Enrichment score: 0.3243851502337266**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_BP_FAT	GO:0030097~hemopoiesis	6	1.07	0.870
David results from RNA-seq cluster 4 (met dDecreased, AMPK independent)				
Annotation cluster 6	**Enrichment score: 1.2202185663855596**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_MF_FAT	GO:0016628~oxidoreductase activity, acting on the CH-CH group of donors, NAD or NADP as acceptor	3	0.88	0.048
Annotation cluster 13	**Enrichment score: 0.8336371317681409**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_MF_FAT	GO:0015662~ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism	4	1.17	0.075
GOTERM_BP_FAT	GO:0006754~ATP biosynthetic process	5	1.47	0.082
GOTERM_BP_FAT	GO:0046034~ATP metabolic process	5	1.47	0.128
GOTERM_MF_FAT	GO:0042626~ATPase activity, coupled to transmembrane movement of substances	5	1.47	0.145
GOTERM_MF_FAT	GO:0043492~ATPase activity, coupled to movement of substances	5	1.47	0.148
GOTERM_MF_FAT	GO:0042625~ATPase activity, coupled to transmembrane movement of ions	4	1.17	0.158
Annotation cluster 41	**Enrichment score: 0.1480055067172435**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_BP_FAT	GO:0006281~DNA repair	6	1.76	0,602
GOTERM_BP_FAT	GO:0006259~DNA metabolic process	9	2.64	0.719
GOTERM_BP_FAT	GO:0033554~cellular response to stress	10	2.93	0.720
GOTERM_BP_FAT	GO:0006974~response to DNA damage stimulus	6	1.76	0.821
David results from RNA-seq cluster 5 (met decreased, AMPK dependent)				
Annotation cluster 30	**Enrichment score: 0.4088079394382511**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_BP_FAT	GO:0033554~cellular response to stress	11	5.09	0.108
GOTERM_BP_FAT	GO:0006259~DNA metabolic process	7	3.24	0.512
GOTERM_BP_FAT	GO:0006974~response to DNA damage stimulus	5	2.31	0.612
GOTERM_BP_FAT	GO:0006281~DNA repair	4	1.85	0.627
David results from RNA-seq cluster 6 (met increased, AMPK independent)				
Annotation cluster 7	**Enrichment score: 1.8688950312990442**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_BP_FAT	GO:0031099~regeneration	3	3.80	0.039
GOTERM_BP_FAT	GO:0031100~organ regeneration	3	3.80	0.006
GOTERM_BP_FAT	GO:0007568~aging	4	5.06	0.013
Annotation Cluster 24	**Enrichment Score: 0.4916356096090675**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_BP_FAT	GO:0030097~hemopoiesis	3	3.80	0,288
David results from RNA-seq cluster 8 (met increased, AMPK dependent)				

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Table 7 cont. Results of DAVID functional annotation of RNAseq. Data from HepG2 treated *in vitro* with metformin [2.5 mM] for 8h [47]

Annotation cluster 21	**Enrichment score: 0.7644313581632808**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_BP_FAT	GO:0033554~cellular response to stress	11	6.67	0.046
GOTERM_MF_FAT	GO:0003684~damaged DNA binding	3	1.82	0.092
GOTERM_BP_FAT	GO:0006281~DNA repair	6	3.63	0.138
GOTERM_BP_FAT	GO:0006974~response to DNA damage stimulus	7	4.24	0.150
GOTERM_BP_FAT	GO:0006259~DNA metabolic process	6	3.63	0.539
GOTERM_BP_FAT	GO:0006260~DNA replication	3	1.82	0.548
Annotation cluster 24	**Enrichment score: 0.5976009129847903**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_BP_FAT	GO:0030097~hemopoiesis	5	3.03	0.192
David results from RNA-seq cluster 9 (met decreased, AMPK independent)				
Annotation cluster 1	**Enrichment score: 4.739401003241343**			
Category	**Term**	**Count**	**%**	**P-value**
GOTERM_BP_FAT	GO:0042775~mitochondrial ATP synthesis coupled electron transport	7	14.58	1,34E-09
GOTERM_BP_FAT	GO:0042773~ATP synthesis coupled electron transport	7	14.58	1,34E-09
GOTERM_BP_FAT	GO:0022904~respiratory electron transport chain	7	14.58	3,06E-09
GOTERM_BP_FAT	GO:0015980~energy derivation by oxidation of organic compounds	8	16.67	1,31E-08
GOTERM_BP_FAT	GO:0045333~cellular respiration	7	14.58	3,86E-08
GOTERM_BP_FAT	GO:0006119~oxidative phosphorylation	7	14.58	4,10E-08
GOTERM_BP_FAT	GO:0022900~electron transport chain	7	14.58	1,02E-07
GOTERM_BP_FAT	GO:0006091~generation of precursor metabolites and energy	8	16.67	2,59E-06
GOTERM_BP_FAT	GO:0006120~mitochondrial electron transport, NADH to ubiquinone	4	8.33	8,64E-05
GOTERM_MF_FAT	GO:0008137~NADH dehydrogenase (ubiquinone) activity	4	8.33	1,29E-04
GOTERM_MF_FAT	GO:0003954~NADH dehydrogenase activity	4	8.33	1,29E-04
GOTERM_MF_FAT	GO:0050136~NADH dehydrogenase (quinone) activity	4	8.33	1,29E-04
GOTERM_MF_FAT	GO:0016655~oxidoreductase activity, acting on NADH or NADPH, quinone or similar compound as acceptor	4	8.33	1,91E-04
GOTERM_MF_FAT	GO:0016651~oxidoreductase activity, acting on NADH or NADPH	4	8.33	9,03E-04
GOTERM_BP_FAT	GO:0016310~phosphorylation	8	16.67	9,76E-04

compound C. The genes were defined as metformin increased, AMPK-dependent. The Gene Ontology (GO) analysis found enrichment for DNA repair and response to DNA damage stimulus and several other terms connected with the proteasomal protein catabolic process (connected with an excess of proteins while aging), organ regeneration, response to cellular stress, and pathways with increased mitochondrial metabolism for these clusters. Cluster 5 with 256 genes contained molecules whose expression decreased after incubation with metformin, but after simultaneous treatment with metformin and compound C, the expression returned to the untreated level. This set of the genes was defined as metformin decreased, AMPK-dependent and was enriched also with the response to DNA damage stimulus and DNA repair. What is more, an upstream regulator analysis of IPA showed enrichment for the AMPK signaling canonical pathway, which is related to anti-aging activity of the compound [47].

Some of the AMPK-independent clusters were found [47]. Cluster 1 contained 194 genes, 6 –74 genes, and 10–57 genes showed molecules with the increased expression after incubation with metformin that remained increased after a simultaneous treatment of metformin

and the compound C. The genes were defined as metformin increased, AMPK-independent. These sets of genes were overrepresented for GO terms as tissue regeneration, DNA repair, hemopoiesis, and others. Cluster 4 containing 365 genes, 9 with 20 genes revealed molecules whose expression decreased under incubation with metformin, which remained decreased after the simultaneous incubation with metformin and the compound C. The set of the genes was defined as metformin decreased, AMPK-independent and the enrichment of genes like DNA repair, cellular respiration, oxidative phosphorylation, and other GO terms [47]. Therefore, by increasing mitochondrial metabolism, metformin reveals anti-aging properties.

A comparative analysis of the gene expression affected by the tested substances with resveratrol [48–51] was performed in order to find similarities in the mechanism of anti-aging action. Vitamin D_3-hydroxyderivatives exhibits photoprotective properties and protect against oxidative stress and DNA damage [52–56]. The compounds revealed also anti-inflammatory action [3], which makes them good candidates for drugs against inflammaging [57, 58].

Analog of vitamin D_3, $20,23(OH)_2D_3$, similarly to resveratrol, caused downregulation of S100 Calcium Binding Protein A9 [59–61], a biomarker of aging [62–66].

Native resveratrol, polyphenolic compounds from a natural source, was used in the study as the control for comparison with five structurally modified resveratrol derivatives, such as isobutyrate, butyrate, acetate, palmitoate, as well as diacetate. The aim was to improve functionality and biological activity at 1% concentration of each substance for 24h in the full thickness cultures of the epidermis. To evaluate gene expression connected with inflammaging the gene array and qPCR, mRNA analysis was used [67]. The expression of the sets of the genes connected with anti-aging and aging properties were evaluated, as well as the markers of inflammation, such as interleukin-1A [IL1A], IL6, IL1R2, IL-8, extracellular factors (collagen 1A1, 4A1, 3A1; tissue inhibitor of matrix metalloproteinase 1, elastin, fibrillin 1, matrix metalloproteinase 9, laminin beta1), silent mating type information regulation 2 homolog 1, antioxidants, such as superoxide dismutase, proliferating cell nuclear antigen, metallothionein 1H/2H, catalase and nerve growth factor. The analogs were evaluated according to the gene expression profile ranking, each from highest-to-lowest: butyrate > isobutyrate > diacetate > acetate > palmitoate. The isobutyrate and butyrate analogs have higher biological activity in comparison to resveratrol and might be used in topical applications for an improvement of dermal condition and for other medical purposes [67].

Conclusion

We have found that resveratrol acts in a similar way to vitamin D_3-hydroxyderivatives, cholecalciferol, SR-9009, atorvastatin and metformin through its anti-inflammatory and against-oxidative-stress action and the consequent reduction of inflammaging process [68–73]. Vitamin D_3-hydroxyderivatives improve the proteasome profile gene expression, as well as metformin, and this finding has also anti-aging properties due to the decrease of the number of aberrant proteins (like amyloid beta, which causes Alzheimer's Disease) accumulated during aging. Interestingly, after incubation with atorvastatin, the Neuroprotective Role of THOP1 in Alzheimer's Disease pathway was enhanced and after treatment with SR-9009 (Agonists of Nuclear Receptor Rev-Erb α/β) an amyloid beta (A4) precursor protein binding family B, member 2 expression was decreased, which suggests these compounds have properties against Alzheimer's Disease, which is recognized as a hallmark of aging [74–78]. We postulate that vitamin D_3-hydroxyderivatives have anti-aging potential because of their anti-inflammatory properties and action against oxidative stress and improvement in DNA repair in cells [79–82]. Their mechanism of action is similar to the action of resveratrol, metformin, SR9009, and native cholecalciferol and is connected with modulation of gene expression involved in particular pathways related to the aging process and anti-aging activity.

Metformin seems to be generally a well-tolerated and safe anti-diabetic drug [83, 84]. However, a well-known complication in metformin treatment is lactic acidosis, especially in cases of renal insufficiency or in intentional overdose [83, 84]. The second medication with anti-aging properties, resveratrol is well tolerated and no marked toxicity was reported [85]. Resveratrol is a substance with a natural origin; it occurs in red wine and dark grapes. However, atorvastatin is a synthetic, the most used drug in the world. The complex toxicological study of atorvastatin was performed on dogs with a histopathologic evaluation which revealed multifocal minimal to slight hemorrhages in the submucosa of the gallbladder, and all the findings were reversible [86].

Potential anti-aging properties and advantages of metformin and resveratrol seem to surpass their weak side effects in healthy adults. However, metformin should not be used as a compound which slows the aging process in the case of patients with renal and liver insufficiency. We found also that atorvastatin (Lipitor medication) is the most widely used drug in the world with promising anti-aging properties due to its effect on gene expression involved in the regulation of the aging process. Vitamin D_3 analogs are endogenously produced, they act like hormones, so in physiological concentrations they reveal no toxic effect, and their anti-aging benefits seem to be worthy of further investigation.

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