

Targeting Nrf2-Mediated Gene Transcription by Triterpenoids and Their Derivatives

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Abstract

Chemoprevention represents a strategy designed to protect cells or tissues against various carcinogens and carcinogenic metabolites derived from exogenous or endogenous sources. Recent studies indicate that plant-derived triterpenoids, like oleanolic acid, may exert cytoprotective functions via regulation of the activity of different transcription factors. The chemopreventive effects may be mediated through induction of the nuclear factor erythroid 2-related factor 2 (Nrf2) transcription factor. Activation of Nrf2 by triterpenoids induces the expression of phase 2 detoxifying and antioxidant enzymes such as NAD(P)H quinone oxidoreductase 1 (NQO1) and heme oxygenase-1 (HO-1) - proteins which can protect cells or tissues against various toxic metabolites. On the other hand, inhibition of other transcription factors, like NF- κ B leads to the decrease in the pro-inflammatory gene expression. Moreover, the modulation of microRNAs activity may constitute a new mechanism responsible for valuable effects of triterpenoids. Recently, based on the structure of naturally occurring triterpenoids and with involvement of bioinformatics and computational chemistry, many synthetic analogs with improved biological properties have been obtained. Data from *in vitro* and *in vivo* experiments strongly suggest synthetic derivatives as promising candidates in the chemopreventive and chemotherapeutic strategies.

Key Words: Anti-oxidant response, Betulin, Chemoprevention, Oleanolic acid, Triterpenoids

CANCER CHEMOPREVENTION

The main goal of chemoprevention is to delay the development of cancer or even to revert the carcinogenesis process. The appropriate use of a chemopreventive agent depends on the understanding of its mechanism of action at different stages, including molecular, cellular, tissue and organ level. Although various compounds are nowadays tested both *in vitro* and *in vivo*, still there is a need for the development of new classes of chemopreventive agents as none of the existing ones is ideal. Triterpenoids have gained much attention as potential chemopreventive agents and their synthetic analogs characterized by lower toxicity, better availability, as well as increased protective effects are nowadays thoroughly examined, some of them are even in the phase I/II clinical trials for cancer treatment.

OLEANOLIC ACID AS AN EXAMPLE OF THERAPEUTIC TRITERPENOID

Terpenoids form a large and structurally diverse family of natural compounds, structure of which is based on five-carbon 2-methyl-1,3-butadiene units (called isoprene units). Depending on the number of isoprene units forming structural skeleton of the molecule and on the form of connection of these units, this group is divided into several classes. One of them is represented by triterpenoids, among which the most important are pentacyclic compounds containing six five-carbon isoprene units. Triterpenoids are biosynthesized in plants by the cyclization of squalene, a chain triterpene hydrocarbon and precursor of all steroids (Phillips *et al.*, 2006) and are widely present in various plants, mostly in wax-like coatings of fruits and medicinal herbs, including apples, cranberries, figs, olives, oregano, rosemary and thyme (Petronelli *et al.*, 2009).

Triterpenoids have been used for medicinal purposes in many Asian countries for ages. During recent 20 years mechanisms responsible for their anti-inflammatory, cytoprotective, antitumorogenic and other effects have been discovered (Fig.

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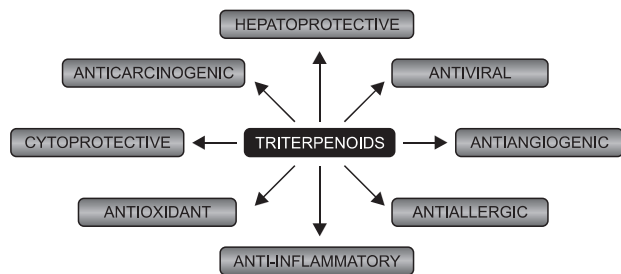


Fig. 1. Schematic representation of a broad action of triterpenoids.

1) (reviewed in: Liby *et al.*, 2007b). Moreover, a variety of additional biological activities including antiviral (anti-HIV) (Mengoni *et al.*, 2002), antiallergic (Yuk *et al.*, 2011) and spasmolytic activity (Begum *et al.*, 2002) have been reported.

One of the best known and characterized triterpenoid is 3 β -hydroxyolean-12-en-28-oic acid, named oleanolic acid (OA), produced by wide range of medicinal herbs and other plants, for example *Olea europaea*, *Calendula officinalis*, and *Viscum album* L. The anti-inflammatory effect of OA was first reported in 1969, when Gupta and coworkers showed its inhibitory effect in carrageenan-induced rat paw edema and formaldehyde-induced arthritis (Gupta *et al.*, 1969). Later, in the late 80's and early 90's, these anti-inflammatory properties were additionally confirmed. OA was reported to inhibit dextran-induced rat paw edema and adjuvant-induced polyarthritis in mice and rats (Singh *et al.*, 1992). It was noted that oral administration of OA was not as effective as intraperitoneal or subcutaneous injection in inhibiting inflammatory reactions (Takagi *et al.*, 1980; Singh *et al.*, 1992). Simultaneously, the experiments were aimed at elucidating the mechanism responsible for anti-inflammatory effect of triterpenoids. The inhibition of lipoxygenase and cyclooxygenase (COX-2) activity was firstly reported for other compound - ursolic acid (Najid *et al.*, 1992) but then also for OA (Suh *et al.*, 1998). Additionally, suppression of inducible nitric oxide synthase (iNOS) activity and the production of NO was demonstrated (Suh *et al.*, 1998). Interestingly, anticancer activities of OA have been reported already more than 25 years ago. It was shown that topical application of OA to mice pretreated by 2-O-tetradecanoylphorbol-13-acetate (TPA) resulted in significantly lower number of papilloma-bearing animals (Tokuda *et al.*, 1986). Later, OA was shown to be effective in treatment of other types of cancer. Three-week long oral treatment of rats with OA resulted in decreased incidence and multiplicity of azoxymethane-induced intestinal tumors (Yoshimi *et al.*, 1992). Fujiwara *et al.* (2011) have recently discovered that OA inhibited the proliferation of human glioblastoma cells via inhibition of signal transducer and activator of transcription-3 (STAT3).

Apart from anti-tumorigenic and anti-inflammatory activity widely described in the literature, OA has also documented hepatoprotective properties. In animal models, it protects against variety of hepatotoxicants, for example carbon tetrachloride, cadmium and bromobenzene (references in: Liu *et al.*, 2008). Moreover, hypoglycemic activity of OA was described. In alloxan-induced diabetic rats, OA caused amelioration of blood glucose elevation and increase of hepatic glycogen and insulin levels (Liu *et al.*, 1994). Recently, the detailed molecular mechanism responsible for the hypoglycemic

activity of OA was reported (Zeng *et al.*, 2012). In the model of streptozotocin-induced type II diabetes in mice, OA administration reversed the hyperglycemia by ~60%. OA was able to modulate the insulin signaling transduction, namely the phosphorylation of Akt and FoxO1 regulating gluconeogenesis was significantly increased in OA-treated mice (Zeng *et al.*, 2012).

The complete list of OA activities is still increasing and probably will be even longer in the future. The results obtained so far indicate that OA interacts with important signaling pathways modulating cell physiology, which has a reflection in its anti-inflammatory and cytoprotective functions.

SYNTHETIC OLEANOLIC TRITERPENIDS AND THEIR PROTECTIVE EFFECTS

Over the past years, a large number of triterpenoids have been synthetically modified in order to increase their bioactivity and protective effects. Based on structure of OA, several hundreds of derivatives called synthetic oleanane triterpenoids (SO) have been obtained. In many cases, it was reported that they possess better anti-inflammatory and cytoprotective properties than OA (reviewed in: Liby *et al.*, 2007b). Three of the most potent known SO are 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO), its methyl ester (CDDO-Me) and its imidazolide (CDDO-Im).

As OA, above mentioned compounds when used at high concentrations, have ability to inhibit cell proliferation and induce apoptosis in ovarian (Gao *et al.*, 2011), prostate (Deeb *et al.*, 2011) and other cancer cells, including leukemia and myeloma cells (reviewed in: Liby *et al.*, 2007b). Another therapeutic agent, CDDO-ethylamide (CDDO-Ea), was shown to extend survival in mouse model of pancreatic cancer (Liby *et al.*, 2010) as well as in lung cancer (Liby *et al.*, 2007a). Some of such derivatives are in clinical trials for treatment of leukemia and solid tumors (Petronelli *et al.*, 2009; Speranza *et al.*, 2012).

Another important mechanism of SO action is the inhibition of blood vessel formation, the critical process for tumor growth and metastasis. CDDO-Me inhibited angiogenesis in matrigel sponge assays *in vivo* and caused significant inhibition of endothelial cell growth *in vitro* (Vannini *et al.*, 2007). The same compound was able to prevent estrogen receptor (ER)-negative mammary tumorigenesis in mice and the inhibition of angiogenesis was suggested to be at least partially responsible for such effect (Liby *et al.*, 2008). Moreover, CDDO-Me blocked angiogenesis and the growth of Kaposi's sarcoma cells in nude mice (Vannini *et al.*, 2007). Recent work by Mu *et al.* (2012) indicates that proangiogenic vascular endothelial growth factor (VEGF) signaling is affected by triterpenoids. The observed anticancer effect of pristimerin, a triterpenoid isolated from *Celastrus* and *Maytenus* spp. was mediated by the suppression of VEGF receptor 2 (VEGFR-2) kinase activity (Mu *et al.*, 2012).

SO are multifunctional drugs reacting with cellular nucleophiles such as -SH groups of cysteines on multiple target proteins. From different possible interaction, the most important in the view of anti-cancer therapy is the regulation of STAT3, retinoid X receptor alpha (RXR α), epidermal growth factor receptor (EGFR), COX-2 and iNOS (reviewed in: Liby *et al.*, 2007b). Moreover, SO may directly bind and inhibit I κ B kinase enzyme complex (IKK) responsible for nuclear translocation

of NF- κ B leading to the silencing of NF- κ B signaling (Ahmad *et al.*, 2006; Yore *et al.*, 2006). CDDO reduced NF- κ B activity in human bronchial epithelial cells and decreased release of NF- κ B dependent gene products like IL-8 (Nichols *et al.*, 2009). In other studies, CDDO-Me was shown to inhibit NF- κ B expression in prostate (Deeb *et al.*, 2010), colorectal (Gao *et al.*, 2010) as well as in ovarian (Gao *et al.*, 2011) cancer cells. The growth-inhibitory and apoptosis-inducing activity of CDDO-Me relies on the inhibition of pro-survival kinase Akt what in turn leads to the inhibition of NF- κ B and phosphomammalian target of rapamycin (p-mTOR) signaling proteins (Gao *et al.*, 2011).

The blockade of the tumor growth and induction of apoptosis are achieved when high concentrations of triterpenoids are used. On the other hand, at low concentrations, SO are rather cytoprotective and anti-inflammatory. The cytoprotective effects are mediated by the induction of the entire set of antioxidative proteins called phase 2 enzymes that protect cells against electrophiles and oxidants. Their upregulation provides a diverse and highly reliable response that enables cells to survive in stressful conditions and it is mediated by the modulation of the activity of nuclear factor erythroid 2-related factor 2 (Nrf2) (Li and Kong, 2009).

Nrf2 AND ITS TRANSCRIPTIONAL ACTIVATION

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that regulates expression of many detoxification and antioxidant enzymes, molecular chaperones, stress response proteins, as well as proteasome subunits (Li and Kong, 2009). Nrf2 belongs to basic leucine zipper (bZIP) transcription factors featuring a cap "n" collar (CNC) structure defined by the presence of a conserved 43-amino acid CNC domain located N-terminally to the DNA binding domain (Sykiotis and Bohmann, 2010).

Like other transcription factors, Nrf2 signaling is regulated by the compartmental segregation (Fig. 2). Activated Nrf2 quickly translocates from the cytoplasm into the nucleus to regulate gene expression. Under normal conditions Nrf2 is anchored within the cytoplasm by Kelch-like-ECH-associated protein 1 (Keap1) (Li and Kong, 2009). When cells are exposed to stressing conditions like chemopreventive agents or oxidative stress, critical cysteine residues (-SH groups) in Keap1 are modified by redox modification. As a result, enzymatic activity of the Keap1-Cullin3-Rbx1 E3 ubiquitin ligase complex (known also as Cullin3-based E3 ligase ubiquitination complex) is inhibited which causes decreased Nrf2 ubiquitination and degradation. Thus, Keap1 functions as a kind of bridge between Nrf2 and above-mentioned ubiquitination complex preventing nuclear accumulation of Nrf2 under normal condition.

Moreover, the activation of Nrf2/ARE signaling may also rely on Nrf2 phosphorylation by multiple cellular kinase pathways: mitogen-activated protein kinases (MAPKs), protein kinase C (PKC), and phosphatidylinositol 3-kinase (PI3K) (reviewed in: Kwak and Kensler, 2010). It was shown that phosphorylation of Nrf2 at Ser 40 facilitated its dissociation from Keap1 (Huang *et al.*, 2002). Bloom and Jaiswal (2003) confirmed this result, however, they demonstrated that Nrf2 phosphorylation is not necessary for Nrf2 stabilization/accumulation in the nucleus. On the other hand, phosphorylation of Nrf2 by the casein ki-

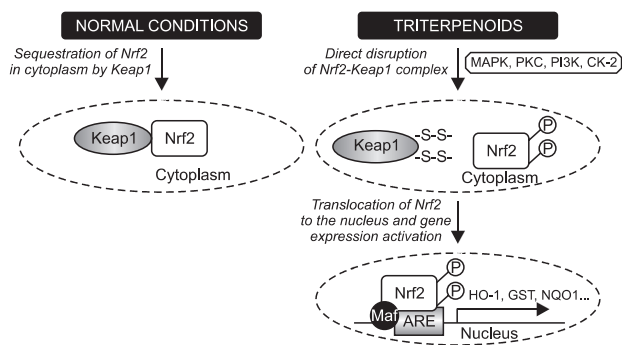


Fig. 2. Nrf2 transcriptional regulation. Under normal conditions, Nrf2 is sequestered in cytoplasm by Keap1. Triterpenoids via oxidative modification of -SH groups in Keap1 or via phosphorylation of Nrf2 by various protein kinase signaling pathways including MAPK, PI3K, PKC and CK-2 facilitate the liberation process of Nrf2 from Keap1 as well as translocation of Nrf2 into the nucleus. After binding Maf proteins and antioxidant response element (ARE) activation, the increased transcription of Nrf2-regulated genes (e.g. HO-1, GST, NQO-1) can be achieved.

nase-2 (CK-2) was shown to play an important role not only in the activation and nuclear translocation of Nrf2 but also in the subsequent hyper-phosphorylation of Nrf2 (Pi *et al.*, 2007).

After translocation into the nucleus, Nrf2 forms heterodimer with a group of nuclear bZIP proteins called small Maf proteins like MafF, G and K. Their heterodimerization with Nrf2 enhances the specificity to bind antioxidant response element (ARE) which represents a common cis-acting regulatory element in the 5' flanking region of promoters of many cytoprotective genes. Binding of Nrf2-Maf dimers to ARE subsequently initiates the transcription of target genes (Li and Kong, 2009). Proteins encoded by genes under control of ARE promoter can be classified into several categories: phase II xenobiotic-metabolizing enzymes, antioxidants and their modulating enzymes, molecular chaperones/proteasome systems, DNA repair enzymes and anti-inflammatory response proteins. Heme oxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase 1 (NQO-1), glutamate cysteine ligase (Gcl) or glutathione-S-transferase (GST) are the examples of Nrf2 target genes. After recovery of cellular redox homeostasis, Keap1 moves into the nucleus and dissociates Nrf2 from ARE. Consequently, cytoprotective genes are no longer transcribed, whereas Nrf2-Keap1 complex is exported out of the nucleus. It associates in the cytoplasm with Cullin3-based E3 ligase ubiquitination complex resulting in degradation of Nrf2 and termination of the Nrf2/ARE signaling pathway (Li and Kong, 2009).

TRITERPENOID AS MODULATORS OF Nrf2/Keap1 PATHWAY

Both naturally occurring triterpenoids, like OA and its synthetic analogs (SO), affect Nrf2/ARE pathway (Yu and Kensler, 2005; Giudice *et al.*, 2010). However, Nrf2 activation and subsequent regulation of the transcription of the plethora of protective genes may be increased thanks to the chemical modifications of the triterpenoid structure.

To show the important role of triterpenoids and their derivatives in the Nrf2/ARE signaling pathway regulation, multiple

experiments using wild-type ($Nrf2^{+/+}$) and knock-out ($Nrf2^{-/-}$) mice/cells have been performed. CDDO-Im activated HO-1 in murine fibroblasts isolated from $Nrf2$ wild-type cells, but this activation was markedly lower in case of cells from $Nrf2^{-/-}$ mice even if higher concentrations of compound were applied (Liby *et al.*, 2005). In another study, CDDO-Im, CDDO-trifluoroethylamide (CDDO-Tfea) and CDDO-Ea were used to examine SO ability to activate $Nrf2$ signaling in ARPE-19 retinal epithelial cells (Pitha-Rowe *et al.*, 2009). All compounds activated $Nrf2$ /ARE pathway in a dose-dependent manner, but CDDO-Im was the most potent. *In vivo*, oral treatment of mice by CDDO-Tfea increased neural retinal levels of the $Nrf2$ -regulated transcripts in comparison to control animals. Also in other tissues, like liver, small intestine and cerebral cortex, these transcripts were induced (Pitha-Rowe *et al.*, 2009).

The induction of $Nrf2$ and its downstream targets by triterpenoids was shown to be protective in different diseases. For example, intraperitoneal injection of OA increased protein expression and activity in livers of wild-type mice, but not in the $Nrf2^{-/-}$ mice and was able to protect against acetaminophen hepatotoxicity (Reisman *et al.*, 2009). CDDO-Me treatment abrogated retinal capillary degeneration induced by ischemia-reperfusion injury in wild-type but not $Nrf2^{-/-}$ mice (Wei *et al.*, 2011). Additionally, CDDO-Im increased $Nrf2$ signaling in kidneys from $Nrf2^{+/+}$ mice and protected them from cisplatin nephrotoxicity (Aleksunes *et al.*, 2010). Interestingly, CDDO-Im was suggested to prevent high-fat diet-induced obesity and in fact it was able to decrease the body weight, adipose mass and hepatic lipid accumulation in $Nrf2$ -dependent manner (Shin *et al.*, 2009). Moreover, neuroprotective activities of OA derivatives dependent on $Nrf2$ have been demonstrated. In mouse model of amyotrophic lateral sclerosis (ALS), treatment with triterpenoids improved motor performance and slowed disease progression (Neymotin *et al.*, 2011). Kaidery *et al.* (2012) investigated the effect of synthetic triterpenoids on Parkinson's disease and showed that in a MPTP-induced preclinical mouse model of this disease the activation of $Nrf2$ by triterpenoids is very effective treatment strategy. Similarly, such compounds were shown to be promising in the treatment of the Huntington's disease. In the brain, CDDO-Ea and CDDO-Tfea upregulated $Nrf2$ /ARE-induced genes, reduced oxidative stress, improved motor impairment and increased longevity (Stack *et al.*, 2010).

Although all above mentioned SO are shown to possess protective effects, still new derivatives of OA are generated. Such derivatives are synthesized by the cyclization of squalene and thanks to sophisticated bioinformatics and structure-activity analysis, the specific carbonyl groups, suggested to be essential for the striking anti-inflammatory potency, are modified (Sporn *et al.*, 2011). Here we investigated the effect of different synthetic analogs of OA, like 28-amino- and 28-hydroxyoleanane derivatives. In our studies we have found that especially three of them, namely ethyl oleanolate A-homo lactam (A-Lac-O-Me), erythrodiol (O-diol) and (succinyloleanolic) succinimide (Sc-O-Sclm) increase $Nrf2$ activity. After transfection of NIH3T3 murine fibroblasts with a construct containing the antioxidant response element sequence driving the expression of reporter gene, luciferase (ARE-luc plasmid), we were able to assess $Nrf2$ binding to ARE using chemiluminescent measurement (Fig. 3). After 24h treatment with both OA and its derivatives we found that all compounds increase $Nrf2$ activity comparable to prostaglandin J2 (PGJ2), a known $Nrf2$

inducer (Fig. 4A). Additionally, $Nrf2$ protein was nicely activated by synthetic analog of OA as shown by immunostaining (Fig. 4B). The induction of HO-1 mRNA, the $Nrf2$ target gene, after 6h stimulation with the compounds was greater than after PGJ2 (Fig. 4C). All those results suggest that OA derivatives have the ability to induce $Nrf2$ /ARE pathway. However, we are still looking for better activators of antioxidant response.

Importantly, the effect of triterpenoids on $Nrf2$ activation might be cell-type dependent. We compared the effect of other triterpenoid, betulin on $Nrf2$ activation in keratinocytes and endothelial cells. We observed greater response of human keratinocytes (HaCaT) than human microvascular endothelial cells (HMEC-1) when the effect of betulin on the $Nrf2$ binding to ARE sequence was tested in the transfection assay (Fig. 4D). Previously, it was shown that in retinal epithelial cells even 10-fold activation was obtained 24h after stimulation with OA derivatives (Pitha-Rowe *et al.*, 2009). However, when human endothelial cells were treated with diterpene lactone called andrographide, only 2-3 times upregulation of $Nrf2$ activity was observed (Yu *et al.*, 2010) similarly to our results. It again suggests that OA derivatives have ability to increase $Nrf2$ activity in various cell types, but the power of this induction is not only dependent on compound tested, but also on cell type.

The protective response of $Nrf2$ induction relies on the effects exerted by the plethora of genes regulated in $Nrf2$ dependent manner. One of the well characterized gene upregulated after $Nrf2$ induction is HO-1, which catalyzes conversion of heme into biologically active products like carbon monoxide (CO), biliverdin and free iron. Biliverdin is quickly reduced to bilirubin by biliverdin reductase, whereas iron is sequestered by ferritin. Bilirubin inhibits NADPH oxidase and is a potent scavenger of free radicals. Decrease of heme content also reduces cellular reactive oxygen species (ROS) generation and CO has antiproliferative and anti-inflammatory properties. Thus, end-products of HO-1 activity demonstrate cytoprotective effects – they attenuate inflammatory response and lower

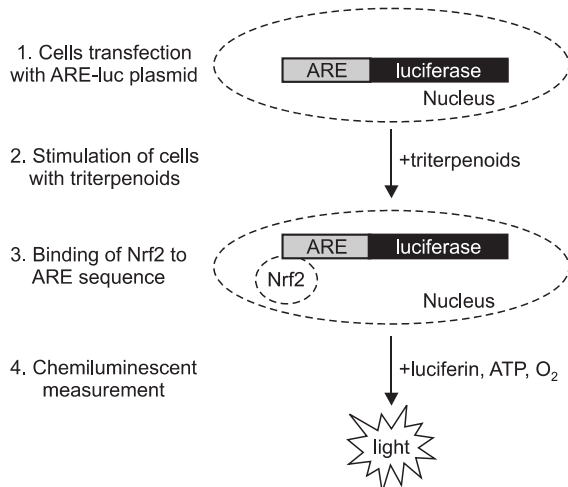


Fig. 3. Schematic representation of the procedure of $Nrf2$ activity assessment. 24h after transfection with a reporter construct containing the antioxidant response element (ARE) sequence driving the expression of reporter gene, luciferase (ARE-luc plasmid), cells are stimulated by $Nrf2$ inducers (e.g. triterpenoids). $Nrf2$ translocates to nucleus and binds to ARE sequence what leads to increased chemiluminescence.

rate of apoptosis (reviewed in: Loboda *et al.*, 2008). However, it has to be remembered that HO-1 increases neovascularization and growing body of evidence suggests that it may play a role in tumor induction and accelerate the growth and spread of tumors (reviewed in: Was *et al.*, 2010).

MAY TRITERPENOIDs REGULATE miRNAs EXPRESSION?

Emerging evidence suggest that microRNAs (miRNAs), small non-coding RNAs regulating gene expression by multi-

ple mechanisms, play important roles in the development and progression of cancer and may be even used as a specific cancer biomarkers (reviewed in: Ciesla *et al.*, 2011). Therefore, targeting specific miRNAs could be a novel therapeutic approach for the treatment of cancers and may be the mechanism of action of chemopreventive agents. In fact, it has been reported that miRNAs could be regulated by different natural chemopreventive compounds, like curcumin, resveratrol or isoflavones, leading to the inhibition of cancer cell growth and metastasis (reviewed in: Li *et al.*, 2010; Karius *et al.*, 2012). However, still little is known about the potency of triterpenoids on miRNAs expression, although such regulation is very probable.

Chinthalapalli *et al.* (2009) showed that methyl 2-cyano-3,11-dioxo-18 β -olean-1,12-dien-30-oate (CDODA-Me), a synthetic derivative of glycyrrhetic acid, a triterpenoid phytochemical found in licorice extracts, decreased expression of miR-27a leading to increased expression of genes responsible for inhibition of the progression of colon cancer cells through G2/M. The downregulation of miR-27a was also observed after stimulation of pancreatic cancer cells with CDDO or CDDO-Me (Jutooru *et al.*, 2010) as well as after betulinic acid treatment of colon cancer cells (Chinthalapalli *et al.*, 2011) or breast cancer cells (Liu *et al.*, 2012). In the mentioned publications, different cell lines have been used and various downstream mechanisms activated by miR-27a inhibition were shown to be responsible for the anticancer activity of triterpenoids, although they were not correlated to Nrf2 activity. miR-27a is oncogenic and its expression is upregulated in different cancers, so it may be a general target of both natural and synthetic triterpenoids. However, there is no data on the effect of triterpenoids on other miRNAs.

A lot of questions still remain to be answered. Is it possible that the synthetic analog which acts more potently than its original compound regulates the specific miRNAs? Is the expression of miRNAs, which binding sites are present within 3'UTR sequence of Nrf2 (e.g. miR-17, miR-129-5p and miR-132, based on miRanda analysis), regulated by triterpenoids? It is reasonable to believe that not only miR-27a can be a target of triterpenoids action. Nevertheless, experiments proving such regulation have to be performed.

CONCLUSIONS

Triterpenoids are multitarget compounds exhibiting concentration-dependent functions: at lower doses they act as an anti-inflammatory and cytoprotective compounds whereas at higher concentrations they possess mostly anticancer activities. The induction of Nrf2 by natural and synthetic triterpenoids has become commonly accepted mechanism responsible for cytoprotection and chemoprevention. Still many questions remain unanswered and the possible involvement of miRNAs in triterpenoids-mediated effects is an intriguing aspect.

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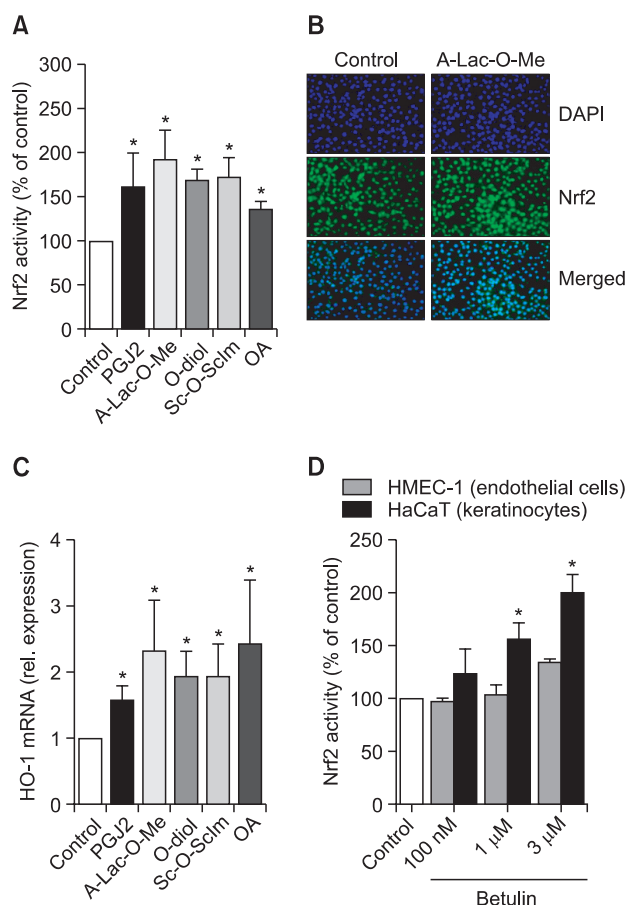


Fig. 4. OA derivatives increase Nrf2 activity and its target gene expression. 24h after transfection with a reporter construct regulated by the antioxidant response element (ARE-luc), NIH3T3 cells were stimulated by PGJ2 (positive control), OA and its derivatives (A-Lac-O-Me, O-diol, Sc-O-Sclm). After next 24h luciferase was measured showing the increased activity of Nrf2 after stimulation by all compounds (A). Moreover, the effect of selected compound (A-Lac-O-Me) on Nrf2 protein level was checked using immunostaining analysis (B). 6h treatment with the OA derivatives led to the increased expression of HO-1 (gene regulated by Nrf2) as shown by real-time PCR (C). Nrf2 activation by triterpenoids might be cell-type dependent. After transfection of human endothelial cells (HMEC-1) and human keratinocytes (HaCaT) with ARE-luc plasmid and stimulation with three different concentrations of betulin, the increase in luciferase activity was observed only in HaCaT cells (D). (A, C, D) - mean of 3 independent experiments, * p <0.05, B - representative immunostaining for Nrf2 (green). DAPI staining was used to visualize nuclei (blue).

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