Bioorganic Chemistry 93 (2019) 103326

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Oleanolic acid oxime derivatives and their conjugates with aspirin modulate the NF-κB-mediated transcription in HepG2 hepatoma cells

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ARTICLE INFO

Keywords:
Oleanolic acid oximes derivatives
Aspirin oleanolate conjugates
NF-κB
HepG2 cells

ABSTRACT

The aim of this study was to evaluate the effect of new oleanolic acid oxime (OAO) derivatives and their conjugates with aspirin (ASP) on the expression and activation of NF-κB in human hepatoma HepG2 cells. OAO derivatives showed a stronger cytotoxic effect against HepG2 cells compared with their conjugates with aspirin. Moreover, conjugation of OAO with ASP led to enhanced downregulation of NF-κB expression and activation. Among the hybrids with ASP, compounds: **19,** 3-(2-acetoxy)benzoyloxyiminoolean-12-en-28-oic acid morpholide and **13,** 3-(2-acetoxy)benzoyloxyiminoolean-12-en-28-oic acid methyl ester, differing, respectively, in morpholide and methyl ester groups at the C-17 position of oleanolic acid (OA) molecule were the most efficient. *COX-2* transcript and protein levels were also diminished after treatment with these compounds. The results of this study indicate that the new derivatives of OAO and particularly their conjugates with ASP, downregulate the expression of *COX-2* in HepG2 cells by modulating the NF-κB signaling pathway and suggest their potential application in the prevention of liver inflammation and cancer.

1. Introduction

Naturally occurring triterpenoids have been shown to possess several biological activities important for cancer chemoprevention and therapy [1]. One of the best known and well-characterized representative of triterpenoids class is oleanolic acid (OA), 3-β-hydroxyolean-12-en-28-oic acid, produced by a variety of medicinal herbs and other plants [1,2]. A number of studies have reported the anticarcinogenic activities of OA in different in vitro and in vivo models [1]. An important factor contributing to these effects of OA is its anti-inflammatory potential. Besides, OA possesses also well documented hepatoprotective properties [3]. However, it has an absolute oral bioavailability of only 0.7% because of its low permeability and aqueous solubility [4]. The very low oral bioavailability of OA could be due to poor absorption and extensive metabolic clearance [5].

Over the past years, a large number of triterpenoids have been chemically modified in order to improve their bioactivity and bioavailability and to enhance their protective and/or therapeutic effects. In many cases, the modified OA has been shown to possess better cytoprotective and anti-inflammatory properties than that of the parent compound [6]. The results of several studies pointed out that the potential therapeutic activities of OA derivatives were associated with their hydrophilicity when compared to the parent compound. In this regard, an earlier study showed that, in comparison to parent compound, the OA oxime (OAO) derivative, methyl 3-octanoyloxyiminoolean-12-en-28-oate, presented better anti-inflammatory activity than that of the parent compound, which was demonstrated by its anti-oedemic effects in rats with carrageenan-induced skin inflammation [7]. Moreover, the pharmacological activity of OA derivatives can be increased by conjugation with drugs such as aspirin (ASP, acetylsalicylic acid). This type of conjugates may enhance the anti-inflammatory activity of OA derivatives, but also allows to avoid ASP-induced gastrointestinal side effects [8]. The results of our previous study demonstrated that in comparison to the parent compound, the conjugate of 3-hydroxyiminoolean-12-en-28-oic acid morpholide with ASP: 3-(2-acetoxy)benzoyloxyiminoolean-12-en-28-oic acid morpholide (**19**) exerts strong anti-inflammatory activity in rats as result of single or subchronic administration of OAO-ASP conjugate. The administration of this compound lowered the blood concentration of interleukin–6 (IL-6) and the level of *IL-6* transcript level in blood lymphocytes, but the mechanism of its anti-inflammatory activity has not been established yet [9]. Multiple signaling pathways might be

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https://doi.org/10.1016/j.bioorg.2019.103326
Received 19 July 2019; Received in revised form 26 September 2019; Accepted 26 September 2019
Available online 27 September 2019

involved in the injury-inflammation-regeneration response. Classical NF-κB signaling is one of the most important pathways that is activated particularly during inflammation-related carcinogenesis. *IL-6* is one of the many genes which is regulated by this transcription factor. NF-κB commonly refers to a p50-p65 heterodimer, which represents the major Rel/NF-κB complex in most of the cells. In the latent state, NF-κB is sequestered in the cytosol by its inhibitor, IκB protein. Stimulation with a variety of signals, including cytokines, pathogens and injuries leads to the activation of IκB kinase (IKK) which phosphorylates IκB. This results in the proteasomal degradation of IKK and the release of NF-κB for nuclear translocation and activation of transcription of genes such as *COX-2*, which encodes cyclooxygenase-2 [10]. Hepatocellular carcinoma (HCC), the major form of primary liver cancer, represents the quintessential example of inflammation-driven cancers. Inflammation is relevant as a risk factor and also as the consequence of carcinogenesis induction in HCC [11].

Overexpression of *COX-2*, related to increased cell growth and invasiveness, is observed in human HCC. Therefore, the suppression of COX-2 activity can be an important approach to prevent hepatic inflammation. Moreover, designing the inhibitors of NF-κB inhibitors, is an interesting approach to develop new cancer chemopreventive agents or therapeutics [12].

Therefore, the aim of this study was the further elucidation of the mechanism of the anti-inflammatory activity of OAO-derivatives and their conjugates with ASP, through the evaluation of their effect on the expression and activation of NF-κB in human hepatoma HepG2 cells. In order to select the most potent modulator of this pathway, a series of new OAO-ASP hybrids were designed and synthesized.

2. Results and discussion

2.1. Synthesis and NMR spectroscopic characteristics of the OAO and their conjugates with aspirin

OAO derivatives and their conjugates were synthesized according to the previously described procedures [7]. Schemes 1 and 2 present an overview of the synthesis. The chemical structures of the synthesized compounds were elucidated based on the analysis of infrared (IR) spectroscopy, ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy, mass spectroscopy (MS) and high-resolution mass spectroscopy (HRMS). The ^{1}H NMR spectra of conjugates **13**, **15**, **17** and **19** show that the protons of aromatic ring of ASP moiety formed two doublet of doublets (observed at about δ 7.97 and 7.14 ppm) and two triplets of doublets (observed at about δ 7.57 and 7.32 ppm). The presence of a strong signal, located at about δ 2.33 ppm, of intensity 3H, confirmed the presence of acetylsalicylic moiety within the molecules of compounds **13**, **15**, **17** and **19**. The chemical structures of the synthesized compounds were elucidated based on ^{1}H and ^{13}C NMR spectra. The ^{1}H NMR spectra of conjugates **13**, **15**, **17** and **19** show that the protons of aromatic ring of acetylsalicylic moiety formed two doublet of doublets (observed at about δ 7.97 and 7.14 ppm) and two triplets of doublets (observed at about δ 7.57 and 7.32 ppm). The presence of a strong signal, located at about δ 2.33 ppm, of intensity 3H, confirmed the presence of acetyl group in acetylsalicylic moiety within the molecules of compounds **13**, **15**, **17** and **19**. This fact was further confirmed by ^{13}C NMR spectra. Aromatic ring carbons formed two quaternary signals (δ about 150 and 123 ppm) and four tertiary signals (δ about 134, 131, 126 and 124 ppm). The presence of acetoxy group within the acetylsalicylic moiety was proved on the basis of two signals: one that was detected at δ 170 ppm (C_q) and the other that was detected at δ 21.0 ppm. In ^{13}C NMR spectra of oximes **12**, **14**, **16** and **18**, a signal derived from C-3 atom was detected at about 167 ppm [13]. Replacement of hydrogen atom within hydroxyimino group of acetylsalicylic moiety resulted in the shifting of value of chemical shift for C-3 atom toward higher values and for compounds **13**, **15**, **17** and **19** signal for C-3 atom was present at δ about 176 ppm. The carbon atom in the carboxyl moiety of ASP provided a signal at δ of about 162 ppm. All signals were present in ^{1}H and ^{13}C NMR spectra of conjugates **13**, **15**, **17** and **19**, which confirm the presence of oleanolate skeleton and its characteristic functional groups.

2.2. Cell viability

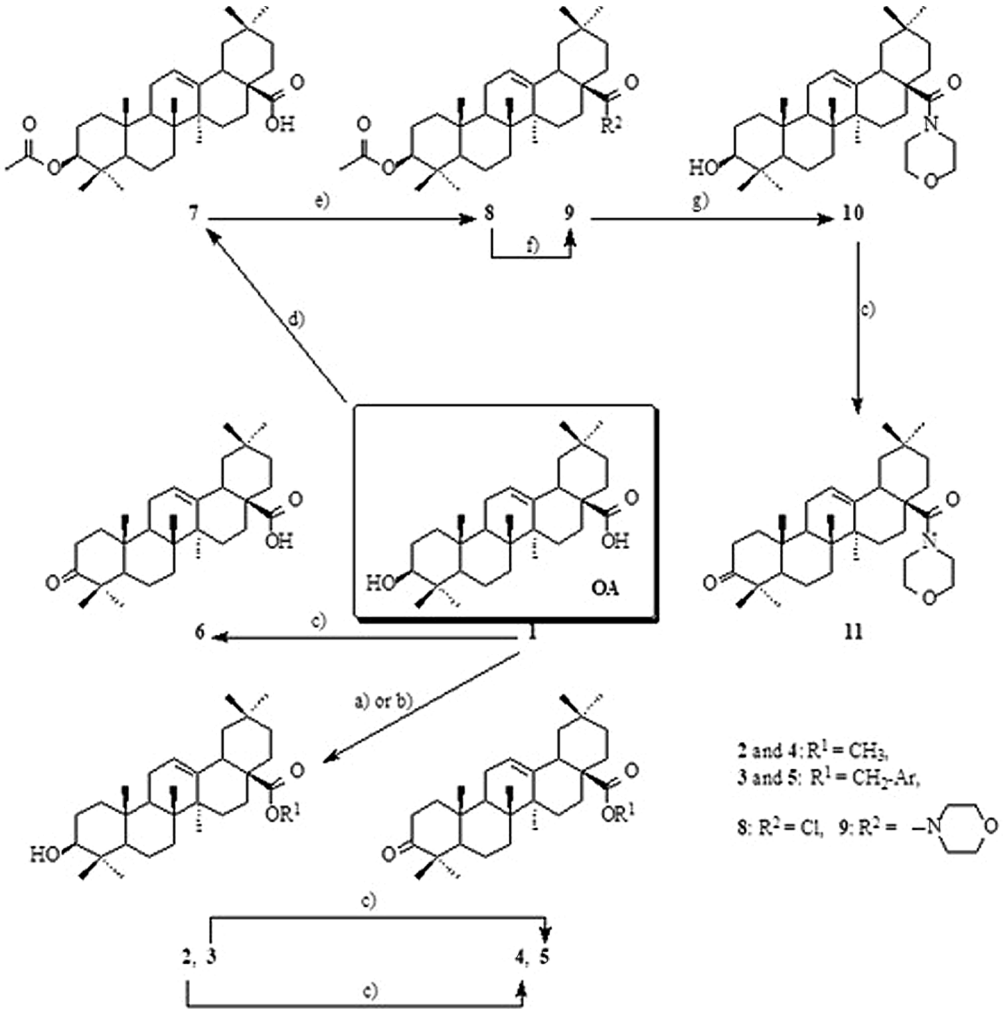
The impact of OA, OAO derivatives and their conjugates with ASP on the viability of human immortalized hepatocytes (THLE-2) and hepatoma cells (HepG2) was evaluated using the MTT assay. In the concentration range 0.2–150 μM, all tested compounds reduced the viability of both cells types in a dose-dependent manner (Fig. 1). As shown in Fig. 1A, most of the synthesized derivatives demonstrated moderate to favorable anti-proliferative activities in HepG2 cells, whereas three compounds (**12**, **16** and **18**) exhibited stronger cytotoxic activity than that of the parent compound (**1**). The cytotoxicity of OAO derivatives conjugates with ASP (**13**, **15**, **17** and **19**) and ASP itself in HepG2 cells was significantly lower than that of OA (**1**) or OAO (**12**, **14**, **16** and **18**). ASP and its OAO derivatives conjugates (**13**, **15**, **17** and **19**) were not toxic in the tested concentration range, although the cytotoxicity of compound **19** was slightly higher than that of other conjugates. Its dose-response curves are presented in Fig. 1B. Fig. 1C and D show the dose–response curves for THLE-2 cells. Basically, the trend was similar in both HepG2 and THLE-2 cells. However, the comparison of IC_{50} values indicates that OA and its oximes are more cytotoxic toward HepG2 cells in comparison to THLE-2 cells. There were no differences in the cytotoxic effects of ASP and its conjugates with OAO on both cell types (Table 1). These data indicate that OAO derivatives themselves, particularly compound **19** (morpholide derivative), may be more efficient as therapeutic agents, while their conjugates with aspirin may be important for chemoprevention purposes.

2.3. Effect of OA, OAO and their ASP conjugates on the expression of NF-κB p50 and p65 genes

Fig. 2 presents the results of quantitative PCR analysis of the transcript levels of *p50* and *p65* NF-κB subunits, the major components of active Rel/NF-κB complex in the cell nucleus. Panel A shows the effects of treatment with OA and OAO, while Panel B illustrates the changes in *NF-κB* subunits expression as the result of treatment of HepG2 cells with OAO conjugates. Incubation with all OAO derivatives decreased the *NF-κB p50* and *NF-κB p65* transcript levels in comparison to the control group. Derivatives **12**, **16** and **18** at a concentration of 20 μM significantly decreased the expression of *NF-κB p50* by ~23%, 20% and 30%, respectively. Similarly the expression of *NF-κB p65* subunit was diminished by compounds **12**, **14**, **16** and **18** by ~27%, 21%, 32% and 45%, respectively. Treatment with OAO conjugates **13** and **19** also decreased the expression of *NF-κB p50* and *NF-κB p65* by ~20–49% at a concentration of 10 and 20 μM, while conjugates **15** diminished the expression of these *NF-κB* subunits by ~20–27% only at a concentration of 20 μM. Thus, the morpholide derivative of OAO and its conjugate with ASP diminished the expression of *NF-κB* subunits to the most extent. Aspirin, in comparable concentrations, showed weaker effect on the transcription of *NF-κB,* suggesting potentially higher anti-inflammatory activity of its hybrid compounds.

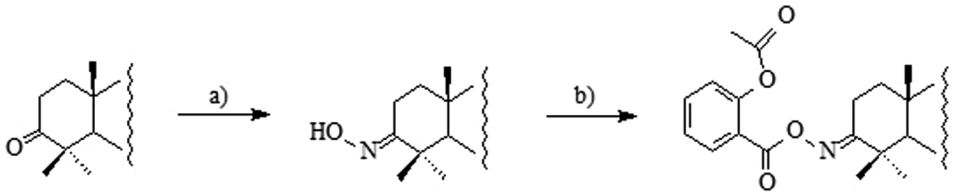
2.4. Effect of OA, OAO derivatives and their ASP conjugates on NF-κB activation

Fig. 3 presents the results of the assessment of the binding of p50 and p65 NF-κB subunits to their immobilized consensus site. All OAO derivatives at the whole range of tested concentrations diminished the content of NF-κB p50 and NF-κB p65 subunits in the DNA-binding complex extracted from the nuclei. However, significant changes (by ~ 20–28% and 25–40% for the binding of p50 and p65, respectively) were observed only for higher concentrations of these



Scheme 1. Synthesis of intermediates **2–11** for the synthesis of oleanolic acid oximes (OAO) and conjugates **12–19**. Reagents and conditions: (a) dimethyl sulfate, NaOH, ethanol, reflux; (b) benzyl chloride, DMF, K_2CO_3, heating; (c) Jones reagent, acetone, r.t.; (d) acetic anhydride, reflux; (e) thionyl chloride, r.t; (f) morpholine, benzene, r.t.; (g) NaOH, ethanol, reflux.

compounds. Again, compound **18** was shown to be the most effective inhibitor of NF-κB activation. ASP, in the highest concentration and its conjugates with OAO derivatives significantly reduced the binding of p65 to its consensus sequence. Conjugate of ASP with morpholide derivative of OAO **(19)** was the most effective and reduced the binding level of p65 by ~ 49%, followed by methyl derivative (**13**). Binding of p50 subunit to its consensus sequence was less affected, but compounds **13** and **19** diminished it by ~ 30% and 25% respectively at the highest concentration. The NF-κB p50 subunit serves only as a helper in NF-κB DNA binding, whereas the p65 subunit is responsible for initiating transcription [14]. The disproportionate increase in the activated p65 and subsequent transactivation of effector molecules is integral to the pathogenesis of many diseases including cancer. Hence, the NF-κB p65 signaling pathway is considered a pivotal point for the drug discovery



Scheme 2. Synthesis of OAO (**12**, **14**, **16** and **18**) and their conjugates with aspirin (ASP) (**13**, **15**, **17** and **19**). Reagents and conditions: (a) $NH_2OH \times HCl$, ethanol, reflux; (b) aspirin, dioxane, DCC, r.t.

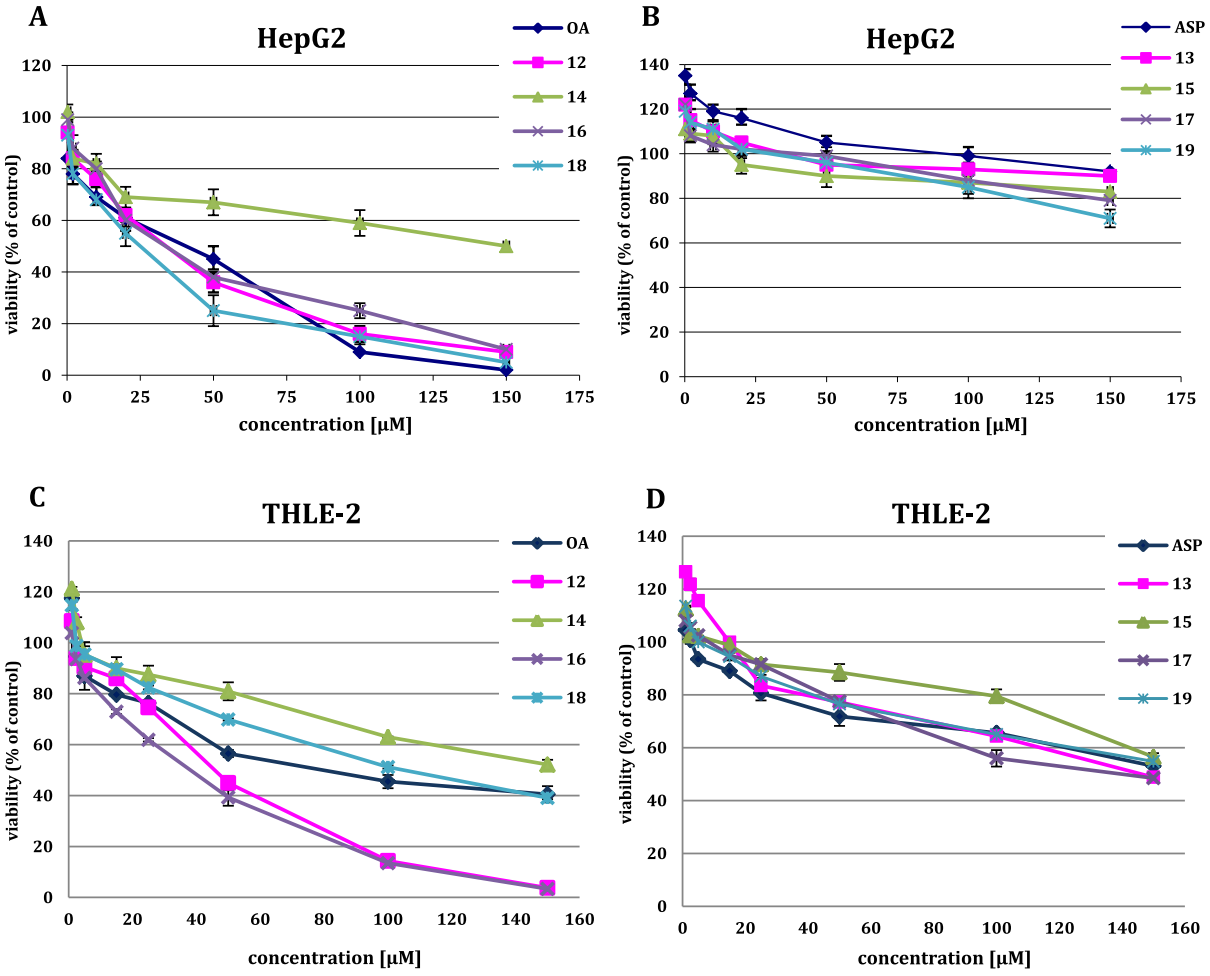


Fig. 1. The effect of the OA, OAO derivatives (**12**, **14**, **16**, and **18**; Panels A and C) and OAO conjugates with ASP (**13**, **15**, **17** and **19**; Panels B and D) on the viability of HepG2 and THLE-2 cells. Data are expressed as means ± SEM from three separate measurements.

Table 1
Comparison of OAO derivatives cytotoxicity in non-tumorigenic (THLE-2) and hepatoma cells (HepG2).

Compound	IC_{50} (μM) ± SEM*	
	THLE-2	HepG2
OA	80 ± 3.71	41 ± 3.31
12	46 ± 1.61	34 ± 1.7
14	> 150	148 ± 1.36
16	38 ± 0.93	34 ± 1.58
18	105 ± 2.13	25 ± 2.55
ASP	> 150	> 150
13	146 ± 0.67	> 150
15	> 150	> 150
17	141 ± 3.53	> 150
19	> 150	> 150

* The IC_{50} values are expressed as mean ± SEM obtained from three independent experiments and calculated based on the dose-response curves assessed by the MTT assay.

and development [15]. Thus, taking the level of cytotoxicity into consideration, the morpholide derivative of OAO might be considered as one of the candidates for new therapeutics, while its conjugate with ASP as a chemopreventive agent. A similar pattern, as in the case of NF-κB p65 DNA binding capability, was observed in the reduction of the p65 nuclear protein level (Fig. 4). This further confirms the effect of these compounds on the activation of NF-κB.

2.5. Effect of OA, OAO derivatives and their ASP conjugates on the IKK protein

NF-κB activation involves IκB kinase (IKK) activation which leads to IκB phosphorylation and subsequent degradation of ubiquitin-dependent IκB by 26S proteasome complex. Therefore, phosphorylation of IκB by IKK complex (α and β) is a critical regulatory step in the activation of NF-κB [16]. Moreover, both IKK α and β can phosphorylate the RelA/p65 subunit to promote transactivation potential [17]. Consequently, in this study, the most active OAO derivatives, **12** and **18**, and their conjugates with ASP (**13** and **19**) significantly (by 20–38%) reduced the IKKα/β protein level (Fig. 5). Thus, decreased activation of NF-κB might result principally from a lower amount of IKK available for IκB phosphorylation and subsequent inhibition of NF-κB transactivation.

2.6. Effect of the OA, OAO derivatives and their ASP conjugates on the COX-2 transcript and protein level

COX-2 gene is under the transcriptional control of NF-κB, thus in the next step the mRNA and protein level of this enzyme was determined. In agreement with the data concerning the effect of new OAO derivatives and their conjugates with aspirin on the activation of NF-κB, COX-2 mRNA transcript and protein levels were the most reduced as result of treatment with OAO derivative **18** and its conjugate with ASP, **19**. The effect of the conjugate was more pronounced on mRNA level, but still conjugates **19** and **13** were more effective than ASP alone (Fig. 6). These results confirm that OAO derivatives, through inhibition of NF-κB

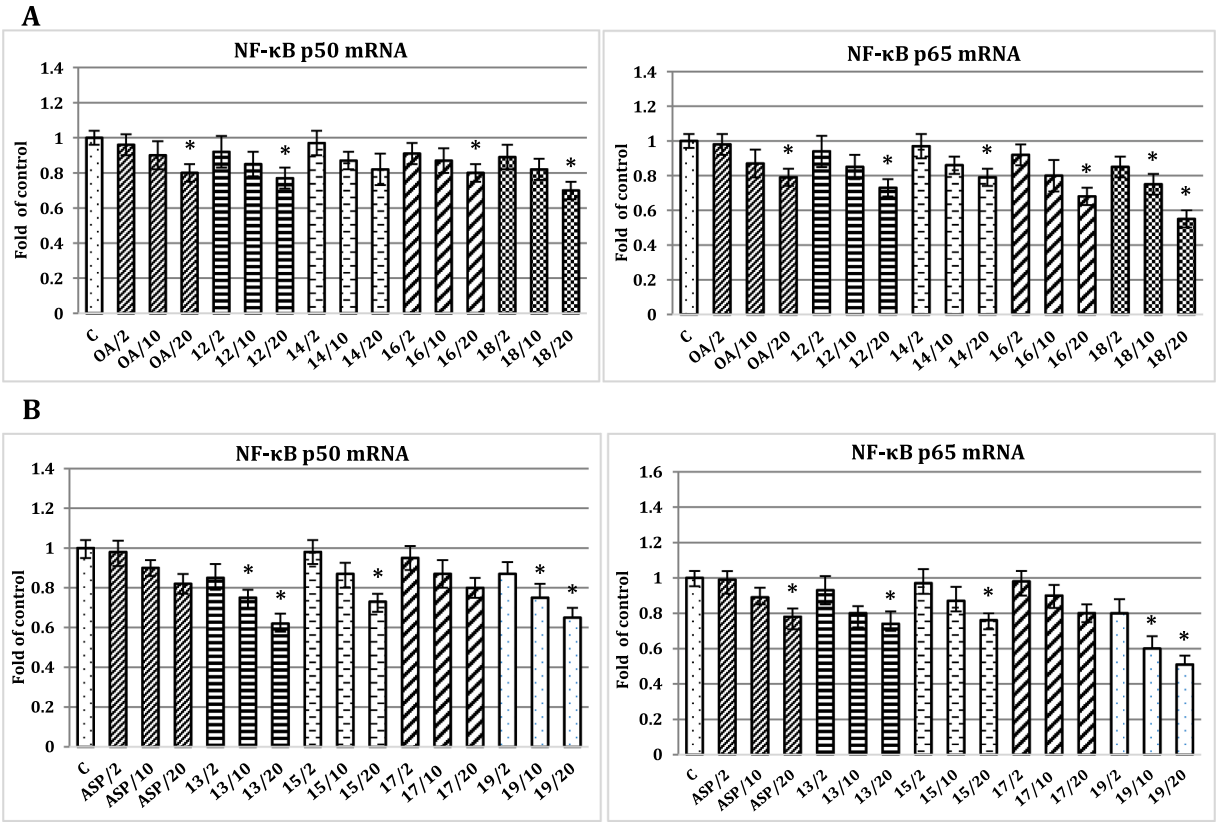


Fig. 2. The effect of the OA, OAO derivatives (**12**, **14**, **16** and **18**, panel A) and OAO conjugates with ASP (**13**, **15**, **17** and **19**, panel B) on the expression of *NF-κB* in HepG2 cells. Data (means ± SEM) from three separate experiments. The values were calculated as mRNA level in comparison with control cells (expression equals 1). The asterisk (*) above the bar denotes statistically significant difference from the control group, $p < 0,05$.

activation, diminish the expression of one of the key elements of inflammatory response. Moreover, conjugation of ASP particularly with OAO morpholide derivatives (**19)** strengthens this effect.

3. Conclusion

The results of this study provided the elucidation of the molecular basis of previously observed in vivo anti-inflammatory activity of oleanolic acid oxime morpholide conjugated with ASP. Inhibition of NF-κB expression and activation, as a result of the reduced amount of IKKα/β, can be responsible for this effect. The specificity of OAO structure substitution seems to be crucial for the NF-κB modulation and subsequent anti-inflammatory effect. In this regard, comparison with parent compound OA (**1**) as well as its oxime (**16**) clearly indicates that the substitution within carboxyl group at position C-17 increases their ability to modulate the NF-κB signaling pathway. Substitution with morpholine (**18**) followed by the substitution with a methyl group (**12**) showed to be the most effective, whereas substitution with benzyl group did not differ in its activity from OA (**1**) or OAO (**16**) in NF-κB modulation. Moreover, conjugation of OAO substituted with morpholine or methyl group at C-28 with ASP inhibited NF-κB activation and subsequently COX-2 expression to a greater extent not only in comparison with OAO, but also ASP.

Conjugation with ASP significantly decreased the cytotoxicity of OAO derivatives in human hepatoma cells. Therefore, the combination of reduced cytotoxicity with diminished NF-κB expression and activity in comparison not only with the OAO, but also ASP might be particularly important for the application of these hybrids in liver cancer chemoprevention and/or in adjuvant therapy. Since the overexpression of *COX-2*, related to increased cell growth and invasiveness, is observed in human HCC, its suppression by OAO derivatives conjugates with ASP further supports the conclusion on the potential application of these compounds in the prevention of liver inflammation and cancer.

4. Experimental section

4.1. Synthesis of triterpenic derivatives 2–19

General information. For reactions in anhydrous conditions, the solvents were purified and dried by using common procedures. Thin layer chromatographic (TLC) analysis (reactions progress and the level of compounds purity) was conducted on HPTLC aluminum sheets covered with silicagel 60 F245. Benzene ethyl acetate (1:1, 2:1, 4:1, or 9:1, v:v) was used as an eluent. The chromatograms were visualized by spraying with 10% ethanolic solution of sulfuric acid and heating the plates at about 110 °C for a few minutes. Column chromatography was performed using silicagel 60 (0.063–0.200 mm, 70–230 mesh) and methylene chloride as eluent. Melting points were measured with a Büchi apparatus in an open capillary and are uncorrected. IR spectra were recorded for KBr disks (0.5% mixture of sample in KBr) using a Specord IR-75 spectrophotometer. NMR spectra for hydrogen atoms (^{1}H) and for carbon atoms (^{13}C) were recorded in deuterated chloroform or deuterated pyridine solutions with tetramethylsilane (TMS) as an internal standard with the application of Varian Gemini 300 VT spectrometer. The multiplicities of signals in ^{1}H NMR spectra were marked as follows: s–singlet, br/s–broad singlet, d–doublet, t–triplet, td–triplet of doublets, dd–doublet of doublets and m–multiplet. The most representative signals are given in the spectral data (^{1}H and ^{13}C NMR) listed below. MS were recorded using an AMD 402 spectrometer with electroionization. The elemental analyses (C, H and N) were performed using a 2400 CHN analyzer (PerkinElmer, MA, USA).

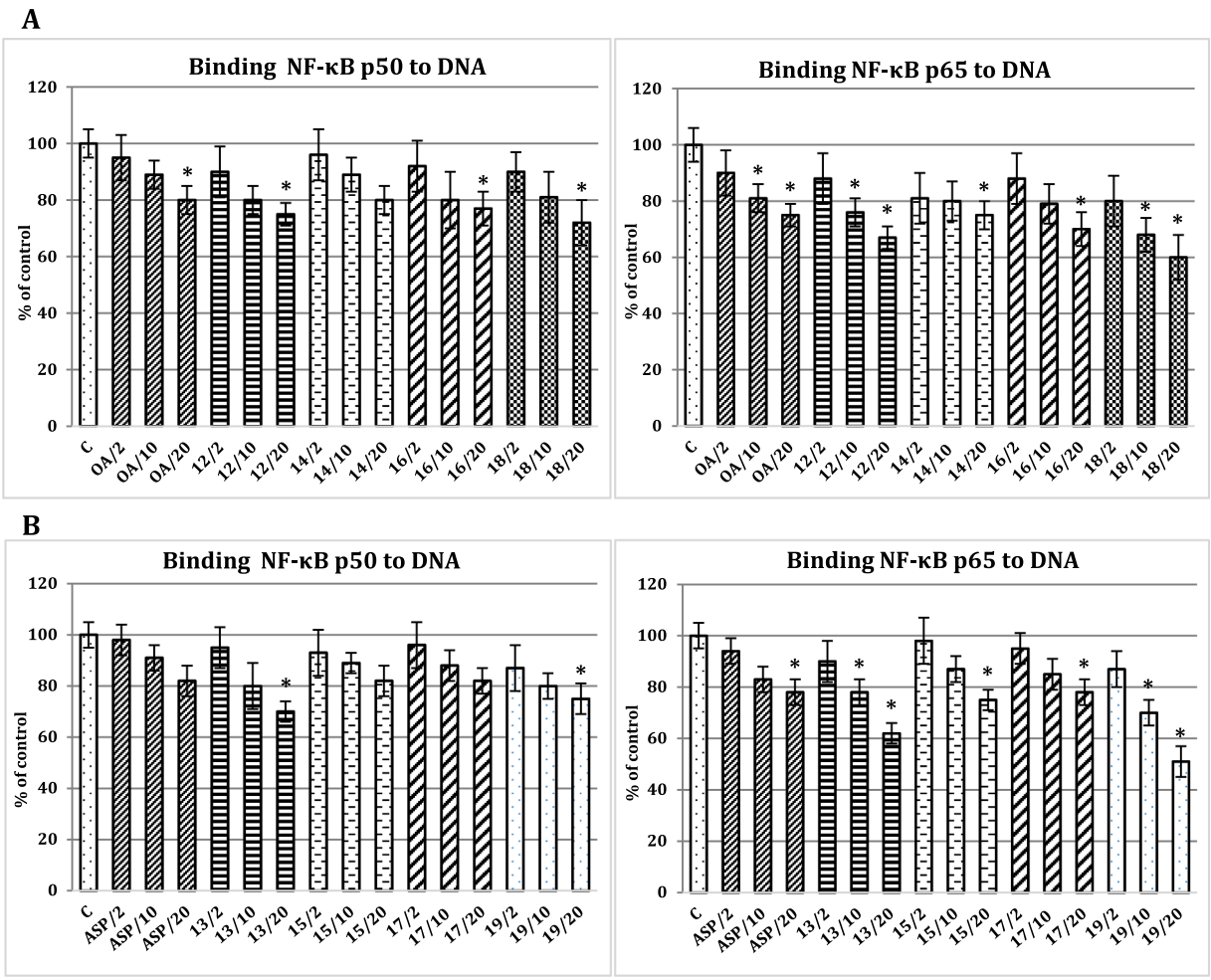


Fig. 3. The effect of the OA, OAO derivatives (**12**, **14**, **16** and **18**, Panel A) and OAO conjugates with ASP (**13**, **15**, **17** and **19**, Panel B) on NF-κB binding to DNA in HepG2 cells. Data (means ± SEM) from three separate experiments. The values were calculated as the level of protein in comparison with control cells (expression equals 100%). The asterisk (*) above the bar denotes statistically significant differences from the control group, $p < 0,05$.

4.1.1. Oleanolic acid (OA) esterification

Methyl oleanolate (2). Synthesis of methyl oleanolate (**2**) from 1.0 mmol of OA (**1**) was performed as presented in the literature. Physical and spectral data agree with those available in the literature data [18].

Benzyl oleanolate (3). K_2CO_3 (210 mg, 1.5 mmol) was added to a stirred and heated (120 °C) solution of OA (**1**) and, (456 mg, 1.0 mmol) in dried N,N-dimethylformamide (DMF) (8.0 mL). The resulted suspension was stirred and heated at 120 °C for 30 min and to this, benzyl chloride (150 mg, 1.2 mmol) was added dropwise. After further stirring and heating for 30 min, the solution was poured into a container with a 5-time excess volume of water. The formed precipitate was filtered off, washed with water and dried. $C_{37}H_{54}O_3$; mol. mass: 546.4073; yield: 520 mg (95.1%); m.p.: 190 °C (EtOH); spectral data agree with those available in the literature [19].

4.1.2. Multistep synthesis of OA morpholide (10)

Acetyloleanolic acid (7). Synthesis of acetyloleanolic acid (**7**) from 1.0 mmol of oleanolic acid (**1**) was performed according to the protocol given in the literature [18]. Physical and spectral data agree with those available in the literature [20].

Acetyloleanolic acid chloride (8). Synthesis of acetyloleanolic acid chloride (**8**) from 1.0 mmol of acetyloleanolic acid (**7**) was performed according to the protocol given in the literature [18].

Acetyloleanolic acid morpholide (9). Synthesis of acetyloleanolic acid morpholide (**9**) from 1.0 mmol of acetyloleanolic acid chloride (**8**) was performed according to the protocol given in the literature. Physical and spectral data agree with those available in the literature [18].

Oleanolic acid morpholide (10). Synthesis of oleanolic acid morpholide (**10**) from 1.0 mmol of acetyloleanolic acid morpholide (**9**) was performed according to the protocol given in the literature. Physical and spectral data agree with those available in the literature [18].

4.1.3. Jones oxidation of compounds 2, 3, 1, and 10

Methyl oleanonate (4). Synthesis of methyl oleanonate (**4**) from 1.0 mmol of methyl oleanolate (**2**) was performed according to the protocol given in the literature [13,18]. Physical and spectral data agree with those available in the literature [13].

Benzyl oleanonate (5). Synthesis of benzyl oleanonate (**5**) from benzyl oleanolate (**3**) was performed according to the protocol given in the literature [13]. Physical and spectral data agree with those available in the literature [21].

Oleanonic acid (6). Synthesis of oleanonic acid (**6**) from 1.0 mmol of oleanolic acid (**1**) was performed according to the protocol given in the literature [13,18]. Physical and spectral data agree with those available in the literature [21].

Oleanonic acid morpholide (11). Synthesis of oleanonic acid morpholide (**11**) from 1.0 mmol of oleanolic acid morpholide (**10**) was performed according to the protocol given in the literature. Physical and spectral data agree with those available in the literature [18].

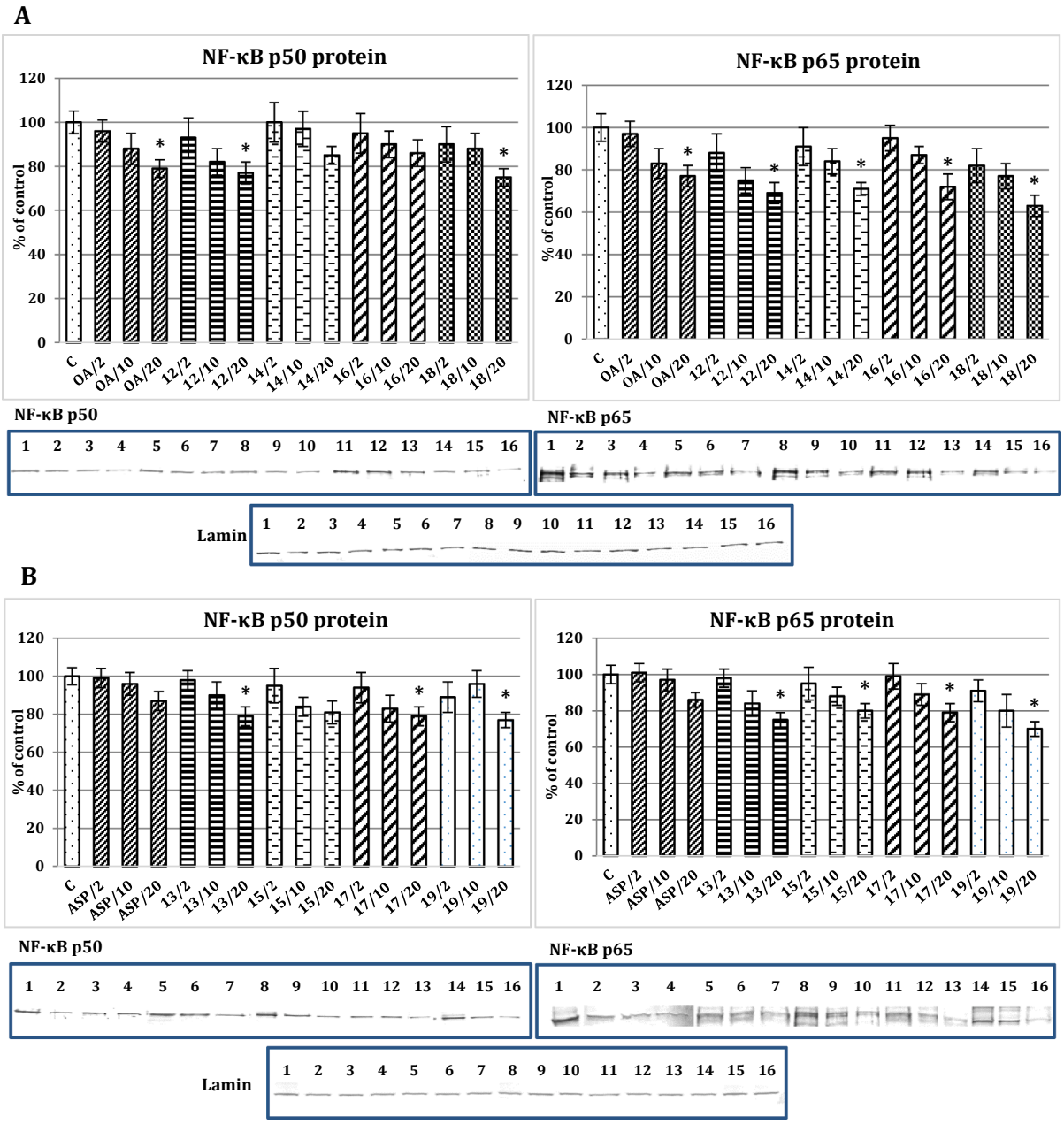


Fig. 4. The effect of the OA, OAO derivatives (**12**, **14**, **16** and **18**, Panel A) and OAO conjugates with ASP (**13**, **15**, **17** and **19**, Panel B) on NF-κB protein level in HepG2 cells. Data (means ± SEM) of Western blot analysis of the nuclear content of p50 and p65 from three separate experiments and representative immunoblots are shown. The sequence of the bands corresponds to the sequence of bars in the graph. Lamin content was used for the normalization of results. The values were calculated as the level of protein in comparison with control cells (expression equals 100%). The asterisk (*) above the bar denotes statistically significant differences from the control group, $p < 0,05$.

4.1.4. Synthesis of oximes 12, 14, 16 and 18

Oxime of methyl oleanonate (12). Synthesis of oxime of methyl oleanonate (**12**) from 1.0 mmol of methyl oleanonate (**4**) was performed according to the protocol given in the literature [13,18]. Physical and spectral data agree with those available in the literature [13].

Oxime of benzyl oleanonate (14). Synthesis of oxime of benzyl oleanonate (**14**) from 1.0 mmol of benzyl oleanonate (**5**) was performed according to the protocol in the literature [13,18]. $C_{37}H_{53}NO_3$; mol. mass: 559.4025; yield: 510.7 mg (91.3%); m.p.: 154–156 °C (EtOH + H_2O). IR (KBr, cm^{-1}): 3240 (OH, NOH), 2905 (CH, -COO-CH_2**Ar**), 1705 (C=O, COOCH$_2$Ar); ^{1}H NMR (400 MHz, $CDCl_3$): 9.34 (1H, br/s, =NOH); 7.36 – 7.30 (5H, m, –COO-CH_2-**Ar**); 5.29 (1H, t, J = 3.5 Hz, C_{12}-H); 5.08 (2H, d, J = 12.6 Hz, –COO-C**H$_2$**-Ar); 2.91 (1H, dd, J = 4.0 and 13.7 Hz, C_{18}-H_β); 1.15; 1.10; 1.05; 1.00; 0.92; 0.89; 0.64 (7 × 3H, 7 × s, 7 × CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): 177.4 (C_q, –**CO**O-CH_2-Ar); 168.4 (C_q, C-3); 143.7 (C_q, C-13); 136.4 (C_q, –COO-CH_2-**Ar**), 128.4 × 2, 127.9 × 2 and 127.8 (5 × CH, –COO-CH_2-**Ar**); 122.3 (CH, C-12); 65.9 (CH_2, –COO-**CH$_2$**-Ar); 47.6 (C_q, C-17). MS/EI (*m/z*): 559.41 (45.1%, $M^{\bullet+}$). Elemental analysis calculated: C 79.38%, H 9.54% and N 2.50%, found: C 79.40%, H 9.55% and N 2.50%.

Oxime of oleanonic acid (16). Synthesis of oxime of oleanonic acid (**16**) from 1.0 mmol of oleanonic acid (**6**) was performed according to the protocol given in the literature [13,18]. Physical and spectral data agree with those available in the literature [22].

Oxime of oleanonic acid morpholide (18). Synthesis of oxime of oleanonic acid morpholide (**18**) from oleanonic acid morpholide (**11**)

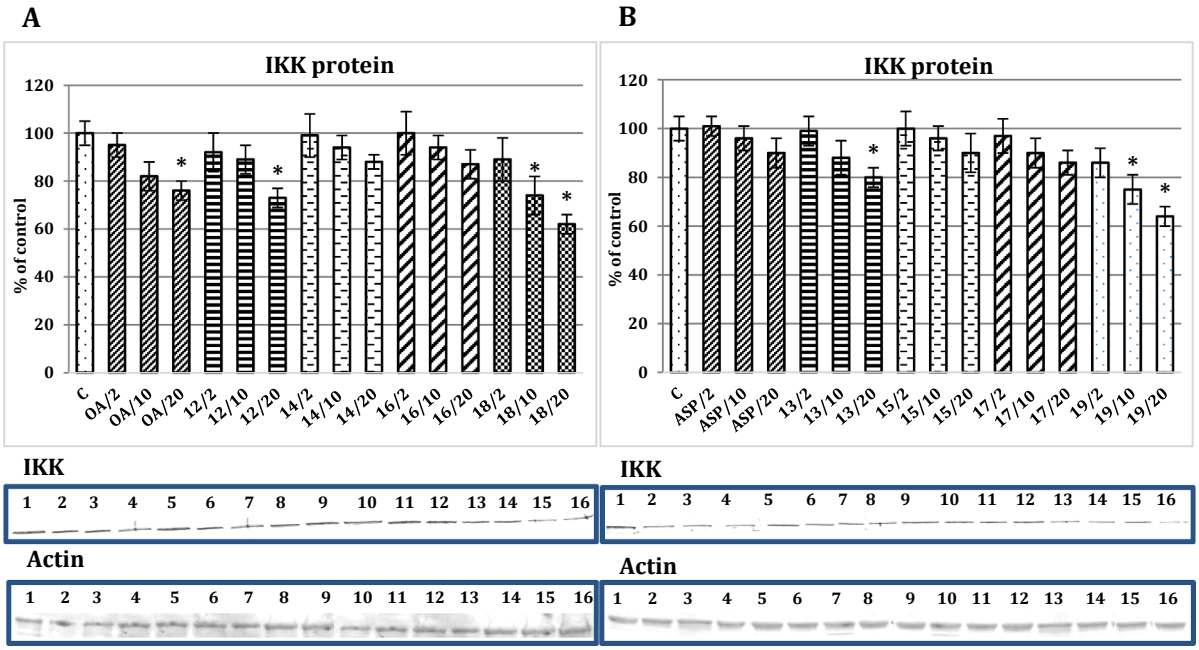


Fig. 5. The effect of the OA, OAO derivatives (**12**, **14**, **16** and **18**, Panel A) and OAO conjugates with ASP (**13**, **15**, **17** and **19**, Panel B) on IκB kinase (IKK) protein level in HepG2 cells. Data (means ± SEM) of Western blot analysis of the cytosolic content of IKK from three separate experiments and representative immunoblots are shown. The sequence of the bands corresponds to the sequence of bars in the graph. Actin content was used for the normalization of results. The values were calculated as the level of protein in comparison with control cells (expression equals 100%). The asterisk (*) above the bar denotes statistically significant differences from the control group, $p < 0,05$.

was performed according to the protocol given in the literature [18]. Physical and spectral data agree with those available in the literature [13,18].

4.1.5. Synthesis of conjugates of oximes 12, 14, 16 and 18 with aspirin (synthesis of compounds 13, 15, 17 and 19)

General method: To a saturated solution of oxime (**12**, **14**, **16,** or **18**, 1.0 mmol) in dioxane, aspirin (1.3 mmol) and dicyclohexylcarbodiimide (DCC, 1.5 mmol) were added and stirred. The stirring was continued at room temperature until the total consumption of oxime (TLC control). The resulting precipitate was filtered, washed with dioxane and rejected. The filtrate was poured into a container with 5-times excess water and the precipitated solid was collected and extracted with methylene chloride (5 × 5 mL). The organic solution was washed with a 5% solution of HCl (5 × 5 mL) and water (3 × 5 mL) and then dried and subjected to column chromatography on silica gel. After evaporating the solvent the crude product was dissolved in ethanol and precipitated with water.

Conjugate of oxime of methyl oleanonate and aspirin (=3-(2-acetoxy)benzoyloxyiminoolean-12-en-28-oic acid methyl ester, **13).** $C_{40}H_{55}NO_6$; mol. mass: 645.403; yield: 606 mg (93.9%), m.p.: 95–100 °C. IR (KBr, cm^{-1}): 1775 (C=O, CH_3**OCO**-Ar-COON = C), 1745 (C=O, CH_3OCO-Ar-**CO**ON = C), 1730 (N=C**,** CH_3OCO-Ar-COO**N**=**C**), 1715 (C=O, –$COOCH_3$); ^{1}H NMR (400 MHz, $CDCl_3$): 7.97 (1H, dd, *J* = 1.6 and 7.8 Hz, CH_3OCO-**Ar**-COON=), 7.57 (1H, td, *J* = 1.2 and 7.8 Hz, CH_3OCO-**Ar**-COON=), 7.32 (1H, td, *J* = 1.2 and 7.6 Hz, CH_3OCO-**Ar**-COON=) and 7.19 (1H, dd, *J* = 1.0 and 8.1 Hz, CH_3OCO-**Ar**-COON=), 5.30 (1H, t, *J* = 3.5 Hz, C_{12}-H); 3.63 (3H, s, –$COOCH_3$); 2.89 (1H, dd, *J* = 4.0 and 13.8 Hz, C_{18}-H_β), 2.34 (3H, s, **CH_3**OCO-Ar-COON=), 1.30, 1.20, 1.15, 1.05, 0.85, 0.81 and 0.76 (7 × 3H, 7 × s, 7 CH_3 groups); Ar = aromatic ring. ^{13}C NMR (100 MHz, $CDCl_3$): 178.2 (C_q, –$COOCH_3$); 176.3 (C_q, C-3); 170.1 (C_q, CH_3**OCO**-Ar-COON=); 162.0 (C_q, CH_3OCO-Ar-**CO**ON=); 150.0 (C_q, CH_3OCO-**Ar**-COON=), 133.7, 131.2, 125.9, and 124.0 (4 × CH, CH_3OCO-**Ar**-COON=) and 122.7 (C_q, CH_3OCO-**Ar**-COON=); 143.8 (C_q, C-13); 121.9 (CH, C-12); 51.5 (CH_3, –$COOCH_3$); 46.6 (C_q, C-17); 21.0 (CH_3, **CH_3**OCO-Ar-COON=); Ar = aromatic ring. MS/EI (*m/z*): 645.45 (13.2%, $M^{\bullet+}$). Elemental analysis calculated: C 74.39%, H 8.58% and N 2.17%, found: C 74.41%, H 8.57% and N 2.16%.

Conjugate of oxime of benzyl oleanonate and aspirin (=3-(2-acetoxy)benzoyloxyiminoolean-12-en-28-oic acid benzyl ester**, 15).** $C_{46}H_{59}NO_6$; mol. mass: 721.434 yield: 673 mg (93.3%), m.p.: 90–95 °C. IR (KBr, cm^{-1}): 2900 (CH, -$COOCH_2$**Ar**), 1775 (C=O, CH_3O**CO**-Ar-COON=C), 1755 (C=O, CH_3OCO-Ar-**CO**ON = C), 1725 (N=C**,** CH_3OCO-Ar-COO**N** = **C**), 1705 (C=O, COOCH_2Ar); ^{1}H NMR (400 MHz, $CDCl_3$): 7.97 (1H, dd, *J* = 1.6 and 7.8 Hz, CH_3OCO-**Ar**-COON=), 7.56 (1H, td, *J* = 1.2 and 7.8 Hz, CH_3OCO-**Ar**-COON =), and 7.13 (1H, dd, *J* = 1.0 and 8.1 Hz, CH_3OCO-**Ar**-COON=); 7.36 – 7.30 (1H + 5H, m, –CH_3OCO-**Ar**-COON = and –COO-CH_2-**Ar**); 5.30 (1H, t, *J* = 3.5 Hz, C_{12}-H); 5.09 (2H, d, *J* = 12.5 Hz, –COO-**CH_2**-Ar); 2.91 (1H, dd, *J* = 4.0 and 13.8 Hz, C_{18}-H_β), 2.32 (3H, s, **CH_3**OCO-Ar-COON=), 1.20, 1.17, 1.14, 1.01, 0.80, 0.77 and 0.68 (7 × 3H, 7 × s, 7 CH_3 groups); Ar = aromatic ring. ^{13}C NMR (100 MHz, $CDCl_3$): 177.1 (C_q, –$COOCH_3$); 176.3 (C_q, C-3); 169.6 (C_q, CH_3**OCO**-Ar-COON=); 161.8 (C_q, CH_3OCO-Ar-**CO**ON=); 150.5 (C_q, CH_3OCO-**Ar**-COON=), 133.6, 131.2, 125.8, 124.0 (4 × CH, CH_3OCO-**Ar**-COON=) and 122.8 (C_q, CH_3OCO-**Ar**-COON=); 143.6 (C_q, C-13); 136.1 (C_q, COO-CH_2-**Ar**), 128.2 × 2, 127.8 × 2 and 127.7 (5 × CH, –COO-CH_2-**Ar**); 122.3 (CH, C-12); 65.7 (CH_2, –COO-**CH_2**-Ar); 47.5 (C_q, C-17); 21.0 (CH_3, **CH_3**OCO-Ar-COON=); Ar = aromatic ring. MS/EI (*m/z*): 721.39 (22.1%, $M^{\bullet+}$). Elemental analysis calculated: C 76.53%, H 8.24% and N 1.94%, found: C 76.52%, H 8.25% and N 1.93%.

Conjugate of oxime of oleanonic acid and aspirin (=3-(2-acetoxy)benzoyloxyiminoolean-12-en-28-oic acid, **17).** $C_{39}H_{53}NO_6$; mol. mass: 631.387; yield: 612 mg (96.9%); m.p.: 125–128 °C, white powder. IR (KBr, cm^{-1}): 3350 (OH, –COOH), 1770 (C=O, CH_3O**CO**-Ar-COON=C), 1750 (C=O, CH_3OCO-Ar-**CO**ON = C), 1725 (N=C**,** CH_3OCO-Ar-COO**N** = **C**), 1700 (C=O, COOH); ^{1}H NMR (400 MHz, $CDCl_3$): 7.97 (1H, dd, *J* = 1.6 and 7.8 Hz, CH_3OCO-**Ar**-COON =), 7.57 (1H, td, *J* = 1.2 and 7.8 Hz, CH_3OCO-**Ar**-COON=), 7.33 (1H, td,

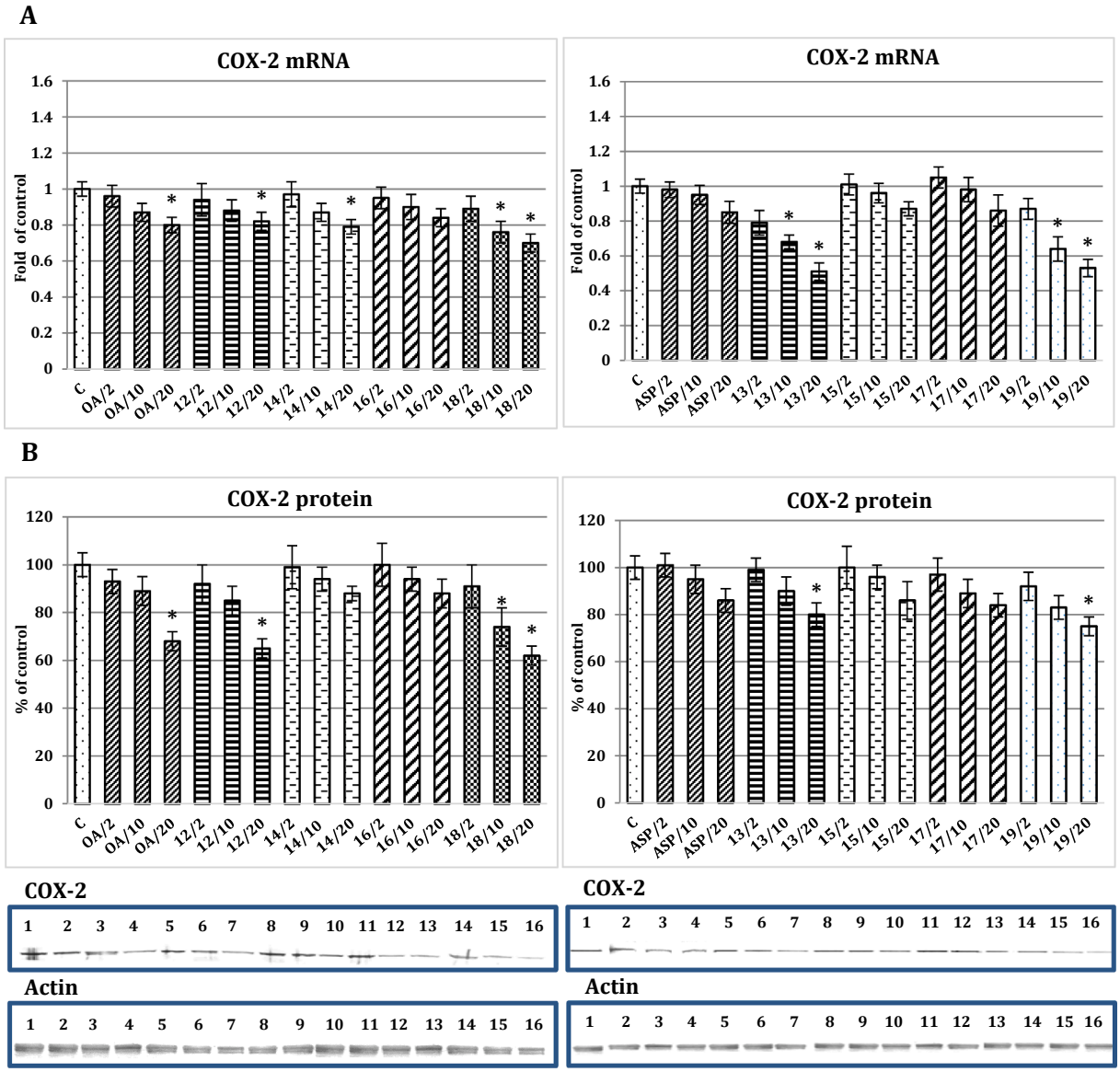


Fig. 6. The effect of the OA, OAO derivatives (**12**, **14**, **16** and **18**) and OAO conjugates with ASP (**13**, **15**, **17** and **19**) on COX-2 mRNA (Panel A) and protein (Panel B) level in HepG2 cells. Panel A - Data (means ± SEM) from three separate experiments. The values were calculated as mRNA level in comparison to control cells (expression equals 1). Panel B - Data (means ± SEM) of Western blot analysis of the cytosolic content of COX-2 from three separate experiments and representative immunoblots are shown. The sequence of the bands corresponds to the sequence of bars in the graph. Actin content was used for the normalization of results. The values were calculated as the level of protein in comparison with control cells (expression equals 100%). The asterisk (*) above the bar denotes statistically significant differences from the control group, $p < 0.05$.

J = 1.2 and 7.5 Hz, CH_3OCO-**Ar**-COON =) and 7.14 (1H, dd, J = 1.1 and 8.1 Hz, CH_3OCO-**Ar**-COON=), 5.30 (1H, t, J = 3.4 Hz, C_{12}-H); 2.84 (1H, dd, J = 3.5 and 13.7 Hz, C_{18}-H_β), 2.33 (3H, s, **CH_3**OCO-Ar-COON=), 1.14, 1.12, 1.10, 1.09, 0.90, 0.86 and 0.77 (7 × 3H, 7 × s, 7 CH_3 groups); Ar = aromatic ring. ^{13}C NMR (100 MHz, $CDCl_3$): 183.5 (C_q, –COOH); 176.2 (C_q, C-3); 170.0 (C_q, CH_3**OCO**-Ar-COON=); 162.0 (C_q, CH_3OCO-Ar-**CO**ON =); 150.0 (C_q, CH_3OCO-**Ar**-COON=), 133.7, 131.3, 125.9, 124.0 (4 × CH, CH_3OCO-**Ar**-COON=) and 122.8 (C_q, CH_3OCO-**Ar**-COON=); 146.8 (C_q, C-13); 122.1 (CH, C-12); 46.5 (C_q, C-17); 21.0 (CH_3, **CH_3**OCO-Ar-COON=); Ar = aromatic ring. MS/EI (*m/z*): 631.31 (15.9%, $M^{\bullet+}$). Elemental analysis calculated: C 74.14%, H 8.45% and N 2.22%, found: C 74.15%, H 8.44% and N 2.21%.

Conjugate of oxime of oleanonic acid morpholide and aspirin (19). Physical and spectral data agree with those available in the literature [9].

4.2. Biological assays

4.2.1. Cell culture and viability assay

HepG2 (ATCC HB 8065) and THLE-2 (ATCC CRL-2706) cells were provided by American Type Culture Collection (ATCC, USA). HepG2 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, USA) containing 10% fetal bovine serum (EURx, Poland) and 1% antibiotics solution (Sigma-Aldrich, USA) while THLE-2 were cultured in BEGM medium supplemented with Bullet Kit (Lonza, Germany) and additionally 10% fetal bovine serum, 5 ng/mL epidermal growth factor (EGF), 70 ng/mL phosphoethanolamine at 37 °C, in a humidified atmosphere with 5% CO_2. To assess the effect of OA, OAO derivatives and ASP with its conjugates on measured parameters, 1×10^6 cells were seeded per 100 mm culture dish and after 24 h of initial incubation the cells were treated with 2 μM, 10 μM and 20 μM of OA, its oximes (**12**, **14**, **16** and **18**), ASP and its conjugates (**13**, **15**, **17**

Table 2
Primers used in RT-PCR.

Gene	Forward primer	Reverse primer
NF-κB p50	5′ATCATCCACCTTCATTCTCAA	5′AATCCTCCACCACATCTTCC
NF-κB p65	5′CGCCTGTCCTTTCTCATC	5′ACCTCAATGTCCTCTTTCTG
COX-2	5′CCTGTGCCTGATGATTGC	5′CAGCCCGTTGGTGAAAGC
PBGD	5′TCAGATAGCATACAAGAGACC	5′TGGAATGTTACGAGCAGTG
TBP	5′GGCACCACTCCACTGTATC	5′GGGATTATATTCGGCGTTTCG

and **19)** and 0.1% dimethyl sulfoxide (DMSO) as the control solution. Incubation lasted for 24 h and the cells were harvested.

The effect of OA and its tested derivatives on cell viability was assessed by MTT assay, following the standard protocol. Briefly, THLE-2 and HepG2 cells were seeded (10^4 per well) in 96-well plates. After 24 h of pre-incubation in the complete medium, compounds were added in various concentrations and cells were incubated for the next 24 h. Later, cells were washed twice with phosphate buffered saline (PBS) and further incubated for 4 h with medium containing 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT). Then, the formazan crystals were dissolved in acidic isopropanol and the absorbance was measured at 570 nm and 690 nm. All experiments were repeated three times. In the following experiments, we used non-toxic concentrations of compounds (with viability level of above 70%): 2, 10, and 20 μM of oleanolic acid (**OA**), its derivatives (**12**, **14**, **16** and **18**), aspirin (ASP) and conjugates of oximes with ASP (**13**, **15**, **17** and **19**).

4.2.2. Nuclear, cytosolic and total protein lysates preparation

The subcellular extracts were prepared using the Nuclear/Cytosol Fractionation Kit (BioVision Research, CA, USA). The concentration of proteins was assessed by the Lowry method and then samples were stored at –80 °C for further analysis.

4.2.3. Total RNA isolation and cDNA synthesis

Extraction of total RNA was performed using GeneMatrix Universal DNA/RNA/Protein Purification Kit (EURx, Poland) and samples were subsequently subjected to reverse transcription by RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, USA) according to manufacturer's instructions. All experiments were repeated three times.

4.2.4. Quantitative real-time PCR

For real-time PCR analyses, the Maxima SYBR Green Kit (Fermentas, USA) and the BioRad Chromo4 thermal cycler were used. Protocol started with 5 min enzyme activation at 95 °C, followed by 40 cycles of 95 °C for 15 s, 56 °C for 20 s and 72 °C for 40 s and elongation at 72 °C for 5 min. Melting curve analysis was used to verify amplicons. The expression of *TBP* (TATA box binding protein) and *PBGD* (porphobilinogen deaminase) was used to normalize data. Primers used in PCR reactions were obtained from the Institute of Biochemistry and Biophysics PAS (Warsaw, Poland). Table 2 lists the sequences of primers used in the analysis of *NF-κB (p65* and *p50 subunits), COX-2, TBP* and *PBGD*.

4.2.5. Western blot analysis

Cytosolic extracts (for IKK, COX-2 and actin), nuclear extracts (for NF-κB p50, NF-κB p65 and lamin), (50–100 μg) were separated on 12% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) slab gels and proteins were transferred to nitrocellulose membranes. After blocking for 2 h with 10% skimmed milk, proteins were probed with rabbit polyclonal anti-IKK, rabbit polyclonal anti-NF-κB p50, rabbit polyclonal anti-NF-κB p65, rabbit polyclonal anti-COX-2, rabbit polyclonal anti-actin, rabbit polyclonal anti-lamin antibodies (Santa Cruz Biotechnology, USA). Actin and lamin expression served as loading controls. Alkaline phosphatase-labeled anti-rabbit IgG was used as the secondary antibody for the staining reaction. Bands were visualized with BioRad AP Conjugate Substrate Kit NBT/BCIP. The amount of immunoreactive product in each lane was determined using Quantity One software (BioRad Laboratories, USA). Values were calculated as relative absorbance units (RQ) per mg protein. All the experiments were repeated three times.

4.2.6. NF-κB binding assay

NF-κB p50 and NF-κB p65 activation was assessed by enzymatic immunoassay (Transcription Factor ELISA Assay Kit Active Motif, Belgium) according to manufacturer's instructions. Activated NF-κB was measured as the amount of p65 and p50 subunits contained in DNA-binding complex. The oligonucleotides containing (5′-GGGACTT TCC-3′) a consensus site for NF-κB were immobilized in/on microplates as bait. Nuclear fractions were incubated with oligonucleotides for 1 h, then wells were washed and DNA-bound subunits were detected by the specific primary antibody and secondary antibody conjugated with the horseradish peroxidase (HRP). The results were expressed as the normalized level of absorbance (OD450 nm per mg of protein).

4.2.7. Statistical analysis

Statistical analysis was performed using the GraphPad Instat. The statistical significance between the experimental groups and their respective controls was assessed by the Student-Newman-Keuls-test.

Acknowledgements

This study was supported by the by Polish National Science Centre, grant no 2016/21/B/NZ7/01758.

Declaration of Competing Interest

The authors declare no conflicts of interest

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