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CYTOTOXIC ACTIVITY OF A-MODIFIED LICORICE TRITERPENOID DERIVATIVES

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The cytotoxic activity of a number of A-modified derivatives of glycyrrhetic acid (GLA) and 11-deoxo- and 18,19-dehydro-GLA against conditionally normal HEK 293 cells, A549 human lung carcinoma, MCF-7 breast adenocarcinoma, and SH-SY5Y neuroblastoma cells was studied. The most active compound was 18,19-dehydro-GLA methyl ester with a pyrazole fragment, which exhibited a pronounced cytotoxic effect against A549, MCF-7, and SH-SY5Y cancer cell lines (IC_{50} 22.57 – 38.11 μM).

Keywords: triterpenoids, glycyrrhetic acid, cytotoxicity.

The search for new antitumor agents is currently an urgent and high-priority task in chemistry and medicine because oncological diseases are second only to cardiovascular diseases among the global causes of mortality. The use of natural compounds of plant origin (secondary metabolites) as platforms is a rapidly developing direction in this area of pharmaceutical chemistry [1]. Secondary metabolites of higher plants belonging to the pentacyclic triterpenoid class are widely distributed in nature. Most of them possess cytotoxic activity against various types of cancer cells and antitumor activity in preclinical *in vivo* models [2, 3].

Glycyrrhetic acid (GLA) (**I**, Fig. 1), the aglycon of glycyrrhizic acid, which is the major triterpene saponin from licorice roots (*Glycyrrhiza glabra* L. and *G. uralensis* Fisher), is a leading representative of pentacyclic triterpenoids with a broad spectrum of pharmacological activity (anti-inflammatory, antiulcer, antiallergic, hepatoprotective, antitumor, antimicrobial, antiviral, etc.) [4, 5]. Synthetic transformations of GLA and related triterpenoids are a promising route for preparing new antitumor agents [6]. During the last decade, >400 GLA derivatives have been synthesized. Among them, >130 compounds with potent cytotoxic activity and IC_{50} values <30 μM have been found. Several derivatives showed high cytotoxic activity (IC_{50} < 1 μM) [5 – 7]. Structural modifications of GLA led to the discovery of lead molecules such as 2-cyano-3,11-dioxo-18β-olean-1,12-dien-30-oic acid (β-CDODA) and its methyl ester (β-CDODA-Me) with high antitumor activity against pancreas, prostate, and colon carcinoma cancer cells [2, 8]. Introduction of a 3-alkoxyimino group into GLA led to a significant increase of antiproliferative and apoptosis-inducing activity of GLA derivatives against HL-60 human leukemia cells [9]. 2-Substituted GLA derivatives containing a 1-en-3-one structure in ring A were active growth inhibitors of bladder and pancreas cancer cells [8]. The mechanism of the cytotoxic activity of GLA and its derivatives is related to the ability to induce apoptosis of tumor cells and to activate production of caspases and cytochrome c [2, 5, 10].

The present work was dedicated to a search for new cytotoxic compounds among GLA derivatives and its analogs. Derivatives of licorice triterpenoids (Fig. 1), i.e., semi- and thiosemicarbazones of GLA (**II**-**IV**), 11-deoxo-GLA (**V** and **VI**), and 18,19-dehydro-GLA (**VII**), which were obtained from the corresponding 3-ketones as before [11, 12], were used in the studies. Pyrazole 18,19-dehydro-GLA (**VIII**) was prepared by reacting the 2-hydroxymethylene-3-one of 18,19-dehydro-GLA methyl ester with hydrazine [13]. Compound **X** with a 1,2-ene fragment in ring A was prepared for the first time.

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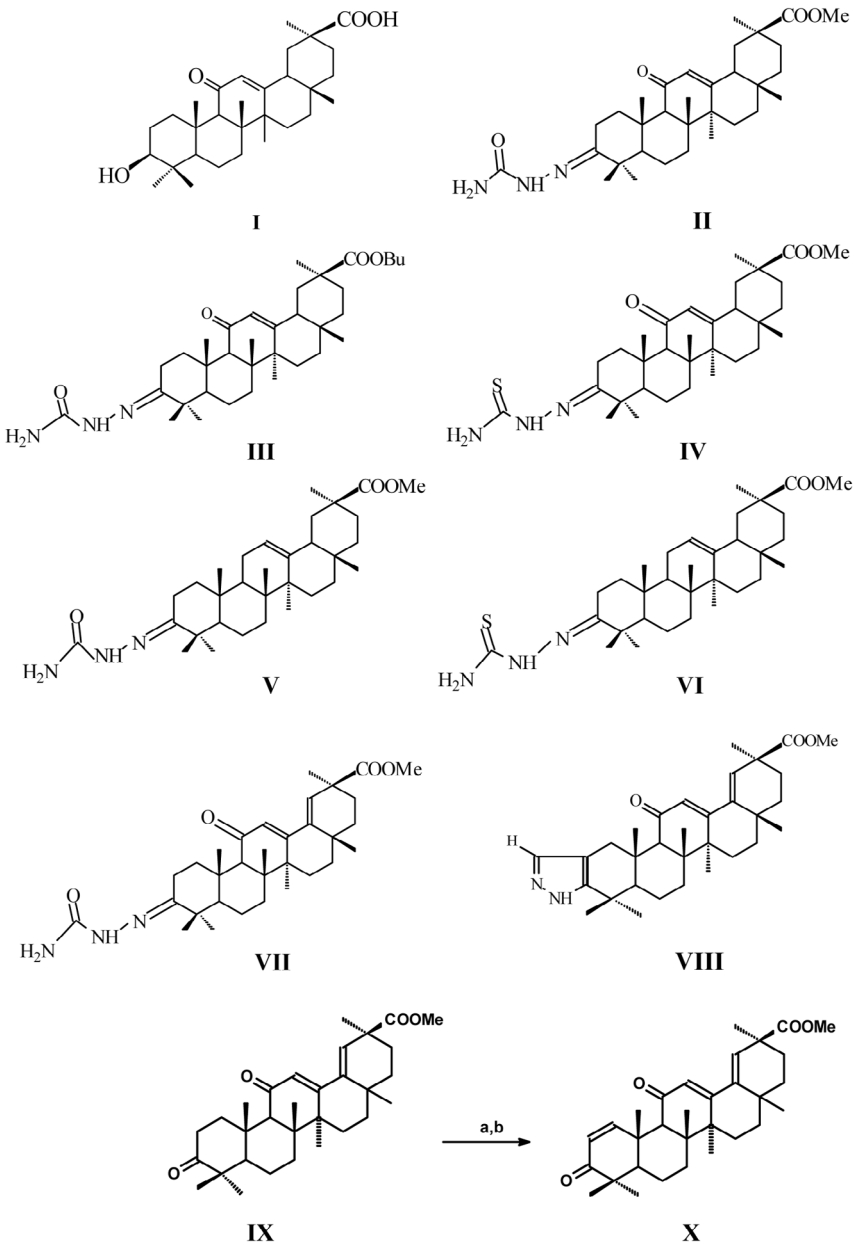

Fig. 1. Glycyrrhetic acid (**I**) and its derivatives **II**-**VIII**; conditions and reagents for preparing **X**: *a*) PhSeCl, EtOAc, 20 – 22°C, 3 h; *b*) *m*-CPBA, Py, EtOAc, 1 h, 20 – 22°C.

EXPERIMENTAL CHEMICAL PART

PMR and ^{13}C NMR spectra were recorded in $CDCl_3$ with TMS internal standard on a Bruker AM 300 spectrometer at operating frequency 300 (^1H) and 75.5 MHz (^{13}C) and a Bruker Avance-III 500 MHz spectrometer at operating frequency 500.13 (^1H) and 125.47 MHz (^{13}C). Optical activity was measured on a Perkin-Elmer 341 polarimeter in a 1-dm tube at 20 – 22°C (λ_{Na} 546 nm). Melting points were determined on a Boetius apparatus.

Molecular ions were determined by liquid-chromatography(mass-spectrometry (LC-MS) on a Shimadzu LCMS-2010 chromatograph(mass-spectrometer in chemical ionization at atmospheric pressure (APCI) mode as solutions in MeCN.

Column chromatography used 50/150 μm silica gel (Sorbpolimer). TLC used Sorbfil plates (Sorbpolimer). Spots of compounds were detected by H_2SO_4 solution (5%) in EtOH followed by heating at 210 – 220°C for 2 – 3 min.

HPLC analysis used a Shimadzu LC-20 liquid chromatograph (Japan) and a Discovery C18 reversed-phase column (250 × 4.6 mm; SUPELCO, USA). The mobile phase was MeCN:H_2O (85:15 vol%) at flow rate 1 mL/min.

A spectrophotometric diode-array detector at λ = 254 nm was used. The purities of the compounds were at least (94 – 96) ± 0.8%. Figure 2 shows an HPLC chromatogram of **VIII** as an example.

GLA (**I**) was obtained via hydrolysis of glycyrrhizic acid by H_2SO_4 (5%) [4]. Mp 292 – 294°C, $[\alpha]_D^{20}$ +166° (s 0,04, $CHCl_3$). Lit. [4]: mp 290 – 292°C, $[\alpha]_D^{20}$ +165°. 11-Deoxo-GLA was produced by reduction of GLA with zinc in HCl solution [4]. Drug substance 18,19-dehydro-GLA [4] was used in the work. 3-Semi- and thiosemicarbazones of methyl esters **II-VII** were prepared from the corresponding triterpenoid 3-oxo-derivatives [4] by refluxing with semicarbazide and thiosemicarbazide in the presence of NaOAc as before [11].

11-Oxo-18βH-olean-12-en-30-oic acid 3-semicarbazone methyl ester (II): mp 293 – 295°C, $[\alpha]_D^{20}$ +166° (c 0.06, CH_2Cl_2); lit. [11]: 295 – 297°C, $[\alpha]_D^{20}$ +65° (c 0.06, CH_2Cl_2).

18βH-Olean-12-en-30-oic acid 3-semicarbazone butyl ester (III): mp 225 – 227°C, $[\alpha]_D^{20}$ +153° (s 0.04, $CHCl_3$); lit. [11]: mp 227 – 230°C, $[\alpha]_D^{20}$ +155° (s 0.06, CH_2Cl_2).

11-Oxo-18βH-olean-12-en-30-oic acid 3-thiosemicarbazone methyl ester (IV): mp 193 – 195°C, $[\alpha]_D^{20}$ +152° (s 0.05, CH_2Cl_2); lit. [11]: mp 192 – 194°C, $[\alpha]_D^{20}$ +155° (s 0.07, CH_2Cl_2).

18βH-Olean-12-en-30-oic acid 3-semicarbazone methyl ester (V): mp 246 – 248°C, $[\alpha]_D^{20}$ +147° (s 0.04, CH_2Cl_3). lit. [11]: 248 – 250°C, $[\alpha]_D^{20}$ +145° (s 0.04, $CHCl_3$).

18βH-Olean-12-en-30-oic acid 3-thiosemicarbazone methyl ester (VI): mp 157 – 159°C, $[\alpha]_D^{20}$ +140° (s 0.06, CH_2Cl_2); lit. [11]: mp 155 – 157°C, $[\alpha]_D^{20}$ +136° (s 0.06, CH_2Cl_2).

11-Oxo-18βH-olean-12(13),18(19)-dien-30-oic acid 3-semicarbazone methyl ester (VII): $[\alpha]_D^{20}$ +185° (c 0.08, $CHCl_3$); lit. [11]: $[\alpha]_D^{20}$ +190° (c 0.12, $CHCl_3$).

11-Oxo-[3, 2*b*]-pyrazolo-18βH-olean-12(13),18(19)-en-30-oic acid methyl ester (VIII) was obtained by the literature method [13]. $[\alpha]_D^{20}$ +365° (c 0.04, $CHCl_3$). LC-MS, *m*/*z*: 505.4 $[M + H]^+$. $C_{32}H_{44}N_2O_3$. M = 504.7.

PMR and ^{13}C NMR spectra of GLA derivatives **II-VIII** agreed with those published earlier [11 – 13].

3,11-Dioxo-18βH-olean-1(2),12(13),18(19)-trien-30-oic acid methyl ester (X). A solution of 3-oxo-triterpenoid **IX** (2.1 mmol) and phenylselenyl chloride (2.5 mmol) in EtOAc (50 mL) was stirred at room temperature (20 – 22°C) for 3 h, treated with saturated aqueous Na_2CO_3 solution, and stirred for 30 min. The organic layer was separated in a separatory funnel. The EtOAc solution was treated with Py (2 mL) and *m*-chloroperbenzoic acid (2.5 mmol). The mixture was stirred at room temperature for 1 h, washed with Na_2CO_3 solution (5%) and saturated NaCl solution, dried over $MgSO_4$, and evaporated. The obtained product was chromatographed over a column of silica gel with elution by C_6H_6(EtOAc (10:1 (5:1, v/v) and TLC monitoring. Yield 58% (toluene(EtOAc, 5:1). $[\alpha]_D^{20}$ + 130° (s 0.08, $CHCl_3$). PMR spectrum (500 MHz, $CDCl_3$, δ, ppm): 7.61 (1H, d, J + 10.2 Hz, H-1), 5.86 (1H, s, H-19), 5.82 (1H, c, H-12), 5.77 (1H, d, J = 10.2 Hz, H-2), 3.66 (3H, s, OCH_3), 2.58 (1H, s, H-9), 2.15 – 2.10 (1H, m, H-5), 2.10 – 2.00 (2H, m, H-6_e, H-22_e), 1.68 – 1.40 (10H, m, H-6_a, 2H-7, 2H-15, 2H-16, 2H-21, H-22_a), 1.47 (3H, s, CH_3-29), 1.28 (3H, s, CH_3-25), 1.21 (3H, s, CH_3-27), 1.19 (3H, s, CH_3-26), 1.13 (3H, s, CH_3-24), 1.10 (3H, s, CH_3-28), 0.96 (3H, s, CH_3-23). ^{13}C NMR spectrum (125 MHz, $CDCl_3$, δ, ppm): 204.17 (C-3), 198.64 (C-11), 176.34 (C-30), 163.75 (C-13), 161.23 (C-1), 142.37 (C-18), 129.95 (C-19), 124.29 (C-2), 123.48 (C-12), 54.53 (C-9), 52.80 (C-5), 51.92 (C-31), 45.24 (C-4), 44.42 (C-8), 44.18 (C-14), 43.41 (C-20), 38.45 (C-10), 35.77 (C-16), 34.66 (C-22), 34.51 (C-17), 32.39 (C-7), 27.66 (C-21), 27.38 (C-23), 25.72 (C-15), 24.76 (C-29), 24.15 (C-28), 21.32 (C-27), 20.28 (C-6), 19.47 (C-24), 18.41 (C-25), 18.04 (C-26). LC-MS, $[M + H]^+$ 479.4. $C_{31}H_{42}O_4$. M = 478.6.

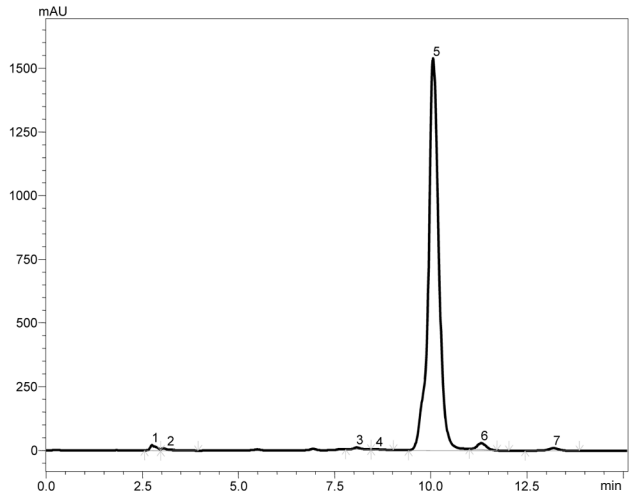

Fig. 2. HPLC chromatogram of **VIII**. Discovery C_{18} column; mobile phase $CH_3CN(H_2O$, 85:15 (vol%); flow rate 1 mL/min; UV detection at λ = 254 nm; retention time (τ) 10.05 min; purity 96.1 ± 0.8%.

CYTOTOXICITY STUDY OF THE COMPOUNDS

The cytotoxic properties of the compounds were determined *in vitro* using the MTT assay [14] in 96-well plates. Cell lines HEK293, SH-SY5Y, A549, and MCF-7 were cultivated for 24 h in DMEM medium (Biolot, Russia) in the presence of 10% fetal bovine serum (Invitrogen, USA), *L*-glutamine (2 mM), and gentamicin sulfate (50 μg/mL). Then, each well was treated with a compound being tested at final concentrations of 1, 10, and 100 μM (in 0.1% DMSO),

incubated for 48 h, and treated with MTT reagent. The optical density at 540 nm was determined by subtracting the background absorption measured at 600 nm using a plate analyzer (2300 EnSpire® Multimode Plate Reader, Perkin-Elmer, USA). The concentration of the compounds causing 50% suppression of cell viability (IC_{50}) was determined from concentration-dependence curves using GraphPad Prism v.5.02 software (GraphPad Software Inc., USA).

Statistical analysis used standard Statistica 6.1 software (StatSoft Inc.). Data were given as means of three measurements for each concentration ± the standard errors of the mean relative to the control (0.1% DMSO) taken as 100%. The significance of differences was found using the Student *t*-criterion for independent sets. Differences were considered statistically significant for $p < 0.05$.

RESULTS AND DISCUSSION

Derivative **X** containing a 1,2-en-3-one fragment in ring A was synthesized in 58% yield via the reaction of 3-ketone **IX** [15] with phenylselenyl chloride (PhSeCl) and *m*-perchlorobenzoic acid (*m*-CPBA) (Fig. 1). The structure of **X** was confirmed by PMR and ^{13}C NMR spectra. Thus, the PMR spectrum of **X** exhibited resonances for C-1 (d 7.61 ppm) and C-2 olefinic protons (d 5.77 ppm) with spin(spin coupling constant $J = 10.2$ ppm. The ^{13}C NMR spectrum showed C-1 and C-2 with chemical shifts of 161.23 and 124.29 ppm, respectively. The 3-C=O resonance (d 204.17 ppm) was shifted to stronger field by 13 ppm as compared to the spectrum of starting 3-ketone **IX** (d 217.2 ppm) [15].

The cytotoxicity of GLA derivatives **II**-**VIII** and **X** was studied against HEK293 human embryonic kidney cells (conditionally normal cells), A549 human lung carcinoma cells, and SH-SY5Y neuroblastoma cells. The reference drug was the native triterpenoid of licorice root GLA (**I**). Table 1 presents the test results.

The cytotoxic properties of the compounds at final concentrations 1, 10, and 100 μM (in 0.1% DMSO) were determined *in vitro* using the MTT assay [14]. The concentration of a compound for which 50% death of cells (IC_{50}) was observed was calculated from dose-dependent curves using GraphPad Prism v.5.02 software (GraphPad Software Inc., USA).

TABLE 1 shows that GLA did not exhibit cytotoxic activity (IC_{50} >100 μM) against the studied cell lines. GLA derivatives **II**-**VIII** and **X** showed various cytotoxicities against normal and cancer cell lines HEK293, SH-SY5Y, A549, and MCF-7. For example, compounds **II**-**IV**, which had C-3 semi- and thiosemicarbazone fragments, were cytotoxic against HEK293 conditionally normal cells. The IC_{50} values for **II**-**IV** were 57.95, 28.58, and 7.6 μM, respectively (Table 1). However, deaths of neuroblastoma, lung carcinoma, and breast adenocarcinoma cells were not observed at these concentrations, like for 11-deoxo-GLA derivatives **V** and **VI** and 18,19-dehydro-GLA (**VII**). New derivative 18,19-dehydro-GLA (**X**) with a 1,2-en-3-one fragment in ring A also exhibited cytotoxic activity against HEK293 cells (IC_{50} 25.52 μM) but was inactive against the tumor cell lines. Pyrazole **VIII** turned out to be the most active of the studied licorice triterpenoid derivatives and showed pronounced cytotoxic activity against A549, MCF-7, and SH-SY5Y cells (IC_{50} 22-57-38.11 μM).

Thus, chemical modification of ring A of GLA by introducing N-containing heterocycles was a promising route to producing new cytotoxic compounds against tumor cells.

TABLE 1. Effect of GLA Derivatives on Metabolic Activity of Cells (PrestoBlue™, Invitrogen) (48-h Incubation of Cells with Compounds)

No.	Solubility (100% DMSO)	IC_{50}, μM			
		HEK293	A549	MCF-7	SH-SY5Y
I	+	> 100	> 100	> 100	> 100
II	+	57.95 ± 6	> 100	> 100	64.21 ± 3.04* ($p = 0.05$)
III	+	28.58 ± 1.5* ($p = 0.03$)	n/d	71.92 ± 3.8* ($p = 0.02$)	51.99 ± 5.4* ($p = 0.04$)
IV	+	7.6 ± 0.5* ($p = 0.01$)	> 100	> 100	n/d
V	+	> 100	86.62 ± 7.8* ($p = 0.01$)	50.56 ± 4.02	> 100
VI	+	n/d	> 100	> 100	51.34 ± 5.2* ($p = 0.01$)
VII	+	86.08 ± 4.1	> 100	> 100	> 100
VIII	+	51.47 ± 6.5* ($p = 0.04$)	22.57 ± 4.9* ($p = 0.05$)	22.98 ± 4.7* ($p = 0.02$)	38.11 ± 2.8
X	+	25.52 ± 3.6* ($p = 0.05$)	> 100	> 100	> 100

n/d, not determined.

* Data given as means and standard errors of the mean ($M \pm s.e.m.$). Differences from control statistically significant ($p \le 0.05$).

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