Modification at A-Ring and Cytotoxic Activity of Betulonic Acid and its N-Methylpyperazinyl Amide

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Abstract

Since cancer remains one of the most prevalent diseases today, there is an urgent need for the development of new agents. Triterpenoids may act in multiple pathways displaying antiproliferative, antiangiogenic, anti-inflammatory, and pro-apoptotic activities that place them as promising multifunctional agents in treating cancer. In this paper a series of betulonic acid and its N-methylpyperazinyl amide derivatives, especially holding C2-nicotinoylidene/furfurylidene/fluorobenzylidene fragments, have been synthesized and evaluated for their cytotoxic activity against the NCI-60 cancer cell line panel. *N*-Methylpiperazinyl amides of betulinic acid **11** and 4-pyridinoylidene-betulinic acid **9** as well as betulonic acid C2-4-pyridinoylidene- **14** or furfurylidene **16** derivatives were found to be the leading compounds with GI_{50} values of 0.49 μM for leukemia CCRF-CEM, 1.60 μM and 1.36 μM for colon cancer HCT-116 and 1.66 μM for melanoma LOX IMVI cell lines, respectively. The activity displayed for these compounds was higher than for the standard drug doxorubicin against colon cancer HCT-15 and ovarian cancer NCI/ADR-RES cell lines. Cell cycle analysis indicates that compound **11** promotes cytotoxic activity through the apoptosis induction both in conditionally normal (HEK293) and in cancer (A549, MCF-7) cells, whereas compound **14** exhibits both cytostatic and cytotoxic activity, dependently on cell line evaluated. In particular, in HEK293 cells the compound **14** induces mainly apoptotic cell death, while in A549 and MCF-7 cells cytostatic effect is dependent on cell cycle arrest in G_2/M phase.Our results suggest that betulinic acid N-methylpyperazinyl amide **11** is the promising compound for the future drug development antitumor studies.

Introduction

Natural products are a rich source of bioactive compounds that can be employed in obtaining substances with great medicinal potential [1]. Attempts to successfully treat cancer are more necessary than ever, as 9.6 million people worldwide died in 2018, and globally about 1 in 6 deaths is due to cancer [2], there is an urgent need for the development of new agents. In cancer treatment, the influence of natural products is quite marked. With more than 50% of all new anticancer drugs (1981-2014), natural products and derivatives thereof still play a key role in treating cancer From the 1940s to the end of 2014 nearly 80% of the 246 anticancer worldwide approved drugs are related with natural sources [3]. Among the naturally occurring compounds, triterpenoids display a wide spectrum of biological activities, including anti-inflammatory, antiviral, anticancer, antimicrobial effects [4-7]. Pentacyclic triterpenoids of lupane, oleanane, ursane, friedelane and dammarane types (among others) have been reported in the literature as possible antitumor agents [8-11]. These substances may act in multiple pathways displaying antiproliferative, antiangiogenic, anti-inflammatory, and pro-apoptotic activities that place them as promising multifunctional agents in treating cancer [12].

Chemical modifications on ring A of triterpenoids may lead to derivatives with cytotoxic activity [13-15]. Recently, raising attention is focused on the introduction of C2-benzylidene moiety into triterpene core which resulted in the derivatives with improved anti-inflammatory [16], anticancer [17-19] and antidiabetic [20] activities. Among them, 2-(4-nitrobenzylidene)-betulinic acid was active against five different human cancer cell lines with IC_{50} range from 1.36 to 3.5 μM [21].

Meanwhile, piperazines are typical saturated nitrogen heterocyclic compounds that are often introduced into drug molecules as synergistic groups, thus beneficial to improve the pharmacokinetics properties, regulate acid-base balance parameters, and lipid-water partition coefficient of drugs [22]. In addition, piperazine moiety can also improve the biological activity of drug molecules by forming hydrogen bonds or ionic bonds with the target protein. The introduction of piperazine fragment into C2, C3 and C28 positions of triterpene core led to high inhibitory activity against different human cancer cell lines [23-26]. Betulonic acid *N*-methyl- and *N*-ethyl-piperazinylamides showed high activity against tumor CEM-13, U-937 and MT-4 cells [27]. Betulonic acid piperazinyl amides also possess antimicrobial activity [28-30], while analogous derivatives of ursolic acid demonstrated antiplasmodial activity [31]. Ursolic acid *N*-methyl-piperazinyl amide showed a selectivity factor of 2.7 for A375 melanoma cells/non-malignant fibroblasts NIH 3T3 [32]. Betulinic acid propylpiperazine and showed stronger inhibitory effects against the four tumor cell lines at a micromolar range [33]. Oleanolic acid rhodamine B-conjugate elicited antiangiogenic effect, without showing irritation effect on the membrane, its ability to inhibit cellular respiration, and therefore can be classified as “mitocan” [34]. Oleanolic acid piperazinylamide spacered with meta substituted carboxylated malachite green was cytotoxic for MCF-7 human breast carcinoma cells (EC_{50} = 0.7 μM) [35]. The safirinium P fluorophores of triterpenoid with piperazinyl-spacered showed moderate cytotoxicity [36]. Betulonic acid *N*-piperazinyl amide displayed mild sensitivity against cell line Leukemia SR (8.04%) [37].

Betulonic acid is easily available from betulin [38] and was used in the synthesis of derivatives with potent cytotoxic activity in different cancer cell lines [39, 40]. In some cases, betulonic acid derivatives displayed higher activity as compared for analogs derived from betulinic acid [41].

Taking all the facts into account, in this study we have focused on the synthesis of C2-nicotinoylidene/furfurylidene/fluorobenzylidene derivatives of betulonic acid and its *N*-methylpyperazinyl amide and evaluation of their cytotoxic activity against the NCI-60 cancer cell line panel and cell cycle analysis for the leading compounds.

Results And Discussion

Chemistry

At first, we have modified betulonic acid and its *N*-methylpiperazinamide **1** [28] by the Claisen–Schmidt reaction with a corresponding aldehyde (4-, 3- or 2-pyridinecarboxaldehyde, 4-trifluoro- or 3,5-difluoro-phenylcarboxaldehyde, or furfural) to afford compounds **2-7** or **14-16** with good yields (Schemes 1, 2). In the ^{1}H NMR spectra of compounds **2-4** and **9**, **10** characteristic signals of the pyridine cycle protons resonated in the region δ 7.09-8.62 ppm, which were observed in the ^{13}C NMR spectra at a weak field in the range from δ 122.2 to 149.7 ppm. The hydrogen atom signals of the furfurylidene fragment in compound **5** and **16** arranged at d 6.49-7.54 ppm; the signals of aromatic protons in compounds **6** and **7** appear at δ 6.42-7.88 ppm in the ^{1}H NMR spectra, and in the ^{13}C NMR spectra they are found in the region δ 121.5-139.5 ppm.

The betulinic acid derivative **9** was obtained by $NaBH_4$ reduction in methanol, while oxo-group of amide **2** was converted to oxime **10** under the treatment of $NH_2OH{\cdot}HCl$ (Scheme 1). Interaction of amide **1** with

ethylformate in the presence of CH_3ONa in benzene led to C2-hydroxymethylene-derivative **8** in yield of 76%. The reaction of compound **11** with diethyl chlorophosphate in pyridine in the presence of 4-dimethylaminopyridine (DMAP) led to diethoxyphosphate **12**. In the ^1H NMR spectra of compound **12** proton signals of two CH_3CH_2O-groups were contained at δ 1.28-1.42 ppm and δ 4.01-4.14 ppm, while four signals of the carbon CH_3 and CH_2 groups appeared at δ 16.1, 16.3 and 61.5, 63.4 ppm in the ^{13}C NMR spectra. Betulinic **11** [28] and azepanobetulinic **13** [43] *N*-methylpiperazinyl amides were also taken into the biological screening.

Biological activity

Evaluation of cytotoxicity activity against NCI-60 cell line panel

Compounds **1-16** were selected by National Cancer Institute and tested at one dose assay (10^{-5} M) towards a panel of approximately sixty cancer cell lines according to the NCI protocol as described elsewhere (see e.g. http://dtp.nci.nih.gov) [43-47].

Compounds **6-8** did not show cytotoxic activity against the studied cell lines (Table 1).. Compound **1** inhibited the cell growth of the leukemia SR, NSC lung cancer NCI-H460, and colon cancer HCT-116 cell lines. Amides with pyridinoylidene fragments **2** and **3** were active against colon cancer HCT-116 and HT29, prostate cancer PC-3, breast cancer MCF-7 cell lines, and all six of leukemia cell lines. Compound **4** inhibited two leukemia MOLT-4 and SR cell lines, whereas furfurylidene derivative **5** showed activity towards leukemia MOLT-4 and SR, colon cancer HT29, CNS cancer SF-295 cell lines and melanoma line LOX IMVI. The oxime **10** and azepanobetulinic acid *N*-methylpiperazinyl amide **13** inhibited only one leukemia cell line SR, as well as compound **15** was active towards one colon cancer cell line HCC-2998. Compound **12** inhibited two leukemia K-562 and SR cell lines. Compounds **11** and **14** demonstrated a broad spectrum of the cell proliferation inhibition against 38 and 54 human tumor cell lines (the growth percent ranging from -98.30 to 128.95 for compound **11** and from -9.50 to 78.00 for compound **14**, respectively). *N*-Methylpiperazinyl amides **9** and **16** showed the greatest antiproliferative effect towards all 60 cell lines, resulting in 50 cases of cancer cell lethality from -0.44 to -90.75% for compound **9** and in 56 cases of cancer cell lethality from -1.95 to -97.61% for compound **16**, respectively (Table 1 and Supp. Material).

Compounds **9**, **11**, **14** and **16** were subjected to five-dose response study and their GI_{50} (growth inhibitory activity) and LC_{50} (cytotoxic activity) values are given in Table 2 and Supp. Material. Compound **9** showed GI_{50} values ranging from 1.60 (colon cancer HCT-116 cell line) to 51.40 μM (CNS Cancer SNB-75 cell line), and LC_{50} - from 31.10 (melanoma MALME-3M) to 97.30 μM (renal cancer A498 cell line). LC_{50} of compound **9** for leukemia subpanel with the exception of HL-60(TB), non-small cell lung cancer subpanel with the exception of HOP-62, NCI-H460 and NCI-H522, COLO 205 and HT29 (colon cancer), SF-268, SNB-19 and SNB-75 (CNS cancer), MDA-MB-435 and UACC-257 (melanoma), IGROV1, OVCAR-4, OVCAR-8 and SK-OV-3 (ovarian cancer), ACHN and TK-10 (renal cancer), PC-3 (prostate cancer) cell lines and breast cancer subpanel with the exception of MCF-7 and MDA-MB-231/ATCC exceeded 100 μM (Supporting Material).

Compound **14** showed GI_{50} values ranging from 1.36 (colon cancer HCT-116 cell line) to 19.40 μM (breast cancer HS 578T cell line), and LC_{50} - from 5.76 (colon cancer HCT-116 cell line) to 86.10 μM (melanoma MDA-MB-435). LC_{50} of compound **14** for leukemia subpanel, EKVX, HOP-62, NCI-H226, NCI-H23 and NCI-H460 (lung cancer), HT29 and SW-620 (colon cancer), SF-268, SF-295 and SF-539 (CNS cancer), MALME-3M (melanoma), IGROV1, OVCAR-4, OVCAR-8, NCI/ADR-RES and SK-OV-3 (ovarian cancer), ACHN, SN12C and TK-10 (renal cancer) cell lines, and prostate cancer subpanel, MCF7, MDA-MB-231/ATCC, HS-578T and T-47D (breast cancer) cell lines exceeded 100 μM.

Table 1. Anticancer screening data at concentration 10 μM

Compound (NSC number)	60 cell lines assay in 1 dose 10 μM concentration					
	Mean growth, %	Range of growth, %	Most sensitive cell lines	Growth % of the most sensitive cell lines	Positive cytostatic effect[a]	Positive cytotoxic effect[b]
1 (797805)	77.51	-12.06 – 103.78	SR (Leukemia)	-12.06	3/58	1/58
2 (797796)	53.95	-21.68 – 89.41	HL-60(TB) (Leukemia)	-21.68	22/59	1/59
3 (797793)	61.75	-4.95 – 106.62	HL-60(TB) (Leukemia)	-4.95	14/59	1/59
4 (822843)	70.91	17.31 – 141.77	SR (Leukemia)	17.31	11/58	0/58
5 (794987)	63.48	-21.86 – 98.77	SR (Leukemia)	-21.86	12/59	1/59
6 (822842)	93.46	72.85 – 107.42	K-562 (Leukemia)	72.85	0/59	0/59
7 (822839)	100.00	81.74 – 111.38	CAKI-1 (Renal cancer)	81.74	0/58	0/58
8 (812002)	104.20	84.62 – 119.64	CAKI-1 (Renal cancer)	84.62	0/58	0/58
9 (804681)	-36.51	-90.75 – 22.04	786-0 (Renal cancer)	-90.75	10/59	48/58
			RXF 393 (Renal cancer)	-88.55		
			SK-MEL-5 (Melanoma)	-82.38		
			HCC-2998 (Colon cancer)	-80.73		
			KM-12 (Colon cancer)	-80.43		
10 (801873)	71.64	3.40 – 98.69	SR (Leukemia)	3.40	8/59	0/59
11	-51.39	-98.30	HCT-116	-98.30	2/58	51/59

(799587)		– 128.95	(Colon cancer)			
			786-0 (Renal cancer)	-95.12		
			OVCAR-3 (Ovarian cancer)	-94.01		
			TK-10 (Renal cancer)	-93.28		
			SNB-75 (CNS cancer)	-93.25		
			ACHN (Renal cancer)	-91.96		
12 (827685)	77.58	-26.57 – 108.48	SR (Leukemia)	-26.57	8/60	1/60
13 (797775)	91.54	24.87 – 121.92	SR (Leukemia)	24.87	2/59	0/59
14 (801872)	28.20	-9.50 – 78.00	RPMI-8226 (Leukemia)	-9.50	47/59	3/59
15 (801871)	63.26	31.97 – 97.48	HCT-116 (Colon cancer)	31.97	16/59	0/59
16 (804713)	-56.00	-98.18 – 31.86	RXF 393 (Renal cancer)	-98.18	4/58	55/58
			UACC-62 (Melanoma)	-97.61		
			KM12 (Colon cancer)	-96.29		
			UO-31 (Renal cancer)	-95.45		
			ACHN (Renal cancer)	-89.82		
			NCI-H322M (NSC lung cancer)	-87.92		
			HOP-92 (NSC lung cancer)	-86.40		

[a]Ratio between number of cell lines with percent growth from 0 to 50 and total number of cell lines.

[b]Ratio between number of cell lines with percent growth of<0 and total number of cell lines

Compound **11** showed GI_{50} values ranging from 0.487 (leukemia CCRF-CEM cell line) to 2.09 μM (colon cancer KM-12) with the exception of cancer lines with LC_{50} > 100 μM. Typical locate of LC_{50} tend to be in the short range of 5.61 to 9.94 μM with the exception of ovarian cancer OVCAR-8 cell line (39.40 μM), and leukemia cancer lines with LC_{50} > 100 μM.

Compound **16** showed GI_{50} values ranging from 1.66 (melanoma LOX IMVI cell line) to 100 μM (CNS cancer SNB-75 cell line) and LC_{50} – from 7.44 (ovarian cancer OVCAR-3) to 53.90 μM (colon cancer HCT-15 cell line). LC_{50} of compound **16** for all subpanel with the exception of cancer lines of colon cancer COLO 205 and HCT-15, ovarian cancer OVCAR-3 and breast cancer MCF-7 exceeded 100 μM (Table 2).

Thus, all compounds exhibited significant antiproliferative effect towards human cancer cell lines, and among them, the highest cytotoxic activity in 5-dose testing mode screening was observed for the betulinic acid *N*-methylpiperazinyl amide **11** with growth inhibitory (GI_{50}) against the most sensitive cell lines at submicromolar (0.487 μM) and micromolar concentrations (1-2 μM), respectively. Cytotoxic activity (LC_{50}) of this compound against the most sensitive cancer cell lines was also high (5-9 μM) (Table 2).

The anticancer activity results showed that the modification of the betulinic, betulonic and azepanbetulinic acid carboxyl groups with the introduction of N-methylpiperazine moiety at C28 position was effective only in the case of betulinic acid and led to compound **11** showing the greatest cytotoxic effect. Amides **1** and **13** were active towards 3 and 1 human tumor cell lines. The presence of fluorobenzylidene residues (compounds **6** and **7**) as well as hydroxymethylene (compounds **8**) at the C2 position of the betulonic acid amide leads to a complete loss of cytotoxic activity, while the presence of pyridinoylidene or furfurylidene fragments at C2 (compounds **2**, **3** and **5**) enhances the antitumor effect with the exception of compound **4** with a 2-pyridinoylidene substituent. The replacement of 3-oxo-group to hydroxyl as in compound **9** leads to the pronounced antiproliferative activity against the all NCI-60 cancer cell line panel, while the oxime group in compound **10** has the opposite effect. The interaction of the initial betulonic acid with aldehydes at C2 is also promising because resulted in the derivatives **14** and **16** with the 4-pyridinoylidene and furfurylidene moieties with high antitumor activity, although the 3-pyridinoylidene derivative **15** does not have activity towards the NCI-60 cancer cell line panel (Table 1).

A raw comparison of the activities of compounds **9**, **11**, **14** and **16** with respect to the activity reported for doxorubicin [48], reflects that the activity displayed for these compounds was lower than for the standard drug except colon cancer HCT-15 and ovarian cancer NCI/ADR-RES. Furthermore, at the LC_{50} level of cytotoxicity, compound **16** was more efficient against 3 cancer cell lines, compounds **9** and **14** – against 12 cell lines, compounds **11** – against 37 cancer cell lines, respectively (Table 2).

Table 2. *In vitro* cytotoxic effects of compounds 9, 11, 14, 16 and standard drug doxorubicin against NCI's human tumor cell line screen

Panel/ Cell Line	Compounds								Standard Drug	
	9		11		14		16		Doxorubicin NSC 123127[c]	
	GI_{50}[a] (μM)	LC_{50}[b] (μM)	GI_{50} (μM)	LC_{50} (μM)	GI_{50} (μM)	LC_{50} (μM)	GI_{50} (μM)	LC_{50} (μM)	GI_{50} (μM)	LC_{50} (μM)
Colon Cancer										
HCT-15	2.65	34.90	1.69	8.82	1.85	10	2.59	53.90	6.46	100.00
Ovarian Cancer										
NCI/ADR-RES	4.81	87.70	1.94	NT	3.99	100	NT	100	7.16	100.00

[a]GI_{50} was the drug concentration resulting in a 50% reduction in the net protein increase (as measured by SRB staining) in control cells during the drug incubation, determined at five concentration levels (100, 10, 1.0, 0.1, and 0.01 μM).

[b]LC_{50} is a parameter of cytotoxicity and reflects the molar concentration needed to kill 50% of the cells;

[c]The values of activity against human tumor cell lines displayed by Adriamycin correspond to the reported by NCI at highest concentration of 10^{-4} M. NT – not tested.

Table 3. The selectivity indexes of compounds 9, 11, 14, 16 on the growth of tumor cell lines subpanel at the GI_{50}, TGI and LC_{50} levels.

Panel	9			11			14			16		
	SI[a]	SI[b]	SI[c]	SI[a]	SI[b]	SI[c]	SI[a]	SI[b]	SI[c]	SI[a]	SI[b]	SI[c]
Leukemia	1.92	0.05	0.84	**3.90**	0.05	NT	2.16	0.05	1.00	NT	0.43	1.01
NSCL cancer	0.72	0.05	0.93	**3.03**	0.30	0.93	1.97	0.06	0.87	2.16	0.43	0.94
Colon Cancer	1.83	0.07	0.84	**3.31**	0.68	0.92	2.7	0.14	0.80	2.26	0.06	0.83
CNS cancer	0.37	0.06	0.94	**3.25**	0.74	1.10	1.93	0.06	0.89	0.20	0.43	1.01
Melanoma	0.86	0.06	0.87	**3.20**	0.80	0.35	2.29	0.09	0.83	1.98	0.43	0.93
Ovarian Cancer	0.75	0.05	0.93	**3.15**	0.45	0.92	0.61	0.06	0.94	1.48	0.05	0.96
Renal Cancer	0.68	0.06	0.91	**3.55**	0.86	0.91	2.10	0.08	0.87	1.54	0.43	0.99
Prostate cancer	0.86	0.06	0.88	**3.20**	0.82	0.92	1.73	0.05	0.95	1.79	0.43	0.91
Breast cancer	0.99	0.05	0.90	**3.23**	0.23	0.92	1.00	0.06	0.93	1.32	0.05	0.93

[a] GI_{50}; [b] TGI; [c] LC_{50}. Bold values represent best results.

The selectivity index (SI) was calculated by dividing the full panel MG_MID_{60} (μM) of the compounds **9**, **11**, **14** and **16** by their individual subpanel MG_MID of the cell line (μM) and is to be considered as a measure of the compounds' selectivity (Table 3). Ratios between 3 and 6 mean moderate selectivity, ratios greater than 6 indicate high selectivity towards the corresponding cell line, while compounds not meeting either of these criteria are rated nonselective [49]. In this context, the compounds **9**, **14**, and **16** in the present study were found to be nonselective at all the GI_{50}, TGI and LC_{50} levels (selectivity indexes 0.37-1.92, 0.05-2.29 and 0.05-2.26, respectively). Compound **11** was moderately selective at the GI_{50} levels towards for the entire panel leukemia, NSCL, colon, CNS, ovarian, renal, prostate, breast cancer and melanoma (selectivity indexes 3.03-3.90).

Compounds **11**, **12** and **14** were additionally evaluated for their cytotoxic activity in conditionally normal (HEK293) and cancer (A549, MCF-7) cell lines (Table 4). Compounds **12** and **14** exhibited the moderate cytotoxicity toward HEK293, A549, MCF-7 cells with IC_{50} values ranging from 28 to 60 μM. Compound **11** has demonstrated the lowest IC_{50} values in all tested cell lines, exerting more remarkably activity against MCF-7 cells.

Table 4.*In vitro* cytotoxic activity of compounds 11, 12 and 14 in human HEK293, A549, MCF-7 cells

Compound	IC_{50} (μM)[a]		
	HEK293	A549	MCF-7
11	20.26 ± 1.76	15.01 ± 1.57	9.62 ± 0.27
12	30.66 ± 3.48	29.88 ± 2.55	40.54 ± 1.36
14	60.90 ± 5.65	28.36 ± 2.14	41.07 ± 1.28

[a] IC_{50} (μM) values obtained from MTT assays. Cells were incubated with compounds for 48 h. Values were the mean ± S.E.M. from two independent experiments, performed in triplicate.

Compounds **11** and **14** have been further evaluated for cell cycle analysis to gain insight into mechanisms of their action. Figure 1 demonstrated that compound **11** caused the increase of apoptotic cells in sub-G_1 compared with the control cells (0.1 % DMSO) in all tested cell lines, suggesting the apoptosis induction. As shown in Figure 2, incubation of HEK293 cells with compound **14** (60.9 μM) increased the proportion of apoptotic cells in sub-G_1without notable changes of cell cycle pattern compared to control (0.1% DMSO-treated) cells, pointing the apoptosis induction. Treatment of cancerous A549 (29.8 μM) and MCF-7 (40.5 μM) cells by compound **14** (at concentrations of 29.8 μM and 40.5 μM respectively) evoked a significant increase in number of cells in the G_2/M, indicating cell cycle arrest in G_2/M phase.

Conclusions

In this study, a series of new betulonic acid and its *N*-methylpyperazinyl amide derivatives, especially containing C2-nicotinoylidene/furfurylidene/fluorobenzylidene fragments, has been synthesized and evaluated for their cytotoxic activity against the NCI-60 cancer cell line panel. *N*-Methylpiperazinyl amides of betulinic **11** and 4-pyridinoylidene-betulinic **9** acids as well as betulonic C2-(4-pyridinoylidene) **14** or C2-(furfurylidene) **16** acids were found to be the leading compounds with GI_{50} values of 0.49 μM for leukemia CCRF-CEM, 1.60 μM and 1.36 μM for colon cancer HCT-116 and 1.66 μM for melanoma LOX IMVI cell lines, respectively. The activity displayed for these compounds was higher than for the standard drug doxorubicin against colon cancer HCT-15 and ovarian cancer NCI/ADR-RES cell lines. Cell cycle analysis indicates that compound **11** promotes cytotoxic activity through the apoptosis induction both in conditionally normal (HEK293) and in cancer (A549, MCF-7) cells, whereas compound **14** exhibits both cytostatic and cytotoxic activity, dependently on cell line evaluated. In particular, in HEK293 cells compound **14** induces mainly apoptotic cell death, while in A549 and MCF-7 cells cytostatic effect is dependent on cell cycle arrest in G_2/M phase. Compound **11** with selectivity indexes 3.03-3.90 at the GI_{50} levels towards for the entire panel is the promising structure for the future drug development antitumor studies.

Experimental

General

The spectra were recorded at the Center for the Collective Use “Chemistry” of the Ufa Institute of Chemistry of the UFRC RAS and RCCU “Agidel” of the UFRC RAS. ^{1}H and ^{13}C-NMR spectra were recorded on a “Bruker AM-500” (Bruker, Billerica, MA, USA, 500 and 125.5 MHz respectively, δ, ppm, Hz) in $CDCl_3$, internal standard tetramethylsilane. Mass spectra were obtained on a liquid chromatograph–mass spectrometer LCMS-2010 EV (Shimadzu, Kyoto, Japan). Single crystal X-ray diffraction study was carried out with SMART APEX II CCD difractometer (λ(Mo-Kα)=0.71073 A, graphite monochromator, ω-scans) at 120 K. Collected data were processed by the SAINT and SADABS programs incorporated into the APEX2 program package (Bruker 2014). The structures were solved by the direct methods and refined by the full-matrix least-squares procedure in anisotropic approximation for non-hydrogen atoms (Sheldrick 2015). Melting points were detected on a micro table “Rapido PHMK05“ (Nagema, Dresden, Germany). Optical rotations were measured on a polarimeter “Perkin-Elmer 241 MC” (Perkin Elmer, Waltham, MA, USA) in a tube length of 1 dm. Elemental analysis was performed on a Euro EA-3000 CHNS analyzer (Eurovector, Milan, Italy); the main standard is acetanilide. Thin-layer chromatography analyses were performed on Sorbfil plates (Sorbpolimer, Krasnodar, Russian Federation), using the solvent system chloroform–ethyl acetate, 40:1. Substances were detected by 10% H_2SO_4 with subsequent heating to 100–120 °C for 2–3 min. Betulonic acid (Flekhter et al. 2002), compounds **1**, **5** and **11** [28], **13** [42], **14** and **16** [50] were obtained according to the methods described previously.

Chemistry

*General procedure for synthesis of compounds (**2-4**), (**6**), (**7**) and (**16**).* 4-, 3- or 2-Pyridinecarboxaldehyde, 4-trifluoro- or 3,5-di-trifluoro-phenylcarboxaldehyde, or furfural (1.3 mmol) and 40% KOH in ethanol (2.5 mL) were added to a solution of compound **1** (0.55 g, 1 mmol) or betulonic acid in EtOH (5 mL) under stirring and cooling (from -5 to 10 °C). The mixture was stirred for 24 h at room temperature, pH was adjusted to neutral values with 5% HCl solution, and the mixture was poured into cold H_2O (50 mL). The residue was filtered, washed with water, and dried, the product was purified by column chromatography on Al_2O_3 using petroleum ether – chloroform (1 : 1 to 1 : 3) as eluent.

N-(2-{4-Pyridinoylidene}-3-oxolup-20(29)-en-28-oyl)-methylpiperazine (2): White solid ($CHCl_3$); Yield 0.57 g (91%); m.p. 138 °C; $[a]_D^{20}$ +25° (*c* 0.01, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 500.13 MHz,): δ = 8.62 (2H, d, *J* = 5.9 Hz, H-4", H-5"), 7.25-7.33 (1H, m, H-1"), 7.22 (2H, d, *J* = 5.9 Hz, H-3", H-6"), 4.72 (1H, s, H-29), 4.61 (1H, s, H-29), 2.81-3.17 (5H, m, H-19, H-1', H-4'), 2.29 (3H, s, H-5'), 2.02-2.41 (7H, m, CH, CH_2, H-2', H-3'), 1.69 (3H, s, H-30), 1.18-2.21 (19H, m, CH, CH_2), 1.14 (3H, s, H-23), 1.12 (3H, s, H-27), 0.98 (3H, s, H-24), 0.95 (3H, s, H-26), 0.77 (3H, s, H-25); ^{13}C NMR ($CDCl_3$, 125.76 MHz,): δ = 207.9 (C, C-3), 173.4 (C, C-28), 151.5 (C, C-20), 150.1 (C, C-2), 143.4 (CH, C-4", C-5"), 138.6 (C, C-2"), 133.8 (CH, C-1"), 124.0 (CH, C-3", C-6"), 109.1 (CH_2, C-29), 55.3, 55.2, 54.6 (CH_2, C-2'), 53.0 (CH_2, C-3'), 52.6 (CH_2, C-1', C-4'), 48.8, 46.0, 45.6, 45.4 (CH_3, C-5'), 42.0, 40.5, 39.3, 36.9, 36.6, 35.9, 34.1, 33.1, 32.4, 31.4, 29.7, 25.7, 22.3, 22.0, 20.3, 19.8, 19.6, 15.9, 15.6, 14.6; EIMS m/z 625 $[M]^+$(calcd. 625.94); Anal. Calcd for $C_{41}H_{59}N_3O_2$: C, 78.67; H, 9.50; N, 6.71. Found: C, 78.51; H, 9.32; N, 6.63.

N-(2-{3-Pyridinoylidene}-3-oxolup-20(29)-en-28-oyl)-methylpiperazine (3)*:* White solid ($CHCl_3$); Yield 0.57 g (91%); m.p. 152 °C; $[a]_D^{20}$ -12 (*c* 0.5, $CHCl_3$); ^1H NMR ($CDCl_3$, 500.13 MHz,): δ = 8.62 (1H, br. s, H-6"), 8.44 (1H, d, *J* = 4.7 Hz, H-5"), 7.63 (1H, d, *J* = 8 Hz, H-3"), 7.32 (1H, br. s, H-4"), 7.21-7.29 (1H, m, H-1"), 4.67 (1H, s, H-29), 4.53 (1H, s, H-29), 2.84-3.12 (5H, m, H-19, H-1', H-4'), 2.29-2.51 (3H, m, CH, CH_2), 2.21 (3H, s, H-5'), 2.12-2.24 (4H, m, H-2', H-3'), 1.64 (3H, s, H-30), 1.11-2.16 (19H, m, CH, CH_2), 1.11 (3H, s, H-23), 1.06 (3H, s, H-27), 0.92 (3H, s, H-24), 0.91 (3H, s, H-26), 0.71 (3H, s, H-25); ^{13}C NMR ($CDCl_3$, 125.76 MHz,): δ = 207.7 (C, C-3), 173.4 (C, C-28), 151.3 (C, C-20), 151.1 (C, C-2), 149.0 (CH, C-5"), 136.9 (CH, C-6"), 136.6 (CH, C-1"), 133.3 (C, C-2"), 131.9 (CH, C-3"), 123.4 (CH, C-4"), 109.3 (CH_2, C-29), 55.3, 55.0, 54.5 (CH_2, C-2'), 53.5 (CH_2, C-3'), 52.6 (CH_2, C-1', C-4'), 48.9, 46.0, 45.6, 45.3 (CH_3, C-5'), 42.0, 40.5, 39.6, 37.0, 36.6, 36.0, 34.1, 33.6, 32.4, 31.3, 29.7, 25.8, 22.4, 22.0, 20.4, 19.7, 19.2, 15.9, 15.6, 14.6; EIMS m/z 625 $[M]^+$(calcd. 625.94); Anal. Calcd for $C_{41}H_{59}N_3O_2$: C, 78.67; H, 9.50; N, 6.71. Found: C, 78.54; H, 9.38; N, 6.59.

N-(2-{2-Pyridinoylidene}-3-oxolup-20(29)-en-28-oyl)-methylpiperazine (4): White solid ($CHCl_3$); Yield 0.58 g (93%); m.p. 159 °C; $[a]_D^{20}$ +7 (*c* 0.5, $CHCl_3$); ^1H NMR ($CDCl_3$, 500.13 MHz,): δ = 8.44 (1H, d, *J* = 4.8 Hz, H-6"), 7.61-7.73 (2H, m, H-4", H-5"), 7.35 (1H, br. s, H-1"), 7.12-7.19 (1H, m, H-3"), 4.65 (1H, s, H-29), 4.55 (1H, s, H-29), 3.49-3.51 (2H, m, H-4'), 2.78-3.10 (3H, m, H-19, H-1'), 2.31-2.49 (3H, m, CH, CH_2), 2.21 (3H, s, H-5'), 1.80-2.14 (4H, m, H-2', H-3'), 1.59 (3H, s, H-30), 1.12-2.14 (19H, m, CH, CH_2), 1.11 (3H, s, H-23), 1.04 (3H, s, H-27), 0.91 (3H, s, H-24), 0.89 (3H, s, H-26), 0.70 (3H, s, H-25); ^{13}C NMR ($CDCl_3$, 125.76 MHz,): δ = 208.9 (C, C-3), 173.4 (C, C-28), 155.6 (C, C-20), 151.5 (C, C-2), 149.6 (CH, C-5"), 138.7 (CH, C-6"), 136.1 (CH, C-1"), 134.4 (C, C-2"), 126.6 (CH, C3"), 122.2 (CH, C-4"), 109.0 (CH_2, C29), 55.1 (CH_2, C3',C2'), 54.6, 52.9, 52.6, 48.6, 45.8, 45.6, 45.3 (CH_3, C-5'), 44.84 (CH_2, C-1', C-4'), 42.0, 40.5, 37.0, 36.3, 35.9, 33.2, 32.4, 31.4, 29.8, 29.4, 25.8, 22.3, 22.0, 20.4, 19.7, 19.2, 15.9, 15.6,14.6; EIMS m/z 625 $[M]^+$(calcd. 625.94); Anal. Calcd for $C_{41}H_{59}N_3O_2$: C, 78.67; H, 9.50; N, 6.71. Found: C, 78.60; H, 9.45; N, 6.68.

N-(2-{4-Fluorobenzylidene}-3-oxolup-20(29)-en-28-oyl)-methylpiperazine (6): White solid ($CHCl_3$); Yield 0.62 g (89%); m.p. 175 °C; $[a]_D^{20}$ -18 (*c* 0.01, $CHCl_3$); ^1H NMR ($CDCl_3$, 500.13 MHz,): δ = 7.63-7.69 (2H, m, H-4", H-6"), 7.39-7.49 (2H, m, H-3", H-7"), 6.42 (1H, br. s, H-1"), 2.81-3.05 (5H, m, H-19, H-1', H-4'), 2.09-2.30 (3H, m, CH, CH_2), 2.49 (3H, s, H-5'), 1.99-2.25 (4H, m, H-2', H-3'), 1.68 (3H, s, H-30), 1.18-1.86 (19H, m, CH, CH_2), 4.53 (1H, s, H-29), 4.60 (1H, s, H-29), 1.11 (3H, s, H-23), 1.06 (3H, s, H-27), 0.94 (3H, s, H-24), 0.93 (3H, s, H-26), 0.73 (3H, s, H-25); ^{13}C NMR ($CDCl_3$, 125.76 MHz,): δ = 208.1 (C, C-3), 173.5 (C, C-28), 151.0 (C, C-20), 139.8 (C, C-2), 136.4 (C, C-2"), 135.4 (CH, C-1"), 132.4 (C, C-5"), 130.2 (CH, C-3"), 128.6 (CH, C-7"), 126.8 (CH, C-4"), 126.1 (CH, C-6"), 125.1 (C, C-8"), 109.3 (CH_2, C29), 55.6, 54.6 (CH_2, C2'), 54.5 (CH_2, C3'), 53.0, 52.6 (CH_2, C-1', C-4'), 48.9, 46.0, 45.6, 45.3 (CH_3, C-5'), 42.0, 40.5, 39.6, 37.0, 36.6, 36.0, 34.1, 33.6, 32.4, 31.3, 29.7, 25.8, 22.4, 22.0, 20.4, 19.7, 19.2, 15.9, 15.6, 14.6; EIMS m/z 692 $[M]^+$(calcd. 692.95); Anal. Calcd for $C_{43}H_{59}F_3N_2O_2$: C, 74.53; H, 8.58; F, 8.23; N, 8.23. Found: C, 74.14; H, 8.38; F, 8.19; N, 8.39.

N-(2-{3,5-Difluorobenzylidene}-3-oxolup-20(29)-en-28-oyl)-methylpiperazine (7): White solid ($CHCl_3$); Yield 0.66 g (87%); m.p. 80 °C; $[a]_D^{20}$ +34 (*c* 0.5, $CHCl_3$); ^1H NMR ($CDCl_3$, 500.13 MHz,): δ = 7.73-7.88 (2H, m, H-3", H-7"), 7.72 (1H, br. s, H-5"), 7.49 (1H, br. s, H-1"), 4.70 (1H, s, H-29), 4.51 (1H, s, H-29), 2.74-3.10 (5H, m, H-19,

H-1', H-4'), 2.31-2.49 (3H, m, CH, CH_2), 2.31 (3H, s, H-5'), 2.05-2.21 (4H, m, H-2', H-3'), 1.55 (3H, s, H-30), 1.12-2.00 (19H, m, CH, CH_2), 1.09 (3H, s, H-23), 1.06 (3H, s, H-27), 0.92 (3H, s, H-24), 0.91 (3H, s, H-26), 0.71 (3H, s, H-25); ^{13}C NMR ($CDCl_3$, 125.76 MHz,): δ = 207.7 (C, C-3), 173.4 (C, C-28), 151.5 (C, C-20), 138.0 (C, C-2), 134.9 (CH, C-1"), 133.6 (C, C2"), 131.7 (C, C-8", C-9"), 131.9 (C, C-6"), 131.7 (C, C-4"), 129.5 (CH, C-3"), 123.5 (CH, C-7"), 121.5 (CH, C-5"), 109.3 (CH_2, C-29), 55.2 (CH_2, C-2', C-3'), 54.5, 53.1, 52.5, 48.8, 45.9, 45.7, 45.6 (CH_3, C-5'), 43.9 (CH_2, C-1', C-4'), 42.0, 40.5, 36.9, 36.8, 35.9, 33.1, 32.2, 31.3, 29.8, 29.5, 29.1, 25.4, 22.4, 21.9, 20.3, 19.7, 15.9, 15.6, 14.6; EIMS m/z 760 $[M]^+$(calcd. 760.95); Anal. Calcd for $C_{44}H_{58}F_6N_2O_2$: C, 69.45; H, 7.68; F, 14.98; N, 3.68. Found: C, 70.03; H, 7.58; F, 15.10; N, 3.69.

2-(Furfurylidene)-3-oxo-lup-20(29)-en-28-oic acid (16): White solid ($CHCl_3$); Yield 0.47 g (88%); m.p. 109 °C; $[a]_D^{20}$ +23° (*c* 0.02, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 500.13 MHz,): δ = 7.59 (1H, d, *J* = 1.0 Hz, H-5"), 7.22–7.38 (1H, m, H-1"), 6.49-6.58 (2H, d, *J* = 3.3 Hz, H-3", H-4"), 4.79 (1H, s, H-29), 4.65 (1H, s, H-29), 3.01-3.08 (1H, m, H-19), 2.24-2.35 (3H, m, CH, CH_2), 1.74 (3H, s, H-30), 1.22-1.84 (20H, m, CH, CH_2, O<u>H</u>), 1.13 (3H, s, H-23), 1.06 (3H, s, H-27), 0.82 (3H, s, H-24), 0.98 (3H, s, H-26), 1.04 (3H, s, H-25); ^{13}C NMR ($CDCl_3$, 125.76 MHz,): δ = 208.3 (C, C-3), 179.5 (C, C-28), 151.4 (C, C-2"), 150.6 (C, C-20), 144.4 (C, C-2), 128.3 (CH, C-5"), 124.2 (CH, C-1"), 115.4 (CH, C-4"), 112.2 (CH, C-3"), 109.6 (CH_2, C-29), 56.4, 52.5, 49.1, 46.8, 44.7, 41.6, 40.3, 39.8, 38.5, 37.0, 35.7, 34.2, 33.0, 32.0, 30.6, 29.6, 25.7, 22.2, 21.7, 20.4, 19.8, 19.5, 16.2, 15.4, 14.6; EIMS m/z 532 $[M]^+$(calcd. 532.77); Anal. Calcd for $C_{35}H_{48}O_4$: C, 78.91; H, 9.08. Found: C, 78.76; H, 8.84.

N-(2-{Hydroxymethylene})-3-oxolup-20(29)-en-28-oyl)-methylpiperazine (8): Ethyl formate (1 mL, 12.38 mmol) was added to a solution of CH_3ONa (0.35 g, 6.5 mmol), the reaction mixture stirred for 20 min, then a solution of compound **1** (0.55 g, 1 mmol) in dry benzene (20 mL) was added. The reaction mixture was stirred for 4 h, the organic layer was diluted with cold H_2O (30 mL) and separated. The aqueous layer was extracted with $CHCl_3$ (2×20 mL) and the combined extracts were washed twice with 5% HCl (2×50mL), water and dried over $CaCl_2$. The solvent was concentrated to dryness *in vacuo* and the residue was purified by column chromatography on Al_2O_3 using $CHCl_3$ as eluent; White solid ($CHCl_3$); Yield 0.43 g (76%); m.p. 210 °C; $[a]_D^{20}$ +73 (c 0.01, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 500.13 MHz,): δ = 14.70 (1H, s, =CH-O<u>H</u>), 8.55 (1H, s, =C<u>H</u>-OH), 4.73 (1H, s, H-29), 4.57 (1H, s, H-29), 2.84-3.20 (5H, m, H-19, H-1', H-4'), 2.32-2.56 (3H, m, CH, CH_2), 2.29 (3H, s, H-5'), 2.06-2.18 (4H, m, H-2', H-3'), 1.67 (3H, s, H-30), 1.22-2.12 (19H, m, CH, CH_2), 1.12 (3H, s, H-23), 1.02 (3H, s, H-27), 0.96 (3H, s, H-24), 0.92 (3H, s, H-26), 0.86 (3H, s, H-25); ^{13}C NMR ($CDCl_3$, 125.76 MHz,): δ = 190.3 (C, C-3),188.8 (CH, C-1"), 173.4 (C, C-28), 151.2 (C, C-20), 109.1 (CH_2, C-29), 55.2, 55.0, 54.5 (CH_2, C-2'), 54.3 (CH_2, C-3'), 52.6 (CH_2, C-1', C-4'), 50.2, 47.3, 45.9, 45.5 (CH_3, C-5'), 41.8, 40.8, 39.6, 38.1, 36.9, 35.9, 34.1, 33.6, 32.4, 31.3, 29.7, 26.5, 25.6, 22.9, 21.6, 20.9, 19.6, 19.3, 15.9, 15.8, 14.5; EIMS m/z 564 $[M]^+$(calcd. 564.86); Anal. Calcd for $C_{36}H_{56}N_2O_3$: C, 76.32; H, 9.68; N, 4.71. Found: C, 76.55, H 9.99, N 4.96.

N-(2-{4-Pyridinoylidene}-3b-hydroxylup-20(29)-en-28-oyl)-methylpiperazine (9): To a solution of compound **2** (0.62 g, 1 mmol) in MeOH (15 mL) $NaBH_4$ (50 mg, 13 mmol) was added. The reaction mixture was stirred at room temperature for 2 h and then neutralized with 10% HCl (10 mL). The precipitate was filtered, washed and dried. The residue was purified by column chromatography on Al_2O_3 using $CHCl_3$ as eluent; White solid

($CHCl_3$); Yield 0.45 g (74%); m.p. 178 °C; $[a]_D^{20}$ +13 (c 0.01, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 500.13 MHz,): δ = 8.53 (2H, d, *J* =5.8 Hz, H-4", H-5"), 7.17-7.28 (1H, m, H-1"), 7.09 (2H, d, *J* = 5.8 Hz, H-3", H-6"), 4.61 (1H, s, H-29), 4.56 (1H, s, H-29), 2.84-3.64 (7H, m, H-19, H-3, H-1', H-4', OH), 2.32-2.56 (3H, m, CH, CH_2), 2.25 (3H, s, H-5'), 2.03-2.12 (4H, m, H-2', H-3'), 1.69 (3H, s, H-30), 1.17-2.01 (19H, m, CH, CH_2), 1.12 (3H, s, H-23), 0.91 (3H, s, H-27), 0.82 (3H, s, H-24), 0.69 (3H, s, H-26), 0.63 (3H, s, H-25); ^{13}C NMR ($CDCl_3$, 125.76 MHz,): δ = 173.4 (C, C-28), 151.4 (C, C-20), 149.7 (CH_2, C-4", C-5"), 137.2 (C, C-2"), 133.0 (CH, C-1"), 123.7 (CH, C-3", C-6"), 109.2 (CH_2, C-29), 81.3 (CH, C-3), 56.0, 55.2, 54.5 (CH_2, C2'), 53.0 (CH_2, C3'), 52.6 (CH_2, C-1', C-4'), 50.2, 45.9, 45.6, 45.5 (CH_3, C-5'),40.9, 40.7, 41.9, 36.9, 36.8, 35.9, 34.2, 33.6, 32.4, 31.3, 29.8, 25.6, 22.9, 21.4, 19.7, 18.4, 16.3, 16.0, 15.6, 14.7; EIMS m/z 627 $[M]^+$(calcd. 627.96); Anal. Calcd for $C_{40}H_{61}N_3O_2$: C, 78.00; H, 9.98; N, 6.82. Found: C, 77.87; H, 9.72; N, 6.69.

N-(2-{4-Pyridinoylidene}-3-oximino-lup-20(29)-en-28-oyl)-methylpiperazine (10): A solution of compound **2** (0.62 g, 1 mmol) and $NH_2OH{\cdot}HCl$ (0.54 g, 8 mmol) in pyridine (15 mL) was refluxed for 2 h, neutralized with 10% HCl (100 mL), the precipitate was filtered, washed and dried. The residue was purified by column chromatography on Al_2O_3 using $CHCl_3$ as eluent; White solid ($CHCl_3$); Yield 0.48 g (76%); m.p. 180 °C; $[a]_D^{20}$ +35 (c 0.01, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 500.13 MHz,): δ = 9.21 (1H, br. s, N-OH), 8.65 (2H, d, *J* = 5.8 Hz, H-4", H-5"), 7.23-7.38 (1H, m, H-1"), 7.20 (2H, d, *J* = 5.8 Hz, H-3", H-6"), 4.73 (1H, s, H-29), 4.59 (1H, s, H-29), 2.84-3.14 (5H, m, H-19, H-1', H-4'), 2.27-2.56 (3H, m, CH, CH_2), 2.29 (3H, s, H-5'), 2.06-2.18 (4H, m, H-2', H-3'), 1.68 (3H, s, H-30), 1.22-2.12 (19H, m, CH, CH_2), 1.12 (3H, s, H-23), 1.02 (3H, s, H-27), 0.96 (3H, s, H-24), 0.92 (3H, s, H-26), 0.79 (3H, s, H-25); ^{13}C NMR ($CDCl_3$, 125.76 MHz,): δ = 173.4 (C, C-28), 167.3 (C, C-3), 151.2 (C, C-20), 142.7 (CH, C-4", C-5"), 138.1 (CH, C-2"), 132.6 (CH, C-1"), 124.2 (CH, C-3", C-6"), 109.1 (CH_2, C-29), 55.2, 55.0, 54.5 (CH_2, C-2'), 53.0 (CH_2, C3'), 52.6 (CH_2, C-2', C-4'), 50.2, 45.9, 45.6, 45.5 (CH_3, C-5'), 41.8, 40.5, 39.6,36.9, 36.6, 35.9, 34.1, 33.6, 32.4, 31.3, 29.7, 25.6, 22.3, 21.6, 20.5, 19.6, 19.3, 15.7, 15.9, 14.5; EIMS m/z 640 $[M]^+$(calcd. 640.96); Anal. Calcd for $C_{40}H_{60}N_4O_2$: C, 76.39; H, 9.62; N, 8.91. Found: C, 76.17, H 9.58, N, 8.83.

N-(3-diethoxyphosphoryl-lup-20(29)-en-28-oyl)-methylpiperazine (12): Diethyl chlorophosphate (0.28 mL, 1.9 mmol) was added dropwise to a stirred solution of compound **11** (0.54 g, 1 mmol) and 4-dimethylaminopyridine (DMAP) in pyridine (6 mL), cooled to 0 °C in an ice-water bath. Mixture was allowed to warm to room temperature and stirred overnight. After completion the reaction, pyridine was removed in vacuum and the residue was dissolved in 50 mL chloroform, washed with 10% HCl (100 mL), saturated $NaHCO_3$ and water, dried and the solvent removed under reduced pressure. The residue was purified by column chromatography on Al_2O_3 using $CHCl_3$/EtOH (50:1) as eluent; White solid (EtOH); Yield 0.49 g (74%); m.p. 122 °C; $[a]_D^{20}$ -26 (c 0.01, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 500.13 MHz,): δ = 4.72 (1H, s, H-29), 4.59 (1H, s, H-29), 4.01-4.14 (4H, m, H-1", H-1'"), 2.68-3.01 (5H, m, H-19, H-1', H-4'), 2.27-2.48 (3H, m, CH, CH_2), 2.39 (3H, s, H-5'), 2.01-2.12 (4H, m, H-2', H-3'), 1.68 (3H, s, H-30), 1.28-1.42 (6H, m, H-2", H-2'"), 1.21-2.07 (22H, m, CH, CH_2), 0.97 (3H, s, H-23), 0.93 (3H, s, H-27), 0.91 (3H, s, H-24), 0.82 (3H, s, H-26), 0.78 (3H, s, H-25); ^{13}C NMR ($CDCl_3$, 125.76 MHz,): δ = 173.6 (C, C-28), 151.2 (C, C-20), 109.3 (CH_2, C-29), 86.4 (CH, C-3), 63.4 (CH_2, C-1"), 61.5 (CH_2, C-1'"), 55.5, 55.0, 54.7 (CH_2, C-3'), 53.0 (CH_2, C-5'), 52.6 (CH_2, C-2', C-6'), 50.7, 45.9, 45.6, 45.5 (CH_3, C7'), 41.8, 40.5, 39.6, 36.9, 36.6, 35.9, 34.1, 33.6, 32.4, 31.3, 29.8, 25.6, 22.3, 21.2, 20.5, 19.6, 19.3, 18.3, 16.2 (CH_3,

C-2''), 16.1 (CH_3, C-2'''), 16.0, 15.8, 14.6; EIMS m/z 674 $[M]^+$(calcd. 674.95); Anal. Calcd for $C_{39}H_{67}N_2O_5P$: C, 69.40; H, 10.01; N, 4.15; P, 4.59. Found: C, 69.28, H 9.93, N, 4.02; P, 4.47.

Pharmacological studies

Culture conditions and treatments

HEK293 (human embryonic kidney 293 cells), A549 (human lung carcinoma), MCF-7 (human breast adenocarcinoma) cell lines were purchased from the Russian Cell Culture Collection (Institute of Cytology Russian Academy of Science, Saint Petersburg, Russia). HEK293, A549 and MCF7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, USA) supplemented with 2 mM *L*-glutamine (Sigma-Aldrich, UK), 10% fetal bovine serum (FBS; Invitrogen, USA), 50 μg/mL gentamicin sulfate (Invitrogen, USA) at 37°C and 5% CO_2. Compounds **11**, **12** and **14** were dissolved in 100% DMSO (Sigma-Aldrich, UK) to 100 mM stock solutions and diluted in completed DMEM immediately before addition to the assay plates. DMSO was maintained at a final concentration of 0.1%.

Cell viability

Cells were cultured at appropriate density in 96-well plates (3×10^4 cells/well for HEK293; 5×10^3 cells/well for A549; 1.2×10^4 cells/well for MCF-7 and allowed to grow for 24 h. Thereafter, cells were treated with compounds **11**, **12** and **14** at a final concentrations of 1, 10, 100 μM for 48 hours and cell viability was measured by conventional MTT assay following manufacturer's instruction (Thermo Fisher Scientific, USA) using «2300 EnSpire® Multimode Plate Reader» (Perkin Elmer, USA) at 590 nm. The concentration of the compound that inhibited 50% cell viability (IC_{50} value) was calculated using nonlinear regression analysis (GraphPad Prism v.5.02; GraphPad Software Inc., USA). The viability of control group (cells treated with 0.1% DMSO) was set at 100%, and viability of treated groups was determined through the comparison of its optical density with control. Data were expressed as mean ± S.E.M. calculated from two independent experiments, performed in triplicate.

Cell cycle analysis

The cell cycle was measured by flow cytometry assay with PI (propidium iodide) staining. Human embryonic kidney HEK293 cells (2×10^5 cells/well), lung carcinoma A549 and, breast adenocarcinoma MCF-7 cells (1×10^5 cells/well) were seeded in 24-well plates in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, USA), containing 50 mg/ml gentamycin, 2 mM *L*-glutamine, 10% fetal bovine serum (FBS) and cultured for 24 h followed by vehicle (0.1% DMSO) or compounds **11** and **14** treatment for 48 hours. Compounds were added at their IC_{50} values, which were previously determined for the aforementioned cell lines in the additional laboratory cytotoxicity screen (Table SX in the Supporting Information files). Thereafter, cells were harvested, fixed in 96% ice-cold ethanol and incubated overnight at -20°C. Subsequently, cells were centrifuged, supernatant was discarded and the pellet was treated with RNase A (0.5 mg/ml; Sigma, USA) for 10 min at room temperature. The treated cells were stained with propidium iodide (50 μg/ml; Sigma, USA) for 15 min at room temperature in the dark. The PI fluorescence of individual cells/nuclei was

measured on Novocyte 2060 flow cytometer (Acea Biosciences, Inc. USA) in linear scale. Data analysis was performed by using the cell cycle module of NovoExpress 1.3.0 software (Acea Biosciences, Inc. USA).

The data are expressed as mean ± S.E.M from 3 experiments performed in triplicate. Comparison of cell cycle phases was performed using Wilcoxon *t*-test (Statistica 6.1 (StatSoft. Inc., USA); * - p□0.05 vs. vehicle for certain cell line.

Declarations

Acknowledgments

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Conflict of interest The authors have declared no conflict of interest.

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Figures

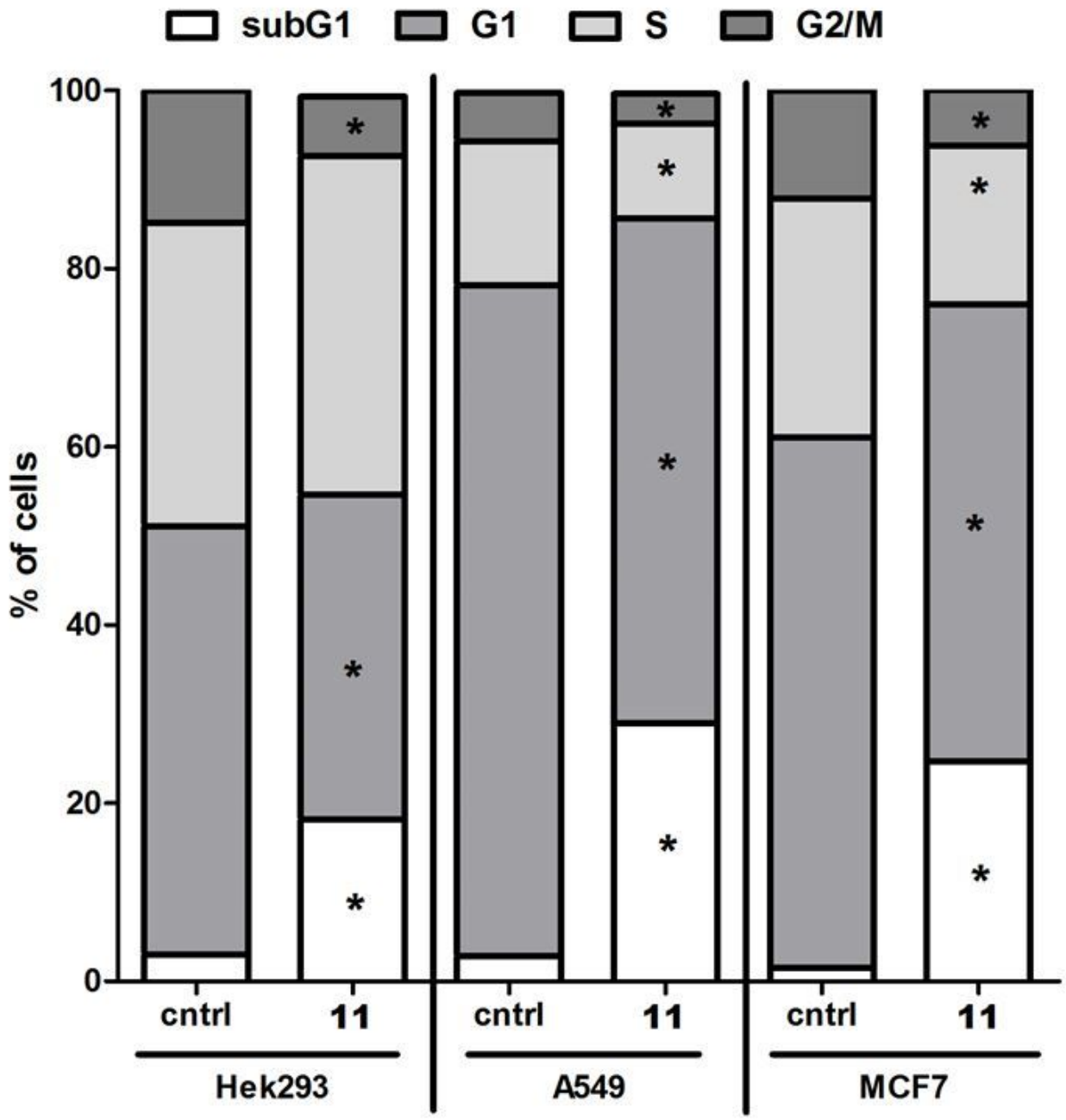

Figure 1

The effect of compound 11 on cell cycle distribution in HEK293, A549 and MCF-7 cells. The data are expressed as mean ± S.E.M from 3 experiments, performed in triplicate. Comparison of cell cycle phases was performed using Wilcoxon t-test; * - p□0.05 vs. vehicle for certain cell line.

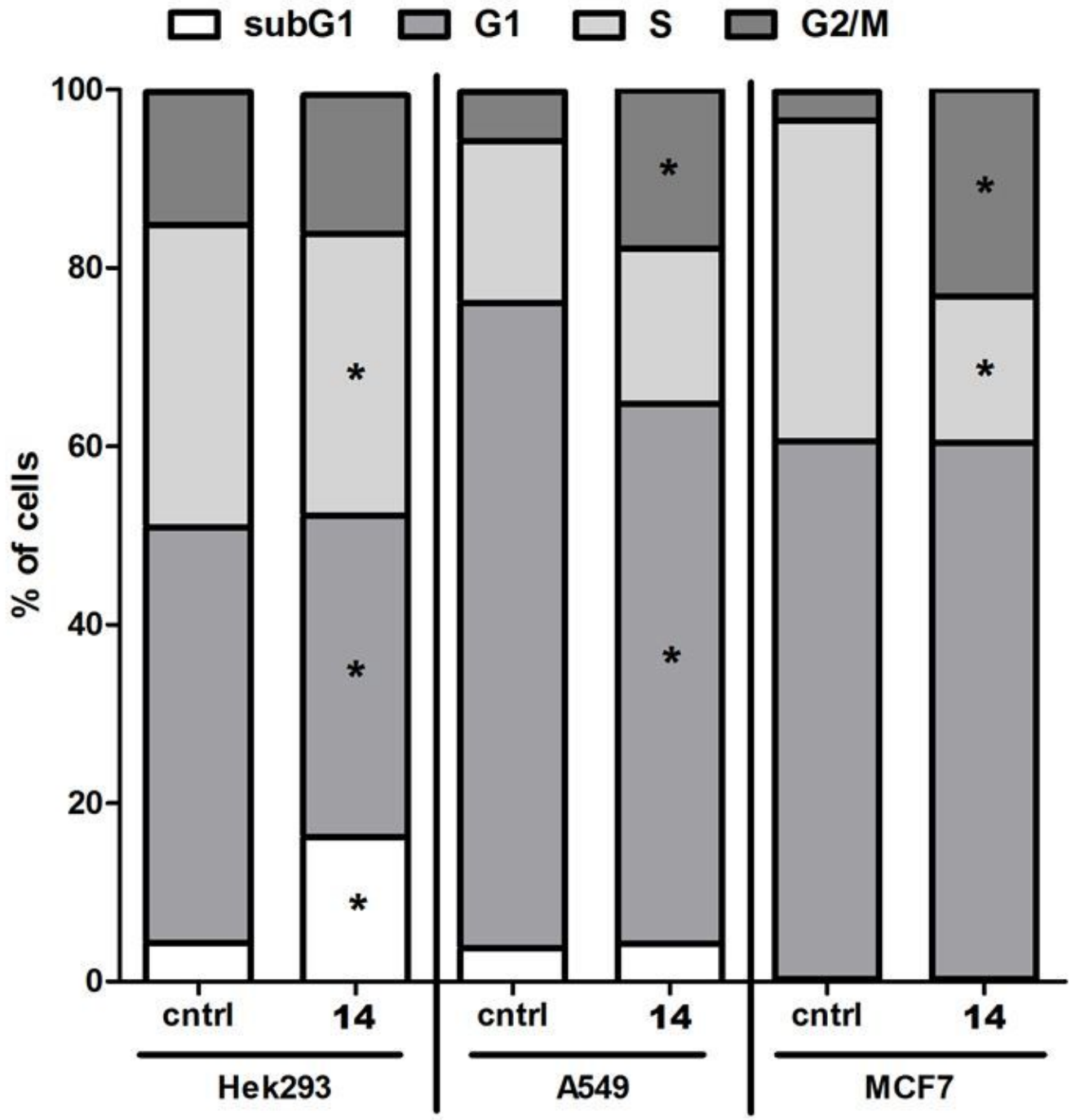

Figure 2

Cell cycle progression of HEK293, A549 and MCF-7 cells upon compound 14 treatment. The data are expressed as mean ± S.E.M from 3 experiments, performed in triplicate. Comparison of cell cycle phases was performed using Wilcoxon t-test; * - p□0.05 vs. vehicle for certain cell line.

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