ISSN 1068-1620, Russian Journal of Bioorganic Chemistry, 2013, Vol. 39, No. 2, pp. 194–201. © Pleiades Publishing, Ltd., 2013.
Original Russian Text © I.V. Sorokina, I.Ya. Mainagashev, N.A. Zhukova, D.V. Korchagina, T.G. Tolstikova, V.P. Nikolin, N.A. Popova, M.A. Pokrovskii, A.G. Pokrovskii, N.F. Salakhutdinov, 2013, published in Bioorganicheskaya Khimiya, 2013, Vol. 39, No. 2, pp. 221–229.

Antitumor Activity of Amides of Dihydrobetulonic Acid in vitro and in vivo

I. V. Sorokina[a, 1], I. Ya. Mainagashev[a], N. A. Zhukova[a], D. V. Korchagina[a], T. G. Tolstikova[a], V. P. Nikolin[b], N. A. Popova[b], M. A. Pokrovskii[c], A. G. Pokrovskii[c], and N. F. Salakhutdinov[a]

[a] *Vorozhtsov Institute of Organic Chemistry, Siberian Branch, Russian Academy of Sciences, pr. Lavrent'eva 9, Novosibirsk, 630090, Russia*

[b] *Institute of Cytology and Genetics, Siberian Branch, Russian Academy of Sciences, Novosibirsk, Russia*

[c] *Novosibirsk State University, Novosibirsk, Russia*

Received August 10, 2012; in final form, October 8, 2012

Abstract—Amides containing homopiperidine and piperazine cycles were synthesized from dihydrobetulonic acid obtained by the oxidation of dihydrobetulin. All substances were shown to have a high antitumor activity ($CCID_{50}$ = 3.5–36.2 μM) in vitro in lymphoid (CEM-13, U-937) and monocytic (MT-4) human cell lines. Amides containing the methyl- and ethylpiperazine residues do not influence the viability of Lung Lewis Carcinoma cells in culture and do not have a significant effect on its transplants in C57BL/6 mice. However, these amides efficiently inhibit the development of metastases in lungs of these mice. The antimetastatic activity of the studied amides increases with changing the methyl by ethyl aliphatic residue in the piperazine cycle.

Keywords: dihydrobetulonic acid, amides, piperazine derivatives, antitumor activity, antimetastatic effect

DOI: 10.1134/S106816201302012X

INTRODUCTION

Over the past decades, pentacyclic triterpenoids of the lupane series have been in the focus of medical chemistry as low-toxicity compounds with a high activity, which stimulate apoptosis of tumor cells [1]. At the same time, they are searching among agent-modifiers, which can be used in traditional chemotherapy to improve the efficiency, and reduce the toxicity, of cytostatic drugs. To obtain libraries of derivatives from starting triterpene compounds, the derivatives of the available plant metabolite, betulin, are often used. We have previously found that the derivatives of betulonic acid containing the fragments of ω-amino acids are active inductors of apoptosis in leukemic and hepatocellular carcinoma cells in vitro [2]. Amides of betulonic acid were shown to be more efficient inductors of apoptosis than the corresponding acids [3]. Betulonic acid and its alanylamide derivatives enhance the antitumor effect of cytostatic polychemotherapy in mice with transplantable tumors [4, 5] and reduce the severity of necrobiotic disorders in the liver, kidney, and myocardium caused by cytostatic drugs [6–8].

Dihydrobetulonic acid (**I**) can be considered as a promising compound in the series of biologically active lupans. Hydrogenation of betulonic acid derivatives in the C20 position increases their antitumor activity as compared to nonhydrogenated analogues [9]. It was shown with an example of 3-*O*-glutaryldihydrobetulin that the use of the hydrogenated analogue of betulin, instead of its common derivative, leads to an increase in the anti-HIV activity by at least three orders of magnitude [10]. The pronounced antitumor activity in colon cancer xenografts was found for the dihydrobetulonic acid derivative containing the 4-nitrobenzyloxyimine substituent in the C3 position [11].

RESULTS AND DISCUSSION

The goal of the work was the synthesis of amides of dihydrobetulonic acid and the study of its antitumor properties in tumor cell cultures and in animals with transplantable tumors. Moreover, we evaluated the antitumor and antimetastatic efficiency of individual lupan derivatives along with cytotoxic polychemotherapy in tumor-bearing mice.

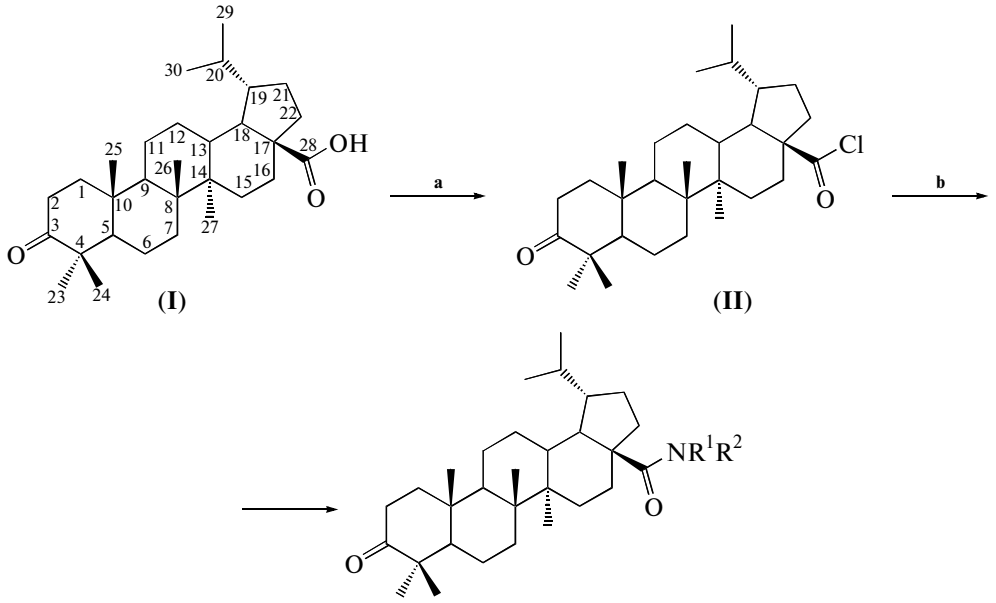
Betulin was obtained by the extraction of birch bark by benzene and used as the starting compound to synthesize amides (**III**)–(**VII**). Hydrogenation of betulin with hydrogen in an autoclave led to dihydrobetulin, which was oxidized with Fieser's solution. At the next

Abbreviations: $CCID_{50}$, concentration leading to the death of 50% of cells; TGI, index of tumor growth inhibition; MII, metastasis inhibition index; MF, metastasis frequency.

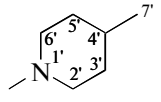
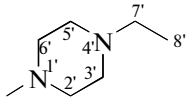
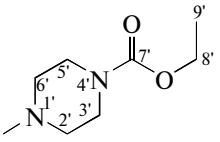
[1] Corresponding author: phone: 7(383)330-07-31; e-mail: sorokina@nioch.nsc.ru.

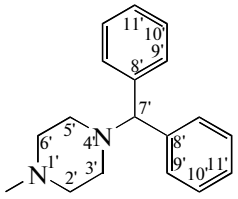
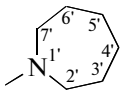
stage, chloroanhydride of dihydrobetulonic acid was obtained, which was then interacted with double excess of the corresponding amine resulting in target compounds (**III**)–(**VII**) (Scheme).

Conditions: **a.** $(COCl)_2$, CH_2Cl_2, 20°C, 4 h; **b.** HNR^1R^2, CH_2Cl_2, 20°C, 18–20 h.



(I) **(II)** **(III)–(VII)**

(III) NR^1R^2 =  **(IV)** NR^1R^2 =  **(V)** NR^1R^2 = 

(VI) NR^1R^2 =  **(VII)** NR^1R^2 = 

Scheme.

The structure of the prepared compounds was confirmed by ^1H and ^{13}C NMR and IR spectra. The specific rotation and melting point values were determined for all synthesized compounds. Brutto formulas of the synthesized compounds were determined from the element analysis or mass spectra of high resolution.

The in vitro screening by the MTT test was used to study the effect of hydrogenated betulonic acid (**I**) and its amides (**III**)–(**VII**) on the viability of human cancer cells: leukemic (CEM-13 and U-937) and monocytic (MT-4) cell lines. Compounds (**I**) and (**III**)–(**VII**) were found to exhibit high antitumor activity in all tumor cultures used. All agents have similar $CCID_{50}$ values in the range of 3.5–36.2 μM (Table 1).

The data allow one to consider betulonic acid (**I**) and its amides (**III**)–(**VII**) as potential promising antitumor drugs with a high activity against carcinoma tumor lines CEM-13, U-937, and MT-4.

Two piperazine derivatives (**III**) and (**IV**), which showed high activity in the MTT test, were chosen for the following in vivo experiments. These experiments were carried out with female mice C57BL/6 inoculated intramuscularly with Lewis lung carcinoma. Compounds (**III**) and (**IV**) were administered intragastrically in a dose of 20 mg/kg daily from the third to the tenth day after transplantation. The dose was chosen according to the data on the antitumor activity of pentacyclic triterpenoids in the dose range of 5 to 50 mg/kg [12]. The measurement of tumor nodules

Table 1. $CCID_{50}$ values of hydrogenated betulonic acid (**I**) and its amides (**III**)–(**VII**) for different human carcinoma cell lines

Compound	$CCID_{50}$, μM		
	CEM-13	U-937	MT-4
(I)	4.4	3.8	3.5
(III)	13.0	7.8	33.4
(IV)	6.9	6.0	7.2
(V)	12.4	23.5	7.7
(VI)	26.0	36.2	10.9
(VII)	10.6	11.9	11.9

Table 2. Indices of tumor growth inhibition and average life span of mice after transplantation of Lewis lung carcinoma and 8-day administration of compounds (**III**) and (**IV**)

Compound	Indexes of tumor growth inhibition, %			Life span (days)
	11th day	13th day	15th day	
Control	–	–	–	19.6 ± 1.7
(III)	1.9	8.7	19.9	22.8 ± 2.4
(IV)	4.7*	7.6	20.3	22.4 ± 1.1

* $P < 0.05$, significant difference from the control.

Table 3. Indices of tumor growth inhibition and lethality in mice with Lewis lung carcinoma during isolated and combined with polychemotherapy 8-day administration of compounds (**III**) and (**IV**)

Compound	Indexes of tumor growth inhibition, %			Lethality, %
	14th day	16th day	18th day	
Control	–	–	–	30
(III)	0	22	12	40
(IV)	0	5	12	40
PCT	11	25	26	20
PCT + **(III)**	11	21	17	20
PCT + **(IV)**	13	9	12	0

started after visualization and continued until the death of animals (from 11 to 15 days after transplantation).

Compounds (**III**) and (**IV**) showed a stable tendency to the tumor growth delay, but this effect was not statistically significant. The index of tumor growth inhibition (TGI) for both compounds was 20% on the 15th day of the experiment. Also noted was a slight increase in the average life expectancy of mice, which may indicate the stimulation of antitumor resistance in the animals (Table 2).

Thus, the eight-day intragastric introduction of methyl- and ethylpiperazine of dihydrobetulonic acid in a dose of 20 mg/kg at the early stage of the neoplastic process had no antitumor effect due, probably, both to an insufficient dose of the agents and the low sensitivity of this tumor to the piperazine derivative of dihydrobetulonic acid. To confirm these assumptions, we performed an additional study of the activity of the compounds in a Lewis carcinoma cell culture, which was obtained from the corresponding solid tumor transplants. After excision of the tumor tissue, cells were washed by saline, crushed and placed in Versen solution. The cells were then washed and placed in a nutrient medium. The compounds were found to have no cytotoxic effects even at the maximum concentration used (100 μM). Thus, in the in vitro and in vivo experiments, no significant cytotoxic effect against Lewis lung carcinoma was found for hydrogenated betulonic acid and its amides.

We evaluated the efficiency of amides (**III**) and (**IV**) when administered at the stage of tumor progression and dissemination, in particular, along with cytotoxic polychemotherapy (PCT). In the latter case, the criterion for efficiency was the increase in the antitumor and antimetastatic activity of the cytostatic preparations. Lewis lung carcinoma was transplanted into female mice C57BL/6. Compounds (**III**) and (**IV**) were intragastrically administered as the water-tween suspension in a dose of 20 mg/kg from day 11 to day 18 after the transplantation. The day before the administration of agents, half of the mice were parenterally administered a one-time cytostatic complex simulating the standard chemotherapy scheme (cyclophosphamide, doxorubicin, vincristine, and prednisolone) [6]. During the administration of the agents, the volume of tumor nodules and the TGI indexes were evaluated. The size of metastatic foci in the lungs was counted morphometrically on 19th day after transplantation.

It was shown that the methyl- and ethylpiperazine derivatives in a stand-alone mode of administration exhibit low antitumor activity and a slightly lower survival rate of the mice compared to the control group. During the course administration along with PCT, methylpiperazine derivative (**III**) insignificantly reduces the cytotoxic effect of chemotherapy and does not affect the mortality percentage of mice. Ethylpiperazine derivative (**IV**) more markedly reduces the efficiency of chemotherapy and improves the survival of animals (Table 3).

The compounds studied were found to exhibit the antimetastatic effect in the stand-alone mode of administration into animals. The more pronounced activity was registered for ethylpiperazine derivative (the 8-fold reduction in the bulk density of metastases) compared with methylpiperazine (reduction by a factor of 2.5) (Table 4). It should be noted that the antimetastataic effect of ethylpiperazine derivative (**IV**)

Table 4. Indicators of metastasis of Lewis lung carcinoma in the lungs of mice after isolated and combined with polychemotherapy 8-day administration of compounds (**III**) and (**IV**)

Compound	Metastasis volume density, μM	Metastasis frequency, %	MII, %
Control	319.78 ± 51.36	100	–
(**III**)	123.93 ± 49.22	100	61.2
(**IV**)	39.08 ± 2.44*	100	87.8
PCT	50.66 ± 9.15*	88	86.0
PCT + (**III**)	78.05 ± 14.91*	100	75.6
PCT+ (**IV**)	40.06 ± 4.14*	100	87.5

* $P < 0.001$ with respect to control.

was not inferior to that of cytostatic PCT, which reduced the area of metastatic foci by a factor of more than six. Nevertheless, the administration of ethylpiperazine derivative along with the standard chemotherapy did not increase its antimetastatic effect. Methylpiperazine derivative (**III**) slightly reduced the corresponding effect of PCT (MII was 75.6 vs 86.0 for PCT).

Thus, ethylpiperazine derivative (IV) at the standalone mode administration exhibits pronounced antimetastatic activity. When used in combination with cytostatic polychemotherapy, this compound's antimetastatic effect is not reduced. The methylpiperazine derivative (**III**) itself exhibits moderate antimetastatic action and when administered along with chemotherapy, slightly decreases its effect.

It can be concluded that methyl- and ethylpiperazine derivatives of dihydrobetulonic acid have a cytostatic effect in vitro on CEM-13, U-937, and MT-4 cells. Lewis lung carcinoma in the form of either cell culture or solid tumor in mice is not very sensitive to the action of the compounds. Administration of amides of dihydrobetulonic acid (**III**) and (**IV**) in mice at the stage of tumor progression causes a significant delay in its dissemination, and has little effect on the growth of transplants. The replacement of the methyl substituent by the ethyl group in the piperazine fragment leads to the enhancement of the antimetastatic effect, which, probably, provides the reduction in mortality of animals during the administration of agent (**IV**). The use of the piperazine derivatives of dihydrobetulonic acid in combination with PCT does not result in the enhancement of the antitumor and antimetastatic efficiency of the latter.

EXPERIMENTAL

IR spectra (ν, cm^{-1}) were recorded on a VECTOR 22 spectrometer (Germany) in KBr. The special rotation ($[\alpha]_D$) was evaluated using a polAAr 3005 spectrometer (Great Britain). Concentrations of solutions (*c*) in $CHCl_3$ are in g/100 mL. Melting points were determined using a Termosystem FP 900 instrument (Mettler Toledo, Switzerland) and on a Kofler table S 30 A/G (Germany). The elemental composition of the substances was determined by elemental analysis and mass spectrometry of a high resolution recorded on a DFS (double Focusing Sector) spectrometer (Thermo Electron Corporation, Germany). ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ on a DRX-500 spectrometer (500.13 MHz and 125.76 MHz for ^1H and ^{13}C, respectively; Bruker, Germany). Chemical shifts (δ) are in ppm, CSSI are in Hz. The residual signals of the solvent were used as the internal standard (δ_H = 7.24 ppm; δ_C = 76.9 ppm). The structure of the compounds was established from the $^1H-^1H$ NMR spectra involving the double-resonance spectra, ^{13}C NMR spectra using standard recording techniques in the *J*-modulation (JMOD) regime with nonresonant and selective suppression of protons, two-dimensional spectra of the $^{13}C-^1H$ heteronuclear correlation of the direct (C-H COSY. $^1J_{C,H}$ 135 Hz) and long-range spin-spin coupling constants (COLOC, $^{2,3}J_{C,H}$ 10 Hz).

Betulin was isolated from the outer bark of birch [13]; dihydrobetulin was synthesized by hydrogenation of betulin over Raney nickel according to the known method [14].

3-Oxolupan-28-ic acid (dihydrobetulonic acid (I)). A suspension of dihydrobetuline (10 g, 22.5 mol) in glacial acetic acid (165 mL) and acetone (110 mL) was cooled to 0°C, and the freshly prepared Fieser's solution (11 g, 0.11 mol of chromic anhydride in 12 mL of glacial acetic acid and 15 mL of H_2O) was added under stirring for 1 h. The reaction mixture was kept at room temperature for 3 h, followed by the addition of methanol (50 mL), benzene (300 mL), and 10% NaCl solution (300 mL). The benzene layer was removed, and the aqueous layer was extracted with benzene (2 × 150 mL). The combined extracts were washed with a 10% solution of NaCl and dried with $MgSO_4$. The mixture was filtered and the benzene evaporated to ~100 mL and poured into a 5% solution of KOH (200 mL). The residue of the potassium salt of dihydrobetulonic acid was filtered and dried. The dry salt was dissolved in ethanol (50 mL), the undissolved portion was filtered, and the filtrate was poured into a 5% solution of HCl (250 mL). The precipitate was filtered, washed with H_2O and

dried. We obtained 5.5 g (54%) of the product, T_m = 253–254°C (lit. T_m = 253–254°C [15]). $[\alpha]_D^{27}$ +10.7 (*c* 0.41). Found: *m/z* 456.3608 $[M]^+$. $C_{30}H_{48}O_3$. Calculated: 456.3603. IR spectrum: 1705 (C=O). ^{1}H NMR spectrum: 0.73 (3 H, d, *J* 7.0, H29); 0.83 (3 H, d, *J* 7.0, H30); 0.90 (3 H, s, H25); 0.93 (6 H, s, H26, H27); 0.98 (3 H, s, H24); 1.04 (3 H, s, H23); 1.11–1.70 (18 H, m, CH_2,CH); 1.76 (1 H, septet d, *J* 7.0, $J_{20,19}$ 2.5, H20); 1.86 (1 H, dd, ^2J 12.4, *J* 7.3, H22α or H22β); 1.88 (1 H, ddd, $J_{1e,1a}$ 13.0, $J_{1e,2a}$ 7.5, $J_{1e,2e}$ 4.3, H1e); 2.15–2.27 (3 H, m, CH_2,CH); 2.38 (1 H, ddd, $J_{2e,2a}$ 15.8, $J_{2e,1a}$ 7.5, $J_{2e,1e}$ 4.3, H2e); 2.47 (1 H, ddd, $J_{2a,2e}$ 15.8, $J_{2a,1a}$ 9.7, $J_{2a,1e}$ 7.5, H2a); 9.48 (1 H, br.s, OH). ^{13}C NMR spectrum: 39.43 (t, C1), 33.97 (t, C2), 218.10 (s, C3), 47.19 (s, C4), 54.75 (d, C5), 19.48 (t, C6), 33.52 (t, C7), 40.49 (s, C8), 49.45 (d, C9), 36.74 (s, C10), 21.25 (t, C11), 26.72 (t, C12), 38.22 (d, C13), 42.48 (s, C14), 29.54 (t, C15), 31.86 (t, C16), 56.72 (s, C17), 48.54 (d, C18), 44.01 (d, C19), 29.59 (d, C20), 22.60 (t, C21), 37.26 (t, C22), 26.50 (q, C23), 20.86 (q, C24), 15.73 and 15.76 (qq, C25 and C26), 14.40 (q, C27), 182.79 (s, C28), 14.53 (q, C29), 22.84 (q, C30).

3-Oxolupan-28-oyl chloride (chloroanhydride of dihydrobetulonic acid (II)). Oxalyl chloride (2.3 mL, 26.36 mmol) was added to the solution of dihydrobetulonic acid (**I**) (5.7 g, 12.48 mol) in anhydrous CH_2Cl_2 (70 mL) and the mixture was kept at room temperature for 4 h. After evaporation of the solvent, the same volume of CH_2Cl_2 was added to the residue, and the mixture was re-evaporated. The residue was treated with anhydrous ether, the precipitate was filtered and washed with ether. We obtained 4.6 g (91%) of chloroanhydride of dihydrobetulonic acid with T_m = 206–207°C. $[\alpha]_D^{27}$ +3.9 (*c* 0.41). Found: *m/z* 474.3258 $[M]^+$. $C_{30}H_{47}ClO_2$. Calculated: 474.3264. IR spectrum: 1811 (COCl), 1705 (C=O). ^{1}H NMR spectrum: 0.71 (3 H, d, *J* 7.0, H29); 0.82 (3 H, d, *J* 7.0, H30); 0.91 (3 H, s, H25); 0.93 (3 H, s, H27); 0.95 (3 H, s, H26); 0.99 (3 H, s, H24); 1.04 (3 H, s, H23); 1.07–1.56 (17 H, m, CH_2,CH); 1.63 (1 H, dm, ^2J 12.6, H12e); 1.76 (1 H, septet d, *J* 7.0, $J_{20,19}$ 2.5, H20); 1.89 (1 H, ddd, $J_{1e,1a}$ 13.0, $J_{1e,2a}$ 7.5, $J_{1e,2e}$ 4.3, H1e); 2.04 (1 H, dddd, $J_{19,18a}$ 11.0, *J* 10.5, *J* 4.1, $J_{19,20}$ 2.5, H19); 2.10 (1 H, ddd, ^2J 12.4, *J* 7.2, *J* 1.3, H22α or H22β); 2.19 (1 H, ddd, $J_{13a,12a}$ 13.0, $J_{13a,18a}$ 10.8, $J_{13a,12e}$ 3.8, H13a); 2.37 (1 H, ddd, $J_{2e,2a}$ 15.8, $J_{2e,1a}$ 7.5, $J_{2e,1e}$ 4.3, H2e); 2.48 (1 H, ddd, $J_{2a,2e}$ 15.8, $J_{2a,1a}$ 9.8, $J_{2a,1e}$ 7.5, H2a). ^{13}C NMR: 39.47 (t, C1), 33.96 (t, C2), 217.86 (s, C3), 47.20 (s, C4), 54.83 (d, C5), 19.46 (t, C6), 33.51 (t, C7), 40.53 (s, C8), 49.52 (d, C9), 36.74 (s, C10), 21.20 (t, C11), 26.60 (t, C12), 37.46 (d, C13), 42.52 (s, C14), 29.42 (t, C15), 31.98 (t, C16), 68.15 (s, C17), 49.02 (d, C18), 43.07 (d, C19), 29.47 (d, C20), 22.00 (t, C21), 36.22 (t, C22), 26.44 (q, C23), 20.91 (q, C24), 15.78 (q, C25), 15.60 (q, C26), 14.43 (q, C27), 177.15 (s, C28), 14.50 (q, C29), 22.68 (q, C30).

General method of the synthesis of amides of dihydrobetulonic acid (III)–(VII). The synthesis was carried out similarly to method [15], except that an equivalent excess of the reacting amine was used instead of triethylamine. The corresponding amine (2.2 mol) was added to the solution of chloroanhydride of dihydrobetulonic acid (**II**) (0.5 g, 1.05 mmol) in anhydrous CH_2Cl_2 (40 mL). The reaction mixture was kept at room temperature for 18–20 h, followed by the dilution with CH_2Cl_2 to 20 mL, washing with H_2O (3 × 20 mL), and drying with $MgSO_4$. The desiccant was filtered off, and the solvent was removed. The residue was separated by column chromatography on silica gel (60–200 μm, Merck, Germany). Different eluents were used for each product (see below). ^{13}C NMR data for compounds (**III**) and (**IV**) are presented in Table 5.

(4'-Methylpiperazine-1'-yl)amid of 3-oxolupan-28-oic acid (III). Eluent: CH_2Cl_2-MeOH (80 : 20). Isolated: 0.41 g (72%) of the product with T_m = 241–242°C. $[\alpha]_D^{22}$ –25.7 (*c* 0.20). Found: *m/z* 538.4486 $[M]^+$. $C_{35}H_{58}N_2O_2$. Calculated: 538.4493. IR spectrum: 1632 (CON); 1710 (C=O). ^{1}H NMR spectrum: 0.68 (3 H, d, J 6.8, H29); 0.78 (3 H, d, J 6.8, H30); 0.88 (3 H, s, H25); 0.89 (3 H, s, H27); 0.92 (3 H, s, H26); 0.97 (3 H, s, H24); 1.01 (3 H, s, H23); 1.02–1.15 (3 H, m, H12a, H15e, H22α or H22β); 1.24 (1 H, dd, $J_{18a,13a}$ 10.8, $J_{18a,19}$ 10.8, H18a); 1.27 (1 H, br.d, $J_{5a,6a}$ 11.5, H5a); 1.23–1.49 (12 H, m, CH_2,CH); 1.64 (1 H, dm, $J_{12e,12a}$ 11.0, H12e); 1.73 (1 H, septet d, *J* 6.8, $J_{20,19}$ 2.5, H20); 1.85 (1 H, dd, ^2J 12.0, *J* 6.1, H22α or H22β); 1.87 (1 H, m, H1a or H1e); 2.05 (1 H, ddd, $J_{16e,16a}$ 13.1, $J_{16e,15a}$ 3.6, $J_{16e,15e}$ 3.0, H16e); 2.14 (1 H, dddd, $J_{19,18a}$ 10.8, *J* 10.3, *J* 3.6, $J_{19,20}$ 2.5, H19); 2.25 (3 H, s, H7'); 2.31 (4 H, m, H3',H5'); 2.35 (1 H, m, H2e); 2.44 (1 H, ddd, $J_{2a,2e}$ 15.5, $J_{2a,1a}$ 9.6, $J_{2a,1e}$ 7.6, H2a); 2.91 (1 H, ddd, $J_{13a,12a}$ 12.8, $J_{13a,18a}$ 10.8, $J_{13a,12e}$ 3.6, H13a); 3.56 (4 H, br.s, 2H2',2H6').

(4'-Ethylpiperazine-1'-yl)amid of 3-oxolupan-28-oic acid (IV). Eluent: CH_2Cl_2-MeOH (90 : 10). Isolated: 0.45 g (78%) of the product with T_m = 264–265°C. $[\alpha]_D^{22}$ –23.8 (*c* 0.23). Found (%): C, 78.26; H,10.93; N, 4.91. $C_{36}H_{60}N_2O_2$ (*M* 552.874). Calculated (%): C, 78.21; H, 10.94; N, 5.07. IR spectrum: 1624 (CON); 1701 (C=O). ^{1}H NMR spectrum: 0.67 (3 H, d, *J* 6.8, H29); 0.77 (3 H, d, *J* 6.8, H30); 0.87 (3 H, s, H25); 0.88 (3 H, s, H27); 0.91 (3 H, s, H26); 0.96 (3 H, s, H24); 1.00 (3 H, s, H23); 1.03 (3 H, t, *J* 7.2, H8'); 0.98–1.13 (3 H, m, H12a, H15e, H22α or H22β) 1.23 (1 H, dd, $J_{18a,13a}$ 10.8, $J_{18a,19}$ 10.8, H18a); 1.26 (1 H, br.d, $J_{5a,6a}$ 11.5, H5a); 1.63 (1 H, dm, $J_{12e,12a}$ 11.0, H12e); 1.27–1.47 (12 H, m, CH_2, CH); 1.72 (1 H, septet d, *J* 6.8, $J_{20,19}$ 2.5, H20); 1.81–1.90 (2 H, m, H1, H22); 2.05 (1 H, ddd, $J_{16e,16a}$ 13.1, $J_{16e,15a}$ 3.4,

Table 5. Data of ^{13}C NMR spectra for amides of dihydrobetulonic acid (**III**)–(**VII**) in $CDCl_3$) δ, ppm)

C atom	(**III**)[a]	(**IV**)[a]	(**V**)[a]	(**VI**)[b]	(**VII**)[b]
C1	39.48 t	39.46 t	39.45 t	39.50 t	39.50 t
C2	33.98 t	33.95 t	33.94 t	34.00 t	33.98 t
C3	217.91 s	217.85 s	217.82 s	217.97 s	217.96 s
C4	47.15 s	47.12 s	47.12 s	47.18 s	47.15 s
C5	54.90 d	54.88 d	54.86 d	54.93 d	54.90 d
C6	19.49 t	19.47 t	19.45 t	19.52 t	19.51 t
C7	33.62 t	33.60 t	33.60 t	33.67 t	33.65 t
C8	40.47 s	40.45 s	40.44 s	40.49 s	40.52 s
C9	49.80 d	49.79 d	49.76 d	49.83 d	49.88 d
C10	36.75 s	36.73 s	36.72 s	36.77 s	36.76 s
C11	21.54 t	21.53 t	21.51 t	21.56 t	21.59 t
C12	27.01 t	26.99 t	26.95 t	27.03 t	27.04 t
C13	36.54 d	36.51 d	36.55 d	36.56 d	36.51 d
C14	41.88 s	41.86 s	41.88 s	41.90 s	41.98 s
C15	29.65 t	29.61 t	29.63 t	29.65 t	29.80 t
C16	32.15 t	32.13 t	32.20 t	32.13 t	31.92 t
C17	54.79 s	54.75 s	54.87 s	54.80 s	55.21 s
C18	52.06 d	52.04 d	52.06 d	52.07 d	52.50 d
C19	42.60 d	42.59 d	42.58 d	42.66 d	42.76 d
C20	29.63 d	29.61 d	29.60 d	29.65 d	29.73 d
C21	23.35 t	23.33 t	23.32 t	23.37 t	23.43 t
C22	35.97 t	35.95 t	35.99 t	35.96 t	36.23 t
C23	26.44 q	26.43 q	26.41 q	26.47 q	26.46 q
C24	20.86 q	20.84 q	20.84 q	20.90 q	20.84 q
C25	15.78 q	15.76 q	15.75 q	15.80 q	15.80 q
C26	15.77 q	15.76 q	15.74 q	15.80 q	15.74 q
C27	14.26 q	14.24 q	14.25 q	14.28 q	14.34 q
C28	173.50 s	173.41 s	173.85 s	173.47 s	173.88 s
C29	14.51 q	14.49 q	14.47 q	14.52 q	14.56 q
C30	22.77 q	22.75 q	22.73 q	22.79 q	22.82 q
C3', C5'	55.06 t	52.90 t	43.64 t	52.16 t	
C7'	45.76 q	52.08 q	155.34 s	76.12 d	
C8'		11.73 q	61.38 t	142.26 s	
C9'			14.45 q	126.66d	

[a] Very broad signals (~45 ppm) are observed for C2' and C6' atoms of compounds (**III**)–(**V**);

[b] Signals of C2' and C6' of compound (**VI**) and C2' and C7' of compound (**VII**) are not observed; chemical shifts of signals of aromatic part in compound (**VI**) are 128.43 (d, C10') and 126.94 (d, C11') ppm.

$J_{16e,15e}$ 3.0, H16e); 2.13 (1 H, dddd, $J_{19,18a}$ 10.7, J 10.0, J 3.8, $J_{19,20}$ 2.7, H19); 2.25–2.38 (5 H, m, H2e, 2H3', 2H5'); 2.34 (2 H, q, $J_{7',8'}$ 7.2, 2H7'); 2.43 (1 H, ddd, $J_{2a,2e}$ 15.7, $J_{2a,1a}$ 9.6, $J_{2a,1e}$ 7.6, H2a); 2.91 (1 H, ddd, $J_{13a,12a}$ 12.8, $J_{13a,18a}$ 10.7, $J_{13a,12e}$ 3.8, H13a); 3.56 (4 H, br.s, 2H2', 2H6').

(4'-Ethoxycarbonylpiperazine-1'-yl)amid of 3-oxo-lupan-28-oic acid (V). Eluent: CH_2Cl_2. Isolated: 0.39 g (63%) of the product with T_m = 185–186°C. $[\alpha]_D^{22}$ – 82.0 (*c* 0.20). Found: *m/z* 596.4551 $[M]^+$. $C_{37}H_{60}N_2O_4$. Calculated: 596.4548. IR spectrum: 1632 (CON); 1707 (C=O). ^1H NMR spectrum: 0.68 (3 H, d, J 6.8, H29); 0.78 (3 H, d, J 6.8, H30); 0.88 (3 H, s, H25); 0.89 (3 H, s, H27); 0.91 (3 H, s, H26); 0.96 (3 H, s, H24); 1.01 (3 H, s, H23); 1.03–1.16 (3 H, m, H12a,

H15e, H22); 1.21 (3 H, t, J 7.1, 3H9'); 1.25 (1 H, dd, $J_{18a,13a}$ 10.6, $J_{18a,19}$ 10.6, H18a); 1.26 (1 H, dd, $J_{5a,6a}$ 11.5, $J_{5a,6e}$ 2.5, H5a); 1.28–1.51 (12 H, m, CH_2,CH); 1.63 (1 H, dm, $J_{12e,12a}$ 11.0, H12e); 1.73 (1 H, septet d, H20, J 6.8, $J_{20,19}$ 2.6); 1.81 (1 H, dd, ^2J 11.7, J 6.3, H22α or H22β); 1.86 (1 H, ddd, $J_{1e,1a}$ 13.2, $J_{1e,2a}$ 7.6, $J_{1e,2e}$ 4.4, H1e); 2.02 (1 H, ddd, $J_{16e,16a}$ 13.2, $J_{16e,15a}$ 3.5, $J_{16e,15e}$ 3.0, H16e,); 2.13 (1 H, dddd, $J_{19,18a}$ 10.6, J 10.2, J 3.6, $J_{19,20}$ 2.6, H19,); 2.34 (1 H, ddd, $J_{2e,2a}$ 15.8, $J_{2e,1a}$ 7.5, $J_{2e,1e}$ 4.4, H2e); 2.44 (1 H, ddd, $J_{2a,2e}$ 15.8, $J_{2a,1a}$ 9.7, $J_{2a,1e}$ 7.6, H2a); 2.88 (1 H, ddd, $J_{13a,12a}$ 13.0, $J_{13a,18a}$ 10.6, $J_{13a,12e}$ 3.7, H13a,); 3.38 (4 H, m, 2H3', 2H5'); 3.52 (4 H, br.s, 2H2', 2H6'); 4.09 (2 H, q, J 7.1, 2H8').

(4'-Diphenylmethylpiperazine-1'-yl)amid of 3-oxolupan-28-oic acid (VI). Eluent: $CHCl_3$. Isolated: 0.68 g (93%) of the product with T_m = 146–147°C. $[\alpha]_D^{22}$ –20.7 (c 0.21). Found: m/z 690.5098 $[M]^+$. $C_{47}H_{66}N_2O_2$. Calculated: 690.5119. IR spectrum: 1630 (CON); 1704 (C=O). ^{1}H NMR spectrum: 0.70 (3 H, d, J 6.8, H29); 0.81 (3 H, d, J 6.8, H30); 0.909 (3 H, s, H25); 0.911 (3 H, s, H27); 0.95 (3 H, s, H26); 1.00 (3 H, s, H24); 1.05 (3 H, s, H23); 1.02–1.14 (3 H, m, H12a, H15e, H22); 1.25 (1 H, dd, $J_{18a,13a}$ 10.7, $J_{18a,19}$ 10.7, H18a); 1.30 (1 H, dd, $J_{5a,6a}$ 11.5, $J_{5a,6e}$ 2.6, H5a,); 1.30–1.51 (12 H, m, CH_2, CH); 1.66 (1 H, dm, $J_{12e,12a}$ 11.0, H12e); 1.76 (1 H, septet d, J 6.8, $J_{20,19}$ 2.4, H20); 1.85 (1 H, br.dd, ^2J 11.7, J 6.1, H22α or H22β,); 1.89 (1 H, ddd, $J_{1e,1a}$ 13.2, $J_{1e,2a}$ 7.6, $J_{1e,2e}$ 4.4, H1e); 2.05 (1 H, dm, $J_{16e,16a}$ 12.8, H16e); 2.17 (1 H, m, H19); 2.31 (4 H, br.s, 2H3', 2H5'); 2.38 (1 H, ddd, $J_{2e,2a}$ 15.6, $J_{2e,1a}$ 7.3, $J_{2e,1e}$ 4.4, H2e); 2.47 (1 H, ddd, $J_{2a,2e}$ 15.6, $J_{2a,1a}$ 9.7, $J_{2a,1e}$ 7.6, H2a); 2.94 (1 H, ddd, $J_{13a,12a}$ 12.8, $J_{13a,18a}$ 10.7, $J_{13a,12e}$ 3.5, H13a); 3.57 (4 H, br.s, 2H2', 2H6'); 4.17 (1 H, s, H7'); 7.15 (2 H, t, J 7.5, 2H11'); 7.25 (4 H, t, J 7.5, 4H10'); 7.39 (4 H, d, J 7.5, 4H9').

(Homopiperidin-1'-yl)amid of 3-oxolupan-28-oic acid (VII). Eluent: $CHCl_3$. Isolated: 0.37 g (65%) of the product with T_m = 242–243°C. $[\alpha]_D^{22}$ –38.0 (c 0.20). Found: m/z 537.4544 $[M]^+$. $C_{36}H_{59}NO_2$. Calculated: 537.4540. IR spectrum: 1622 (CON); 1709 (C=O). ^{1}H NMR spectrum: 0.69 (3 H, d, J 6.8, H29); 0.78 (3 H, d, J 6.8, H30); 0.88 (3 H, s, H25); 0.90 (3 H, s, H27); 0.94 (3 H, s, H26); 0.96 (3 H, s, H24); 1.01 (3 H, s, H23); 1.00–1.13 (3 H, m, H12a, H15e, H22α or H22β); 1.22 (1 H, dd, $J_{18a,13a}$ 10.8, $J_{18a,19}$ 10.8, H18a); 1.27 (1 H, dd, $J_{5a,6a}$ 11.2, $J_{5a,6e}$ 2.5, H5a); 1.28–1.49 (12 H, m, CH_2, CH); 1.71 (1 H, septet d, J 6.8, $J_{20,19}$ 2.5, H20); 1.54–1.76 (4 H, m, CH_2, CH); 1.86 (1 H, m, H1); 1.88 (1 H, dd, ^2J 11.7, J 6.1, H22α or H22β); 2.14 (1 H, dm, $J_{16e,16a}$ 12.6, H16e); 2.17 (1 H, m, H19); 2.35 (1 H, ddd, $J_{2e,2a}$ 15.7, $J_{2e,1a}$ 7.5, $J_{2e,1e}$ 4.3, H2e); 2.44 (1 H, ddd, $J_{2a,2e}$ 15.7, $J_{2a,1a}$ 9.6, $J_{2a,1e}$ 7.6, H2a); 3.02 (1 H, ddd, $J_{13a,12a}$ 12.8, $J_{13a,18a}$ 10.8, $J_{13a,12e}$ 3.6, H13a); 3.10–3.8 (4 H, m, 2H2', 2H7').

Antitumor activity of compounds (I), (III)–(VII) in vitro. The T-cell leukemia cell lines CEM-13 and U-937 and the tumor TM-4 cell line of human monocytes were cultured in RPMI 1640 containing 10% fetal calf serum and antibiotics (100 U/mL of penicillin and 0.1 mg/mL of streptomycin) in 5% CO_2 at 37°C.

The viability of cells after the incubation with compounds (**I**), (**III**)–(**VII**) was evaluated by the MTT test, which allows for the spectrophotometric determination of the amount of living cells in a sample. For this, the cells were planted in 96-well plates (100 μL of cells at a concentration of 500000 cells/mL), followed by the addition of the solution of compounds (**I**), (**III**)–(**VII**) in DMSO to the final concentration in the medium of 0.1–100 μg/mL, and incubated with these compounds for three days under the same conditions. The MTT solution (5 mg/mL) in phosphate-saline buffer was then added to the cells without changing the medium to a concentration of 0.5 mg/mL, and the incubation continued for 3 h under the same conditions. The medium was removed, DMSO (100 μL) was added, and the optical absorption was measured on a multichannel spectrophotometer at 570 nm and 630 nm, where A_{570} is the absorption of formazan and A_{630} is the background of cells. The data are presented as the amount of living cells vs. the control. The number of cells in the control was 100% when cells were incubated only in the presence of the solvent (DMSO).

Antitumor and antimetastatic activity of compounds (III) and (IV) in vivo. The study was performed on mice with transplanted Lewis lung carcinoma in two series of experiments: the agents were introduced in the early or late stages of cancer.

We used female mice of the C57BL/6 line weighing 20–25 g, which were kept under normal vivarium conditions with natural light regime and fed with a standard feed and water. All manipulations with animals were carried out in accordance with the European Convention on humane treatment of laboratory animals. Transplantation of Lewis lung carcinoma cells was performed intramuscularly (2×10^6 cells in 0.1 mL of physiological solution). Measurement of each tumor nodule was performed with a caliper in three mutually perpendicular directions. The antitumor activity was expressed as an index of tumor growth inhibition (TGI), which was defined as the ratio of the difference of the tumor mass in the control and experimental groups to the tumor mass in the control. Each group contained at least 10 mice.

In the first experimental series, amides of dihydrobetulonic acid (**III**) and (**IV**) were administered intragastrically as the water-tween suspension in a dose of 20 mg/kg for 8 days, starting from the third day after the tumor transplantation (the total dose was 160 mg/kg). The control group of animals with the tumor intragastrically received the water-tween mixture. The dynamics of the growth of tumor transplants

was evaluated from the day after withdrawal of the tested compounds or from 11 to 18 days after the transplantation (before the mass deaths of animals in all groups). The state of the animals was assessed by the length of life span during the whole experiment, which was calculated as the average per animal.

In the second experimental series, the administration of the agents was carried out on 11th day after the transplantation of Lewis lung carcinoma. Each of the agents was administered into two groups of animals, one of which received cytostatic polychemotherapy (PCT) a day before, while the other remained untreated. Compounds (**III**) and (**IV**) were administered intragastrically in a dose of 20 mg/kg as the water-tween suspension for 8 days. When using PCT, the standard scheme was used adapted to laboratory animals: doxorubicin in a dose of 4 mg/kg intravenously, cyclophosphan, vincristine, and prednisolone intraperitoneally in doses of 50, 0.4 and 5 mg/kg, respectively [6]. The control and the PCT group received the equivalent amount of water with tween-80 in the same periods of time. The size of tumor transplants was measured during the period of administration of agents; after the administration the percentage of dead animals was evaluated. After the administration of compounds (**III**) and (**IV**), the remaining animals were sacrificed by the dislocation; the lungs were removed and studied by light microscopy after standard histological processing. The antimetastatic effect was evaluated by the morphometric analysis of sections of both lungs. The volume density (Vv% of metastases was calculated by the Avtandilov method [16] using an ocular grid (289 points). The intensity of the process of metastasis was assessed by the metastasis frequency (MF) (the ratio of the number of animals with metastases to the total number of animals per group) and metastasis inhibition index (MII):

$$\frac{(A_cB_c) - (AB)}{(A_cB_c)} \times 100\%,$$

where A_c and A are MF in the control and experimental groups, respectively; B_c and B are the metastasis density in the control and experimental groups, respectively.

ACKNOWLEDGMENTS

The work was supported by the Interdisciplinary integration project no. 41 of the Siberian Branch of the Russian Academy of Sciences.

REFERENCES

1. Tolstikova, T.G., Sorokina, I.V., Tolstikov, G.A., Tolstikov, A.G., and Flekhter O.B, *Russ. J. Bioorg. Chem.*, 2006, vol. 32, no. 3, pp. 261–276.
2. Shintyapina, A.B., Shults, E.E., Petrenko, N.I., Uzenkova, N.V., Tolstikov, G.A., Pronkina, N.V., Kozhevnikov, V.S., and Pokrovsky, A.G., *Russ. J. Bioorg. Chem.*, 2007, vol. 33, pp. 579–583.
3. Pokrovskii, A.G., Shintyapina, A.B., Pronkina, N.V., Kozhevnikov, V.S., Plyasunova, O.A., Shul'ts, E.E., and Tolstikov, G.A., *Dokl. Biochem. Biophys.*, 2006, vol. 407, pp. 94–97.
4. Sorokina, I.V., Tolstikova, T.G., Zhukova, N.A., Petrenko, N.I., Shul'ts, E.E., Uzenkova, N.V., Grek, O.R., Pozdnyakova, S.V., and Tolstikov, G.A., *Dokl. Akad. Nauk SSSR*, 2004, vol. 399, pp. 274–277.
5. Sorokina, I.V., Zhukova, N.A., Tolstikova, T.G., Pozdnyakova, S.V., Grek, O.R., Popova, N.A., Kaledin, V.I., and Nikolin, V.P., *Vopr. Biol. Med. Farm. Khim.,* 2006, pp. 29–31.
6. Zhukova, N.A., Semenov, D.E., Sorokina, I.V., Tolstikova, T.G., Pozdnyakova, S.V., and Grek, O.R., *Byull. Eksp. Biol. Med.*, 2005, vol. 14, pp. 348–351.
7. Pozdnyakova, S.V., Grek, O.R., Funtikov, A.S., Danil'cheva, T.V., Zhukova, N.A., Sorokina, I.V., and Tolstikova, T.G., *Byull. SO RAMN*, 2007, vol. 127, pp. 117–121.
8. Zhukova, N.A., Sorokina, I.V., Tolstikova, T.G., Lushnikova, E.L., Nepomnyashchikh, L.M., and Semenov, D.E., *Byull. Eksp. Biol. Med.*, 2010, vol. 150, pp. 108–112.
9. Mukherjee, R., Kumar, V., Srivastava, S.K., Agarwal, S.K., and Burman, A.C., *Anti-cancer Agents Med. Chem.*, 2006, vol. 6, pp. 271–279.
10. Kashiwada, Y., Sekiya, M., Ikeshiro, Y., Fujioka, T., Kilgore, N.R., Wild, C.T., Allaway, G.P., and Lee, K.-H., *Bioorg. Med. Chem. Lett.*, 2004, vol. 14, pp. 5851–5853.
11. Rajendran, P., Jaggi, M., Singh, M.K., Mukherjee, R., and Burman, A.C., *Invest. New Drugs*, 2008, vol. 26, pp. 25–34.
12. Laszczyk, M.N., *Planta Med.*, 2009, vol. 75, pp. 1549–1560.
13. Krasutsky, P.A., *Nat. Prod. Rep.*, 2006, vol. 23, pp. 919–942.
14. Ruzicka, L., Brener, M., and Rey, E., *Helv. Chim. Acta,* 1941, vol. 24, pp. 515–529.
15. Dzhemilev, U.M., Tolstikov, G.A., Pokrovsky, A.G., Salakhutdinov, N.F., Tolstikova, T.G., and Shults, E.E., US Patent No. US2010/87539 A1, 2010.
16. Avtandilov, G.G., *Meditsinskaya morfometriya. Rukovodstvo* (Medical Morphometry: A Guide), Moscow: Meditsina, 1990.