Acta Poloniae Pharmaceutica – Drug Research, Vol. 70 No. 5 pp. 823–832, 2013 ISSN 0001-6837
Polish Pharmaceutical Society

NEW PYRIDOCARBAZOLE DERIVATIVES. SYNTHESIS AND THEIR *IN VITRO* ANTICANCER ACTIVITY

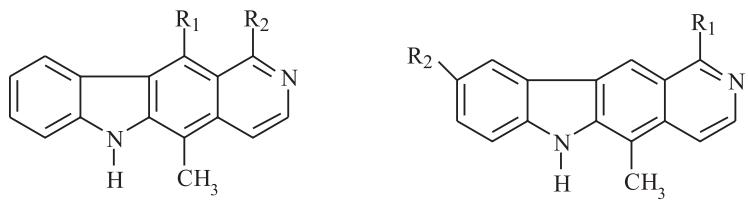
RYSZARD JASZTOLD-HOWORKO[1], BEATA TYLIŃSKA[1*], BOGUSŁAWA BIADUŃ[1], TOMASZ GĘBAROWSKI[2] and KAZIMIERZ GĄSIOROWSKI[2]

[1]Department of Organic Chemistry, [2]Department of Basic Medical Sciences, Wrocław Medical University, Faculty of Pharmacy, Borowska 211, 50-556 Wrocław, Poland

Abstract: In this paper, we describe our results of the synthesis and biological testing of analogues of the natural alkaloids olivacine and ellipticine. We have synthesized fourteen new 1-substituted pyrido[4,3-b]carbazole derivatives. All of them were tested *in vitro* for their anticancer activity on three human tumor cell lines: CCRF/CEM (T lymphoblast leukemia), A549 (lung adenocarcinoma), and MCF7 (breast cancer). Cytotoxicity to non-cancer cells was estimated in cultures of the mice fibroblast cell line 3T3 BALB. The anticancer activity of 9-methoxy-5,6-dimethyl-1-[(1,1-bis-hydroxymethyl-propylamino)-methyl]-6*H*-pyrido[4,3-b]carbazole (compound **9**) was the strongest amongst compounds tested on the three cancer cell lines; it was about 5 times higher than ellipticine and about 10% higher than doxorubicin.

Keywords: olivacine, ellipticine, pyridocarbazole, cytotoxic, 3T3 BALB, CCRF/CEM, A549, MCF7

Researchers looking for effective cancer treatment are often interested in natural alkaloids with cytostatic properties such as olivacine (1,5-dimethyl-6*H*-pyrido[4,3-b]carbazole) (**1**) and ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-b]carbazole) (**2**) (Fig. 1). Olivacine was isolated from *Aspidosperma olivaceum* Müll. Arg. (1) in 1958 and ellipticine was isolated from *Ochrosia elliptica* Labill. (2) in 1959. Their anticancer activity was discovered in the 1960s (3-5). To date, scientists have synthesized a number of derivatives of these natural alkaloids. Some of them exhibit improved antitumor properties. The first synthetically obtained derivatives of ellipticine which entered clinical trials were elliptinium and celiptium (6-10). 9-Hydroxy-5,6-dimethyl-*N*-[2-(dimethylamino)ethyl]-6*H*-pyrido(4,3-b)-carbazole-1-carboxamide (S 16020-2) was a derivative of olivacine which was selected for clinical trials (11, 12). Based on previous studies (13, 14) we synthesized new olivacine derivatives (**3**) (Fig. 1). In this paper, we describe our results of the synthesis and biological properties testing of newly obtained 1-substituted pyrido[4,3-b]carbazoles in cultures of normal mouse 3T3 fibroblasts and 3 human cancer cell lines. Three of the tested derivatives (**9, 12, 16**) showed stronger antitumor properties in *in vitro* tests when compared to the reference drug ellipticine, whereas two of our compounds (**15, 17**) were not active in our biological tests.



1 Olivacine $R_1 = H$, $R_2 = CH_3$ **3**

2 Ellipticine $R_1 = CH_3$, $R_2 = H$

Figure 1. Olivacine, ellipticine and new olivacine derivatives

* Corresponding author: e-mail: beata.tylinska@umed.wroc.pl; phone: +48 71 7840345

EXPERIMENTAL

All melting points were determined on an Electrothermal Melting Point Apparatus Model 9100 and were uncorrected. ^{1}H NMR spectra were recorded on a Bruker 300 at 300.14 MHz (Bruker, Rheinstetten, Germany), using TMS as the internal standard. Column chromatography was carried out on silica gel (Merck Kieselgel 100; Merck, Darmstadt, Germany). All of the newly obtained compounds were analyzed for C, H, and N and the analytical results were within ±0.4% of the theoretical values. The starting compound 5,6-dimethyl-1-formyl-9-methoxy-6*H*-pyrido[4,3-b]carbazole (**4**) (Scheme 1) was prepared according to a known procedure (15).

Chemistry

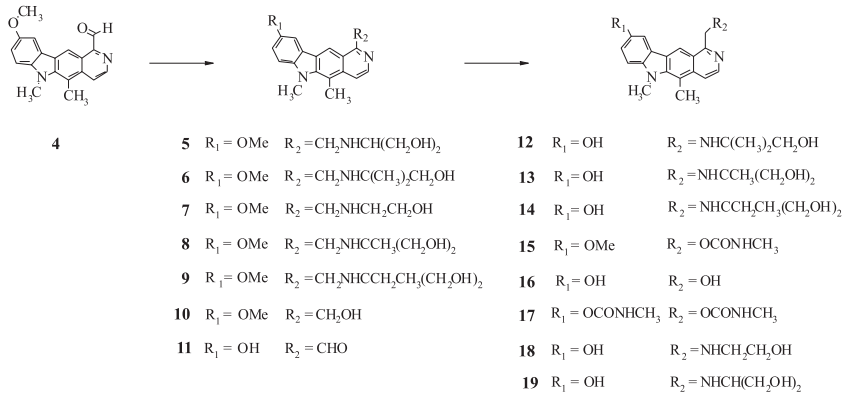
General procedure for the synthesis of 5,6-dimethyl-9-methoxy-6H-pyrido[4,3-b]carbazole derivatives 5-9

The mixture of 5,6-dimethyl-1-formyl-9-methoxy-6*H*-pyrido[4,3-b]carbazole (**4**) (0.91 g, 3 mmol), toluene-4-sulfonic acid (0.86 g, 5 mmol), appropriate amine (5 mmol): (2-amino-1,3-propanediol or 2-amino-2-methyl-1-propanol or 2-aminoethanol or 2-amino-2-methyl-1,3-propanediol or 2-amino-2-ethyl-1,3-propanediol) and toluene (300 mL) was refluxed for 4 h. After this time, the solvent was evaporated to 1/3 volume. Sodium borohydride (0.91 g) was added to the residue and the mixture was stirred for 24 h. Next, the solvent was evaporated, the solid was dissolved in water (100 mL), and hydrochloric acid was added to pH = 4. After 0.5 h, conc. aq. ammonia (or sodium hydrogen carbonate) was added to the mixture to pH = 9. The residue was extracted with chloroform and the extract was dried with magnesium sulfate. After evaporation of solvent, the solid residue was purified by chromatography on a silica gel column with chloroform : methanol, in an appropriate proportion.

5 Yield: 48%, m.p.: 188-190°C (chloroform : methanol 95 : 5, v/v). ^{1}H NMR (DMSO-d_6, δ, ppm): 3.09 (s, 3H, 5-CH_3), 3.50 (s, 1H, CH), 3.90 (s, 4H, 2×-CH_2), 3.86 (s, 3H, 9-OCH_3), 4.17 (s, 3H, 6-CH_3), 5.24 (s, 2H, 1-CH_2), 7.25 (dd, $J_{8\text{-}1}$ = 2.4 Hz, $J_{8\text{-}7}$ = 8.7 Hz, 1H, 8-H), 7.61 (d, $J_{7\text{-}8}$ = 8.7 Hz, 1H, 7-H), 8.12 (d, $J_{10\text{-}8}$ = 2.4 Hz, 1H, 10-H), 8.25 (d, $J_{4\text{-}3}$ = 6.2 Hz, 1H, 4-H), 8.44 (d, $J_{3\text{-}4}$ = 6.2 Hz, 1H, 3-H), 9.31 (s, 1H, 11-H), 9.45 (s, 1H, NH). Analysis: calcd. for $C_{22}H_{25}N_3O_3$: C, 69.64; H, 6.64; N, 11.07%; found C, 69.23; H, 6.95; N, 10.68%.

6 Yield: 40%, m.p.: 245°C (chloroform : methanol 9 : 1, v/v). ^{1}H NMR (DMSO-d_6, δ, ppm): 1.44 (s, 6H, 2×CH_3), 3.12 (s, 3H, 5-CH_3), 3.69 (s, 2H, -CH_2OH), 3.91 (s, 3H, 9-OCH_3), 4.19 (s, 3H, 6-CH_3), 5.06 (s, 2H, 1-CH_2), 7.26 (dd, $J_{8\text{-}10}$ = 2.3 Hz, $J_{8\text{-}7}$ = 8.7 Hz, 1H, 8-H), 7.63 (d, $J_{7\text{-}8}$ = 8.7 Hz, 1H, 7-H), 8.10 (d, $J_{10\text{-}8}$ = 2.4 Hz, 1H, 10-H), 8.21 (d, $J_{4\text{-}3}$ = 6.2 Hz, 1H, 4-H), 8.44 (d, $J_{3\text{-}4}$ = 6.2 Hz, 1H, 3-H), 9.24 (s, 2H, 11-H + NH). Analysis: calcd. for $C_{23}H_{27}N_3O_2$: C, 73.18; H, 7.21; N, 11.13%; found C, 72.97; H, 7.28; N, 10.98%.

7 Yield: 35%, m.p.: 244-245°C (chloroform : methanol 95 : 5, v/v). ^{1}H NMR (DMSO-d_6, δ, ppm): 3.04 (s, 3H, 5-CH_3), 3.22 (s, 2H, α-CH_2), 3.73 (s, 2H, β-CH_2), 3.94 (s, 3H, 9-OCH_3), 4.19 (s, 3H, 6-CH_3), 4.21 (s, 2H, 1-CH_2), 5.30 (s, 1H, OH), 7.23



Scheme 1. Synthesis of 1-substituted pyrido[4,3-b]carbazole derivatives **5-19**

(dd, J_{8-10} = 2.4 Hz, J_{8-7} = 8.7 Hz, 1H, 8-H), 7.62 (d, J_{10-8} = 2.4 Hz, 1H, 10-H), 7.70 (d, J_{7-8} = 8.7 Hz, 1H, 7-H), 8.51 (d, J_{4-3} = 6.2 Hz, 1H, 4-H), 8.75 (d, J_{3-4} = 6.2 Hz, 1H, 3-H), 9.17 (s, 1H, 11-H), 9.33 (s, 1H, NH). Analysis: calcd. for $C_{21}H_{23}N_3O_2$: C, 72.18; H, 6.63; N, 12.03%; found C, 71.86; H, 6.40; N, 12.23%.

8 Yield: 65%, m.p.: 188-189°C (chloroform : methanol 9 : 1, v/v). ^{1}H NMR (DMSO-d_6, δ, ppm): 1.16 (s, 3H, -CH_3), 3.06 (s, 3H, 5-CH_3), 3.25 (s, 2H, 2×-OH), 3.55 (s, 4H, 2×-CH_2), 3.89 (s, 3H, 9-OCH_3), 4.13 (s, 3H, 6-CH_3), 4.64 (s, 2H, 1-CH_2), 7.20 (dd, J_{8-10} = 2.4 Hz, J_{8-7} = 8.8 Hz, 1H, 8-H), 7.54 (d, J_{7-8} = 8.8 Hz, 1H, 7-H), 7.94 (m, 2H, 10-H + 4-H), 8.33 (d, J_{3-4} = 6.2 Hz, 1H, 3-H), 9.02 (s, 1H, 11-H), 9.15 (s, 1H, NH). Analysis: calcd. for $C_{23}H_{27}N_3O_3$: C, 70.21; H, 6.92; N, 10.68%; found C, 70.51; H, 7.12; N, 10.46%.

9 Yield: 25%, m.p.: 113-114°C (chloroform : methanol 9 : 1, v/v). ^{1}H NMR (DMSO-d_6, δ, ppm): 0.81 (m, 3H, -CH_3), 1.75 (m, 2H CH_2), 2.97 (s, 3H, 5-CH_3), 3.23 (s, 2H, 2×-OH), 3.69 (s, 4H, 2×-CH_2), 3.93 (s, 3H, 9-OCH_3), 4.17 (s, 3H, 6-CH_3), 4.21 (s, 2H, 1-CH_2), 5.37 (m, 1H, NH), 7.11 (dd, J_{8-10} = 2.3 Hz, J_{8-7} = 8.7 Hz, 1H, 8-H), 7.79 (d, J_{7-8} = 8.7 Hz, 1H, 7-H), 7.79 (d, J_{10-8} = 2.4 Hz, 1H, 10-H), 8.53 (d, J_{4-3} = 6.2 Hz, 1H, 4-H), 8.73 (d, J_{3-4} = 6.2 Hz, 1H, 3-H), 9.37 (s, 1H, 11-H). Analysis: calcd. for $C_{24}H_{29}N_3O_3$: C, 70.74; H, 7.17; N, 10.31%; found C, 70.94; H, 7.24; N, 10.39%.

General procedure for the synthesis of 5,6-dimethyl-9-hydroxy-6H-pyrido[4,3-b]carbazole derivatives 11-14, 16

Compound **4** (0.21, 0.7 mmol) or **6** (0.26 g, 0.7 mmol) or **8** (0.27 g, 0.7 mmol) or **9** (0.28 g, 0.7 mmol) or **10** (0.21 g, 0.7 mmol) was dissolved in 150 mL of methylene chloride, chilled to -70°C and boron tribromide (15 mL) was added dropwise to the reaction mixture at this temperature. Then, the mixture was stirred under nitrogen at normal pressure for 2 h, maintaining the temperature of -70°C, additionally stirred at room temperature for 12 h and finally evaporated to dryness. The residue was taken up in water (100 mL), with appropriate addition of methanol to dissolve solid, basified with conc. aq. ammonia (or sodium hydrogen carbonate), extracted with methylene chloride, and the extract was dried with magnesium sulfate. After evaporation of the solvent, the solids remained were purified by chromatography on a silica gel column with chloroform : methanol, in an appropriate proportion.

11 Yield: 42%, m.p.: 276-277°C (chloroform : methanol 8 : 2, v/v). ^{1}H NMR (DMSO-d_6, δ, ppm): 3.03 (s, 3H, 5-CH_3), 4.09 (s, 3H, 6-CH_3), 7.06 (dd, J_{8-10} = 2.4 Hz, J_{8-7} = 8.7 Hz, 1H, 8-H), 7.43 (d, J_{7-8} = 8.7 Hz, 1H, 7-H), 7.53 (d, J_{10-8} = 2.4 Hz, 1H, 10-H), 8.01 (d, J_{4-3} = 6.2 Hz, 1H, 4-H), 8.31 (d, J_{3-4} = 6.2 Hz, 1H, 3-H), 9.10 (s, 1H, 11-H), 9.34 (s, 1H, 9-OH), 10.30 (s, 1H, 1-CHO). Anal. calcd. for $C_{18}H_{14}N_2O_2$: C, 74.47; H 4.86; N, 9.65%; found C, 74.26; H 4.96; N, 9.80%.

12 Yield: 42%, m.p.: 227°C (chloroform : methanol 9 : 1, v/v). ^{1}H NMR (DMSO-d_6, δ, ppm): 1.21 (s, 6H, 2×-CH_3), 3.04 (s, 3H, 5-CH_3), 3.34 (s, 1H, 1-OH), 3.42 (s, 2H, -CH_2OH), 4.10 (s, 3H, 6-CH_3), 4.59 (s, 2H, 1-CH_2), 5.21 (m, 1H, NH), 7.07 (dd, J_{8-10} = 2.3 Hz, J_{8-7} = 8.7 Hz, 1H, 8-H), 7.43 (d, J_{7-8} = 8.7 Hz, 1H, 7-H), 7.72 (d, J_{10-8} = 2.4 Hz, 1H, 10-H), 7.93 (d, J_{4-3} = 6.2 Hz, 1H, 4-H), 8.32 (d, J_{3-4} = 6.2 Hz, 1H, 3-H), 8.92 (s, 1H, 11-H), 9.21 (s, 1H, 9-OH). Analysis: calcd. for $C_{22}H_{25}N_3O_2$: C, 72.70; H, 6.93; N, 11.56%; found C, 72.38; H, 7.14; N, 11.37%.

13 Yield: 35%, m.p.: 199-200°C (chloroform : methanol 9 : 1, v/v). ^{1}H NMR (DMSO-d_6, δ, ppm): 1.11 (s, 3H, -CH_3), 3.03 (s, 3H, 5-CH_3), 3.34 (s, 2H, 2×-OH), 3.60 (s, 4H, 2×-CH_2), 4.09 (s, 3H, 6-CH_3), 4.51 (s, 2H, 1-CH_2), 5.37 (m, 1H, NH), 7.05 (dd, J_{8-10} = 2.2 Hz, J_{8-7} = 8.7 Hz, 1H, 8-H), 7.42 (d, J_{7-8} = 8.7 Hz, 1H, 7-H), 7.71 (d, J_{10-8} = 2.2 Hz, 1H, 10-H), 7.90 (d, J_{4-3} = 6.2 Hz, 1H, 4-H), 8.33 (d, J_{3-4} = 6.2 Hz, 1H, 3-H), 8.93 (s, 1H, 11-H), 9.28 (s, 1H, 9-OH). Analysis: calcd. for $C_{22}H_{25}N_3O_3$: C, 69.64; H, 6.64; N, 11.07%; found C, 69.38; H 6.75; N, 11.18%.

14 Yield: 41%, m.p.: >300°C (chloroform : methanol 9 : 1, v/v). ^{1}H NMR (DMSO-d_6, δ, ppm): 0.78 (m, 3H, -CH_3), 1.79 (m, 2H CH_2), 2.97 (s, 3H, 5-CH_3), 3.21 (s, 2H, 2×-OH), 3.70 (s, 4H, 2×-CH_2), 4.15 (s, 3H, 6-CH_3), 4.17 (s, 2H, 1-CH_2), 5.30 (m, 1H, NH), 7.10 (m, 1H, 8-H), 7.49 (m, 1H, 7-H), 7.63 (s, J_{10-8} = 2.2 Hz, 1H, 10-H), 8.51 (d, J_{4-3} = 6.2 Hz, 1H, 4-H), 8.73 (d, J_{3-4} = 6.2 Hz, 1H, 3-H), 9.33 (s, 1H, 11-H,) 9.42 (s, 1H, 9-OH). Analysis: calcd. for $C_{23}H_{27}N_3O_3$: C, 70.21; H, 6.92; N, 10.68%; found C, 69.96; H, 6.72; N, 10.79%.

16 Yield: 84%, m.p.: >300°C (chloroform : methanol 9 : 1, v/v). ^{1}H NMR (DMSO-d_6, δ, ppm): 3.05 (s, 3H, 5-CH_3), 4.10 (s, 3H, 6-CH_3), 5.18 (s, 2H, 1-CH_2), 5.42 (s, 1H, 1-OH), 7.06 (dd, J_{8-10} = 2.2 Hz, J_{8-7} = 8.7 Hz, 1H, 8-H), 7.43 (d, J_{7-8} = 8.7 Hz, 1H, 7-H), 7.65 (d, J_{10-8} = 2.2 Hz, 1H, 10-H), 7.95 (d, J_{4-3} = 6.3 Hz, 1H, 4-H), 8.31 (d, J_{3-4} = 6.3 Hz, 1H, 3-

H) 8.86 (s, 1H, 11-H), 9.22 (s, 1H, 9-OH). Analysis: calcd. for $C_{18}H_{16}N_2O_2$: C, 73.96; H, 5.52; N, 9.58%; found C, 73.76; H 5.59; N, 9.66%.

General procedure for the synthesis of 5,6-dimethyl-1-hydroxy-6H-pyrido[4,3-b]carbazole derivatives 18, 19

A mixture of 5,6-dimethyl-1-formyl-9-hydroxy-6*H*-pyrido[4,3-b]carbazole **11** (0.14 g, 0.05 mmol), appropriate amine (2 mmol of ethanolamine or 2-amino-1,3-propanediol) and 200 mL of toluene was refluxed with stirring for 4 h. After evaporation to dryness, the residue was taken up to ethanol (100 mL), basified with sodium borohydride and the mixture was stirred for 24 h. Next, the solvents were evaporated, the solid residue was dissolved in water (100 mL), and hydrochloric acid was added to pH = 4. After 0.5 h, sodium hydrogen carbonate was added to the mixture to pH = 9. The solution was extracted with chloroform and the extract was dried with magnesium sulfate. After evaporation of solvent, the solid residue was purified by chromatography on a silica gel column with chloroform : methanol 9:1, v/v.

18 Yield: 35%, m.p : 238°C. ^{1}H NMR (DMSO-d_6, δ, ppm): 3.06 (s, 3H, 5-CH_3), 3.17 (s, 2H, α-CH_2), 3.77 (s, 2H, β-CH_2), 4.12 (s, 3H, 6-CH_3), 4.97 (s, 2H, 1-CH_2), 5.44 (s, 2H, 2×OH), 7.07 (dd, $J_{8\text{-}10}$ = 2.4 Hz, $J_{8\text{-}7}$ = 8.7 Hz, 1H, 8-H), 7.46 (d, $J_{7\text{-}8}$ = 8.7 Hz, 1H, 7-H), 7.71 (d, $J_{10\text{-}8}$ =2.4 Hz, 1H, 10-H), 8.04 (d, $J_{4\text{-}3}$ = 6.2 Hz, 1H, 4-H), 8.38 (d, $J_{3\text{-}4}$ = 6.2 Hz, 1H, 3-H), 8.82 (s, 1H, 11-H), 9.35 (s, 2H, 9-OH + NH). Analysis: calcd. for $C_{20}H_{21}N_3O_2$: C, 71.62; H, 6.31; N, 12.53%; found C, 71.37; H, 6.60; N, 12.24%.

19 Yield: 30%, m.p.: 230°C. ^{1}H NMR (DMSO-d_6, δ, ppm): 3.07 (s, 3H, 5-CH_3), 3.58 (s, 1H, CH), 3.84 (s, 4H, 2×-CH_2), 4.12 (s, 3H, 6-CH_3), 5.16 (s, 2H, 1-CH_2), 5.47 (s, 2H, 2×OH), 7.11 (dd, $J_{8\text{-}10}$ = 2.4 Hz, $J_{8\text{-}7}$ = 8.7 Hz, 1H, 8-H), 7.45 (d, $J_{7\text{-}8}$ = 8.7 Hz, 1H, 7-H), 7.77 (d, $J_{10\text{-}8}$ = 2.4 Hz, 1H, 10-H), 8.07 (d, $J_{4\text{-}3}$ = 6.2 Hz, 1H, 4-H), 8.41 (d, $J_{3\text{-}4}$ = 6.2 Hz, 1H, 3-H), 8.89 (s, 1H, 11-H), 9.19 (s, 1H, NH), 9.37 (s, 1H, 9-OH). Analysis: calcd. for $C_{21}H_{23}N_3O_3$: C, 69.02; H, 6.34; N, 11.50%; found C, 68.82; H 6.44; N, 11.33%.

5,6-Dimethyl-1-hydroxymethyl-9-methoxy-6H-pyrido[4,3-b]carbazole 10

Compound **4** (0.24 g, 0.8 mmol) was dissolved in 100 mL of methanol : chloroform 5 : 1, v/v mixture and $NaBH_4$ (0.2 g) was added. Then, the reaction mix-

Table 1. Cytotoxicity of the tested compounds to non-cancer 3T3 Balb mouse fibroblasts. Cell cultures were stained with 0.4% aqueous solution of the trypan blue stain.

Tested compounds	4 hours	18 hours
	TD_{50} (±SD)	
5	not toxic	50.9 (±8.32)
6	39.2 (±5.11)	36.8 (±5.46)
7	not toxic	not toxic
8	41.2 (±6.49)	41.7 (±6.82)
9	not toxic	67.0 (±2.40)
10	31.8 (±9.03)	34.3 (±8.04)
12	not toxic	27.5 (±4.70)
13	not toxic	47.5 (±6.60)
14	not toxic	not toxic
15	not toxic	not toxic
16	not toxic	25.9 (±1.08)
17	not toxic	not toxic
18	not toxic	not toxic
19	not toxic	42.1 (±6.29)
ellipticine	38.8 (±8.37)	20.3 (±6.27)
doxorubicin	51.6 (±11.63)	33.1 (±2.72)

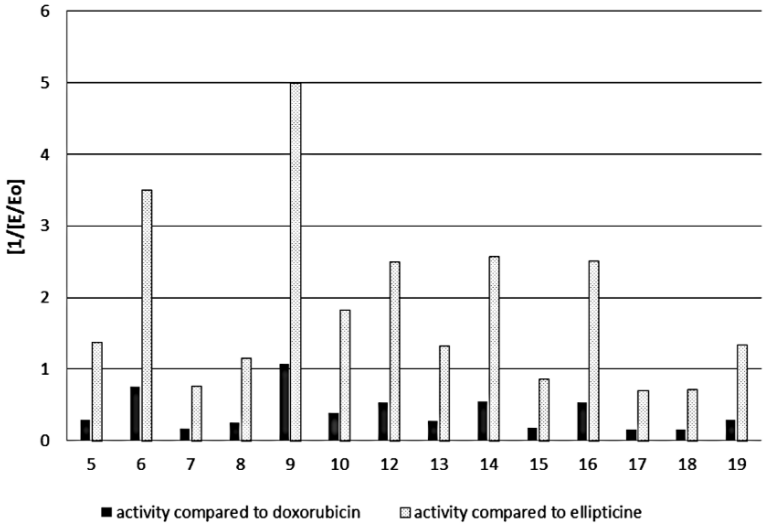


Figure 2. Anticancer activity of the tested compounds in the SRB assay. Sum of the results of cell growth inhibition parameters (GI_{50}, TGI_{50}, LC_{50}) obtained with three cell lines was calculated in the case of each compound then, compared to sum of the SRB test with the standard compounds: doxorubicin and ellipticine. Results obtained in cultures incubated with the tested compounds (E) were compared to those estimated in control cultures (without tested compounds, Eo) and given in Figure 2 as 1/[E/ Eo] ratios.

Table 2. Influence of the tested compounds on frequency of apoptosis in CCRF / CEM cell cultures; A_{20} – a calculated concentration of the compound which caused an increase of apoptotic cell frequency by 20% after 4 h of culture, and A_{50} – a calculated concentration which caused an increase of apoptotic cell frequency by 50% after 18 h culture in the presence of the tested compounds.

Tested compounds	4 hours	18 hours
	A_{20} (±SD)	A_{50} (±SD)
5	not active	not active
6	not active	not active
7	0.520 (±0.223)	1.329 (±0.554)
8	not active	not active
9	0.296 (±0.095)	0.520 (±0.185)
10	not active	not active
12	0.743 (±0.305)	0.442 (±0.062)
13	not active	0.815 (±0.185)
14	not active	not active
15	not active	not active
16	not active	0.359 (±0.109)
17	not active	not active
18	not active	not active
19	not active	not active
ellipticine	not active	not active
doxorubicin	1.377 (±0.118)	1.005 (±0.241)

ture was stirred at room temperature for 2 h and evaporated to dryness. The residue was taken up to the water (100 mL) and hydrochloric acid was added to pH = 5. The solid was filtered off and the filtrate was basified with conc. aq. ammonia and extracted with chloroform. The extract was dried with magnesium

Table 3. The SRB test results with human cancer cell line CCRF/CEM.

Tested compounds	CCRF / CEM		
	GI_{50} µM (±SD)	TGI µM (±SD)	LC_{50} µM (±SD)
5	0.586 (±0.497)	3.080 (±1.532)	12.352 (±2.025)
6	0.680 (±0.851)	2.053 (±1.659)	12.709 (±2.722)
7	5.269 (±0.170)	7.528 (±2.255)	11.723 (±1.768)
8	0.660 (±0.369)	3.903 (±1.610)	14.091 (±1.264)
9	0.225 (±0.588)	0.820 (±0.629)	13.681 (±0.771)
10	0.247 (±0.214)	1.623 (±1.164)	13.987 (±0.621)
12	0.164 (±0.161)	1.501 (±0.747)	9.446 (±1.071)
13	0.364 (±0.544)	1.573 (±1.480)	8.757 (±0.146)
14	3.927 (±1.467)	not active	not active
15	1.364 (±0.789)	3.511 (±0.844)	13.536 (±1.295)
16	0.166 (±0.241)	1.484 (±0.392)	6.169 (±1.965)
17	1.827 (±0.886)	4.299 (±1.117)	10.410 (±0.209)
18	1.339 (±0.219)	3.872 (±0.785)	11.499 (±4.408)
19	1.543 (±0.241)	3.746 (±0.498)	9.214 (±1.919)
ellipticine	1.162 (±0.906)	2.929 (±1.493)	13.586 (±3.446)
doxorubicin	0.241 (±0.426)	0.864 (±0.968)	10.925 (±2.513)

sulfate. After evaporation of solvent, the solid residue was purified by chromatography on a silica gel column with methylene chloride : methanol 95 : 5, v/v.

Yield: 83%, m.p.: 173-174°C. ^{1}H NMR (DMSO-d_6, δ, ppm): 3.05 (s, 3H, 5-CH_3), 3.90 (s, 3H, 9-OCH_3), 4.13 (s, 3H, 6-CH_3), 5.20 (s, 2H, 1-CH_2), 5.40 (s, 1H, 1-OH), 7.18 (dd, $J_{8\text{-}10}$ = 2.2 Hz, $J_{8\text{-}7}$ = 8.8 Hz, 1H, 8-H), 7.53 (d, $J_{7\text{-}8}$ = 8.8 Hz, 1H, 7-H), 7.95 (m, 2H, 10-H, 4-H), 8.32 (d, $J_{3\text{-}4}$ = 6.2 Hz, 1H, 3-H), 8.98 (s, 1H, 11-H). Analysis: calcd. for $C_{19}H_{18}N_2O_2$: C, 74.49; H, 5.92; N, 9.14%; found C, 74.19; H, 5.73; N, 9.05%.

General procedure for the synthesis of 5,6-dimethyl-1-methylcarbamoyloxymethyl-6H-pyrido[4,3-b]carbazole derivatives 15, 17

A solution of **10** (0.15 g, 0.5 mmol) (or **16** 0.15 g, 0.5 mmol) and 4-N,N-dimethylaminopyridine (0.12 g, 1 mmol) in dry chloroform (100 mL) was stirred for 0.5 h, then, methyl isocyanate (1 mL) was added. The mixture was stirred at room temperature for 24 h. After evaporation of solvent, the solid residue was purified by chromatography on a silica gel column with methylene chloride : methanol 98 : 2, v/v.

15 Yield: 91%, m.p.: 96-97°C. ^{1}H NMR (DMSO-d_6, δ, ppm): 2.07 (s, 3H, -NHCH_3), 3.06 (s, 3H, 5-CH_3), 3.89 (s, 3H, 9-OCH_3), 4.13 (s, 3H, 6-CH_3), 5.74 (s, 2H, 1-CH_2), 7.16 (s, 1H, NH), 7.21 (dd, $J_{8\text{-}10}$ = 2.4 Hz, $J_{8\text{-}7}$ = 8.8 Hz, 1H, 8-H), 7.54 (d, $J_{7\text{-}8}$ = 8.8 Hz, 1H, 7-H), 7.99 (m, 2H, 10-H + 4-H), 8.34 (d, $J_{3\text{-}4}$ = 6.2 Hz, 1H, 3-H), 8.99 (s, 1H, 11-H). Analysis: calcd. for $C_{21}H_{21}N_3O_3$: C, 69.41; H, 5.82; N, 11.56%; found C, 69.71; H 6.03; N, 11.39%.

17 Yield: 72%, m.p.: 208-210°C. ^{1}H NMR (DMSO-d_6, δ, ppm): 2.60 (d, J = 4.4 Hz, 3H, 1-NH*C*H_3), 2.70 (d, J = 4.5 Hz, 3H, 9-NH*C*H_3), 3.07 (s, 3H, 5-CH_3), 4.15 (s, 3H, 6-CH_3), 5.74 (s, 2H, 1-CH_2), 7.15 (q, J = 4.4 Hz, 1H, 1-*NH*CH_3), 7.29 (dd, $J_{8\text{-}10}$ = 2.2 Hz, $J_{8\text{-}7}$ = 8.7 Hz, 1H, 8-H), 7.57 (d, $J_{7\text{-}8}$ = 8.8 Hz, 1H, 7-H), 7.66 (q, J = 4.5 Hz, 1H, 9-*NH*CH_3), 8.00 (d, $J_{4\text{-}3}$ = 6.2 Hz, 1H, 4-H), 8.14 (d, $J_{10\text{-}8}$ = 2.1 Hz, 1H, 10-H), 8.35 (d, $J_{3\text{-}4}$ = 6.2 Hz, 1H, 3-H), 9.02 (s, 1H, 11-H). Analysis: calcd. for $C_{22}H_{22}N_4O_4$: C, 65.01; H, 5.46; N, 13.78%; found C, 65.27; H 5.76; N, 13.52%.

Biological testing

Cell culture media RPMI 1640, EMEM, DMEM as well as fetal bovine serum, L-glutamine-penicillin-streptomycin solution and trypsin-EDTA solution were purchased from Lonza (Switzerland). The apop-

tosis detection kit (APO-AF) and sulforhodamine B were obtained from Sigma-Aldrich (St. Louis, MO, USA). The reference cytostatic drugs doxorubicin and ellipticine were also from Sigma-Aldrich. Plastic culture dishes 24-well and 48-well were obtained from Becton-Dickinson (MA, USA). All other chemicals were from POCH (Gliwice, Poland).

Tested compounds

Tested compounds were initially dissolved in DMSO and just before their addition to cell cultures were diluted with culture medium to make the final concentration desired (range: 0.5–50 μM).

Cell lines

Anticancer activity of the tested compounds was evaluated in cultures of three human cell lines selected from the panel of tumor cell lines recommended by the National Cancer Institute: CCRF/CEM (T lymphoblast leukemia), A549 (lung adenocarcinoma), and MCF7 (breast cancer). Cytotoxicity to non-cancer cells was estimated in cultures of the mouse fibroblast cell line 3T3 BALB. Cell lines were obtained from the Department of Histology and Embryology, Wroclaw Medical University. CCRF/CEM cells were grown in RPMI 1640 medium, A549 and MCF7 cells were grown in EMEM medium and mouse fibroblasts (3T3 BALB) were cultured in DMEM medium. All the culture media were supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

Bioassays

The SRB test

The SRB test was carried out in accordance with the method given in the literature (16). After 24 h of cell conditioning in culture medium on 48-well plastic dishes, the tested compounds were added to the cultures (T0) for 48 h. Finally, cell cultures were fixed in 10% TCA, stained for 10 min with a 0.4% (w/v) solution of sulforhodamine B in 1% acetic acid and absorbance (A_{540nm}) was measured with a Victor X2 microspectrophotometer (Perkin-Elmer, USA). The results were calculated in accordance with the NCI guidelines (17) and expressed as: the concentration of the tested compounds in which cell proliferation was reduced by half in comparison to the control (GI_{50}), concentration of the compounds which completely inhibited cell proliferation (total growth inhibition, TGI) and concentration of the compounds in which the number of cells was reduced by 50% (LC_{50}).

Table 4. The SRB test results with human cancer cell line A549.

Tested compounds	A549		
	GI_{50} μM (±SD)	TGI μM (±SD)	LC_{50} μM (±SD)
5	52.643 (±0.538)	14.607 (±3.520)	not active
6	0.891 (±0.397)	3.985 (±0.970)	8.104 (±0.381)
7	2.993 (±0.656)	4.646 (±0.525)	8.122 (±0.456)
8	2.472 (±0.341)	12.566 (±0.931)	not active
9	0.377 (±0.159)	4.741 (±0.547)	18.789 (±1.032)
10	1.625 (±0.473)	5.785 (±0.866)	18.178 (±5.918)
12	0.885 (±0.071)	6.399 (±0.703)	10.665 (±0.610)
13	1.129 (±0.367)	5.023 (±0.923)	15.762 (±1.984)
14	not active	not active	not active
15	not active	not active	not active
16	0.962 (±0.520)	3.743 (±0.457)	10.391 (±1.064)
17	not active	not active	not active
18	2.461 (±0.884)	9.768 (±2.215)	not active
19	4.415 (±1.310)	9.526 (±1.615)	not active
ellipticine	2.441 (±0.666)	13.381 (±0.602)	not active
doxorubicin	0.580 (±0.087)	5.039 (±0.626)	13.970 (±0.936)

Table 5. The SRB test results with human cancer cell line MCF7.

Tested compounds	MCF7		
	GI_{50} μM (±SD)	TGI μM (±SD)	LC_{50} μM (±SD)
5	3.503 (±2.674)	8.459 (±2.259)	22.146 (±4.284)
6	0.465 (±0.085)	1.754 (±0.320)	6.619 (±1.213)
7	3.921 (±1.580)	8.388 (±2.076)	18.256 (±1.967)
8	4.945 (±2.611)	11.421 (±2.495)	17.807 (±4.540)
9	0.322 (±0.095)	1.580 (±0.363)	7.819 (±1.803)
10	3.173 (±0.471)	5.837 (±0.619)	19.327 (±1.887)
12	2.403 (±0.319)	5.043 (±0.190)	7.683 (±0.194)
13	6.238 (±0.552)	8.575 (±0.675)	19.516 (±2.188)
14	not active	not active	not active
15	9.824 (±2.680)	16.282 (±2.171)	22.740 (±1.705)
16	2.586 (±0.332)	4.644 (±0.457)	12.049 (±2.446)
17	12.613 (±4.716)	20.302 (±3.632)	not active
18	10.669 (±1.779)	17.791 (±2.134)	24.913 (±2.512)
19	2.469 (±0.452)	5.450 (±0.468)	8.431 (±0.618)
ellipticine	5.238 (±0.201)	18.220 (±2.003)	22.602 (±4.761)
doxorubicin	0.351 (±0.027)	2.050 (±0.399)	12.342 (±4.966)

Cytotoxic activity

Cytotoxicity of tested compounds to non-cancer cells was carried out with the line BALB 3T3 mouse fibroblast after 4 and 18 h of cell culture incubation in the presence of the compounds. Viable and dead cell counts were estimated under a microscope after staining of cell suspensions with 0.4% aqueous solution of the trypan blue stain and inspected under a regular light microscope. The concentration of the tested compounds which increased frequency of necrotic cells in the culture to 50% (TD_{50}) were calculated.

Frequency of apoptosis in cell cultures.

The influence of the tested compounds on frequency of apoptosis in CCRF/CEM cell cultures was evaluated under a fluorescent microscope (ECLIPSE E600, Nikon) after staining of the cell suspension with a mixture of fluorochromes (annexin V-FITC/PI) for 15 min. The frequencies of viable cells (not stained), early apoptotic (green fluorescence), late apoptotic (green on a cell surface with red cell nucleus) and dead (red fluorescence) cells were counted. The concentration of the tested compounds in which the frequency of apoptotic cells increased to 20% (A_{20}) and to 50% (A_{50}) were calculated in 4 h cultures and in 18 h cultures, respectively. The tested compounds exhibited autofluorescence, which could interfere with the results of fluorescent staining used in detection of apoptosis; therefore, the influence of the tested compounds on frequency of apoptosis in cell cultures was studied in low concentration of the compounds (up to 1 μM), since in low concentration the autofluorescence of the compounds was almost negligible.

RESULTS AND DISCUSSION

Synthesis

The starting compound - 5,6-dimethyl-1-formyl-9-methoxy-6*H*-pyrido[4,3-b]carbazole (**4**), was prepared according to a previously described procedure (15).

Compounds **5, 6, 7, 8** and **9** were obtained by heating of 6*H*-pyrido[4,3-b]carbazole derivative **4** with appropriate amines in toluene with addition of a small amount of *p*-toluenesulfonic acid. Newly obtained amines were 9-*O*-demethylated using boron tribromide to give derivatives **12, 14, 16** and **13**. The reduction of aldehyde **4** by sodium borohydride resulted in derivative **10**. The alcohols **10** and **16** were treated with methyl isocyanate yielding compound **15** and **17**, respectively. Pyrido[4,3-b]carbazole derivative **4** was 9-*O*-demethylated by

heating with boron tribromide forming 5,6-dimethyl-1-formyl-9-hydroxy-6*H*-pyrido[4,3-b]carbazole **11**. Aldehyde **11** was heated with appropriate amines in toluene to give **18** or **19**, respectively.

Biology

The tested compounds did not exhibit acute toxicity to the 3T3 mouse fibroblasts (Table 2) after 4 h of incubation, except for three compounds (**6**, **8** and **10**); the TD_{50} coefficient was calculated for these compounds, and in each case the coefficient was lower in comparison to the reference cytostatic drug, doxorubicin, suggesting the marked cytotoxic activity of these compounds. After 18 h of cell incubation with the tested compounds TD_{50} could be calculated for 9 of the compounds and the TD_{50} coefficient was higher when compared to the cytotoxic drugs ellipticine and doxorubicin, except for two compounds: **12** and **16**. These compounds increased the number of dead cells in a lower concentration when compared to doxorubicin.

As may be seen in Figure 2, all the tested compounds exhibited growth inhibitory activity in the SRB test, being stronger both in comparison to doxorubicin and to ellipticine. The inhibitory activity was especially strong with compounds **6**, **9**, **12** and **16**. The anticancer activity of compound **9** was the strongest in cultures of three cancer cell lines, about 5 times more active than ellipticine and by 10% more active than doxorubicin.

Incubation of CCRF/CEM cells for 4 h with the tested compounds led to an increase of apoptotic cell number in the cases of compounds **7**, **9**, **12**. The pro-apoptotic effect was the strongest with compound **9** – the concentration of compound **9** which caused an increase of apoptotic cell number by 20% (A_{20}) was almost 4.6 times lower than the concentration of doxorubicin. After 18 h incubation a marked elevation of apoptotic cell number was observed in cultures carried out in the presence of compounds **7**, **9**, **12**, and **13**. Their pro-apoptotic activity was stronger than doxorubicin – the A_{50} concentration calculated with these four compounds was lower by 40-70% than the A_{50} concentration of doxorubicin. It can be seen in Table 2 that compound **9** exhibited the strongest pro-apoptotic activity, both fast and continuously persistent during 18 h of cell culture.

CONCLUSION

Our group has focused on synthesis of new chemical analogues of olivacine with potential anticancer activity (18, 19). Several previously synthesized compounds exhibited a markedly stronger cytostatic effect in comparison to the reference drug ellipticine and also to the standard cytostatic drug cisplatin in cultures of human kidney and lung cancer cell lines (19). In this paper, we describe pathways of the synthesis and anticancer activity of fourteen analogues of olivacine. The SRB test results obtained with three human cancer cell lines (Tables 3-5) indicated four compounds (**6**, **9**, **12** and **16**) exhibiting especially strong inhibitory activity against cancer cell growth. Analysis of pro-apoptotic activity revealed that compounds **9**, **12** and **16** increased the frequency of apoptotic cells in CCRF/CEM cultures and the effect was markedly stronger than that of the reference anticancer drug doxorubicin. Taken together, the results obtained with the SRB test and the apoptosis detection test suggest that compounds **9**, **12** and **16** are worth studying further as promising candidates for future anticancer drugs.

Acknowledgments

Studies on anticancer activity with presented bioassays were supported by a Wroclaw Medical University research grant (project number PBMN 56).

The authors have declared no conflict of interest.

REFERENCES

1. Schmutz J., Hunzicker F.: Pharm. Acta Helv. 33, 341 (1958).
2. Goodwin S., Smith A.F. Horning E.C.: J. Am. Chem. Soc. 81, 1903 (1959).
3. Mosher C.W., Crews O.P., Acton E.M., Goodman L.: J. Med. Chem. 9, 237 (1966).
4. Dalton L.K., Demerac S., Elmes B.C., Loder J.W., Swan J.M., Teitei T.: Aust. J. Chem. 20, 2715 (1967).
5. Svoboda G.H., Poore G.A., Montifort M.L.: J. Pharm. Sci. 57, 1720 (1968).
6. Le Pecq J.B., Gosse C., Nguyen-Dat-Xuong, Paoletti C.: Acad. Sci. Hebd. Seances Acad. Sci. D 281, 1365 (1975).
7. Juret P., Tanguy A., Girara A., Le Talaer J.Y., Abbatucci J.S., Nguyen-Dat-Xuong, Le Pecq J.B., Paoletti C.: Eur. J. Cancer 14, 205 (1978).
8. Paoletti C., Le Pecq J.B., Nguyen-Dat-Xuong, Juret P., Garnier H., Amiel J.L., Rouesse J.: Recent Results Cancer Res. 74, 107 (1980).
9. Buzdar A.U., Hortobagyi G.N., Esparza, L.T., Holmes F.A., Ro J.S., Fraschini G., Lichtiger B.: Oncology. 47, 101 (1990).

10. Rouëssé J., Spielmann M., Turpin F., Le Chevalier T., Azab M., Mondésir J.M.: Eur. J. Cancer 29A, 856 (1993).
11. Giacchetti S., Cornez N., Eftekhari P., Awada A., Cuvier C., Bleiberg H., Hidvegi E. et al.: Proc. Am. Assoc. Cancer Res. 39, 324 (1998).
12. Awada A., Giacchetti S., Gerard B., Eftekhary P., Lucas C., De Valeriola D., Poullain M.G. et al.: Ann Oncol. 13, 1925 (2002).
13. Tylińska B., Jasztold-Howorko R., Mastalarz H., Kłopotkowska D., Filip B., Wietrzyk J.: Acta Pol. Pharm. Drug Res. 67, 495 (2010).
14. Tylińska B., Jasztold-Howorko R., Mastalerz H., Szczurska-Nowak K., Materek P., Wietrzyk J.: Acta Pol. Pharm. Drug Res. 68, 31 (2011).
15. Jasztold-Howorko R., Bisagi E.: Eur. J. Med. Chem. Chim. Ther. 19, 6, 541 (1984).
16. Skehan P., Storeng R., Scudiero D., Monks A., McMahon J., Vistica D., Warren J. et al.: J. Natl. Cancer Inst. 82, 1107 (1990) .
17. Boyd M.R., Paull K.D.: Drug Develop. Res. 34, 91 (1995).
18. Jasztold-Howorko R., Pelczynska M., Nasulewicz A., Wietrzyk J. Opolski A.: Arch. Pharm. Chem. Life Sci. 338, 556 (2005).
19. Tylińska B., Jasztold-Howorko R., Mastalarz H., Szczaurska-Nowak K., Wietrzyk J.: Arch. Pharm. Chem. Life Sci. 341, 351 (2008).

Received: 04. 12. 2012