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NEW BENZIMIDAZOLE DERIVATIVES AS TOPOISOMERASE I INHIBITORS – SYNTHESIS AND FLUOROMETRIC ANALYSIS

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Abstract: A series of new benzimidazole derivatives with potential anticancer activity were tested as a new topoisomerase I inhibitors. The fluorometric method was used to determine *in vitro* the quantitative level of plasmid DNA relaxation by these compounds. Optimization of the fluorometric system and validation of the established analytical method were performed. Out of benzimidazole derivatives which were analyzed, in the case of five derivatives inhibition of topoisomerase I was greater than camptothecin (compounds **11, 12, 15, 21, 22**).

Key words: anticancer activity, benzmidazole, inhibitors of topoisomerase I, nitrobenzimidazole

Neoplastic diseases, apart from cardiovascular system diseases, are the major cause of deaths all over the world. According to WHO data, 7.6 million people died of neoplasms in 2008, which accounted for 13% of the total number of deaths. It is estimated that the number of deaths caused by cancer will be growing and by 2030 it might be up to 11 million. Despite a rapid development of knowledge in the field of diagnostics and prophylaxis of neoplastic diseases, as well as more profound insight into the molecular basis for the process of forming neoplasms, the current therapy which mainly includes cytotoxic and antiproliferative medicines is hardly selective and not always effective.

In order to minimize systemic effects of administered medicines and reduce drug resistance, scientists conduct research into targeted therapy with the use of specific conditions which appear in neoplastic microenvironment. What scientists are striving to invent are preparations which are characterized with great selective toxicity under conditions of oxygen deficiency, which results from hypoxia of tumor cells at an early stage of the neoplasm development. The medicines which have a bioreductive mechanism of action include nitro compounds (CB 1954) and heterocyclic N-oxides (tirapazamine. AQ4N) (1-4). Inhibitors of topoisomerase I make up a group of compounds with potential anticancer properties (5-8). Inhibition of topoisomerase I, at the level of relaxed DNA is at present one of the most important factors in combating neoplastic diseases. A group of benzimidazole derivatives i.e., inhibitors of the enzyme, should be particularly paid attention to. These compounds are intensively being worked on as they might have anticancer properties (9-16).

Pharmacological activity of these two groups of chemical compounds was the reason for initiating our experiments in the group of new benzimidazole and N-oxide benzimidazole derivatives.

We worked out indirect and direct conditions for obtaining new benzimidazole derivatives (**9**-**15**), whose structural formula is presented in Figure 1a (17-20). In the course of the synthesis of benzimidazole derivatives an intermediate product - compound **8** - Schiff base was isolated. Its cyclocondensation led to receiving a final product – compound **9**. An indirect way of synthesis of compounds **9**-**15** was confirmed. Compounds **9**-**15** were also obtained by direct condensation of proper diamine (**1**-**2**) with proper aldehyde (**3**–**7**) in anhydrous solvent at its boiling point. The compounds whose structure resembled N-oxide benzimidazoles (**16**-**22**) (Fig. 1b) were obtained by direct reaction of 30% solution of hydrogen peroxide on benzimidazole derivatives, obtained in the first stage (**9**–**15**), in glacial acetic acid. The synthetic process included the reactions presented in Scheme 1.

Because the isolated compounds were biochemically tested and their cytotoxic properties were determined, we wanted to explain their mech-

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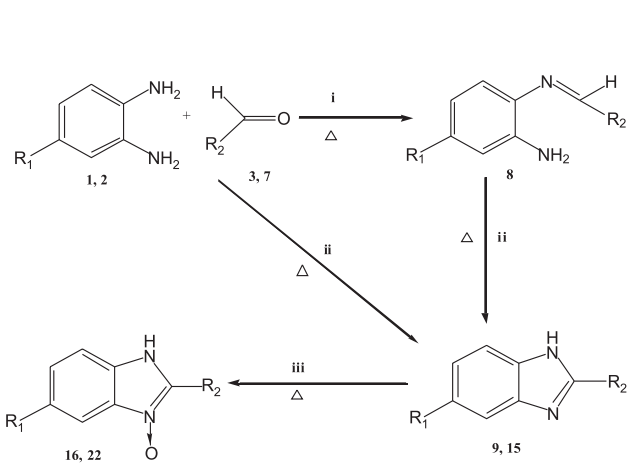
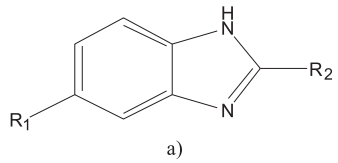
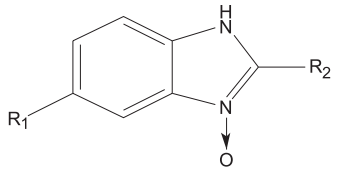


	R_1	R_2
1	Cl	-
2	NO_2	-
3	-	NAPH
4	-	PIP
5	-	NO_2B
6	-	ClB
7	-	COOHB
8	NO_2	PIP
9	NO_2	PIP
10	NO_2	NAPH
11	NO_2	NO_2B
12	NO_2	ClB
13	NO_2	COOHB
14	Cl	NAPH
15	Cl	PIP
16	NO_2	PIP
17	NO_2	NAPH
18	Cl	NAPH
19	Cl	PIP
20	NO_2	COOHB
21	NO_2	ClB
22	NO_2	NO_2B

Scheme 1. Synthesis of compounds **9**-**22**. Reagents: i - anhydrous ethanol, ii - anhydrous ethanol + nitrobenzene, iii - anhydrous acetic acid + hydrogen peroxide. NAPH – naphthyl, PIP – piperonyl, NO_2B – nitrophenyl, ClB –chlorophenyl, COOHB – carboxyphenyl



	R_1	R_2
9	NO_2	PIP
10	NO_2	NAPH
11	NO_2	NO_2B
12	NO_2	ClB
13	NO_2	COOHB
14	Cl	NAPH
15	Cl	PIP



	R_1	R_2
16	NO_2	PIP
17	NO_2	NAPH
18	Cl	NAPH
19	Cl	PIP
20	NO_2	COOHB
21	NO_2	ClB
22	NO_2	NO_2B

Figure 1. Structural formulas of the obtained compounds: benzimidazole derivatives, b) N-oxide benzimidazole derivatives. NAPH – naphthyl, PIP – piperonyl, NO_2B – nitrophenyl, ClB –chlorophenyl, COOHB – carboxyphenyl

anism of action (20, 21). The suggested mechanism of action is the inhibition of human topoisomerase I, the enzyme playing an essential role in cell metabolic processes such as translation, transcription and replication of DNA. The level of the inhibition of human topoisomerase I was determined with fluorometric method. The assays were performed with indirect method with the use of ethidium bromide (EtBr), which allowed to determine how much of DNA is supercoiled, as well as with the use of camptothecin serving as reference compound. (21)

EXPERIMENTAL

Procedures of synthesis

The IR spectra (KBr discs) were registered using the Mattson Infinity Series FT-IR spectrophotometer (USA). ^{1}H and ^{13}C NMR spectra were

recorded on a 300 MHz Varian Mercury spectrometer (Germany) in DMSO or $CDCI_3$ as solvent and tetramethylsilane (TMS) as an internal reference. The MS spectra (FAB method, M + 1, matrix - glycerin) were recorded on a Finnigan Mat 95 spectrometer (Brema, Germany). Carbon, hydrogen and nitrogen elemental analyses were performed using the Perkin Elmer 2400 series II CHNS/O (Madison, USA) apparatus and agreed with the proposed structures within ± 0.3% of theoretical values.

Chromatographic purification was performed on HPTLC and silica gel plates (Merck F_{254} Darmstadt, Germany) with indicated eluents. Chemicals and solvents were obtained from commercial sources. The HPLC analysis was used for compounds **9**, **10** and **16**, **17** (20). The HPLC system consisted of Waters 600 LC system. Chromatographic separation was achieved by analysis on Supelco RP-18 column (15 cm × 4 mm × 5 µm plus symmetry C18 guard, Waters) held at 20°C. Chromatographic peaks were identified with a UV detector (Waters).

General procedure for preparation of compounds 8

A mixture of equimolar portions of the appropriate 4-nitro-o-phenylenediamine (10 mmol) and piperonal (10 mmol) was dissolved in anhydrous ethanol (50 mL) and heated under reflux for 24 h. After this time, the reaction mixture was concentrated to the half of its initial volume and the precipitated crude product was recrystallized from isopropanol. Schiff base was isolated with chromatographic purity (chloroform/methanol 6.25% v/v).

General procedure for preparation of compounds 9

Starting from (N-benzo[1,3]dioxol-5-yl-methylidene)-4-nitro-o-phenylenediamine (**8**)

Schiff base (10 mmol) (**8**) was dissolved in 50 mL of anhydrous ethanol, 3 mL nitrobenzene was added and the mixture was heated for 24 h. Next, the solution was concentrated to the half of its initial volume and crude precipitate was filtered off. The dry solid was recrystallized from isopropanol. Compound was isolated with chromatographic purity (chloroform/methanol 6.25% v/v).

General procedure for preparation of compounds **9**-**22** by direct cyclocondensation was described previously (19).

N^1-Benzo[1,3]dioxol-5-ylmethylidene-4-nitrophenylene-1,2-diamine (8)

Yield 65%. IR (KBr, cm^{-1}): 3434 (NH asym.), 3379 (NH sym.), 3033 (ArH), 1640 (CH=N), 1594 (NO_2 asym.), 1312 (NO_2 sym.); ^{1}H NMR (DMSO-d_6, δ, ppm): 8.7 (s, 1H, CH=N), 8.0 (s, 1H, CH), 7.9 (dd, 2H, *J* = 2.6 Hz, CH), 7.8 (s, 1H, CH), 7.5 (d, 1H, *J* = 1.6 Hz, CH), 7.1 (d, 1H, *J* = 7.9 Hz, CH), 6.8 (s, 2H, NH_2), 6.2 (s, 2H, CH_2); ^{13}C NMR (DMSO-d_6, δ, ppm): 160.7, 149.1, 147.7, 145.7, 141.1, 123.9, 123.7, 120.3, 115.5, 115.1, 115.0, 111.3, 90.3; MS m/z: 286.1. Analysis: calcd. for $C_{14}H_{11}N_3O_4$: C 58.95, H 3.89, N 14.73%; found: C 58.72, H 3.8, N 14.79%. R_f (chloroform/methanol 6.25% v/v) = 0.45.

Syntheses of 2-naphthyl-5-nitro-1H-benzimidazole (**9**) and 2-benzo[1,3]dioxol-5-yl-5-nitro-1H-benzimidazole (**10**) were described previously [19].

5-Nitro-2-(2-nitrophenyl)-1H-benzimidazole (11)

Yield 55%. IR (KBr, cm^{-1}): 3418 (NH), 1516 (NO_2 asym.), 1472 (C=N), 1342 (NO_2 sym); ^{1}H NMR (DMSO-d_6, δ, ppm): 14 (s, 1H, NH), 8.6 (s, 1H, CH), 8.1 (dd, 2H, *J* = 0.8 Hz, CH), 8.0 (dd, 2H, *J* = 1.4 Hz, CH), 7.8 (dd, 2H, *J* = 1.6 Hz, CH); ^{13}C NMR (DMSO-d_6, δ, ppm): 148.8, 142.9, 133.9, 133.1, 131.9, 129.6, 128.0, 126.4, 124.6, 119.6, 116.5, 113.0: MS m/z: 285.2, 283.3. Analysis: calcd. for $C_{13}H_8N_4O_4$: C 54.93, H 2.83, N 19.71%; found: C 54.78, H 2. 84, N 19.67%. R_f (chloroform/methanol 6.25% v/v) = 0.50.

2-(4-Chlorophenyl)-5-nitro-1H-benzimidazole (**12**) was described previously [19].

5-Nitro-2-carboxyphenyl-1H-benzimidazole (13)

Yield 60%. IR (KBr, cm^{-1}): 3401 (NH), 3177 (OH in COOH), 1684 (C=O), 1522 (NO_2 asym.), 1490 (C=N), 1300 (NO_2 sym.); ^{1}H NMR (DMSO-d_6, δ, ppm): 14.0 (s, 1H, OH), 8.4 (s, 1H, CH), 8.1 (dd, 2H, *J* = 1.8 Hz, CH), 7.8 (dd, 2H, *J* = 7.3 Hz, CH,), 7.6 (dd, 2H, *J* = 7.7 Hz, CH) 6.8 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, δ, ppm): 168.5, 153.0, 143.3, 135.8, 133.2, 132.4, 132.0, 130.7, 130.0, 128.1, 127.4, 121.8, 118.2, 114.5; MS m/z: 282.4, 282.1. Analysis: calcd. for $C_{14}H_9N_3O_4$: C 59.37, H 3.20, N 14.84%; found: C 59.54, H 3.19, N 14.79%. R_f (chloroform/methanol 6.25% v/v) = 0.53.

5-Chloro-2-naphthyl-1H-benzoimidazole (14)

Yield 60%. IR (KBr, cm^{-1}): 3311 (NH), 3043 (ArH), 1474 (C=N); ^{1}H NMR (DMSO-d_6, δ, ppm): 8.9 (s, 1H, CH), 8.3 (d, 1H, *J* = 1.6 Hz, CH), 8.2 (d, 1H, *J* = 8.7 Hz, CH), 8.1 (dd, 2H, *J* = 6.1 Hz, CH), 7.8 (dd, 2H, *J* = 8.7 Hz, CH,), 7.6 (dd, 2H, *J* = 5.0 Hz, CH), 7.5 (d, 1H, *J* = 2.0 Hz, CH), 4.5 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, δ, ppm): 152.5, 137.9, 133.7, 132.7, 128.7, 128.5, 128.2, 127.5, 127.1, 126.8, 126.5, 126.4, 125.5, 123.8, 122.8, 117.8, 116.0.; MS m/z: 279.1, 277.1. Analysis: calcd. for

$C_{17}H_{11}ClN_2$: C 73.25, H 3.98, N 10.50%; found: C 72.98, H 3.99, N 10.47%. R_f (chloroform/methanol – 6.25% v/v) = 0.53.

2-Benzo[1,3]dioxol-5-yl-5-chloro-1H-benzimidazole (15)

Yield 65%. IR (KBr, cm^{-1}): 3356 (NH), 2963 (CH_2), 1469 (C=N), 1261 (C-O-C sym.), 1095 (C-O-C asym.); ^{1}H NMR (DMSO-d_6, δ, ppm): 7.9 (dd, 2H, *J* = 1.8 Hz, CH), 7.7 (dd, 2H, *J* = 8.7 Hz, CH), 7.5 (d, 1H, *J* = 2.0 Hz, CH), 7.3 (d, 1H, *J* = 8.3 Hz, CH), 6.2 (s, 2H, CH_2), 4.5 (s, 1H, NH); ^{13}C NMR (DMSO-d_6, δ, ppm): 152.9, 149.3, 148.8, 132.8, 130.9, 129.2, 124.1, 123.9, 122.6, 116.6, 115.8, 112.3, 111.0; MS m/z: 273.1, 271.1. Analysis: calcd. for $C_{14}H_9ClN_2O_2$: C 61.66, H 3.33, N 10.27%; found: C 61.43, H 3.34, N 10.24%. R_f (chloroform/methanol 6.25% v/v) = 0.52.

2-Naphthyl-5-nitro-1H-benzimidazole N-oxide (16)

Yield 60%. IR (KBr, cm^{-1}): 3380 (NH), 3100 (ArH), 1523 (NO_2 asym.), 1474 (C=N), 1344 (NO_2 sym.), 1261 (N-O), ^{1}H NMR (DMSO-d_6, δ, ppm): 13.8 (s, 1H, NH), 8.8 (s, 1H, CH), 8.5 (s, 1H, CH), 8.3 (dd, 2H, *J* = 1.4,1.6 Hz, CH), 8.2 (d, 1H, *J* = 2.2 Hz, CH), 8.1 (d, 1H, *J* = 2.8 Hz, CH), 8.0 (d, 1H, *J* = 3.4 Hz, CH), 7.8 (d, 1H, *J* = 8.7 Hz, CH), 7.6 (dd, 2H, *J* = 2.9 Hz, CH); ^{13}C NMR (DMSO-d_6, δ, ppm): 172.1, 155.8, 142.8, 133.9, 132.7, 128.9, 128.7, 127.9, 127.8, 127.2, 126.9, 126.4, 123.9, 118.2; MS m/z: 306.1, 304.1. Analysis: calcd. for $C_{17}H_{11}N_3O_3$: C 66.88, H 3.63, N 13.76%; found: C 66.65, H 3.64, N 13.72%. R_f (chloroform/methanol 6.25% v/v) = 0.53.

2-Benzo[1.3]dioxol-5-yl-5-nitro-1H-benzimidazole N-oxide (17)

Yield 75%. IR (KBr, cm^{-1}): 3330 (NH), 1482 (C=N), 1504 (NO_2 asym.), 1300 (NO_2 sym.), 1237 (C-O-C asym.), 1258 (N-O), 1037 (C-O-C sym.). ^{1}H NMR (DMSO-d_6, δ, ppm): 13.5 (s, 1H, NH), 8.4 (s, 1H, CH), 8.2-8.1 (dd, 2H, *J* = 8.5, 8.9 Hz, CH,), 7.8-7.7 (dd, 2H, *J* = 7.7, 8.3 Hz, CH), 7.1 (d, 1H, *J* = 0.4 Hz, CH,), 6.1 (s, 2H, CH_2); ^{13}C NMR (DMSO-d_6, δ, ppm): 155.6, 149.6, 147.9, 142.4, 135.3, 129.8, 123.3, 123.3, 122.9, 121.8, 117.8, 108.8, 106.7, 101.9.; MS m/z: 300.2, 298.1. Analysis: calcd. for $C_{14}H_9N_3O_5$: C 56.19, H 3.03, N 14.04%; found: C 56.40, H 3.03, N 13.99%. R_f (chloroform/methanol 6.25% v/v) = 0.51.

5-Chloro-2-naphthyl-1H-benzimidazole N-oxide (18)

Yield 75%. IR (KBr, cm^{-1}): 3385 (NH), 3059 (ArH), 1449 (C=N), 1230 (N-O); ^{1}H NMR (DMSO-d_6, δ, ppm): 4.0 (s, 1H, NH), 7.5 (d, 1H, *J* = 3.9 Hz, CH), 7.7 (d, 1H, *J* = 3.6 Hz, CH), 7.8 (dd, 2H, *J* = 2.6 Hz, CH). 7.9 (d, 1H, *J* = 2.6 Hz, CH), 8.1 (d, 1H, *J* = 2.4 Hz, CH), 8.20 (d, 1H, *J* = 6.3 Hz, CH), 8.3 (dd, 2H, *J* = 1.8 Hz, CH), 8.90 (s, 1H, CH); ^{13}C NMR (DMSO-d_6, δ, ppm): 168.5, 153.0, 143.3, 135.8, 133.2, 132.4, 132.0, 130.7, 130.0, 128.1, 127.4, 121.8, 118.2, 114.5; MS m/z: 295. Analysis: calcd. for $C_{17}H_{11}ClN_2O$: C 69.28, H 3.76, N 9.50%; found: C 69.05, H 3.75, N 9.48%. R_f (chloroform/methanol 6.25% v/v) = 0.49.

2-Benzo[1,3]dioxol-5-yl-5-chloro-1H-benzimidazole N-oxide (19)

Yield 65%. IR (KBr, cm^{-1}): 3303 (NH), 2908 (CH_2), 1468 (C=N), 1257 (C-O-C asym); 1358 (N-O), 1095 (C-O-C sym); ^{1}H NMR (DMSO-d_6, δ, ppm): 4.0 (s, 1H, NH), 6.2 (s, 2H, CH_2), 7.2 (d, 1H, *J* = 8.1 Hz, CH), 7.4 (d, 1H, *J* = 7.7 Hz, CH), 7.7 (d, 1H, *J* = 9.5 Hz, CH), 7.75 (s, 1H, CH), 7.8 (s, 1H, CH), 7.85 (d, 1H, *J* = 8.3 Hz, CH); ^{13}C NMR (DMSO-d_6, δ, ppm): 165.0, 150.8, 150.2, 148.0, 128.6, 123.2, 118.2, 116.1, 115.2, 114.9, 113.5, 109.1, 107.1, 102.4; MS m/z: 289.1, 287.1. Analysis: calcd. for $C_{14}H_9ClN_2O_3$: C 58.25, H 3.14., N 9.70%; found: C 58.03, H 3.1, N 9.68%. R_f (chloroform/methanol 6.25% v/v) = 0.50.

2-(5-nitro-1H-benzimidazol-2-yl)-benzoic acid N-oxide (20)

Yield 70%. IR (KBr, cm^{-1}): 3327 (NH), 3178 (OH in COOH), 1684 (C=O), 1517 (NO_2 asym.), 1498 (C=N), 1331 (NO_2 sym.); ^{1}H NMR (DMSO-d_6, δ, ppm): 13.3 (s, 1H, NH), 8.5 (s, 1H, CH), 8.0 (dd, 2H, *J* = 2.6 Hz, CH), 7.9 (dd, 2H, *J* = 5.3 Hz), 7.8-7.7 (dd, 1H, *J* = 7.5 Hz, CH), 7.6 (dd, 2H, J = 7.5 Hz, CH); MS m/z: 300.1. Analysis: calcd. for $C_{14}H_9N_3O_5$: C 56.19, H 3.03, N 14.04%; found C 55.96, H 3.02, N 14.07%. R_f (chloroform/methanol 6.25% v/v) = 0.51.

2-(4-Chlorophenyl)-5-nitro-1H-benzimidazole N-oxide (21)

Yield 55%. IR (KBr, cm^{-1}): 3287 (NH), 1536 (NO_2 asym.), 1331 (NO_2 sym.), 1498 (C=N), 1290 (N-O); ^{1}H NMR (DMSO-d_6, δ, ppm): 13.7 (s, 1H, NH), 8.5 (s, 1H, CH), 8.3 (d, 1H, *J* = 2.6 Hz, CH), 8.2 (dd, 2H, *J* = 4.9 Hz, CH), 7.7 (dd, 2H, *J* = 2.4 Hz. CH), 7.6 (d, 1H, *J* = 4.9 Hz, CH); ^{13}C NMR (DMSO-d_6, δ, ppm): 151.8, 150.1, 147.8, 144.3, 139.8, 135.3, 133.9, 129.6, 126.2, 124.4, 118.6, 116.1, 112.9; MS m/z: 288.1. Analysis: calcd. for $C_{13}H_8ClN_3O_3$: C 53.90, H 2.78, N 14.51%; found: C 53.71, H 2.77, N 14.57%. R_f (chloroform/methanol 6.25% v/v) = 0.51.

5-Nitro-2-(2-nitrophenyl)-1H-benzimidazole N-oxide (22)

Yield 45%. IR (KBr, cm^{-1}): 3380 (NH), 2963 (CH_2), 1518 (NO_2 asym.), 1467 (C=N), 1342 (NO_2 sym.), 1261 (N-O): ^{1}H NMR (DMSO-d_6, δ, ppm): 13.8 (s, 1H, NH), 8.5 (s, 1H, CH), 8.1 (d, 1H, *J* = 1.4 Hz, CH), 8.0 (d, 1H, *J* = 7.5 Hz, CH), 7.90 (dd, 2H, *J* = 1.2 Hz, CH), 7.83 (s, 1H, CH), 7.81 (d, 1H, *J* = 1.4 Hz, CH); ^{13}C NMR (DMSO-d_6, δ, ppm): 151.8, 150.1, 147.8, 144.3, 139.8, 135.3, 133.9, 129.6, 126.2, 124.4, 118.6, 116.1, 112.9; MS m/z: 301.0, 299.0. Analysis: calcd. for $C_{13}H_8N_4O_5$: C 52.01, H 2.69, N 18.66%; found: C 52.14, H 2.70, N 18.60%. R_f (chloroform/methanol 6.25% v/v) = 0.50.

Procedures of biochemistry experiments

Fluorometric analysis

The level of inhibition of topoisomerase I by benzimidazole derivatives was determined with fluorometric method. DNA relaxation level was measured with EtBr. Fluorescence gets more intense after EtBr has joined DNA and EtBr tends to intercalate into helical DNA rather than into relaxed DNA [22]. EtBr was purchased in Sigma-Aldrich Co. To perform assays, topoisomerase I and plasmid DNA pBR322 were also purchased in Sigma-Aldrich Co.

Validation of fluorometric method

Precision: Five water solutions of EtBr, having the following concentrations: 0.1, 0.5, 1.0, 1.5 and 2.0

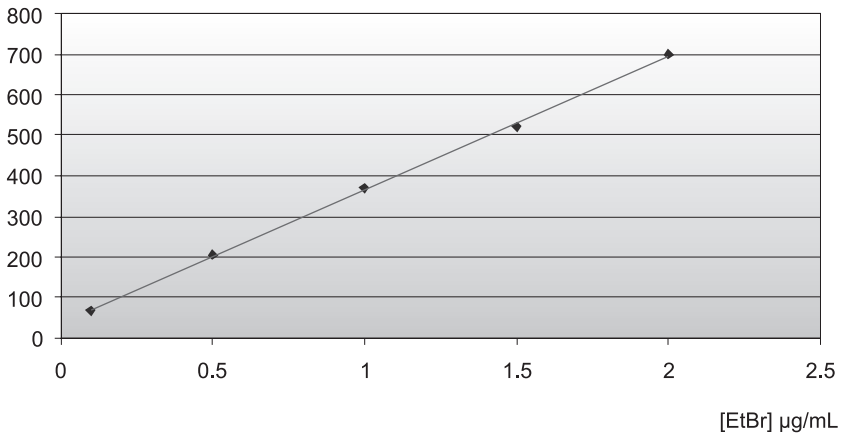


Figure 2. Fluorescence intensity I as a function of EtBr. (I = 329.88x + 35.427 r^2 = 0.9994)

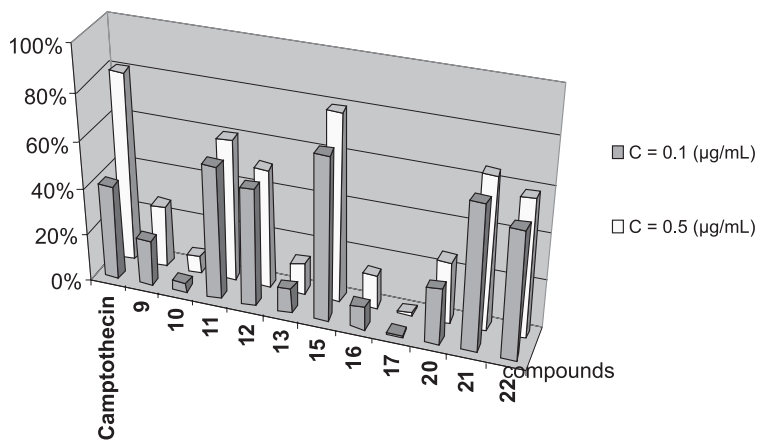


Figure 3. The degree of inhibition (%) topoisomerase I by a particular compounds **9**-**13**, **15**-**17** and **20**-**22** and camptothecin at concentration: 0.1 and 0.5 µg/mL

μg/mL were prepared. To determine precision of the method the following parameters were set: $\bar{x}$, s, μ, %RSD.

Accuracy: We prepared five water solutions of EtBr having the following concentrations: 0.1, 0.5, 1.0, 1.5 and 2.0 μg/mL. The following parameters were set: $\bar{x}$, s, μ, %RSD.

Linearity: We drafted analytical curve for five different concentrations of EtBr. Figure 2 presents a linear dependence between the intensity of fluorescence and the concentration of EtBr. The data obtained allowed to calculate straight line coefficient $y = bx + a$ and correlation coefficient r.

Limit of detection (LOD): Solution of EtBr (0.1 μg/mL) was prepared. The limit of detection was calculated.

Limit of quantitation (LOQ): Solution of ethidium bromide (0.1 μg/mL) was prepared. The limit of quantitation was calculated.

Fluorometric measurements

Dilutions of the analyzed compounds (0.5, 0.25, 0.1, 0.05 and 0.01 μg/mL) prepared in 0.2% DMSO solution were added to the mixture containing 0.5 μg DNA, 1 unit of topoisomerase I and 1 μg/mL of EtBr. The whole mixture was incubated at 37°C for 1 h. Emission at 618 nm was determined with the use of excitation at 285 nm. The width of the slit, both for the excitation and emission was 5 nm. Assays were performed at 20°C. Intensity of fluorescence was measured with spectrofluometer Cary Eclipse. The obtained result showed the growth of intensity ΔI of EtBr. The value of fluorescence I was decreased by the intensity of blind trial I_{sl} containing 0.5 μg DNA, 1 unit of topoisomerase I and 1 μg/mL of EtBr. The blind trial after 1 h incubation contained maximum amount of relaxed DNA, which confirms 100% activity of the enzyme. The received results were compared to those of camptothecin, the compound whose inhibition properties were confirmed. Figure 3 presents calculated percentage of enzyme inhibition by a particular compound.

RESULTS AND DISCUSSION

Synthesis

The aim of our study was to determine the method of the synthesis of benzimidazole deriva-

Table 1. Precision of the analytical method.

c (μg/mL)	0.1	0.5	1	1.5	2
I_1	65.146	202.972	375.927	517.804	688.043
I_2	65.110	203.503	375.610	517.233	688.492
I_3	65.317	203.340	376.142	516.130	689.411
$\bar{x}$	65.191	203.272	375.893	517.06	688.649
s	0.203	0.269	0.267	0.850	0.697
%RDS	0.312	0.132	0.071	0.164	0.101
μ	65.691 ± 0.743	203.400 ± 0.386	375.893 ± 0.142	517.06 ± 0.329	688.649 ± 0.202

arithmetic mean ($\bar{x}$), standard deviation (s), the mean ± SD (μ), relative standard deviation [%] (RSD)

Table 2. Accuracy of the analytical method.

c (μg/mL)	0.1	0.5	1	1.5	2
I_1 determined	65.274	203.955	379.283	514.531	691.841
I_2 determined	65.159	203.421	379.283	514.655	691.715
I_3 determined	65.105	203.495	378.927	515.203	690.492
$\bar{x}$ determined	65.179	203.624	379.164	514.796	691.349
I real	65.430	203.738	377.238	515.722	689.649
Mean recovery %	99.61	99.94	100.51	99.82	100.25
s	0.086	0.290	0.205	0.357	0.743
%RDS	0.132	0.142	0.054	0.069	0.107

arithmetic mean ($\bar{x}$), standard deviation (s), relative standard deviation [%] (RSD)

tives (**9**-**22**). During the experiments, two methods of synthesis of benzimidazole derivatives (**9**-**15**) were defined. The first method was a direct cyclocondensation of 5-chlorodiamine (**1**) or 5-nitrodiamine (**2**) with: 2-naphthaldehyde (**3**), piperonal (**4**), 2-nitrobenzaldehyde (**5**), p-chlorobenzaldehyde (**6**) or 2-carboxybenzaldehyde (**7**). The other synthetic way involved cyclization of previously isolated Schiff base (**8**). In this way we determined the conditions for the synthesis of benzimidazole derivatives, obtained in direct and indirect condensation. Additionally, we isolated an indirect product (**8**). Next, the product underwent cyclocondensation. As a result, we obtained a benzimidazole derivative – 9. All the received benzimidazole derivatives (**9**-**15**) were oxidized during the reaction with 30% solution of hydrogen peroxide in anhydrous acetic acid to N-oxide benzimidazole derivatives (**16**-**22**) – a new group of planned chemical bonds. All the syntheses were monitored by TLC. Selected assays of compounds were analyzed with HPLC (20, 23).

Fluorometric analysis

Precision: To determine precision of the method the following parameters were determined: $\bar{x}$, s, μ, %RSD. Table 1 presents the results.

Accuracy: To determine accuracy of the method the following parameters were determined: $\bar{x}$, s, μ, %RSD. The mean percentage of recovery was in the range 99.61-100.51%, mean and relative standard deviations are presented in Table 2.

Linearity: Calibration curve characterizes linear dependence with equation of a straight line: I = 329.88x + 35.427. The correlation coefficient was: r^2 = 0.9994. (Fig. 2)

LOD: The limit of detection (arithmetically calculated) was 0.004 μg/mL for EtBr.

LOQ: The limit of quantitation (arithmetically calculated) was 0.012 μg/mL for EtBr.

Camptothecin, the reference compound, with confirmed activity of topoisomerase I inhibition exhibits 40.68% the enzyme inhibition at the concentration 0.1 μg/mL. At this concentration compounds **11**, **12**, **15**, **21**, **22** exhibited a higher level of enzyme inhibition than camptothecin. At the concentration of 0.5 μg/mL, five of the analyzed 5-nitrobenzimidazoles exhibited inhibition of topoisomerase I greater than 50%. N-oxide 2-(4-chlorophenyl)-5-nitrobenzimidazole (**21**) as well as N-oxide 2-(2-carboxyphenyl)-5-nitrobenzimidazole (**22**) proved to be more effective in inhibiting topoisomerase I (at the concentration of 0.5 μg/mL the percentage of the inhibition was 65.31% and 58.86%, respectively) than corresponding benzimidazole derivatives: 2-(4-chlorophenyl)-5-nitrobenzimidazole (**12**) 50.98% and 2-(2-carboxyphenyl)-5-nitrobenzimidazole (**13**) 12.99%.

CONCLUSION

The inhibitory effects of presented benzimidazole derivatives - compounds **11**, **12**, **15**, **21** and **22**, are significant, as these compounds can be used as new potential DNA topoisomerase I inhibitors. They have better effect at lower concentration than camptothecin. Additionally, N-oxide benzimidazole derivatives – compounds **21** and **22**, deserve special attention because of their stronger activity than their analogues without N-oxide bond. Because we determined the cytotoxic effects of presented benzimidazole derivatives, we know that compound **12** (IC_{50} 22.42 ± 0.75 μM) and **15** (IC_{50} 33.15 ± 1.43 μM) have activity similar to cisplatin, which is clinically used as anticancer drug in the treatment of human solid tumors. The IC_{50} determinated experimentally for cisplatin against WM 115 was 18.2 ± 4.3 μM. (20, 24) These all conclusions are subject to our further investigation, to check the activity of the investigated compounds against hypoxia.

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