

# New benzimidazole derivatives with potential cytotoxic activity — study of their stability by RP-HPLC

Katarzyna Błaszczak-Świątkiewicz<sup>1</sup>✉, Marek Mirowski<sup>2</sup>, Katarzyna Kaplińska<sup>2</sup>, Rafał Kruszyński<sup>3</sup>, Agata Trzęsowska-Kruszyńska<sup>3</sup> and Elżbieta Mikiciuk-Olasik<sup>1</sup>

<sup>1</sup>Department of Pharmaceutical Chemistry and Drug Analysis, Medical University, Lodz, Poland; <sup>2</sup>Department of Pharmaceutical Biochemistry, Medical University, Lodz, Poland; <sup>3</sup>Department of X-ray Crystallography and Crystal Chemistry, Institute of General and Ecological Chemistry, Technical University of Lodz, Poland

Obtained benzimidazole derivatives, our next synthesized heterocyclic compounds, belong to a new group of chemical bondings with potential anticancer properties (Błaszczak-Świątkiewicz & Mikiciuk-Olasik, 2006, *J Liquid Chrom Rel Tech* 29: 2367–2385; Błaszczak-Świątkiewicz & Mikiciuk-Olasik, 2008, *Wiad Chem* 62: 11–12, in Polish; Błaszczak-Świątkiewicz & Mikiciuk-Olasik, 2011, *J Liquid Chrom Rel Tech* 34: 1901–1912). We used HPLC analysis to determine stability of these compounds in 0.2% DMSO (dimethyl sulfoxide). Optimisation of the chromatographic system and validation of the established analytical method were performed. Reversed phases (RP-18) and a 1:1 mixture of acetate buffer (pH 4.5) and acetonitrile as a mobile phase were used for all the analysed compounds at a flow rate 1.0 mL/min. The eluted compounds were monitored using a UV detector, the wavelength was specific for compounds 6 and 9 and compounds 7 and 10. The retention time was specific for all four compounds. The used method was found to have linearity in the concentration range of (0.1 mg/mL–0.1 µg/mL) with a correlation coefficient not less than  $r^2=0.9995$ . Statistical validation of the method proved it to be a simple, highly precise and accurate way to determine the stability of benzimidazole derivatives in 0.2% DMSO. The recoveries of all four compounds examined were in the range 99.24–100.00%. The developed HPLC analysis revealed that the compounds studied remain homogeneous in 0.2% DMSO for up to 96 h and that the analysed *N*-oxide benzimidazole derivatives do not disintegrate into their analogues — benzimidazole derivatives. Compounds 8, 6 and 9 exhibit the best cytotoxic properties under normoxic conditions when tested against cells of human malignant melanoma WM 115.

**Key words:** anti-cancer drugs, benzimidazole, RP-HPLC, hypoxia, nitrobenzimidazole

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## INTRODUCTION

According to recent studies, modern anti-cancer drugs used to treat early stages of neoplastic diseases have a mechanism of action based on characteristic the hypoxia for cancer cells (McKeown *et al.*, 2007). These compounds belong to the group of drugs with bioreductive

mechanism of action (Albertella *et al.*, 2008). They are activated in hypoxic conditions influenced by specific biochemical mechanisms (Denny, 2000). Nitro compounds, such as CB 1954 and those containing *N*-oxide groups such as tirapazamine and banoxantrone AQ4N are the most effective (Lin *et al.*, 1972; Cowan *et al.*, 1994; McKeown *et al.*, 1996; Koch, 1993; Brown, 1999). Moreover, derivatives containing benzimidazole ring are active as human DNA topoisomerase I inhibitors (Selcen *et al.*, 2007; 2009; Coban *et al.*, 2009; Singh & Tandon, 2011). In the first part of our experimental study we decided to investigate the potential usefulness of a range of heterocyclic compounds by us. We started our analytical experiments in order to establish stability of the obtained benzimidazole derivatives in a typical solvent used in cytotoxicity studies (0.2% DMSO) for up to 96 h. That time was chosen as it corresponded to the duration of our cytotoxicity studies performed on human melanoma (WM-115) cells lines. The structures of the analysed compounds are presented in Fig. 1.

The reaction of diamine with aldehydes is known and described in the literature (Jerchel *et al.*, 1952; Preston, 1974; Panieres *et al.*, 2000). We worked out conditions for obtaining new benzimidazole derivatives and *N*-oxide benzimidazole derivatives, expected to possess anticancer properties and selective affinity for cells under hypoxic conditions.

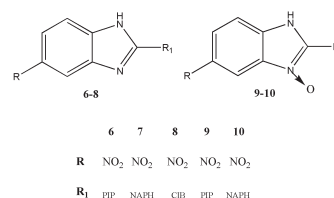


Figure 1. Structural formula of benzimidazole derivatives.

✉ e-mail: katarzyna.blaszczak-swiatkiewicz@umed.lodz.pl

**Abbreviations:** AR, analytical reagent; DMEM, Dulbecco's Modified Eagle Medium; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; HL-60, human leukemia cells; IC50, inhibitory concentration; LOD, limit of detection; LOQ, limit of quantification; MS, mass spectrum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NALM-6, human, peripheral blood, leukemia, pre-B cell; RP-HPLC, reversed-phase high-performance liquid chromatography; RSD, relative standard deviation; S.D., standard deviation; SDS, sodium dodecyl sulfate; TMS, tetramethylsilane; WM-115, human melanoma cell; Rt, retention time; k, retention coefficient; n, theoretical plates; r, correlation coefficient;  $\bar{x}$ , arithmetic mean.

Our research aimed at the synthesis of new benzimidazole derivatives (6–10). They were obtained by direct condensation of a proper diamine (1, 2) with a proper aldehyde (3–5) in an anhydrous solvent at its boiling point. Compounds whose structure resembled *N*-oxide benzimidazoles (9, 10) (Fig. 1) were obtained by direct reaction of 30% solution of hydrogen peroxide with the benzimidazole derivatives obtained in the first stage (6–8), in glacial acetic acid. The synthetic process included the following reactions (Scheme 1).

The designed analytical investigations conducted by means of high performance liquid chromatography (HPLC) concerned:

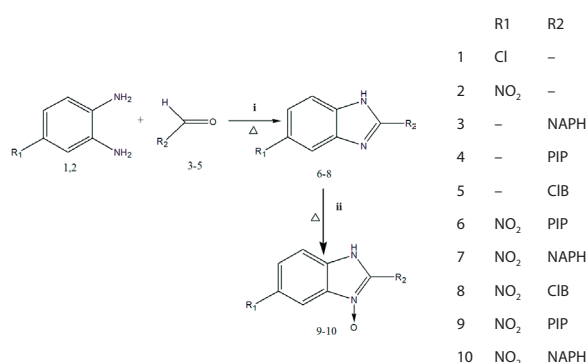
- optimisation of the chromatographic system for the analysed compounds,
- validation of the established analytical method,
- examination of the stability of the benzimidazole derivatives in of 0.2% DMSO with the validated HPLC method.

The obtained compounds were biochemically tested for cytotoxic properties (Mikiciuk-Olasik, 2004). The cytotoxic activity of benzimidazole derivatives (6–8) and *N*-oxide benzimidazole derivatives (9, 10) was determined on human malignant melanoma cell line WM 115 in chronic exposition for three days with the use of trypan blue test. The reference compound was tirapazamine I. Cell survival was determined in normoxia. At present similar experiments are conducted under hypoxic conditions.

## EXPERIMENTAL

### Procedures of synthesis

IR spectra (KBr discs) were registered using a Mattson Infinity Series FT-IR spectrophotometer (USA). <sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded on a 300 MHz Varian Mercury spectrometer (Germany) in DMSO-*d*<sub>6</sub> or CDCl<sub>3</sub> as solvent and tetramethylsilane (TMS) as internal reference. MS spectra (FAB method, M+1, matrix-glycerine) were recorded on a Finnigan Mat 95 spectrometer (Brema, Germany). Carbon, hydrogen and nitrogen elemental analyses were performed using a Perkin Elmer 2400 series II CHNS/O analyzer (Madison, USA), and agreed with proposed structures within ±0.3% of theoretical values. Silica gel 60 F<sub>254</sub> on aluminium sheets was used for TLC.



**Scheme 1. Synthesis of compounds 6–10.**

Reagents: i, anhydrous ethanol + nitrobenzene, ii, anhydrous acetic acid + hydrogen peroxide. NAPH, naphthyl; PIP, piperonyl; CIB, chlorobenzyl.

### General procedure for preparation of compounds 6–8 by directed cyclocondensation

A mixture of equimolar portions of 4-nitro-1,2-phenylenediamine (10 mmole) (2) or 4-chloro-1,2-phenylenediamine (10 mmole) (1) and the appropriate aldehyde (3–5) (10 mmole) were dissolved in 50 ml anhydrous ethanol and heated for 24 h under reflux. Next nitrobenzene (3 mL) was added and the mixture was heated for another 24 h. Next, the reaction mixture was concentrated to the half of its initial volume and a crude precipitate was filtered off. As a result, in this reaction the following compounds were obtained with chromatographic purity:

#### 2-benzo[1,3]dioxol-5-yl-5-nitro-1H-benzimidazole (6)

Yield 65%, IR (KBr)  $\nu/\text{cm}^{-1}$ : 3331 (NH), 2914 (CH<sub>2</sub>), 1505 (NO<sub>2</sub>asym), 1300 (NO<sub>2</sub>sym) 1482 (C=N), 1257 (C-O-Csym), 1036 (C-O-Casym); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>)  $\delta$ : 4.4 (s, 1H, NH), 6.1 (s, 2H, CH<sub>2</sub>), 7.2 (d, 1H, CH, J=8.1 Hz), 7.6 (s, 2H, CH), 7.8 (d, 1H, CH, J=1.8 Hz), 8.1 (d, 1H, CH, J=2.2 Hz), 8.4 (s, 1H, CH); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>)  $\delta$ : 149.7, 147.9, 143.6, 142.5, 136.7, 135.3, 133.2, 129.8, 123.3, 122.8, 121.9, 117.8, 116.0, 101.9; MS  $m/z$ : 284.2, 282.1; calculated for C<sub>14</sub>H<sub>9</sub>N<sub>3</sub>O<sub>4</sub>: C 59.37, H 3.20, N 14.84; found C 59.15, H 3.21, N 14.90. R<sub>f</sub> (chloroform/methanol – 6.25% v/v) = 0.48

#### 2-naphthyl-5-nitro-1H-benzimidazole (7)

Yield 60%, IR (KBr)  $\nu/\text{cm}^{-1}$ : 3422 (NH), 3043 (ArH), 1523 (NO<sub>2</sub>asym), 1343 (NO<sub>2</sub>sym), 1474 (C=N); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>)  $\delta$ : 6.0 (s, 1H, NH), 7.6 (d, 1H, CH, J=1.6 Hz)  $\delta$ : 7.8 (d, 1H, CH, J=8.9 Hz), 8.0 (dd, 2H, CH, J=8.3 Hz), 8.1 (s, 1H, CH), 8.2 (dd, 2H, CH, J=2.2 Hz) 8.4 (s, 1H, CH), 8.5 (s, 1H, CH), 8.8 (s, 1H, CH); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>)  $\delta$ : 159.6, 151.2, 142.7, 135.9, 135.3, 134.6, 133.9, 133.8, 132.6, 131.8, 129.8, 128.8, 128.7, 128.2, 127.7, 124.4, 112.9; MS  $m/z$ : 290.1, 288.2; calculated for C<sub>17</sub>H<sub>11</sub>N<sub>3</sub>O<sub>2</sub>: C 70.58, H 3.83, N 14.53; found C 70.30, H 3.82, N 14.49. R<sub>f</sub> (chloroform/methanol – 6.25% v/v) = 0.49

#### 2-(4-chlorophenyl)-5-nitro-1H-benzimidazole (8)

Yield 70%, IR (KBr)  $\nu/\text{cm}^{-1}$ : 3289 (NH), 1536 (NO<sub>2</sub>asym), 1332 (NO<sub>2</sub>sym), 1499 (C=N); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>)  $\delta$ : 14.0 (s, 1H, NH), 7.6 (dd, 2H, CH, J=2.6 Hz), 7.8 (dd, 2H, CH, J=0.4 Hz), 8.1 (dd, 2H, CH, J=2.2 Hz), 8.4 (s, 1H, CH); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>)  $\delta$ : 143.4, 142.8, 136.7, 135.7, 135.4, 129.8, 129.3, 128.7, 127.9, 125.5, 123.3; MS  $m/z$ : 274.2, 272.2; calculated for C<sub>13</sub>H<sub>8</sub>ClN<sub>3</sub>O<sub>2</sub>: C 57.05, H 2.95, N 15.35; found C 56.83, H 2.94, N 15.29. R<sub>f</sub> (chloroform/methanol – 6.25% v/v) = 0.52.

### General procedure for preparation of compounds 9 and 10

15 mL of anhydrous acetic acid and 10 mmole hydrogen peroxide were added to 10 mmole appropriate derivatives of benzimidazole. The mixture was heated under reflux at 50–60°C. After six hours another 5 mmole of hydrogen peroxide was added. After 24h heating, the mixture was concentrated in vacuum to a small volume, diluted with methylene chloride, and washed with sodium carbonate solution. The organic layer was dried, concentrated in vacuum and diluted with diethyl ether. The solid precipitate was filtered off and recrystallized from isopropanol. Chromatographic purity of the obtained compound was confirmed using chloroform/methanol 6.25% (v/v) as eluent. As a result, in this reaction the

**Table 1.** Crystallographic and structure refinement data

Compound	8
Empirical formula	C <sub>29</sub> H <sub>25</sub> Cl <sub>2</sub> N <sub>3</sub> O <sub>6</sub>
Formula weight	638.46
Crystal system, space group	monoclinic, <i>P</i> 2 <sub>1</sub> / <i>c</i> (No. 14)
Unit cell dimensions [Å, deg]	<i>a</i> = 15.9047(14) <i>b</i> = 13.5433(12) <i>c</i> = 14.0439(12) $\beta$ = 91.570(9)
Volume [Å <sup>3</sup> ]	3023.9(5)
Z, Calculated density [Mg/m <sup>3</sup> ]	4, 1.402
Absorption coefficient [mm <sup>-1</sup> ]	2.399
F(000)	1320
Crystal size [mm]	0.130, 0.126, 0.107
<i>q</i> range for data collection [°]	2.78 to 67.37
Index ranges	-18 ≤ <i>h</i> ≤ 19, -16 ≤ <i>k</i> ≤ 16, -16 ≤ <i>l</i> ≤ 14
Reflections collected / unique	33674/5332 [ <i>R</i> <sub>int</sub> ] = 0.0285]
Completeness [%]	98.5 (to $\theta = 67^\circ$ )
Min. and max. transmission	0.765 and 0.782
Data/restraints/parameters	5332/0/399
Goodness-of-fit on <i>F</i> <sup>2</sup>	1.078
Final <i>R</i> indices [ <i>I</i> > 2 $\sigma$ ( <i>I</i> )]	<i>R</i> <sub>1</sub> = 0.0345, <i>wR</i> <sub>2</sub> = 0.0868
<i>R</i> indices (all data)	<i>R</i> <sub>1</sub> = 0.0454, <i>wR</i> <sub>2</sub> = 0.0989
Largest diff. peak and hole [e·Å <sup>-3</sup> ]	1.076, -0.567

following compounds were obtained with chromatographic purity:

#### 2-benzo[1,3]dioxol-5-yl-5-nitro-1H-benzimidazole N-oxide (**9**)

Yield 75%, IR (KBr)  $\nu$ /cm<sup>-1</sup>: 3330 (NH), 1482 (C=N), 1504 (NO<sub>2</sub>asym), 1300 (NO<sub>2</sub>sym), 1237 (C-O-Casym), 1258 (N-O), 1037 (C-O-Csym), <sup>1</sup>H NMR (DMSO-d<sub>6</sub>)  $\delta$ : 13.5 (s, 1H, NH), 8.4 (s, 1H, CH), 8.2-8.1 (dd, 2H, CH, *J* = 8.5, 8.9 Hz), 7.8-7.7 (dd, 2H, CH, *J* = 7.7, 8.3 Hz), 7.1 (d, 1H, CH, *J* = 0.4 Hz), 6.1 (s, 2H, CH<sub>2</sub>); <sup>13</sup>C NMR (DMSO-d<sub>6</sub>)  $\delta$ : 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; calculated for C<sub>14</sub>H<sub>9</sub>N<sub>3</sub>O<sub>5</sub>: C 56.19, H 3.03, N 14.04; found C 56.40, H 3.03, N 13.99. *R*<sub>f</sub> (chloroform/methanol — 6.25% v/v) = 0.51.

#### 2-naphthyl-5-nitro-1H-benzimidazole N-oxide (**10**)

Yield 60%, IR (KBr)  $\nu$ /cm<sup>-1</sup>: 3380 (NH), 3100 (ArH), 1523 (NO<sub>2</sub>asym), 1474 (C=N), 1344 (NO<sub>2</sub>sym), 1261 (N-O), <sup>1</sup>H NMR (DMSO-d<sub>6</sub>)  $\delta$ : 13.8 (s, 1H, NH), 8.8 (s, 1H, CH), 8.5 (s, 1H, CH), 8.3 (dd, 2H, CH, *J* = 1.4, 1.6 Hz), 8.2 (d, 1H, CH, *J* = 2.2 Hz), 8.1 (d, 1H, CH, *J* = 2.8 Hz), 8.0 (d, 1H, CH, *J* = 3.4 Hz), 7.8 (d, 1H, CH, *J* = 8.7 Hz), 7.6 (dd, 2H, CH, *J* = 2.9 Hz); <sup>13</sup>C NMR (DMSO-d<sub>6</sub>)  $\delta$ : 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, calculated for C<sub>17</sub>H<sub>11</sub>N<sub>3</sub>O<sub>5</sub>: C 66.88, H 3.63, N 13.76; found C 66.65, H 3.64, N 13.72. *R*<sub>f</sub> (chloroform/methanol — 6.25% v/v) = 0.53.

#### Preparation of crystals

Compound **8** (500 mg) was dissolved in 8 mL of dimethylformamide (DMF). The solution was heated to 67°C and 1.2 mL of distilled water was added by 0.05 mL portions every 2 minutes delay between each portion addition (addition of larger amount of water leads to precipitation of amorphous form of **8**). During the whole process (48 min) the temperature remained between 66°C and 67.5°C. Next the crystallisation vessel was transferred to a hermetic thermally-isolated semiautomatic crystalliser heated to 67°C. The following parameters were applied to the crystallisation process: temperature decreasing speed 2°C/24h, vessel internal volume increase: 1 mL/24 h during first 19 days of crystallisation and 3 mL /24 h during next 14 days. After 25 days the temperature was fixed to 21°C and vessel volume enlargement was set to 5 mL/24 h. After the next 11 days small but relatively good quality crystals of **8** grew. The crystals were filtered off, dried by a stream of dry helium and stored in sealed vessels filled with dry helium. Note: Usage of pure DMF for crystallisation leads to amorphous form of **8** (according to XRPD).

#### Crystal structure determination

A light yellow (almost colourless) prism crystal of compound **8** was sealed in glass capillary filled with helium and next it was mounted on a KM-4-CCD automatic diffractometer equipped with a CCD detector, and used for data collection. X-ray intensity data were collected with graphite monochromated CuK $\alpha$  ( $\lambda$  = 1.54178 Å) radiation at a temperature 291.0(3) K, with  $\omega$  scan mode. The exposure of 27 seconds time was used, and reflections inside Ewald sphere were collected up to  $\theta$  = 67.37°. The unit cell parameters were determined from 2463 strongest reflections. Details concerning crystal data and refinement are given in Table 1. Examination of reflections on two reference frames monitored after each 20 frames measured showed 7.19% loss of the intensity during measurement. During the data reduction Lorentz, polarization, decay and numerical absorption (STOE&Cie GmbH, 1999) corrections were applied. The structure was solved by partial structure expansion procedure. All non-hydrogen atoms were refined anisotropically using full-matrix, least-squares technique on *F*<sup>2</sup>. All the hydrogen atoms were found from difference Fourier synthesis after four cycles of anisotropic refinement, and refined as “riding” on the adjacent atom with geometric idealisation after each cycle of refinement and individual isotropic displacement factors equal 1.2 times the value of equivalent displacement factor of the par-

**Table 2.** Selected structural data for compound **12** [Å, °].

N1—C8	1.369(6)
N2—C13	1.375(6)
C7—N1	1.323(6)
C7—N2	1.364(6)
N51—C58	1.278(11)
N52—C63	1.332(11)
C57—N51	1.354(10)
C57—N52	1.332(9)
N1—C7—N2	112.8(5)
N51—C57—N52	112.4(7)

ent non-methyl carbon or nitrogen atoms and 1.5 times of parent methyl group carbon atoms and oxygen atom. The methyl groups were allowed to rotate about their local three-fold axis. The SHELXS97, SHELXL97 and SHELXTL (Sheldrick, 2008) programs were used for all the calculations. Atomic scattering factors were those incorporated in the computer programs. Selected interatomic bond distances and angles are listed in Table 2 and intermolecular interactions are listed in Tables 3 and 4.

### Procedures of chromatography

**Equipment.** An HPLC Waters 600 LC system was used with a Supelco RP-18 column (15 cm × 4 mm × 5 μm plus symmetry C18 guard, Waters) run at 20°C. Chromatographic peaks were identified with a UV detector (Waters). A computer programme Millennium 32 version 4.0 (Waters) was used for processing chromatograms.

**Chemicals and reagents.** We used ammonium acetate (AR grade), acetonitrile (HPLC grade) and acetic acid (AR grade).

**Chromatographic conditions.** Compound samples were weighed on analytical scales with an accuracy of 0.01 mg. They were dissolved in a mobile phase consisting of solvents of chromatographic purity. The gradient mobile phase consisted of acetate buffer and acetonitrile (1:1, v/v, pH 4.5). 10 mmole acetate buffer solution pH 4.5 was prepared. pH of the whole was adjusted to 4.5 ± 0.05 with diluted acetic acid and filtered through 0.45 μm membrane filter. The analysis was started at a ratio of 4:1, v/v of acetate buffer pH 4.5 and acetonitrile mixture. The proportion of acetonitrile was increased linearly to 50% v. The mobile phase flow rate was 1 mL/min. Compounds **6** and **9** were monitored at 281 nm and compounds **7** and **10** at 262 nm.

### Standard Preparation

Samples of all the compounds at various concentrations (mg/mL) dissolved in the mobile phase, i.e. acetate buffer pH 4.5 and acetonitrile at a ratio 4:1 v/v, were prepared in 5 mL, 10 mL, 25 mL volumetric flasks. Solution injections of 6 μL and 10 μL were performed with the use of an autosampler (Waters). Each sample was analysed three times. The final result was presented as an arithmetic mean.

### Optimisation and Validation of HPLC system

**Specificity.** Solutions of 0.1 mg/mL of compounds **6** and **7**, **9** and **10** were prepared by being dissolved in mobile phase. The initial mobile phase used for the analysis of the compounds **6**, **7**, **9**, **10** contained the mixture of acetate buffer and acetonitrile at a ratio of 4:1, v/v and then the proportion of the mixture increased to 1:1, v/v. A chromatogram for a control sample ( $T_0$ ) was determined as well.

**Precision.** The prepared solutions contained 25% to 100% (v/v) of the studied compounds content.

**Accuracy.** The prepared solutions contained 25% to 100% (v/v) of the studied compounds content.

**Linearity.** Solutions of particular compounds (**6** and **7**, **9** and **10**) were prepared at 0.1 mg/mL, 0.05 mg/mL, 0.01 mg/mL, 0.005 mg/mL, 0.001 mg/mL, 0.5 μg/mL, 0.1 μg/mL.

**Limit of Detection (LOD).** Concentration of 0.1 μg/mL of a particular compound in the solvent was prepared.

**Limit of Quantification (LOQ).** Concentration, μg/mL, of a given compound in the solvent was prepared.

**Cytotoxicity.** Human malignant melanoma cell line WM 115 was used. Cells were cultured in DMEM medium containing stabilized L-glutamine supplemented with 10% heat-inactivated fetal bovine serum and 5 μg/mL gentamycin, at 37°C in a 95% air/5% CO<sub>2</sub> atmosphere.

WM 115 cells were seeded at 1 × 10<sup>4</sup> per well of a 96-well plate. After 24 hours cells were exposed to the compounds (range concentration 500–1 μM) prepared in DMSO. Cells were incubated for 72 hours. Then MTT assay was used to evaluate cytotoxic activity of the analyzed compounds. Briefly, MTT solution was added to cells and the plate was incubated for additional 3 hours. The purple formazan crystals formed by alive cells were solubilized in DMF/SDS solution and optical density was measured at 572 nm. Control cells were incubated with DMSO only. Tirapazamine was used as a reference compound.

### Statistical analysis of the data

The results are expressed as mean ± S.D. Statistical analysis was made by using Student's *t*-test. *P* < 0.05 was considered significant.

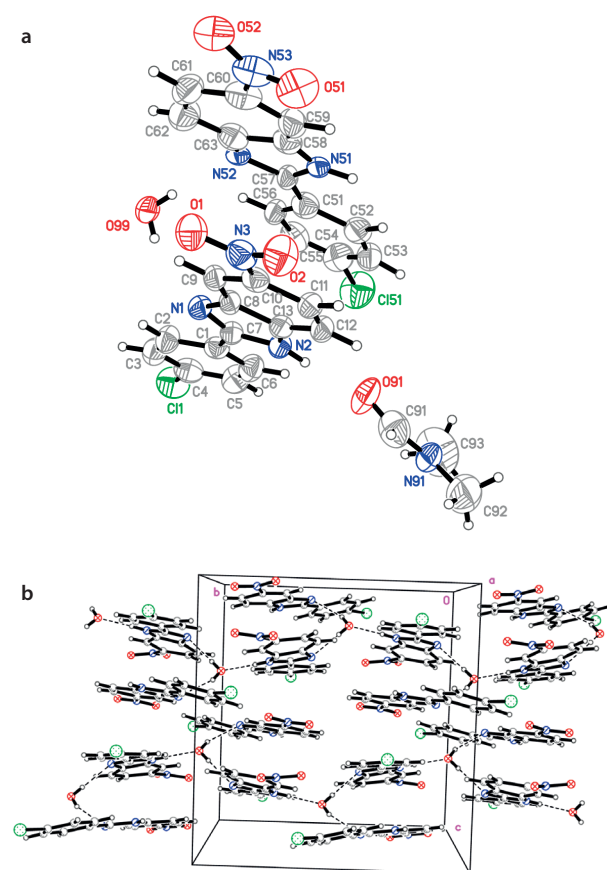
### Study on benzimidazole derivative stability in 0.2 % DMSO

**Sample preparation.** A sample of the analysed compound was weighed on analytical scales with an accuracy of 0.01 mg, 1 mL of 0.2% DMSO was added to a 10 mL volumetric flask with the weighed substance. After 96 h the flask was filled up to 10 mL with a mixture of acetate buffer and acetonitrile at a ratio of 4:1, v/v.

## RESULTS AND DISCUSSION

### X-ray structure analysis

A perspective view of compound **8** structure and packing is shown in Fig. 2a and 2b, respectively. All atoms occupy the general positions. The asymmetric part of the unit cell contains two molecules of **8** possessing slightly different conformations, one water molecule and one DMF molecule. Some of the atoms in both heterocyclic molecules (mainly terminal ones) show differences in magnitude between components of the anisotropic displacement parameters along chemical bonds and in the equivalent isotropic displacement parameters in comparison to those of adjacent bonded atoms. This originates primarily from the flat character of molecules of **8** and thus more privileged motion and static disorder in the plane of the molecule. Both molecules of **8** are close to planarity, however, slight but obvious deviations exist. The nitro and chlorobenzene substituents are inclined at 1.4(4) and 7.3(2)° to the 6-nitro-1H-benzimidazol-2-yl moiety containing C1 atom (molecule A hereafter) and at 18.2(14) and 9.3(3)° to the one containing C51 atom (molecule B hereafter). The maximum deviation from weighed least squares plane calculated thought all non hydrogen atoms of fused 9-membered ring system is found for the C8 atom (0.020(4) Å, molecule A) and N52, C57 atoms (-0.064(6) Å, molecule B). The bond lengths of the imidazole moieties (Table 2) suggest strong delocalisation of bonds within the 5-membered rings. Noteworthy is the fact that in one molecule (A) the NH group of imidazole moiety can be considered as existing in *trans* configuration to the nitro group whereas



**Figure 2a.** Molecular structure of compound **8** with atom numbering scheme.

Plotted with 30% probability of displacement ellipsoids.

**Figure 2b.** Molecular packing showing two-dimensional supra-molecular net of **8**.

Hydrogen bonds are indicated by dashed lines.

in the second one in *cis* configuration (molecule B) and thus formally it can be stated that one molecule possesses a 6-nitro substituent (*cis* one) and a 5-nitro substituent (*trans* one).

The molecules of **8** and solvent molecules are connected by N—H...O and O—H...N intermolecular interactions (Table 3) to the hydrogen-bonded ribbon extending along the crystallographic [010] axis (Fig. 2b). The interactions form the  $N_1$ DDDD zero-dimensional unitary graph set, which becomes one-dimensional on the second level, i.e.  $N_2C_2^2(6)DD$ . In the structure one intramolecular C—H...N and one intermolecular C—H...O short contact can be found (Table 3) which can be classified as weak hydrogen bonds (Desiraju 1999). The molecules of compound **8** are connected by  $\pi$ ... $\pi$  stacking interactions (Table 4) to the piles extending along the crystallographic [001] axis. In this way a supramolecular two-dimensional network interconnected by different kinds of interaction, is formed along the crystallographic (100) plane (Fig. 2b).

### Validation

**Specificity results.** An analytical interpretation of chromatographic data was made by determining retention time ( $R_t$ ), retention coefficient ( $k'$ ) and number of theoretical plates ( $n$ ). (Table 5).

**Table 3.** Hydrogen bonds geometry of compound **12** [ $\text{\AA}$ ,  $^\circ$ ].

D—H...A	d(D—H)	d(H...A)	d(D...A)	<(DHA)
N2—H2N...O91	0.86	1.86	2.706(7)	167
N51—H51N...O99 <sup>i</sup>	0.86	1.86	2.695(6)	162
O99—H99O...N52	0.77	2.03	2.807(7)	179
O99—H99P...N1	0.74	2.14	2.847(5)	159
C2—H2...N1	0.93	2.59	2.904(8)	100
C52—H52...O99 <sup>i</sup>	0.93	2.59	3.466(8)	157

Symmetry transformations used to generate equivalent atoms: (i)  $-x+1, y+1/2, -z+1/2$

**Precision results.** We determined the compound content in the sample, with a calibration curve for a given substance at concentrations ranging from 0.1 mg/mL to 0.1  $\mu\text{g/mL}$ . The following parameters:  $\bar{x}$ ,  $s$ ,  $\mu$ ,  $RSD$  were used to define accuracy of the method for a particular compound. The results are presented in Table 6.

**Accuracy results.** We determined the compound content in the sample with a calibration curve for a given substance at concentrations ranging from 0.1 mg/mL to 0.1  $\mu\text{g/mL}$ . The recovery degree defined for a given compound content was expressed in mg/mL and the following parameters:  $\bar{x}$ ,  $s$ ,  $\mu$ ,  $RSD$  were used to determine accuracy of the method for a particular compound. The results are presented in Table 7.

**Linearity results.** A linear relationship between a particular sample concentration and a peak area was established. A coefficient of a straight line displacement value (b) and an inclination parameter value (a) expressed by the equation  $y=ax-b$  for the analysed substance was calculated. Correlation coefficients were also determined. The results are presented in Fig. 3.

**Limit of Detection (LOD) results.** The LOD parameter was determined as an arithmetic mean from three individual measurements, each one an arithmetic mean from three subsequent injections. The limits of detection at a signal-to-noise ratio of 3 were: 0.03  $\mu\text{g/mL}$ , 0.03  $\mu\text{g/mL}$ , 0.03  $\mu\text{g/mL}$  and 0.045  $\mu\text{g/mL}$  for compounds **6**, **7**, **9** and **10**, respectively.

**Limit of Quantification (LOQ) results.** The LOQ parameter was determined as an arithmetic mean from three individual measurements, each one an arithmetic mean from three subsequent injections. The limits of quantification at a signal-to-noise ratio of 10, were: 0.09  $\mu\text{g/mL}$ , 0.1  $\mu\text{g/mL}$ , 0.1  $\mu\text{g/mL}$ , 0.1  $\mu\text{g/mL}$  for compounds **6**, **7**, **9** and **10** respectively.

### Optimisation of the chromatographic system for the analysed benzimidazole derivatives

Compounds assigned for the chromatographic analysis are derivatives of benzimidazole (**6** and **7**, **9** and **10**). The study was performed on columns with a reversed phase (RP-18) due to the necessity of analysing two groups of compounds (benzimidazole derivatives **6** and **7** and N-oxide of benzimidazole derivatives **9** and **10**) with different polar properties. We analyzed these two groups of compounds in the same chromatographic system in which disintegration of N-oxide of benzimidazole derivatives into their analogues of the benzimidazole structure could be confirmed or excluded. Chromatograms in the mixture of acetate buffer pH 4.5/acetonitrile in a ratio 4:1 v/v initiated optimisation of the system for chro-

**Table 4. Stacking interactions (distances in Å and angles in °).**

Cg(1), Cg(2), Cg(3), Cg(4), Cg(5), Cg(6) indicates the centroids of aromatic rings (R) containing N(1), C(1), C(10), N(51), C(51), C(60) atoms respectively, a is a dihedral angle between planes I and J, b is an angle between Cg(I)-Cg(J) vector and normal to plane I and dp is a perpendicular distance of Cg(I) on ring J plane.

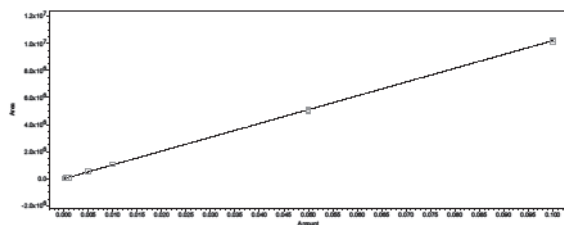
R(I)•••R(J)	Cg•••Cg	a	b	d <sub>p</sub>
Cg(1)•••Cg(5)	4.276(4)	5.2(3)	37.14	-3.5828(19)
Cg(1)•••Cg(6)	3.757(3)	2.9(3)	22.14	-3.5028(19)
Cg(1)•••Cg(6) <sup>i</sup>	3.718(3)	11.1(3)	17.70	3.2600(19)
Cg(2)•••Cg(6)	4.850(4)	10.2(3)	46.84	-3.667(3)
Cg(3)•••Cg(5)	3.690(3)	4.5(3)	21.04	-3.537(2)
Cg(3)•••Cg(5) <sup>i</sup>	4.279(3)	16.1(3)	33.86	3.293(2)
Cg(3)•••Cg(6)	4.733(3)	2.8(3)	43.56	-3.4772(19)
Cg(3)•••Cg(6) <sup>i</sup>	3.877(3)	10.6(3)	23.66	3.4698(19)
Cg(3)•••Cg(7)	4.496(4)	7.5(3)	43.99	-3.594(2)
Cg(5)•••Cg(1)	4.276(4)	5.2(3)	33.08	3.409(3)
Cg(5)•••Cg(3)	3.690(3)	4.5(3)	16.56	3.444(3)
Cg(5)•••Cg(3) <sup>ii</sup>	4.279(3)	16.1(3)	39.67	-3.553(3)
Cg(6)•••Cg(1)	3.758(3)	2.9(3)	21.20	3.480(3)
Cg(6)•••Cg(1) <sup>ii</sup>	3.717(3)	11.1(3)	28.74	-3.541(3)
Cg(6)•••Cg(2)	4.850(4)	10.2(3)	40.88	3.317(3)
Cg(6)•••Cg(3)	4.733(3)	2.8(3)	42.71	3.430(3)
Cg(6)•••Cg(3) <sup>ii</sup>	3.876(3)	10.6(3)	26.49	-3.550(3)
Cg(7)•••Cg(3)	4.496(4)	7.5(3)	36.92	3.234(3)

Symmetry transformations used to generate equivalent atoms: (i) x, -y+3/2, z-1/2; (ii) x, -y+3/2, z+1/2

matographic separation of the described benzimidazole derivatives. A good linear relationship between area of peak and amount of substance was obtained for a ratio of 1:1 v/v of the mixture consisting of acetate buffer, pH 4.5 and acetonitrile.

#### Results of the analysis in acetate buffer, pH 4.5 and acetonitrile 1:1 v/v for all compounds

The conducted study aimed at analysing stability of new benzimidazole derivatives (**6**, **7**, **9**, **10**) in the aqueous environment of 0.2% DMSO. The obtained chromatograms allowed us to conclude that, due to unsatisfactory results of separating all benzimidazole (**6–10**) derivatives with the mixture of water/acetonitrile as a mobile phase, the composition of the mobile phase should be modified. Low values of retention coefficient *k* and very low values of the number of theoretical plates *n*,



com. 6	$y=1.10 \text{ e}+006x-1.15 \text{ e}+006, r^2=0.9999$
com. 7	$y=1.85 \text{ e}+005x-1.94 \text{ e}+005, r^2=0.9995$
com. 9	$y=5.33 \text{ e}+005x-5.64 \text{ e}+005, r^2=0.9995$
com. 10	$y=4.35 \text{ e}+005x-4.47 \text{ e}+005, r^2=0.9997$

**Figure 3. Regression curve  $y = ax - b$  for compound **6**, **7**, **9**, **10****

as well as broadened peaks, appearing on the chromatograms rendered this system useless for optimisation of the studied compounds. The parameters of separation of four compounds (**6**, **7**, **9** and **10**) achieved with the use of the mixture of water/acetonitrile as a mobile phase were not regarded satisfactory and did not provide the basis for identification of benzimidazole and N-oxide benzimidazole in their mixture. We decided to use a mixture of acetate buffer pH 4.5 and acetonitrile in the ratio: initially 4:1, v/v and finally 1:1, v/v. The proportion of acetonitrile was increased linearly to 50%. Further analyses with the use of the modifier in the ratio acetate buffer/acetonitrile — 1:1,

v/v allowed us to determine retention times of all the analysed compounds. This eluent allowed for obtaining satisfactory separation parameters of four analysed compounds (**6**, **7**, **9**, **10**) for all prepared concentrations within  $10^{-1}$ – $10^{-4}$  mg/mL. The signals characteristic for the analysed sample are sharp, the number of theoretical plates for compound **6** equals  $n=5332$ , for compound **7** — equals  $n=5636$ , for compound **9** — equals  $n=3683$  and for compound **10** — equals  $n=3208$ . Retention time is specific for a given compound. The system can be utilised in further analytical experiments with these substances (Table 5).

The HPLC analysis of the selected compounds in the same system allowed us to draw the following conclusions:

- compounds **6** and **7**, **9** and **10** should be analysed in a system of reversed phases with 1:1, v/v acetate buffer pH 4.5/acetonitrile as an eluent,
- in selected systems, retention times are specific for the analysed substances.

**Table 5. The obtained values of  $R_t$ ,  $k$ ,  $n$  for the system of acetate buffer pH 4.5/ acetonitrile 1:1% (v/v)**

concentration $10^{-1}$ [mg/mL]	$R_{t\text{mean}}$ [min]	$k_{\text{mean}}$	$n_{\text{mean}}$
com. 6	7.475	3.7759e+00	5332
com. 7	7.793	3.7202e+00	5636
com. 9	4.611	1.9690e+00	3683
com. 10	5.064	2.2607e+00	3208

Retention time ( $R_t$ ), retention coefficient ( $k$ ), number of theoretical plates ( $n$ )

Table 6. Precision of the analytical method

sample mg/mL	Concentration % (v/v) of com. 6					
	25.00	37.50	50.00	60.00	75.00	100.00
1	0.047	0.071	0.095	0.114	0.142	0.190
2	0.048	0.070	0.094	0.114	0.141	0.190
3	0.048	0.071	0.095	0.115	0.142	0.191
$\bar{X}$	0.048	0.071	0.095	0.114	0.142	0.190
s	0.0007	0.0007	0.0007	0.0007	0.0007	0.002
$\mu$	0.048±0.0014	0.071±0.0014	0.095±0.0014	0.114±0.0014	0.142±0.0014	0.190±0.0007
RSD (%)	1.47	0.99	0.74	0.62	0.49	0.37
sample mg/mL	Concentration % (v/v) of com. 7					
	25.00	37.50	50.00	60.00	75.00	100.00
1	0.042	0.064	0.084	0.102	0.127	0.169
2	0.042	0.064	0.085	0.103	0.126	0.170
3	0.041	0.064	0.084	0.102	0.127	0.169
$\bar{X}$	0.042	0.064	0.084	0.102	0.127	0.169
s	0.0007	0.00	0.0007	0.0007	0.0007	0.0007
$\mu$	0.042±0.0014	0.064±0.00	0.084±0.0014	0.102±0.0014	0.127±0.0014	0.169±0.0014
RSD(%)	1.68	0	0.84	0.69	0.56	0.42
sample mg/mL	Concentration % (v/v) of com. 9					
	25.00	37.50	50.00	60.00	75.00	100.00
1	0.050	0.075	0.100	0.121	0.150	0.200
2	0.049	0.075	0.101	0.121	0.150	0.201
3	0.050	0.076	0.101	0.121	0.151	0.201
$\bar{X}$	0.050	0.075	0.101	0.121	0.150	0.201
s	0.0007	0.0007	0.0007	0.00	0.0007	0.0007
$\mu$	0.050±0.0014 048	0.075±0.0014	0.101±0.0014	0.121±0.00	0.150±0.0014	0.201±0.0014
RSD (%)	1.4	0.93	0.93	0	0.47	0.35
sample mg/mL	Concentration % (v/v) of com. 10					
	25.00	37.50	50.00	60.00	75.00	100.00
1	0.051	0.077	0.102	0.123	0.154	0.205
2	0.051	0.078	0.103	0.123	0.155	0.205
3	0.051	0.077	0.103	0.124	0.155	0.206
$\bar{X}$	0.051	0.077	0.103	0.123	0.155	0.205
s	0.00	0.0007	0.0007	0.0007	0.0007	0.0007
$\mu$	0.051±0.00	0.077±0.0014	0.103±0.0014	0.123±0.0014	0.155±0.0014	0.205±0.0014
RSD (%)	0	0.92	0.69	0.57	0.46	0.34

Validation of the established HPLC method for the analysis of compound stability in 0.2% DMSO medium.

Determining HPLC method and its optimisation results were essential for applying this method in the study on stability of the obtained benzimidazole derivatives which might have potential anticancer activity.

Validation of the established HPLC method was conducted through determining individual process parameters:

specificity, precision, linearity, accuracy, detectability and determinability.

The elaborated method was:

- specific, as additional peaks on the sample chromatogram are well separated from the main peak and also from one another, and a control sample chromatogram does not show peaks within the studied ranges (Table 5),
- precise —  $s \leq 0.001$  and  $RSD \leq 2\%$  (Table 6),

Table 7. Accuracy of the analytical method

Sample	Contents of the determined substance 7 in relation to the declared (mg/mL)											
	25.00%		37.50%		50.00%		60.00%		75.00%		100.00%	
	Real value	Value determined	Real value	Value determined	Real value	Value determined	Real value	Value determined	Real value	Value determined	Real value	Value determined
1	0.042	0.042	0.064	0.064	0.085	0.085	0.102	0.103	0.129	0.128	0.170	0.170
2	0.040	0.040	0.064	0.065	0.081	0.081	0.105	0.104	0.127	0.127	0.168	0.168
3	0.041	0.041	0.065	0.065	0.083	0.083	0.105	0.104	0.127	0.127	0.172	0.172
Mean recover (%) = 99.99. $s = 0.47\%$ . $\mu = 99.99\% \pm 0.93\%$ . RSD = 0.47%												
Sample	Contents of the determined substance 6 in relation to the declared (mg/mL)											
	25.00%		37.50%		50.00%		60.00%		75.00%		100.00%	
	Real value	Value determined	Real value	Value determined	Real value	Value determined	Real value	Value determined	Real value	Value determined	Real value	Value determined
1	0.053	0.053	0.084	0.083	0.116	0.116	0.121	0.121	0.149	0.149	0.199	0.199
2	0.055	0.055	0.088	0.088	0.110	0.110	0.127	0.127	0.155	0.155	0.198	0.199
3	0.053	0.053	0.084	0.084	0.116	0.116	0.121	0.121	0.147	0.147	0.200	0.199
Mean recover (%) = 100.07. $s = 0.26\%$ . $\mu = 100.07\% \pm 0.27\%$ . RSD = 0.26%												
Sample	Contents of the determined substance 10 in relation to the declared (mg/mL)											
	25.00%		37.50%		50.00%		60.00%		75.00%		100.00%	
	Real value	Value determined	Real value	Value determined	Real value	Value determined	Real value	Value determined	Real value	Value determined	Real value	Value determined
1	0.064	0.064	0.111	0.110	0.128	0.128	0.158	0.159	0.213	0.213	0.277	0.277
2	0.061	0.061	0.107	0.107	0.122	0.122	0.151	0.151	0.214	0.214	0.275	0.275
3	0.061	0.061	0.106	0.106	0.121	0.121	0.150	0.150	0.212	0.212	0.273	0.273
Mean recover (%) = 100.02. $s = 0.56\%$ . $\mu = 100.02\% \pm 1.12\%$ . RSD = 0.56%												
Sample	Contents of the determined substance 9 in relation to the declared (mg/mL)											
	25.00%		37.50%		50.00%		60.00%		75.00%		100.00%	
	Real value	Value determined	Real value	Value determined	Real value	Value determined	Real value	Value determined	Real value	Value determined	Real value	Value determined
1	0.090	0.090	0.111	0.111	0.151	0.152	0.183	0.183	0.201	0.201	0.302	0.302
2	0.095	0.095	0.118	0.118	0.161	0.161	0.205	0.205	0.206	0.206	0.301	0.301
3	0.093	0.093	0.115	0.115	0.177	0.177	0.200	0.200	0.201	0.201	0.304	0.304
Mean recover (%) = 99.96. $s = 0.14\%$ . $\mu = 99.96\% \pm 0.28\%$ . RSD = 0.25%												

Standard deviation ( $s$ ); mean  $\pm$ S.D. ( $\mu$ ); relative standard deviation [%] (RSD)

– accurate, as the mean percentage recovery is between 99.24 and 100.00% and the results range between 95% and 105% (Table 8),  
 – linear, as the determined values fall regressive curve  $y = ax - b$  and the correlation coefficient  $r$  approach 1 (Fig. 3).

Detectability (LOD) and determinability (LOQ) levels for the particular compound, defined with the worked-out analytical method, were also been determined.

#### Stability of benzimidazole derivatives in of 0.2% DMSO

The established analytical method allowed a study of the stability of benzimidazole derivatives **6** and **7**, **9** and **10** in 0.2% DMSO with satisfactory precision, accuracy and specificity. The biochemical experiment which aimed at evaluating cytotoxicity of the studied compounds and their stability in a given environment were performed within the same period of time, i.e. 96 h. We started to

count down the time of 96 h after dissolving the sample in 0.2% DMSO.

We expected that the chromatographic analysis would indicate disintegration of the N-oxide benzimidazole derivatives into benzimidazole derivatives, which would be confirmed by the presence of additional significant peaks on the chromatogram as well as the relation between sample recovery and the real content. We were particularly interested in chromatograms of N-oxide benzimidazole derivatives (compounds **9**, **10**) because of the possibility of their disintegration in this environment.

The chromatographic analysis, performed with the use of validated HPLC method of N-oxide benzimidazole derivatives (compounds **9**, **10**) and their structural analogues, i.e. benzimidazole derivatives (compounds **6**, **7**), showed that these compounds do not undergo disintegration in 0.2% DMSO and for up to 96 h. The compound recoveries ranged from 99.24% to 100.00%, v/v. Homogeneity of compounds **6**, **7**, **9** and **10** was confirmed by analytical interpretation of the chromatograms.



**Table 8. Sample recovery after 96h of incubation in 0.2% DMSO at room temperature**

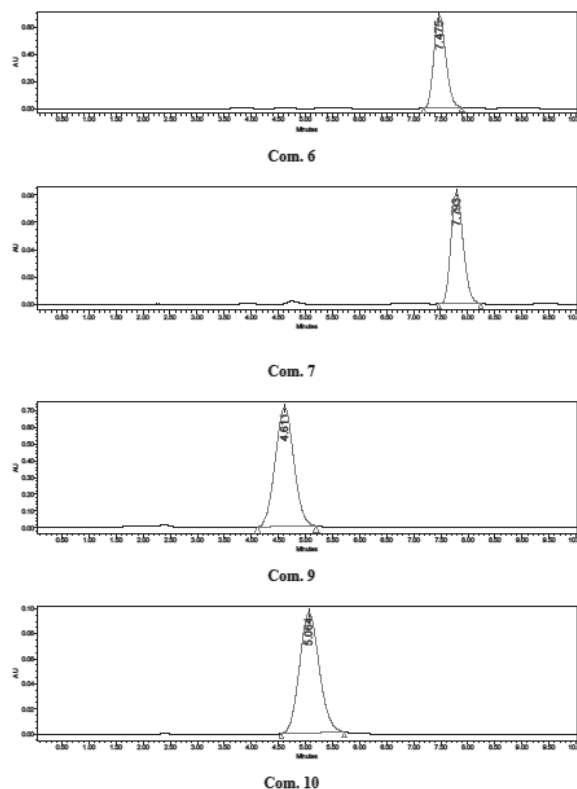
Compound	content (mg/mL)	Value determined (mg/mL)	Recovery $\bar{x}$ n=3
6	0.110	0.110	$\bar{x}$ = 0.110 100.00%
		0.110	
		0.109	
6	0.126	0.126	$\bar{x}$ = 0.126 100.00%
		0.126	
		0.126	
7	0.150	0.149	$\bar{x}$ = 0.149 99.33%
		0.149	
		0.149	
7	0.131	0.130	$\bar{x}$ = 0.130 99.24%
		0.130	
		0.131	
9	0.144	0.144	$\bar{x}$ = 0.144 100.00%
		0.144	
		0.144	
9	0.153	0.153	$\bar{x}$ = 0.153 100.00%
		0.153	
		0.153	
10	0.142	0.142	$\bar{x}$ = 0.142 100.00%
		0.142	
		0.142	
10	0.120	0.120	$\bar{x}$ = 0.120 100.00%
		0.120	
		0.120	

Retention times and UV spectra for the additional peaks appearing on the chromatograms were compared with the retention times and UV spectra characteristic for the analysed derivatives. The recovery degree of the analysed compound was defined. The retention times of the additional peaks appearing on the chromatograms allowed us to draw the conclusion that they do not derive from the compound analysed and are analytically unimportant. The most important fact is that chromatograms of N-oxide benzimidazole derivatives did not demonstrate the presence of an appropriate benzimidazole in the studied environment (Table 8, Fig. 4).

### Cytotoxicity in normoxia

The results of biochemical experiments allowed us to claim that compounds **8**, **6** and **9** demonstrate the best cytotoxic properties in normoxia. All benzimidazole derivatives which have a substituent of piperonyl inhibit the growth of human malignant melanoma WM 115 more than analogous benzimidazole derivatives which contain naphthyl. We also found that N-oxide benzimidazole (compound **10**) is not as strong its analogue compound **9**. This is caused by a specific activity of N-oxide benzimidazole derivatives directed at hypoxic cells more than normoxia cells. An identical activity correlation in normoxia and hypoxia can be observed for tirapazamine — a compound characterized by strong cytotoxic properties in oxygen deficiency conditions (Table 9).

Compounds containing naphthyl group or piperonyl group were synthesized and analysed by our team in previous experiments (Mikiciuk-Olasik *et al.*, 2004). Experiments were performed in oxic and hypoxic conditions with leukemia cells, namely HL-60 and NALM-6. We have proved that quinazoline derivatives with a naphthyl, especially 6-chloro-2-(2-naphthyl)-

**Figure 4. Chromatograms showed stability of compounds 6, 7 and 9, 10 in 0.2% DMSO**

4-phenyl-1,2-dihydroquinazoline N<sup>3</sup>-oxide, had a higher antiproliferative effect on HL-60 cells in oxic conditions than the derivatives containing a piperonyl group. IC<sub>50</sub> estimated experimentally for this compound was 48.8 ± 13.9 μM and it is less than that for tirapazamine (87.8 ± 3.0 μM) (Mikiciuk-Olasik *et al.*, 2004). Our current studies have demonstrated that treatment with compounds containing a chlorobenzyl and piperonyl but not naphthyl group significantly decreased the number of viable malignant melanoma cells. We presume that this discrepancy could be the result of different cell lines used in these two experiments.

In sum, the cytotoxic effects of presented benzimidazole derivatives indicate that they could be useful as anticancer agents, compound **8** being the most effective (IC<sub>50</sub> 22.42 ± 0.75). An important aspect is activity of N-oxide benzimidazole derivatives which demonstrate

**Table 9. Cytotoxicity of compounds for human malignant melanoma cell line WM 115 in chronic exposure under normoxia**

Compound	IC <sub>50</sub> (μmole)
6	85.88±4.60
7	490.47±27.45
8	22.42±0.75
9	89.34±10.44
10	387.14±4.05
Tirapazamine	66.09±0.58

IC<sub>50</sub> is mean ± S.D. (n≥3). Cells were exposed for 72 h to the tested compounds then the viability assay was done.

less specific activity for normoxia. The antiproliferative effect of this newly synthesized compound seems to be similar to, e.g., cisplatin, which is clinically used as anticancer drug in the treatment of human solid tumors. The  $IC_{50}$  determined experimentally for cisplatin against WM 115 was  $18.2 \pm 4.3 \mu\text{M}$  (Budzisz *et al.*, 2010). On the other hand camptothecin, a potent topoisomerase I inhibitor, reduces cancer cell proliferation effectively at a much lower concentration ( $IC_{50}$  from 3.5 nM to 0.3  $\mu\text{M}$ ) (Tanizawa *et al.*, 1994; Gupta *et al.*, 1997; Kaczirek *et al.*, 2004).

These all conclusions are subject to our further investigation, to check they activity for hypoxia and to explain their mechanism of action, for example as a inhibitors of topoisomerase I.

### Acknowledgments

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### Supplementary Data

Tables of crystallographic data and structure refinement, anisotropic displacement coefficients, atomic coordinates and equivalent isotropic displacement parameters for non-hydrogen atoms, H-atom coordinates and isotropic displacement parameters, bond lengths and interbond angles have been deposited with the Cambridge Crystallographic Data Centre under No. CCDC837324.

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