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Original article

Synthesis, characterization and cytotoxicity of some novel 1,3-disubstituted-2,3-dihydro-2-iminobenzimidazoles

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ABSTRACT

Some new 1,3-disubstituted-2,3-dihydro-2-iminobenzimidazoles were synthesized using 1-(un) substituted-2-aminobenzimidazoles as precursors in order to determine their cytotoxicity. The structures of the compounds were confirmed by IR, ^{1}H NMR, ^{13}C NMR and elemental analysis.

Compounds **4**, **7**–**11** and **13**–**14** were evaluated for their cytotoxical effect on two cancer cell lines: human colorectal cancer cell line HT-29, breast cancer cells MDA-MB-231 and as well as normal spleen cells. The distinctly marked antiproliferative activity of 1,3-bis(3-phenylpropyl-1)-1,3-dihydro-2H-benzimidazol-2-imine hydro bromide **7**, N-(aminopropyl)-2-(3-{2-[(aminopropyl)-amino]-2-oxoethyl}-2-imino-2,3-dihydro-1H-benzimidazol-1-yl)acetamide **9** and 1,3-bis[2-(4-methylpiperazin-1-yl)-2-oxoethyl]-2,3-dihydro-2H-benzimidazol-2-imine **11** against human colorectal cancer cell line HT-29 was ascertained and the calculated IC_{50} were 9.26, 0.56 and 0.013 nM respectively. Compounds **4**, **9**, **10** and **13** exhibited relative high cytotoxic activity against MDA-MB-231 cells. The calculated IC_{50} values were in the range 0.123–1.65 nM. All tested compounds excluding compound 1,3-bis[2-(4-methylpiperazin-1-yl)-2-oxoethyl]-2,3-dihydro-2H-benzimidazol-2-imine (**11**) revealed proliferative activities to normal spleen cells. The computed EC_{50} values varied from 0.05 to 16.91 nM.

1. Introduction

2-Aminobenzimidazole is one of the well known biologically accepted pharmacophores. The polyfunctionality resulting from the cyclic guanidine residue has made it a building block for the synthesis of a wide diversity of benzimidazole derivatives of pharmacological interest. The demonstrated potent antihelminthic activities of different substituted 2-aminobenzimidazoles as well as the development of various drugs support further the importance of this heterocycle in generating better chemotherapeutical agents against parasitic diseases [1–3]. On the other hand a number of 2-aminobenzimidazoles have exhibited antiproliferative properties [4–7]. Albendazole, a benzimidazole carbamate with extensive clinical use as an antihelminthic drug, can also inhibit hepatocellular carcinoma cell proliferation under both *in vitro* and *in vivo* experimental conditions [8]. It was reported that carbendazim (FB642) is an anticancer agent that induces apoptosis of cancer cells [9] and together with benomyl show interesting and diverse cytotoxic mechanisms of action and seem suitable as leading compounds for the development of new anticancer drugs [10], while mebendazole was identified as a potent, melanoma-specific cytotoxic agent [11]. The 2-aminoderivatives revealed cytotoxicity against human colorectal cancer cell line (HT-29) and a human prostate cancer cell line (PC-3) [12]. There are data in the literature that the antihelminthic flubendazole inhibits microtubule function through a mechanism distinct from Vinca alkaloids and displays preclinical activity in leukemia and myeloma [13]. Series of Schiff bases of 2-aminobenzimidazole and substituted aromatic aldehydes exhibited cytotoxic activity against the cells of human cancer cell lines as SW707 (rectal), HCV29T (bladder), A549 (lung) and T47D (breast cancer) [14]. Despite advances in the field of cancer treatment the finding of cytotoxic agents that have specificity for cancer and tumor cells is indispensable.

On the basis of all above observations and following our aim to synthesize new benzimidazole derivatives we undertook investigation over the interaction of 2-aminobenzimidazole with different alkylation agents both under solid–liquid phase transfer catalysis conditions and in the presence of DBU. In this paper we report the synthesis of new 1,3-disubstitutet-2,3-dihydro-2-iminobenzimidazoles and their cytotoxicity against human colorectal cancer cell line HT-29, breast cancer cells MDA-MB-231 and normal spleen cells.

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Because of the structural similarity of benzimidazole nucleus to naturally occurring compounds as purines it could be expected the benzimidazole derivatives easily to interact with biological targets in the living systems. As 2-aminobenzimidazole compounds are usually associated with antiparasitic, anticancer, antiviral and some other pharmacological activities, it may be anticipated 1,3-disubstitutes-2-iminobenzimidazoles to display potential biological activity not only against parasites but also against tumor cells.

2. Chemistry

The synthesis of 1,3-disubstituted-2,3-dihydro-2-iminobenzimidazoles is illustrated and outlined in Fig. 1.

The starting 1-(un)substituted-2-aminobenzimidazoles **1**–**3** were synthesized according to the method described by us early in [15]. The nucleophilic substitution between the 2-aminobenzimidazoles and the appropriated halogen derivatives under solid–liquid phase transfer catalysis conditions in dry acetonitrile as well as in the presence of DBU resulted in the formation of 1,3-disubstituted-2,3-dihydro-2-iminobenzimidazoles **4**–**8**.The condensation of ethyl [3-(2-ethoxy-2-oxoethyl)-2-imino-2,3-dihydro-1H-benzimidazol-1-yl] acetate hydrogen bromide **4** with hydrazine hydrate afforded the hydrazide **12**, which was reacted with the corresponding arylaldehydes yielding hydrazones **13**–**14**.

The reaction of ethyl [3-(2-ethoxy-2-oxoethyl)-2-imino-2,3-dihydro-1H-benzimidazol-1-yl]acetate **4** with 1,3-diaminopropane, benzyl amine and 1-methylpiperazine led to the formation of 2-imino-2,3-dihydrobenzimidazol-1,3-diacetamides **10**–**12**.

The chemical structures of the compounds were established by elemental analyses, IR-, ^{1}H NMR and ^{13}C NMR spectra and the results are presented in the Experimental part. The elemental analyses indicated by the symbols of the elements were within ±0.4% of theoretical values.

3. Pharmacology

3.1. Cytotoxicity

Compounds **4**, **7**–**8** and **10**–**13** were evaluated for their cytotoxicity to human colorectal cancer cell line HT-29, breast cancer

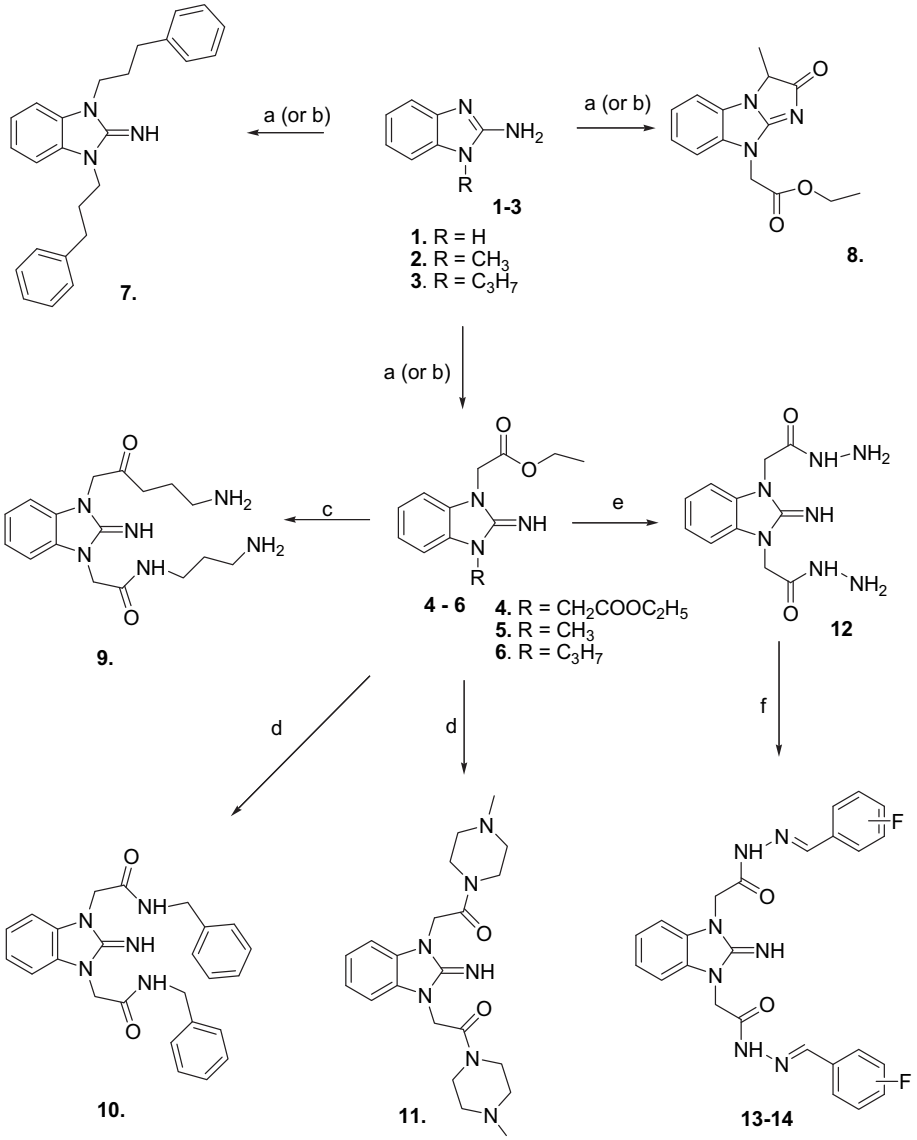

Fig. 1. Synthesis of 1,3-disubstituted-2,3-dihydro-2-iminobenzimidazoles. Regents and conditions: a) DBU, acetonitrile, halogen derivative, 20 °C; acetonitrile, TBAB, dry K_2CO_3, halogen derivative, 25 °C; c) ethanol, 1,3-diaminopropane, 25 °C; d) ethanol, benzyl amine or 1-methylpiperazine, refluxing; e) hydrazine hydrate, ethanol, refluxing; f) ethanol, 4- or 3-floro-benzaldehyde, refluxing.

cells MDA-MB-231 and normal spleen cells by using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrasolium inner salt) – test [16].

4. Results and discussion

We have already reported the synthesis of 1-(un)substituted-2-aminobenzimidazoles [15]. In order to synthesize new derivatives of 2-aminobenzimidazole and study their cytotoxicity we undertook investigation on the reaction of 1-(un)substituted-2-aminobenzimidazoles with halogen containing reagents in the presence of DBU in dry acetonitrile. It was established that the reaction led to the formation of 1,3-disubstituted-2,3-dihydro-2-iminobenzimidazoles in good yields, 66–75%. The same interaction was studied under solid-liquid phase transfer catalysis using anhydrous potassium carbonate and tetrabutyl ammonium bromide as catalyst. It was established that the reaction runs best at mol ratio of 2-aminobenzimidazoles **4**–**6**, halogen compounds and potassium carbonate 1:2:2 and has led to a range of new 1,3-disubstituted derivatives being prepared easily and in good yields.

The synthesis of 1,3-disubstituted-2,3-dihydro-2-iminobenzimidazoles was reported earlier but by using the reduction of 2-halogen-nitrobenzenes and condensation with BrCN [17–19].

The reaction performed with ethyl 2-bromopropanoate resulted in formation of fused imidazo [1,2-a]benzimidazole ring, possessing on third position the ethyl propionate residue. The IR-spectral data characteristics of both carbonyl groups are – υ C=O –1737, 53 cm^{-1} and υ C=O –1695, 32 cm^{-1}.

The test for estimating the *in vitro* cytotoxicity against human colorectal cancer cell line HT-29, breast cancer cells MDA-MB-231 and normal spleen cells was performed with compounds **4**, **7**–**10**, as well as **13**–**14** according to MTS method, as described in [16]. The compounds were dissolved in DMSO at the concentration of 0.5 mg/ml. The investigation was carried out by dilution of the stock solution in ratio 1:10, 1:100, 1:1000 and 1:10 000. Samples of cells, grown in non-modified medium served as a control. After 24 h of incubation of the samples MTS colorimetric assay of cell survival was performed. The wells were treated with MTS solution and incubated for 2 h at 37 °C under 5% carbon dioxide and 95% air atmosphere. The absorbance of each well at 490 nm was read by an automatic microplate reader ("Tecan", Austria). Relative cell viability, expressed as a percentage of the untreated control (100% viability), was calculated for each concentration. All data points represent an average of three independent assays and are given in Figs. 2–4. The obtained results were plotted and IC_{50} and EC_{50} were calculated. The data are given in Table 1 and Table 2.

Among all tested compounds only compounds **7**, **9** and **11** revealed cytotoxicity against HT-29 cells. The substituted with 4-methylpiperazine-1,3-diacetamide **11** showed highest cytotoxic effect on HT-29 cells, IC_{50} = 0.013 nM, followed by the compound **9**, IC_{50} = 0.56 nM. Compounds **4**, **9**, **10** and **13** exhibited relative high cytotoxic activity against MDA-MB-231 cells. Most toxic were N-(aminopropyl)-2-(3-{2-[(aminopropyl) amino]-2-oxoethyl}-2-imino-2,3-dihydro-1H-benzimidazol-1-yl) acetamide **9** and the hydrazone **13** with IC_{50} – 0.123 nM and IC_{50} – 0.78 nM respectively.

As it can be seen from the results, given in Table 2, all tested compounds excluding compound **11** revealed proliferative effects to the normal spleen cells. The EC_{50} values varied in the range from 0.05 nM for compound **9** to 16.91 nM for compound **13**. If the results, obtained for compound **9** are taken in consideration it should be noted that N-(aminopropyl)-2-(3-{2-[(aminopropyl) amino]-2-oxoethyl}-2-imino-2,3-dihydro-1H-benzimidazol-1-yl) acetamid revealed proliferative activity against normal spleen cells at lower concentration in comparison to the concentration

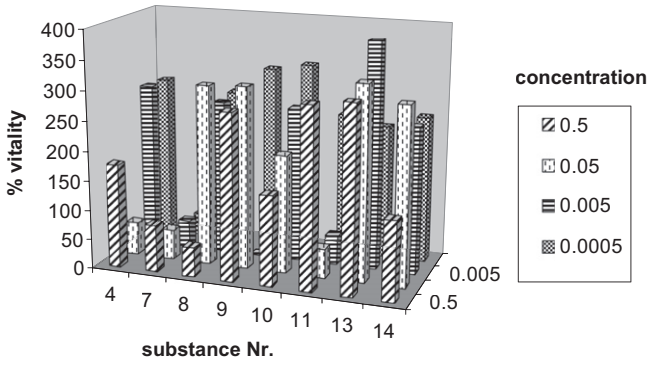

Fig. 2. Relative HT-29 cells viability (%).

at which the compound excited cytotoxic effect on HT-29 cells. That fact is an indicator for the selectivity effect of the compound **9**. It could be underlined, that excluding compound **11**, all compounds possessing cytotoxicity against HT-29 and MDA-MB-231 revealed proliferative activities to normal spleen cells. Stimulating effect on the three types of cells showed ethyl 2-(3-methyl-2-oxo-2,3-dihydro-9H-imidazo[1,2-a]benzimidazol-9-yl) propanoate **8**. Statistical significant differences in the level of cells in both control and experimental groups were determined ($p \leq 0.05$).

To make a conclusion about the mechanism of the action of the studied compounds, it is necessary to perform additional investigations, but it can be pointed out that the cell proliferation MTS-assay is based on the fact that the MTS tetrazolium compound is bioreduced by cells into a colored formazan product that is soluble in the tissue culture medium. This conversion is presumably accomplished by NADPH or NADH, produced by dehydrogenase in metabolically active cells [20]. The bigger release amount of formazan indicates to a higher vitality of the cells (proliferation). The low vitality demonstrates a cytotoxic influence of the experimental compounds.

5. Conclusion

Improved conditions have been developed and optimized for the synthesis of new 1,3-substituted-2,3-dihydro-2-iminobenzimidazoles using 1-(un)substituted-2-aminobenzimidazoles as precursors and different halogen derivatives under solid–liquid PTC as well as by means of DBU. This methodology, combined with a "catch and release" purification strategy, has led to a range of new derivatives being prepared easily and in excellent yield.

The initial biological screening *in vitro* showed that the studied compounds **7**, **9**, **11** possessed relative high cytotoxicity against

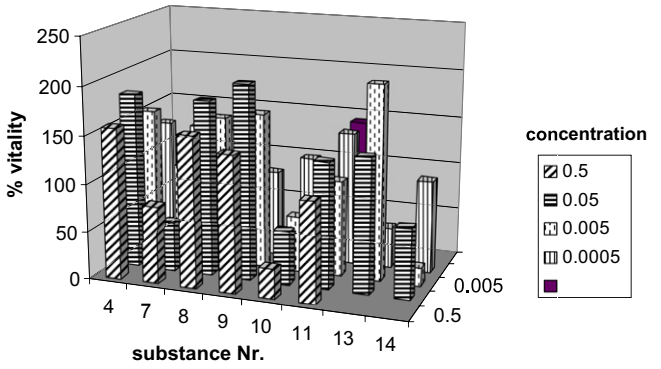

Fig. 3. Relative MDA-MB-231 cells viability (%).

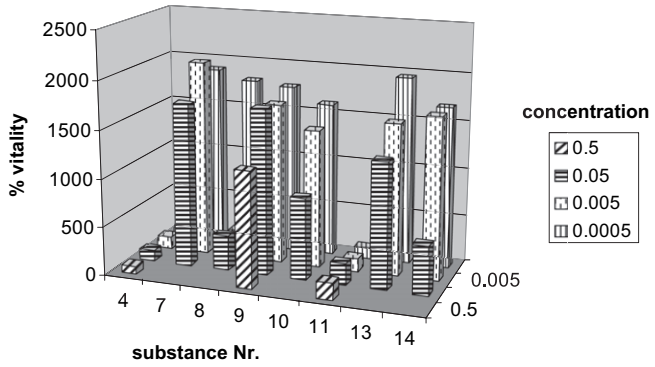

Fig. 4. Relative normal spleen cells viability (%).

HT-29 cells. IC_{50} values were in the range 0.013–9.26 nM. In respect to MDA-MB-231 cells most cytotoxic was compound **9** with IC_{50} – 0.123 nM. All investigated compounds excluding compound **11** revealed proliferative effects on normal spleen cells, the EC_{50} values were in the range 0.05–16.91 nM.

The above results confirmed also the hypothesis that the introduction of different substituents at 1 and 3-th position in the structure of 2-aminobenzimidazole are auspicious to the interaction of these molecules with the biological targets.

6. Experimental part

Melting points (mp) were determined on an Electrothermal AZ 9000 3MK4 apparatus and were uncorrected. The thin layer chromatography (TLC, Rf values) was performed on silica gel plates F_{254} (Merck, 0.2 mm thick) and visualization was effected with ultraviolet light. IR spectra were recorded on a Bruker Equinox 55 spectrophotometer as potassium bromide discs. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance II + 600 MHz NMR instrument. The spectra are referred to the solvent signal. Chemical shifts are expressed in ppm and coupling constants in Hz. The precise assignment of the ^1H and ^{13}C NMR spectra was accomplished by measurement of 2D homonuclear correlation (COSY), DEPT-135 and 2D inverse detected heteronuclear (C–H) correlations (HMQC and HMBC). The microanalyses for C, H, N and S were performed on Perkin–Elmer elemental analyzer.

2-Amino-1H-benzimidazoles **1**–**3** were synthesized by heating of 1-(un)substituted-1*H*-benzimidazol-2-sulphonic acid and 25% ammonium hydroxide as described in [15].

6.1. General procedures for preparation of compounds ***4***–***8***

6.1.1. Method A

To a solution of 1-(un)substituted-2-aminobenzimidazole (0.004 mol) and 0.016 mol of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in 20 ml dry acetonitrile 0.024 mol of the corresponding halogen derivative was dropped by cooling. The solution was stirred for 2–4 h at ambient temperature and the obtained precipitate was filtered. Additional quantity of the compounds was received through refluxing the filtrate for 1 h, removing the solvent and crystallizing the obtained oil product with suitable solvent.

Table 1
In vitro cytotoxicity against HT-29, MDA-MB-231 and normal spleen cells.

Comp	IC_{50} ± SE (nM)		
	HT-29	MDA-MB-231	Normal spleen cells
4	–	1.15 ± 0.12	–
7	9.26 ± 0.11	–	–
8	–	–	–
9	0.56 ± 0. 28	0.123 ± 0.43	
10	–	1.65 ± 0.23	
11	0.013 ± 0.81	–	1.38 ± 0.04
12	–	0.78 ± 0.29	–
13	–	–	–

Table 2
Proliferative effects *in vitro*.

Comp.	EC_{50} ± SE (nM)		
	HT-29	MDA-MB-231	Normal spleen cells
4	2.37 ± 0.65	–	14.32 ± 0.02
7	–	1.388 ± 0.22	14.13 ± 0.59
8	5.68 ± 0.17	2.019 ± 0.13	0.542 ± 0.56
9	–	–	0.05 ± 0.29
10	0.205 ± 0.36	–	7.74 ± 0.26
11	–	1.59 ± 0.12	–
13	4.30 ± 0.14	–	16.91 ± 0.21
14	3.51 ± 0.45	0.002 ± 0.20	11. 02 ± 0.50

6.1.2. Method B

To a solution of 1-(un)substituted-2-aminobenzimidazole (0.01 mol) in dry acetonitrile (50 ml) was added anhydrous potassium carbonate (2.7 g, 0.02 mol) and tetrabutyl ammonium bromide (TBAB) (0.9 g, 0.003 mol) and 0.02 mol of the halogen organic reagent was dropped by cooling. The mixture was stirred for 2–5 h vigorously at 25 °C and monitored by TLC over the reaction period. After completion of the reaction, the mixture was filtered to separate the solid K_2CO_3, and the organic solvent was evaporated. The residue was then crystallized from appropriate solvent to give products **4**–**8**.

6.1.3. *Ethyl[3-(2-ethoxy-2-oxoethyl)-2-imino-2,3-dihydro-1H-benzimidazol-1-yl]acetate hydro bromide (**4**)*

After removing of acetonitrile the compound was crystallized with ethanol. Yield – 66.48% (method A); 82.5% (method B); Mp – 245–246 °C; Rf = 0.68, mobile phase: benzene/ethanol – 1:1; ^1H NMR (DMSO-d6) δ (ppm): 1.245 (t, J = 7.1 Hz, 6H, CH_3), 4.201 (q, J = 7.1 Hz, 4H, OCH_2), 5.250 (s, 4H, NCH_2), 7.34 (AA' part of AA'XX' system, 2H, 5-H and 6-H) and 7.63 (XX' part of AA'XX', 2H, 4-H and 7-H), 9.200 (bs, 2H, NH.HBr). ^{13}C NMR (DMSO-d6) δ (ppm): 14.02 (CH_3), 44.24 (NCH_2), 61.84 (OCH_2), 110.61 (4-C and 7-C), 124.03 (5-C and 6-C), 129.56 (3a-C and 7a-C), 150.71 (C=N), 166.49 (C=O). Analysis: Calc. for $C_{15}H_{20}BrN_3O_4$; C, 46.64; H, 5.22; Br, 20.69; N, 10.88; O, 16.57; Found: C, 46.61; H, 5.25; Br, 20.72; N, 10.86; O, 16.54.

6.1.4. *Ethyl [3-(2-ethoxy-2-oxoethyl)-2-imino-2,3-dihydro-1H-benzimidazol-1-yl]acetate (**4.1**)*

Some quantity of compound **4** was washed with water to afford **4.1**. Mp – 161–162 °C; Rf = 0.66, mobile phase: benzene/ethanol – 1:1; ^1H NMR (DMSO-d6) δ (ppm): ^1H NMR (DMSO-d6) δ (ppm): 1.208 (t, J = 7.1 Hz, 6H, CH_3), 4.138 (q, J = 7.1 Hz, 4H, OCH_2), 4.758 (s, 4H, NCH_2), 6.91–6.94 (AA' part of AA'BB' system, 2H, 5-H and 6-H) and 6.96–6.99 (BB' part of AA'BB', 2H, 4-H and 7-H); ^{13}C NMR (DMSO-d6) δ (ppm): 14.04 (CH_3), 42.44 (NCH_2), 60.91 (OCH_2), 106.79 (4-C and 7-C), 120.56 (5-C and 6-C), 131.31 (3a-C and 7a-C), 152.41 (C=N), 168.16 (C=O); Analysis: Calc. for $C_{15}H_{19}N_3O_4$; C, 59.01; H, 6.27; N, 13.76; O, 20.96; Found: C, 59.04; H, 6.23; N, 13.78; O, 20.91.

6.1.5. *Ethyl (2-imino-3-methyl-2,3-dihydro-1H-benzimidazol-1-yl) acetate hydro bromide (**5**)*

Yield – 75% (method A), 88% (method B); re-crystallized with ethanol; Mp. 242–244 °C; Rf = 0.60, mobile phase: benzene/ethanol – 1:1.

^{1}H NMR (DMSO-d6) δ (ppm): ^{1}H NMR (DMSO-d6) δ (ppm): 1.246 (t, J = 7.1 Hz, 6H, CH_3), 3.704 (s, 3H, NCH_3), 4.200 (q, J = 7.1 Hz, 4H, OCH_2), 5.204 (s, 2H, NCH_2), 7.323 (dt, J = 1.1, 8.0 Hz, 1H) and 7.364 (dt, J = 1.1, 8.0 Hz, 1H, 5-H and 6-H), 7.59–7.61 (m, 2H, 4-H and 7-H), 8.986 (s, 2H, NH). ^{13}C NMR (DMSO-d6) δ (ppm): 13.99 (CH_3), 29.67 (NCH_3), 44.05 (NCH_2), 61.78 (OCH_2), 110.38 (4-C and 7-C), 123.62 and 123.80 (5-C and 6-C), 129.62 and 130.00 (3a-C and 7a-C), 150.37 (C=N), 166.66 (C=O); Analysis: Calc. for $C_{12}H_{15}N_3O_2$; C, 61.79; H, 6.48; N, 18.01; O, 13.72; Found: C, 61.82; H, 6.45; N, 18.04; O, 13.69.

6.1.6. Ethyl (2-imino-3-propyl-2,3-dihydro-1H-benzimidazol-1-yl) acetate hydro bromide (**6**)

The compound was crystallized with ethanol; Yield: 73 % (method A), 85.0% (method B), the compound was crystallized with ethanol; Mp. 213–215 °C; Rf = 0.46, mobile phase: benzene/ethanol – 2:1. ^{1}H NMR (DMSO-d6) δ (ppm): 0.907 (t, J = 7.1 Hz, 3H, CH_3), 1.238 (t, J = 7.1 Hz, 3H, CH_3), 1.726 (sextet, J = 7.1 Hz, 2H, CH_2), 4.185 (t, J = 7.1 Hz, 2H, NCH_2), 4.194 (q, J = 7.1 Hz, 2H, OCH_2), 5.196 (s, 2H, NCH_2), 7.324 (dt, J = 1.1, 7.6 Hz, 1H) and 7.357 (dt, J = 1.1, 7.5 Hz, 1H, 5-H and 6-H), 7.616 (dd, J = 1.1, 7.5 Hz, 1H) and 7.381 (dt, J = 1.1, 7.6 Hz, 1H, 4-H and 7-H), 8.977 (s, 2H, NH). ^{13}C NMR (DMSO-d6) δ (ppm): 10.47 (CH_3), 13.96 (CH_3), 20.79 (CH_2), 43.84 (NCH_2), 44.02 (NCH_2), 61.79 (OCH_2), 110.48 and 110.59 (4-C and 7-C), 123.66 and 123.88 (5-C and 6-C), 129.35 and 129.60 (3a-C and 7a-C), 149.92 (C=N), 166.63 (C=O). Analysis: Calc. for $C_{14}H_{19}N_3O_2$; C, 64.35; H, 7.33; N, 16.08; O, 12.25; Found: C, 64.32; H, 7.35; N, 16.04; O, 12.27.

6.1.7. 1,3-Bis(3-phenylpropyl-1)-1,3-dihydro-2H-benzimidazol-2-imine hydro bromide (**7**)

The compound crystallized after addition of ethyl acetate. Yield – 84% (method B); Mp – 222–224 °C; Rf = 0.67, mobile phase: benzene/ethanol – 2:1; ^{1}H NMR (DMSO-d6) δ (ppm): 1.990 (m, 4H, 2-CH_2), 2.686 (m, 4H, 3-CH_2), 4.224 (t, J = 7.4 Hz, 4H, 1-CH_2), 7.154 (t, J = 7.2 Hz, 2H, p-Ph), 7.178 (d, J = 7.4 Hz, 4H, o-Ph), 7.246 (t, J = 7.6 Hz, 4H, m-Ph), 7.32 (AA' part of AA'XX' system, 2H, 5-H and 6-H) and 7.56 (XX' part of AA'XX', 2H, 4-H and 7-H), 8.875 (bs, 2H, NH.HBr). ^{13}C NMR (DMSO-d6) δ (ppm): 29.19 (2-CH_2), 31.84 (3-CH_2), 42.52 (1-CH_2), 110.37 (4-C and 7-C), 123.47 (5-C and 6-C), 125.96 (p-Ph), 128.07 (o-Ph), 128.34 (m-Ar), 129.56 (3a-C and 7a-C), 140.93 (i-Ph), 149.03 (C=N); Analysis: Calc. for $C_{25}H_{27}N_3$; C, 81.26; H, 7.37; N, 11.37; Found: C, 81.29; H, 7.39; N, 11.35.

6.1.8. Ethyl 2-(3-methyl-2-oxo-2,3-dihydro-9H-imidazo[1,2-a]benz imidazol-9-yl)propanoate (**8**)

The compound crystallized after addition of water. Yield: 86% (Method B); Mp. 128–130 °C; Rf = 0.39, mobile phase: benzene/ethanol – 5:1.

^{1}H NMR (DMSO-d6) δ (ppm): 1.114 (t, J = 7.0 Hz, 3H, CH_3), 1.637 (d, J = 7.1 Hz, 3H, CH_3), 1.744 (d, J = 7.4 Hz, 3H, CH_3), 4.118 (q, J = 7.0 Hz, 2H, OCH_2), 5.090 (q, J = 7.1 Hz, 1H, CH), 5.105 (q, J = 7.4 Hz, 1H, CH), 7.14 (t, J = 7.1 Hz, 1H) and 7.16 (t, J = 7.2 Hz, 1H, 6-H and 7-H), 7.50 (d, J = 7.0 Hz, 1H) and 7.54 (d, J = 6.8 Hz, 1H, 5-H and 8-H); ^{13}C NMR (DMSO-d6) δ (ppm): 13.93 (CH_3), 14.11 (CH_3), 15.73 (CH_3), 49.06 (CH), 54.98 (CH), 109.27 and 118.43 (5-C and 8-C), 121.45 and 121.50 (6-C and 7-C), 131.07 and 144.40 (4a-C and 8a-C), 152.45 (C=N), 169.19 (OC=O), 175.06 (NC=O). Analysis: Calc. for $C_{15}H_{17}N_3O_3$; C, 62.71; H, 5.96; N, 14.63; O, 16.71; Found: C, 62.74; H, 5.91; N, 14.65; O, 16.69.

6.2. Procedure for preparation of N-(aminopropyl)-2-(3-{2-[(amino propyl)amino]-2-oxoethyl}-2-imino-2,3-dihydro-1H-benzimidazol-1-yl)acetamide (**9**)

To 1 g (0.003 mol) ethyl [3-(2-ethoxy-2-oxoethyl)-2-imino-2,3-dihydro-1H-benzimidazol-1-yl]acetate **4** diluted in 10 ml ethanol 1.5 ml (0.018 mol) 1,3-diaminopropane was added and the solution was stirred at ambient temperature for 6 h. After completion of the reaction the solvent was removed under reduced pressure. The oily residue was crystallized with 5 ml chloroform and re-crystallized with ethyl acetate.

Yield – 68%; Mp – 146–148 °C; Rf = 0.32, mobile phase: benzene/methanol – 3:1; ^{1}H NMR (D_2O) δ (ppm): 1.710 (sextet, J = 7.1 Hz, 4H, CH_2), 2.794 (t, J = 7.1 Hz, 4H, NCH_2), 3.204 (q, J = 7.1 Hz, 4H, NCH_2), 4.564 (s, 4H, NCH_2), 6.93–6.97 (AA' part of AA'BB' system, 2H, 5-H and 6-H) and 7.04–7.07 (BB' part of AA'BB', 2H, 4-H and 7-H); ^{13}C NMR (D_2O) δ (ppm): 27.41 (CH_2), 36.30 (NCH_2), 37.26 (NCH_2), 44.21 (NCH_2), 107.58 (4-C and 7-C), 122.28 (5-C and 6-C), 130.67 (3a-C and 7a-C), 155.07 (C=N), 161.23 (C=O).

6.2.1. Analysis

Calc. for $C_{17}H_{27}N_7O_2$; C, 56.49; H, 7.53; N, 27.13; O, 8.85; Found: C, 56.46; H, 7.51; N, 27.17; O, 8.87.

6.3. General procedure for preparation of compounds **10** and **11**

To solution of 0.5 g (0.0013 mol) ethyl [3-(2-ethoxy-2-oxoethyl)-2-imino-2,3-dihydro-1H-benzimidazol-1-yl]acetate **4** in 10 ml ethanol 0.0052 mol of the corresponding amine was added. The mixture was refluxed for 2–6 h. After cooling the obtained precipitate was filtered and re-crystallized with ethanol.

6.3.1. N-Benzyl-2-{3-[2-(benzylamino)-2-oxoethyl]-2-imino-2,3-di hydro-1H-benzimidazol-1-yl}acetamide (**10**)

Yield – 94%; Mp – 178–181 °C, re-crystallized with ethanol; Rf = 0.45, mobile phase: benzene/ethanol – 2:1; ^{1}H NMR (DMSO-d6), δ (ppm): 4.395 (s, 4H, 2CH_2); 4.565 (s, 4H, 2CH_2); 7.211–7.248 (m, 10H, 2Ph); 7. 391–7.448 (dd, J = 7.57 Hz, 2H, 2CH); 8.145 (d, J = 7.65, 2H, 2CH); Analysis: Calc. for $C_{25}H_{25}N_5O_2$: C, 70.24; H, 5.89; N, 16.38; O, 7.49; Found: C, 70.22; H, 5.87; N, 16.41; O, 7.46.

6.3.2. 1,3-Bis[2-(4-methylpiperazin-1-yl)-2-oxoethyl]-2,3-dihydro-2H-benzimidazol-2-imine (**11**)

Yield – 65%; Mp – 265–268 °C (decomp.); Rf = 0.50, mobile phase: benzene/ethanol – 1:1; ^{1}H NMR (DMSO-d6) δ (ppm): 2.174 (s, 6H, CH_3), 2.37 (m, 8H, NCH_2), 2.89 (m, 8H, NCH_2), 4.54 (s, 4H, CH_2), 6.84–6.94 (AA' part of AA'BB' system, 2H, 5-H and 6-H) and 7.02–7.12 (BB' part of AA'BB', 2H, 4-H and 7-H). Analysis: Calc. for: $C_{21}H_{31}N_7O_2$; C, 61.00; H, 7.56; N, 23.71; O, 7.74; Found: C, 61.04; H, 7.53; N, 23.74; O, 7.70.

6.4. Procedure for preparation of 2-[3-(2-hydrazino-2-oxoethyl)-2-imino-2,3-dihydro-1H-benzimidazol-1-yl]acetohydrazide (**12**)

To a solution of 1 g (0.002 mol) ethyl [3-(2-ethoxy-2-oxoethyl)-2-imino-2,3-dihydro-1H-benzimidazol-1-yl]acetate hydro bromide (**4**) in 20 ml ethanol were added 0.6 ml 98% hydrazine hydrate and the mixture was refluxed for 2 h. After cooling the obtained precipitation was filtered and re-crystallized ethanol. Yield – 95%; Mp – 254–256 °C (decomp.); Rf = 0.31, mobile phase: benzene/diethyl ether/ethanol = 8:8:1; ^{1}H NMR (DMSO-d6), δ (ppm): 4.43 (ds, 2H, CH_2); 4.52 (s, 2H CH_2); 6.93–7.10 (m, 4H, Ar); 9.28 (bs, 1H, NH); 10.21 (s, 2H, 2NH); ^{13}C NMR (DMSO-d6): 40.10 (2CH_2); 107.8 (CH); 131.35 (CH); 143.68 (C=N); 152.62 (C); 157.45 (C); 165.84 (C); Analysis: Calc. for $C_{11}H_{15}N_7O_2$; C, 47.65; H, 5.45; N, 35.36; O, 11.54; Found: C, 47.68; H, 5.43; N, 35.38; O, 11.51.

6.5. General procedure for preparation of compounds **13**–**14**

0.3 g (0.001 mol) acetohydrazide (**12**) and 0.003 mol of the relevant aldehyde were refluxed in 10 ml absolute ethanol for

4–5 h. After completion of the reaction the solution was cooled, the obtained precipitate was filtered and re-crystallized with ethanol.

*6.5.1. 2-(3-{2-[2-(4-Fluoro)-benzylidenehydrazino]-2-oxoethyl}-2-imino-2,3-dihydro-1H-benzimidazol-1-yl)-N′-[(4-fluoro)-benzylidene]acetohydrazide (**13**)*

^{1}H NMR (DMSO-d6) δ (ppm): 5.543 (s, 4H, NCH_2), 7.29–7.32 (AA' part of AA'XX' system, 2H, 5-H and 6-H) and 7.60–7.62 (XX' part of AA'XX', 2H, 4-H and 7-H), 7.340 (t, J = 8.8 Hz, 4H, 3-Ar and 5-Ar), 7.879 (dd, J = 5.6, 8.8 Hz, 4H, 2-Ar and 6-Ar), 8.096 (s, 2H, CH), 8.96 (bs, 1H, NH), 11.96 (bs, 2H, NNH); ^{13}C NMR (DMSO-d6) δ (ppm): 44.84 (NCH_2), 110.68 (4-C and 7-C), 116.10 ($^2J_{CF}$ = 22.0 Hz, 3-Ar and 5-Ar), 123.86 (5-C and 6-C), 129.49 ($^3J_{CF}$ = 8.6 Hz, 2-Ar and 6-Ar), 130.34 (3a-C and 7a-C), 130.69 ($^4J_{CF}$ = 2.7 Hz, 1-Ar), 143.454 (CH), 151.48 (C═N), 163.32 ($^1J_{CF}$ = 248.0 Hz, 4-Ar), 166.67 (C═O); ^{19}F NMR ($CDCl_3$) δ (ppm): −110.4; Analysis: Calc. for $C_{25}H_{25}N_5O_2$: C, 70.24; H, 5.89; N, 16.38; O, 7.49; Found: C, 70.21; H, 5.91; N, 16.41; O, 7.47.

*6.5.2. 2-(3-{2-[2-(3-Fluoro)-benzylidenehydrazino]-2-oxoethyl}-2-imino-2,3-dihydro-1H-benzimidazol-1-yl)-N′-[(3-fluoro)-benzylidene]acetohydrazide (**14**)*

^{1}H NMR (DMSO-d6) δ (ppm): 5.565 (s, 4H, NCH_2), 7.301 (dd, J = 6.1, 8.8 Hz, 1H, 4-Ar), 7.29–7.31 (AA' part of AA'XX' system, 2H, 5-H and 6-H) and 7.59–7.61 (XX' part of AA'XX', 2H, 4-H and 7-H), 7.619 (d, J = 7.7 Hz, 1H, 6-Ar), 7.702 (ddd, J = 1.4, 2.3, 9.2 Hz, 1H, 2-Ar), 7.530 (dt, J = 6.1, 7.9 Hz, 1H, 5-Ar), 8.100 (s, 2H, CH), 9.00 (bs, 1H, NH), 12.05 (bs, 2H, NNH); ^{13}C NMR (DMSO-d6), δ (ppm): 44.76 (NCH_2), 110.51 (4-C and 7-C), 112.90 ($^2J_{CF}$ = 22.6 Hz, 2-Ar), 117.00 ($^2J_{CF}$ = 21.5 Hz, 4-Ar), 123.87 (5-C and 6-C), 124.88 ($^4J_{CF}$ = 2.5 Hz, 6-Ar), 130.31 (3a-C and 7a-C), 131.02 ($^3J_{CF}$ = 8.2 Hz, 5-Ar), 136.59 ($^3J_{CF}$ = 7.9 Hz, 1-Ar), 142.92 ($^4J_{CF}$ = 2.8 Hz, CH), 151.46 (C═N), 162.51 ($^1J_{CF}$ = 243.8 Hz, 3-Ar), 166.88 (C═O). ^{19}F NMR ($CDCl_3$) δ (ppm): −112.8; Analysis: Calc. for $C_{25}H_{25}N_5O_2$: C, 70.24; H, 5.89; N, 16.38; O, 7.49; Found: C, 70.27; H, 5.85; N, 16.36; O, 7.51.

6.6. MTS test

The cells were seeded in 96-well flat-bottomed microplates (Orange scientific) at a concentration of 2×10^4 cells/well. At the 24th h cells from monolayers were washed and covered with media modified with different concentrations of the compound tested. Samples of cells grown in non-modified medium served as a control. After 2 h of incubation MTS colorimetric assay of cell survival was performed as described by the protocol of "Promega" [CellTiter 96 Non-Radioactive Cell proliferation assay Technical Bulletin #TB112, Promega Corporation]. This consisted of 1–3 h incubation with MTS solution at 37 °C under 5% carbon dioxide and 95% air. The absorbance of each well at 490 nm was read by an automatic microplate reader (Absorbance Reader "Tecan", Austria). Relative cell viability, expressed as a percentage of the untreated control (100% viability), was calculated for each concentration. All data points represent an average of three independent assays.

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