European Journal of Medicinal Chemistry 63 (2013) 696–701

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Design, synthesis and antiproliferative properties of some new 5-substituted-2-iminobenzimidazole derivatives

Anelia Ts. Mavrova [a,*], Diana Wesselinova [b], Nikolay Vassilev [c], Jordan A. Tsenov [c]

[a] University of Chemical Technology and Metallurgy, Department of Organic Synthesis, 8 Kliment Ohridski Blvd., 1756 Sofia, Bulgaria
[b] Institute of Parasitology and Experimental Pathology, Bulgarian Academy of Science, 1113 Sofia, Bulgaria
[c] Institute of Organic Chemistry, Bulgarian Academy of Science, 1113 Sofia, Bulgaria

ARTICLE INFO

Article history:
Received 30 October 2012
Received in revised form
3 March 2013
Accepted 5 March 2013
Available online 18 March 2013

Keywords:
2-Aminobenzimidazoles
5-Substituted-2,3-dihydro-2-iminobenzimidazoles
Solid–liquid phase transfer catalysis
Cytotoxicity
Anticancer activity
Proliferation

ABSTRACT

Some new 1,3,5-substituted-2,3-dihydro-2-imino-benzimidazoles were synthesized under solid–liquid phase transfer catalysis conditions using 5-substituted-2-aminobenzimidazoles as precursors in order to assess their cytotoxicity respectively proliferative activity. The structures of the compounds were confirmed by IR, ^{1}H NMR, ^{13}C NMR and elemental analysis.

Compounds **9**–**10**, **12** and **16**–**17** were evaluated for their cytotoxical effect on four cancer cell lines: HT-29, breast cancer cells MDA-MB-231, HeLa, HepG2 and as well as human diploid cell line Lep-3.

Significant cytotoxicity of hydrazone **16** against MDA-MB-231 was established by biologically study, the IC_{50} was 6.2 nM while the EC_{50} value to Lep 3 is 0.21 nM. Relative high antiproliferative effects of the acetate **12** and compound **16** against HT-29 were ascertained and the calculated IC_{50} values were IC_{50} – 0.85 nM and IC_{50} – 2.83 nM respectively. Cytotoxic activity against HeLa and HepG2 cells was demonstrated by hydrazone **17**, IC_{50} was 7.2 nM and 117 nM respectively. All tested compounds revealed proliferative activities to human diploid cell line Lep-3. The EC_{50} values were in the range from 0.05 to 16.91 nM. The obtained results prove the selective cytotoxicity of the tested compounds and are promising for further evaluation of the investigated compounds in vivo experiments using experimentally induced tumors in laboratory animals.

© 2013 Elsevier Masson SAS. All rights reserved.

1. Introduction

The 2-aminobenzimidazole occurs as pharmacophore in the structure of many clinically useful chemotherapeutic agents and is of crucial importance as precursor to generating new derivatives with a wide spectrum of biological activity as antiviral, diuretic, as H3 agonist and selective NOD1-inhibitors [1–5]. It is known, that 2-aminobenzimidazole anthelmintics block microtubule function in cells, so that research efforts are aimed at testing their activity against cancer cells and elucidation of the mechanism of action. Albendazole, a benzimidazole carbamate (methyl 5-propylthio-1*H*-benzimidazol-2-yl carbamate) with extensive clinical use as an anthelmintic drug, can also inhibit hepatocellular carcinoma cell proliferation under both in vitro and in vivo experimental conditions [6]. The cytotoxicity of albendazole derivatives was evaluated against a human colorectal cancer cell line (HT-29) and a human prostate cancer cell line (PC-3) and some of them were up to ten times more cytotoxic than the parent drug albendazole [7]. Flubendazole induced cell death in leukemia and myeloma cell lines and primary patient samples at nanomolar concentrations and inhibited tubulin polymerization by binding tubulin at a site distinct from vinblastine [8]. Cytotoxicity assay on HEp-2 (Human larynx cancer cell line) was performed with 2-aminobenzimidazole derivatives. Some of the tested compounds showed cytotoxicity, which is comparable to that of the standard drug 5-Fluorouracil [9]. Antiparasitic mebendazole showed survival benefit in 2 preclinical models of glioblastoma multiforme and it was also reported that mebendazole induces apoptosis via Bcl-2 inactivation in chemoresistant melanoma cells [10,11]. Many other 2-aminobenzimidazole derivatives were synthesized and studied for antitumor activity [12,13]. On the other hand several new nitrobenzimidazoles were created as substrates for DT-diaphorase and reported to possess cytotoxic activity [14,15].

On the base of the above mentioned facts, the present work is an extension of our ongoing efforts toward a development of new potent anticancer agents using 5-substituted-2-aminobenzimida zoles as precursors. Our previous study on the 1,3-substituted-2,3-dihydro-2-imino-benzimidazoles showed that some of the studied

* Corresponding author. Tel.: +359 28163207; fax: +359 2685488.
E-mail addresses: anmav@abv.bg, mavrova_anelia@abv.bg (A.Ts. Mavrova).

0223-5234/$ – see front matter © 2013 Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.ejmech.2013.03.010

compounds exhibited remarkable activity in vitro against HT-29 and MDA-cancer cell lines and in the same time the tested compound revealed proliferative effect against human spleen cells [16]. Stimulated by these encouraging and promising results we undertook investigation on the synthesis of some novel 5-nitro- and 5-benzoyl-1,3-disubstituted-2,3-dihydro-2-imino-benzimidazoles and pharmacological evaluation of the compounds against four human cancer cell lines and human diploid cell line.

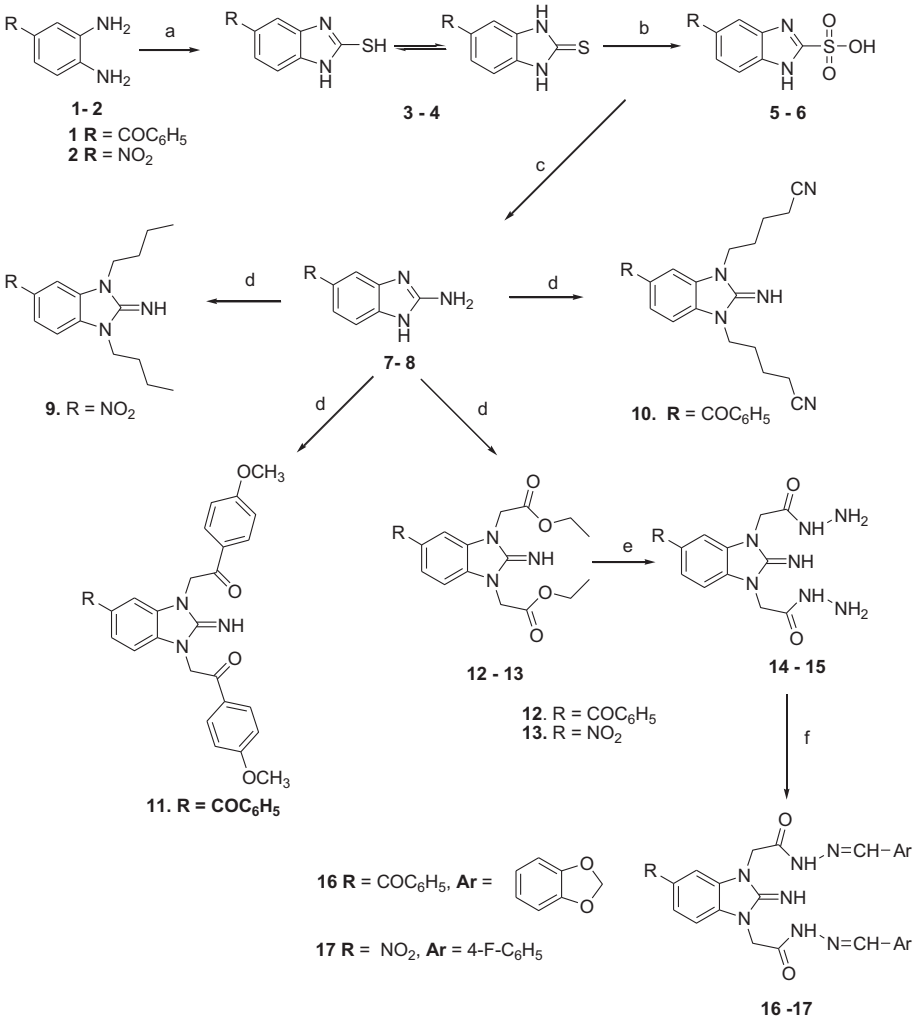
2. Chemistry

The synthesis of 1,3-disubstituted-2,3-dihydro-2-iminobenzimi dazoles is illustrated and outlined in Scheme 1.

The starting 5-substituted-2-aminobenzimidazoles were synthesized in three steps starting with the obtaining of the corresponding benzimidazol-2-thiols **3**–**4**, subsequent oxidation of the thiols to the corresponding sulfonic acids **5**–**6** and substitution of sulfonic-group by interaction with ammonium hydroxide according to the method described by us early in Ref. [17]. The refluxing of the solution of 4-substituted-1,2-diaminobenzen, carbon disulfide and sodium hydroxide in ethanol medium afforded the corresponding thiols. The oxidation of the thiols with potassium permanganate in water solution of sodium hydroxide led to the obtaining of benzimidazol-2-sulfonic acids. The yields of thiols were in the range of 84–98%, while these of the sufonic acids varied from 54% to 69%. The lower yields of the sulfonic acids can be explained with the fact that the reaction took place in heterogenous medium. The nucleophilic substitution between the 2-aminobenzimidazoles and the appropriated halogen derivatives under solid–liquid phase transfer catalysis conditions in dry acetonitrile resulted in the formation of 1,3,5-substituted-1,3-dihydro-2-imino-benzimidazoles **9**–**13**. The condensation of ethyl [3-(2-ethoxy-2-oxoethyl)-5-nitro-2-imino-5-nitro-2,3-dihydro-1*H*-benzimidazol-1-yl]acetate respectively ethyl [3-(2-ethoxy-2-oxoethyl)-2-imino-5-benzoyl-2,3-dihydro-1*H*-ben zimidazol-1-yl]acetate with hydrazine hydrate in molar ratio afforded the hydrazides **14**–**15**, which were reacted with the corresponding arylaldehydes yielding hydrazones **16**–**17**.

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

In the ^{1}H NMR spectra, particularly meaningful are characteristic NCH_2CO signals (singlets) of compounds **11**–**17**, shifted downfield due to deshielding effect of both N-atom and the CO group. The chemical shift values varied in the range from 4.3 to



Scheme 1. Synthesis of 1,3,5-substituted-2,3-dihydro-2-iminobenzimidazoles., Regents and conditions: a) sodium hydroxide, ethanol–water solution, carbon disulphide, refluxing; b) $KMnO_4$, 25% NaOH, reflux; c) 25% NH_4OH, 145 °C, in welded ampoule; d) acetonitrile, TBAB, dry K2CO3, halogen derivative, 25 °C; e) hydrazine hydrate, ethanol, refluxing; f) ethanol, substituted benzaldehydes, refluxing.

5.3 ppm depending of the substituents. The aromatic protons in the ^{1}H NMR spectra show typical pattern for mono-, di- and three-substituted phenyls. The labile NH protons are not characteristic and their chemical shifts depend on the water quantity in the solvent. In the ^{13}C NMR spectra, the characteristic signals are NCH_2 (43–45 ppm), C=N (~158 ppm), N–C=O (~166 ppm) and C=O (~195 ppm). The ^{19}F NMR spectra of fluorine-containing compounds were useful to check the purity. Some ^{1}H NMR and ^{13}C NMR spectra are given in the Supplementary material.

3. Pharmacology

3.1. Cytotoxicity

Compounds**9**–**10**, **12** and **16**–**17** were evaluated for their cytotoxicity to human colorectal cancer cell line HT-29, breast cancer cells MDA-MB-231, cervical cancer cells HeLa, human liver carcinoma cell line HepG2 and human normal diploid cell line Lep3 by using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy methoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrasolium inner salt) – test [18].

4. Results and discussion

In order to synthesized new 5-substituted-2,3-dihydro-2-aminobenzimidazole derivatives and to compare their effects on human cancer cell lines to these of the no-substituted in 5-position derivatives we improved the method for unsubstituted benzimidazoles, described by us earlier [16,17]. The required 2-amino benzimidazoles were obtained by the reaction of the relevant 5-substituted-1,2-diaminobenzene, carbon disulfide, sodium hydroxide in ethanol, followed by the oxidation of the synthesized thiols to the corresponding sulfonic acids. The reaction of 5-substituted-2-aminobenzimidazoles with halogen containing reagents under solid–liquid phase transfer catalysis using anhydrous potassium carbonate and tetrabutyl ammonium bromide as catalysis led to the obtaining of 1,3-disubstituted-2,3-dihydro-2-iminobenzimidazoles. It was established that the reaction runs best at mol ratio of 2-aminobenzimidazoles **7**–**8**, halogen compounds and potassium carbonate 1:2:2 and has led to obtaining of a range of new 1,3-disubstituted derivatives being prepared easily and in good yields – 75–87%.

The cytotoxicity respectively proliferative effect was assessed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrasolium inner salt (MTS) assay, which is based on the reduction of yellow tetrazolium salt by metabolically active viable cells to a formazan product that can be measured spectrophotometrically. Hence, the intensity of the color in the solution is directly proportional to cell viability [19,20]. In order to evaluate the effects of some of the newly synthesized compounds on cell proliferation, four cancer cell lines: human colorectal cancer cell line HT-29, breast cancer cells MDA-MB-231, cervical cancer cells HeLa, human liver carcinoma cell line HepG2 and human diploid cell line Lep-3 were used. The test was performed with compounds **9**, **10**, **12** as well as **16**–**17** according to MTS method, as described in Refs. [18,19]. 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. 1–3. The obtained results were plotted and IC_{50} and EC_{50} were calculated. Statistical significant differences in the level of cells in both control and experimental groups were determined ($p \leq 0.05$).

Among all tested compounds only compound **16** revealed cytotoxicity against MDA-MB-231 cells. The calculated IC_{50} value was 6.2 nM. The highest cytotoxicity against HT29 cell lines showed

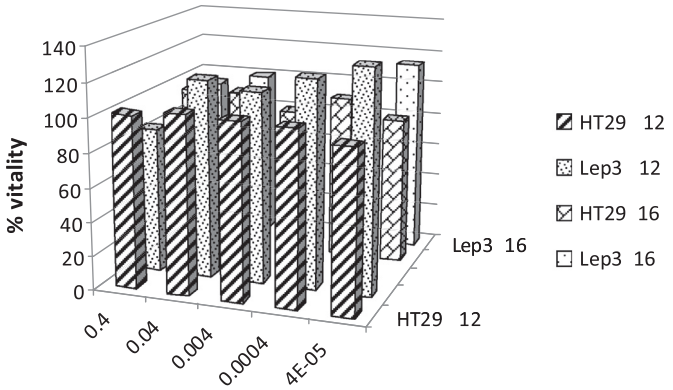


Fig. 1. Relative viability of HT-29 and Lep 3 cells (%) after treatment with, compounds **12** and **16**.

compounds **12** and **16** with IC_{50} – 0.64 nM and IC_{50} – 2.83 nM respectively. As regards to the effects of the compounds on HeLa and Hep G2-cell lines it may be pointed out, that only compound **17** revealed cytotoxic activity and the IC_{50} values were 117 nM and 7.27 nM respectively.

All studied compounds manifested proliferative effects to the human diploid cell line Lep-3. The EC_{50} values varied in the range from 0.21 nM for compound **16** to 11.40 nM for compound **9**. Substances **9** and **10** exhibited proliferative activity against all others cell lines (Table 1; Supplementary material – Figs. 12–16).

If the results, obtained for compound **16** are taken in to consideration it should be noted that *N*′-[1,3-benzodioxol-5-ylmethylene]-2-(3-{2-[2-(1,3-benzodioxol-5-ylmethylene)]-2-oxoethyl}-5-benzoyl-2-imino-2,3-dihydro-1*H*-benzimidazol-1-yl) acetohydrazide revealed proliferative activity against human diploid cell line Lep-3 at lower concentration in comparison to the concentration at which the compound excited cytotoxic effect on HT-29 cells. That fact is an indicator for the selectivity effect of the compound **16**. Similar results were observed also for compounds 17, which showed cytotoxicity effect at higher concentration on HeLa cells and proliferative effect on Lep-3 at lower concentration. The relation between cell viability and the concentrations of compounds **12**, **16** and **17** was plotted to obtain the survival curve of MDA-MB 231, HT-29, HeLa and Hep G2-cell lines as well as that of Lep-3, Figs. 1–3. As it can be seen from figures, the viability of the treated with the above mentioned compounds cancer cells was many times lower than the vitality of Lep-3 cells.

The biological data of the synthesized compounds highlighted that the nature and position of the substituent on the benzene ring of benzimidazolone moiety as well as these at 1,3-position greatly influence both the cytotoxicity to the cancer cells and the proliferation properties of Lep3 of the tested compounds. The introduction of the benzoyl group in position 5 and the disubstitution in 1 and 3 positions by means of butyl or cyanopentyl groups of the benzimidazole ring generally led to a proliferation activity as

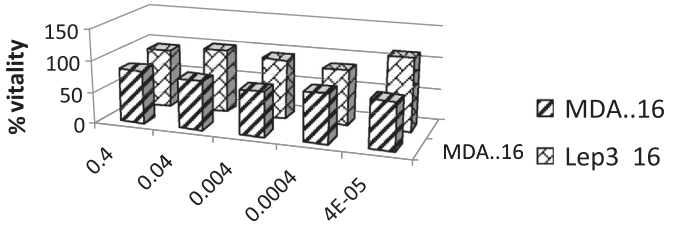


Fig. 2. Relative viability of MDA-MB-231 and Lep 3 cells (%) after treatment with, compound **16**.

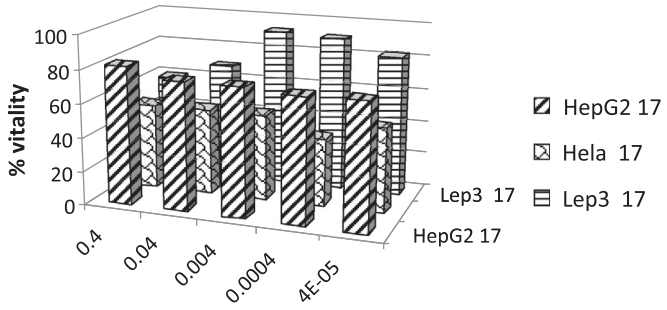


Fig. 3. Relative viability of HepG2, Hela and Lep 3 cells (%) after treatment, with compound **17**.

regards to all tested cells. In particular, the presence of a benzoyl group at the 5 position and ethoxycarbonylmethyl groups at the 1 and 3 position of the 2-imino-benzimidazole system provoked the appearance of cytotoxicity and provided the most active compound **12** against HT-29 (IC50 − 0.85 nM), while the unsubstituted analog showed EC 50 to HT-29 cells [16]. The hydrazone 17 containing a nitro group in the 5 place revealed cytotoxicity to HeLa and Hep and in comparison to the unsubstituted analog showed proliferative properties to HT-29 [16]. To display more precise the relationship between structure and activity of the synthesized compounds it is necessary to be carried out further research on the rest of the synthesized compounds.

Moreover, we have planned further structural modifications such as the conversion of the imino group of the benzimidazole nucleus into the isosteric thiocarbonyl group and as well as the introduction of different substituents at the 1, 3 and 5-th position.

5. Conclusion

Optimized reaction conditions for the synthesis of new 1,3-substituted-2,3-dihydro-2-iminobenzimidazoles were developed using 5-substituted-2-aminobenzimidazoles and different halogen derivatives as precursors under solid–liquid PTC and a range of new derivatives have being prepared easily and in good yields.

The initial biological screening in vitro showed that the studied compound **16** possessed relative high cytotoxicity against MDA-MB-231 as well as against HT-29 cell lines, the IC50 values were 6.63 nM and 2.83 nM, but the compound **12** was more toxic to HT-29 cells than compound **16** with a IC_{50} value of 0.64 nM. Only compound **17** revealed cytotoxic activity to HeLa and Hep G2-cell lines and the IC_{50} values were 117 nM and 7.27 nM respectively. All investigated compounds revealed proliferative effects on human diploid cells, the EC_{50} values were in the range 0.2–114 nM.

The above results confirmed also the hypothesis that the presence of different substituents at 5-th position in the structure of 1,3-disubstituted-2,3-dihydro-2-imino-benzimidazoles not only contribute to the expression of cytotoxicity, but also to the manifestation of proliferative activity to human diploid cells. That fact could be regarded as a sign of selectivity. The in vitro effect of the examined substances is indicating that their biological role can be examined further in in vivo experiments using experimentally induced tumors in laboratory animals. In case these results become similar, we could suppose that exactly that derivatives could be proceed further as potential oncotherapeutics.

Table 1
The proliferative activity (EC_{50}) of the studied compounds.

No	EC_{50} ± SE (nM)				
	MDA-MB231	HT-29	HeLa	HepG2	Lep3
9.	86 ± 0.23	177 ± 0.196	158 ± 0.05	142 ± 0.073	114 ± 0.012
10.	119 ± 0.06	46.7 ± 0.22	0.015 ± 0.016	94.1 ± 0.04	68 ± 0.013
12.	0.28 ± 0.023	–	0.002 ± 0.001	70.1 ± 0.068	73 ± 0.077
16.	–	–	0.25 ± 0.02	10.2 ± 0.056	0.21 ± 0.102
17.	17 ± 0.023	24.5 ± 0.08	–	–	12.2 ± 0.0170

6. Experimental part

The reactions were monitored by thin layer chromatography, which was performed on Merck pre-coated plates (silica gel. 60 F254, 0.25 mm) and was visualized by fluorescence quenching under UV light (254 nm).

Melting points (mp) were determined on an Electrothermal AZ 9000 3MK4 apparatus and were uncorrected. IR spectra were recorded on a Bruker spectrophotometer as potassium bromide discs. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance II+ 250 MHz and 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) and as well as ^{19}F NMR for the compounds containing F atom. The microanalyses for C, H, N and S were performed on Perkin–Elmer elemental analyzer.

The starting 4-substituted-1,2-diaminobenzenes **1**–**2** were commercially available.

6.1. General procedure for compounds *3*–*4*

4-Substituted-1,2-diaminobenzene (0.019 mol) and water (3 ml) were added to a solution of sodium hydroxide (0.022 mol) in ethanol (20 ml) and carbon disulphide (0.022 mol). The mixture was heated under reflux for 3 h. Charcoal was added cautiously and removed by filtration after the mixture has been refluxed for 10 min more. The filtrate was heated to 60–70 °C and quenched with warm water (70°, 20 ml), and then 50% acetic acid (9 ml) was added by good stirring. After cooling the solution in refrigerator for 3 h the crystallization was completed and the relevant thiol was separated.

6.1.1. (2-Mercapto-1H-benzimidazol-5-yl)(phenyl)methanone *3*

Yield − 96,8%; Mp − 248–250 °C; $R_f = 0.68$, mobile phase: ^1H NMR (DMSO-d_6) δ (ppm): 1.877 (s, 1H, SH), 7.251 (d, $J = 8.2$ Hz, 1H, 7-H), 7.462 (d, $J = 1.6$ Hz, 1H, 4-H), 7.545 (dd, $J = 8.2$ Hz, $J = 1.6$ Hz, 1H, 6-H), 7.558 (t, $J = 7.7$ Hz, 2H, m-Ph), 7.658 (dt, J = 7.4 Hz, $J = 1.4$ Hz, 1H, p-Ph), 7.700 (dd, $J = 8.1$ Hz, $J = 1.4$ Hz, 2H, o-Ph), 12.75 (br s, 1H, NH);

^{13}C NMR (DMSO-d_6) δ (ppm): 109.30 (7-C), 110.81 (4-C), 125.12 (6-C), 128.48 (m-Ph), 129.35 (o-Ph), 130.51 (3a-C), 132.12 (p-Ph), 132.92 (7a-C), 137.14 (5-C), 137.99 (i-Ph), 170.78 (C=N), 195.08 (C=O).

Analysis: Calc. for $C_{14}H_{10}N_2OS$; C, 66.12; H, 3.96; N, 11.02; O, 6.29; S, 12.61; Found: C, 66.09; H, 3.91; N, 11.12; O, 6.32; S, 12.64;

6.1.2. 5(6)-Nitro-1H-benzimidazole-2-thiol *4*

Yield − 84%; Mp − 272 °C (decomp); $R_f = 0.41$, mobile phase: $CHCl_3$/ethyl acetate = 6:1; ^1H NMR (DMSO-d_6): 7.94 (dd, $J = 9.1$ Hz, 1H, CH), 8.13 (m, 1H, CH), 8.34 (d, 1H), 12.32 (s, 1H, SH). Analysis: Calc for $C_7H_5N_3O_2S$: C, 43.07; H, 2.58; N, 21.53; O, 16.39; S, 16.43; Found: C, 43.17; H, 2.53; N, 21.57; O, 16.41; S, 16.42;

6.2. General procedure for compounds *5*–*6*

To a boiling solution of 4-substituted-1*H*-benzimidazol-2-yl-tiol (0.04 mol) in water (25 ml) and 50% sodium hydroxide (15 ml) a

solution of potassium permanganate (0.08 mol) in 200 ml was added in small portions by stirring. After the complete addition of potassium permanganate the reaction solution was refluxed for 30 min more. The formed manganese dioxide was filtered. The filtrate was treated with hydrochloric acid to pH = 1 by cooling. The obtained precipitate of 1-(un)substituted-1*H*-benzimidazol-2-yl-sulphonic acid was filtered and washed with water.

6.2.1. 5-Benzoyl-1H-benzimidazole-2-sulfonic acid ***5***

Yield – 69.4%; Mp – 275–277 °C; R_f = 0.53, mobile phase: dichloromethane/methanol = 5: 1; ^{1}H NMR (DMSO-d_6) δ (ppm): 7.59 (m, J = 7.2 Hz, 2H, H-18, H-20), 7.67 (m, J = 1.9 Hz, 1H, H-9), 7.88 (m, J = 7.4 Hz, 2H, H-17, H-21), 7.98 (dd, 1H, H-7), 8.04 (s, 1H, H-9), 8.14 (dd, J = 8.6 Hz, 1H, H-6), 12.36 (br s, 1H, OH). Analysis: Calc. for $C_{14}H_{10}N_2O_4S$; C, 55.62; H, 3.33; N, 9.27; O, 21.17; S, 10.61; Found: C, 55.68; H, 3.31; N, 9.30; O, 21.14; S, 10.68;

6.2.2. 5-Nitro-1H-benzimidazole-2-sulfonic acid ***6***

Analysis: Yield – 52.4%; Mp –310 °C (decomp.); R_f = 0.58, mobile phase: dichloromethane/methanol = 8:1; ^{1}H NMR (DMSO-d_6) δ (ppm): 8.07 (dd, 1H, H-7), 8.36 (d, J = 9.1 Hz, 1H, H-6), 8.51 (d, J = 2.3, 1H, H-9). Analysis: Calc. for $C_7H_5N_3O_5S$; C, 34.57; H, 2.07; N, 17.28; O, 32.89; S, 13.18; Found: C, 34.61; H, 2.010; N, 17.24; O, 32.85; S, 13.14;

6.3. General procedure for compounds ***7–8***

4-Substituted-1*H*-benzimidazol-2-sulphonic acid (0.002 mol) and 25% ammonium hydroxide (1 ml) were heated in welded ampoule for 5 h at 145–150 °C. After cooling the formed crystals of 2-aminobenzimidazole were filtered and washed with ammonium hydroxide (0.3 ml) and water (4 ml) and re-crystallized with ethanol.

6.3.1. (2-Amino-1H-benzimidazol-5-yl)(phenyl)methanone ***7***

Yield – 64.5%; Mp – 172–174 °C (destruction); R_f = 0.44, mobile phase: dichloromethane/methanol = 8:1; ^{1}H NMR (DMSO-d_6) δ (ppm): 6.91 (bs, 1H, NH), 7.18 (dd, J = 9.1 Hz, 1H, H-6), 7.65 (m, 1H, H-7), 7.70 (dd, J = 2.1 Hz, 1H, H-9); Analysis. Calc. for $C_{14}H_{11}N_3O$; C, 70.87; H, 4.67; N, 17.71; O, 6.74; Found: C, 70.80; H, 4.71; N, 17.74; O, 6.73;

6.3.2. 5-Nitro-1H-benzimidazol-2-amine ***8***

Analysis: Yield – 77.4%; Mp – 137–139 °C; R_f = 0.64, mobile phase: dichloromethane/methanol = 8:1; ^{1}H NMR (DMSO-d_6) δ (ppm): 7.26 (bs, 1H, NH), 7.98 (dd, J = 9.1 Hz, 1H, H-6), 8.05 (dd, 1H, H-7), 7.70 (dd, J = 2.2 Hz, 1H, H-9). Analysis: Calc. for $C_7H_6N_4O_2$; C, 47.19; H, 3.39; N, 31.45; O, 17.96; Found: C, 47.23; H, 3.42; N, 31.41; O, 17.92.

6.4. General procedure for compounds ***9–13***

0.004 mol of 2-aminobenzimidazole was diluted in 20 ml dry acetonitrile and TBAB (tetrabutylammonium bromide) (0.0012 mol) was added to the solution. The halogen derivative (0.008 mol) was added (in the case of the halogen esters drop by drop) by intensive stirring and after that dry K_2CO_3 (0.008 mol) was placed. The reaction mixture was allowed to be stirred for 3–6 h at room temperature. After completing of the reaction, which was determined through TLC, K_2CO_3 was filtrated and the solvent was removed under reduced pressure. The obtained residue was crystallized through the addition of suitable solvent to obtained compounds **9**–**13**.

6.4.1. 1,3-Dibutyl-5-nitro-1,3-dihydro-2H-benzimidazol-2-imine ***9***

After removing of acetonitrile the compound was crystallized with ethanol. Yield – 81%; Mp – 245–247 °C; R_f = 0.62, mobile phase: dichloromethane/methanol = 8:1; ^{1}H NMR (DMSO-d_6) δ (ppm): 0.919 (t, J = 7.4 Hz, 6H, CH_3), 1.292 (sextet, J = 7.4 Hz, 4H, CH_2), 1.544 (pentet, J = 7.4 Hz, 4H, CH_2), 3.138 (m, 4H, CH_2), 6.315 (s, 1H, NH), 7.012 (d, J = 8.6 Hz, 1H, 7-Ar), 7.733 (dd, J = 2.2, 8.6 Hz, 1H, 6-Ar), 7.82 (d, J = 2.2 Hz, 1H, 4-Ar); ^{13}C NMR (DMSO-d_6) δ (ppm): 13.60 (CH_3), 19.30 (CH_2), 23.14 (CH_2), 57.59 (CH_2), 106.11 (4-Ar), 110.63 (7-Ar), 115.57 (6-Ar), 128.22 (3a-Ar), 128.50 (7a-Ar), 137.99 (5-Ar), 164.19 (C═N). Analysis: Calc. for $C_{25}H_{27}BrN_4O_2$; C, 60.61; H, 5.49; Br, 16.13; N, 11.31; O, 6.46; Found: C, 60.55; H, 5.52; Br, 16.15; N, 11.29; O, 6.49;

6.4.2. 5-[3-(4-Cyanobutyl)-5-benzoyl-2-imino-2,3-dihydro-1H-benzimidazol-1-yl]pentanenitrile ***10***

Yield –75%; Mp – 180 °C; R_f = 0,48, mobile phase: dichloromethane/methanol = 8:1; NMR (DMSO-d_6) δ (ppm): 1.792 (m, 8H, 4CH_2, J = 7.35 Hz); 2.62 (t, 4H CH_2CN, J = 6.41); 4.15 (t. 4H, CH_2–N, J = 6.4 Hz); 7.52 (dd, 2H, CH, J = 7.21 Hz) 7.82 (dd, 2H, 2CH, J = 7.63 Hz); 8.61 (br s, 1H, NH exchangeable with D_2O);

6.4.3. 1,3-Bis(4-methoxyphenyl)-2-oxoethyl)-5-benzoyl-2,3-dihydro-1H-benzimidazol-2-imine ***11***

After removing of acetonitrile the compound was crystallized with dichloromethane. Yield – 75%; Mp – 206–208 °C, recrystallized with dichloromethane; R_f = 0.46 phase: dichloromethane/methanol = 8:1; NMR (DMSO-d_6) δ (ppm): 3.95 (s, 3H, 2O–CH_3); 6.25 (s, 4H, 2CH_2); 7.18 (dd, 4H, 4CH, J = 8.56); 7.65 (m, 2H, CH, J = 7.15); 7.82 (m, 6H, 6CH, J = 7.28); 8.18 (m, 4H, CH, J = 8.57). Analysis: Calc. for $C_{22}H_{27}N_3O_5$; C, 72.03; H, 5.10; N, 7.88; O, 14.99; Found: C, 72.07; H, 5.13; N, 7.85; O, 14.97;

6.4.4. Ethyl [3-(2-ethoxy-2-oxoethyl)-2-imino-5-benzoyl-2,3-dihydro-1H-benzimidazol-1-yl]acetate ***12***

Yield - 86.4%; Mp – 138–140 °C; R_f = 0.66, mobile phase: dichloromethane/methanol = 8:1; ^{1}H NMR (DMSO-d_6) δ (ppm): 1.191 (t, J = 7.1 Hz, 6H, CH_3), 4.152 (q, J = 7.1 Hz, 4H, OCH_2), 4.262 (s, 4H, NCH_2), 7.107 (d, J = 8.1 Hz, 1H, 7-Ar), 7.346 (d, J = 8.1 Hz, 1H, 6-Ar), 7.446 (s, 1H, 4-H), 7.536 (t, J = 7.7 Hz, 2H, m-Ph), 7.640 (t, J = 7.4 Hz, 1H, p-Ph), 7.678 (dd, J = 8.2, 1.2 Hz, 2H, o-Ph); ^{13}C NMR (DMSO-d_6) δ (ppm): 13.97 (CH_3), 47.48 (NCH_2), 60.14 (OCH_2), 105.77 (7-C), 107.92 (4-Ar), 109.58 (6-Ar), 128.37 (m-Ph), 129.10 (7a-C), 129.30 (o-Ph), 131.95 (p-Ph), 132.25 (3a-C), 138.06 (i-Ph), 152.22 (C═N), 167.77 (O–C═O), 171.52 (C═O). Analysis: Calc. for $C_{22}H_{23}N_3O_5$; C, 64.54; H, 5.66; N, 10.26; O, 19.54; Found: C, 64.59; H, 5.61; N, 10.28; O, 19.49;

6.4.5. Ethyl [3-(2-ethoxy-2-oxoethyl)-2-imino-5-nitro-2,3-dihydro-1H-benzimidazol-1-yl]acetate hydro bromide ***13***

Yield – 78%; Mp – 240–242 °C; R_f = 0.48, mobile phase: dichloromethane/methanol = 8:1; ^{1}H NMR (DMSO-d_6) δ (ppm): 1.225 (t, J = 6.9 Hz, 6H, CH_3), 4.168 (q, J = 7.1 Hz, 4H, CH_2), 5.072 (s, 4H, CH_2), 7.045 (s, 1H, NH), 7.207 (d, J = 8.8 Hz, 1H, 4-Ar), 7.953 (dd, J = 2.3, 8.8 Hz, 1H, 5-Ar), 8.131 (d, J = 2.3 Hz, 1H, 7-Ar); ^{13}C NMR (DMSO-d_6) δ (ppm): 14.12 (CH_3), 43.59 (NCH_2), 61.40 (OCH_2), 104.11 (7-Ar), 113.66 (4-Ar), 118.33 (5-Ar), 134.36 (3a-Ar), 142.23 (7a-Ar), 149.72 (6-Ar), 159.50 (C═N). 167.88 (C═O). Analysis: Calc. for $C_{15}H_{19}BrN_4O_6$; C, 41.78; H, 4.44; Br, 18.53; N, 12.99; O, 22.26; Found: C, 41.73; H, 4.40; Br, 18.60; N, 12.90; O, 22.22;

6.5. General procedure for preparation of acetohydrazides ***14–15***

To a solution of 0.002 mol of the corresponding ethyl [3-(2-ethoxy-2-oxoethyl)-2-imino-2,3-dihydro-1*H*-benzimidazol-1-yl]acetate hydro bromide (**4**) in 20 ml ethanol were added 0.6 ml (0.012) mol 98% hydrazine hydrate and the mixture was refluxed

for 2 h. After cooling the obtained precipitation was filtered and re-crystallized with ethanol.

6.5.1. 2-[3-(2-Hydrazino-2-oxoethyl)-5-benzoyl-2-imino-2,3-dihydro-1H-benzimidazol-1-yl] acetohydrazide ***14***

Yield - 81%; Mp 254–255 °C, re-crystallized with ethanol; $R_f = 0.41$, mobile phase: dichloromethane/methanol = 8:1; ^{1}H NMR (DMSO-d_6) δ (ppm): 4.3 (bs, 4H, NH_2), 4.451 (s, 2H) and 4.531 (s, 2H, NCH_2), 7.108 (d, $J = 8.2$ Hz, 1H, 7-Ar), 7.426 (dd, $J = 8.2$, 1.6 Hz, 1H, 6-Ar), 7.462 (d, $J = 1.6$ Hz, 1H, 4-H), 7.552 (t, $J = 7.7$ Hz, 2H, m-Ph), 7.652 (tt, $J = 7.5$, 1.3 Hz, 1H, p-Ph), 7.695 (dd, $J = 8.3$, 1.3 Hz, 2H, o-Ph), 9.351 (s, 1H, C═NH), 10.351 (s, 2H, HNC═O); ^{13}C NMR (DMSO-d_6) δ (ppm): 42.76 and 43.95(NCH_2), 106.12 (7-C), 107.69 (4-Ar), 126.48 (6-Ar), 128.56 (m-Ph), 128.76 (7a-C), 129.42 (o-Ph), 137.65 (5-C), 138.13 (p-Ph), 131.09 (3a-C), 138.13 (i-Ph), 157.78 (C═N), 165.68 (N–C═O), 194.72(C═O). Analysis: Calc. for $C_{18}H_{19}N_7O_3$; C, 56.69; H, 5.02; N, 25.71; O, 12.59; Found: C, 56.62; H, 5.06; N, 25.75; O, 12.53;

6.5.2. 2-[3-(2-Hydrazino-2-oxoethyl)-2-imino-5-nitro-2,3-dihydro-1H-benzimidazol-1-yl]acetohydrazide ***15***

Yield – 88,2%; Mp 284 °C (decomp), re-crystallized with ethanol; $R_f = 0.41$, mobile phase: dichloromethane/methanol = 8:1; ^{1}H NMR (DMSO-d_6) δ (ppm): 4.700 (s, 2H) and 4.730 (s, 2H, NCH_2), 6.974 (bs, 2H) and 7.244 (br s, 2H, NH_2), 7.18 (d, $J = 8.7$ Hz, 1H, 7-Ar), 7.865 (dd, $J = 8.7$, 2.0 Hz, 1H, 6-Ar), 7.922 (d, $J = 2.0$ Hz, 1H, 4-H), 8.76 (s, 1H, C═NH), 9.44 (s, 2H, HNC═O); ^{13}C NMR (DMSO-d_6) δ (ppm): 43.49 and 43.68 (NCH_2), 103.60 (7-C), 107.34 (4-Ar), 109.52 (6-Ar), 114.90 (5-C), 134.40 (7a-C), 138.93 (3a-C), 158.25 and 159.79 (C═N), 165.63 and 165.72 (N–C═O). Analysis: Calc. for $C_{11}H_{14}N_8O_4$; C, 40.99; H, 4.38; N, 34.77; O, 19.86; Found: C, 40.91; H, 4.45; N, 34.81; O, 19.80;

6.6. General procedure for preparation of compounds ***16–17***

0.3 g (0.001 mol) acetohydrazide (**14** or **15**) 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.

6.6.1. N′-[1,3-Benzodioxol-5-ylmethylene]-2-(3-{2-[2-(1,3-benzodioxol-5-ylmethylene)]-2-oxoethyl}-5-benzoyl-2-imino-2,3-dihydro-1H-benzimidazol-1-yl)acetohydrazide ***16***

Yield – 76.7%; Mp 263–265 °C; $R_f = 0.73$, mobile phase: dichloromethane/methanol = 8:1; NMR (DMSO-d_6) δ (ppm): ^{1}H NMR (DMSO-d_6) δ (ppm): 4.586 (bs, 2H) and 5.056 (bs, 2H, NCH_2), 6.079 (s, 8H, OCH_2), 6.979 (d, $J = 8.0$ Hz, 2H, 5-Ar), 7.153 (dd, $J = 1.5$, 8.0 Hz, 2H, 6-Ar), 7.207 (d, $J = 8.3$ Hz, 1H, 7-Ar), 7.363 (d, $J = 1.5$ Hz, 2H, 2-Ar), 7.428 (dd, $J = 1.4$, 8.2, 1H, 6-Ar), 7.482 (d, $J = 1.6$ Hz, 1H, 4-Ar), 7.553 (t, $J = 7.7$ Hz, 2H, m-Ph), 7.653 (dt, $J = 1.3$, 7.5 Hz, 1H, p-Ph), 7.709 (dd, $J = 1.3$, 7.7 Hz, 2H, o-Ph), 7.940 (s, 2H, CH), 9.35 (s, 1H, C═NH), 10.342 (s, 2H, HNC═O); ^{13}C NMR (DMSO-d_6) δ (ppm): 42.72 and 43.89 (NCH_2), 101.63 (OCH_2), 105.21 (2-Ar), 106.33 (7-Ar), 107.72 (4-Ar), 108.52 (5-Ar), 123.37 (6-Ar), 126.57 (6-Ar), 128.44 (7a-C), 128.54 (m-Ph), 129.30 (i-Ar). 129.41 (o-Ph), 130.86 (3a-C), 132.07 (p-Ph), 134.29 (5-Ar), 138.00 (3-Ar), 138.17 (i-Ph), 143.81 (4-Ar), 144.04 (CH). 157.64 (C═N). 167.62 (NC═O), 194.70 (C═O). Analysis: Calc for $C_{34}H_{27}N_7O_7$; C, 63.25; H, 4.22; N, 15.19; O, 17.35; Found: C, 63.30; H, 4.27; N, 15.15; O, 17.31;

6.6.2. N′-[4-Fluorophenyl)methylene]-2-(3-{2-[2-(4-fluorophenyl) methylenehydrazino]-2-oxoethyl}-2-imino-5-nitro-2,3-dihydro-1H-benzimidazol-1-yl)acetohydrazide ***17***

Yield 72%; Mp – 273–275 °C; $R_f = 0.57$, mobile phase: dichloromethane/methanol = 8:1; ^{1}H NMR (DMSO-d_6) δ (ppm): 4.700 and 4.729 (s, 4H, NCH_2), 7.205 (d, $J = 8.7$ Hz, 1H, 4-Ar), 7.943 (d, $J = 8.7$ Hz, 1H, 5-Ar), 7.316 (t, $J = 8.8$ Hz, 4H, 3-Ar and 5-Ar), 7.865 (dd, $J = 2.2$, 8.8 Hz, 4H, 2-Ar and 6-Ar), 7.952 (s, 1H, 7-C), 8.05 (s, 2H, CH), 8.76 (br s, 1H, NH), 9.44 (br s, 2H, NNH); ^{13}C NMR (DMSO-d_6) δ (ppm): 43.49 (NCH_2), 103.72 (7-C), 109.53 (4-C), 115.98 ($^2J_{CF} = 21.4$ Hz, 3-Ar and 5-Ar), 118.27 (5-C), 129.32 ($^3J_{CF} = 8.3$ Hz, 2-Ar and 6-Ar), 130.77 ($^4J_{CF} = 2.7$ Hz, 1-Ar), 134.40 (3a-Ar), 140.20 (7a-Ar), 142.78 ($^5J_{CF} = 2.8$ Hz, CH), 149.83 (6-C), 159.80 (C═N), 163.15 ($^1J_{CF} = 247.7$ Hz, 4-Ar), 165.72 (C═O). ^{19}F NMR (DMSO-d_6) δ (ppm): –111.2; Analysis: Calc. for $C_{23}H_{20}F_2N_8O_4$; C, 54.12; H, 3.95; F, 7.44; N, 21.95; O, 12.54; Found: C, 54.06; H, 3.89; F, 7.47; N, 21.90; O, 12.57.

6.7. MTS test

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 cells with the compounds MTS colorimetric assay of cell survival was performed. The wells were treated with MTS solution and incubated for 4 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).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.03.010.

References

[1] K. Andries, M. Moeremans, T. Gevers, R. Willebrords, C. Sommen, J. Lacrampe, F. Janssens, P.R. Wyde, Antivir. Res. 60 (3) (2003) 209–219.
[2] H. Wahe, P.F. Asobo, R.A. Cherkasov, A.E. Nkengfack, G.N. Folefoc, Z.T. Fomum, D. Doepp, Arkivoc xiv (2003) 170–177.
[3] M. Mor, F. Bordi, C. Silva, S. Rivara, V. Zuliani, F. Vacondio, M. Rivara, E. Barocelli, S. Bertoni, V. Ballabeni, F. Magnanini, M. Impicciatore, P.V. Plazzi, Bioorg. Med. Chem. 12 (4) (2004) 663–674.
[4] M. Rivara, V. Zuliani, G. Cocconcelli, G. Morini, M. Comini, S. Rivara, M. Mor, F. Bordi, E. Barocelli, V. Ballabeni, S. Bertoni, P.V. Plazzi, Bioorg. Med. Chem. 4 (5) (2006) 1413–1424.
[5] R.G. Correa, P.M. Khan, N. Askari, D. Zhai, M. Gerlic, B. Brown, G. Magnuson, R. Spreafico, S. Albani, E. Sergienko, P.W. Diaz, G.P. Roth, J.C. Reed, Chem. Biol. 18 (7) (2011) 825–832.
[6] M.H. Pourgholami, L. Woon, R. Almajd, J. Akhter, P. Bowery, D.L. Morris, Cancer Lett. 165 (2001) 43–49.
[7] Y. Zhao, M.H. Pourgholami, D.L. Morris, J.G. Collins, A.I. Day, Org. Biomol. Chem. 8 (2010) 3328–3337.
[8] P.A. Spagnuolo, J. Hu, R. Hurren, X. Wang, M. Gronda, M.A. Sukhai, A. Di Meo, J. Boss, I. Ashali, R.B. Zavareh, N. Fine, C.D. Simpson, S. Sharmeen, R. Rottapel, A.D. Schimmer, Blood 115 (2010) 4824–4833.
[9] N. Siddiqui, B. Bhrigu, D. Pathak, M.S. Alam, R. Ali, Ann. Biol. Res. 2 (3) (2011) 194–199.
[10] R.-Y. Bai, V. Staedtke, C.M. Aprhys, G.L. Gallia, G.J. Riggins, Neuro-Oncology 13 (9) (2011) 974–982.
[11] N. Doudican, A. Rodriguez, I. Osman, S.J. Orlow, Mol. Cancer Res. 6 (2008) 1308–1315.
[12] M. Hranjec, K. Starčević, S.K. Pavelić, P. Lučin, K. Pavelić, G.K. Zamola, Eur. J. Med. Chem. 46 (6) (2011) 2274–2279.
[13] S. Singh, H. Ojha, A.K. Tiwari, N. Kumar, B. Singh, A.K. Mishra, Cancer Biother. Radiopharm. 25 (2) (2010) 245–250.
[14] J. Sarlauskas, E. Dickancaite, A. Nemeikaite, Z. Anusevicius, H. Nivinskas, J. Segura-Aguilar, N. Cenas, Arch. Biochem. Biophys. 346 (2) (1997) 219–229.
[15] M.M. Ramla, M.A. Omar, A.M. EL Khamry, H.I. EL Diwani, Bioorg. Med. Chem. 14 (2006) 7324–7332.
[16] A.Ts. Mavrova, D. Wesselinova, N. Vassilev, J.A. Tsenov, Eur. J. Med. Chem. 46 (8) (2011) 3362–3367.
[17] A.Ts. Mavrova, P. Denkova, J.A. Tsenov, K. Anichina, D.I. Vutchev, Bioorg. Med. Chem. 15 (18) (2007) 6291–6297.
[18] CellTiter 96 Non-Radioactive Cell proliferation assay, Technical Bulletin #TB112, Promega Corporation USA, Revised 12/99.
[19] M.V. Berridge, A.S. Tan, Arch. Biochem. Biophys. 303 (1993) 474–482.
[20] R. Jover, X. Ponsoda, J.V. Castell, M.J. Gómez-Lechón, Toxicol. In Vitro 8 (1994) 47–54.