Arch. Pharm. Res.
DOI 10.1007/s12272-013-0197-0

Archives of Pharmacal Research
www.springer.com/12272

RESEARCH ARTICLE

Synthesis and cytotoxic evaluation of novel *N*-substituted amidino-1-hydroxybenzimidazole derivatives

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Received: 22 March 2013 / Accepted: 23 June 2013

Abstract A new class of *N*-substituted amidino-1-hydroxybenzimidazole derivatives (**15–24**) were synthesized and evaluated for their in vitro cytotoxic activities against human leukemia cell lines, HL-60 and K562. The preliminary results showed that compounds **16**, **20**, **21** and **23** had moderate antitumor activity against HL-60 cell line. Further investigation on the mechanism of the observed cytotoxic effects demonstrated that compound **21** increased the expression of autophagic and apoptotic genes and induced apoptosis of HL-60 cells.

Keywords 1*H*-benzimidazole · Amidinobenzimidazole · Cytotoxic activity · K562 · HL-60

Introduction

Pentamidine, an aromatic diamidine, is used against early stage human African tryponosomiasis (HAT) (Apted 1980), antimony-resistant leishmaniasis (Bryceson et al. 1985) and AIDS-related P. jiroveci pneumonia (Ivady and Paldy 1958). Pentamidine is also reported to exert anticancer activity against different cancers such as human melanoma (Pathak et al. 2002) and human leukemia (Qiu et al. 2012).

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Anticancer activity of various benzimidazoles (Fig. 1) **I** (Karpinska et al. 2011), **II** (Romero-Castro et al. 2011), **III** (Gowda et al. 2009) and amidinobenzimidazoles **IV** (Starcevic et al. 2007) have been reported. These results prompted us to investigate the cytotoxic activity of a new series of 1-hydroxybenzimidazole derivatives carrying *N*-substituted amidine groups against acute myeloid leukemia (AML) cell line HL-60 and chronic myeloid leukemia (CML) cell line K562. Effects of these novel compounds on expression of apoptotic (Tzifi et al. 2012) and autophagic (Wirawan et al. 2012) genes were also evaluated.

Materials and methods

Chemistry

Experimental

Uncorrected melting points were measured on a Büchi B-540 capillary melting point apparatus. ^{1}H NMR spectra were recorded in deuterated dimethyl sulfoxide (DMSO-d_6) on a Varian Mercury 400 MHz FT spectrometer, chemical shifts (δ) are in ppm relative to tetramethylsilane (TMS), and coupling constants (*J*) are reported in Hertz. Mass spectra were taken on a Waters Micromass ZQ connected with Waters Alliance HPLC, using ESI(+) method, with C-18 column. Elemental analyses were performed by Leco CHNS-932. Some HCl salts of compounds were prepared by using dry HCl gas in MetOH. All the reagents and solvents were purchased from Sigma–Aldrich Chemical Co. or Fischer Scientific. Compounds **1, 2** (Göker et al. 2002), **3** (Göker et al. 2005a), **4**, **5**, **7**, **12**, **13** (Göker et al. 2005b), **9** and **14** (Ates-Alagoz et al. 2006) were synthesized as described in literature (Scheme 1).

Published online: 09 July 2013

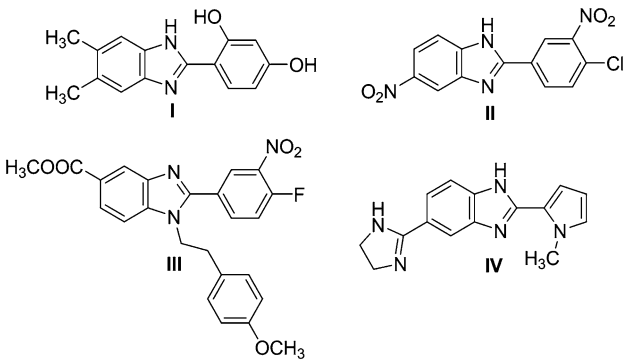


Fig. 1 The structures of some benzimidazoles having anticancer activity

*General synthesis of **5–14***

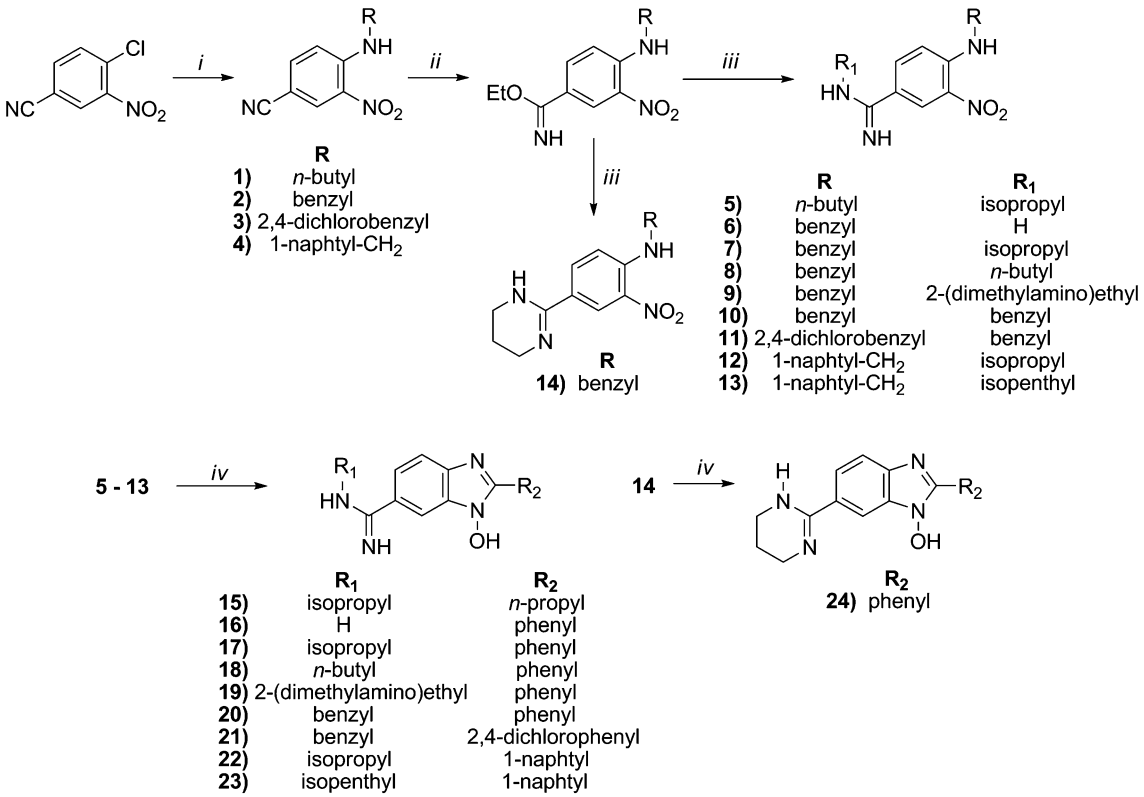
Compound **1–4** (2 mmol) were suspended in absolute EtOH, cooled in an ice-salt bath, and dry HCl gas was passed through the solution for 40 min. The solution was stirred in a stoppered flask at room temperature for 3 days and then diluted with dry ether. The imidate esters precipitated as yellow solids, which were washed with ether then dried under vacuum at room temperature. All imidate esters were used directly without characterisation. A suspension of imidate ester HCl in absolute EtOH was stirred with corresponding the amines (1.5- to 2-fold excess) overnight at 25–30 °C. The reaction mixture was evaporated and diluted with ether, the precipitate was filtered, washed with ether, then dried. Compounds **5–14** were used without purification as HCl salts for the next steps since they were prepared completely pure.

*4-(Benzylamino)-3-nitrobenzamidine (**6**)*

Yield 74 %, mp 268–269 °C; ^{1}H NMR (DMSO-d_6) δ: 4.70 (d, 2H, $J = 6.4$ Hz), 7.03 (d, 1H, $J = 9.6$ Hz), 7.22 (t, 1H, $J = 7.0$ Hz), 7.29–7.36 (m, 4H), 7.81 (dd, 1H, $J = 9.2$, $J = 2$ Hz), 8.67 (d, 1H, $J = 2.4$ Hz), 8.93 (s), 9.18 (t). MS (ESI+) *m*/*z* (rel intensity): 271 (M+H, 100), $C_{14}H_{14}N_4O_2$.

*4-(Benzylamino)-N-butyl-3-nitrobenzamidine (**8**)*

Yield 65 %, mp 280–282 °C(bubbling), ^{1}H NMR (DMSO-d_6) δ: 0.90 (t, 3H, $J = 7.2$ Hz), 1.35 (m, 2H), 1.59 (m, 2H), 3.37 (overlapped with DMSO), 4.73 (d, 2H, $J = 6.4$ Hz), 7.05 (d, 1H, $J = 9.2$ Hz), 7.24–7.40 (m, 5H), 7.80 (dd, 1H, $J = 9.2$, $J = 2.4$ Hz), 8.59 (d, 1H, $J = 2.4$), 9.17 (t), MS (ESI+) *m*/*z* (rel intensity): 327 (M+H, 100), $C_{18}H_{22}N_4O_2$.



Scheme 1 General synthesis of novel 1-hydroxybenzimidazole carboxamidines. Reagents and condition: *i)*. Corresponding amines, DMF. *ii)*. HCl (g), EtOH. *iii)*. Corresponding amines, EtOH. *iv)*. NaH, DMF

*N-benzyl-4-(benzylamino)-3-nitrobenzamidine (**10**)*

Yield 70 %, mp 278–279 °C, ^{1}H NMR (DMSO-d_6) δ: 4.63(s, 2H), 4.70 (d, 2H, $J = 6.4$ Hz), 7.03 (d, 1H, $J = 9.6$ Hz), 7.22 (t, 1H, $J = 6.8$ Hz), 7.31–7.41 (m, 9H), 7.82 (dd, 1H, $J = 6.8$, $J = 2.4$ Hz), 8.62 (d, 1H, $J = 2.4$ Hz), 9.15(t), MS (ESI+) *m/z* (rel intensity): 361 (M+H, 100), $C_{21}H_{20}N_4O_2$.

*N-benzyl-4-(2,4-dichlorobenzylamino)-3-nitrobenzamidine (**11**)*

Yield 68 %, mp 268–270 °C, ^{1}H NMR (DMSO-d_6) δ: 4.60 (s, 2H), 4.73 (d, 2H, $J = 6$ Hz), 6.91 (d, 1H, $J = 9.2$ Hz), 6.28–6.38 (m, 7H), 7.67 (d, 1H, $J = 2$ Hz), 7.79 (d, 1H, $J = 9.6$ Hz), 8.62 (d, 1H, $J = 2.4$ Hz), 9.08(t), MS (ESI+) *m/z* (rel intensity): 429 (M+H, 100), 431(M+H+2, 66), 433 (M+H+4, 10), $C_{21}H_{18}Cl_2N_4O_2$.

*General synthesis of **15–24***

Compound **5–14** (0.5 mmol) were dissolved in dimethylformamide (DMF) (2–3 ml) and the solution was then treated with NaH (0.5 mmol, 60 % suspension in paraffin oil). The mixture was stirred overnight at room temperature (Bowser et al. 2007). At the end of this time, the solution was added to H_2O (2 ml) and EtOH (2 ml) and all solvents were evaporated in vacuo. The residue was purified by silica gel column chromatography using chloroform/methanol/ammonium hydroxide solution (100:20:1) as eluent.

*1-Hydroxy-N-isopropyl-2-propyl-1H-benzo[d]imidazole-6-carboxamidine HCl (**15**)*

Yield 53 %, mp 207–209 °C (bubbling), ^{1}H NMR (DMSO-d_6) δ: 0.99 (t, 3H, $J = 7.6$ Hz), 1.30 (d, 6H, $J = 6$ Hz), 1.88 (m, 2H), 3.03 (t, 2H, $J = 7.2$ Hz), 4.10 (m, 1H), 7.67 (d, 1H, $J = 8.8$ Hz), 7.85 (d, 1H, $J = 8.4$ Hz), 8.014 (s, 1H), 9.12 (s, 1H), 9.51 (s, 1H), 9.66 (d, 1H, $J = 8$ Hz). MS (ESI+) *m/z* (rel intensity): 261 (M+H, 82), 172 (100), Anal. for $C_{14}H_{20}N_4O{\cdot}1{,}5HCl{\cdot}H_2O$, Calcd. C, 50,49 H, 7.11 N, 16.82 Found C, 50.11, H, 7.22 N, 16.66.

*1-Hydroxy-2-phenyl-1H-benzo[d]imidazole-6-carboxamidine HCl (**16**)*

Yield 24 %, mp 267–269 °C (bubbling), ^{1}H NMR (DMSO-d_6) δ: 7.62 (m, 3H), 7.76 (dd, 1H, $J = 8.8$, $J = 2$ Hz), 7.90 (d, 1H, $J = 8.4$ Hz), 8.17 (d, 1H, $J = 1.2$ Hz), 8.35 (m, 2H), 9.17 (s), 9.46 (s), MS (ESI+) *m/z* (rel intensity): 253 (M+H, 60), 189(100), Anal. for $C_{14}H_{12}N_4O{\cdot}2HCl{\cdot}0{,}5H_2O$, Calcd. C, 50,31, H, 4.52 N, 16.76 Found C, 50.08 H, 4.74 N, 16.36.

*1-Hydroxy-N-isopropyl-2-phenyl-1H-benzo[d]imidazole-6-carboxamidine HCl (**17**)*

Yield 60 %, mp 271–272 °C (bubbling), ^{1}H NMR (DMSO-d_6+D_2O) δ: 1.33 (d, 6H, $J = 6.8$ Hz), 4.05 (m, 1H), 7.59–7.64 (m, 4H), 7.86 (d, 1H, $J = 8.8$ Hz), 7.95 (d, 1H, $J = 1.6$ Hz), 8.29 (m, 2H), MS (ESI+) *m/z* (rel intensity): 295 (M+H, 100), Anal. for $C_{17}H_{18}N_4O{\cdot}HCl{\cdot}0{,}75H_2O$, Calcd. C, 59.29 H, 6.00 N, 16.27 Found C, 59.09 H, 6.24 N, 16.19.

*N-butyl-1-hydroxy-2-phenyl-1H-benzo[d]imidazole-6-carboxamidine HCl (**18**)*

Yield 55 %, mp 221–223 °C (bubbling), ^{1}H NMR (DMSO-d_6) δ: 0.95 (t, 3H, $J = 7.6$ Hz), 1.41 (m, 2H), 1.67 (m, 2H), 3.45 (q, 2H), 7.60–7.67 (m, 4H), 7.89 (d, 1H, $J = 8$ Hz), 8.04 (d, 1H, $J = 1.6$ Hz), 8.35 (m, 2H), 9.09 (s), 9.53 (s), 9.83 (s), MS (ESI+) m/z (rel intensity): 309 (M+H, 100), Anal. for $C_{18}H_{20}N_4O{\cdot}1HCl{\cdot}1{,}66H_2O$, Calcd. C, 57.69 H, 6.54 N, 14.95 Found C, 57.42 H, 6.36 N, 14.96.

*N-(2-dimethylamino)ethyl-1-hydroxy-2-phenyl-1H-benzo[d]imidazole-6-carboxamidine (**19**)*

Yield 53 %, mp 198–200 °C (bubbling), ^{1}H NMR (DMSO-d_6+D_2O) δ: 2.19 (s, 6H), 2.52 (t, 2H, $J = 6$ Hz), 3.44 (t, 2H, $J = 6$ Hz), 7.26 (dd, 1H, $J = 8.4$, $J = 2$ Hz), 7.35–7.39 (m, 3H), 7.47 (d, 1H, $J = 8.4$ Hz), 7.67 (s, 1H), 8.57 (d, 2H, $J = 7.2$ Hz), MS (ESI+) *m/z* (rel intensity): 324 (M+H, 100), Anal. for $C_{18}H_{21}N_5O{\cdot}1{,}75{\cdot}H_2O$, Calcd. C, 60.91 H, 6.96 N, 19.73 Found C, 60.77 H, 6.83 N, 19.34.

*N-benzyl-1-hydroxy-2-phenyl-1H-benzo[d]imidazole-6-carboxamidine HCl (**20**)*

Yield 30 %, mp 240–242 °C (bubbling), ^{1}H NMR (DMSO-d_6+D_2O) δ: 4.74 (s, 2H), 7.39–7.49 (m, 5H), 7.65 (m, 3H), 7.73 (dd, 1H, $J = 8.4$, $J = 1.6$ Hz), 7.92 (d, 1H, $J = 8.8$ Hz), 8.08 (d, 1H, $J = 2$ Hz), 8.28 (m, 2H), MS (ESI+) *m/z* (rel intensity): 343 (M+H, 100), Anal. for $C_{21}H_{18}N_4O{\cdot}2HCl{\cdot}1{,}75H_2O$, Calcd. C, 56.44 H, 5.30 N, 12.54 Found C, 56.18 H, 5.18 N, 12.82.

*N-benzyl-2-(2,4-dichlorophenyl)-1-hydroxy-1H-benzo[d]imidazole-6-carboxamidine (**21**)*

Yield 22 %, mp 279–280 °C (bubbling), ^{1}H NMR (DMSO-d_6+D_2O) δ: 4.67 (s, 2H), 7.36–7.67 (m, 11H), MS (ESI+) *m/z* (rel intensity): 411 (M+H, 100), 413 (M+H+2, 63), 415 (M+H+4, 12), Anal. for $C_{21}H_{16}Cl_2N_4O{\cdot}2HCl{\cdot}2{,}75H_2O$, Calcd. C, 47.25 H, 4.43 N, 10.50 Found C, 47.28 H, 4.16 N, 10.11.

*1-Hydroxy-N-isopropyl-2-(naphthalen-1-yl)-1H-benzo[d]imidazole-6-carboxamidine (**22**)*

Yield 24 %, mp 233–235 °C (bubbling), ^{1}H NMR (DMSO-d_6+D_2O) δ: 1.14 (d, 6H, J = 6 Hz), 3.82 (m, 1H), 7.21 (d, 1H, J = 8.8 Hz), 7.49–7.59 (m, 4H), 7.79 (s, 1H), 7.96–8.01 (m, 2H), 8.10 (d, 1H, J = 6.8 Hz), 8.35 (d, 1H, J = 7.6 Hz), MS (ESI+) *m/z* (rel intensity): 345 (M+H, 100), Anal. for $C_{21}H_{20}N_4O{\cdot}2HCl{\cdot}2H_2O$, Calcd. C, 55.63 H, 5.78 N, 12.35 Found C, 55.30 H, 6.02 N, 12.28.

*1-Hydroxy-N-isopentyl-2-(naphthalen-1-yl)-1H-benzo[d]imidazole-6-carboxamidine (**23**)*

Yield 21 %, mp 245–247 °C (bubbling), ^{1}H NMR (DMSO-d_6+D_2O) δ: 0.92 (d, 6H, J = 6.4 Hz), 1.53 (m, 2H), 1.67 (m, 1H), 3.28 (t, 2H, J = 7.2 Hz), 7.39 (dd, 1H, J = 8.4, J = 2 Hz), 7.47–7.57 (m, 3H), 7.66 (d, 1H, J = 8.8 Hz), 7.73 (s, 1H), 7.95 (d, 1H, J = 7.2 Hz), 7.99 (d, 1H, J = 8.4 Hz), 8.03 (d, 1H, J = 8.8 Hz), 8.22 (d, 1H, J = 8 Hz), MS (ESI+) *m/z* (rel intensity): 373 (M+H, 45), 208 (100), Anal. for $C_{23}H_{24}N_4O{\cdot}3HCl{\cdot}2{,}5H_2O$, Calcd. C, 52.43 H, 6.12 N, 10.63 Found C, 52.59 H, 6.00 N, 10.48.

*2-Phenyl-6-(1,4,5,6-tetrahydropyrimidin-2-yl)-1H-benzo[d]imidazol-1-ol (**24**)*

Yield 58 %, mp 255–257 °C, ^{1}H NMR (DMSO-d_6+D_2O) δ: 1.93 (m, 2H), 3.45 (m, 4H), 7.26 (dd, 1H, J = 8.4 Hz), 7.40–7.47 (m, 3H), 7.52 (d, 1H, J = 8.4 Hz), 7.75 (s, 1H), 8.65 (d, 2H, J = 6.4 Hz), MS (ESI+) *m/z* (rel intensity): 293 (M+H, 45), 188(100), Anal. for $C_{17}H_{16}N_4O{\cdot}2{,}75H_2O$, Calcd. C, 59.72 H, 6.33 N, 16.39 Found C, 59.51 H, 6.48 N, 16.13.

Biological tests

Cell culture

Human leukemia cell lines (HL-60, K562) were grown in RPMI 1640 supplemented with 10 % fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 mg/ml) in a 5 % CO_2 atmosphere at 37 °C. Sterile bovine serum was inactivated in 60 °C water bath for 30 min before preparing medium. Cells were counted with trypan blue to seed equal number of cells to each well of 6, 24 and 96 well plates.

Preparation of compounds

Compounds were dissolved in DMSO in a stock solution at a concentration of 20 mM, stored at −20 °C and protected from light. In each experiment DMSO concentration never exceeded 0.5 % and this percentage did not interfere with cell growth. Camptothecin was used as a positive control in all viability experiments.

Cell viability test/cytotoxicity test

MTT test was used to determine cell viability. Briefly, cells (4×10^4 cells/well) were seeded to 96-well plates. Cell proliferation assay was carried out using the Cell Proliferation Kit I [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; (MTT)] (Roche, Germany) as described by the manufacturer using cells treated at various concentrations (1, 25, 50, 75, 100 μM) of new synthesized benzimidazoles (compounds **15**–**23**) for 72 h. Mitochondrial succinate dehydrogenase of living cell reduces tetrazolium dye and constitutes formazan crystals which can be measured colorimetrically and correlate with number of living cells (Mosmann 1983). The spectrophotometric absorbance was measured using a microplate reader (Biotek, USA) at wavelength 550 nm with a reference wavelength of 690 nm. Number of viable cells was calculated from untreated cells, and the data were expressed as percent cell viability. All the experiments were conducted in triplicate and DMSO was used as negative control in corresponding concentrations.

Quantitative RT-PCR

Total RNA was extracted from HL-60 cell line by using the High Pure RNA kit (Roche, Mannheim, Germany) according to the manufacturer's protocols. cDNA was generated from RNA by reverse transcriptase (Transcriptor High Fidelity cDNA Synthesis Kit; Roche). Quantitative real-time RT-PCR was performed using SYBR Green PCR Master Mix (Roche) on LC480 instrument. mRNA was measured relative to HPRT as an endogenous control. Experiments were performed in biological triplicates. For primer sequences see Table 1.

Cell morphology determination

Cells (4×10^5 cells/well) were seeded to 6-well plates and treated with different concentrations of compounds for 72 h. After treatment protocol, cells were washed with PBS two times and then fixed with 1:3 volume of acetic acid/methanol. Cells on slides were then soaked into 1:20 diluted Giemsa solution for 15 min and then washed with distilled water and visualized under a microscope.

Annexin V–propidium iodide staining for apoptosis determination

HL-60 cells (4×10^5 cells/well) were seeded in 6 well-plates. Cells were incubated with different concentrations of

Table 1 Primer sequences used in RT-PCR

Genes	Primer Sequences	Products (bp)
HPRT	Forward: TGACACTGGCAAAACAATGCA	94
	Reverse: GGTCCTTTTCACCAGCAAGCT	
Atg5	Forward: TGGGCCATCAATCGGAAACTC	129
	Reverse: TGCAGCCACAGGACGAAACAG	
Atg7	Forward: GATCCGGGGATTTCTTTCACG	181
	Reverse: CAGCAATGTAAGACCAGTCAAGT	
Atg12	Forward: TCCGAGCCAGCGGCCTAACT	206
	Reverse: AAGGAGGCGCCGGAGTAGGG	
Beclin 1	Forward: AGCTGCCGTTATACTGTTCTG	185
	Reverse: ACTGCCTCCTGTGTCTTCAATCTT	
Bad	Forward: GATGAGTGACGAGTTTGTGGA	130
	Reverse: CAAGTTCCGATCCCACCAG	
Bax	Forward: GACGGCAACTTCAACTGGG	182
	Reverse: AGGAGTCTCACCCAACCAC	
Bim	Forward: ATCTCAGTGCAATGGCTTCC	111
	Reverse: CATAGTAAGCGTTAAACTCGTCTCC	
Bcl-2	Forward: CGCCCTGTGGATGACTGAGT	93
	Reverse: GGGCCGTACAGTTCCACAA	

compounds for 72 h. After all incubation period, cells were collected, washed with PBS two times and resuspended in binding buffer according to suppliers instructions (BD Biosciences). 5 µl FITC-Annexin V and 5 µl PI was added to 100 µl of cell suspension. After a brief vortex, cells were incubated for 15 min at room temperature. After this incubation, 400 µl 1 × binding buffer was added to cells and cells were measured in flow cytometry (Accuri C6).

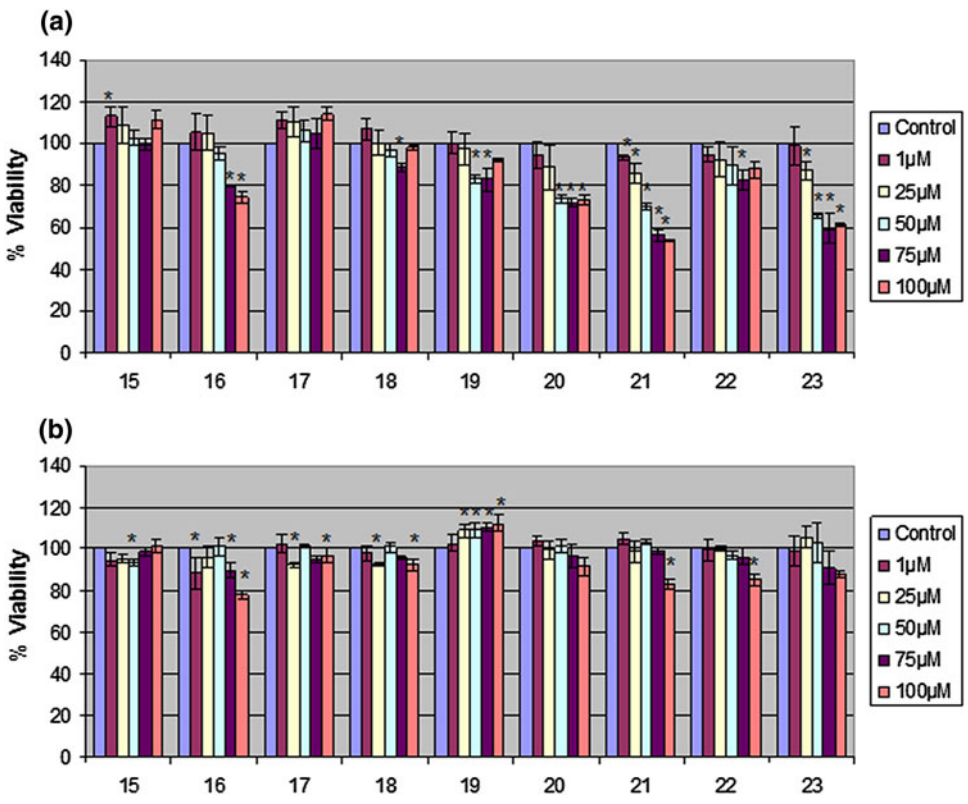


Fig. 2 **a** Cytotoxic activity results of compounds **15–23** against HL-60 cells **b** Cytotoxic activity results of compounds **15–23** against K562 cells Experiments were conducted in triplicate. Error bars indicate ±s.d.,* significantly different ($p < 0.05$) from control. Anticancer activities of compound **24** could not be determined due to solubility problem in DMSO

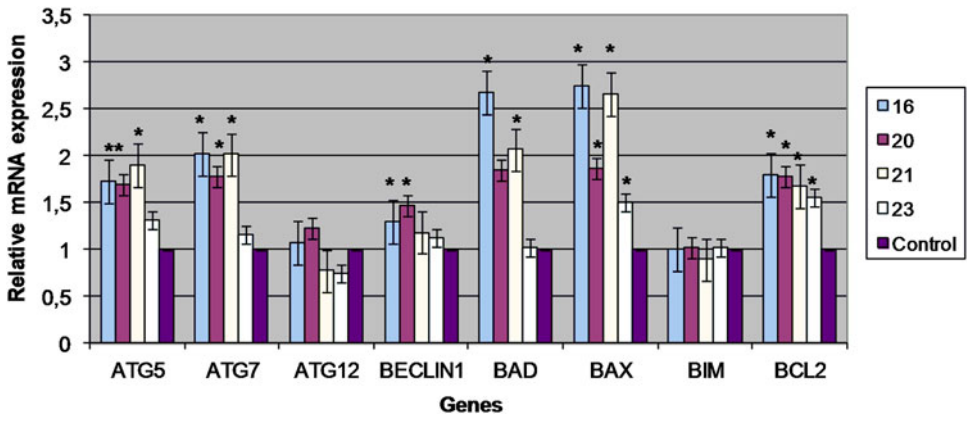


Fig. 3 Relative gene expressions of compounds **16**, **20**, **21** and **23** at 100 μM concentration. Experiments were conducted in triplicate. Error bars indicate ±s.d.,* significantly different ($p < 0.05$) from control

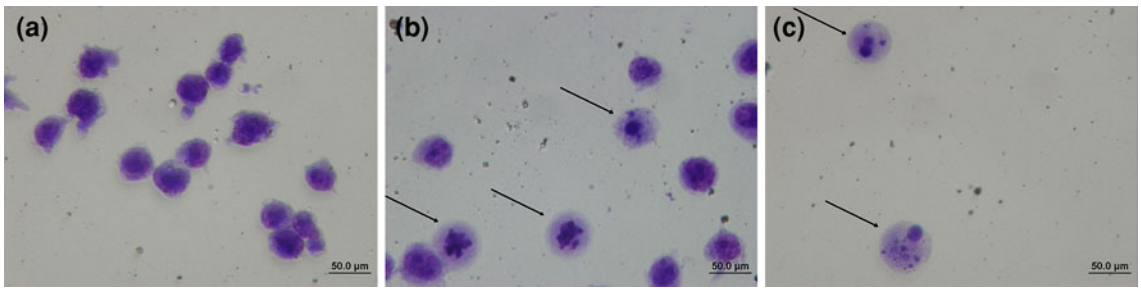
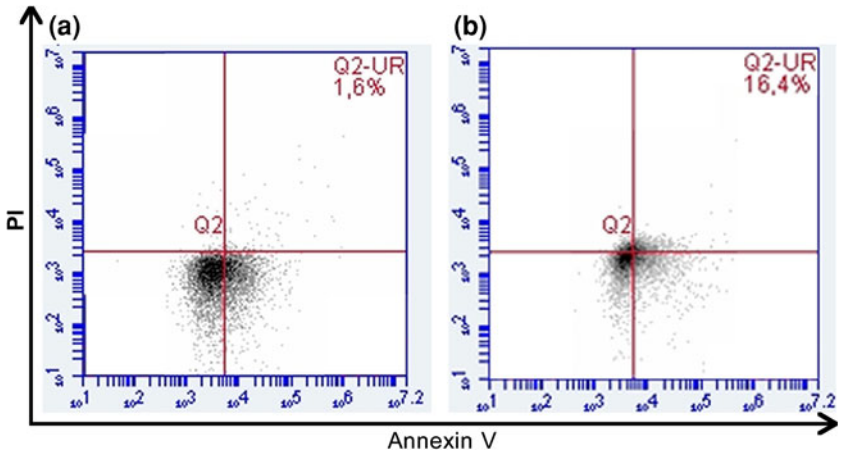


Fig. 4 Giemsa staining for apoptotic cells. **a** Control **b** Compound **21** (100 μM) **c** Camptothecin (1 μM)

Fig. 5 Annexin V/PI staining of HL-60 cells. **a** Control **b** Compound **21** (100 μM)



Results and discussion

The synthetic pathway for the preparation of the targeted benzimidazoles **15–24** are shown in Scheme 1. Nucleophilic displacement of the chloro group of 4-chloro-3-nitrobenzonitrile, by the reaction with several amines in DMF gave **1–4**. The nitrile group of **1–4** was converted to imidate esters (they were not characterized because of instability), with dry HCl gas in absolute ethanol, by Pinner reaction, and then treatment of the imidate esters with several amines in ethanol gave **5–14**. Cyclocondensation reaction of these compounds in DMF in the presence of NaH afforded the corresponding benzimidazoles, **15–24**.

The novel *N*-substituted amidino-1-hydroxybenzimidazole derivatives (**15–24**) were investigated for their in vitro anticancer activities in leukemia cell lines, HL-60 and K562, using MTT assay, flow cytometry and RT-PCR. Cell viability test results were reported in Fig. 2. Compounds **16, 20, 21** and **23** exhibited remarkable cytotoxic activity against HL-60 cell line. Cell viability for these compounds at 100 μM concentration were determined as 74, 73, 54 and 61 %, respectively when compared with the control group. *N*-isopropyl (**17**) or *N*–*n*-butyl (**18**) substitution of amidino group decreased anticancer activity. Compound **21** which has electron-withdrawing halogens substituted on phenyl ring was the

most cytotoxic derivative in this series. Most of the compounds displayed higher cytotoxicity to HL-60 cells than K562 cells. Expressions of apoptotic and autophagic genes were investigated to test the mechanism of cytotoxicity in HL-60 cell line. Apoptosis (type 1 cell death), which is activated either through intrinsic (mitochondrial) or extrinsic (death receptor) pathway is defined as a major type of programmed cell death (Okada and Mak 2004). Morphological changes such as membrane blebbing, cell membrane asymmetry loss, chromatin condensation and DNA fragmentation are among characteristics of apoptosis (Danial and Korsmeyer 2004). Autophagy (type 2 cell death), an intracellular degradation process which includes transport of cytoplasmic constituents to lysosomes is thought to play roles in cell growth, development and energy balance. Autophagy is also considered as a mechanism of cancer cell suicide activated by chemotherapy or radiation (Kuma et al. 2004). Since apoptosis and autophagy are the two major cell death signalling pathways, chemical agents interfering these two pathways may have the potential to possess anticancer activity. Therefore, we investigated the effects of compounds **16**, **20**, **21** and **23** on the expression of genes related to autophagy (Atg5, Atg7, Atg12, Beclin-1) and apoptosis (Bad, Bim, Bax, Bcl-2) (Fig. 3). According to expression results of autophagic genes, compounds **16** and **20** increased expressions of Atg5, Atg7 and Beclin 1 and compound **21** increased expressions of Atg5 and Atg7. We could not find any significant change in expression of Atg12. Compounds **16**, **20**, **21** and **23** increased expression of proapoptotic Bax and also increased antiapoptotic Bcl-2 but did not exert any effect on Bim expression. Compounds **16** and **21** enhanced proapoptotic bad expression. Compound **21** induced the highest gene expression ratio of Bax/Bcl-2. Compound **21** was shown to induce apoptotic morphology of HL-60 cells (Fig. 4). Compound **21** exhibited 16.4 % more late apoptotic profile than control group (Fig. 5). Considering the expression changes of apoptotic and autophagic genes, it is thought that apoptosis and autophagy play important role in cytotoxic activity of compound **21**. Late apoptosis rates of cells treated with compounds **16**, **20** and **23** were found as 3.1, 2.3 and 4.3 % respectively. Increased Atg5 and Atg7 expressions and low late apoptotic rate of cells treated with compounds **16** and **20** implied that autophagy played more important role than apoptosis in cytotoxic activity of these compounds.

These results indicate that some of the *N*-substituted amidino-1-hydroxybenzimidazole derivatives have cytotoxic activity in HL-60 cell line. More active derivatives against AML can be obtained by chemical modification of these compounds.

Acknowledgments Central Instrumental Analysis Lab in Faculty of Pharmacy, Ankara University provided the support for acquisition of the NMR, Mass and Elemental analysis data used in this work.

References

Apted, F.I. 1980. Present status of chemotherapy and chemoprophylaxis of human trypanosomiasis in the Eastern Hemisphere. *Pharmacology & Therapeutics* 11: 391–413.

Ates-Alagoz, Z., M. Alp, C. Kus, S. Yildiz, E. Buyukbingol, and H. Goker. 2006. Synthesis and potent antimicrobial activities of some novel retinoidal monocationic benzimidazoles. *Archiv der Pharmazie* 339: 74–80.

Bowser, T.E., V.J. Bartlett, M.C. Grier, A.K. Verma, T. Warchol, S.B. Levy, and M.N. Alekshun. 2007. Novel anti-infection agents: Small-molecule inhibitors of bacterial transcription factors. *Bioorganic & Medicinal Chemistry Letters* 17: 5652–5655.

Bryceson, A.D., J.D. Chulay, M. Mugambi, J.B. Were, G. Gachihi, C.N. Chunge, R. Muigai, S.M. Bhatt, M. Ho, H.C. Spencer, J. Meme, and G. Anabwani. 1985. Visceral leishmaniasis unresponsive to antimonial drugs. II. Response to high dosage sodium stibogluconate or prolonged treatment with pentamidine. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 79: 705–714.

Danial, N.N., and S.J. Korsmeyer. 2004. Cell Death: Critical Control Points. *Cell* 116: 205–219.

Gowda, N.R.T., C.V. Kavitha, K.K. Chiruvella, O. Joy, K.S. Rangappa, and S.C. Raghavan. 2009. Synthesis and biological evaluation of novel 1-(4-methoxyphenethyl)-1*H*-benzimidazole-5-carboxylic acid derivatives and their precursors as antileukemic agents. *Bioorganic & Medicinal Chemistry Letters* 19: 4594–4600.

Göker, H., M. Alp, and S. Yildiz. 2005a. Synthesis and potent antimicrobial activity of some novel N-(alkyl)-2-phenyl-1*H*-benzimidazole-5-carboxamidines. *Molecules* 10: 1377–1386.

Göker, H., C. Kuş, D.W. Boykin, S. Yildiz, and N. Altanlar. 2002. Synthesis of some new 2-substituted-phenyl-1*H*-benzimidazole-5-carbonitriles and their potent activity against Candida species. *Bioorganic & Medicinal Chemistry* 10: 2589–2596.

Göker, H., S. Özden, S. Yildiz, and D.W. Boykin. 2005b. Synthesis and potent antibacterial activity against MRSA of some novel 1,2-disubstituted-1*H*-benzimidazole-N-alkylated-5-carboxamidines. *European Journal of Medicinal Chemistry* 40: 1062–1069.

Ivady, G., and L. Paldy. 1958. Ein neuseus behandlungsverfahren der interstitiellen plasmazelligan pneumonie fruhgeborener mit funfwertigem stibium und aromatischen diamidinen. *Monatsschrift fur Kinderheilkunde* 106: 10–14.

Karpinska, M.M., J. Matysiak, and A. Niewiadomy. 2011. Synthesis of novel 4-(1*H*-benzimidazol-2-yl)benzene-1,3-diols and their cytotoxic activity against human cancer cell lines. *Archives of Pharmacal Research* 34: 1639–1647.

Kuma, A., M. Hatano, M. Matsui, A. Yamamoto, H. Nakaya, T. Yoshimori, Y. Ohsumi, T. Tokuhisa, and N. Mizushima. 2004. The role of autophagy during the early neonatal starvation period. *Nature* 432: 1032–1036.

Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods* 65: 55–63.

Okada, H., and T.W. Mak. 2004. Pathways of apoptotic and non-apoptotic death in tumour cells. *Nature Reviews Cancer* 4: 592–603.

Pathak, M.K., D. Dhawan, D.J. Lindner, E.C. Borden, C. Farver, and T. Yi. 2002. Pentamidine is an inhibitor of PRL phosphatases

with anticancer activity. *Molecular Cancer Therapeutics* 1: 1255–1264.

Qiu, G., J. Jiang, and X.S. Liu. 2012. Pentamidine sensitizes chronic myelogenous leukemia K562 cells to TRAIL-induced apoptosis. *Leukemia Research* 36: 1417–1421.

Romero-Castro, A., I. Leon-Rivera, L.C. Avila-Rojas, G. Navarrete-Vazquez, and A. Nieto-Rodriguez. 2011. Synthesis and preliminary evaluation of selected 2-aryl-5(6)-nitro-1*H*-benzimidazole derivatives as potential anticancer agents. *Archives of Pharmacal Research* 34: 181–189.

Starcevic, K., M. Kralj, K. Ester, I. Sabol, M. Grce, K. Pavelic, and G. Karminski-Zamola. 2007. Synthesis, antiviral and antitumor activity of 2-substituted-5-amidino-benzimidazoles. *Bioorganic & Medicinal Chemistry* 15: 4419–4426.

Tzifi, F., C. Economopoulou, D. Gourgiotis, A. Ardavanis, S. Papageorgiou, and A. Scorilas. 2012. The role of BCL2 family of apoptosis regulator proteins in acute and chronic leukemias. *Advances in Hematology* 2012: 524308.

Wirawan, E., T. Vanden Berghe, S. Lippens, P. Agostinis, and P. Vandenabeele. 2012. Autophagy: For better or for worse. *Cell Research* 22: 43–61.