Bioorganic & Medicinal Chemistry Letters 22 (2012) 6297–6300

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Newly synthesized bis-benzimidazole derivatives exerting anti-tumor activity through induction of apoptosis and autophagy

Xiu-Jun Wang [a], Na-Ying Chu [b], Qin-He Wang [a], Chao Liu [a], Chun-guo Jiang [a], Xiao-Yu Wang [a], Takashi Ikejima [b,*], Mao-Sheng Cheng [a,*]

[a] Key Laboratory of Structure-Based Drugs Design & Discovery of Ministry of Education, School of Pharmaceutical Engineering, Shenyang Pharmaceutical University, Shenyang 110016, PR China

[b] China-Japan Research Institute of Medical and Pharmaceutical Sciences, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang 110016, PR China

ARTICLE INFO

Article history:
Received 30 April 2012
Revised 21 June 2012
Accepted 29 June 2012
Available online 7 July 2012

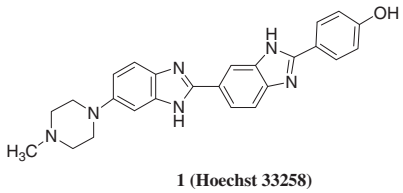
Keywords:
Bis-benzimidazole
Anti-tumor
Apoptosis
Autophagy
PBMC

ABSTRACT

In this study, a new series of bis-benzimidazole derivatives were designed and synthesized. Most of these new compounds showed significant anti-tumor activity in vitro compared to Hoechst 33258. Among them, the most potent compound **8** had the IC_{50} values of 0.56 μM for HL60 (Human promyelocytic leukemia cells) tumor cell line and 0.58 μM for U937 (Human leukemic monocyte lymphoma cells) tumor cell line. Subsequent toxicity study on human peripheral blood mononuclear cells (PBMC) showed that compound **8** exhibited less toxicity than 5-FU. We also found that apoptosis and autophagy were simultaneously induced by compound **8** in HL60 cells, and inhibition of autophagy by 3-MA decreased compound **8**-induced apoptosis, indicating that they acted in synergy to exert tumor cell death.

Apoptosis (type I cell death) and autophagy (type II cell death) are maior types of programmed cell death.[1] Apoptosis (type I cell death) and autophagy (type II cell death) are maior types of programmed cell death. Apoptosis is induced either through mitochondrial (intrinsic) pathway or death receptor (extrinsic) pathway in multicellular organisms.[2] It is characterized by a series of morphological changes including membrane blebbing, loss of cell membrane asymmetry and attachment, cell shrinkage, chromatin condensation and chromosomal DNA fragmentation and others.[3] Deregulation of apoptosis is a hallmark of all cancer cells, and the agents that activate apoptosis in cancer cells could be used as valuable anti-cancer therapeutics.[4] As type II programmed cell death, autophagy is an intracellular degradation system that delivers cytoplasmic constituents to the lysosome. It is involved in cell growth, development and homeostasis, helping to maintain a balance between synthesis and degradation. Autophagy is not only a survival response to either growth factor or nutrient deprivation but also a mechanism for tumor cell suicide induced by chemotherapy or radiation.[5] Recent studies have demonstrated that the co-regulation of both apoptosis and autophagy may participate in mammalian cell death.[6]

Bis-benzimidazole derivatives are one of the most extensively studied classes of heterocyclic compounds. They have been proven to be potent anti-tumor agents via DNA minor groove binding. A representative compounds, Hoechst 33258, is listed in Scheme 1 Hoechst 33258[7], a fluorescent compound with a head-to-tail bis-benzimidazole structure, was initially found to be cytotoxic against L1210 murine leukemia. Phase I clinical trials of this compound showed positive pancreatic cancer activity. However, a subsequent phase II clinical trial failed to yield any objective results.[8] Due to the synthetic accessibility and high binding affinity of Hoechst 33258, several groups have focused on a strategy to utilize the pharmacophore-like benzimidazole motif derived from Hoechst 33258.[9–12] In 2011, Singh et al. synthesized several 2-aryl-5-substituted-2,5-bisbenzimidazole derivatives and evaluated their antitumor activity in vitro.[13]



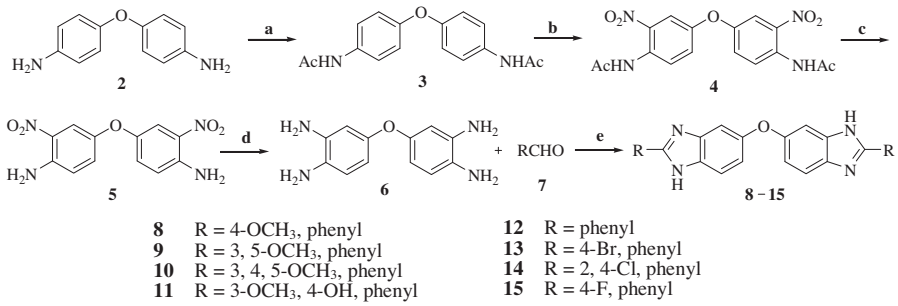
1 (Hoechst 33258)

Scheme 1. Chemical structures of Hoechst 33258.

* Corresponding authors. Fax: +86 24 23844463 (T.I.); fax: +86 24 23995043 (M.-S.C.).

E-mail addresses: ikejimat@vip.sina.com (T. Ikejima), mscheng@syphu.edu.cn (M.-S. Cheng).

0960-894X/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmcl.2012.06.102



8 R = 4-OCH_3, phenyl
9 R = 3, 5-OCH_3, phenyl
10 R = 3, 4, 5-OCH_3, phenyl
11 R = 3-OCH_3, 4-OH, phenyl
12 R = phenyl
13 R = 4-Br, phenyl
14 R = 2, 4-Cl, phenyl
15 R = 4-F, phenyl

Scheme 2. Reagents and conditions: (a) $(CH_3CO)_2O/NEt_3$, acetone, 0 °C, 2 h, 98%; (b) Conc. HNO_3, acetic acid, 0–50 °C, 5 h, 98%; (c) 42% KOH aq, MeOH, reflux, 2 h, 97%; (d) Pd-C, H_2, MeOH, rt, 6 h, 89%; (e) $NaHSO_3$, methanol, rt, 8 h, 54–93%.

Table 1
The anti-tumor activities of the compounds (**8**–**15**)

Compound	IC_{50} (μM)		
	HeLa	HL60	U937
8	4.96 ± 0.53	0.56 ± 0.04	0.58 ± 0.06
9	8.59 ± 0.88	6.35 ± 1.03	7.09 ± 1.20
10	9.37 ± 1.67	7.56 ± 0.94	6.92 ± 0.65
11	10.61 ± 1.13	8.17 ± 0.71	5.60 ± 0.53
12	18.35 ± 2.16	16.47 ± 2.01	17.11 ± 3.64
13	20.39 ± 3.78	17.22 ± 1.37	21.67 ± 2.15
14	17.24 ± 2.19	15.78 ± 1.96	20.52 ± 2.68
15	22.76 ± 2.72	23.19 ± 2.47	22.63 ± 3.50
H. 33258	51.31 ± 4.56	32.43 ± 3.27	15.42 ± 2.16
5-FU	0.28 ± 0.03	0.06 ± 0.01	1.07 ± 0.08
Paclitaxel	2.93 ± 0.61 (nM)	2.82 ± 0.41 (nM)	3.37 ± 0.89 (nM)

The data were the mean ± SD obtained from three independent experiments.
5-Fluorouracil and Paclitaxel were used as the positive control.

To our knowledge, most derivatives of Hoechst 33258 kept whole molecules to planar. In 2009, Hu et al. synthesized and evaluated the antibacterial activities of a new series of bisbenzimidazole derivatives with various two atom or one-atom as a linker.[14] Alpan et al. synthesized a new series of bis-benzimidazole derivatives with alkyl chain as a linker which were found to be cytotoxic against MCF7 and A431 cells.[15] No literature related adding an oxygen atom between two benzimidazoles has been reported so far. In this study, we combined two benzimidazole derivatives into one molecule with an oxygen atom. All of these compounds were screened for anti-tumor activity in vitro. Among them, the most compound **8** had the good activity for three tumor cell lines (HeLa, HL60 and U937). We also compared the toxic effects of compound **8** and 5-FU on human PBMC. The result indicated that compound **8** had lower toxicity than 5-FU.

In the subsequent study, inhibition of autophagy by 3-MA promoted compound **8**-induced apoptosis, indicating that compound **8** could induce autophagy and apoptosis simultaneously and these PCD both exerted HL60 cells death.

Compound **8**–**15** were achieved with an efficient synthetic route (Scheme 2). Protection of the amino group of commercially available 4,4′-diaminodiphenyl ether with acetic anhydride at 0 °C gave 4,4′-diacetamido-diphenyl (**3**). Nitration of compound **3** with nitric acid in acetic acid formed compound **4**. Treatment of compound **4** with a solution of potassium hydroxide in water and ethanol gave 3,3′-dinitro-4,4′-diaminodiphenyl ether (**5**), followed by reduction of the nitro group to afford 3,3′, 4,4′-tetraaminodiphenyl ether (**6**). Treatment of compound **6** with various substituted benzaldehyde in methanol at reflux gave the desired compounds.

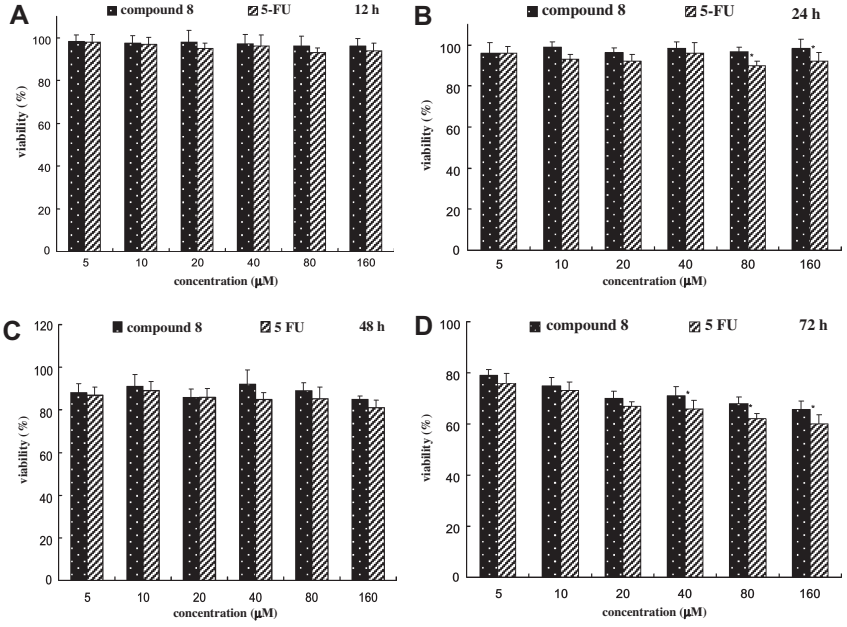


Figure 1. Effects of compound **8** and 5-FU on human PBMC cells. The PBMC were obtained from three healthy adult volunteers and were seeded on 96-well culture plates at a density of 5×10^5 cells/well. The cells were cultured for 12, 24, 48 and 72 h with various doses of compound **8**, 5-FU. n=3, mean ± SD. $^*p < 0.05$, comparison with 5-FU.

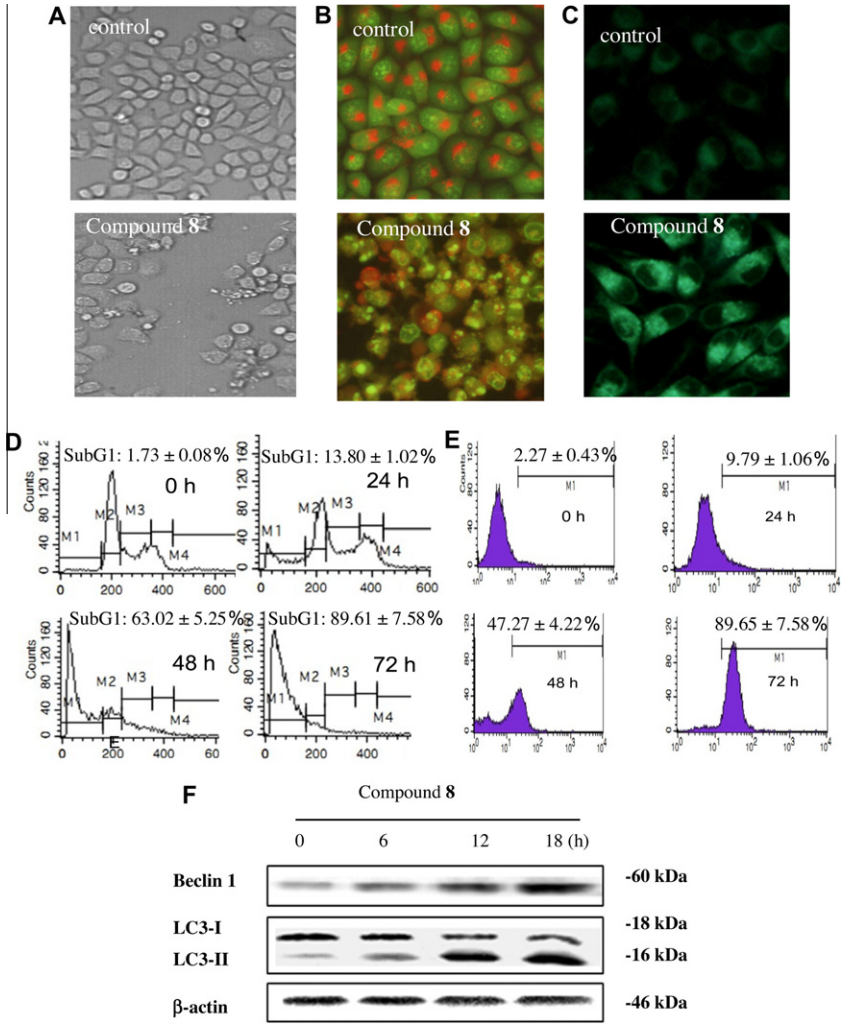


Figure 2. (A) The morphologic changes of compound **8**-treated cells were observed. (B) The apoptosis changes were observed under a fluorescence microscope by AO staining. (C) The autophagic changes were observed under a fluorescence microscope by MDC staining. (D) Flow cytometric analysis of compound **8**-induced apoptosis (SubG1 fraction). (E) The cells were treated with 0.5 μmol L^{-1} compound **8** for 0, 24, 48 or 72 h, respectively. The MDC fluorescent intensity was analyzed by flow cytometry. (F) The cells were treated with 0.5 μmol L^{-1} compound **8** for indicated time period, followed by Western blot analysis for detection of Beclin-1 and LC3 levels. β-actin was used as an equal loading control.

All the newly synthesized compounds were investigated for their anti-tumor activities in three cancer cell lines using MTT assay.[16] In respect to the in vitro cytotoxic activities listed in Table 1, almost all of the synthesized compounds exhibited high anti-tumor activity when compared with known DNA minor groove agent Hoechst 33258 in Table 1. As shown in Table 1, compounds **8**–**11** with electronic-donating substituents (methoxy group) on the benzene ring showed more potent anti-tumor activities than compounds **13**–**15**, which only contains electronic-withdrawing halogen substituents (Cl, F or Br) on benzene ring. Compounds **8**–**11** showed low cytotoxicity at concentration of 10 μM while compounds **13**–**15** were less potent with IC_{50} values more than 10 μM. Among them, compound **8** was most potent with IC_{50} values of 0.56 μM for HL60 tumor cell line and 0.58 μM for U937 tumor cell line. The standard drugs used for comparison were 5-FU and paclitaxel. In terms of U937 tumor cell line, compound 8 showed similar anticancer activity with 5-FU. On the other hand, 5-FU is widely used as a clinical anti-tumor drugs.[17] Its low PBMC toxicity was an important indicator for its use. By now, bis-benzimidazole compounds have no yet went to the clinicals. So we compared compound **8**'s PBMC toxicity with that of 5-FU (Fig. 1).[18]

When human peripheral blood mononuclear cells (PBMC) were cultured with 5 μM compound **8** at 12, 24, 48 and 72 h, the viabilities of PBMC were about 98%, 96%, 88% and 79%, respectively. In the case of 5-FU (5 μM) were about 98%, 94%, 87% and 76%, respectively. In other concentrations, the viabilities of compound **8** were also higher than 5-FU. Especially, when human peripheral blood mononuclear cells (PBMC) were cultured with 160 μM compound **8** at 24 and 72 h, compound **8** has lower toxicity to normal PBMC from healthy volunteers than 5-FU with a significant level (p <0.05).

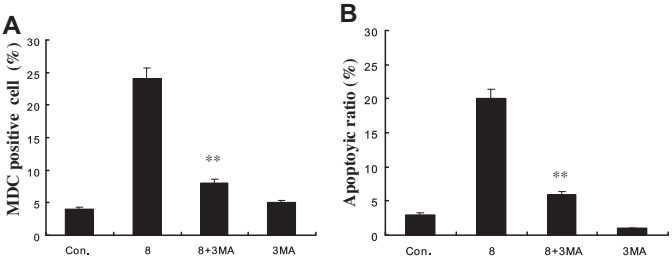


Figure 3. Effects of 3-MA on compound **8**-induced autophagy and apoptosis in HL60 cells. The cells were treated with 0.5 μM compound **8** for 24 h in the presence or absence of 1 mM 3-MA, the autophagic ratio (A) and the apoptotic ratio (B) were measured by flowcytometric analysis. n = 3, mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

To determine the characteristics of HL60 cell death, we examined the morphological changes and DNA fragmentation of the compound **8** treated cells. When the cells were cultured with 0.5 μM compound **8** for 24, 48 and 72 h, marked morphological changes were observed as compared with the untreated control. Compound **8**-treated HL60 cells underwent retraction of cellular processes and became round in shape at 24 h. By 48 h, the majority of HL60 cells had become round, with shrunken nuclei. Some of these cells showed membrane blebbing and the nuclei were fragmented into apoptotic bodies. Eventually, dead cells became non-adherent. Untreated cells did not show these apoptotic characteristics (Fig. 2A). The quantitative analysis of apoptosis by flow-cytometry also showed that compound **8** induced a significant time-dependent increase in the percentage of subG1 cells (Fig. 2D). When we stained the HL60 cells with acridine orange, the cells in control medium were stained homogeneously with AO, whereas treatment with compound **8** resulted in marked chromatin condensation and nuclear fragmentation in HL60 cells, a hallmark of apoptosis (Fig. 2B).

Autophagy has been reported to co-exist with apoptosis in many experimental systems,[19–21] therefore we tried to examine the effect of compound **8** on autophagy induction. The formation of autophagic vacuoles was assessed by staining cells with autophagosomal fluorescent dye MDC (monodansylcadaverine). As shown in Figure 2C, treatment with compound **8** induced marked increase in the number of MDC-labeled fluorescent particles in HL60 cells compared to control cells. For quantitative analysis of autophagy, the MDC fluorescent intensity of compound **8** treated cells for the indicated time periods was analyzed by flow-cytometry. As shown in Figure 2E, compound **8** induced HL60 cell autophagy in a time-dependent manner. Accordingly, the expression level of Beclin 1 and the conversion from LC3-I to LC3-II, as autophagic markers, were increased with time after administration of compound **8** (Figure 2F), suggesting that compound **8** induces autophagy as well as apoptosis in HL60 cells.

We further investigated the role of autophagy in compound **8**-induced apoptosis in HL60 cells. Treatment with 3-MA (a specific autophagy inhibitor), prior to the addition of compound **8**, significantly decreased the percentage of MDC-positive cells as well as the apoptotic ratio compared with the group treated with compound **8** alone (Fig. 3A and B), indicating that autophagy played a apoptosis-promoting role in compound **8**-treated HL60 cells. All these results demonstrated that compound **8** induced both apoptosis and autophagy in HL60 cells.

For the first time, we found that bis-benzimidazole derivatives could induced both autophagy and apoptosis in cancer cells. Such dual properties have never been reported for bis-benzimidazole derivatives as far as we know. We also found that inhibition of autophagy by 3-MA decreased compound **8**-induced apoptosis, indicating that they act together to exert cell death.

In conclusion, this is the first report focused on adding an oxygen atom between two benzimidazoles as novel anti-tumor agents. This work enriched the structural types of bis-benzimidazole. On the other hand, compound **8** exerted a strong anti-tumor effect and had a lower toxity on PBMC in comparison to paclitaxel and 5-FU. Thus, compound **8** can be considered to become an appropriate lead compound to develop more potent agents with anti-tumor promoting activity for clinical use.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.06.102. These data include MOL files and InChiKeys of the most important compounds described in this article.

References and notes

1. Savill, J.; Fadok, V. *Nature* **2000**, *407*, 784.
2. Okada, H.; Mak, T. W. *Nat. Rev. Cancer* **2004**, *4*, 592.
3. Danial, N. N.; Korsmeyer, S. J. *Cell* **2004**, *116*, 205.
4. Debatin, K. M. *Adv. Exp. Med. Biol.* **1999**, *457*, 237.
5. Kuma, A.; Hatano, M.; Matsui, M.; Yamamoto, A.; Nakaya, H.; Yoshimori, T.; Ohsumi, Y.; Tokuhisa, T.; Mizushima, N. *Nature* **2004**, *432*, 1032.
6. Paglin, S.; Hollister, T.; Delohery, T.; Hackett, N.; McMahill, M.; Sphicas, E.; Domingo, D.; Yahalom, J. *Cancer Res.* **2001**, *61*, 439.
7. Reddy, B. S.; Sondhi, S. M.; Lown, J. W. *Pharmacol. Ther.* **1999**, *84*, 1.
8. Baraldi, P. G.; Bovero, A.; Fruttarolo, F.; Preti, D.; Tabrizi, M. A.; Pavani, M. G.; Romagnoli, R. *Med. Res. Rev.* **2004**, *24*, 475.
9. Mann, J.; Baron, A.; Opoku-Boahen, Y.; Johansson, E.; Parkinson, G.; Kelland, L. R.; Neidle, S. *J. Med. Chem.* **2001**, *44*, 138.
10. Neidle, S. *Nat. Prod. Rep.* **2001**, *18*, 291.
11. Singh, M.; Tandon, V. *Eur. J. Med. Chem.* **2011,** *46*, 659.
12. Hosamani, K. M.; Shingalapur, R. V. *Arch Pharm (Weinheim)* **2011**, *344*, 311.
13. Singh, M.; Tandon, V. *Eur. J. Med. Chem.* **2011**, *46*, 659.
14. Hu, L.; Kully, M. L.; Boykin, D. W.; Abood, N. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 3374.
15. Alpan, A. S.; Zencir, S.; Zupko, I.; Coban, G.; Rethy, B.; Gunes, H. S.; Topcu, Z. *J. Enzyme Inhib. Med. Chem.* **2009**, *24*, 844.
16. Zhou, W. J.; Deng, R.; Zhang, X. Y.; Feng, G. K.; Gu, L. Q.; Zhu, X. F. *Mol. Cancer Ther.* **2009**, *8*, 3203.
17. Lu, D. Y.; Huang, M.; Xu, C. H.; Yang, W. Y.; Hu, C. X.; Lin, L. P.; Tong, L. J.; Li, M. H.; Lu, W.; Zhang, X. W.; Ding, J. *BMC Pharmacol.* **2005**, *5*, 11.
18. Fei, X. F.; Wang, B. X.; Li, T. J.; Tashiro, S.; Minami, M.; Xing, D. J.; Ikejima, T. *Cancer Sci.* **2003**, *94*, 92.
19. Cheng, Y.; Qiu, F.; Ikejima, T. *Autophagy* **2009**, *5*, 430.
20. Tan, C.; Lai, S.; Wu, S.; Hu, S.; Zhou, L.; Chen, Y.; Wang, M.; Zhu, Y.; Lian, W.; Peng, W.; Ji, L.; Xu, A. *J. Med. Chem.* **2010**, *53*, 7613.
21. Zhang, Y.; Wu, Y.; Wu, D.; Tashiro, S.; Onodera, S.; Ikejima, T. *Arch. Biochem. Biophys.* **2009**, *489*, 25.