B1, a novel amonafide analogue, overcomes the resistance conferred by Bcl-2 in human
premyelocytic leukemia HL60 cells

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Abbreviations:

B1, N-2-(Dimethylamino)ethyl)-2-aminothiazonaphthalimide; DMSO, dimethyl sulfoxide; MTT,
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI, propidium iodide; BCIP,
5-bromo-4-chloro-3-indolyl-phosphate; NBT, nitro blue tetrazolium; PMSF,
phenylmethylsulfonyl fluoride

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Abstract

In the course of screening for novel anticancer compounds, B1 (N-(2-(Dimethylamino)ethyl)-2-aminothiazonaphthalimide), a novel amonafide analogue, was generated as a new anticancer candidate. In the present study, B1 displayed stronger antitumor effects than amonafide in HL60 cells. We further examined whether B1 overcomes the resistance conferred by Bcl-2, since overcoming the resistance conferred by Bcl-2 represents an attractive therapeutic strategy against cancer. Our viability assay showed that B1 overcomes the resistance conferred by Bcl-2 in human premyelocytic leukemia HL60 cells. Various apoptosis assessment assays demonstrated that B1 overcomes the resistance conferred by Bcl-2 in HL60 cells by inducing apoptosis. Noticeably, we elucidated the marked downregulation of 14-3-3σ protein by B1, indicating that B1 overcomes the resistance conferred by Bcl-2 in HL60 cells via 14-3-3σ.

Analysis of ChIP assay indicated that MBD2 was associated with the methylated 14-3-3σ promoter-associated CpG Island, and thus, interfered with transcriptional activity of the methylated promoter. Furthermore, knock-down of MBD2 using siRNA transfection inhibited B1-induced apoptosis and overcome the resistance conferred by Bcl-2. Accordingly, these data demonstrated the involvement of MBD2 in B1-induced apoptosis and overcome the resistance conferred by Bcl-2, which suggested that MBD2 might guide the development of future anticancer agents.

Keywords: B1; Leukemia; Apoptosis; Bcl-2; MBD2
1. Introduction

Many antitumor agents, which interact with different targets, can kill chemosensitive leukemic cells via an apoptotic process [1,2]. Apoptosis is a genetically encoded cell death program characterized by distinct set of morphological and biochemical changes [3-5]. The apoptotic pathway, in general, can be affected by different factors such as the tumor cell type, differentiation status, growth factors, or oncogenes [4]. Several genes have been identified as either inducers or repressors of apoptosis. Bcl-2 functions as an anti-apoptotic protein [6]. Bcl-2, in general, regulates mitochondrial outer membrane permeabilization and thereby determines the cellular commitment to apoptosis [6-8]. The anti-apoptotic function of the Bcl-2 protein depends, at least in part, on its ability to dimerize with another member of the Bcl-2 family, Bax [9].

Overexpression of Bcl-2 has been reported in a wide variety of cancers.

In some preclinical systems, Bcl-2 overexpression has been shown to induce survival, or to restore the clonogenic potential of malignant progenitor cells [10,11]. On the other hand, anti-apoptotic factors, including Bcl-2, impair the ability to achieve remission and cure with chemotherapy, protecting the tumor cells from the apoptotic effects of various anti-neoplastic agents [12–14]. Therefore, overcoming the resistance conferred by anti-apoptotic factors such as Bcl-2 represents an attractive therapeutic strategy against leukemia cells [8].

Amonafide, a naphthalimide derivative, although selected for exploratory clinical trials for its potent anticancer activity, has long been challenged by its unpredictable side effects. In the course
of screening a series of amonafide analogues, B1

(N-(2-(Dimethylamino)ethyl)-2-aminothiazonaphthalimide) in this series that significantly caused
damage on DNA and cancer cell growth was identified [15]. Moreover, It has been reported that
B1 induced cell cycle arrest and apoptosis in HeLa cells via p53 activation [16].

In the present study, we have found that B1 induces p53-independent apoptosis and overcomes
the resistance conferred by Bcl-2 in HL60 (p53 null) cells through MBD2.
2. Materials and methods

2.1. Materials

B1 (N-(2-(Dimethylamino)ethyl)-2-aminothiazonaphthalimide) and amonafide (>99% pure) were synthesized and provided as described previously [15]. A 25 mM stock solution of B1 was prepared with dimethyl sulfoxide (DMSO) and freshly diluted in culture media for all *in vitro* experiments. The control cells were treated with the same amount of vehicle alone. The final DMSO concentration never exceeded 0.1% (v/v), in either control or treated samples.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst33258 and propidium iodide (PI) and 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) were from Sigma Chemical Co. (St. Louis, MO); human reactive monoclonal antibodies anti-14-3-3σ, anti-Caspase 3, anti-PARP, anti-Bcl-2, anti-Bad, anti-p-bad at Ser155, and anti-β-actin were from Cell signaling Technology (Beverly, MA).

2.2. Cell culture and establishment of Bcl-2-overexpressing HL60 cells

HL60 human premelocytic leukemia cells, K562 human chronic myelogenous leukemia cells and U937 human myelomonocytic leukemia cells were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The culture medium used...
throughout these experiments was RPMI-1640 medium, containing 10% heat-inactivated bovine serum, penicillin (100 U/ml) and streptomycin (100 U/ml). Bcl-2-overexpressing cells were generated using a pMAX vector containing the human bcl-2 gene (provided by Dr. Rakesh Srivastava, NIH/NIA). HL60, K562 or U937 cells (400 ml) in RPMI 1640 (20 ×10⁶ cells/ml) were transfected by pre-incubating with 15 mg Bcl-2 plasmid for 10 min at room temperature and then electroporating at 500 V, 700 μF. The sample was immediately placed on ice for 10 min, then 10 ml of complete medium was added, and the cells were incubated at 37 °C for 24 h. The cells were selected in a medium containing 0.7 mg/ml geneticin (G418) for 4 weeks. Single cell clones were obtained by limiting dilution and subsequently analyzed for an increase in Bcl-2 protein expression relative to identically cloned empty vector controls.

2.3. Cytotoxicity assay

The cytotoxicity assay was performed by MTT method [17]. Cells were seeded into 96-well culture plates (10⁴ × cells/well) respectively, and allowed to attach for 12 h before treatment. The cells were treated with B1 (0-5 μM) or amonafide (0-120 μM). Cell viability was evaluated by MTT assay after 48 h treatment. The optical density (OD) in control and drug-treated wells were measured in an Automated Microplate Reader (Multiskan Ex, Lab systems, Finland) at a test wavelength of 570 nm. The cytotoxicity of B1 was expressed as IC₅₀ (concentration of 50% cytotoxicity, which was extrapolated from linear regression analysis of experimental data).

2.4. Colony forming assay
Cells were plated into 24-well culture plate with culture medium and agarose mixture (according to the ratio of culture medium : agarose = 3:1). Culture medium containing B1 (0-5μM) or amonafide (0-120μM) was added to cells and incubated for 7 days. After that, wells were fixed with methanol and stained with 5% Gimsa solution and colonies (>50 cells) were counted under an inverted microscope. The anti-proliferation activity was expressed as EC50 (concentration of 50% inhibitory colony number).

2.5. Flow cytometric analysis of apoptosis and necrosis

Extent of apoptosis was measured through Annexin V-FITC apoptosis detection kit (Invitrogen, USA) as described by the manufacture’s instruction. Briefly, cells (1×10⁶/2 ml/well) were seeded in 6-well plates and treated with B1 at 0, 0.5, 1 and 2 μM. After 48 h cells were collected, washed with cold PBS twice, gently resuspended in 400 μl 1×binding buffer. Added 5 μl of Annexin V-FITC, gently vortex the cells and incubated for 10 min at 4-8 °C in the dark. Added 10 μl of PI to tube for another 5 min at 4-8 °C in the dark. And then it was analyzed by flow cytometry using FACScan flow cytometer (Becton Dickinson, USA). The fraction of cell population in different quadrants was analyzed using quadrant statistics. Cells in the lower right quadrant represented early apoptosis and in the upper right quadrant represented late apoptotic cells [18].

2.6. Nuclear morphology analysis of apoptosis

Forty-eight hours after treatment, the cell suspension was cytopspun onto a clean fat-free glass slide with a cytocentrifuge. Cytocentrifuged samples were fixed in MeOH-HOAc (3:1, v/v) for 10 min
at 4 °C, and stained with Hoechst 33258 (5 μg/ml in PBS) for 5 min at room temperature and then examined in a LEICA DMIRB fluorescent microscope at 356 nm. To quantitate apoptosis, an average of 1000 nuclei from random fields were analyzed, and apoptotic cell counts were expressed as a percentage of the total number of nuclei counted.

2.7. Immunocytochemistry using confocal microscopy

Cytocentrifuged cells were fixed for 10 min in 10% formaldehyde, incubated with the mouse anti-cytochrome c antibody for 1 h, washed 3× for 5 min each and then incubated with donkey anti-rabbit IgG FITC antibody for 1 h at room temperature. Images were obtained using confocal laser scanning microscopy (LSM 410; Zeiss, Jena, Germany).

2.8. Mitochondrial membrane potential

Cells were washed twice with 500 μL of PBS. Then, 0.5 μL of rhodamine 123 solution (5 mg/mL diluted in ethanol – Sigma, USA) was added to each cell group and incubated for 15 min at room temperature. The cells were washed twice with PBS and analyzed using a FACScan flow cytometer (Becton Dickinson, USA). A total of 10,000 events were collected per sample.

2.9. Western blotting analysis

Conducted as described previously [19,20]. In brief, cell lysates were centrifuged at 14,000 rpm for 15 min at 4 °C. Protein concentrations of cell lysates were determined with the BCA assay (Pierce, Rockford, IL, USA) and 50 μg of proteins was loaded onto 7.5-15% SDS-polyacrylamide
gels. The gels were transferred to Nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The blot was blocked in blocking buffer (5% non-fat dry milk/1% Tween 20 in PBS) for 1 h at room temperature, and then incubated with appropriate primary antibodies in blocking buffer overnight at 4 °C. The blot was then incubated with appropriate secondary antibody alkaline phosphatase (AP) conjugated and detected in 5 ml AP buffer containing 16.5 μl BCIP and 33 μl NBT at room temperature for 10-20 min, and then photographed. β-Actin was used as a loading control.

2.10. Coimmunoprecipitation assay

Conducted as described previously [21,22]. In brief, cells were collected and lysed in 1 ml of immunoprecipitation lysis buffer (300 mM NaCl, 50 mMTris-Cl [pH 7.6], 0.5% Triton X-100, protease inhibitors, 10 mM Na₃P₂O₇, 1 mM Na₃VO₄, 25 mM NaF and 1 mM β-glycerophosphate). Protein concentrations of cell lysates were determined using the Bradford method and 500 μg of protein was precleared and then incubated with PML or 14-3-3σ antibody in extraction buffer at 4 °C overnight. The immune complexes were precipitated with protein A/G-agarose beads (Sigma) for 2 h and washed 5 times with extraction buffer prior to boiling in SDS sample buffer. Immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis and Western blot analysis was performed as described above.

2.11. Methylation-specific PCR (MSP)

Methylation-specific PCR was performed with 25 μL using 3 units of platinum Taqpolymerase
(Invitrogen, Carlsbad, CA) per reaction. We used the following MSP primers: (1) for unmethylated 14-3-3σ sequence: 5′-TTATTAGAGGTGGGTTAAG-3′ (sense) and 5′-CAACCCCAAACCACAACCATAA-3′ (antisense); (2) for methylated 14-3-3σ sequence: 5′-GGTTTTTTCGGTTAGTTGCGCGG-3′ (sense) and 5′-CCAACGAAAACCTCGCGACCTCCG-3′ (antisense). After denaturation at 95°C for 5 min, 40 PCR-cycles were completed with the bisulfite-treated genomic DNA as a template. 20 μL of the PCR-amplified fragments were loaded onto 2% agarose gels for analysis. Positive controls used for methylation-specific PCR included DNA from normal prostate tissues as unmethylated DNA control and CpGenome Universal methylated DNA as methylated DNA control (Chemicon International). Negative control MS-PCR reactions were performed using water only as template.

2.12. Chromatin immunoprecipitation assay

The ChIP assay was performed using the EZ ChIP kit according to the manufacturer’s directions (Upstate, Lakeplacid, NY). After 12 h treatment with 10 nM NVB, 2.5 × 10⁶ cells were harvested, cross-linked with 1% formaldehyde and the reaction was quenched by glycine 125 mM. Cells were lysed and sonicated to generate chromatin fragments between 200 and 1000 bp. Sheared chromatin fractions were incubated overnight with anti-MBD2 antibody (Upstate Biotechnology) on rotating device at 4°C. Immunocomplexes were then washed and eluted with elution buffer. The eluates and the input sample (1% of the amount used in the IP procedure) were reverse-crosslinked by incubating at 65°C overnight in presence of 0.2 M NaCl. After Rnase and
proteinase K digestion, the DNA fragments were extracted using phenol–chloroform and purified using Qiagen PCR purification kit. Presence of selected DNA sequences was assessed by quantitative PCR with primers specific to the 14-3-3σ promoter:

5′-CATGAAAGGCCGTGGGAGAA-3′ (sense) and 5′-GCTGATGTCCATGGCCCTG-3′ (anti-sense).

2.13. Real-Time Quantitative Reverse Transcription -PCR Analysis

Total RNA of B1- treated cells was extracted using TRIZOL™ reagent (Promega Corporation) according to the supplier’s instruction. RNA was quantitated by optical density measurement at 260 and 280 nm using a spectrophotometer (all RNA samples had an A260/A280 ratio >1.8), and integrity was confirmed by running RNA on a 1.2% agarose gel. Reverse transcription was performed with 1 μg of total RNA using Reverse Transcription System (Takara Shuzo, Shiga, Japan). Real-time PCR was performed using SYBR Green Supermix with an iCycler® thermal cycler (Bio-Rad). Primers were obtained from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China) and their sequences were:

5′-ACGACAAGAGCCGCATCTGTG-3′ (sense) and 5′-GGCATCTCCTTCTTGCTGATG-3′ (anti-sense) for 14-3-3σ, and 5′-GGTCGGAGTCAACGGATTTG-3′ (sense) and 5′-ATGAGCCCCAGCCTTCTCCAT-3′ (anti-sense) for GAPDH. The data were collected and analyzed using the comparative Ct (threshold cycle) method using GADPH as the reference gene.

The cells were plated at $5 \times 10^5$ cells/mL in a 6-well plate. 48 h after plating, the cells were transfected with either the control siRNA or the MBD2 siRNA (siMBD2-1, 5'-AAGAGGATGGATTGCCCGGCC-3' and siMBD2-2, 5'-AAGCAGAGACTCCGGAATGAC-3'). “Smart pool” siRNAs that combined the above MBD2 siRNAs 1-2 targeted against different regions of the MBD2 mRNA sequence (NM_003927) were used for transfection to increase the knockdown effect. The transfection of the siRNA was carried out according to the manufacturer’s instructions (Cell Signaling Technology). Subsequently, the cells were collected and analyzed using PCR analysis, ChIP assay and western blotting.

2.15. Statistical analysis

Each experimental value was expressed as means ± standard deviation (SD). Statistical analysis was performed using the Origin7.0 software to evaluate the significance of differences between groups considered as $^* p < 0.05; ^*^* p < 0.01; ^*^*^* p < 0.001$. All data points represented the mean of triplicates.
3. Results

3.1. B1 overcomes the resistance conferred by Bcl-2 in human premyelocytic leukemia HL60 cells

Overexpression of Bcl-2 has been associated with tumor development and chemoresistance in some tumors, including leukemia [12-14]. We examined the cytotoxicity of B1 and amonafide in three Bcl-2-overexpressing cells: HL60/Bcl-2, K562/Bcl-2, and U937/Bcl-2 (Table 1). For each of these cell lines, we calculated the resistance factor (RF) as the ratio of the IC50 value of resistant cells to that of the parental cells. B1 overcame the resistance by Bcl-2 in the three Bcl-2-overexpressing cell lines with the RF values of 0.96, 1.23 and 1.66, which were much lower than amonafide (6.35, 7.66, and 6.22). B1 displayed approximately the same cytotoxicity against
HL60/vector and HL60/Bcl-2 cells, which showed that HL60/Bcl-2 cells were most sensitive to B1. Although amonafide at 1.56-25 μM significantly reduced the viability of HL60/vector cells compared to the controls (control HL60 and HL60/vector cells), the overexpression of Bcl-2 significantly attenuated the amonafide-induced reduction of viability. On the contrary, B1 at 0.062-1 μM reduced the viability to a similar extent in both the HL60/vector and HL60/Bcl-2 cells (Fig. 2A). Long-term survival as determined by a colony forming assay revealed a significant decline in clonogenic survival after treatment with B1 (>50 nM) in HL60, HL60/vector and HL60/Bcl-2 cells (Fig. 2B). These data suggest that B1 overcomes the resistance conferred by Bcl-2 in HL60 cells.

3.2. B1 overcomes the resistance conferred by Bcl-2 in HL60 cells by inducing apoptosis

We next examined the mechanism by which B1 overcomes the resistance conferred by Bcl-2. The IC80 and IC50 values of B1 (0.05 μM and 0.3 μM) and amonafide (2 μM and 5 μM) were used as the experimental doses.

Hoechst staining showed that the percentage of apoptotic cells with condensed or fragmented nuclei was not significantly reduced in 0.3 μM B1-treated HL60/Bcl-2 cell compared to 0.3 μM B1-treated HL60 cells while the percentage of these apoptotic cells was significantly reduced in 5 μM amonafide treated HL60/Bcl-2 cells compared to the 5 μM amonafide treated HL60 or HL60/vector cells (Fig. 3A). Immunofluorescent staining to cytochrome c showed that translocation of cytochrome c from the mitochondria to the cytosol in 0.3 μM B1-treated HL60, HL60/vector and HL60/Bcl-2 cells and 5 μM amonafide-treated HL60 and HL60/vector cells, but
not in 5 \( \mu \)M amonafide-treated HL60/Bcl-2 cells (Fig. 3B). Flow cytometry demonstrated that apoptotic population was increased in 0.3 \( \mu \)M B1-treated HL60/vector and HL60/Bcl-2 cells, and 5 \( \mu \)M amonafide-treated HL60/vector cells but not in 5 \( \mu \)M amonafide-treated HL60/Bcl-2 cells (Fig. 3C). Mitochondrial membrane potential assay also showed that reduction of \( \Delta \Psi_m \) in 0.3 \( \mu \)M B1-treated HL60, HL60/vector and HL60/Bcl-2 cells and 5 \( \mu \)M amonafide-treated HL60 and HL60/vector cells, but not in 5 \( \mu \)M amonafide-treated HL60/Bcl-2 cells (Fig. 3D). Western blot assay showed the cleavage of caspase-3 and PARP in 0.3 \( \mu \)M B1-treated HL60/vector and HL60/Bcl-2 cells and 5 \( \mu \)M amonafide-treated HL60/vector cells, but not in 5 \( \mu \)M amonafide-treated HL60/Bcl-2 cells (Fig. 3E). These data indicate that B1 overcomes the resistance conferred by Bcl-2 by inducing apoptosis.

3.3. B1 overcomes the resistance conferred by Bcl-2 in HL60 cells through 14-3-3σ

We next examined whether B1 alters the expression levels of apoptosis regulatory factors. Western blot assays showed that anti-apoptotic proteins, such as Bcl-2 and 14-3-3σ, were downregulated in cells undergoing apoptosis in response to amonafide or B1 treatment (Fig. 4A). Meanwhile up-regulation of Bax was observed, suggesting an increase of Bax/Bcl-2 ratios which might be involved in apoptosis induced by amonafide or B1 (Fig. 4A). Most previous reports showed that the expression level of the 14-3-3σ protein was constantly sustained, although cells undergo apoptosis through the 14-3-3σ/Bad pathway. Thus, it is noticeable that B1 (0.3 \( \mu \)M) and amonafide (5 \( \mu \)M) markedly downregulated 14-3-3σ protein compared with the controls. Importantly, whereas B1 (0.3 \( \mu \)M) reduced 14-3-3σ protein expression in HL60/Bcl-2 cells.
compared with the controls, amonafide (5 μM) had no significant effect on 14-3-3σ protein expression in HL60/Bcl-2 cells. Since downregulation of 14-3-3σ protein is bound to result in decreasing the opportunity of the association between Bad and 14-3-3σ, this downregulation of 14-3-3σ and subsequent modulation of the 14-3-3σ/Bad pathway can be speculated to be involved in overcoming the resistance conferred by Bcl-2. Thus, our further investigation was focused on 14-3-3σ.

Time-sequenced western blot assays showed that downregulation of 14-3-3σ protein in cells undergoing apoptosis is evident at early time points after amonafide or B1 treatment (Fig. 4B). Conversely, the reduction of cell viability begins at later time points (Fig. 4C). These data indicate that the downregulation of 14-3-3σ precedes the reduction of cell viability.

We next examined whether B1 exerts its antitumor activity via Bad, a representative client protein of 14-3-3σ. Neither B1 (0.3 μM) nor amonafide (5 μM) altered the expression level of total Bad protein both in HL60/vector and HL60/Bcl-2 cells. Notably, downregulation of phosphor-Bad (Ser 155) was observed in HL60/vector cell apoptosis in response to B1 (0.3 μM) or amonafide (5 μM) treatment. B1 (0.3 μM), but not amonafide (5 μM), showed the corresponding downregulation in HL60/Bcl-2 cells (Fig. 5A). Co-immunoprecipitation assays showed that the interaction between 14-3-3σ and Bad is reduced in HL60/vector cells in response to B1 (0.3 μM) or amonafide (5 μM) treatment. B1 (0.3 μM), but not amonafide (5 μM), showed the corresponding reduction of the interaction in HL60/Bcl-2 cells (Fig. 5B). Time-sequenced western blot assays showed that the up-regulation of Bax and the downregulation of phosphor-Bad and Bcl-2, like 14-3-3σ, precedes the reduction of viability (Fig. 5C).
Taken together, the downregulation of 14-3-3σ, subsequent modulation of the 14-3-3σ/Bad pathway and concomitantly the augment of the Bax/Bcl-2 ratios presumably plays a pivotal role in amonafide- or B1-induced apoptosis in HL60 cells.

3.4. B1 downregulates 14-3-3σ via Methyl-CpG-Binding Protein MBD2 in HL60/Bcl-2 cells

We examined whether downregulation of 14-3-3σ protein by amonafide or B1 is regulated at a transcriptional level. Since we found 14-3-3σ is a relatively well-known isoform to exert an antiapoptotic function, our real-time PCR data indicated that the gene expression level of 14-3-3σ was substantially downregulated in HL60/vector cells in response to amonafide or B1 treatment. B1, but not amonafide, showed the corresponding downregulation in HL60/Bcl-2 cells (Fig. 6A). To determine whether methylation of the 14-3-3σ promoter region was responsible for the downregulation of expression of this gene, we next examined whether amonafide and B1 can affect the methylation level of the 14-3-3σ promoter region using MSP. Two MSP primer sets were designed to specifically amplify either methylated (M) or unmethylated (U) bisulfite-modified sequence in the 14-3-3σ promoter-associated CpG islands [23]. Amplification with either the methylated or unmethylated set of primers results in either the presence or absence of a PCR product depending on the methylation status of the CpG dinucleotides interrogated by that primer pair. PCR products obtained with the two MSP primer sets, M and U, on bisulfite-modified DNA from amonafide- or B1- treated cells are shown (Fig. 6B). These MSP analysis results suggest the presence of dense methylation of the 14-3-3σ promoter in 0.3 μM B1-treated HL60/vector and HL60/Bcl-2 cells and 5 μM amonafide-treated HL60/vector cells, but not in 5 μM
amonafide-treated HL60/Bcl-2 cells.

Silencing mediated by CpG methylation correlates with the binding of MBD2 on methylated promoters [24]. The 14-3-3σ promoter region contains dense CpG dinucleotides and this region could potentially bind to MBD2 [23]. It is possible that methylation-mediated transcriptional silencing of the 14-3-3σ gene promoter may involve interaction with a methyl-CpG binding protein MBD2. To assess whether MBD2 is involved in CpG methylation-dependent 14-3-3σ gene silencing, we performed ChIP assays in amonafide- or B1- treated cells. ChIP is a powerful technique used to test for the presence of certain DNA-binding proteins that modulate transcriptional characteristics of the specific region of DNA with which they are associated. Using antibodies against MBD2 protein, formaldehyde cross-linked protein-chromatin complexes were immunoprecipitated from 14-3-3σ expressing or non-expressing cells. After immunoprecipitation, the DNA was released, and the amount of 14-3-3σ specific DNA that was released from the immunoprecipitates was analyzed by PCR. As shown in Fig. 6C, ChIP assay showed that MBD2 is strongly associated with the methylated CpG island in 0.3 μM B1-treated HL60/vector and HL60/Bcl-2 cells and 5 μM amonafide-treated HL60/vector cells, but not in 5 μM amonafide-treated HL60/Bcl-2 cells. Western blot assays demonstrated that the expression level of MBD2 protein were substantially upregulated in HL60/vector cells in response to amonafide or B1 treatment. B1, but not amonafide, showed the corresponding upreguation in HL60/Bcl-2 cells (Fig. 6D). These findings indicate that MBD2 was associated with the methylated 14-3-3σ promoter-associated CpG Island, and thus, interfered with transcriptional activity of the methylated promoter.
3.5. Inhibition of B1-induced apoptosis and overcome the resistance conferred by Bcl-2 by knock-down of MBD2 using siRNA transfection

To further confirm the role of MBD2 in B1-induced apoptosis and overcome the resistance conferred by Bcl-2, the siRNA technique was used to selectively knock-down the MBD2 gene. In 0.3 μM B1-treated HL60/vector and HL60/Bcl-2 cells, and 5 μM amonafide-treated HL60/vector cells transfected with MBD2 siRNA, the expression of MBD2 and Bax were reduced and the expression of 14-3-3σ, phosphor-Bad and Bcl-2 were increased compared to that without siRNA transfection (Fig. 7A). Furthermore, the apoptotic induction in 0.3 μM B1-treated HL60/vector and HL60/Bcl-2 cells, and 5 μM amonafide-treated HL60/vector cells transfected with MBD2 siRNA were significantly decreased, as demonstrated using flow cytometry (Fig. 7B).

Accordingly, these data demonstrated the involvement of MBD2 in B1-induced apoptosis and overcome the resistance conferred by Bcl-2.

4. Discussion

Although amonafide, a naphthalimide derivative, has long been challenged by its unpredictable side effects, the simplicity of amonafide, associated with its interesting anticancer activity, offers
promise for the rational design of new chemotherapeutic agents. Previous studies reported several amonafide analogues showing stronger antitumor effects than amonafide [25,26]. In our previous study, a novel amonafide analogue B1 was designed to aim at improving the antitumor efficiency and in particular, to alleviate the toxicity of the parent compound amonafide [15]. Recently, the amonafide analogue B1 has been reported to induce cell cycle arrest and apoptosis in HeLa cells via p53 activation [16]. To understand the mechanism by which B1 exerts potent antitumor activity on p53-null HL60 cells, further future studies are required.

14-3-3σ is a member of the 14-3-3 family of proteins which play critical roles in the cell cycle progression, mitogenic signal transduction, metabolism, oncogenesis and apoptosis [27,28]. Importantly, 14-3-3σ plays a critical role in cellular survival by the interaction of numerous proteins such as Bad [29]. Bad is a representative 14-3-3 client protein. Apoptotic stimuli separate Bad from 14-3-3. Bad isolated from 14-3-3 heterodimerizes with Bcl-2, liberating Bax from Bcl-2 to induce apoptosis [29]. Through previous studies, targeting 14-3-3 proved to provide an effective strategy to sensitize tumor cells for therapy-induced cell death. Most chemotherapeutic agents have been delineated not to alter the expression level of 14-3-3σ protein, although they exert antitumor activity via the 14-3-3σ/Bad pathway. Only a few chemotherapeutic agents have been documented to downregulate 14-3-3σ in cells undergoing apoptosis via the 14-3-3σ/Bad pathway [30-33]. In the present study, we first revealed that the antitumor activity of B1, as well as amonafide, is associated with the downregulation of 14-3-3σ which seems, at least in part, to play a role in amonafide- or B1-induced apoptosis in HL60 cells.

Previous studies indicated downregulation of 14-3-3σ expression, often due to the methylation
level of promoter associated CpG islands [34]. Transcriptional silencing via methylation of promoter-associated CpG islands has been shown to be caused by two mechanisms, one of which is direct interference with the binding of transcriptional factors [35,36], and the other, recruiting of methyl-CpG-binding proteins (MeCPs), which inhibit the binding of transcriptional factors to the promoter regions [37-40]. Since most transcriptional factors do not have CpG dinucleotides within their binding sites, silencing by methylation of promoter-associated CpG islands is believed to be largely mediated by the binding of MeCPs to methylated CpG dinucleotides. Furthermore, since MBD2 acts to inhibit transcription of 14-3-3σ and other tumor-suppressor genes inactivated by CpG methylation in cancers, new cancer treatments or preventive strategies should potentially target the MBD2 transcriptional repression pathway. A recent study lends further support to our results indicating that MBD2 is not only a repressive regulator, but also an active transcriptional regulator [41]. Our data obtained after transfection with siRNA of MBD2 support the notion that B1 treatment triggers MBD2 activation and leads to a MBD2-dependent downregulation of 14-3-3σ. ChIP assays further confirmed MBD2 role, showing that MBD2 is strongly associated with the methylated CpG island of 14-3-3σ promoter in 0.3 μM B1-treated HL60/vector and HL60/Bcl-2 cells and 5 μM amonafide-treated HL60/vector cells, but not in 5 μM amonafide-treated HL60/Bcl-2 cells.

In conclusion, our study provides further insight into the molecular mechanism underlying B1-induced apoptosis and overcome the resistance conferred by Bcl-2 in HL60 cells, by showing the downregulation of 14-3-3σ, subsequent modulation of the 14-3-3σ/Bad pathway and concomitantly the augment of the Bax/Bcl-2 ratios. Furthermore, we highlight the pivotal role of
MBD2 in B1-induced apoptosis and overcome the resistance conferred by Bcl-2, such as MBD2 was associated with the methylated 14-3-3σ promoter-associated CpG Island, and thus, interfered with transcriptional activity of the methylated promoter. Given the strong relationship between apoptosis and drug effectiveness, reinforcement of MBD2 activity and/or abrogation of 14-3-3σ expression could represent attractive ways to promote B1-induced apoptosis and overcome the resistance conferred by Bcl-2.
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Figure legends

Fig. 1. Chemical structure of amonafide and B1.

Fig. 2. B1 overcomes the resistance conferred by Bcl-2 in HL60 cells. (A) Cell viability assay 48 h after treatment with amonafide or B1 shows that the overexpression of Bcl-2 significantly attenuated the amonafide-induced reduction of viability whereas B1 reduced the viability to a similar extent in both the HL60/vector and HL60/Bcl-2 cells. **p < 0.01, ***p < 0.001 comparing to controls (HL60 control and HL60/vector cells). (B) Determination of cellular survival with colony forming assay. Survival curves as determined by the colony forming assay 7 days after amonafide or B1 treatment.

Fig. 3. B1 efficiently induces apoptosis in HL60/Bcl-2 cells. C, control; V, vehicle; B1, B1; Am, amonafide. (A) (a) Hoechst staining showing nuclear morphology of HL60 cells 48 h after amonafide or B1 treatment. (b) Quantitation of hoechst-stained condensed nuclei was performed. (B) Immunofluorescent stating to cytochrome c shows that translocation of cytochrome c from the
mitochondria to the cytosol in 0.3 μM B1-treated HL60, HL60/vector and HL60/Bcl-2 cells and 5 μM amonafide-treated HL60 and HL60/vector cells, but not in 5 μM amonafide-treated HL60/Bcl-2 cells. (C) Flow cytometry demonstrated that apoptotic population was increased in 0.3 μM B1-treated HL60/vector and HL60/Bcl-2 cells, and 5 μM amonafide-treated HL60/vector cells but not in 5 μM amonafide-treated HL60/Bcl-2 cells. (D) Mitochondrial membrane potential assay shows that reduction of ΔΨ_m is observed in 0.3 μM B1-treated HL60, HL60/vector and HL60/Bcl-2 cells and 5 μM amonafide-treated HL60 and HL60/vector cells, but not in 5μM amonafide-treated HL60/Bcl-2 cells. (E) Western blot assay showing activation of caspase-3. Degradation of caspase-3 and PARP are shown in 0.3 μM B1-treated HL60/vector and HL60/Bcl-2 cells and 5 μM amonafide-treated HL60/vector cells, but not in 5 μM amonafide-treated HL60/Bcl-2 cells. β-actin is shown as a loading control.

Fig. 4. B1 overcomes the resistance conferred by Bcl-2 in human leukemic HL60 cells through 14-3-3σ. (A) Western blot assay showing up-regulation of Bax and downregulation of Bcl-2 and 14-3-3σ proteins in cells undergoing apoptosis in response to amonafide or B1 treatment. (B) Time-sequenced western blot assay showing that downregulation of 14-3-3σ protein in cells undergoing apoptosis is evident at early time points after amonafide or B1 treatment. β-actin is shown as a loading control. (C) Time-sequenced viability assay showing that the reduction of viability begins at later time points after amonafide or B1 treatment.

Fig. 5. B1 exerts its antitumor activity via Bad. (A) Western blot assay showing total Bad and
phosphor-Bad (Ser 155) proteins expression levels. 48 h after treatment. (B)

Co-immunoprecipitation assay showing the interaction between 14-3-3σ and Bad. IB, immunoblot; IP, immunoprecipitation. IgG is shown as a loading control. (C) Time-sequenced western blot assay showing the alteration of phosphor-Bad (Ser 155), Bcl-2 and Bax expression levels after amonafide or B1 treatment.

Fig. 6. B1 downregulates 14-3-3σ via Methyl-CpG-Binding Protein MBD2. (A) Real-time PCR data showing the gene expression level of 14-3-3σ in cells undergoing apoptosis in response to amonafide or B1 treatment. *p < 0.05, **p < 0.01, ***p < 0.001 comparing to controls of HL60/vector cells; ##p < 0.01, ###p < 0.001 comparing to controls of HL60/Bcl-2 cells. (B) A representative DNA methylation specific PCR analysis 14-3-3σ gene. Quantification of the bands was performed. M: reaction with primers specific for the methylated target. U: reaction with primers specific for the unmethylated target. **p < 0.01 comparing to controls of M; #p < 0.05, ##p < 0.01 comparing to controls of U. (C) Assessment of MBD2 binding on the 14-3-3σ promoter by ChIP assay coupled to detection by qRT-PCR. The fold enrichment of the immunoprecipitation was calculated by dividing the quantities of DNA in the anti-MBD2-precipitated samples by that of the matched control antibody (IgG2a). *p < 0.05, **p < 0.01 comparing to controls of HL60/vector cells; #p < 0.05, ##p < 0.01 comparing to controls of HL60/Bcl-2 cells.

Fig. 7. Inhibition of B1-induced apoptosis and overcome the resistance conferred by Bcl-2 by knock-down of MBD2 using siRNA transfection. The cells were transfected with a control and
MBD2 siRNA duplex for 24 h plus recovery, followed by treatment with 0.3 μM B1 or 5 μM amonafide for 48 h. (A) MBD2, 14-3-3σ, phosphor-Bad, Bcl-2 and Bax protein levels checked based on western blot analysis. (B) Apoptosis assessed using Annexin V-FITC and PI staining with flow cytometry.

Fig. 1

![Amonafide and B1 structures](image)
Fig. 2A

![Bar chart showing cell viability (%)](image)

- **Cell viability (%)**
  - HL60
  - HL60/vector
  - HL60/Bcl-2

**B1 (μM)**

- 0
- 0.0625
- 0.125
- 0.25
- 0.5
- 1
Fig. 2B

Cell viability (%) for different concentrations of Amonafide in HL60, HL60/vector, and HL60/Bcl-2 cells.
Fig. 3A(a)
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<tr>
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<th>HL60/Bcl-2</th>
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Fig. 3A(b)

Condensed nuclei (%)
Table 1: Western Blot Analysis of Cytochrome C Levels in HL60, HL60/vector, and HL60/Bcl-2 Cells

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Fig. 3C

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<th>HL60/Bcl-2</th>
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<td>9.49%</td>
<td>5.9%</td>
<td>6.98%</td>
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</tbody>
</table>
Fig. 3D

Mitochondria Membrane Potential (%)

Control  Vehicle  B1 0.05  B1 0.3  Am 2  Am 5

HL60/vector

Normal  Reduced

*  #  ***  *  #  **  ###
Fig. 3E

Mitochondria Membrane Potential (%)

Control  Vehicle  B1 0.05  B1 0.3  Am 2  Am 5

HL60/Bcl-2

Mitochondria Membrane Potential (%)

Control  Vehicle  B1 0.05  B1 0.3  Am 2  Am 5

HL60/Bcl-2

Caspase 3

PARP

β-actin

Normal

Reduced

Fig. 3E

Mitochondria Membrane Potential (%)

Control  Vehicle  B1 0.05  B1 0.3  Am 2  Am 5

HL60/Bcl-2

Caspase 3

PARP

β-actin

Normal

Reduced

Fig. 3E

Mitochondria Membrane Potential (%)

Control  Vehicle  B1 0.05  B1 0.3  Am 2  Am 5

HL60/Bcl-2

Caspase 3

PARP

β-actin

Normal

Reduced

Fig. 3E

Mitochondria Membrane Potential (%)

Control  Vehicle  B1 0.05  B1 0.3  Am 2  Am 5

HL60/Bcl-2

Caspase 3

PARP

β-actin

Normal

Reduced

Fig. 3E

Mitochondria Membrane Potential (%)

Control  Vehicle  B1 0.05  B1 0.3  Am 2  Am 5

HL60/Bcl-2

Caspase 3

PARP

β-actin

Normal

Reduced

Fig. 3E
Fig. 4A

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<td>B1</td>
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<tr>
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- Bax
- Bcl-2
- 14-3-3σ
- β-actin
**Fig. 4B**

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HL60/vector

HL60/Bcl-2
Fig. 5A

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<td>B1</td>
</tr>
<tr>
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<td>V</td>
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</table>

- Bad
- p-Bad
- β-actin
Fig. 5B

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Fig. 5C

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<tbody>
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- **p-Bad**
- **Bcl-2**
- **Bax**
- **β-actin**
- **p-Bad**
- **Bcl-2**
- **Bax**
- **β-actin**

Comparison between HL60/vector and HL60/Bcl-2.
Fig. 6A

![Bar graph showing relative mRNA levels for HL60/vector and HL60/Bcl-2](image)

- Control
- Vehicle
- B1 0.05
- B1 0.3
- Am 2
- Am 5

Legend:
- HL60/vector
- HL60/Bcl-2

Significance levels:
- *: p < 0.05
- **: p < 0.01
- ***: p < 0.001
- ###: p < 0.0001

The graph compares the relative mRNA levels of HL60/vector and HL60/Bcl-2 under different treatments.
Fig. 6B

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<th>Am 2</th>
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<tr>
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<td>HL60/Bcl-2</td>
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</table>

Relative methylation levels:

- **M**: Methylation
- **U**: Unmethylated

- Control
- Vehicle
- B1 0.05
- B1 0.3
- Am 2
- Am 5

Graph showing relative methylation levels for HL60/vector and HL60/Bcl-2.
**Fig. 6C**

- **Relative methylation Levels**
  - **HL60/Bcl-2**
  - **Fold increase over IgG**
    - **HL60/vector**
    - **HL60/Bcl-2**
Fig. 6D

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<tr>
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<tr>
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<td>B1</td>
<td>Am</td>
<td>B1</td>
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<tr>
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<td>V</td>
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<tr>
<td>MBD2</td>
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<tr>
<td>β-actin</td>
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### Fig. 7A

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Fig. 7B

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Table 1. Effects of B1 and amonafide on the Bcl-2-overexpressing cells.

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<th>IC₅₀(µM)</th>
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<td>cl-2</td>
<td></td>
<td>l-2</td>
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<tr>
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<td>0.9</td>
<td>6</td>
<td>0.61±0.0</td>
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<tr>
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Each value represented means ± standard deviation (SD) of three independent experiments. The resistance factor (RF) was calculated as the ratio of the IC₅₀ value of the Bcl-2-overexpressing cells to that of the corresponding parental cells.
B1, a novel amonafide analogue, overcomes the resistance conferred by Bcl-2 in human premyelocytic leukemia HL60 cells

Xin Liang, Yufang Xu, Ke Xu, et al.

Mol Cancer Res Published OnlineFirst November 12, 2010.

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