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3-*m*-Bromoacetylamino Benzoic Acid Ethyl Ester: A New Cancericidal Agent That Activates the Apoptotic Pathway through Caspase-9

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ABSTRACT. The mechanism underlying the cancericidal activity of 3-*m*-bromoacetylamino benzoic acid ethyl ester (3-BAABE) was investigated. 3-BAABE exerted a strong cancericidal effect on human leukemia and lymphoma cells (IC_{50} < 0.2 μg/mL) and on cell lines of prostate, colon, ductal, and kidney cancer (IC_{50} 0.8 to 0.88 μg/mL). Multiple drug resistance (MDR) had no effect on the susceptibility of human lymphoma cells to 3-BAABE, since Daudi/MDR_{20} and wild-type Daudi cells had a similar susceptibility to the cytotoxic effect of 3-BAABE. The cancericidal effect of 3-BAABE, which was not associated with changes in the cell cycle, was mediated by apoptosis. Thus, cells exposed to 3-BAABE displayed the DNA fragmentation ladder characteristic for apoptosis, associated with a marked increase of the activity of apoptosis effector caspases-3 and -6, which was followed by proteolytic cleavage of DNA fragmentation factor (DFF) and poly(ADP-ribose) polymerase (PARP). Exposure of tumor cells to 3-BAABE increased the activity of apical caspase-9, but had no effect on caspase-8. Complete inhibition of 3-BAABE-induced apoptosis was exerted by LEHD-FMK, a caspase-9 inhibitor. DEVD-FMK, a caspase-3 inhibitor, and VEID-FMK, a caspase-6 inhibitor, partially inhibited 3-BAABE-induced apoptosis, whereas exposure to IETD-FMK, a caspase-8 inhibitor, had no effect. The fragmentation and elevated activity of caspase-9 in 3-BAABE-treated cells and the fact that only an inhibitor of caspase-9 abrogated 3-BAABE-induced apoptosis indicate that 3-BAABE is a distinctive compound that elicits apoptosis through a pathway that is limited specifically to activation of apical caspase-9. BIOCHEM PHARMACOL **60**;11:1693–1702, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. apoptosis; cancericidal drug; caspases

In recent years it has become clear that most of the anti-cancer chemotherapeutic drugs that are used clinically can induce apoptosis in sensitive tumor cell lines [1, 2]. Apoptotic death of cells following their exposure to damaging agents results from a complex interaction of pro-apoptotic and anti-apoptotic genes that set into motion the cleavage and activation of a cascade of proteolytic enzymes that, in turn, affect various cytoplasmic and nuclear targets [3].

The Bcl-2 system plays a critical role in determining the sensitivity of cells to the apoptotic effect induced by chemotherapeutic agents [4, 5]. Bcl-2 and related proteins (Bcl-xL and Bag) that exert an anti-apoptotic effect interact with proteins that promote apoptosis (such as Bax, Bcl-xs, and Bak) to form heterodimers [6]. The pro-apoptotic molecules may act by binding to Bcl-2 homologues, thereby allowing APAF-1** and its homologues to induce cell death via activation of caspase-9 [7, 8]. The expression of Bcl-xL in tumor cells confers resistance to a wide range of anti-cancer agents, such as ionizing radiation and chemotherapeutic drugs [9–11]. Conversely, the transfer of the *bax*-α gene to tumor cells sensitizes the cells to radiation and drug-induced apoptosis [10, 11]. Further studies indicated that the ratio of pro-apoptotic to anti-apoptotic proteins, rather than their absolute concentra-

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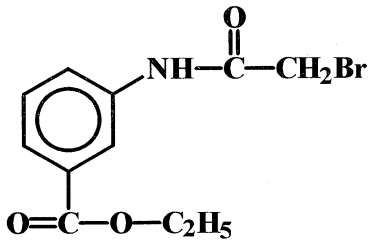
** *Abbreviations*: APAF-1, apoptotic protease activating factor-1; 3-BAABE, 3-m-bromoacetylamino benzoic acid ethyl ester; 3-BAABU, 3-bromoacetylamino benzoylurea; MDR, multiple drug resistance; 3-IAABU, 3-iodoacetamido benzoylurea; MAP, microtubule-associated protein; MTT, microculture tetrazolium; PBL, peripheral blood lymphocytes; DFF, DNA fragmentation factor; PARP, poly(ADP-ribose) polymerase; TNF, tumor necrosis factor; FADD, Fas associated death domain; and ECL, enhanced chemiluminescence.



(3-BAABE)

SCHEME 1.

tions, determines the sensitivity of tumor cells to various chemotherapeutic agents. The Bax:Bcl-2 ratio was found to be correlated with susceptibility to apoptosis in various human tumors and leukemias [12, 13]. Phosphorylation of Bcl-2 at serine residues abrogates its anti-apoptotic activity by increasing the level of free Bax, which, in turn, leads to apoptosis [14]. Phosphorylation of Bcl-2 was elicited in tumor cells exposed to drugs that affect microtubules, but not by DNA-damaging agents [15]. A different pathway of apoptosis is induced via "death receptors" such as CD95/Fas and other members of the TNF receptor family. Activation of apoptosis by ligation of receptors of the TNF family is mediated via the FADD adaptor protein through activation of caspase-8 [16]. Apoptosis induced in cancer cells by chemotherapeutic drugs was shown to be associated with expression of CD95 ligand (CD95-L) [17] or with induction of both CD95 receptor and CD95-L [18].

A number of 3-*m*-haloacetyl aminobenzoyl ureas and their analogues were synthesized in our laboratory, and shown recently to exert pronounced cancericidal activity [19, 20]. The cancericidal activity of 3-BAABU [19] and of 3-IAABU [20] on tumor cell lines was mediated by inhibition of the assembly of microtubules from tubulin, resulting in mitotic arrest of the tumor cells predominantly in the M-phase, followed by apoptosis. In contrast, preliminary results showed that *in vitro* treatment of cancer cells with 3-BAABE (Scheme 1) resulted in apoptosis without eliciting a mitotic block. Animal experiments showed that 3-BAABE inhibits the growth of lymphoma in tumor-bearing mice [21]. The aim of the present study is to provide detailed information about the cancericidal effect of 3-BAABE, and about the mechanism of action of this new compound.

MATERIALS AND METHODS

Reagent

3-BAABE, a compound with a molecular weight of 253 Da, was synthesized in our laboratory (patent pending). The details of the synthesis and confirmation of the compound were reported previously [22]. The compound was dissolved in a mixture of *N*,*N*-dimethylacetamide, propylene glycol, and Tween 80 (1:2:1, by vol.). Since the stock solution of 3-BAABE was 10 mg/mL and the drug concentrations used to treat tumor cells were between 0.01 and 10 μg/mL, the solvent was diluted 1,000- to 1,000,000-fold and was without cytotoxicity in all tested cell lines.

TABLE 1. *In vitro* cancericidal activity of 3-BAABE against various human and murine tumor cell lines

Cell type	Cell line	IC_{50} (μ/mL)
Normal lymphocytes (human)		5.50 ± 0.91
Lymphoma (mouse)	EL4	0.07 ± 0.012
T-cell leukemia	CEM	0.08 ± 0.008
Biphenotypic MDS leukemia	SP	0.11 ± 0.009
Burkitt's lymphoma	Daudi/wt	0.11 ± 0.005
Burkitt's lymphoma	Daudi/MDR_{20}	0.14 ± 0.006
Histiocytic lymphoma	U-937	0.18 ± 0.02
Prostate cancer	PC-3	0.80 ± 0.07
Colon cancer	HCT 116	0.83 ± 0.09
Ductal cancer	MDA-MB-134	0.83 ± 0.08
Kidney cancer	ACHN	0.88 ± 0.05
Melanoma	SK.Mel-5	1.10 ± 0.09
Breast cancer	MCF-7	1.40 ± 0.11

Tumor cell cultures and normal lymphocytes were treated with 3-BAABE concentrations ranging from 0 to 25 μg/mL for 48 hr. The MTT assay was employed to determine cell viability for each concentration of 3-BAABE. At least five experiments were carried out for each cell type. The IC_{50} values were calculated on the basis of the best mathematical (polynomial) curve fitting ($r > 0.98$) by computer. With the exception of EL4, all the cells tested were of human origin. The leukemias and lymphomas grew in suspension, whereas the other cells adhered to the plates. Values are means ± SD.

Cells

The cell lines used in the present study are listed in Table 1. With the exception of the SP line [23] and of two Daudi sublines [24], all cell lines were acquired from the American Type Culture Collection. Two sublines of the Daudi Burkitt's lymphoma were obtained from Dr. T. Ohnuma, Mount Sinai School of Medicine. The Daudi/MDR_{20} subline, carrying the *MDR* gene, is resistant to treatment with either vincristine or doxorubicin and expressed P-glycoprotein, whereas the Daudi wild-type line (Daudi/wt) is sensitive to the chemotherapeutic drugs and does not express P-glycoprotein [24]. The culture media in which each line was cultivated were described previously [19, 20]. Normal human PBL were isolated from the blood of healthy individuals using Ficoll-Hypaque gradients and were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotics. All cell lines were maintained in 5% CO_2 at 37°.

Cytotoxicity and IC_{50} Determination

Cells in suspension were seeded into 96-well microplates (Falcon) at 10^5 cells/well, followed by the addition of 3-BAABE at a final concentration of 0–10 μg/mL. Total culture volume was 250 μL/well. Cell viability was assessed by MTT assay, after incubation for 48 hr at 37°. For monolayer cultures, cells were placed in 96-well microplates at 5×10^4 cells/well, 0–10 μg/mL of 3-BAABE was added, and the cultures were incubated for 48 hr at 37°. To

obtain single-cell suspensions, the supernatant fluids were removed gently, and the cells in the wells were treated with EDTA–trypsin. At least five experiments were performed under identical conditions with each cell type. The drug concentration that induced cellular death in 50% of the treated cells as compared with untreated control cells (IC_{50}) was calculated as described previously [19].

Cancericidal Activity after Pulse Exposure

Cells of the CEM T-cell leukemia line were pulse-exposed to 0.5 μg/mL of 3-BAABE, at 37° for 15, 30, 60, 120, and 240 min, respectively. After incubation, the cells were washed twice in PBS. The cell pellets were resuspended in drug-free medium and incubated at 37° for 48 hr of post-pulse exposure, and the cell viability was determined by MTT assay.

Cell Cycle Analysis

The DNA content of treated cells was measured using the Cycle TEST kit (Becton Dickinson). Light scattering and DNA luminescence of individual cells were examined with a FACScan flow cytometer (Becton Dickinson), using Cellfit software for gating analysis (Becton Dickinson).

Detection of Bcl-2 Phosphorylation

Cells were treated with different concentrations of 3-BAABE for 72 hr. Thereafter, the cells were lysed with a lysis buffer as described previously [20]. Lysate samples with equal protein concentrations, determined by a DC protein assay kit (Bio-Rad), were electrophoresed in 0.1% SDS–10% polyacrylamide gel. The blots were transferred onto nitrocellulose membranes. After blocking of the membrane with 5% non-fat milk in TBST buffer (Tris-buffered saline, Tween-20) at room temperature for 1 hr, the Bcl-2 protein was probed with anti-Bcl-2 monoclonal antibody (PharMingen).

Detection of the Catalytic Activity of Caspases

The activity of various caspases was studied using commercial kits. The ApoAlert™ CPP32/caspase-3 colorimetric assay kit was from ClonTech, while caspase-9/Mch6, caspase-8/FLICE, and caspase-6/Mch2 colorimetric assay kits were from BioVision. The assays are based on spectrophotometric measurement of the chromophore *p*-nitroanilide (*p*NA) after cleavage from the *p*NA-labeled peptide substrates, of which DEVD-*p*NA, VEID-*p*NA, IETD-*p*NA, and LEHD-*p*NA were for caspase-3, -6, -8, and -9 activity, respectively. CEM cells were treated with 3-BAABE at 0.75 μg/mL for 0, 1, 3, 6, or 12 hr. The cells were collected, washed twice in PBS, and aliquotted at 4×10^6 cells per test. The experiments were performed following the steps recommended by the vendors. Raw data (the optical density at 405 nm) were calibrated according to the protein concentrations (150 μg/test).

Western Blot for Caspases

CEM cells (4×10^6/test) were taken from the culture treated with 3-BAABE (0.75 μg/mL) for 0, 1, 3, 6, 12, and 24 hr, washed twice with PBS, and then suspended in 30 μL of lysis buffer containing 50 mM Tris–HCl (pH 7.5), 250 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 20 μg/mL of leupeptin, 20 μg/mL of aproptinin, 0.1% Triton X-100, and 1% SDS at 0–4° for 15 min. After centrifugation at 1500 *g* for 10 min at 0°, the supernatants were collected, and the protein was separated by 15% SDS–PAGE. Following electrophoresis, protein blots were transferred to a nitrocellulose membrane. The membrane was blocked with 5% non-fat milk in TBST solution, and incubated with the corresponding primary antibodies against caspase-9, caspase-8, and caspase-3 (PharMingen), respectively, in block solution overnight in a cold room. After washing three times with TBST solution, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma) diluted with TBST solution (1:1000) at room temperature for 1 hr. The signals of detected protein were visualized by the ECL system (Amersham).

Western Blot Analysis for DFF and PARP

Cleavage of DFF and of PARP was determined as described previously [25]. Cells were treated for different times with 0.75 μg/mL of 3-BAABE, then washed once in PBS, and pellets were frozen. Following resuspension of the pellets in lysis buffer [1% Triton X-100, 150 mM NaCl, 25 mM Tris (pH 7.4), 1 μg/mL of leupeptin, antipain, aproptinin, benzamidine HCl, chymostatin, pepstatin, and 0.5 mM phenylmethylsulfonyl fluoride] and centrifugation, the supernatants were used for analysis of DFF. The pellets were extracted [62.5 mM Tris (pH 6.8), 6 M urea, 10% glycerol, 2% SDS, 0.003% bromophenol blue, 5% β-mercaptoethanol], sonicated for 20 sec, and incubated for 15 min at 65°C. For DFF, 50 μg of the lysis buffer supernatant was resolved by 15% SDS–PAGE, transferred to nitrocellulose, probed with anti-DFF antibody (Upstate Biotechnology) followed by goat anti-rabbit HRP, and visualized by ECL. For PARP, 2 μg of the cell pellet extract was resolved by 10% SDS-PAGE, transferred to nitrocellulose, probed with anti-PARP antibody (mouse anti-calf thymus PARP, BIOMOL) followed by anti-mouse IgG HRP, and visualized by ECL.

Inhibition of Caspase Activity

Tumor cells were incubated for 24 hr at 37° in the absence or presence of caspase inhibitors, at a concentration of 10 μM, prior to their exposure to various concentrations of

3-BAABE. Forty-eight hours later, the viability of the cells was assessed by the MTT assay. The tetra-peptide caspase inhibitors, purchased from BioVision, were the following: IETD-FMK (caspase-8 inhibitor), LEHD-FMK (caspase-9 inhibitor), VEID-FMK (caspase-6 inhibitor), and DEVD-FMK (caspase-3 inhibitor).

DNA Fragmentation

Cells were incubated in 1 mL of lysis buffer [0.01 M Tris–HCl (pH 8.0), 0.01 M NaCl, 0.01 EDTA (pH 8.0), and 5% SDS] containing 1 μg/mL of protease K for 1 hr at 50°. DNA was extracted with phenol and chloroform: isoamyl alcohol (v/v, 24:1), and precipitated with ethanol. The ethanol was removed, and the dry pellet of the samples was resuspended in 10 mM Tris–HCl/1 mM EDTA solution. Then the DNA samples were treated with RNase (Sigma Chemical Co.) at a final concentration of 0.1 μg/μL at 37° for 20 min and loaded on 1.5% horizontal agarose gels, prepared with 1% ethidium bromide. Electrophoresis was performed at 75 V for 2 hr, and the DNA migration was visualized under UV light.

Microtubule Polymerization Assay

The method used for inhibition of microtubule assembly was described previously [20]. The cyto-DYNAMIX Microtubule Polymerization Screens 1 and 2 (CDSO and CDSO2) from Cytoskeleton, Inc. were used to determine the potency of 3-BAABE to inhibit microtubule polymerization either with purified tubulin or with MAP-rich tubulin preparation. Lyophilized protein was resuspended (120 μg/well) in wells of a 96-well plate with 125 μg/mL of G-PEM [80 mM PIPES buffer (pH 6.9), 1.0 mM $MgCl_2$, 0.5 mM EGTA, and 1.0 mM GTP] containing 3-BAABE at concentrations of 0–100 μM. The optical density (O.D.), which is proportional to the concentration of polymerized tubulin was read (Spectramax 250, Molecular Devices, Inc.) at 350 nm.

RESULTS

Cancericidal Activity

The anti-cancer activity of 3-BAABE was demonstrated in various human tumor cell lines and in a mouse leukemia line (Table 1). Leukemia and lymphoma cells displayed the highest susceptibility to 3-BAABE, as indicated by the IC_{50} values, which were lower than 0.2 μg/mL. Cell lines of prostate, colon, ductal, and kidney cancer were less susceptible, having IC_{50} values of 0.8 to 0.88 μg/mL. Melanoma and breast cancer cells had the lowest susceptibility to 3-BAABE, although they too were about four times more sensitive to the cytotoxic effect of 3-BAABE than peripheral blood lymphocytes. The large differential between the susceptibility of CEM leukemic cells and PBL is illustrated in Fig. 1. Daudi/MDR_{20} cells and wild-type Daudi cells had

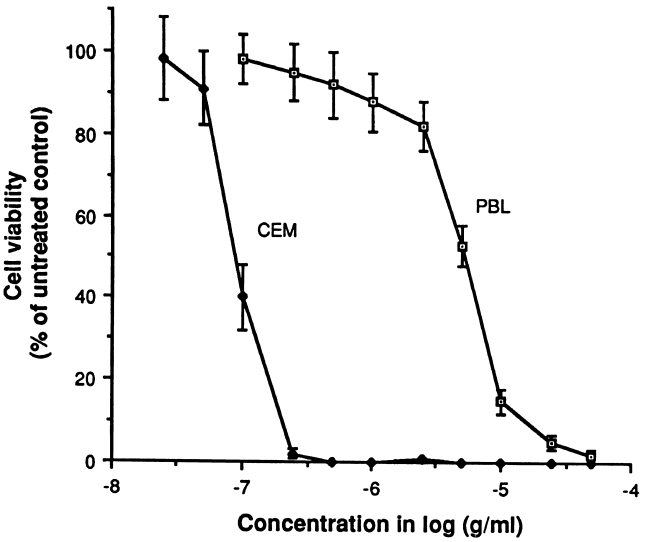


FIG. 1. Comparison of the cytotoxic effects of 3-BAABE on normal human lymphocytes and CEM human leukemia cells. Suspensions of normal human lymphocytes and of CEM human leukemia cells (both at a concentration of 2.5×10^5/mL) were exposed to various concentrations of 3-BAABE for 48 hr at 37°. Cell viability was assessed by MTT assay. Values are means ± SD (N = 5).

a similar susceptibility to the cytotoxic effect of 3-BAABE (Table 1).

To determine the time of exposure to 3-BAABE required for exertion of its cytotoxic effect, leukemic cells were exposed to the drug for 15–240 min, followed by further incubation, up to 48 hr, and the viability of the cells was compared with that of untreated cells (Fig. 2). Fifteen minutes of exposure of leukemic cells to 3-BAABE at a concentration of 0.5 μg/mL sufficed to render over 50% of the cells nonviable at 48 hr. Practically none of the cells remained viable at 48 hr after 4 hr of exposure to the drug.

Cell Cycle and Microtubule Polymerization

In marked contrast to 3-IAABU and 3-BAABU [19, 20], 3-BAABE had no effect whatsoever on the cell cycle. The proportion of cells in each phase of the dividing cycle remained unaltered during 24 hr of exposure to 3-BAABE.

While 3-BAABE failed to affect the cell cycle, it remained of interest to determine whether it could exert an inhibitory effect on the assembly of microtubules from tubulin *in vitro*. At concentrations of 1, 5, 10, and 20 μM, which exerted a strong cytotoxic effect on cancer cells, 3-BAABE had no effect on microtubule polymerization (not shown). At a concentration of 40 μM, 3-BAABE inhibited microtubule polymerization, clearly detectable with both purified and MAP-rich tubulin, as shown in Fig. 3.

DNA Fragmentation

To determine whether the cytotoxic effect of 3-BAABE was associated with apoptosis, DNA was extracted from

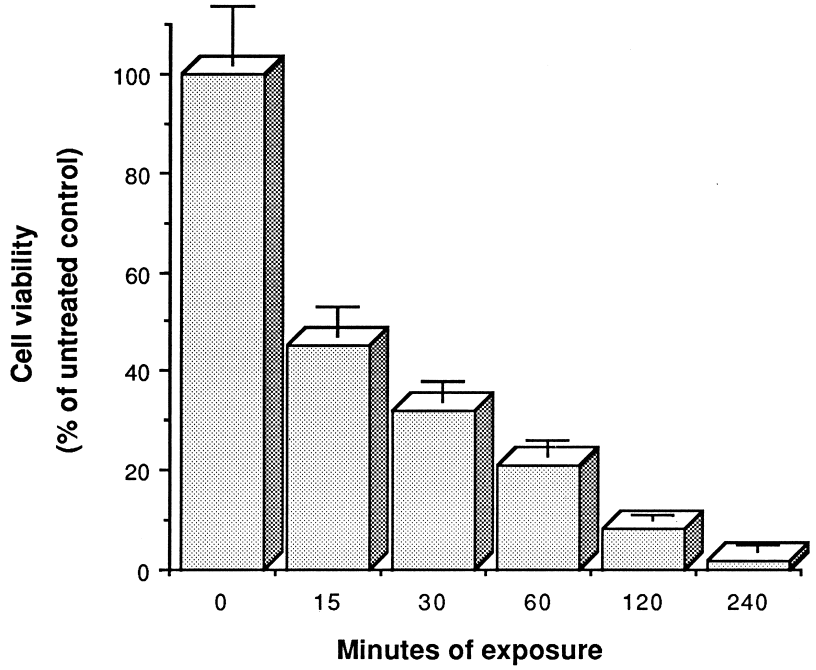


FIG. 2. Kinetics of the cancericidal effect of 3-BAABE. 3-BAABE at a concentration of 0.5 μg/mL was added to CEM leukemia cells (2.5×10^5/mL) for time periods ranging from 0 to 240 min at 37°. Thereafter, the cells were washed, resuspended in fresh culture medium, and incubated at 37°C for up to 48 hr. Cell viability was determined as mentioned above. Values are means ± SD (N = 5).

CEM tumor cells at various time intervals after exposure to 3-BAABE and electrophoresed in agarose gel. DNA fragmentation characteristic for apoptosis could be detected in cells exposed to 3-BAABE for 12 and for 24 hr (Fig. 4). The intensity of the DNA ladder increased with time, and the strongest signals were obtained at 24 hr after exposure to 3-BAABE.

Apoptotic Pathways Involved in the Activity of 3-BAABE

It was of interest to determine whether exposure of CEM cells would lead to phosphorylation of Bcl-2. In CEM cells treated with 3-BAABE for 12 hr, a weak slow-migrating band of phosphorylated Bcl-2 protein became apparent (not shown).

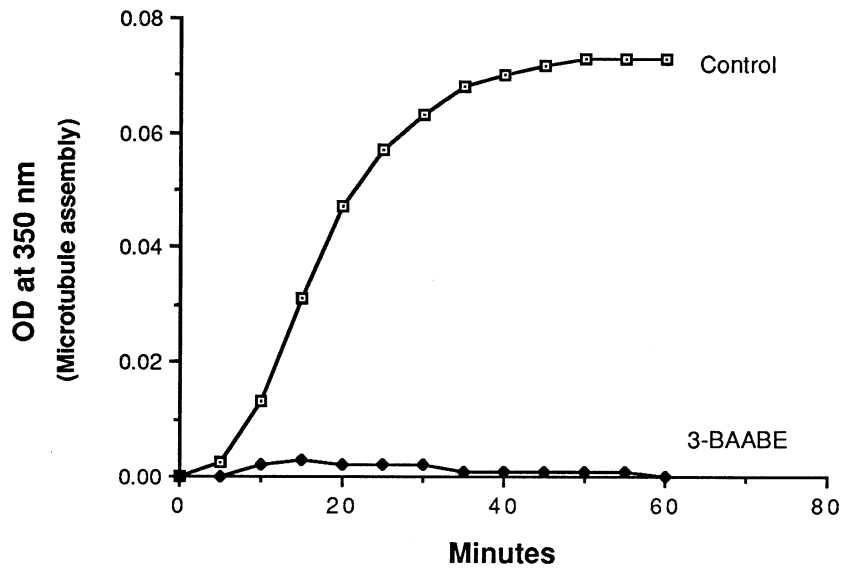


FIG. 3. Effect of 3-BAABE on polymerization of microtubules in a cell-free system. MAP-rich tubulin in reaction buffer was incubated with GTP and Mg^{2+} at 37° in the absence or presence of 3-BAABE (40 μM). The level of microtubule assembly was measured every 5 min by determining the absorbance at 350 nm. The experiment was performed three times.

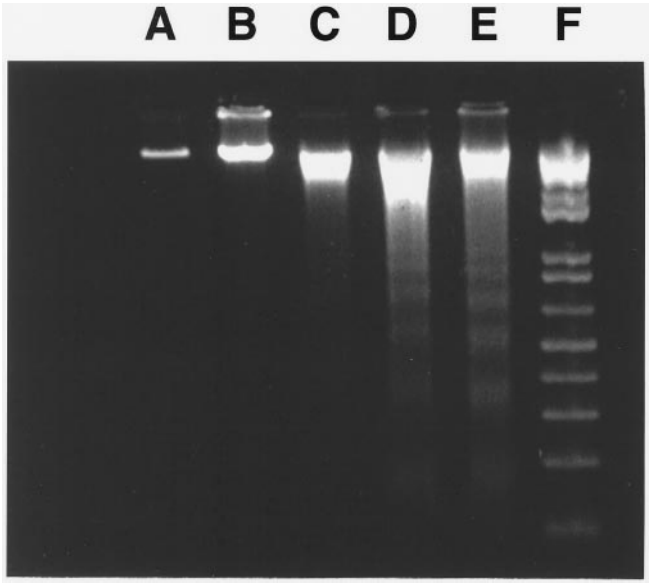


FIG. 4. DNA fragmentation in CEM cells exposed to 3-BAABE. Lane A: DNA from untreated CEM cells. Lanes BñE: DNA obtained from CEM cells exposed to 0.5 μg/mL of 3-BAABE for 1, 4, 12, or 24 hr, respectively. Lane F: standard markers of DNA ladder (75–12,216 bp).

To determine which apoptotic pathway is involved in the cytotoxic activity of 3-BAABE, the catalytic activity of a number of caspases was determined by colorimetric protease assays following exposure of CEM cells to 3-BAABE. The activity of caspase-9 was increased within 1 hr after exposure to 3-BAABE, reaching a peak 6 hr after exposure (Fig. 5, *top panel*). Caspases-3 and -6 were likewise increased in CEM cells exposed to 3-BAABE. The activity of caspases-3 and -6, like that of caspase-9, reached a peak at 6 hr after the beginning of exposure to 3-BAABE (Fig. 5, *bottom panel*). In contrast, the activity of caspase-8 remained unchanged.

To verify the results obtained with catalytic activity assays, western blots *were also used to analyze the activities of* caspases-3, -8, and -9. As shown in Fig. 6, clear bands of activated caspase-3 (Fig. 6A) *were* found after 6 hr, and the strongest signal was after 9 hr of treatment with 3-BAABE. Caspase-9 (Fig. 6B) was processed into subunits (which is considered as an active form) 3 hr after treatment. The signal of the activated caspase-9 *was* enhanced with time. We did not see active fragments of caspase-8 (Fig. 6C) at any time points examined, confirming the results shown in Fig. 5. It appeared to us that the colorimetric protease assays had higher sensitivity than the western blot in our experimental systems, probably because the colorimetric protease assay measured enzymatic activity, but western blot measured protein fragmentation.

To further delineate the contribution of various caspases to the apoptosis induced by 3-BAABE, CEM cells were incubated for 24 hr with various tetrapeptide caspase inhibitors prior to exposure to 3-BAABE. The strongest inhibition of 3-BAABE-induced apoptosis was exerted by LEHD-FMK, a caspase-9 inhibitor (Table 2). Exposure of CEM cells to either DEVD-FMK, a caspase-3 inhibitor, or VEID-FMK, a caspase-6 inhibitor, resulted in partial inhibition of 3-BAABE-induced apoptosis, whereas exposure to IETD-FMK, a caspase-8 inhibitor, had no effect on 3-BAABE-induced apoptosis. For solid tumor cell lines, a breast cancer cell line, MCF-7, was tested in this system. Again, inhibitors to caspases-9, -6, and -3 showed significant protection of cell death from 3-BAABE treatment, among which the caspase-9 inhibitor was the strongest one. Caspase-8 inhibitor did not show measurable inhibitory effect of cell death caused by 3-BAABE (Table 2).

Cleavage of DFF and PARP

Cleavage of DFF, evidenced by disappearance of the 45-kDa intact protein, occurred by 6 hr of exposure of CEM cells to 3-BAABE and progressed almost to completion by 12–24 hr (Fig. 7A). Similarly, cleavage of intact PARP from the 116-kDa active enzyme to the 85-kDa death signature fragment occurred by 12 hr of treatment, with disappearance of the intact enzyme by 24 hr (Fig. 7B).

DISCUSSION

Our laboratory has shown recently that a number of 3-*m*-haloacetyl aminobenzoyl ureas and their derivatives exert a strong cytotoxic effect on tumor cells [19, 20]. 3-BAABE was cytotoxic for tumor cells at low concentrations, similar to those required for the cytotoxic effect of 3-BAABU and 3-IAABU. The sequence of events set into motion by 3-BAABE differed, however, from that set into motion by 3-BAABU and 3-IAABU. Both 3-BAABU and 3-IAABU inhibited the mitotic machinery of tumor cells, leading to mitotic arrest of the cells at the M-phase, and both abrogated the polymerization of tubulin into microtubules [20]. In contrast, treatment of tumor cells with 3-BAABE did not alter the cell cycle, nor did it elicit mitotic arrest of the cells. Unlike the situation with 3-BAABU and 3-IAABU, low concentrations of 3-BAABE that were sufficient for eliciting its cancericidal effect (1 μM) had no detectable influence on the polymerization of microtubules. However, 3-BAABE was not completely devoid of anti-tubulin activity, since at a high concentration (40 μM) 3-BAABE inhibited the polymerization of microtubules. Ten 3-*m*-haloacetyl aminobenzoyl ureas and their analogues, other than 3-BAABE, 3-BAABU, and 3-IAABU, that were tested in parallel at concentrations of 29–48 μM, had no effect on microtubule assembly (unpublished data).

The cancericidal effect of 3-BAABE was mediated by apoptosis as indicated by the characteristic DNA fragmentation ladder found in cells exposed to 3-BAABE. The apoptosis in 3-BAABE-treated cells was associated with a marked increase of the activity of apoptosis effector caspases-3 and -6, which, in turn, was followed by cleavage of the apoptotic death substrates DFF and PARP. 3-BAABE had a similar cytotoxic effect on Daudi/MDR_{20}

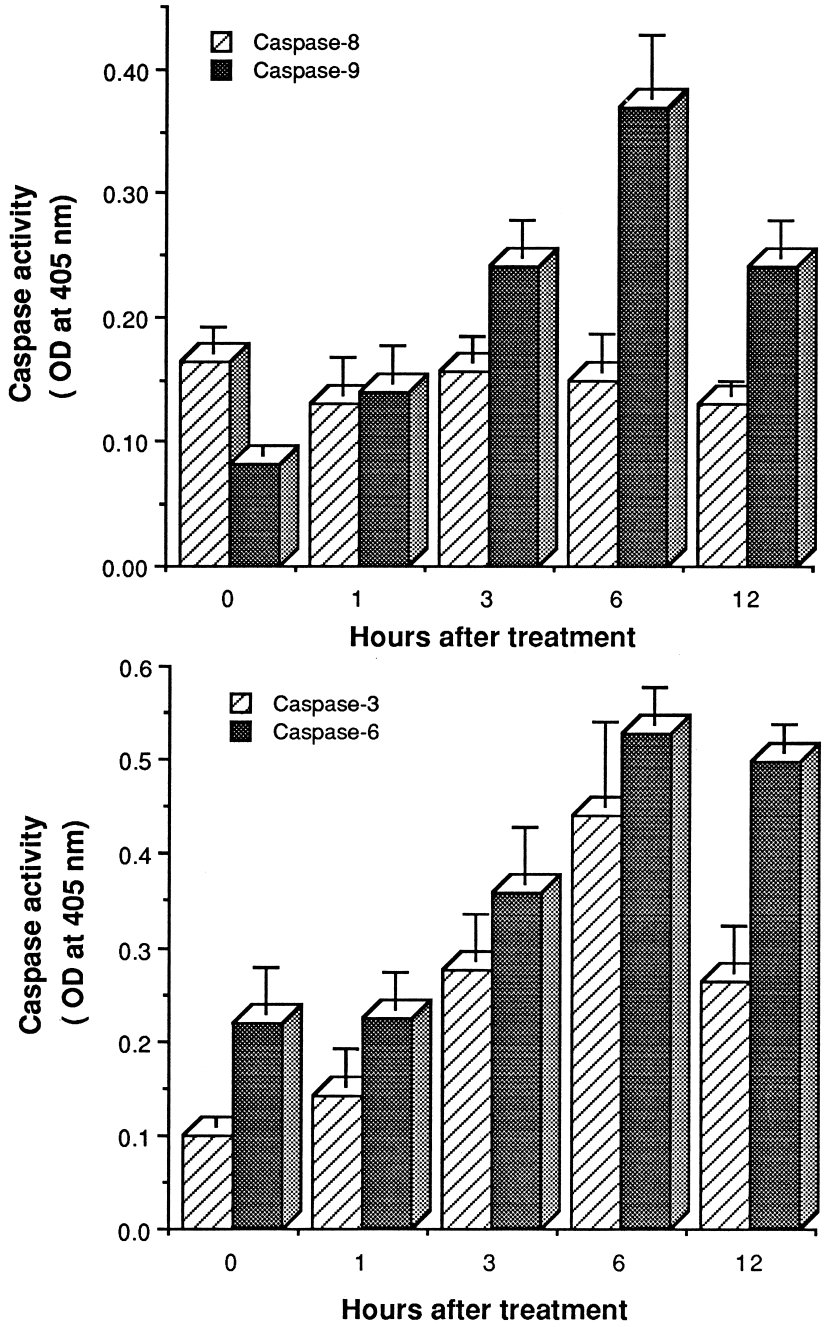


FIG. 5. Activity of caspases elicited by exposure of CEM to 3-BAABE for various time intervals. CEM cells (5×10^5/mL) were treated with 3-BAABE at 0.75 μg/mL for 0, 1, 3, 6, or 12 hr. The activity of apical caspases-8 and -9 (top panel) and of effector caspases-3 and -6 (bottom panel) was determined by colorimetric (*p*NA) protease assay. The final concentration of DEVD-*p*NA (caspase-3) was 50 μM, and that of VEID-*p*NA (caspase-6), IETD-*p*NA (caspase-8), and LEHD-*p*NA (caspase-9) was 200 μM. Values are means ± SD (N = 3).

cells and wild-type Daudi cells, indicating that MDR had no effect on the susceptibility of cells to 3-BAABE. In a number of experimental systems, P-glycoprotein-expressing multidrug-resistant cells had a diminished susceptibility to induction of apoptosis, suggesting that P-glycoprotein expression may interfere with the apoptotic process [22, 26, 27]. Expression of P-glycoprotein and reduced sensitivity to apoptosis, however, could represent two independent resistance mechanisms, which can occur together [28]. Alternatively, P-glycoprotein may prevent cells from attaining the minimal intracellular concentration of agents required for the triggering of apoptosis. Come *et al.* [28] showed that treatment of MDR-resistant and non-resistant HL-60 cells with equitoxic concentrations, rather than with equal concentrations of daunorubicin, induced similar death pathways. In the present study, the fact that Daudi/MDR_{20} cells, which are resistant to vincristine and doxorubicin and expressed P-glycoprotein [24], were as sensitive to the

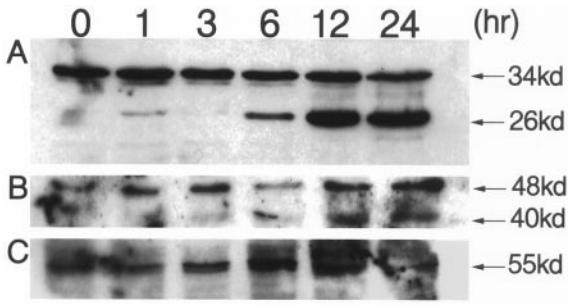


FIG. 6. Cleavage of caspases-3, -8, and -9 in CEM cells treated with 3-BAABE. CEM cells were treated with 3-BAABE at 0.75 μg/mL for 0, 1, 3, 6, 12, and 24 hr. Cleavage of caspase-3 (A), -9 (B), and -8 (C) was detected by western blot (see Materials and Methods).

cancericidal activity of 3-BAABE as Daudi/wt cells shows clearly that MDR-resistant cells can maintain a high sensitivity to apoptotic compounds such as 3-BAABE. The susceptibility of various cells to induction of apoptosis by dexamethasone, staurosporine, and DNA-damaging drugs is controlled by the *bcl-2/bax* genes [11]. The pro-apoptotic activity of Bax and of related proteins is held at bay by the formation of complexes with proteins of the Bcl-2 family. It seems, however, that different drugs may affect the Bcl-2/Bax system and elicit apoptosis through disparate mechanisms. The exposure of dividing tumor cells to drugs that affect the assembly of microtubules [15], including 3-BAABU and 3-IAABU [20], leads to phosphorylation of Bcl-2 molecules, which, in turn, abrogates their anti-apoptotic activity by breaking up their association with Bax. In contrast, DNA-damaging anticancer drugs, such as 5′-fluorouracil, methotrexate, cisplatin, and doxorubicin, do not induce Bcl-2 phosphorylation and therefore, presumably, affect the Bcl-2/Bax system in a different way. In the present study, treatment of tumor cells with 3-BAABE elicited a weaker phosphorylation of Bcl-2 than previously shown to be induced by either 3-BAABU or 3-IAABU [20]. It is possible that phosphorylation of Bcl-2 may contribute to the apoptotic effect of 3-BAABE.

The apoptotic pathway induced by either chemical compounds or receptors differs in that the first apical caspase to be activated is either caspase-9 or caspase-8, respectively [29]. Following activation of the apical caspase, a cascade of additional caspases are activated. Apoptotic signals induced via members of the TNF receptor family, such as the CD95/Fas receptors, recruit CD95 receptor-associated adaptor proteins with death domains, which, in turn, bind to the death effector domains of caspase-8, leading to the activation of caspase-8 [30]. The central role of caspase-8 for this pathway was shown by the fact that cells of mice lacking caspase-8 are resistant to apoptosis induced by the TNF receptors CD95 and DR3 [31]. An alternative pathway of apoptosis, regulated by the Bcl-2/Bax system, controls the conversion of pro-caspase-9 into activated caspase-9. Activation of caspase-9 is brought about by the interaction of pro-caspase-9 with Apaf-1 in the presence of cytochrome *c* and dATP [28, 32]. Following activation of caspase-9, cytochrome *c* and ATP activated not only effector caspases-3, -6, and -7 but also caspases-2, -8, and -10 [33]. Similarly, the induction of apoptosis by exposure to etoposide was accompanied by activation of both apical caspases-8 and -9, as well as effector caspases-3 and -7 [29]. Several studies indicate that caspase-9 is involved in the activation of caspase-8. Thymus cells of *Apaf-1* null mice show impaired activation of caspase-8 in response to pro-apoptotic stimuli [34]. Similarly, the processing of caspase-8 following exposure to dexamethasone was impaired in thymocytes of caspase-9-deficient mice [35].

TABLE 2. Effect of caspase inhibitors on the *in vitro* cancericidal activity of 3-BAABE

Treatment of cells prior to addition of 3-BAABE	IC_{50} (μg/mL)	
	CEM*	MCF-7†
Saline	0.081 ± 0.01	1.36
Caspase-3 inhibitor	0.26 ± 0.04	4.1
Caspase-6 inhibitor	0.25 ± 0.04	3.8
Caspase-8 inhibitor	0.092 ± 0.015	1.42
Caspase-9 inhibitor	0.36 ± 0.07	5.9

Human tumor cells were incubated for 24 hr at 37°C in the presense of either saline or several caspase inhibitors at a concentration of 10 μM. Thereafter, various concentrations of 3-BAABE were added, and the cells were incubated for an additional 48 hr at 37°C. The MTT assay was employed to determine cell viability for each concentration of 3-BAABE. The IC_{50} values were calculated by computer.

* Values are means ± SD (N = 3).

† Values are means of two independent experiments.

In contrast, using colorimetric protease assay and western blot, the present study demonstrated that exposure of tumor cells to 3-BAABE increased the activity of apical caspase-9 and of effector caspases-3 and -6, but had no effect on caspase-8. To further delineate the involvement of various caspases in 3-BAABE-induced apoptosis, several tetrapeptide caspase inhibitors were tested for their ability to inhibit the capacity of the drug to induce apoptosis. The strongest inhibition of 3-BAABE-induced apoptosis was exerted by LEHD-FMK, a caspase-9 inhibitor. Exposure of tumor cells (CEM cells or MCF-7 cells) to either a caspase-3 or a caspase-6 inhibitor resulted in partial inhibition of 3-BAABE-induced apoptosis, while exposure to a caspase-8 inhibitor had no effect on 3-BAABE-induced apoptosis. The various caspase inhibitors tested exerted a similar inhibitory effect on apoptosis elicited by 3-BAABU (unpublished data). The elevated activity of caspase-9 in 3-BAABE-treated cells and the fact that only an inhibitor of caspase-9 abrogated apoptosis indicate that 3-BAABE and probably also 3-BAABU are distinctive compounds that elicit apoptosis through a caspase cascade that is limited specifically to activation of the caspase-9 apical caspase. Analysis of apoptotic events in cells derived from mice with knockout of either the caspase-3 or caspase-9 genes led to the conclusion that at least four different apoptotic pathways exist among various mammalian cells [35]. It was suggested that apoptotic pathways could differ according to whether they depended on the activity of both

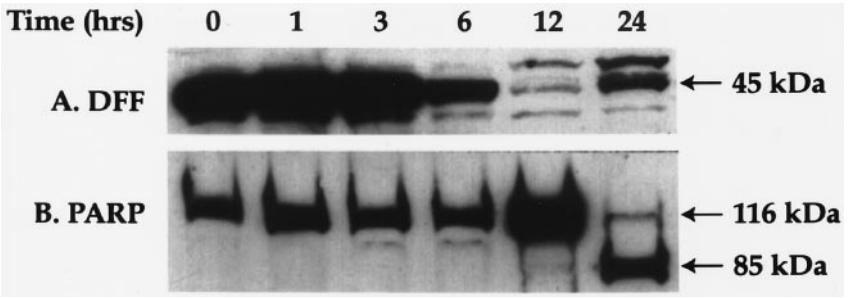


FIG. 7. Time course of the effect of 3-BAABE on apoptotic death substrates. CEM cells were treated with 0.7 μg/mL of 3-BAABE for the indicated time intervals and analyzed for DFF (panel A) and PARP (panel B) by western blot.

caspase-3 and caspase-9, on either one of them alone, or on neither. By the same token it may be suggested that although all chemicals that induced apoptosis initially activate caspase-9, some compounds set into motion a pathway that also results in activation of caspase-8, such as noticed after exposure to cytochrome *c* [33] and etoposide [29]. The present study indicates that other compounds, such as 3-BAABE, may activate caspase-9, without additional activation of caspase-8.

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