Bisindolylmaleimide IX Facilitates Tumor Necrosis Factor Receptor Family-mediated Cell Death and Acts as an Inhibitor of Transcription*

Received for publication, May 10, 2002, and in revised form, June 24, 2002
Published, JBC Papers in Press, June 28, 2002, DOI 10.1074/jbc.M204612200

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Bisindolylmaleimides (Bis) were originally described as protein kinase C inhibitors. Several studies have shown that Bis potentiate tumor necrosis factor (TNF) receptor family-mediated apoptosis in lymphoid and dendritic cells, but the inhibition of protein kinase C cannot account for these effects (Zhou, T., Song, L., Yang, P., Wang, Z., Lui, D., and Jope, R. S. (1999) Nat. Med. 5, 42–48). We investigated the effect of four Bis derivatives (I, II, VIII, and IX) in human prostatic carcinoma cell lines and found that Bis IX was the most potent inducer of apoptosis under simultaneous treatment with TNF-α, agonistic anti-Fas monoclonal antibody, and TNF-related apoptosis-inducing ligand (TRAIL). Bis IX synergistically induced caspase activity in combination with apoptosis-inducing ligands and converted the phenotype of cell lines from apoptosis-resistant to -sensitive. Bis IX induced p53 accumulation in LNCaP (lymph node carcinoma of prostate), which expresses wild-type p53 that was not accompanied by the induction of p58-responsive genes, p21/WAF1, and Mdm2. Moreover, the induction of p21/WAF1 and Mdm2 by doxorubicin was abrogated by simultaneous treatment with Bis IX. These effects apparently result from general inhibition of transcription by Bis IX. We have shown by Northern blot analysis that the transcription activity of the hygromycin gene after transient transfection of pcDNA3.1-Hygro plasmid in 293 and HeLa cells was inhibited by Bis IX in a dose-dependent manner. Moreover, DNA binding activity of Bis IX was prevented by actinomycin D, suggesting that actinomycin D and Bis IX have similar mechanisms of interaction with DNA. In addition, we found that actinomycin D and Bis IX induced caspase activity to the same extent during TRAIL-mediated apoptosis. In summary, these results suggest that Bis IX potentiates TNF receptor family-mediated cell death in part as an inhibitor of transcription.

Several bisindolylmaleimide (Bis)1 derivatives were originally described as protein kinase C (PKC) inhibitors (1–3). Zhou et al. (4) estimated the potentiating effect of nine Bis derivatives on Fas-mediated apoptosis in astrocytoma 1321N1 cells and found that some Bis derivatives were capable of substantially facilitating Fas-mediated apoptosis in these cells. The greatest potentiation was found with Bis VIII and IX; Bis III, X, and XI produced intermediate potentiation, whereas Bis I, II, and IV did not potentiate Fas-mediated apoptosis. The Bis derivatives tested were described as potent inhibitors of PKC, but three other PKC inhibitors structurally unrelated to Bis were tested and were ineffective in potentiating Fas-mediated apoptosis. Based on these results, the authors concluded that the inhibition of PKC could not account for the potentiation of Fas-mediated apoptosis by Bis. The authors also showed that Bis VIII potentiated TNF-mediated apoptosis in 1321N1 cells and greatly facilitated Fas-mediated apoptosis in several human T cell lines and in activated T cells. In contrast, Bis VIII did not facilitate apoptosis in 1321N1 cells treated with dexamethasone or irradiation. They concluded that Bis VIII selectively facilitates apoptosis induced by activation of the TNF receptor family. In addition, several Bis derivatives (I, II, III, IV, and VIII) have been found to facilitate Fas- and TRAIL-mediated apoptosis in human dendritic cells (5). Bis VIII has also been described to potentiate TNF-α-, Fas-, and TRAIL-mediated apoptosis in Jurkat cells (6). However, the critical factors involved in the effects of Bis are still not understood.

The objective of the current study was to determine whether Bis can potentiate TNF receptor family-mediated apoptosis in human prostatic carcinoma cell lines. We examined the effect of Bis I, II, VIII, and IX in three human prostatic carcinoma cell lines, DU145, LNCaP, and PC3. These experiments showed that Bis IX was the most potent inducer of apoptosis under simultaneous treatment with TNF-α, agonistic anti-Fas mAb (IPO-4), and TRAIL. Further, Bis IX binds to the mitochondria and inhibits transcription by binding with DNA to the actinomycin D (act D)-related sites. These results provide new insight into the mechanisms by which Bis IX potentiates TNF receptor family-mediated apoptosis.

EXPERIMENTAL PROCEDURES

Cell Lines and Estimation of Cell Viability—The human prostatic carcinoma cell lines LNCaP, PC3, and DU145 were cultured in RPMI 1640 as has been described previously (7). Human TRAIL was purified from human embryonic kidney (293) cells infected with a recombinant replication-defective adenovirus expressing a TNF-related apoptosis-inducing ligand; LNCaP, lymph node carcinoma of prostate; mAb, monoclonal antibody; PBS, phosphate-buffered saline; CDL, cardiolipin; AFC, androgen-free conditions; FCS, fetal calf serum; DHT, dihydrotestosterone; Dox, doxorubicin; act D, actinomycin D; CHX, cycloheximide; DISC, death-signaling complex; FADD, Fas-associated polypeptide with death domain; Ac, acetyl; FLIP, flice-like inhibitory protein.

* This work was supported by National Institutes of Health Grant CA 67171 (to M. B. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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1 The abbreviations used are: Bis, bisindolylmaleimide; PKC, protein kinase C; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; LNCaP, lymph node carcinoma of prostate; mAb, monoclonal antibody; PBS, phosphate-buffered saline; CDL, cardiolipin; AFC, androgen-free conditions; FCS, fetal calf serum; DHT, dihydrotestosterone; Dox, doxorubicin; act D, actinomycin D; CHX, cycloheximide; DISC, death-signaling complex; FADD, Fas-associated polypeptide with death domain; Ac, acetyl; FLIP, flice-like inhibitory protein.
chased from PeproTech (Rocky Hill, NJ), TNF-α was purchased from R&D Systems (Minneapolis, MN), and agonistic anti-Fas (IgM) mAb was described previously (7). Bis I, II, VIII, and IX were purchased from Alexis Biochemicals (San Diego, CA). Cycloheximide and actinomycin D were purchased from Sigma. To measure cell viability, we used calcein AM assay (Molecular Probes, Eugene, OR), as described previously (8).

Assessment of Apoptosis—Apoptosis was estimated by caspase activation using both fluorogenic substrates and Western blot analysis and proteolysis of poly(ADP-ribose)/polymerase as described previously (9). In order to measure caspase activity with fluorogenic substrates, cytosolic extracts were prepared in 1% Triton X-100 buffer, pH 7.2 containing 0.1 mM phenylmethylsulfonyl fluoride and 2 μg/ml pepstatin, leupeptin, and aprotinin. 40 μg of protein lysate was incubated for 60 min in assay buffer (20 mM PIPES, pH 7.2, 100 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, 0.1% CHAPS, and 10% sucrose) with 40 μM fluorogenic substrates (Biomol): Ac-DEVD-AMC as a substrate that is cleaved by caspase-3, -7, and to some extent -8; Ac-VEID-AMC as a substrate for caspase-6; and Ac-LEHD-AMC as a substrate for caspase-9. Western blot detection of proteins was performed as described previously (7). Membranes were blocked with 5% nonfat dry milk in PBS containing 0.1% Tween 20 and then incubated with the corresponding mouse monoclonal or rabbit polyclonal antibodies: anti-cytochrome c, anti-poly(ADP-ribose)/polymerase (PharMingen), cytochrome oxidase (subunit IV; Molecular Probes), anti-caspase-3 (Transduction Laboratories, San Diego, CA), anti-caspase-2, (R&D Systems), anti-caspase-8, (Upstate, Lake Placid, NY), anti-caspase-7, and anti-caspase-9 (Oncogene, Uniondale, NY). The blots were counterstained with goat anti-mouse or anti-rabbit IgG conjugated with horseradish peroxidase (Pierce). The immunoreactive bands were visualized by incubation of the membrane with enhanced chemiluminescence reagent (Pierce).

Detection of Bis IX in Mitochondrial and Cytosolic Fractions—Mitochondria and mitochondria-free cytosol were prepared as described (10). Briefly, cells were lysed in ice-cold Mito-buffer (20 mM HEPES pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 250 mM sucrose, 0.1% phenylmethylsulfonyl fluoride, and 2 μg/ml pepstatin, leupeptin, and aprotinin) by homogenization in a small glass homogenizer with Teflon pestle. The homogenates were first spun at 800 × g to remove nuclei and cell debris and were then spun twice at 16,000 × g for 20 min at 4 °C to pellet the mitochondria. The supernatants were used as mitochondria-free cytosol, and the absence of mitochondrial and cytosolic fractions in 200 μl of Mito buffer, and fluorescence was measured with an FL600 fluorimeter using an excitation wave length at 485 nm and emission wave length at 545 nm. In addition, different amounts of cardiolipin (CDL) were incubated with Bis IX for 30 min, and fluorescence was then measured.

Detection of Bis IX in LNCaP Cells Using Fluorescent Microscopy—LNCaP cells were grown on glass slides coated with poly-l-lysine (10 μg/ml) and treated with Bis IX (10 μM) for 1 h. Cells were washed with PBS and fixed in 1% paraformaldehyde in PBS, and fluorescent Bis IX was observed in cells using either a Zeiss LSM 510 confocal microscope or Zeiss Axioskop 2 (Carl Zeiss, Inc., Thornwood, NY) with a fluorescence microscopy filter (no. 14) and Spot 2 digital camera (Diagnostic Instruments, Sterling Heights, MI). 293 and HeLa cells were transfected with 4 μg of pcDNA3.1-Hygro vector (Invitrogen) using LipofectAMINE Plus transfection protocol (Invitrogen). Four hours after transfection, serum-free medium was changed to medium containing serum. After 20 h of incubation with drugs (24 h after transfection), cell death was estimated by calcine assay. Cells were plated at a density of 7,000 cells/well in 96-well flat-bottomed plates. Reagents were added at the time of plating, and cell death was estimated 24 h later. Each point represents mean values of six replicates in one of two separate experiments, which gave similar results.

RESULTS

Bis IX Converts LNCaP and DU145 from Fas-resistant to Fas-sensitive cells—We have shown previously that LNCaP and DU145 are resistant to Fas-mediated apoptosis (7). We therefore investigated whether Bis could modulate this resistance. LNCaP and DU145 were treated with four different Bis derivatives: I, II, VIII, and IX. Cells were treated either with Bis alone or in combination with agonistic anti-Fas antibodies (IPO-4). As shown in Fig. 1, the most prominent effect was detected with Bis IX. This compound in combination with IPO-4 induced cell death in almost 80% of the cells. Importantly, Bis IX alone induced cell death in only 20% of the cells, whereas anti-Fas alone induced cell death in 5–10% of the cells. These results indicate that of the four different Bis derivatives tested, Bis IX was the most active compound in converting LNCaP and DU145 cells from Fas-resistant to Fas-sensitive.

Bis IX Potentiates Cell Death Mediated by TNF-α, Anti-Fas mAb, and TRAIL in Human Prostatic Carcinoma Cell Lines—We subsequently investigated whether Bis IX could potentiate TNF-α- and TRAIL-induced cell death. We have previously reported that PC3 is sensitive to Fas- and TRAIL-mediated apoptosis (7), LNCaP is sensitive to TNF-α treatment but resistant to Fas- and TRAIL-mediated cell death, and DU145 is resistant to treatment with all three ligands (9, 11). We also investigated whether Bis IX could potentiate cell death in sensitive cell lines under treatment with low concentrations of death-inducing ligands that by themselves cannot induce cell death. As shown in Fig. 2, Bis IX alone can kill only 20–30% of cells after 24 h of treatment. Importantly, Bis IX in combination with TNF-α, anti-Fas mAb, and TRAIL was found to be capable of enhancing cell death in up to 60–90% of all cell lines tested after 24 h of treatment. Only in DU145 did we not observe the potentiating effect of Bis IX under simultaneous
treatment with TNF-α. The highest potentiating effect of Bis IX was observed after treatment of cells in the presence of TRAIL. However, the killing effect of Bis IX alone was found to be sharply increased after 48 h of treatment. Although the potentiating effect of Bis IX was still observed after 48 h of treatment in combination with death-inducing ligands, this effect was not as prominent after 48 h as it was after 24 h of treatment. These data suggest that Bis IX affects some factor(s) that is responsible for resistance to all three death-inducing ligands.

Synergistic Effect of Bis IX on Caspase Activity under Simultaneous Treatment with Apoptosis-inducing Ligands—We next investigated the activation of caspases under Bis IX treatment in combination with TNF-α, anti-Fas mAb, and TRAIL. Caspase activity was estimated using three different fluorogenic substrates. PC3, LNCaP, and DU145 were treated for 2 and 8 h either with Bis IX and ligands separately or simultaneously. Low levels of caspase activity were detected after 2 h of treatment (data not shown), but caspase activity was sharply increased after 8 h of treatment (Fig. 3). As seen in Fig. 3, Bis IX alone only induced VEIDase activity in LNCaP. However, Bis IX dramatically increased DEVDase activity under simultaneous treatment with TNF-α, anti-Fas, and TRAIL in all cell lines. Bis IX also increased VEIDase activity in PC3 and LNCaP, but VEIDase activity in DU145 was found to be low. Although LEHDase activity was lower in PC3 and LNCaP compared with DEVDase and VEIDase activities, Bis IX clearly increased LEHDase activity under simultaneous treatment with apoptosis-inducing ligands. These results indicate that Bis IX potentiates activation of caspases and suggest that the mechanism of cell death is caspase-dependent.

Bis IX Becomes Fluorescent in Cells—Some cytotoxic drugs induce cell death via up-regulation of death receptors (12). To
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Fluorescence was measured by fluorimeter using excitation wave length at 485 nm and emission wave lengths at 530, 590, and 645 nm. Flow cytometric analysis was performed using FITC and PE channels (excitation wave length at 488 nm). The values presented are mean values derived from at least three different experiments.

| Emission | Fluorimeter | Flow cytometer |
|----------|-------------|----------------|
|          | 530 | 590 | 645 | FITC(530/20) | PE(575/26) |
| Bis I    | 400 | 13900 | 25200 | 0.6 | 3.6 |
| Bis II   | 220 | 12200 | 22500 | 0.3 | 2.5 |
| Bis VIII | 150 | 26900 | 46900 | 0.2 | 8.2 |
| Bis IX   | 0   | 40900 | 66400 | 0.8 | 16.9 |

**Bis IX Is Detected in Mitochondria**—Because Bis IX was fluorescent inside cells, we next analyzed the distribution of Bis IX inside the cells by fluorescent microscopy using Zeiss Axioskop 2. Fig. 4 reveals that Bis IX was found in a punctate pattern within the cells, both perinuclear and extending down the long processes of the cell, which is typical of a mitochondrial-staining pattern. To further analyze the localization of Bis IX in the cells, we treated cells with Bis IX for 2 h, isolated mitochondrial and cytosolic fractions, and examined the fluorescence in both fractions. We found fluorescence in the mitochondrial fraction but not in the cytosol (data not shown). However, these experiments were difficult to quantitate because it is not known how much Bis-IX is retained in the cell mitochondria. Therefore, it is reasonable to conclude that some other compound(s) in addition to CDL determine whether Bis IX-mediated cell death occurs by this mechanism. We attempted to investigate the levels of death receptor expression under Bis IX treatment by flow cytometric analysis. During these experiments we unexpectedly found very high levels of fluorescence in the cells treated with Bis IX but not stained with phycoerythrin (PE)-labeled Abs. This high level of fluorescence was noted only on the PE channel (emission at 575/26 nm) but not on the fluorescein isothiocyanate channel (emission at 530/30 nm); red fluorescence (PE channel) of LNCaP treated with Bis IX increased 50× compared with untreated cells. Cells were incubated with 10 μM Bis for 1 h at room temperature, washed twice with PBS, and resuspended in PBS, and the fluorescence of Bis I, II, VIII, and IX was measured using both flow cytometric analysis and a fluorimetric assay (FL600, Bio-Tek Instruments). The fluorescence was excited at 485 nm and analyzed at 530, 590, and 645 nm. We found that after incubation with cells the red fluorescence of Bis IX (emission at 590 and 645 nm) sharply increased, whereas the fluorescence of Bis I, II, and VIII remained at lower levels (Table I). These data show that all Bis samples tested became fluorescent in the cells, and the highest level of fluorescence was noted for Bis IX.

**Fig. 4.** Bis IX becomes fluorescent in cells and shows a mitochondrial staining pattern. LNCaP cells were grown on glass slides, and fluorescent Bis IX was observed in cells using Zeiss Axioskop 2. Bis IX can be found in a punctate pattern within the cells, both perinuclear and extending down the long processes of the cell, which is a typical mitochondrial staining pattern.

**Expression of p53, p21/WAF1, and Mdm2 during Bis IX-induced Cell Death in the Presence and Absence of Androgens**—We have shown previously that LNCaP is sensitive to TNF-α treatment and that TNF-α-mediated apoptosis in LNCaP is p53-dependent (9). In this study, we found that LNCaP became resistant to TNF-α treatment after culturing in androgen-free conditions (AFC) (Fig. 5). As can be seen from Fig. 5, 35% of LNCaP cells were killed by TNF-α when they were cultured in medium supplemented with FCS, but no cell death was observed after culturing in AFC for 14 days. To test whether Bis IX was capable of potentiating cell death in AFC, we treated LNCaP with Bis IX either alone or in combination with TNF-α after culturing LNCaP in AFC. As shown in Fig. 5, Bis IX dramatically increased the sensitivity to TNF-α treatment in LNCaP cultured in AFC. In the presence of 4 μM Bis IX the sensitivity of LNCaP was similar to LNCaP cultured in FCS when cells were treated with 20 ng/ml TNF-α.

We then investigated the levels of p53 in LNCaP after Bis IX treatment during culturing of these cells in both FCS and in AFC. Surprisingly, we found that Bis IX treatment increased...
p53 levels in cells cultured in medium supplemented with FCS but not in LNCaP cultured in AFC. To further examine whether Bis IX-induced p53 expression is androgen-dependent, we investigated p53 expression in LNCaP cultured in AFC in the presence of different concentrations of dihydrotestosterone (DHT). As shown in Fig. 6A, the induction of p53 by Bis IX treatment was found to be androgen-dependent because 10 nm DHT restored the capacity of Bis IX to increase the levels of p53.

We next investigated whether p53 accumulation is androgen-dependent specifically in cells treated with Bis IX or if p53 induction by other well known inducers of p53, doxorubicin (Dox), and gamma irradiation is androgen-dependent as well. As shown in Fig. 6B, Dox induced p53 expression regardless of the presence of androgens. The same results were obtained with gamma irradiation (data not shown). These data suggest that androgen-dependent accumulation of p53 is not a general phenomenon in response to inducers of p53 but rather that it is Bis IX-specific. The next question we asked was whether p53 accumulation after Bis IX treatment was capable of transactivating the target genes p21/WAF1 and Mdm2. As shown in Fig. 6C, Bis IX induced increased expression of p21/WAF1 and Mdm2. In contrast, the treatment of LNCaP with Bis IX did not result in induction of p21/WAF1 and Mdm2 despite the accumulation of p53. Most importantly, the increased expression of p21/WAF1 and Mdm2 mediated by Bis IX was eliminated by simultaneous treatment with Bis IX and Dox. These unexpected data raise the possibility that Bis IX mediated its effect as an inhibitor of transcription.

**Bis IX Is an Inhibitor of Transcription**—Different inhibitors of RNA polymerase II, including act D at high doses, can trigger accumulation of p53 with concomitant inhibition of p21/WAF1 and Mdm2 protein levels because of an inhibition of mRNA synthesis (13, 14). To test the hypothesis that Bis IX acts as an inhibitor of transcription, we treated LNCaP with Bis IX (4 μM), act D (4 μM), and cycloheximide (CHX, 10 μg/ml) and examined the levels of p53, p21/WAF1, and Mdm2 by Western blot analysis. As shown in Fig. 7, both act D and Bis IX induced accumulation of p53 to the same extent and decreased the levels of p21/WAF1 and Mdm2. Compared with act D and Bis IX, CHX modulated the levels of these proteins differently. These differences cannot be explained by increased proteolysis of p21/WAF1 and Mdm2 in the presence of p53 accumulation. CHX did not induce accumulation of p53, but the levels of Fig. 6. Bis IX and Dox effects on expression of p53, p21/WAF1, and Mdm2. A, Bis IX-induced accumulation of p53 is androgen-dependent. LNCaP cells were cultured in medium supplemented with FCS or in AFC for 14 days in the absence of DHT or in the presence of 10 pt and 10 NM DHT. Cells were treated with Bis IX (4 μM) for 8 h, cells were lysed in 2× sample buffer, 20 μg of proteins were separated on 4–20% SDS-PAGE, and expression of p53 was examined by Western blot analysis. B, Dox-induced accumulation of p53 is androgen-independent. LNCaP cells were cultured either in normal medium or in AFC for 14 days, cells were treated with Bis IX (4 μM) or Dox (1 μg/ml) for 8 h, and expression of p53 was examined by Western blot analysis. C, Bis IX eliminates Dox-induced accumulation of p21/WAF1 and Mdm2. LNCaP cells were cultured in medium supplemented with FCS, were treated for 8 h with Bis IX (4 μM) and Dox (1 μg/ml) either separately or simultaneously, and expression of p21/WAF1 and Mdm2 were examined by Western blot analysis.

Fig. 7. Expression of p21/WAF1, p53, and Mdm2 is affected similarly by act D and Bis IX but differently from CHX. LNCaP cells were treated with act D (4 μM), CHX (10 μg/ml), and Bis IX (4 μM) for 8 h, and expression of p21/WAF1, p53, and Mdm2 was examined by Western blot analysis.
A, Bis IX became fluorescent in the presence of DNA, and this fluorescence was found to be dose-dependent. We then investigated whether act D could interfere with Bis IX binding to DNA. As shown in Fig. 9B, act D prevented the binding of Bis IX to DNA. These data suggest that act D and Bis IX interact with overlapping or identical sites on DNA. Despite the finding that Bis IX binds DNA and became fluorescent in the presence of DNA in solution, we did not observe the Bis IX fluorescence in nuclei (Fig. 4). Bis IX, as an inhibitor of transcription, appears to interact only with DNA sites that are not covered by chromosomal proteins. The number of available Bis IX binding sites on chromosomal DNA may be too few to make this interaction detectable with fluorescence microscopy. In contrast, Bis IX can be readily detected in mitochondria because Bis IX became fluorescent not only in the presence of DNA, that is

9A, Bis IX became fluorescent in the presence of DNA, and this fluorescence was found to be dose-dependent. We then investigated whether act D could interfere with Bis IX binding to DNA. As shown in Fig. 9B, act D prevented the binding of Bis IX to DNA. These data suggest that act D and Bis IX interact with overlapping or identical sites on DNA. Despite the finding that Bis IX binds DNA and became fluorescent in the presence of DNA in solution, we did not observe the Bis IX fluorescence in nuclei (Fig. 4). Bis IX, as an inhibitor of transcription, appears to interact only with DNA sites that are not covered by chromosomal proteins. The number of available Bis IX binding sites on chromosomal DNA may be too few to make this interaction detectable with fluorescence microscopy. In contrast, Bis IX can be readily detected in mitochondria because Bis IX became fluorescent not only in the presence of DNA, that is

“naked” in mitochondria but also in the presence of mitochondria-specific cardiolipin.

To compare the effects of Bis IX and act D on caspase activity, we treated the TRAIL-resistant cell line DU145 for 8 h with act D (4 μM), Bis IX (4 μM), and TRAIL (100 ng/ml) either separately or in a combination of Act D + TRAIL and Bis IX + TRAIL. Caspase activity was measured in fluorogenic substrates (A) as described under “Experimental Procedures” and in the legend to Fig. 3. B, 20 μg of proteins were separated on 4–20% SDS-PAGE, and activation of caspase-3 and -7 was estimated by Western blot analysis. Arrows indicate the activated subunits of caspase-3 and -7.

**DISCUSSION**

One promising approach for treating apoptosis-resistant tumor cells is to use cytophagic drugs to sensitize cells to death-inducing ligands (15, 16). From this point of view, the study by Zhou et al. (4) is very promising. That Bis VIII potentiated Fas-mediated apoptosis in human astrocytoma 1321N1 cells, different tumor T cell lines, and activated T cells is intriguing. Moreover, Bis VIII has activity in vivo by preventing the development of symptoms of T cell-mediated autoimmune diseases. Although Bis derivatives were originally synthesized as PKC inhibitors (1–3), Zhou et al. (4) found that the inhibition of PKC cannot account for the potentiation of Fas-mediated apoptosis by Bis.

Based on our own previous work in receptor/ligand-induced

**FIG. 8. Bis IX and act D inhibit expression of hygromycin gene.** HeLa cells were transfected with 4 μg of pcDNA3.1-Hygro vector. Four hours after transfection, cells were treated with act D or Bis IX. Bar 1, untransfected cells; bar 2, transfected no treatment with drugs; bar 3, Bis IX (4 μM); bar 4, Bis IX (10 μM); bar 5, Bis IX (20 μM); bar 6, act D (4 μM). After 20 h of treatment with drugs, RNAs were isolated, resolved on a formaldehyde-agarose gel, blotted onto a nylon membrane, and probed with labeled hygromycin-encoding sequence. The right panel shows the amount of transcript as a percent of control.

**FIG. 9. Act D prevents Bis IX fluorescence in the presence of DNA.** A, Bis II and Bis IX (both 10 μM) were incubated with different concentrations of DNA in Tris-buffered saline buffer for 30 min, and fluorescence was measured with excitation at 485 nm and emission at 645 nm. DNA was isolated from LNCaP cells. The same results were obtained with three different plasmid DNA (data not shown). B, Bis IX was incubated with 5 μg of DNA either in the absence or presence of act D (5 μM). Values shown are mean ± S.D.

**FIG. 10. Act D and Bis IX induce caspase activity to the same extent.** DU145 cells were treated for 8 h with act D (4 μM), Bis IX (4 μM), and TRAIL (100 ng/ml) either separately or in a combination of Act D + TRAIL and Bis IX + TRAIL. Caspase activity was measured with fluorogenic substrates (A) as described under “Experimental Procedures” and in the legend to Fig. 3. B, 20 μg of proteins were separated on 4–20% SDS-PAGE, and activation of caspase-3 and -7 was estimated by Western blot analysis. Arrows indicate the activated subunits of caspase-3 and -7.
cell death (7–11), it was of great interest to investigate whether Bis could modulate the resistance of human prostatic carcinoma cells to TNF receptor family-induced apoptosis. Certain cytotoxic drugs can induce cell death through up-regulation of death receptors (12). However, we found that the expression of Fas receptor (CD 95), TRAIL-R1, and TRAIL-R2 was decreased by 30–50% under Bis IX treatment for 24 h (data not shown). These results show that, in contrast to the effect of cytotoxic drugs that increase the surface expression of death receptors (12), Bis IX mediates its effect by another mechanism(s). Our data suggest that Bis IX inhibits the production of some factor(s) that is responsible for the resistance to all three death-inducing ligands: TNF-α, Fas, and TRAIL.

The capacity of different Bis derivatives to overcome the resistance of tumor cells to TNF family death ligands is not cell type-specific. Bis VIII enhanced apoptosis after treatment of Jurkat cells with agonistic antibodies to Fas, TNF-α, and TRAIL (6). Bis III was found to inhibit FLIP<sub>L</sub> accumulation in dendritic cells and sensitize Fas- and TRAIL-mediated apoptosis (5), and Bis VIII potentiated both Fas- and TNF-α-mediated apoptosis in human astrocytoma 1321N1 cells (4). At the same time the mechanisms by which Bis acts in different cell types may be different. For example, Bis I, II, and IV were active on dendritic cells (5), but T cells were not affected by these Bis derivatives (4). Bis VIII and IX showed the same activity in potentiating Fas-mediated apoptosis in 1321N1 cells (4), whereas we found that in DU145 Bis IX was 3 × more active than Bis VIII. Bis VIII was shown to potentiate Fas-mediated apoptosis in Jurkat cells without enhancing DISC formation (6). Although Bis VIII can alter the phosphorylation status of Fas-associated polypeptide with death domain (FADD), the total amount of FADD in the DISC was unchanged after treatment (6). Therefore, Bis VIII apparently accomplishes its effect downstream of the DISC.

We exploited the fluorescent properties of Bis IX to examine the distribution of Bis IX in cells. Bis IX was found to bind to mitochondria. The ability of Bis to bind to mitochondria has also been shown in one other study (17). Localization of fluorescent derivatives of bisindolylmaleimide (fim-1, fim-2, rim-1), synthesized by Chen and Poenie (17), were investigated and compared with staining patterns with antibodies to PKC. Fim-1 stained mitochondria, but no staining was identified with antibodies to PKC. Thus, the ability of Bis to interact with mitochondria does not appear to be linked to the capacity to interact with PKC.

To further investigate the mechanism of Bis IX-mediated cell death, we examined the effect of Bis IX using the androgen-dependent cell line LNCaP. We have previously shown that TNF-α-mediated apoptosis in LNCaP is p53-dependent (9). In this study, we observed that LNCaP became resistant to treatment with TNF-α after culturing in AFC. We therefore compared the effect of Bis IX on LNCaP cultured in regular medium and in AFC. We found that Bis IX dramatically increased the sensitivity to TNF-α treatment in LNCaP cultured in AFC. This effect was not dependent on an accumulation of p53, and Bis IX was found to mediate its effect as an inhibitor of transcription. We have shown previously that cycloheximide converted the phenotype of prostate cancer cell lines from Fas- and TNF-α-resistant to -sensitive (7). We have also found that resistance to Fas- and TNF-α-mediated apoptosis dominates over-sensitivity in cell hybrids, and these findings suggest that the resistance may be regulated by an apoptosis suppressor factor(s) (18). Here, we found that Bis IX potentiates cell death in prostate tumor cell lines that are resistant to Fas-, TNF-α-, and TRAIL-mediated apoptosis. Bis IX alone cannot induce caspase activity. However, eight hours of treatment with Bis IX in the presence of death-inducing ligands was found to be sufficient to induce caspase activity in resistant cells. These results suggest that this resistance is dependent on the presence of a labile protein(s) that determines the resistance to TNF receptor family-mediated killing. We have examined the levels of FLIP<sub>L</sub>, FLIP<sub>S</sub>, IAP1, IAP2, and XIAP, which are known to inhibit apoptosis in some cell lines (19, 20). However, the levels of these proteins were unchanged after treatment with Bis IX (data not shown). It remains to be determined which protein(s) is modulated under Bis IX treatment.

It is well known that an effective therapy for androgen-independent prostate cancer is urgently needed. There are a limited number of agents that are cytotoxic to prostate tumor cells. However, these agents typically induce death in proliferating cells. Bis IX (or other Bis derivatives) may be good candidates for treatment of prostate tumor cells for the following reasons. First, Bis IX is capable of potentiating TNF receptor family-induced apoptosis in LNCaP even after androgen withdrawal when cells stop proliferating. Second, the effect of Bis IX is p53-independent; Bis IX potentiated TNF receptor family-induced apoptosis not only in LNCaP, which expresses wild-type p53, but also in PC3, which is p53-null, and in DU145, which expresses two different p53 mutants. Third, inhibition of transcription by Bis IX in normal cells should not have significant consequences. This point of view is supported by Zhou et al. (4); in their experiments rats were injected with 250 µg of Bis VIII every other day for five doses without side effects. In addition, they showed that Bis VIII in vivo did not have a substantial effect on activated T cells from Fas-deficient lpr/lpr mice. Moreover, Bis VIII selectively potentiated apoptosis in activated T cells while having little effect on non-activated T cells. TRAIL-based therapy is a promising approach (15, 16), and simultaneous treatment with bisindolylmaleimides and TRAIL may be useful therapeutically, specifically for treatment of androgen-independent prostate cancer.

REFERENCES

1. Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Lorilolle, F., Duhamel, L., Charon, D., and Kirovsky, J. (1991) J. Biol. Chem. 266, 15771–15781
2. Bit, H. A., Jarvis, P. D., Elliott, L. H., Harris, W., Hill, C. H., Keech, E., Kumar, A. Lawton, G., Maw, A., and Nixon. J. S. (1993) J. Med. Chem. 36, 21–29
3. Jacobson, P. E., Kuchera, S. L., Metz, A., Schachtele, C., Imre, K., and Schrier, D. J. (1995) J. Pharmacol. Exp. Ther. 275, 995–1002
4. Zhou, T., Song, L., Yang, P., Wang, Z., Lui, D., and Jope, R. S. (1999) Nat. Med. 5, 42–48
5. Willers, F., Amraoui, Z., Vanderheynde, N., Verhasselt, V., Askoy, E., Scaffidi, C., Peter, M. E., Kramer, P. H., and Goldman, M. (2000) Blood 95, 3478–3482
6. Meng, X. W., Helderbrant, M. P., Kaufmann, S. H. (2002) J. Biol. Chem. 277, 3776–3783
7. Rohklin, O. W., Bishop, G. A., Hostager, B. S., Gudkov, A. V., Kwek, S., Glover, R. A., Gewies, A. S., and Cohen, M. B. (1997) Cancer Res. 57, 1758–1768
8. Rohklin, O. W., Guseva, N. V., Tagiyev, A., Koudoun, M. C., and Cohen, M. B. (2001) Oncogene 20, 2836–2843
9. Rohklin, O. W., Gudkov, A. V., Kwek, S., Glover, R. A., Gewies, A. S., and Cohen, M. B. (2000) Oncogene 19, 1959–1963
10. Gewies, A., Rohklin, O. W., and Cohen, M. B. (2000) Cancer Res. 60, 2163–2168
11. Rohklin, O. W., Guseva, N. V., Tagiyev, A. F., Glover, R. A., and Cohen, M. B. (2002) Prostate 52, 1–11
12. Micheau, O., Solary, E., Hamann, A., and Dimanche-Boitrel, M. T. (1999) J. Biol. Chem. 274, 7987–7992
13. Ljungman, M., Zhang, F., Chen, F., rainbow, A. J., and McKay, B. C. (1999) Oncogene 18, 583–592
14. An, W. G., Chuman, Y., Fojo, T., and Blagosklonny, M. V. (1998) Exp. Cell Res. 244, 54–60
15. El-Deiry, W. S. (2001) Cell Death Differ. 8, 1066–1075
16. Ashkenazi, A. (2002) Nat. Rev. Cancer 2, 420–430
17. Chen, C.-S., and Poenie, M. (1993) J. Biol. Chem. 268, 15812–15822
18. Rohklin, O. W., Hostager, B. S., Bishop, G. A., Koudoun, S. P., Glover, R. A., Gudkov, A. V., and Cohen, M. B. (1997) Cancer Res. 57, 3941–3943
19. Kim, K., Fisher, M. J., Xu, S.-O., and El-Deiry, W. S. (2000) Clin. Cancer Res. 6, 335–346
20. Reed, J. C. (2000) Am. J. Pathol. 157, 1415–1430