ARTICLE

BMD188, A Novel Hydroxamic Acid Compound, Demonstrates Potent Anti-Prostate Cancer Effects *in vitro* and *in vivo* by Inducing Apoptosis: Requirements for Mitochondria, Reactive Oxygen Species, and Proteases

Dean G TANG,[1-3] Li LI,[1] Zhenyu ZHU,[1] Bindu JOSHI,[1] Carl R JOHNSON,[4] Lawrence J MARNETT,[5] Kenneth V HONN,[2,3] John D CRISSMAN,[3,6] Stanislaw KRAJEWSKI,[7] John C REED,[7] József TIMAR,[8] and Arthur T. PORTER[2,3]

[1]Biomide Laboratories, and Departments of [2]Radiation Oncology, [4]Chemistry, and [6]Pathology, Wayne State University, Detroit, [3]Karmanos Cancer Insitute, Detroit, [5]Department of Biochemistry, Vanderbilt University Medical Center, Nashville, [7]Apoptosis & Cell Death Program, The Burham Institute, La Jolla, [8]1st Institute of Pathology and Experimental Cancer Research, Semmelweis University of Medicine, Budapest, Hungary

A newly synthesized cyclic hydroxamic acid compound, BMD188 [cis-1-hydroxy-4-(1-naphthyl)-6-octylpiperidine-2-one], was found to induce the apoptotic death of cultured prostate cancer cells by activating caspase-3. Orally administered BMD188 significantly inhibited the primary growth of prostate cancer cells (Du145) orthotopically implanted into SCID mice. Mechanistic studies indicated that BMD188 did not alter the protein levels of several Bcl-2 family members. In contrast, the BMD188 effect required three essential factors: reactive oxygen species (ROS), the mitochondrial respiratory chain function, and proteases. First, the apoptosis-inducing effect of BMD188 could be blocked by ROS scavengers such as Desferal. Second, both BMD188-induced PARP cleavage as well as PC3 cell apoptosis could be dramatically inhibited by several complex-specific mitochondrial respiration blockers. The involvement of mitochondria was also supported by the observations that BMD188 dramatically altered the mitochondrial distribution and morphology without affecting the cellular ATP levels. Finally, the apoptosis-inducing effect of BMD188 in PC3 cells could be significantly inhibited by serine protease inhibitors (TPCK and TLCK) as well as by caspase inhibitors (zVAD-fmk and DEVD-CHO). Collectively, the present study suggests that BMD188 and its analogs may find clinical applications in the treatment of prostate cancer patients by inducing apoptotic death of prostate cancer cells. (Pathology Oncology Research Vol 4, No 3, 179–190 1998)

Key words: hydroxamic acid, prostate cancer, apoptosis, free radical, mitochondria, chemotherapy

Introduction

Hormone-refractory prostate cancer cells could be induced to undergo programed cell death or apoptosis by various chemotherapeutic drugs or radiation. Unfortunately, these treatment regimens could rarely eradicate the whole tumor mass due to the evolution of apoptosis-resistant clones. So far at least three new approaches (reviewed in ref. 1) are being developed with aim to eliminating the whole tumor population: identification of novel, more potent chemicals or natural products (e.g., Linomide and β-lapachone;[2,3]), p53 and bcl-2-targeted gene therapy,[4-6] and novel mechanism-based drug development, such as modulators of apoptosis signal transduction pathways.[7] We have recently synthesized a series of cyclic hydroxamates (~200 compounds; US patents 5,292,884 and

Received: May 26, 1998, *accepted:* August 18, 1998

Correspondence: Dean G TANG,[1] Department of Radiation Oncology Wayne State University, 407 Life Science Bldg., Detroit, MI 48202; Tel: (313) 577-2184; fax: (313) 577-2375; e-mail: dtang@sun.science.wayne.edu; dtang@ucl.ac.uk

© 1998 W. B. Saunders & Company Ltd on behalf of the Arányi Lajos Foundation 1219-4956/98/030179+12 $ 12.00/0

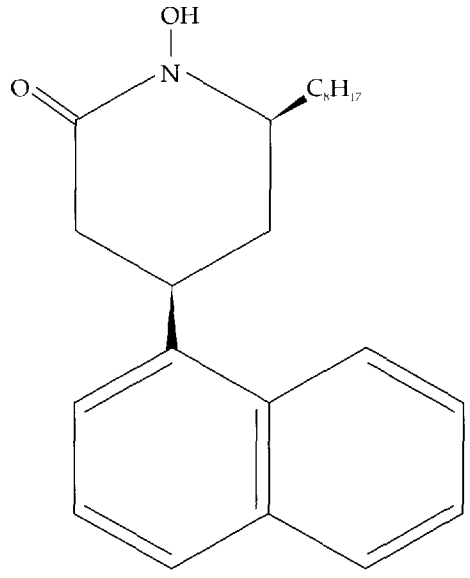

Figure 1. *Chemical structure of BMD188.*

5,234,933) that inhibit the arachidonate leukocyte-type 12-lipoxygenases. Since we have previously demonstrated a potential role of lipoxygenases in regulating tumor cell survival and apoptosis,[8-10] we thus screened these novel chemical entities for their biological effects on prostate cancer cells. The results indicate that some hydroxamates induce a strong apoptotic response (up to 100% cell killing) in multiple prostate cancer cells and many other types of cancer cells *in vitro*.[11] In this study, we thoroughly characterized the biological effect of one lead compound termed BMD188 on human prostate cancer cells. We further studied its mechanism of action and observed that the effect of BMD188 involved mitochondrial dysfunction, free radical accumulation, and protease activation. Finally, BMD188 given by oral gavage inhibited both primary tumor growth as well as the invasion of orthotopically implanted Du145 human prostate cancer cells in SCID mice.

Materials and Methods

Cell culture, chemicals, and reagents

PC3 and Du 145 cells were obtained from ATCC and cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and antibiotics. HL60 cells were cultured in RPMI medium as previously described.[11] The following primary antibodies were used in the immunoblotting experiments: mouse monoclonal anti-human Bcl-2 (clone 124; Dako), rabbit polyclonal anti-Bax (one batch obtained from PharMingen and the other batch prepared as previously described;[12]), anti-Bad, and anti-Bak antibodies (Santa Cruz Biotech., CA), rabbit polyclonal anti-poly-(ADP-ribose) polymerase (Boehringer Mannheim), monoclonal anti-caspase-3 (Transduction Laboratories, KY), and monoclonal anti-actin (ICN). The secondary antibody was either goat anti-rabbit or anti-mouse IgG (whole molecule) conjugated to horseradish peroxidase (Biorad). ECL reagents were bought from Amersham. Protease inhibitors TPCK (tosyl-L-phenylalanyl chloromethyl ketone), TLCK (tosyl-L-lysyl chloromethyl ketone), ALLN (N-Ac-Leu-Leu-normethioninal), and iodoacetamide were obtained from Calbiochem. Peptide inhibitor zVAD-fmk (benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone) and a control peptide Phe-Ala-fmk were purchased from Enzyme Systems, Inc. (Dublin, CA). Cell permeable tetrapeptides DEVD-CHO (N-Ac-Asp-Glu-Val-Asp aldehyde) and YAVD-CHO (N-Ac-Tyr-Val-Ala-Asp aldehyde), which were linked at the N-terminal to the hydrophobic signal sequence from k-FGF, were obtained from Biomol.

Chemical synthesis and characterization of BMD18

The synthesis of hydroxamic acids was previously detailed (US patents 5,292,884 and 5,234,933). The chemical synthesis and characterization of BMD188 *(Figure 1)* were previously detailed.[13]

Effects of free radical scavengers on BMD188-induced PC3 cell apoptosis

PC3 cells ($0.5x10^6$/T25 flask) were pretreated for 1 h with lactoferrin (1-20 μM) or Desferal (0.1–1 mM), followed by BMD188 treatment. At the end of the treatment, cells in the whole flask were collected and cell survival was determined by the trypan blue dye exclusion/apoptosis morphology assays.[8-10] The results were expressed as the % of the vehicle control. Each condition was run in triplicate and the experiments were repeated a minimum of three times.

Effects of protease inhibitors on BMD188-induced PC3 cell apoptosis

PC3 cells ($1x10^6$) cultured in 35 mm^2 culture dishes were pretreated with ethanol, TPCK (0.02–0.2 mg/ml), TLCK (0.01–0.1 mg/ml), iodoacetamide (5–50 μg/ml), ALLN (50–500 μM), zVAD-fmk (5–50 μM), DEVD-CHO (5 μM), YVAD-CHO (5–50 μM), or control peptide Phe-Ala-fmk (50 μM) for 45 min, followed by treatment with BMD188 (50 μM) for 2 h. Then all cells were harvested and used for PARP cleavage assays (see below). To quantitate the effect of these protease inhibitors on BMD188-induced apoptosis, PC3 cells cultured in flat-bottom 24-well plates ($2.5x10^4$ cell/well) were similarly pretreated and treated as described above and, at the end of 2 h, apop-

tosis was quantitated by counting apoptotic cells based on typical morphology.[8-10] Each condition was run in triplicate and 500-1,000 cells were counted for each condition.

PARP [poly-(ADP-ribose) polymerase] cleavage and Western blotting

Western blotting protocols were detailed before.[8-10] Whole cell lysates were prepared by directly lysing control and drug-treated PC3 cells in TNC (10 mM Tris-acetate, 0.5% NP-40, and 5 mM $CaCl_2$, pH 7.4) buffer and solubilized proteins analyzed on 4–20% gradient PAGE. Isolated proteins were transferred to nitrocellulose and probed with individual primary antibodies. The secondary antibody used was goat anti-rabbit or -mouse IgG conjugated to horseradish peroxidase. For PARP cleavage assays, an identical number ($2.5x10^5$/well) of PC3 cells were plated onto 6-well petri dishes and treated with different doses of BMD188 for various time intervals. At the end of the treatment, cells were directly scraped into 2x sample buffer followed by boiling. Thereafter, equal amounts of samples were loaded onto a 7.5% SDS-PAGE and the 116 kd-PARP and the 85 kd cleavage product were detected with a polyclonal antibody using ECL.[11]

Mitochondrial labeling with MTT staining

The MTT ((3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazodium bromide) assay works by being metabolized by mitochondrial dehydrogenases to form formazan dyes. $5x10^4$ PC3 cells/well cultured in 24-well plates were treated with 40 µM BMD188 for 30 min and then, after washing, incubated (at 37°C) with 0.5 mg/ml MTT prepared in RPMI for 1.5 h. Viable cells with active mitochondria cleave the tetrazolium ring into a visible dark blue formazan reaction product while dead cells are not stained. The mitochondrial distribution and morphology were monitored under a phase contrast microscope.

ATP assays

The intracellular ATP levels were determined using the ATP Assay kit obtained from Sigma (Cat# 366-A). The procedure was based on the reaction: ATP + 3-phosphoglycerate → ADP + 1,3-diphosphoglycerate (catalyzed by phosphoglyceric phosphokinase or PGK) and 1,3-diphosphoglycerate + NADH → Glyceraldehyde-3-P + NAD + Pi (catalyzed by glyceraldehyde phosphate dehydrogenase or GAPD). Formation of NAD was then quantitated by measuring the change in absorbance at 340 nm. By determining the decrease in absorbance at 340 nm that results when NADH is oxidized to NAD, a measure of the amount of ATP originally present is obtained. PC3 cells or HL60 cells ($3x10^6$ total) treated with BMD188 for various time intervals were resuspended in 1 ml PBS at the end of the treatment. 1 ml of 12% TCA was then added to the cell suspension and the mixture allowed to stand in ice for 5 min. After centrifugation, ATP levels were determined by following the Instruction Manuals from the manufacturer. The results were plotted as % of the time 0 (i.e., without BMD188).

Activation of caspase-3 (CPP32)

Two independent methods were used to measure the caspase-3 activation. In the first, PC3 cells ($3x10^6$) were treated with BMD188 (40 µM) for 5, 15, 30 min, 1 h, or 2 h and then the whole cell lysates were harvested using the TNC lysis buffer, as described above. One hundred µg/lane of proteins was separated on a denaturing 15% SDS-PAGE and proteins were transferred to nitrocellulose membrane. Western blotting was carried out as described above, using the monoclonal anti-CPP32. The activation of caspase-3 was monitored by the appearance of the cleavage product(s). In the second method, the caspase-3 activity was measured using the fluorogenic substrate DEVD-AFC. Briefly, PC3 cells ($2x10^6$/10 cm dish) were treated with 40 µM BMD188 for 0, 5, 15, 30, 45, 60, 90, and 120 min. At the end of the treatment, whole cell lysates were prepared in cell lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM $NaH_2PO_4/NaHPO_4$, pH 7.5, 130 mM NaCl, 1% Triton X-100, 10 mM NaPPi). After protein concentration measurement (Lowry), 100 µg protein was coincubated with 30 µM DEVD-AFC substrate in a total of 2 ml reaction buffer (20 mM HEPES, pH 7.5, 10% glycerol, 2 mM DTT) at 37°C for 1 h. Subsequently, the activation of caspase-3 was monitored by reading the samples on a Perkin-Elmer LS-5 fluorescence spectrophotometer at excitation wavelength of 400 nm and emission wavelength of 505 nm. All the samples were blanked against the reaction buffer with the substrate alone. The results were expressed as the fold increase in activation compared to time 0, at which time the CPP32 activity was considered as 1. Each experiment was run in duplicate the experiments were repeated three times.

In vivo anti-tumor activity of BMD188 on Du145 human prostate cancer cells orthotopically implanted into SCID mice

Du145 cells (10^6) were injected microsurgically into the two prostate lobes of SCID (C57BL/CBA hybrid) mice under anesthesia (Nembutal) in 50 µl of serum-free RPMI.[14] Tumor cells were then allowed to grow for 2 weeks before treatment. BMD188 was first solubilized in ethanol and then mixed with corn oil. Then BMD188 was given to animals by oral gavage at 0, 25, 100, 250, and 500 mg/kg every other day from day 14. In total 12 oral admin-

istrations were carried out. At the end of treatment (i.e., day 39 since tumor cell inoculation), animals were sacrificed by Nembutal inhalation. Primary tumors were isolated and measured with a caliper and the sizes were expressed as mm^3. Local tumor invasion into pelvic organs (prostate capsules, testis, urinary bladder, and rectum) and distant metastasis (liver, kidney, lung, and intestine) were examined both macroscopically and microscopically by tissue sectioning and HE staining. All animals were maintained in the SCID Laboratory according to standard animal welfare/husbandry conditions. The statistical analysis was carried out using the SigmaPlot software.

Results

BMD188 Induces PC3 Cell Apoptosis by Activating Caspase-3 (or CPP32)

Our previous studies[11,13] have established that: *a*) Hydroxamic acid compounds induce apoptotic death (as revealed by morphology, MTS assays, DAPI staining and PARP cleavage assays) of multiple types of prostate cancer cells; *b*) Hydroxamic acid compounds induce a much stronger apoptotic response in PC3 prostate cancer cells than several standard chemotherapeutic drugs (including adriamycin, bleomycin, cisplatin, camptothecin, taxol, methotrexate, VP16, vinblastine, and vincristine); *c*) Hydroxamates kill both androgen-dependent and –independent prostate cancer cells in a p53- and cell cycle-independent manner. They also kill prostate cancer cells with MDR (multidrug resistance) phenotype; *d*) Hydroxamates preferentially kill prostate cancer cells compared to various normal cells including normal prostate epithelial cells; *e*). *In vivo* experiment with small number of animals has indicated the antitumor efficacy with BMD188, a hydroxamate lead compound; *f*) Preliminary data suggest that the BMD188 specifically targets mitochondria to induce apoptosis.

In this study, we performed a series of new experiments either to confirm or extend our previous observations. As shown in *Figure 2A*, BMD188 activated caspase-3 in PC3 cells, as evidenced by the cleavage of the protein from its parental 32kd form to the P17 form. Using a more sensitive assay, i.e., the cleavage of a fluorogenic substrate (DEVD-AFC), it was shown that a low level (i.e., ~3-fold) of caspase-3 activation was observed at 45 min post BMD188 treatment *(Figure 2B)*. More than 10-fold activation of caspase-3 was observed by 60 min and the maximum activation (~25 fold) was noticed at ~90 min post stimulation *(Figure 2B)*. As CPP32 was activated at 45-60 min post BMD188 stimulation, PARP, a known caspase-3 substrate, was also cleaved (from 116kd to 85 kd; *Figure 2A*, lower panel). By 2 h when >90% PC3 cells were apoptotic,[11,13] significant CPP32 activation and nearly complete PARP cleavage were observed (*Figure 2*).

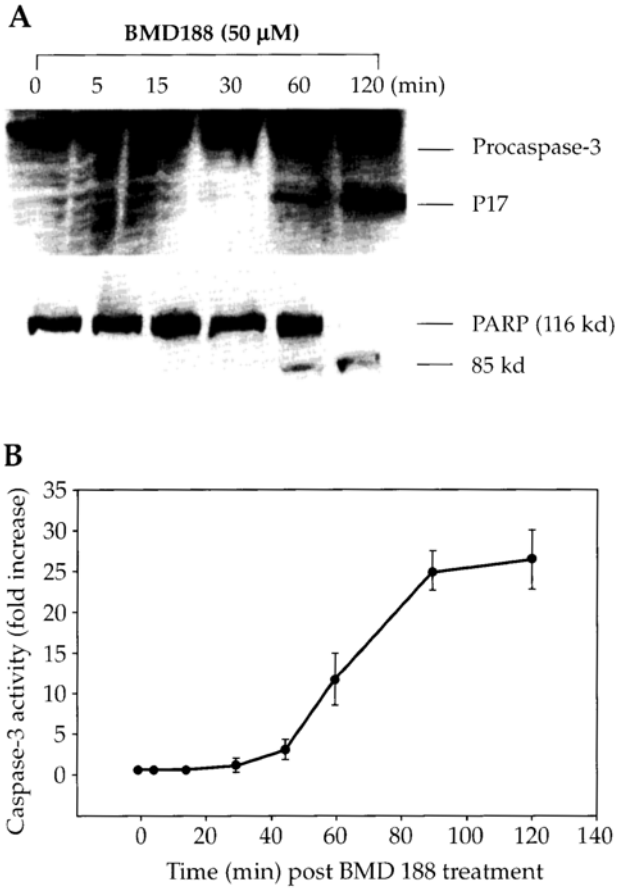

Figure 2. *BMD188 induces PC3 cell apoptosis by activating caspase-3. A. Upper panel, caspase-3 activation as revealed by Western blotting. The 32 kd pro-caspase-3 and the complete cleavage product (P17) were indicated on the right. Lower panel, PARP cleavage. The 116 kd-band represents the parental PARP protein and the 85 kd-band the cleavage product. B. Caspase-3 measurement using the fluorogenic substrate DEVD-AFC. The data were expressed as the fold activation of caspase-3 over the control (i.e., the ethanol-treated PC3 cells). Shown are the mean ± SEM derived from three independent experiments.*

Inhibition by BMD188 of primary tumor growth and invasion of Du145 prostate tumor cells orthotopically implanted into SCID mice

Preliminary studies indicated that PC3 tumor cells orthotopically implanted into SCID mice did not metastasize very well;[14] *Timár et al.*, unpublished observations). Therefore, in all of our *in vivo* studies we used another human prostate cancer cell line, Du145. Du145 cells were more malignant than PC3 cells in the sense that these cells survive the longest in the absence of trophic factors and they were extremely resistant to apoptosis induction by serum withdrawal.[15] Previous studies with a small set of animals demonstrated that BMD188 given by i.p. injection

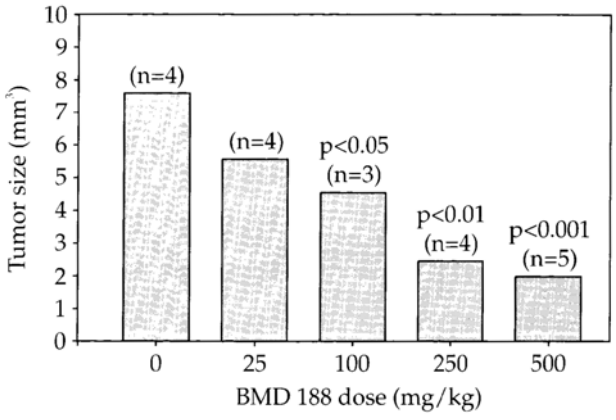

Figure 3. *BMD188 administered p.o. inhibits the primary tumor growth of Du145 prostate cancers orthotopically transplanted into SCID mice. Shown were the mean values of each group. The S.Ds were <10%. Indicated p values were obtained by comparing with the control (i.e., 0) group.*

inhibited the *in vivo* growth and invasion of Du145 human prostate cancer tumors orthotopically implanted into SCID mice.[13] The *in vivo* anti-tumor effect of BMD188 was further evaluated in this study by oral administration. The experiment was initially started with 25 animals in each group. The animals were sacrificed after 12 oral deliveries of the BMD188. As shown in *Figure 3*, BMD188, in a dose-dependent manner, inhibited the growth of the primary tumor. The unequal number of animals in each group (*Figure 3*) was due to loss prior to drug delivery (accidental death) or cumulative toxicity. BMD188 also appeared to inhibit the local invasion of Du145 tumor cells. The metastasis incidence was 4/4 (the control group), 0/4 (the 25 mg/kg) group), 2/3 (the 100 mg/kg group), (the 250 mg/kg group) and 0/5 (the 500 mg/kg group), respectively. No distant metastasis in lung, liver, spleen, and kidney was observed in any animal during this experiment.

BMD188-Induced PC3 Cell Apoptosis Involves Free Radical Generation

We next explored the molecular mechanism(s) of action for BMD188-induced PC3 apoptosis. Hydroxamate compounds were initially synthesized as leukocyte 12-lipoxygenase inhibitors. However, PC3 and Du145 cells do not express 12-lipoxygenase mRNA as revealed by Northern blotting and nested RT-PCR (data not shown). These cells also do not express the lipoxygenase protein or metabolize exogenous arachidonic acid to 12(S)-HETE, the natural metabolic product of 12-lipoxygenase (data not shown). These observations suggest that BMD188 induces prostate cancer cell apoptosis independently of its lipoxygenase-inhibitory activity. We next examined, by quantitative Western blotting using the protocol we recently developed,[15] the effect of BMD188 on several Bcl-2 family proteins including Bcl-2, Bad, Bak, and Bax. The results did not reveal any significant changes in the expression levels of these proteins following BMD188 treatment *(Figure 4)*.

Previous observations by others[16,17] have demonstrated that hydroxamates may perturb the cellular redox homeostasis. This possibility was thus tested by pretreating PC3 cells with a panel of free radical scavengers prior to BMD188 treatment. The results indicated that the BMD188-triggered PC3 cell apoptosis could be significantly blocked by PDTC (a general free radical scavenger), SOD (a superoxide scavenger), and tryptophan (a scavenger of singlet oxygen), in a dose-dependent manner, thus suggesting that 188 treatment led to free radical (e.g., superoxide and singlet oxygen) generation. Subsequent experiments confirmed the generation of ROS upon BMD188 treatment (Joshi et al., manuscript in preparation).

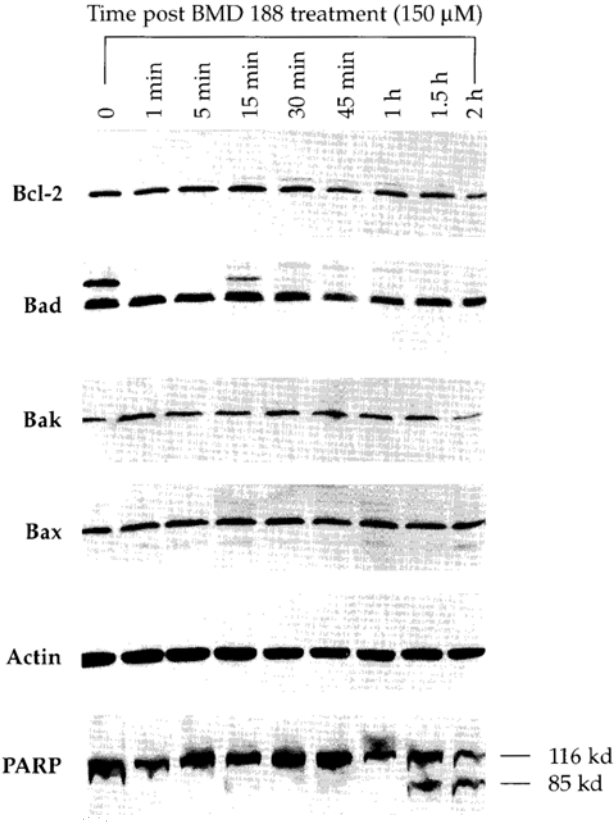

Figure 4. *BMD188 does not alter the levels of the Bcl2-family proteins. Cultured PC3 cells were treated with BMD188 for various time intervals and then harvested for Western blot analysis of individual Bcl-2 family proteins or for PARP. The blot was probed and then reprobed for Bcl-2, Bad, Bak, and Bax. Actin levels were used as the internal control. In this specific experiment, PARP was cleaved at ~1.5 h post BMD188 treatment. Note there were no dramatic alterations in any of the proteins examined after normalizing to the actin levels.*

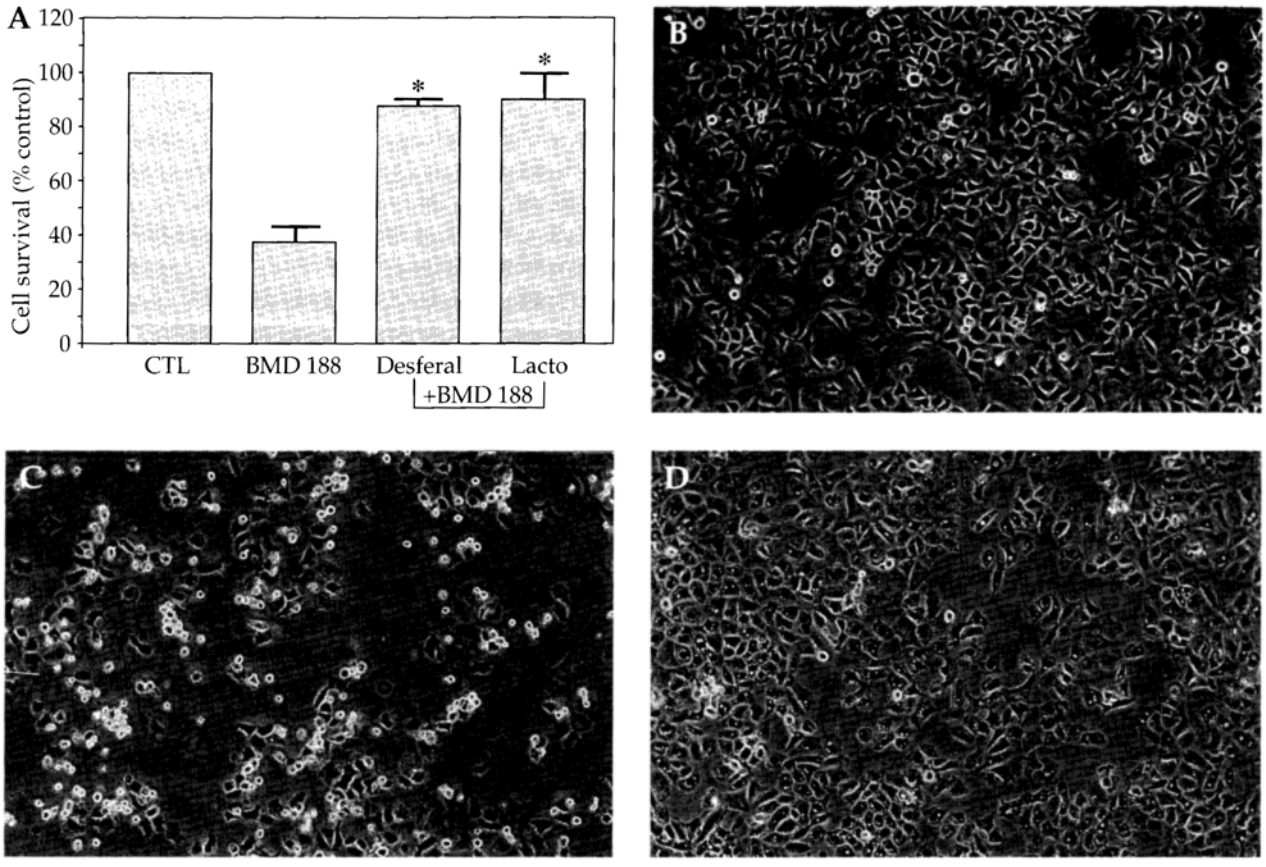

Figure 5. *BMD188-induced PC3 cell apoptosis can be inhibited by free radical scavengers. A. Quantitation of cell survival in PC3 cells pretreated with free radical inhibitors (i.e., iron chelators). PC3 cells (2.5x10⁴/well) cultured in flat-bottom 24-well plates were pretreated with Desferal (100 μM) or lactoferrin (Lacto; 10 μM) for 1 h prior to BMD188 treatment (50 μM; 2 h). Apoptotic cells were scored with the trypan blue dye exclusion/apoptotic morphology assays as described.[8-10,13] The results were expressed as the % cell survival (the control as 100%). Shown are the mean ± S.E from three independent experiments. *, p<0.01 compared with BMD188 alone. B–D, photomicrographs of PC3 cells treated with ethanol (B), BMD188 (C; 50 μM x 2 h) or lactoferrin (10 μM x 1 h) followed by BMD188 (D; 50 μM x 2 h). Original magnification: x200.*

Previous studies have also suggested that hydroxamates disrupt the cellular redox homeostasis by interfering with iron metabolism,[16,17] therefore, the involvement of iron in BMD188-induced cell death was examined. PC3 cells were pretreated with Desferal, an iron-selective chelator[18] or lactoferrin (which blocks the conversion of Fe^{2+} to Fe^{3+};[19]) followed by BMD188 treatment. As shown in *Figure 5A*, both inhibitors, at the doses tested (see Materials & Methods), delayed apoptotic response and significantly extended PC3 cell survival. Morphological observations demonstrated that in BMD188-treated cultures there existed numerous apoptotic cells with shrunken cell bodies and membrane blebbings *(Figure 5C)*. In contrast, cells pretreated with lactoferrin *(Figure 5D)* or Desferal (not shown) mostly showed normal morphologies except for easily recognizable cytoplasmic vacuoles, which represented the earliest microscopic changes upon BMD188 treatment.

BMD188 Alters the Morphological Distribution of Mitochondria prior to Apoptosis Induction

Our previous experiments (Joshi et al., manuscript in preparation) have documented that BMD188-induced apoptosis *absolutely* requires functional mitochondria. This conclusion is based on the following observations: 1). BMD188 induces rapid ROS generation in mitochondria. This increased ROS production is required for apoptosis induction since ROS inhibitors/scavengers significantly retard the BMD188-induced cell death. 2). The BMD188-induced apoptosis can be blocked by mitochondrial inhibitors such as cyclosporine A and ruthenium red as well as complex-specific chemical inhibitors including rotenone (complex I or NADH-ubiquinone reductase inhibitor), antimycin A (complex III or ubiquinol-cytochrome c reductase inhibitor), cyanide (complex IV or cytochrome c oxidase inhibitor), and oligomycin B

(complex V or ATP synthase inhibitor). In contrast, TTFA, a complex II or succinate-ubiquinone reductase inhibitor, does not affect the BMD188-induced cell death. These results indicate that the BMD188 acts on the NADH pathway (but not the succinate-$FADH_2$ pathway) of the electron transport. 3). BMD188 induces an early-phase (i.e., within 10 min) increase in the mitochondrial membrane potential, which appears to be important for apoptosis induction. Thus, prior treatment of cells with valinomycin which dissipates the mitochondrial membrane potential blocks the apoptotic effect of BMD188. Finally, mitochondrial respiration-deficient cells are much more resistant to apoptosis induction by BMD188.

To support for the involvement of mitochondria in the BMD188-induced apoptotic response, PC3 cells treated with the compound demonstrated dramatic morphological alterations *(Figure 6)*. In control PC3 cells, mitochondria, which were labeled as dark brown precipitates due to the metabolism of MTT, generally were distributed in the perinuclear region *(Figure 6A*, arrowheads). In sharp contrast, there was a drastic decrease in the intensity of the mitochondria labeling 30 min post BMD188 treatment *(Figure 6B)*, suggesting the impairment of the mitochondrial function. Furthermore, the morphology of mitochondria appeared different: it became more dot-like *(Figure 6B)* instead of fibrous *(Figure 6A)*. The physiological function of mitochondria is to generate ATP via electron transport. Therefore, we measured ATP levels following BMD188 treatment. As a positive control, HL60 cells were simultaneously treated with BMD188 and measured for the intracellular levels of ATP as a function of time. As shown in *Figure 7*, BMD188 induced PC3 cell apoptosis without affecting the ATP levels. By contrast, HL60 cells demonstrated a significant decrease in the ATP levels upon BMD188 treatment.

BMD188-Induced PC3 Cell Apoptosis Requires Protease Activation

Proteases (serine proteases, caspases, and endonucleases) are intimately associated with apoptosis induction in various models. To explore what protease(s) are involved in BMD188-induced cell death, PC3 cells were pretreated for 45 min with the following specific chemical/peptide inhibitors: TPCK (an irreversible inhibitor of chymotrypsin-like serine protease), TLCK (an irreversible inhibitor of trypsin-like serine protease), iodoacetamide (a conventional cysteine protease inhibitor), or ALLN (an inhibitor of calpains). Then PC3 cells were treated with BMD188 (50 μM; 2 h). As shown in *Figure 8A*, BMD188-induced PC3 cell PARP cleavage was effectively inhibited by TPCK and TLCK, but not significantly by iodoacetamide or ALLN. Expectedly, BMD188-induced PC3 cell apoptosis was effectively inhibited by TPCK and TLCK, but not by iodoacetamide or ALLN.[13] On the other hand, all three caspase inhibitors, i.e., zVAD-fmk (a broad-spectrum inhibitor of caspases), DEVD-CHO (a relatively specific inhibitor of caspase-3), YVAD-CHO (a relatively specific inhibitor of ICE or caspase-1), inhibited the PARP cleavage induced by BMD188 *(Figure 8B)*. Quantitation of apoptosis revealed that zVAD at 5 μM reduced apopto-

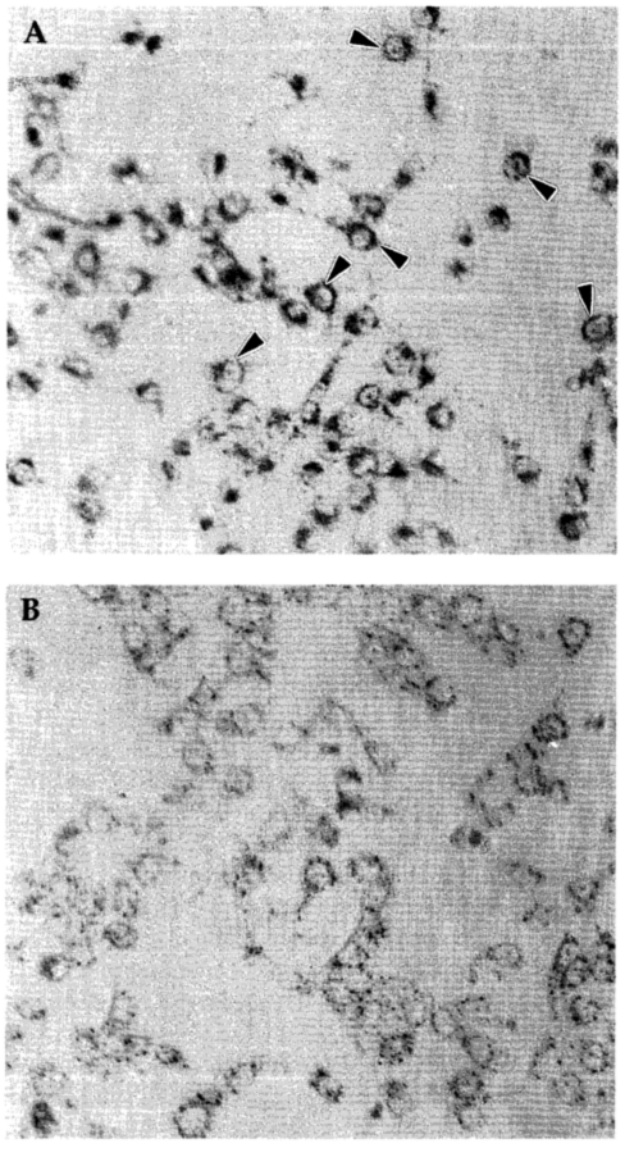

Figure 6. *BMD188 effect on the PC3 cell mitochondrial activity. PC3 cells were treated with either ethanol (A) or BMD188 (40 μM) for 30 min (B). Following washing, cells were incubated with MTT as described in Materials and Methods. Note that in control cells the mitochondria were mostly labeled as dark brown precipitates in the perinuclear regions (A, arrowheads) while in the BMD188-treated cells there was a significant decrease in the mitochondrial labeling (B). Original magnification: x100.*

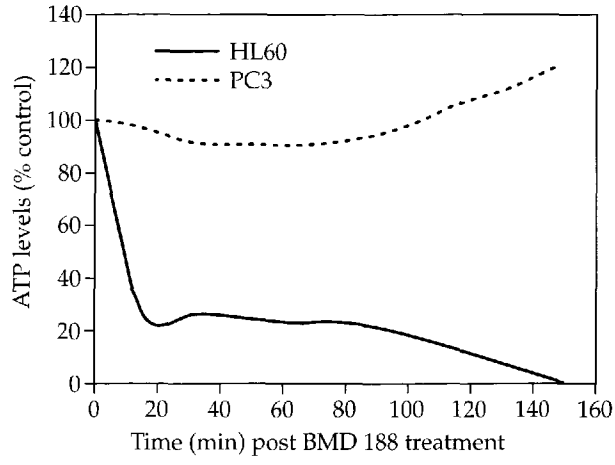

Figure 7. BMD188 decreases the intracellular ATP levels of HL 60 cells but not PC3 cells. ATP levels were determined as detailed in Materials and Methods.

sis to 30% and at 50 μM to 20%. DEVD-CHO at 5 μM reduced PC3 cell apoptosis to 25% and YVAD-CHO at 5 and 50 μM reduced apoptosis to 46% and 15%, respectively ([13]; data not shown).

Discussion

The present study, together with previous observations ([11,13], Joshi et al., manuscript submitted), demonstrates that BMD188, a newly synthesized cyclic hydroxamic acid compound, inhibits the growth of prostate cancer cells *in vitro* and *in vivo* by inducing caspase-3-dependent apoptosis. Subsequent studies of the molecular mechanism(s) of action revealed three essential parameters in the apoptosis-inducing effects of BMD188: mitochondria, ROS, and proteases, which have all previously been implicated in apoptosis.[20-28] Detailed time course studies using various inhibitors as well as by direct measurement of individual parameters (i.e., electron transport, ROS and caspase) have allowed us to compile the sequence of molecular events in PC3 cells upon BMD188 treatment, culminating in apoptotic cell death (*Figure 9*). BMD188, a 6 member-ring hydroxamic acid with a 8-C hydrocarbon tail which is structurally related to a fatty acid side chain *(Figure 1)*, upon entering cells, appears to target mitochondria. More specifically, the chemical appears to target or "activate" the NADH pathway of the electron transport since the BMD188-induced PARP cleavage and apoptosis can be effectively prevented by inhibitors of complex I, III, and IV *(Figure 9)*. In contrast, the complex II inhibitor (TTFA) does not affect the BMD188 effect (unpublished observations) suggesting that the $FADH_2$ as electron donors is not involved. Suffice it to say that the electron transport function of mitochondria is required for the apoptotic effect of BMD188. This conclusion gains further support from the observations that respiration-deficient (i.e., ρ^0) cells whose mitochondria lack eletron transfer capacities are resistant to BMD188-induced caspase-3 activation and apoptosis (Joshi et al., manuscript submitted). Many apoptosis inducers such as TNF-α cause cell death by directly damaging mitochondria.[29] The BMD188 effect, in contrast, appears to be very unique since the mitochondria function is required for cell death. In this regard, the BMD188 effect resembles that of short-chain fatty acid, butyrate, whose apoptosis-inducing effect on colon epithelial cells also can be prevented by several mitochondrial respiration inhibitors.[30]

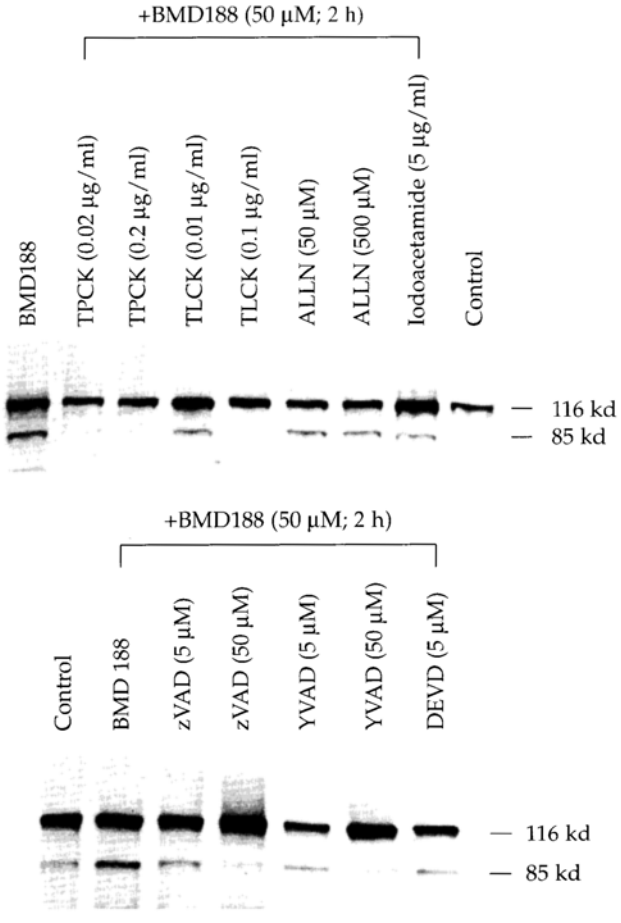

Figure 8. Protease involvement in BMD188-induced PC3 cell death. A. Effects of protease inhibitors on BMD188-induced PC3 cell apoptosis. PC3 cells were pretreated with various concentrations of TPCK, TLCK, ALLN, Iodoacetamide, or with ethanol (control) for 45 min followed by BMD188 treatment (50 μM; 2 h). At the end of the treatment cells were harvested for PARP cleavage assays. B. Effects of caspase inhibitors on BMD188-induced PC3 cell apoptosis. PC3 cells were pretreated with various concentrations of zVAD, DEVD, or YVAD, or with ethanol (control) for 1 h followed by BMD188 treatment (50 μM; 2 h). At the end of the treatment cells were harvested for PARP cleavage assays. Note that a low level of PARP cleavage was detected in the control.

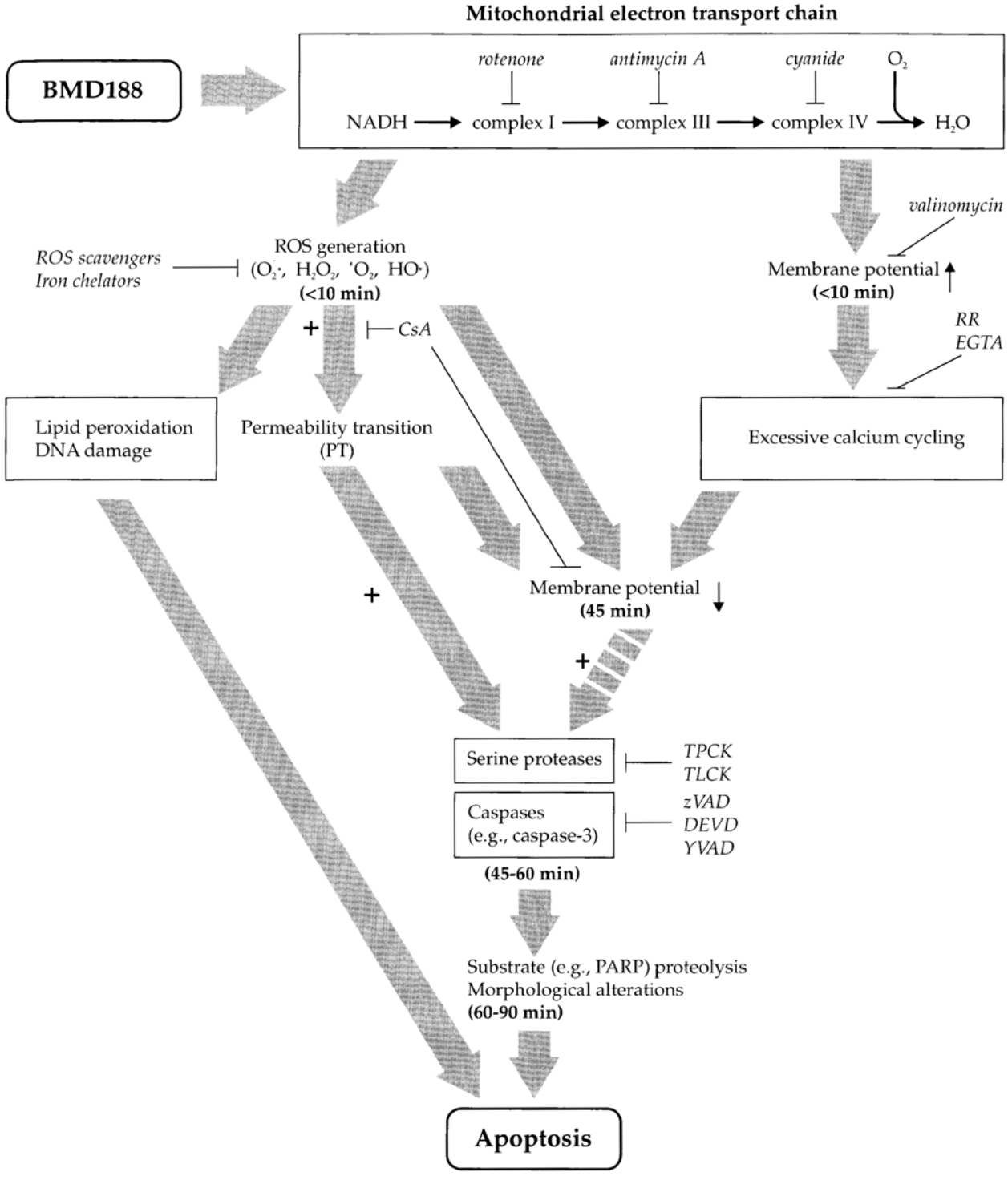

Figure 9. *The sequence of molecular events for BMD188-induced PC3 cell apoptosis. See text for details. The sites of inhibitors (italicized) are indicated. "+", increase.*

Fow cytometric analysis with $\Delta\Psi_m$-sensitive fluorochrome rhodamine123 reveals that BMD188 induces a rapid (within 1 min) increase in PC3 cell $\Delta\Psi_m$ which peaks at ~10 min and then gradually returns to the basal level (*Figure 9*; Joshi et al., manuscript submitted). It is well accepted that the $\Delta\Psi_m$ is established from the "pumping out" of protons from matrix to intermembrane space, as a result of the electron transport through the respiratory

chain complexes. Therefore, the increased $\Delta\Psi_m$ at early time point, again, suggests that BMD188 is enhancing the electron transport activity, which is consistent with the inhibition of the BMD188 effect by complex-specific inhibitors (*Figure 9*). Significantly, the early increase in the $\Delta\Psi_m$ apparently is essential for the apoptotic process since prior dissipation of the membrane potential with valinomycin eliminates the apoptotic effect of BMD188 (*Figure 9*, Joshi et al., manuscript submitted). Interestingly, oligomycin, which reduces the ATP levels by inhibiting the F_1F_0 ATPase, also blocks the BMD188-induced apoptosis. This suggests that BMD188 is initiating an apoptotic program not by decreasing intracellular ATP levels, which is also supported by the direct measurement (*Figure 7*). In fact, depleting ATP in PC3 cells by longer exposure to oligomycin never triggers apoptosis but rather induces homogeneous necrosis (unpublished observation), which is consistent with observations on other cell systems.[31,32] The precise mechanism whereby oligomycin inhibits BMD188-induced PC3 cell apoptosis merits further scientific exploration. However, it is quite likely that this inhibitory effect results from decreased proton motive force (inhibition of the ATP synthase will decrease the number of protons flowing back to the matrix which eventually will result in the stagnation and collapse of proton gradient) and/or from inhibition of some ATP-dependent steps (e.g., caspase activation) in the apoptotic process.[31,32]

At the same time frame (i.e., within 10 min) when the $\Delta\Psi_m$ is increased, enhanced ROS generation is also observed, most likely due to BMD188-induced increase in the mitochondrial respiratory chain activities (*Figure 9*; Joshi et al., manuscript submitted) since mitochondrial respiration normally involves the generation of various species of ROS and mitochondria represent one of the major sources of ROS production inside cells.[33-35] Direct measurement by cytochrome c reduction assays and by DHR123 flow cytometric analysis confirmed increased ROS production (Joshi et al., manuscript submitted). The overproduction in mitochondrial ROS apparently is important for the apoptotic response since tryptophan (which preferentially scavenges singlet oxygen), lactoferrin (which prevents production of hydroxyl radicals by blocking the Fenton reaction), and several general free radical scavengers or inhibitors (such as PDTC) significantly delay the 188-induced PC3 cell apoptosis (this study and Joshi et al., manuscript submitted). That BMD188-induced PC3 apoptosis can be partially blocked by lactoferrin and Desferal also suggests the involvement of iron in the death process. Intracellular "free" iron (i.e., non-protein bound) is an active transition metal thought to contribute to (e.g., in Fenton reactions) the formation of highly reactive free radicals of multiple species.[36,37] Regardless, the fact that various general or specific ROS scavengers/inhibitors block the apoptosis-inducing effect of BMD188 strongly suggests that increased ROS generation in mitochondria is a key "death" factor (*Figure 9*).

The BMD188-induced PC3 cell death can also be blocked by other inhibitors of mitochondrial functions such as cyclosporine A (CSA) and ruthenium red (RR) (Joshi et al., manuscript submitted). RR blocks the uptake of Ca^{2+} into mitochondria while CSA inhibits the membrane permeability transition.[20,38-40] The results with RR suggest that BMD188 may induce excessive calcium cycling across the mitochondria inner membrane which may have resulted from the increased $\Delta\Psi_m$ since the uptake of calcium into mitochondria by the uniporter is driven by the membrane potential.[38-40] The possibility of excessive calcium entry into mitochondria (or calcium cycling) is also supported by the inhibition of BMD188-induced PC3 cell death by EGTA (*Figure 9;* unpublished observations). The results with CSA suggest that BMD188 induces the opening of the mitochondrial membrane permeability transition (or PT) pores, most likely as a consequence of increased ROS generation (*Figure 9*). PT has been proposed as a central regulator of nuclear as well as cytoplasmic apoptosis.[41,42] CSA may also inhibit the BMD188 effect by preventing the decrease of $\Delta\Psi_m$, which is observed at ~45 min following stimulation (*Figure 9*). Decreased $\Delta\Psi_m$ may result from a combination of excessive calcium cycling, ROS generation, and PT (*Figure 9*;[41,42]).

Proteolysis plays a crucial role in cell death. Multiple proteolytic enzymes such as serine proteases,[22] calpains,[23] endonucleases,[24] proteosomes,[25,26] as well as various caspases[27,28] are involved in the execution stage of apoptosis. Recent experimental data implicate protease cascade in the apoptotic process.[28] Not surprisingly, BMD188-induced PC3 cell apoptosis requires activation of several different types of proteases. Specifically, the BMD188 effect can be blocked by TPCK, TLCK, DEVD, YVAD, and zVAD, suggesting the requirement for serine proteases and caspases (*Figure 9*). Caspase activation during BMD188-triggered apoptosis is also supported by specific cleavage of PARP, a substrate for most caspases. Eventually, the substrate proteolysis by activated caspases, together with ROS-induced membrane peroxidation and DNA damage, results in full-blown apoptotic phenotype (*Figure 9*). It is interesting to note that BMD188 demonstrates a 2–5 fold preferential killing of prostate cancer cells versus normal cells.[13] This relative specificity of action may be related to well-documented observations that tumor cells very frequently exhibit abnormal mitochondrial activities. The present work demonstrates that BMD188, in a mitochondria-, ROS-, and protease-dependent fashion, triggers potent apoptotic death of hormone-refractory human prostate cancer cells. The compound also demonstrates good *in vivo* efficacy against human prostate cancer cells orthotopically implanted into SCID mice. Together, the data suggest that BMD188 and

its structural analogs may represent novel chemical entities that are potentially useful in the clinical treatment of prostate cancer patients.

Acknowledgement

The authors are indebted to Drs. S. Garde, S-Y. Yang, C.P. Lee, B. Taffe, and C. Zhang for their excellent technical help and very enlightening discussions. This work was supported, in part, by National Institute of Health grant CA 72256 to D.G.T

References

1. *Tang DG, and Porter AT:* Target to apoptosis: A hopeful weapon for prostate cancer. Prostate 32:284-293, 1997.
2. *Vukanovic J, and Isaacs JT:* Human prostate cancer cells are sensitive to programmed (apoptotic) death induced by the antiangiogenic agent Linomide. Cancer Res 55:3517-3520, 1995.
3. *Li CJ, Wang C and Pardee AB:* Induction of apoptosis by -lapachone in human prostate cancer cells. Cancer Res. 55:3712-3715, 1995.
4. *Reed JC, Stein C, Subasinhe C et al:* Antisense-mediated inhibition of BCL-2 protooncogene expression and leukemic cell growth and survival: comparison of phosphodiester and phosphorothioate oligodeoxynucleotides. Cancer Res 50:6565-6570, 1990.
5. *Yang C, Cirielli C, Capogrossi MC et al:* Adenovirus-mediated wild-type p53 expression induces apoptosis and suppresses tumorigenesis of prostatic tumor cells. Cancer Res 55:4210-4213, 1995.
6. *Eastman JA, Hall SJ, Sehgal I et al:* In vivo gene therapy with p53-p21 adenovirus for prostate cancer. Cancer Res 55:5151-5155, 1995.
7. *Furuya Y, Lundmo P, Short AD et al:* The role of calcium, pH, and cell proliferation in the programmed (apoptotic) death of androgen-independent prostate cancer cells by thapsigargin. Cancer Res 54:6167-6175, 1994.
8. *Tang DG, Chen YQ, and Honn KV:* Arachidonate lipoxygenases as essential regulators of cell survival and apoptosis. Proc Natl Acad Sci USA 93:5241-5246, 1996.
9. *Tang D, and Honn KV:* Apoptosis of W256 carcinosarcoma cells of the monocytoid origin induced by NDGA involves lipid peroxidation and depletion of GSH: Role of 12-lipoxygenase in regulating tumor cell survival. J Cell Physiol 172:155-170, 1997.
10. *Tang D, Guan K-L, Li L, et al:* Suppression of W256 carcinosarcoma cell apoptosis by arachidonic acid and other unsaturated fatty acids. Int J Cancer 72:1078-1087, 1997.
11. *Tang DG, Li L, Zhu Z et al:* Apoptosis in the absence of cytochrome c accumulation in the cytosol. Biochem Biophys Res Commun 242:380-384, 1998.
12. *Krajewski S, Zapata JM, and Reed JC:* Detection of multiple antigens on Western blots. Anal Biochem 236:221-228, 1996.
13. *Li L, Zhu Z, Joshi B, et al:* A novel hydroxamic acid compound, BMD188, demonstrates anti-prostate cancer effects by inducing apoptosis. I: *In vitro* studies. Anticancer Res Submitted.
14. *Timar J, Raso E, and Fazakas Zs, et al:* Multiple use of a signal transduction pathway in tumor cell invasion. Anticancer Res 16:3299-3306, 1996.
15. *Tang DG, Li L, Chopra D, et al:* Extended survivability of prostate cancer cells in the absence of trophic factors: Increased proliferation, evasion of apoptosis and the role of apoptosis proteins. Cancer Res 58, 3466-3479, 1998.
16. *Summers JB, Mazdiyasni H, Holms JH, et al:* Hydroxamic acid inhibitors of 5-lipoxygenase. J Med Chem 30:574-580, 1987.
17. *Jackson WP, Islip PJ, Kneen G, et al:* Acetohydroxamic acid inhibitors of 5-lipoxygenase. J Med Chem 31:499-500, 1988.
18. *Van Reyk DM, and Dean RT:* The iron-selective chelator desferal can reduce chelated copper. Free Rad Res 24:55-60, 1996.
19. *Lanerdal B, and Iyer S:* Lactoferrin: Molecular structure and biochemical function. Annu Rev Nutr 15:93-110, 1995.
20. *Richter, C:* Pro-oxidants and mitochondrial Ca^{2+}: their relationship to apoptosis and oncogenesis. FEBS Lett 325:104-107, 1993.
21. *Marchetti P, Susin SA, Decaudin D, et al:* Apoptosis-associated derangement of mitochondrial function in cells lacking mitochondrial DNA. Cancer Res 56:2033-2038, 1996.
22. *Zhivotovsky B, Gahm A, Ankarcrona M, et al:* Multiple proteases are involved in thymocyte apoptosis. Exp Cell Res 221:404-412, 1995.
23. *Brown SB, Bailey K, and Savill J:* Actin is cleaved during constitutive apoptosis. Biochem. J 323:233-237, 1997.
24. *Deng G, and Podack ER:* Deoxyribonuclease induction in apopototic cytotoxic T lymphocytes. FASEB J 9:665-669, 1995.
25. *Grimm LM, Goldberg AL, Poirier GG, et al:* Proteosomes play an essential role in thymocyte apoptosis. EMBO J 15:3835-3844, 1996.
26. *Drexler HCA:* Activation of the cell death program by inhibition of the proteosome function. Proc Natl Acad Sci USA 94:855-860, 1997.
27. *Faleiro L, Kobayashi R, Fearnhead H, et al:* Multiple species of CPP32 and Mch2 are the major active caspases present in apoptotic cells. EMBO J 16:2271-2281, 1997.
28. *Porter AG, Ng P, and Janicke RU:* Death substrates come alive. BioEssays 19:501-507, 1997.
29. *Schulze-Osthoff K, Bakker AC, Vanhaesebroeck B, et al:* Cytotoxic activity of tumor necrosis factor is mediated by early damage of mitochondrial functions. J Biol Chem 267:5317-5323, 1992.
30. *Heerdt BG, Houston MA, and Augenlicht LH:* Short-chain fatty acid-initiated cell cycle arrest and apoptosis of colonic epithelial cells is linked to mitochondrial function. Cell Growth Differ 8:523-532, 1997.
31. *Eguchi Y, Shimizu S, and Tsujimoto Y:* Intracellular ATP levels determine cell death fate by apoptosis or necrosis. Cancer Res 57:1835-1840, 1997.
32. *Shimizu S, Eguchi Y, Kamiiki W, et al:* Retardation of chemical hypoxia-induced necrotic cell death by Bcl-2 and ICE inhibitors: possible involvement of common mediators in apoptotic and necrotic signal transductions. Oncogene 12:2045-2050, 1996.
33. *Buttke TM, and Sandstrom PA:* Oxidative stress as a mediator of apoptosis. Immunol Today 15:7-10, 1994.

34. *Glinn MA, Lee CP, and Ernster L:* Pro- and anti-oxidant activities of the mitochondrial respiratory chain: factors influcing NAD(P)H-induced lipid peroxidation. Biochim Biophys Acta 1318:246-254, 1997.

35. *Lander HM:* An essential role for free radicals and derived species in signal transduction. FASEB J 11:118-124, 1997.

36. *Hentze MW and Kuhn LC:* Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide, and oxidative stress. Proc Natl Acad Sci USA 93:8175-8182, 1996.

37. *Lash A, and Saleem A:* Iron metabolism and its regulation. A review. Ann Clin Lab Sci 25:20-30, 1995.

38. *Zoeteweij JP, van de Water B, de Bont HJGM, et al:* Involvement of intracellular Ca^{2+} and K^{+} in dissipation of the mitochondrial membrane potential and cell death induced by extracellular ATP in hepatocytes. Biochem J 288:207-213, 1992.

39. *van de Water B, Zoeteweij JP, de Bont HJGM, et al:* Role of mitochondrial Ca^{2+} in the oxidative stress-induced dissipation of the mitochondrial membrane potential: Studies in isolated proximal tubular cells using the nephrotoxin 1,2-dichlorovinyl-L-cysteine. J Biol Chem 269:14546-14552, 1994.

40. *Kowaltowski AJ, Castilho RF, and Vercesi AE:* Opening of the mitochondrial permeability transition pore by uncoupling or inorganic phosphate in the presence of Ca^{2+} is dependent on mitochondrial-generated reactive oxygen species. FEBS Lett 378:150-152, 1996.

41. *Marchetti P, Castedo M, Susin SA, et al:* Mitochondrial permeability transition is a central coordinating event of apoptosis. J Exp Med 184:1155-1160, 1996.

42. *Zamzami N, Susin SA, Marchetti P, et al:* Mitochondrial control of nuclear apoptosis. J Exp Med 183:1533-1544, 1996.