Published in final edited form as:

Bioorg Med Chem Lett. 2017 June 01; 27(11): 2286–2291. doi:10.1016/j.bmcl.2017.04.047.

Brefeldin A enhances docetaxel-induced growth inhibition and apoptosis in prostate cancer cells in monolayer and 3D cultures

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Abstract

Docetaxel is a commonly used chemotherapeutic drug for patients with late stage prostate cancer. However, serious side effect and drug resistance limit its clinical success. Brefeldin A is a 16-membered macrolide antibiotic from mangrove-derived Fungus *Aspergillus* sp. (9Hu), which exhibited potent cytotoxicity against human cancer cells. In the present study, we determined the effect of brefeldin A on docetaxel-induced growth inhibition and apoptosis in human prostate cancer PC-3 cells. Brefeldin A in combination with docetaxel inhibited the growth of PC-3 cells in monolayer and in three dimensional cultures. The combination also potently stimulated apoptosis in PC-3 cells as determined by propidium iodide staining and morphological assessment. Mechanistic studies showed that growth inhibition and apoptosis in PC-3 cells treated with brefeldin A and docetaxel were associated with decrease in the level of Bcl-2. The present study indicates that combined brefeldin A with docetaxel may represent a novel approach for improving the efficacy of docetaxel, and Bcl-2 may serve as a target for brefeldin A to enhance the effects of docetaxel chemotherapy.

Graphical abstract

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Prostate cancer is one of the most commonly diagnosed visceral malignancies and the second leading cause of cancer-related deaths among men in the United States.¹ Androgen deprivation therapy (ADT) remains the main treatment for advanced and metastatic prostate cancer.² Despite initial response, nearly all patients on ADT progress to castration-resistant prostate cancer (CRPC) in 18–24 months.³ Although the newer and more potent anti-androgen treatment have shown some promise, resistance is already being encountered in the clinic.⁴ Chemotherapy with docetaxel is currently a standard treatment for metastatic and CRPC and remains a backbone in current development of treatment regimens.⁵

Docetaxel (Fig. 1), an anti-microtubule agent, was approved by the US FDA as the mainstay treatment against CRPC.^{5, 6} Docetaxel is produced semi-synthetically from the needles of the Pacific yew tree (*Taxus brevifolia*). Studies had shown that docetaxel was more effective against progressive human prostate cancers than other conventional anti-cancer agents.^{6–8} Although initially effective, docetaxel-based regimen has only shown a median survival of 18–20 months and response rate of only 50%.^{7–8} In addition, there are severe side effects associated with docetaxel treatment including the suppression of bone marrow function leading to immune dysfunction and anemia.⁹ The development of chemoresistance to docetaxel is observed in some patients which limits its clinical success.^{10, 11} Clearly, it holds high clinical significance to enhance the efficacy of docetaxel at lower doses in a less-toxic manner and to reduce the resistance to this chemotherapeutic drug.

Marine-derived fungi have become a rich source of biologically active natural products.¹² We have focused on screening out natural antitumor metabolites from mangrove-derived fungi.^{13, 14} Among the isolated metabolites, brefeldin A (Fig. 1), identified as an antitumor macrolide-compound, was newly found to be the most abundant metabolite in the extract of the fungus *Aspergillus* sp. (No. 9Hu), derived from the root of the mangrove *kandelia candel*.¹⁵ Brefeldin A, isolated previously from the *Penicillium decumben* by Singleton et al.,^{16, 17} has been initially known to play a regulatory role in the intracellular transport system.^{18, 19} It induces the reversible disassembly of the Golgi complex, resulting in the interruption of protein transport from the endoplasmic reticulum (ER) to the Golgi.¹⁸ Brefeldin A has also been shown to collapse the Golgi complex into the ER, redistributing Golgi-associated proteins/enzymes to the ER.¹⁹ In addition, brefeldin A had been found to induce apoptosis and growth inhibition in several human cancer cells, including leukemia, colon, and prostate cancer cells.^{20–23} An earlier study in showed that brefeldin A had anti-proliferative activity in vivo in animal models.²⁴

Although earlier studies have demonstrated the anticancer activity of brefeldin A in prostate cancer cells,^{22, 25, 26} the effects of brefeldin A in combination with commonly used chemotherapeutic drugs on growth and apoptosis of prostate cancer cells have not been reported. If brefeldin A can enhance the efficacy of docetaxel at lower doses in a less-toxic manner, it will significantly improve the docetaxel-based chemotherapy and be of great benefit for patients. We hypothesized that brefeldin A will enhance the effectiveness of docetaxel in prostate cancer cells. To test our hypothesis, we determined the effects of brefeldin A isolated from mangrove-derived Fungus *Aspergillus* sp. and docetaxel alone or in combination on growth and apoptosis of human prostate cancer cells.

The extraction and isolation of brefeldin A from mangrove-derived fungus *aspergillus* sp. (No. 9Hu) were carried out as previous described.¹⁵ The chemical structure of brefeldin A was elucidated by comparing the MS, ¹H and ¹³C NMR, and X-ray diffraction spectroscopic data (crystallographic data for BFA has been deposited with the Cambridge Crystallographic Data Centre, CCDC number 1525069, see supplementary material) with reported values.^{16, 17, 27} The effects of brefeldin A and docetaxel alone or in combination on the growth of human prostate cancer cells were determined using the trypan blue exclusion assay. As shown in Figure 2A, treatment of human prostate cancer LNCaP (androgen-dependent) and PC-3 (androgen-independent) cells with docetaxel or brefeldin A resulted in concentration-dependent decrease in the number viable cells. The effects of docetaxel or brefeldin A on apoptosis in LNCaP and PC-3 cells were determined by propidium iodide staining and morphological assessment. As shown in Figure 2B, treatment of the cells with docetaxel or brefeldin A resulted in concentration-dependent increase in apoptosis. The androgen-dependent LNCaP cells were more sensitive than the androgen-independent PC-3 cells to the growth inhibition and apoptosis induced by docetaxel (Fig. 2A&B). Docetaxel is clinically used in patients with advanced androgen-independent prostate cancer. Therefore, we choose the androgen-independent PC-3 cells to determine the effect of brefeldin A and/or docetaxel on cell growth and apoptosis.

Previous pharmacokinetic studies showed that patients received intravenous infusion of 20 mg/m² docetaxel had a median plasma level of 2.8 nM at 72 hours post infusion,²⁸ and the mean $\pm$ SD concentration of docetaxel on day 8 after drug intravenous infusion was 1.79 $\pm$ 0.53 nM.²⁹ Prolonged circulation of low concentrations of docetaxel may contribute to the inhibitory effect of the drug on tumors through suppression of microtubule dynamics.²⁹ We did not find any report on pharmacokinetic study of brefeldin A in patients. Results of a study using mouse model showed that plasma level of brefeldin A was ~700 nM at 60 min after intravenous injection of the drug at a dose of 26.3 mg/kg.³⁰ Based on the results of these pharmacokinetic studies, we investigated the effects of docetaxel and brefeldin A on cell viability in prostate cancer PC-3 cells and in normal prostate epithelial RWPE-1 cells. We found that brefeldin A at 100 nM and higher concentrations had significant toxicity to RWPE-1 cells, and that docetaxel was toxic to RWPE-1 cells at doses of 5 and 10 nM (Figure 3A). Therefore, we used brefeldin A and docetaxel at concentrations that were not toxic to RWPE-1 cells for the subsequent studies of drug combination. Effects of combinations of brefeldin A and docetaxel at different ratios on PC-3 cells were determined. As shown in Figure 3B, the combination of brefeldin A (50 nM) and docetaxel (2 or 3 nM) had potent effects on decreasing the number of viable PC-3 cells. We further determined the

effect of these combinations on viability of RWPE-1 cells. The combination of brefeldin A (50 nM) and docetaxel (2 nM) had a small effect on decreasing the number of viable RWPE-1 cells while the combination of brefeldin A (50 nM) and docetaxel (3 nM) had moderate to strong effect on decreasing RWPE-1 cell viability. The effect of brefeldin A (50 nM) in combination with docetaxel (2 nM) was around 4 times stronger in PC-3 cells than in RWPE-1 cells. This result indicates that the drug combination at certain concentrations has high selective toxicity on prostate cancer cells.

The effect of brefeldin A (50 nM) and docetaxel (2 nM) in combination on apoptosis of PC-3 cells was determined. As shown in Table 1, treatment with docetaxel or brefeldin A alone resulted in a moderate increase in the number of apoptotic cells. Combinations of docetaxel and brefeldin A had more potent effects on increasing apoptosis than either agent alone (Table 1). Representative micrographs of propidium iodide staining in PC-3 cells treated with docetaxel and/or brefeldin A are shown in Figure 4. To the best of our knowledge, this is the first report indicating a combined effect of brefeldin A and docetaxel in prostate cancer cells.

Docetaxel is a commonly used and effective chemotherapeutic treatment for cancers.⁵ However, docetaxel exhibits significant adverse effects in patients with comorbid conditions, which mandate dose reduction which increases the possibility of selection for resistant clones.⁹ Novel combinations to reduce dose-limiting toxicity of docetaxel and/or to increase its efficacy are highly desirable. The concentrations of docetaxel used in studies in cultured prostate cancer cells ranged between 1 and 100 nM, and the IC₅₀ for docetaxel using different assays in cultured PC-3 cells ranged between 5 and 24 nM.^{31–35} In the present study, we found that the IC₅₀ for PC-3 cells was around 8 nM. A lower dose of docetaxel (2 nM) in combination with brefeldin A had similar effects on growth inhibition and apoptosis as compared with that in cells treated with a higher dose of docetaxel (10 nM) alone. This result indicates that combination of docetaxel with brefeldin A may reduce the toxic side effect of docetaxel by lowering the dose of this drug.

A 3D cell culture model was used to determine the effects of docetaxel and brefeldin A alone or in combination on the formation and growth of three dimensional tissue-like structures. Figure 5 shows representative micrographs of monolayer (A–D) and 3D (E–H) cultures of PC-3 cells treated with docetaxel (2 nM) or brefeldin A (50 nM) alone or in combination. Cell viability in PC-3 cells treated with brefeldin A and docetaxel in monolayer cultures was determined by the trypan blue exclusion assay and the result is presented in Table 1. Representative micrographs of trypan blue staining are shown in Figure 4(E–H). As shown in Figure 5E, PC-3 cells formed a tissue-like morphology in 3D culture in extra cellular matrix gel. Treatment with brefeldin A or docetaxel alone had inhibitory effect on the formation and growth of tissue-like structure (Fig. 5F and G). Brefeldin A and docetaxel in combination had a more potent effect on inhibiting the formation of tissue-like structure (Fig. 5H). Result of the average size of tissue-like structure relative to the control is shown in Table 1. Comparing to the conventional 2D monolayer cell cultures, the 3D culture system mimics the structural architecture and differentiation function of the tumor tissues. It is well known that cell-cell interactions and cell-matrix interactions within the 3-dimensional microenvironment are important to the physiological function and response of

cancer cells to anticancer agents.^{36,37} In the present study, we found that PC-3 cells formed 3D tissue-like morphology in extra cellular matrix Matrigel. Treatment of PC-3 cells with docetaxel and brefeldin A in combination had stronger inhibitory effect than either agent alone on the formation of tissue-like morphology in 3D cultures suggesting that the drug combination may have more potent inhibitory effect on tumor growth in vivo.

To investigate the possible mechanisms of actions for brefeldin A and docetaxel, we determined the effects of these two drugs alone or in combination on the levels of Bcl-2, phospho-Akt and phospho-Erk by the Western blot analysis. As shown in Figure 6, docetaxel alone had little or no effect on the level of Bcl-2. Treatment of PC-3 cells with brefeldin A in combination with docetaxel resulted in a strong decrease in the level of Bcl-2 (Fig. 6). Brefeldin A alone or in combination with docetaxel had no effect on the levels of phospho-Akt and phospho-Erk1/2 (Fig. 6). The density of the bands relative to control in Western blot is shown in Figure 6. It is identified that apoptosis plays a significant role in the response of prostate cancer to chemotherapy.³⁸ Bcl-2 family members are important in the regulation and control of apoptosis pathway. Overexpression of Bcl-2 has been found to stabilize the outer mitochondrial membrane and prevent release of cytochrome c and the initiation of apoptosis.^{39,40} Brefeldin A was found to induce apoptosis in human cancer cells.^{20–23} It has been found that brefeldin A-mediated apoptosis in T-cell leukemia Jurkat cells was inhibited by Bcl-2.⁴¹ A recent study demonstrated that brefeldin A-induced apoptosis in breast cancer cells was mediated through down-regulation of anti-apoptotic proteins Bcl-2 and Mcl-1.⁴² In the present study, we found that the combination of brefeldin A and docetaxel strongly decreased the level of Bcl-2. This result indicates that decrease in the level of this anti-apoptotic protein may contribute to the combined effects of brefeldin A and docetaxel on prostate cancer cells.

In summary, we showed in the present study that brefeldin A in combination with docetaxel inhibited the growth of prostate cancer cells in monolayer and 3D cultures. The combination of docetaxel and brefeldin A also more potently induced apoptosis in prostate cancer cells than either agent used individually. The effects of brefeldin A in combination with docetaxel were associated with a marked decrease in the level of Bcl-2 in PC-3 cells. Our study suggests that docetaxel in combination with brefeldin A may represent an effective approach for improving the efficacy and reducing the toxic side effect of docetaxel chemotherapy in prostate cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The present study was supported by grants from the Guangdong Province Leadership Grant, the Rutgers Cancer Institute of New Jersey (CCSG P30-CA072720) and the China National Science Foundation (81272452 and 21102020). Dr. Xi Zheng is the Unilever Chair in Nutrition and Disease Prevention Research at Rutgers University.

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Brefeldin A **Docetaxel**

Figure 1.
Structures of docetaxel and brefeldin A.

Figure 2.

Effects of docetaxel and brefeldin A on human prostate cancer cells. LNCaP and PC-3 cells were seeded at a density of 0.2×10^5 cells/ml in cell culture dishes and incubated for 24 h. The cells were then treated with different concentrations of docetaxel or brefeldin A for 72 h. The number of viable cells was determined by the trypan blue exclusion assay. Apoptosis was determined by propidium iodide staining and morphological assessment.

Figure 3.

Effects of brefeldin A and docetaxel on human prostate epithelial RWPE-1 and prostate cancer PC-3 cells. The cells were seeded at a density of 0.2×10^5 cells/ml in cell culture dishes and incubated for 24 h. The cells were then treated with different concentrations of brefeldin A (BFA) or docetaxel (DTX) alone or in combination for 72 h. The number of viable cells was determined by the trypan blue exclusion assay. (A) Percent viable cells in RWPE-1 and PC-3 cells treated with different concentrations of BFA or DTX. (B) Percent viable cells in RWPE-1 and PC-3 cells treated with combinations of BFA and DTX. Different dose combinations of BFA and DTX are shown in the table under (B).

Figure 4.

Micrographs of PC-3 cells stained with propidium iodide and trypan blue. For the propidium iodide staining, the cells after drug treatment were fixed in acetone/methanol (1:1) for 10 min and stained with propidium iodide (1 $\mu\text{g}/\text{ml}$ in phosphate buffered saline).

Representative micrographs of cells stained with propidium iodide from three separate experiments are shown (scale bar = 25 μm ; arrows indicate apoptotic cells). (A) Control cells. (B) Cells treated with brefeldin A (50 nM). (C) Cells treated with docetaxel. (D) Cells treated with (2 nM) a combination of docetaxel (2 nM) and brefeldin A (50 nM). For the trypan blue staining, the cells were trypsinized after drug treatment, 80 μl of cell suspension was mixed with 20 μl of 0.4% trypan blue solution for 2 min. The number of live and dead cells was determined using the Countess cell counter (Life Technology Ltd, USA).

Representative micrographs of trypan blue staining are shown (scale bar = 100 μm ; arrows indicate dead cells). (E) Control cells. (F) Cells treated with brefeldin A (50 nM). (G) Cells treated with docetaxel (2 nM). (H) Cells treated with a combination of docetaxel (2 nM) and brefeldin A (50 nM).

Figure 5.

Effects of docetaxel and/or brefeldin A on prostate cancer cells in monolayer and 3D culture. A–D: PC-3 cells were seeded at a density of 0.2×10^6 cells/ml of medium in 35 mm culture dishes (2 ml/dish) and incubated for 24 h. The cells were then treated with brefeldin A (50 nM) or docetaxel (2 nM) alone or in combination for 72 h. Representative micrographs of PC-3 cells in the control (A), brefeldin A-treated (B), docetaxel-treated (C) and docetaxel + brefeldin A-treated (D) groups in monolayer culture are shown. E–H: Morphology of PC-3 cells in 3D cultures. PC-3 cells were seeded at a density of 0.5×10^5 cells/ml in Matrigel in 12-well plate (1 ml/well) and incubated for 2 h to let the gel solidified. Culture medium was added on top of the gel (1 ml/well) and the cells were incubated for 24 h. The cells were then treated with docetaxel (2 nM) and brefeldin A (50 nM) alone or in combination once every other day for 10 days. Representative micrographs of 3D cultures in the control (E), brefeldin A-treated (F), docetaxel-treated (G) and docetaxel + brefeldin A-treated (H) groups are shown. Scale bar = 50 μ m.

Figure 6.

Effect of docetaxel and brefeldin A on the level of Bcl-2, total Akt, total Erk1/2, phospho-Erk1/2 and phospho-Akt in PC-3 cells. PC-3 cells were seeded at a density of 1×10^5 cells/ml of medium and incubated for 24 h. The cells were then treated with docetaxel or brefeldin A alone or in combination for 24 h. The levels of Bcl-2, total Akt, total Erk1/2, phospho-Akt and phospho-Erk1/2 were determined by Western blotting. The NIH image J software was used to measure the bend density. The density of the bends relative to control in Western blot is shown.

Table 1

Effects of brefeldin A and docetaxel on growth, apoptosis and formation of tissues-like structure in PC-3 cells

Treatment	% Apoptotic cells	Viable cells (% of control)	Size of tissue-like structure (% of control)
Control	1.9 ± 0.8	100	100
Docetaxel (2 nM)	14.5 ± 1.8	86.7 ± 1.6	83.1 ± 4.6
Brefeldin A (50 nM)	18.0 ± 3.4	83.4 ± 4.6	79.3 ± 4.4
Docetaxel (2 nM) + Brefeldin A (50 nM)	51.2 ± 6.8 *	48.4 ± 7.5 *	38.8 ± 5.7 *

PC-3 cells were seeded at a density of 0.2×10^5 cells/ml in cell culture dishes and incubated for 24 h. The cells were then treated with docetaxel and brefeldin A alone or in combination for 72 h. Viable cells were determined by the trypan blue exclusion assay. Apoptotic cells were determined by propidium iodide staining and morphological assessment. The 3D cultures of PC-3 cells were examined using a light microscope. The size of tissue-like structure in 3D cultures was measured by the NIH image J software. Statistical analysis using ANOVA with Tukey-Kramer multiple comparison test showed that the differences in the number of viable cells, apoptotic cells and the average size of tissue-like structure between the combination-treated group and the docetaxel- or brefeldin A-treated group were statistically significant.

* $p < 0.001$.