Novel Quinuclidinone Derivative 8a Induced Apoptosis in Human MCF-7 Breast Cancer Cell Lines

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Abstract. Novel quinuclidinone derivatives that cause cytotoxicity in human non-small lung carcinoma epithelial cells null for p53 (H1299) have been previously reported. The current study investigates the effect of these derivatives on cytotoxicity of human MCF-7 cells and normal breast epithelial cells (MCF-12a). This study shows that quinuclidinone derivatives 8a and 8b induce growth inhibition mainly through apoptosis of breast cancer cells (MCF-7) with less cytotoxic effect in normal breast epithelial cells (MCF-12a). Derivative 8a was chosen for further investigation. 8a induced G1 phase arrest, presumably sensitizing the breast cancer cells to apoptosis by increasing expression level of p21 and cyclin E. Moreover, 8a increased expression level of ERK1, p53 and BAX, and it reduced expression level of AKT and BCL-2. By investigating the sphingomyelinase apoptosis pathway, it was observed that 8a significantly increased sphingomyelinase activity and increased formation of ceramide as well as increased expression levels of JNK phosphorylation, caspase-8 and caspase-9. Based on previous results it is proposed that quinuclidinone derivative 8a provokes apoptosis in human breast cancer cells (MCF-7) via the sphingomyelinase pathway.

Breast cancer is the leading cause of cancer death in women in the United States and Europe (1, 2). In the United States, it is estimated that one out of eight women develop breast cancer in their lifetime and there is a growing rate in many industrialized nations (3). Breast cancer is a metastatic cancer which affects bone, liver and lung, and metastasis occurs in one-third in all patients of breast cancer, who ultimately die (4). Despite the combined efforts of scientists and clinicians worldwide, there has been a constant increase in the incidence of breast cancer in the last two decades (5). Chemoresistance often accompanies the progression of breast cancers (6), thus efficient therapies are needed to increase the effectiveness of radiation and chemotherapy.

Cancer chemotherapeutics and chemopreventives exert their effects by triggering either apoptotic cell death or cell cycle transition, and accordingly, the induction of tumor cell apoptosis is used to predict tumor treatment response (7). Novel quinuclidinone analogs that induce cytotoxicity in human non-small lung carcinoma cell line (H1299) null for p53, a tumor suppressor protein, have previously been reported (8). However, the mechanisms by which these analogs induce cytotoxicity are poorly understood. Here, this study investigates the effect of these derivatives on proliferation and apoptosis of breast cancer cells (MCF-7) and normal breast epithelial cells (MCF-12a).

Most chemotherapeutic drugs, and radiotherapy, exert their effects through induction of apoptosis. However, apoptosis susceptibility does not always correlate with the status of p53 expression (9-11). It was previously reported for the first time that diallyl trisulfide induced apoptosis via p53 in human breast cancer cells (12). The molecular mechanism induced by most chemotherapeutics is apoptosis, involving interaction between many cellular components including ceramide, caspases and BCL-2 family. Ceramide is a hydrolyzed product of sphingomyelin catalyzed by the key enzyme sphingomyelinase (Smase). Intracellular ceramide is a second messenger that induces apoptosis in many cellular types in chemotherapy (13-15). Ceramide is implicated to induce apoptosis in lung cancer cells through the JNK pathway (16). BCL-2 family proteins interactions are important in carrying the apoptosis signal forward. Two important members of BCL-2 family are BCL-2 and BAX, having opposing function in controlling apoptosis. A simplification of the roles would be that BAX forms homodimers at the mitochondrial outer membrane altering the...
mitochondrial function to allow the release of cytochrome c, while BCL-2 prevents mitochondrial release of cytochrome c that can activate caspase 9 by forming heterodimers with BAX, preventing the formation of bax/bax (17-19).

In this study the effects of two novel analogs (8a and 8b) on breast cancer cells (MCF-7) were investigated by MTT assay, clonogenic survival assay, ELISA based apoptotic assay, TUNEL assay, dot blot, flow cytometry and Western blot analysis. 8a induced apoptosis more in MCF-7 than MCF-12a while 8b induced apoptosis equally in both cells. 8a induced apoptosis by arresting cells in G1 phase and increasing expression levels of cyclin E and p21. It affects survival and apoptotic protein by up-regulating p53, BAX and ERK1/2 while it down-regulates AKT and BCL-2. It also provokes sphingomyelinase signaling by increasing sphingomyelinase activity and increased ceramide levels, JNK1 phosphorylation and caspase 9. Based on previous results, it is concluded that 8a induced apoptosis in human breast cancer cells possibly via sphingomyelinase pathway.

Materials and Methods

Cell culture and drug treatment. MCF-7, breast cancer cells with wild type p53 gene were obtained from (ATCC, USA). MCF-7 cells were maintained in Dulbecco’s modified essential medium (DMEM) (Gibco, CA, USA) supplemented with 10% fetal bovine serum, 100 Units/ml penicillin and 100 μg/ml streptomycin at 37˚C in a 5% CO2 atmosphere (Gibco). Quinuclidinone analogs were prepared in 100 μM concentration and dissolved in suitable media.

Methyl tetrazolium (MTT) bromide mitochondrial activity assay. Cell viability was measured by the methyl tetrazolium (MTT) bromide mitochondrial activity assay (ATCC, Manassas, VA, USA). Briefly, 4,000-5,000 cells/well in 100 μl of medium were seeded in a 96-well plate for 24 h prior to drug treatment. The media was then changed to media with analogs (100 μM) and cells were treated with gamma radiation 4 Gy. After 24 h, 10 μl of 5 mg/ml MTT reagent was added to each well and incubated for 4 h. After incubation, 100 μl of detergent reagent was added to each well to dissolve the formazan crystals. The absorbance was determined at 570 nm. Cells treated only with irradiation were used as controls. Each assay was performed in triplicate and the standard deviation was determined.

Clonogenic survival assay. Cell survival following gamma radiation was measured by clonogenic assays in monolayer on 10 cm plates. Cells were plated in triplicate and were grown for two days to approximately 70% confluency. Quinuclidinone analogs were dissolved in culture media at a final concentration of 100 μM and added to the plated cells 30 min prior to irradiation. Cells were irradiated with different doses of gamma radiation ranging from 2.5-10 Gy from a 137Cs source (J. L. Shepherd and Associates, CA, USA). After 24 h, the media containing the analogs was removed and replaced with fresh media without the derivatives. The cells were grown for 14 days to monitor colony formation. Colonies of at least 50 cells were scored as clonogenic survivors. The surviving fraction was determined by the proportions of seeded cells that formed colonies after drug and radiation treatment relative to the control cells, which were irradiated with gamma radiation without prior drug treatment. Each data point was derived from the results of three independent experiments and expressed as mean±standard deviation.

Enzyme linked immunosorbent apoptosis assay. Cells were seeded at a density of 2x10^4/well in a 96-well plate and incubated for 24 h. Media was changed to media containing the different analogs (100 μM) 30 min before irradiation. Cells then incubated for 24 h. An ELISA assay was performed using Cell Death Detection ELISA PLUS kit (Roche-Applied Science, Indianapolis, USA) that measures histone release from fragmented DNA in apoptosing cells. Briefly, cells were lysed with 200-μl lysis buffer for 30 min at room temperature. The lysate was centrifuged at 200 xg for 10 min then 150 μl of supernatant was collected, of which 20 μl was incubated with anti-histone biotin and anti-DNA peroxidase at room temperature for 2 h. After washing with incubation buffer three times, 100 μl of substrate solution (2,2’azino-di(3-ethylbenzthiazolin-sulphuric acid) was added to each well and incubated for 15-20 min at room temperature. The absorbance was measured using an ELISA reader (Spectra Max Plus) at 405 nm. The control group were cells treated with either UV or gamma radiation. Each assay was carried out in triplicate and the standard deviation was determined.

TUNEL and DAPI staining. For in situ detection of apoptotic cells, TUNEL assay was performed using DeadEnd™ fluorometric terminal deoxynucleotidyl transferase (rTdT)-catalyzed reaction and nucleotide mixture for 60 min at 37˚C in a humidified atmosphere and then immersed in stop/wash buffer for 15 min at room temperature. Cells were then washed with PBS to remove unincorporated fluorescein-12-dUTP. After washing with PBS, cells were incubated in 1 μg/ml DABI solution for 15 min in the dark (data not shown). Cells were observed with fluorescence microscopy (RT slider Spot, Diagnostic Instruments, Inc) and photographed at 100x magnification.

Flow cytometric analysis. Cells were seeded at a density of 3-5x10^5/10 cm plate and incubated for 24 h before radiation. Media was changed to media containing 100 μM 8a and 24 h after drug administration cells were harvested by trypsinization. The cells were washed with PBS and fixed with ice-cold 70% ethanol while vortexing. Finally, the cells were washed with PBS and fixed with anti-histone biotin and anti-DNA peroxidase at room temperature. The absorbance was measured using an ELISA reader (Spectra Max Plus) at 405 nm. The control group was cells treated with 8a, 8b and untreated cells.

Pro-apoptotic and anti-apoptotic protein determination using Western blot analysis. Total protein was extracted from treated and untreated cells using lysis buffer (10 mM Tris HCl pH 7.5, 1mM EDTA, 1% Triton X-100, 150 mM NaCl, 1 mM dithiothreitol, 10% glycerol, 0.2 mM phenylmethylsulphonyl fluoride and protease inhibitors) for 30-50 min on ice. The extracts were centrifuged at
13,000 rpm for 15 min at 4°C to remove cell debris. Folin Lowry (Pierce, USA) protein assay was used to determine the protein concentration in the cell lysates. Proteins were resolved by electrophoresis on 8-10% sodium dodecyl sulphate–polyacrylamide gel. The resolved proteins were transferred onto PVDF membrane and then probed with primary antibody against the protein of interest prepared in 5% milk/PBS-T. The membrane was washed using PBS-T and then appropriate secondary antibody conjugated to HRP was used for visualization of the bands using ECL chemiluminescence kit (GE, USA). Anti-caspase-8, anti-BAX, anti-JNK-1P, anti-caspase 9, anti-BCL-2, anti-pro-caspase-3 and anti-PARP-1 were purchased from SantaCruz, USA. Pixel density of the proteins studied was calculated using Image J, version 1.41o, NIH. The values obtained were first normalized to loading control (GAPDH/β-actin) and folds increase was measured by normalizing to the control (0 hr) value. At least two independent experiments were performed.

Analysis of sphingomyelinase activity. Sphingomyelinase activity was determined in vitro by Amplex Red sphingomyelinase assay (Molecular probes, USA) in a 96-well microplate reader according to manufacturer’s protocol. Briefly, 4000-5000 cells/well in 100 μl of medium were seeded in a 96-well plate for 24 h prior to drug treatment. The media was then changed to media with 100 μM of 8a. Samples were diluted with reaction buffer and pipetted into 96-well microplate. 100 μl of 100 μM Amplex Red reagent containing 2 U/ml horse radish peroxidase (HRP), 0.2 U/ml choline oxidase, 8 U/ml alkaline phosphatase and 0.5 mM sphingomyeline working solution was added to each sample and incubated for 30 min, protected from light. Fluorescence was measured in a fluorescence microplate reader (Spectra Max Plus) using excitation range of 530-560 and emission detection at 590 nm. Each experiment was performed in triplicate and standard deviation determined.

Ceramide measurement using dot blot assay. Cell pellets of untreated and treated samples were collected and lysed on ice for 30 min using lysis buffer (10 mM Tris HCl pH 7.5, 1 mM EDTA, 1% Triton X-100, 150 mM NaCl, 1 mM dithiothreitol, 10% glycerol, 0.2 mM phenylmethysulphonyl fluoride and protease inhibitors). To remove cell debris, the extracts were centrifuged at 13,000 rpm for 15 min at 4°C. To detect ceramide level in the treated and untreated cells, the centrifuged extracts were applied to PVDF membrane placed in a Dot Blot apparatus (Biorad, USA). Protein concentration was first determined using Folin-Lowry protein assay and equivalent volumes were then spotted. Ceramide level was analyzed using primary anti-ceramide antibody and then probed using secondary antibody conjugated to HRP.

Statistical analyses. All the assays described above were repeated more than once. The quantification assays were performed in triplicate and data were calculated and presented as mean±standard deviation (mean±std). Data were either analyzed by unpaired Student t-test or one-way ANOVA with Tukey’s post-hoc test depending on the nature of the assays. Significant level was taken for p<0.05.

Results

8a reduced cell proliferation and induced apoptosis in breast cancer cells. An MTT assay was used to screen several cancer cell lines including breast, melanoma, prostate cancer and hepatoma (data not shown). Initial screening showed that 8a and 8b were the most potent derivatives in breast cancer cells (MCF-7), which were selected for further analysis and compared with normal breast cells (MCF12a). The cell proliferation assay indicated that 100 μM 8a and 8b reduced cell viability to approximately 60% and it further decreased to approximately 30% after treating with gamma radiation. On the other hand, 8b reduced viability of normal breast epithelial cells to 30% while the viability for was approximately 66% for 8a (Figure 1A). To confirm that the decreased viability was due to apoptosis, ELISA was used, which detects histone release from apoptotic cells, revealed that addition of 100 μM 8a increased apoptosis in MCF-7 (approximately 2-fold increase), compared to non-treated controls while 8b induced apoptosis equally in both MCF-7 and MCF-12a cells (Figure 1B). Based on this observation, 8a was chosen for further investigation. TUNEL assays were performed in order to ascertain induction of apoptosis by 100 μM 8a (Figure 1C). In MCF-7 cells, the assay revealed the presence of nuclear condensation and TUNEL-positive cells after treating cells with 100 μM 8a, while fewer TUNEL-positive cells were found in MCF-12a (Figure 1D).

8a sensitized breast cancer cells to gamma radiations. Cell survival was measured by clonogenic assays after drug treatment and gamma radiation. As expected, 8a sensitized cells in MCF-7 and MCF12a to gamma radiation (2.5-10 Gy) (Figure 2A, B) in a dose-dependent manner. The effects of 100 μM 8a were compared to the control group that consisted of cells treated only with irradiation. 8a had less effect on clonogenic survival in MCF-12a cells (Figure 2B). In both cell lines, the percentage of clonogenic survival at higher doses of gamma radiations (7.5 and 10 Gy) was greatly reduced to less than 10%.

Impact of quinuclidinone analogs on cell cycle checkpoints using flow cytometry. Flow cytometry was used to examine the effects of the derivatives on cell cycle checkpoints, as well as cell proliferation and apoptosis. The percentage of cells in G1, S, G2, and apoptosis were determined after treating cells with 100 μM 8a (Figure 3A); however, no significant difference was found in the percentage of cells in S phase. MCF-7 cells were treated with 100 μM of 8a and harvested after 24 h and showed a significant increase in the percentage of G1 cells (50%) compared to control group (30%). The G1 arrest was consistent with increased expression of p21 (4-fold increase) which regulates the G1 checkpoint and also increased cyclin E levels (2.4-fold) (Figure 3B).

Impact of 8a on apoptotic and survival signals. Apoptosis occurs due to an imbalance between pro and anti-apoptotic proteins. 8a increased expression level of p53 (2.5-fold) and it also up-regulated its downstream target pro-apoptotic
protein BAX (2.2-fold) in MCF-7 cells after 24 h. ERK belongs to a family of mitogen-activated protein kinases (MAPKs) that can be activated in response to various stress stimuli. ERK phosphorylation increased on 8a treatment (3-fold), while the expression level of AKT (by half) and BCL-2 was reduced (2.4-fold) with treatment of 8a (Figure 4).

8a increased sphingomyelinase and ceramide formation during apoptosis. The sphingomyelin pathway has been implicated in chemotherapeutic based apoptosis; sphingomyelinase is involved in the catabolism of sphingomyelin present in the plasma membrane to produce ceramide, an important second messenger triggering
Figure 1. 8a reduces cell proliferation and induced apoptosis in MCF-7 cells. (A, B) Cell proliferation assay was performed to detect living cells. Each data point was an average of results from three independent experiments performed in triplicate and presented as Mean±SD. ELISA assay was applied for apoptotic cell detection. Data are presented as Mean±SD. (C) TUNEL assay was used to confirm induction of apoptosis between treated and non-treated cells. MCF-7 cells were treated with 100 μM 8a for 24 h and induction of apoptosis was confirmed by appearance of TUNEL-positive cells. (D) Presence of nuclear condensation and TUNEL-positive cells with 100 μM 8a in MCF-7 cells, while fewer TUNEL-positive cells were found in MCF-12a.
downstream apoptotic targets (20). 8a significantly increased sphingomyelinase activity in MCF-7 cells. In keeping with the sphingomyelinase activation trend, 8a induced significant ceramide formation in MCF-7 cells after 24 h treatment (Figure 5A).

8a impact on downstream targets of ceramide-mediated apoptosis. Sphingomyelinase can be activated by death receptors and the ceramide formed can cause clustering of these death receptors, resulting in further activation of downstream targets of these receptors (21, 22). To examine whether death receptors are involved in 8a-induced cell death, activated caspase-8 protein was probed using Western blotting. Procaspase-8 was cleaved on activation of death receptors to form activated caspase-8 after 8a treatment. Additionally, 8a increased JNK-1 phosphorylation and caspase-9 activation in MCF-7 cells (Figure 5B).

Discussion

Chemoprevention is the most efficient way to treat and control cancer (7). Most chemotherapeutics induce tumor regression by causing apoptosis. Apoptosis, programmed cell death, is a conserved sequence of events that is characterized by the formation of nuclear and cytoplasmic blebbing, DNA fragmentation and cell shrinkage (23, 24). In chemotherapeutic induced apoptosis these paths are indistinguishable as both are activated due to interplay of molecules boosting the apoptosis signal by the chemical. The data from this study show that 8a can be used to treat breast cancer cell lines and it has less effect on normal cells. The progression of cell cycle requires activation of cyclin-dependent kinases, whose activation is dependent upon their association with corresponding cyclins. Cyclin E, one of the G1 cyclins, is a critical regulator of the G1/S transition of the cell cycle and initiation of DNA replication (25). Cyclin E levels increased under stress condition, and increased cyclin E expression sensitizes cells to apoptotic stimuli (26). The current data indicate that MCF-7 cells are arrested at G1 Phase (50%) after 8a treatment as indicated in Figure 2. Furthermore, the arrest in G1 phase was confirmed by increased expression of p21 (4-fold), which is a key regulator of G1 phase; these data are consistent with those of Yongxian and Keith (27). These results suggest that increased G1 phase arrest does sensitize the cells to chemotherapeutic apoptosis. The current results showed that 8a reduced expression level of survival signals generated by both BCL-2 and AKT while it up-regulated expression level of p53 and BAX.

Ceramide is considered as a second messenger that carries the signal of apoptosis forward (28, 29). The role of sphingomyelinase activation and subsequent release of ceramide was reported in some of the initial studies of doxorubicin, cisplatin and gemitabine but was activated in normal and cancer cells at similar levels (13-15). Intriguingly, sphingomyelinase activity in quinuclidinone derivatives 8a treated cells was significantly affected in breast cancer cells with a significant increase in the level of ceramide compared to control group.

Ceramide activates down stream protein kinases involved in apoptosis (30). SAPK/JNK is involved in ceramide triggered apoptosis in cancer cells (16). JNK-1 appears to play an important role in activating BAX, allowing the release of cytochrome c and activating caspase-9, which cleaves procaspase-3 to effector caspase-3. It also regulates anti-apoptotic proteins such as BCL-2 (31-33). Many studies indicate that when death receptors, such as TNF-α, FAS and TRAIL, are involved, procaspase-8 is cleaved to form active caspase-8 which either activates effector caspase-3 directly or triggers the mitochondrial apoptosis pathway through the BCL-2 related family members (34). Ceramide amplifies the death receptor apoptotic signals by forming platforms to cluster the
Figure 3. 8α arrests MCF-7 cells in G₁ phase. Cells were treated as described under the Materials and Methods. The % of cell cycle phases was determined in MCF-7 cells after treatment with 100 μM 8α for 24 h (A). Non-treated cells for the same time interval were used as control. Total protein was extracted and used for Western blotting analysis to determine the differences in p21 and cyclin E expression levels between treated and non-treated cells with 100 μM 8α for 24 h. β-Actin was used as loading control (B, C). Each data point is the mean of three independent experiments and expressed as mean±SD.

Figure 4. 8α affects survival and apoptotic signals in MCF-7 human breast cancer cells. MCF-7 cells were treated with 100 μM 8α for 24 h. Total protein was extracted and used for Western blotting analysis to determine the differences in p53, Bax, Bcl-2, Akt and ERK expression levels between treated and non-treated cells. Each data point is the mean of three independent experiments and expressed as mean±SD.
receptors through the activation of sphingomyelinase (35, 25). In the current study, JNK-1 was activated followed by the formation of caspase 8 after drug treatment in lung cancer cells, suggesting the involvement of death receptor in apoptosis with the mediated through sphingomyelinase activation and formation of ceramide. The p53, a tumor suppressor protein, is an essential mediator in the cellular response to DNA damage by causing G1/G2 cell cycle arrest and/or apoptosis (36). The current data indicated that 8a increased expression levels of p53 and its downstream target BAX which receives apoptotic signals from both extrinsic and intrinsic sources, and usually plays a role in mitochondria-mediated apoptosis (37, 38). The activation of BAX by 8a, which leads to the apoptosis of MCF-7 cells, may be mediated through p53 or some other upstream factors (39). ERK1/2 also is activated in response to various stress stimuli through divergent mechanisms involving the RAS-RAF-MEK pathway (40, 41) Depending upon the cell type, the stimulus, and the duration of activation, a variety of biological responses (i.e., cell proliferation, migration, differentiation, and apoptosis) are associated with ERK.
activation (42, 43). With the increase in BAX protein levels, an increase in caspase-9 levels, caspase-8, JNK-P and ceramide level suggest the mitochondrial apoptosis pathway involvement of sphingomyelinase pathway. Here a potential novel anticancer derivative 8a that mediates apoptosis via activation of sphingomyelinase in human breast cancer cells is presented.

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References


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