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In Vitro and *In Vivo* Efficacy of a Novel Quinuclidinone Derivative Against Breast Cancer

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Abstract. *Previously, our laboratory reported on novel quinuclidinone derivatives that cause cytotoxicity in human non-small lung carcinoma epithelial cells null for p53 (H1299). The current study aims to investigate the effect of novel designed quinuclidinone derivatives on cytotoxicity towards human MCF-7 breast cancer cells, normal breast epithelial cells (MCF-12a) and an animal model of breast cancer. Quinuclidinone 2 induced growth inhibition mainly through apoptosis in breast cancer cells (MCF-7), with less cytotoxic effects towards normal breast epithelial cells (MCF-12a) compared to the other derivatives. Our novel quinuclidinone-2 increased expression of p53 and cyclin-D and reduced expression levels of (Mdm2), (Bcl-2) and (Akt). It also reduced expression of (Bax) as down stream target of p53 at both RNA and protein levels. Additionally, quinuclidinone 2 induced G_1 phase arrest presumably sensitizing breast cancer cells to apoptosis by increasing expression of p21. In vivo studies were performed to assess the anticancer effect of quinuclidinone 2 on N-Nitroso-N-methylurea-induced breast cancer in female rats by evaluating physiological processes and the expression levels of β-catenin and E-cadherin. The approximate lethal dose of quinuclidinone 2 was determined to be 90 mg/kg and it led to significant reduction in tumor size compared to the untreated group. In vivo studies revealed that quninuclidinone derivative 6 does not induce any apparent toxicity towards the treated hosts and under the present experimental set up seems to be a promising candidate for further evaluation in cancer therapy*

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Breast cancer is still the most common cancer among women worldwide and the second leading cause of cancer-related death in women accounting for 14% of deaths (1). Despite the fact that mortality rates of breast cancer seem to reduce during the past two decades, incidence rates continue to increase in the world (2). Although breast cancer is often curable when treated at an early stage, metastatic disease is almost uniformly fatal due to the development of therapeutic resistance (3). Most 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 for tumor treatment response (4).

Previously, our laboratory reported novel quinuclidinone analogs that induce apoptosis in lung cancer *via* sphingomyelinase pathways (5). However, the mechanisms by which these analogs induce cytotoxicity were poorly-understood. Here we optimized the ring structures to investigate their effect on proliferation and apoptosis of breast cancer cells (MCF-7) and normal epithelial cells (MCF-12a) as well as N-Nitroso-N-methylurea -induced breast cancer rats.

Apoptosis is cellular suicide or programmed cell death which is mediated by activation of an evolutionary-conserved intracellular pathway. The relation of apoptosis in cancer has been emphasized and increasing evidence suggests that the processes of neoplastic transformation, progression and metastasis involve alteration of normal apoptotic pathway (6). Apoptosis is a selective process of physiological cell deletion that plays an important role in the balance between cellular replication and death (7). Apoptotic signaling can proceed through two pathways, *i.e.*, *via* death receptors expressed on the plasma membranes of cells or alternatively *via* mitochondria, which contain several proteins that regulate apoptosis (8-11). In a typical eukaryotic cell cycle, successful completion of cell-cycle events and genetic information prior to proceeding to the next phase of the cell cycle is strictly regulated by key cyclin-dependent kinases. These regulatory pathways are commonly called cell-cycle checkpoints (12).

p53 is activated and stabilized by a host of post translational modification in response to external and internal stress signals,

such as genotoxic stress and anticancer drugs, these signals promote its nuclear transformation into an active form. However, in response to genotoxic stresses, such as exposure to ultraviolet light or γ-irradiation, p53 protein levels increase and trigger either cell-cycle arrest or apoptosis (13). p53 plays a critical role in cancer prevention because it can suppress tumorigenesis by inducing cell-cycle arrest and apoptosis through its transcriptional activity. Activation of p53 leads to activation of the intrinsic pathway by increasing the expression of many pro-apoptotic proteins such as (Fas), (DR5), (Apaf-1), procaspase-6, (Bax) and (Bid). After reception of the death signal, BAX increases mitochondrial permeability to increase the release of cytochrome-*c*, which also activates caspase-3, which in turn stimulates apoptosis by attacking many valuable proteins required by the cell (14-16). However, p53 is one of the tumor suppressor genes that is most frequently found to be inactivated in cancer (17).

In this study, we investigated the impact of the novel quinuclidinone 2 on breast cancer cells (MCF-7) by (MTT) assay, (ELISA) based apoptotic assay, (TUNEL) assay, (RT-PCR) and western blot analysis. We also investigated the protective effect of our novel quinuclidinone derivative on NMU-induced breast cancer in female rats by evaluating the expression level of β-catenin and E cadherin.

Materials and Methods

Cell culture and drug treatment. MCF-7, breast cancer cells with wild type p53 gene were purchased from the AmericanType Culture Collection (VA, USA). MCF-7 cells were maintained in Dulbecco's modified essential media (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% CO_2 atmosphere (Gibco). MCF-12a human mammary epithelial cells, were maintained in DMEM-F12. (Invitrogen, CA, USA) supplemented with 0.5 μg/ml hydrocortisone (Sigma-Aldrich), 10 μg/ml insulin (Sigma-Aldrich, St Louis, MO, USA), and 20 ng/ml recombinant human (EGF) (Peprotech, Rocky Hill, NJ, USA). Quinuclidinone analogs (1-3) were prepared at 20 μM concentration and dissolved in suitable media.

MTT bromide mitochondrial activity assay. Cell viability was measured by MTT bromide mitochondrial activity assay as described previously (ATCC). 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 medium was then changed to medium with analogs (20 μM). After 24 h, 10 μL of 5 mg/ ml MTT reagent was added to each well and cells were 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 without analogs were used as controls. Each assay was performed in triplicate and the standard deviation was eternined.

ELISA-based apoptosis assay. Cells were seeded at a density of 2×10^4/well in a 96-well plate and incubated for 24 h. Media were changed to media containing analogs (1-3) at 20 μM 30 min and incubated for 24 hours. An ELISA assay was performed using Cell Death Detection ELISAPLUS kit (Roche-Applied Science, 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 × *g* 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 plates were 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 was treated with fresh media. Each assay was done in triplicate and standard deviation determined.

TUNEL and (DAPI) staining. For *in situ* detection of apoptotic cells, TUNEL assay was performed using DeadEnd™ fluorimetric tunnel system (Promega, USA). Cells were cultured on 4-chamber slides (VWR, USA) at a density of 2×10^4 cells/chamber. After treatment with 20 μM of quinuclidinone derivative 2, cells were washed with PBS and fixed by incubation in 4% paraformaldehyde (PFA) for 20 min at 4˚C. The fixed cells were then incubated with digoxigenin-conjugated dUTP in terminal deoxynucleotide transferase recombinant (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 DAPI 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 x100 magnification.

Flow cytometric analysis. Cells were seeded at a density of $3\text{-}5\times10^5$/10 cm plate and incubated for 24 h before treatment. Media were changed to media containing 20 μM quinuclidinone derivative 2; 24 h after treatment, 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 and re-suspended in PBS containing 5 μg/mL RNase A (Sigma, St. Louis, MO USA) and 50 μg/ml propidium iodide (Sigma) for analysis. Cell-cycle analysis was performed using FACScan Flow Cytometer (Becton Dickson) according to the manufacturer's protocol. Windows multiple document interfaces (WinMD) software was used to calculate the cell-cycle phase distribution from the resultant DNA histogram, and data are expressed as a percentage of cells in the G_0/G_1 and G_2/M phases. The apoptotic cells were determined on the DNA histogram as a subdiploid peak.

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), 1 mM EDTA, 1% triton X-100, 150 mM NaCl, 1 mM dithiothretol, 10% glycerol, 0.2 mM phenylmethysulphonyl fluoride and protease inhibitors for 30-50 min on ice. The extracts were centrifuged at 13,000 × *g* 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- polycrylamide 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-(p53), anti-(Bcl-2), anti-(MDM2), anti-(Akt), anti-(p21), anti-(cyclinD), Texas red conjugated secondary antibody, anti-E-cadherin and anti-β-catenin were purchased from SantaCruz, (USA). Pixel density of the proteins blots studied was calculated using Image J, version 1.41o, NIH. The values obtained were first normalized to loading control β-actin and the fold change was measured by normalizing to that of the control (0 h) value. At least two independent experiments were performed.

Animal treatment and in vivo chemotherapy. Virgin female Wistar Albino rats (animal house, Faculty of Medicine, Egypt) were obtained at 60 days of age, weighing ~120 g. The animals were housed in plastic cages (five rats/cage) in a controlled environment with temperature maintained at 25-27˚C and a 12 h light / dark cycle. They were acclimatized for seven days before the start of the experiment, with food and water provided *ad libitum*. Rats were housed four per cage in conditioned rooms at 20±2˚C, kept under an automatic 12 h light/12 hours darkness schedule, and given pellets and tap water *ad libitum*. All animal studies were conducted in accordance with the NIH Guide for the Care and the Use of Laboratory Animal. Crystalline NMU (Sigma) was dissolved in 0.85% NaCl solution and acidified (acetic acid) to pH 5.0. The concentration was then adjusted to 5 mg/100 g body weight/rat. Heparin-treated blood (0.2 ml) was obtained by cardiac puncture from rats lightly anesthetized with ether. Animals were palpated for detection of mammary tumors after injection and the animals were examined by palpation for tumor masses thrice weekly post administration of carcinogen. Tumor sizes were measured with a caliper and their growth and histological parameters were recorded.

Experimental design. At 65 days of age, the female rats were divided into the following groups: I- Healthy Controls (n=10) without NMU-induction; II- NMU controls (n=20) received weekly an injection of NMU (5 mg/100 g body weight/rat); III- NMU and treated with quinuclidinone derivative 2 (n=20) received weekly an injection of NMU5 mg/100 g body weight/rat) and observed for tumor development, at which quinuclidinone derivative 2 was injected at different time intervals for 60 days.

Histopathological examination and biochemical analysis of serum. The organs were prepared as paraffin-embedded tissue glass slides stained with hematoxylin and eosin and evaluated according to the National Toxicology Program standards. A complete cross-section of each liver and kidney, when possible, was evaluated. For liver, two cross-sections, one of each of the two largest liver lobes were examined. For kidneys, an entire cross-section (left longitudinal, right transverse) was evaluated. The entire sections on the slides (all fields) were evaluated under blinded conditions for lesions and scored (graded) on a subjective basis compared to control animals. The grades were as follows: 1=minimal, 2=mild, 3=moderate, 4=marked, and –=no pathological changes. Serum biochemistry parameters analyzed included BUN, AST, ALT, CK and AP. Blood was collected from the posterior vena cava of control rats that were either treated with water and treated rats administered 90 mg/kg quinuclidinone derivative 2 after 60 days of treatment.

Chemical synthesis of quinuclidinone derivative 2. A solution of 1 (1.73 mmole) in water (10 mL) was treated with anhydrous K2CO3 (1.73 mmole) followed by thiourea (1.78 mmole). The reaction mixture was heated under reflux whereupon a solid product was separated after 60 min. The reflux was continued for further 30 min and the product was filtered while hot, washed with water then crystallized from ethanol to give 1a in 88% yield (based on the hydrochloride); mp 215-216˚C lit mp.

NMR spectral analysis. 1.28-1.31, 1.50-1.54, 1.70-1.78 and 1.83-1.92 (4m, 4H, H-7, 9),2.39-2.44 (m,1H,N-CH), 2.47 (d,1H,H-4), 2.57-2.59 (m,1H,N-CH), 2.57-2.59 (m,1HN-CH), 2.69-2.74 (m, 2H, N-CH2), 2.9 (2bt, 1H, CH2-4), 3.23, 3.26 (dd, 1H, CH2-4), 5.81 (S, 1H, D2O exchangeable, OH), 7.94, 8.27 (2S,2H, D2O exchangeable, 2NH).
Statistical analyses. All the assays described above were repeated more than once. The quantification assays were performed in triplicate and data were calculated and are presented as means±standard deviation. Data was either analyzed by unpaired Student *t*-test or one-way ANOVA with Tukey's post-hoc test, depending on the nature of the assays. Significance was set at $p<0.05$.

Results

Quinuclidinone derivative 2 reduced proliferation and induced apoptosis of MCF-7cells greater than in MCF-12a cells. Based on previous observation, we optimized the ring structures for quinuclidinone analogs as shown in (Figure 1A). We treated both MCF-7 and MCF-12a cells with 20 μM of quinuclidinone derivatives and we determined the percentage of cell viability. Based on the data shown in Figure 1B, quinuclidinone derivative 2 was less cytotoxic towards MCF-12 cells than the other two derivatives. To confirm that the decrease in viability was due to apoptosis, ELISA was used and revealed that addition of 20 μM derivatives increased apoptosis in MCF-7 compared to non-treated controls. The results indicated that quinuclidinone derivative 2 was the most potent in inducing apoptosis of MCF-7 cells with an approximately four-fold increase while it had less apoptotic effects on MCF-12a cells (approximately three-fold increase) (Figure 1C). Based on this observation quinuclidinone derivative 2 was chosen for further investigation. TUNEL assays were performed in order to ascertain induction of apoptosis by 20 μM of quinuclidinone derivative 2 (Figure 1D). In MCF-7 cells, the assay revealed the presence of nuclear condensation and TUNEL-positive cells after treating cells with 20 μM quinuclidinone derivative 2, while fewer TUNE-positive were found in MCF-12a (Figure 1D).

Impact of quinuclidinone derivative 2 on apoptotic and survival signals. The tumor suppressor gene, p53, is as a key component of a cellular emergency response system to induce cell growth arrest or apoptosis. Figure 2A shows that p53 expression was up-regulated (approximately four-fold increase) in MCF-7 cells after treatment with 20 μM quinuclidinone derivative 2 compared to the control untreated group. Figure 2B shows that there was a five-fold increase in the expression of cyclin-D1. The anti-apoptotic signals represented by MDM2, AKT and BCL-2 were also determined by western blot analysis; the results in Figure 2A indicate that

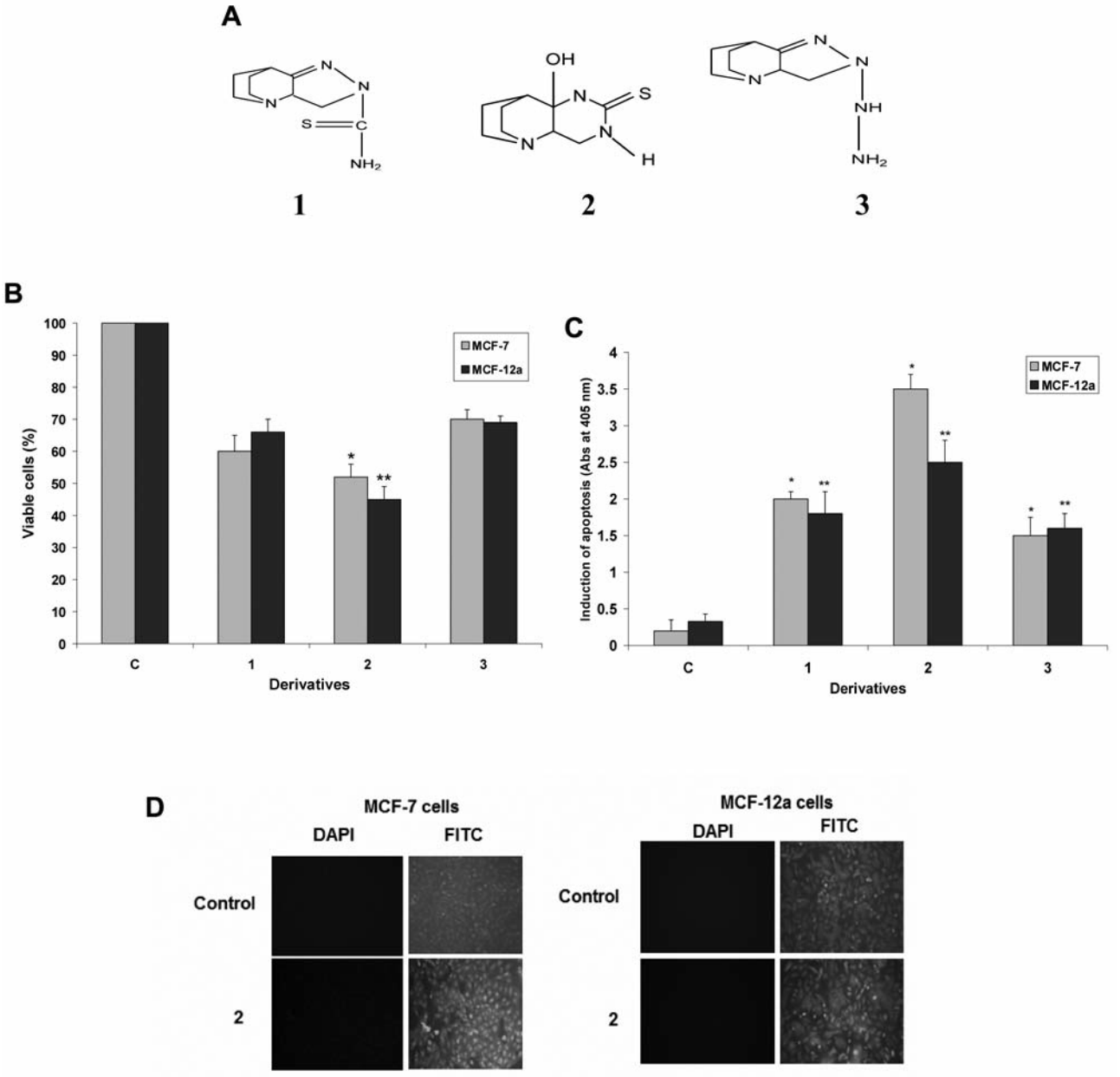

Figure 1. *Impact of quinuclidinone analogs on apoptosis. Structures of designed quinuclidinone analogs(1-3) (A). MCF-7 and MCF-12a cells were treated with 20 μM of derivatives and the percentage of cell viability was determined (B). Induction of apoptosis in MCF-7 cells and MCF-12 cells was determined after treating cells with quinuclidinone analogs for 24 h (C). Each data point is the mean±SD of three independent experiments. Induction of apoptosis is represented as absorbance at 405 nm. TUNEL assay was used for derivative 2 to determine induction of apoptosis in treated and non-treated cells. Both MCF-7 and MCF-12a cells were treated with quinuclidinone derivative 2 for 24 h and induction of apoptosis was confirmed by the presence of TUNEL-positive cells (D).*

quinuclidinone derivative 2 reduced expression of MDM2, AKT and BCL-2 by approximately 5-, 2- and 1.8-fold, respectively (Figure 2C).

Quinuclidinone derivative 2 induced overexpression of BAX in MCF-7 cells. BAX, a BCL-2 family protein, induces cell death though disruption of mitochondrial permeability and subsequent release of cytochome *c*. MCF-7 cells were treated with 20 μM quinuclidinone derivative 2 for 24 h and non-treated cells were used as control. As shown in Figure 3A, using real-time PCR quinuclidinone derivative 2 significantly increased BAX expression in MCF-7 cells at the mRNA level

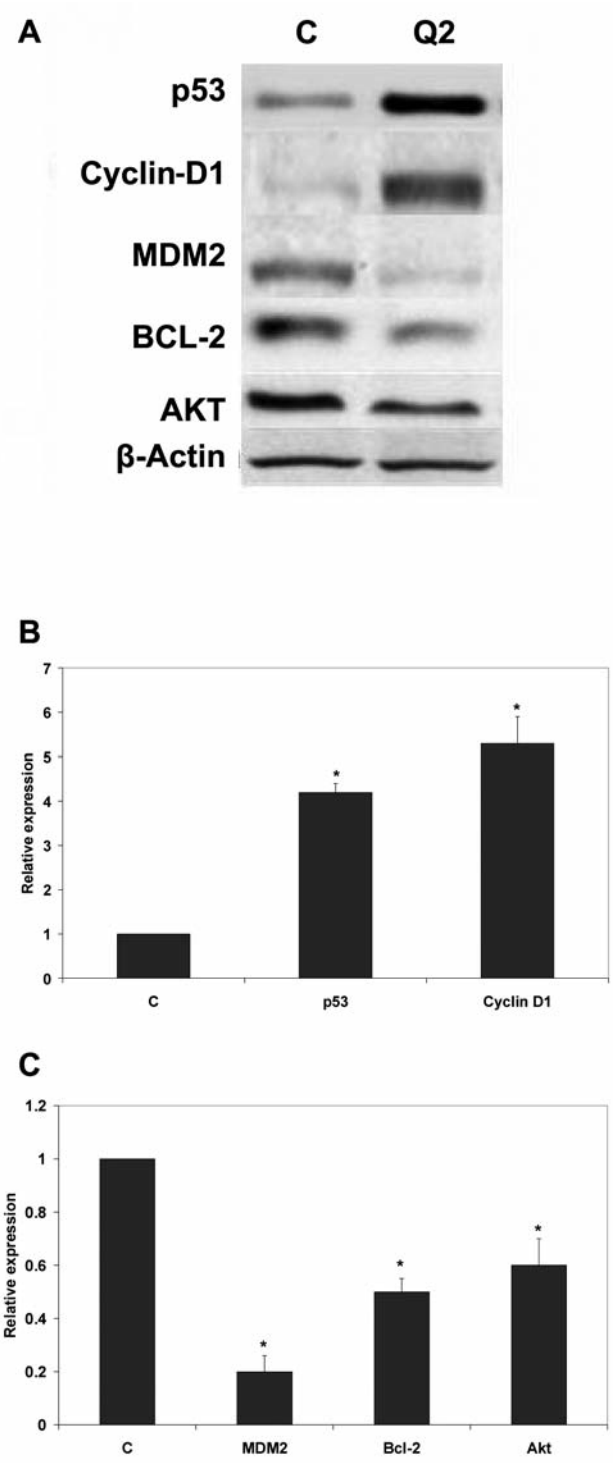

Figure 2. *Quinuclidinone derivative 2 modulates survival and apoptotic signals in MCF-7 human breast cancer cells. MCF-7 cells were treated with 20 μM of quinuclidinone derivative 2 for 24 h. Western blot analysis was performed to determine p53, cyclin-D1, AKT, BCL-2 and MDM2 protein expression levels in treated and non-treated control cells. β-Actin was used as a loading control. Each data point is the mean±SD of three independent experiments (p<0.05) compared to control group.*

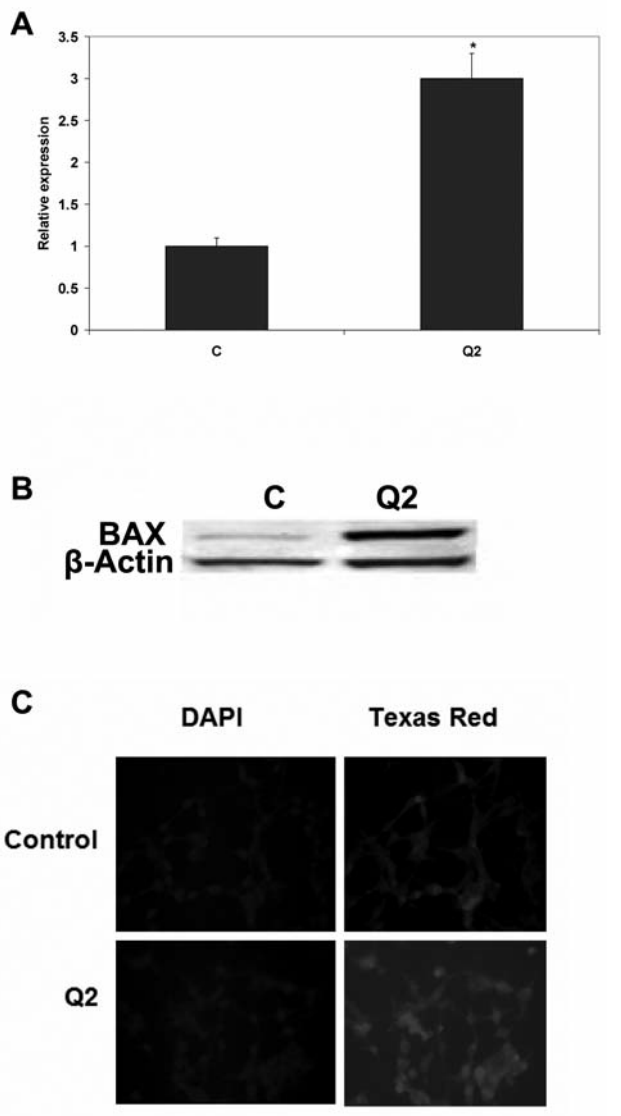

Figure 3. *Quinuclidinone derivative 2 treatment induced BAX expression in MCF-7 human breast cancer cells. MCF-7 cells were treated with 20 μM quinuclidinone derivative 2 for 24 h. Non-treated cells were used as control. mRNA was extracted and reversed-transcribed to cDNA for real-time PCR analysis of Bax mRNA levels p<0.05 (A). BAX expression was determined after treating MCF-7 cells with quinuclidinone derivative 2 using Western blot analysis((B). BAX in MCF-7 cells was immuno stained using BAX (B-9) as the primary antibody and Texas Red – conjugated IgG as the secondary antibody(C). The individual images for BAX were taken using an immunofluorescence microscope. Data of representative three independent experiments are shown.*

by three-fold, as indicated in Figure 3A ($p<0.05$) compared with non-treated control cells. The expression of BAX increased by four-fold as compared with the controls, using western blot analysis (Figure 3B). These results were further confirmed by immunofluorescence microscopic study, which showed that 20 μM quinuclidinone derivative 2 increased

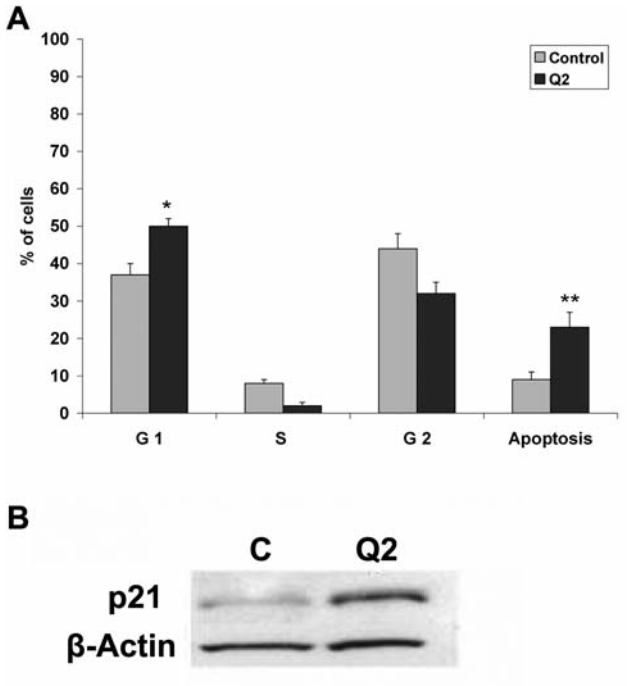

Figure 4. *Impact of quinuclidinone derivative 4 on cell cycle in MCF-7 cells. The percentage of cells in each cell cycle phase was determined in MCF-7 cells after treatment with 20 μM quinuclidinone derivative 2 for 24 h (A). Each data point is the mean±SD of three independent experiments. Total protein was extracted and used for western blot analysis to determine the differences in p21, between treated and non-treated cells with 20 μM quinuclidinone derivative 2 for 24 h. β-Actin was used as loading control (B).*

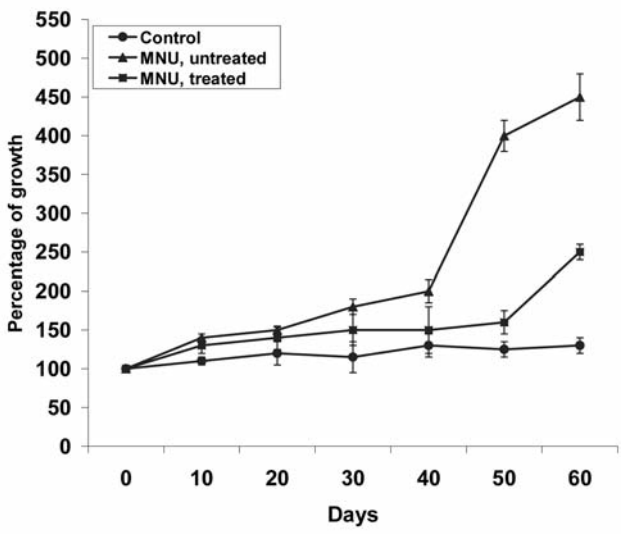

Figure 5. *Tumorigenicity in breast cancer was inhibited by quinuclidinone derivative 2. Data was plotted as mean±SD tumor volume at different time points during the course of treatment with quinuclidinone derivatives 2. Each group comprised of ten rats and the percentage of tumor growth is determined.*

BAX expression in MCF-7 cells after a 24 h treatment up on staining with Texas Red conjugated IgG (Figure 3C).

Quinuclidinone derivative 2 arrested MCF-7 cells in the G_1 phase. Apoptosis is frequently associated with proliferating cells, implying the existence of molecules in late G_1 and S phase whose activities facilitate execution of the apoptotic process. Flow cytometry was used to examine the effects of the quinuclidinone derivative 2 on cell-cycle checkpoints, as well as cell proliferation and apoptosis. The percentage of cells in G_1, S, G_2, and apoptosis were determined after treating MCF-7 cells with 20 μM of quinuclidinone derivative 2 (Figure 4A); however no significant difference was found in the percentage of cells in the S phase. Quinuclidinone derivative 2 induced apoptosis by arresting cells in the G_1 phase (55%) compared with the untreated group (30%). The arrest in the G_1 phase was consistent with an increased expression of p21 (3-fold increase) in MCF-7 cells treated with 20 μM of quinuclidinone derivative 2 compared to the untreated group (Figure 4B).

In vivo study. LD_{50} determination: Investigation of the acute toxicity is the first step in the toxicological investigation of an unknown substance. LD_{50} was determined by arithmetic method of Karbar. To assess the preventive effect of quinuclidinone derivative 2 on development and progression of breast cancer, quinuclidinone derivative 2 was examined for toxic effects. The results of this study are shown in Table I. After 14 days, there were no deaths recorded in animals given 100 mg/kg body weight oral dose of quinuclidinone derivative 2. During the observation period, animals did not exhibit any variations in general appearance and motor activity, and there was no death in rats tested during the period of observation. Oral administration of quinuclidinone derivative 2 at all given doses (higher than 100 mg/kg) caused death in the tested groups during the first 24 h of observation. The median lethal dose LD_{50}, was calculated using the following equation: LD_{50}=Least lethal dose -Σ (a×b)/N, where N is the number of animals in each group, a: the dose difference and b the mean mortality.

Histopathological examination and serum biochemical analysis: The data shown in Table II show that derivative 2 at sublethal doses only led to mild liver atrophy while using lethal dose led to mild fatty liver change and moderate liver atrophy. There were no morphological changes in kidney upon using lethal and sublethal doses.

Effect of quinuclidinone derivative 2 on liver function markers: The activities of serum AST, ALT, ALP and GGT were found to be significantly higher in NMU- treated rats when compared to control animals. Elevated activities of serum AST, ALT, ALP and GGT observed in NMU-treated rats may be due to NMU-induced hepatic damage and the subsequent leakage of these enzymes into the circulation. Data in Table III

Table I. *Lethal median dose (LD_{50}) determination for Q2 by the arithmetic method of Karbar.*

Group	Dose (mg/kg)	Number of rats	No. of animals dead	Dose difference (a)	Mean mortality (b)	Probit (a×b)
1	Control	5	0	0	0	0
2	100	5	0	100	0	0
3	200	5	1	100	0.5	50
4	300	5	1	100	1	100
5	400	5	2	100	1.5	150
6	500	5	3	100	2.5	250

Sum of the product=550, LD_{50}=200 - (550/5)=90 mg/kg.

show that rats with breast cancer and untreated (group II) had a significant ($p<0.01$) increase in serum levels of AST, ALT and ALP compared to normal rats in group I (Table III). Quinuclidinone derivative 2 administration to group III caused significant reduction ($p<0.05$) in serum level of AST, ALT and ALP as compared to the positive control group (group II). Results in Table IV showed that rats with breast cancer (group II) had significant decrease ($p<0.001$) in serum level of total protein and albumin compared to normal rats (group I). Quinuclidinone derivative 2 administration caused significant ($p<0.05$) increase in serum level of total protein and albumin in group III as compared to the untreated group (group II) (Table V). NMU administration significantly ($p<0.001$) increased total bilirubin in group II, compared to their control counterparts. Quinuclidinone derivative 2 significantly reduced total bilirubin, ($p<0.05$) when compared to the NMU-treated group (table IV). Significant increase ($p<0.001$) was observed in serum levels of the tumor marker protein AFP and GGT in group (II) as compared to control group (group I). Quinuclidinone derivative 2 supplementation for 5 weeks resulted in significant decrease of AFP and GGT levels ($p<0.05$) (Table IV).

Quinuclidinone derivative 2 reduced tumorigenicity in NMU-treated rats: Quinuclidinone derivative 2 was evaluated at the maximum tolerated dose and administration was scheduled for five injections from day 1 of maximum tumor size until day 25. Tumor volume (V) was recorded and determined by the equation (LXW^2)/2 in which L is the length and W is the width of the tumor. Data in Figure 5 reveal that there was approximately two-fold reduction in tumor size after day 25 after treatment with quinuclidinone derivative 2 compared to the untreated group (Figure 5).

Impact of quinuclidinone derivative 2 treatment on β-catenin and E-cadherin expression in breast cancer rat model: β-Catenin plays a significant role in the regulation of mammary development and tumorigenesis. At the plasma membrane, β-catenin aids cadherins in maintaining mammary epithelial integrity. In the nucleus, β-catenin regulates gene expression programs that are essential for mammary stem cell biology during mammary development. Our data in Figure 6 show that

Table II. *Summary of histopatholigcal examination of animals after administration of Q2.*

Organ lesion grade	Control group	Q2 sublethal dose	Q2 lethal dose
Liver fatty change	-	-	2±0.5
Liver atrophy	-	2±1	3±0.6
Kidney tubular dilation	-	-	-
Kidney glomerulopathy	-	-	-

The experiment was performed with three animals per dose. Tissue samples were taken at necropsy and preserved in 10% buffered neutral formalin (average severity grade 1-4) 1 minimal, 2 mild, 3 moderate, 4 marked, – no pathological changes.)

treatment with quinuclidinone derivative 2 led to a strong staining for β-catenin and E-cadherin in cell membrane and cellular junctions and revealed a weak cytoplasmic expression.

Discussion

Breast cancer ranks first among the most commonly diagnosed and cancer-related deaths in women worldwide (18). Despite significant advances toward targeted therapy and screening techniques, breast cancer continues to be the leading cause of cancer-related death. Identification of new non-toxic agents can delay breast cancer progression, which is the main leading cause of cancer-related deaths among women (19, 20), is highly warranted. In the present study, we investigated the effect of three novel quinuclidinone derivatives on proliferation of two cell lines, MCF-7 human breast cancer cells and non-tumorigenic MCF12a mammary epithelial cells. Our data showed that treatment with 20 μM quinuclidinone derivative 2 for 24 h suppressed viability of cultured MCF-7 cells, with less cytotoxic effects on normal breast epithelial cells.

Apoptosis is an important mechanism to kill tumor cells (21). It can be induced by increase in mitochondrial calcium that results in loss of membrane potential expansion of the matrix and rupture of the outer mitochondrial membrane (22). Quinuclidinone derivative 2 induced apoptosis of MCF-7 cells

Table III. *Effect of Q2 on serum activities of liver marker enzymes in experimental groups of rats. Data are presented as mean±S.E.M (n=10).*

Group	Treatment	ALT (U/l)	AST(U/l)	ALP(U/L)	GGT (U/l)
I	Control	65.5±1	120±2.5	195±2	28±2
II	NMU-untreated	180±5***	260±7***	320±5***	190±8***
III	NMU-treated	140±4*	179±3.2*	242±2*	129±2*

ALT: Alanine transaminase, AST: aspartate transaminase, ALP: alkaline phosphatase, GGT: gamma-glutamyl transpeptidas. NMU: N-nitroso-N-methylurea. NMU- untreated: represent cancer control group. *$p<0.05$ as compared to group II (NMU-treated), **$p<0.01$ as compared to group I (control), ***$p<0.001$ as compared to group I (control).

Table IV. *Effect of Q2 on serum levels of total protein, albumin, total bilirubin and (AFP) in experimental groups of rats. Data are presented as mean±S.E.M (n=10).*

Group	Treatment	Total protein (g/dl)	Albumin (g/dl)	Total bilirubin (g/dl)	AFP (ng/ml)
I	Control	7±0.004	4.4±0.04	0.85±0.01	4.5±0.8
II	NMU-untreated	5±0.05**	2.6±.04**	3.4±0.1**	90±4***
III	NMU-treated	6.8±0.04*	3.1±0.09*	1.7±0.19*	62±2.4*

*$p<0.05$ as compared to group II (NMU-treated), **$p<0.01$ as compared to group I (control), ***$p<0.001$ as compared to group I (control).

as shown by the TUNEL assay. The progression of cell cycle requires activation of cyclin-dependent kinases, whose activation is dependent upon their association with corresponding cyclins. Cyclin E is a critical regulator of the G_1/S transition of the cell cycle and initiation of DNA replication (23). Cyclin E levels increased under stress condition, and increased cyclin E expression sensitizes cells to apoptotic stimuli (24). Our data indicated that MCF-7 cells are arrested at the G_1 phase after quinuclidinone 2 treatment as compared to the untreated group. $p21^{WAF1/CIP1}$, a CDK inhibitor, is a p53-inducible protein that blocks the cell-cycle progression in the G_1/S phase (25). Thus, up-regulation of p21 by quinuclidinone derivative 2 might have resulted in activation of downstream effectors of p53-dependent G_1/S arrest. The hypophosphorylated form of retinoblastoma protein (pRb), a tumor suppressor, forms a complex with E2F transcription factor resulting in repression of cell proliferative genes (26). Based on our data, the arrest in G_1 phase was confirmed by increased expression of p21 (by four-fold). p53 is a tumor suppressor, mutated in 50% of human cancers (27), which regulates cell growth and the sensitivity to γ-radiation and multiple anticancer agents (28) However, many p53-deficient cell lines retain the ability to undergo radiation–induced apoptosis, suggesting that there are p53-independent pathways of apoptosis (29). The p53 and p21WAF1 tumor suppressor genes are known to be involved in apoptosis (30). Functional p53 can down-regulate BCL-2, which allows cells to survive a variety of fatal cellular events and protects cells from apoptosis (31).

p53 can also induce $p21^{WAF1}$, and an increased level of $p21^{WAF1}$ can, in turn, decrease the activity of cyclin-dependent kinases (CDKs), resulting in growth arrest (32, 32). We investigated BAX as a downstream target for p53 and our results revealed that quinuclidinone derivative 2 treatment increased Bax expression at both RNA and protein levels. Several pathways have been described to mediate p53-induced apoptosis, Bax receives apoptotic signals from both extrinsic and intrinsic sources, and usually plays a role in mitochondria-mediated apoptosis (33, 34). The activation of Bax by quinuclidinone derivative 2, which leads to the apoptosis of MCF-7 cells, may be mediated through p53 or some other upstream factors.

After evaluating the effects of our derivative on proliferation, apoptosis, and cell-cycle progression, apoptotic and survival proteins, we chose to study the impact of the quinuclidinone derivative 2 on NMU-induced mammary tumors in the Wistar albino model because the histological structure of mammary gland tumors in this animal closely resembles that of human mammary tumors. Induction of mammary carcinomas by NMU in female rats is one of the most frequently used animal models for the investigation of breast carcinogenesis and mammary tumor treatment (35-37). Liver damage caused by NMU generally reflects instability of liver cell metabolism, which leads to distinctive changes in serum enzyme activities. The biomarkers used in this study provide the measures of carcinogen exposure in rats as an area of high risk for development of hepatocellular carcinoma. Serum AST, ALT, ALP and GGT are sensitive indicators of hepatic injury. Results of this study showed that NMU-treated rats (group II) presented significant increase in serum levels of AST, ALT, ALP, as well as GGT with respect to their control counterparts. The increase in these parameters in rats can be attributed to injury to the structural integrity of the liver and the subsequent leakage of these enzymes into the circulation after cellular damage induced by NMU. In the present investigation, treatment with quinuclidinone derivative 2 significantly lowered the activities of these enzymes to near normal values suggesting that it may eliminate the hepatotoxic effect of the NMU.

The WNT/β-catenin signaling pathway plays an important role in carcinogenesis and tumor metastasis, which is involved in many cancer types including breast cancer (38, 39). Thus, targeting the WNT/ β-catenin-TCF signaling is of great significance for chemoprevention and chemotherapy of cancer. Deregulation of the WNT/β-catenin signaling has been implicated in the pathogenesis of many human cancers, including breast cancer (40-42). Our data clearly showed that quinuclidinone derivative 2 treatment reduced the localization and expression of β-catenin and E-cadherin in the nucleus and

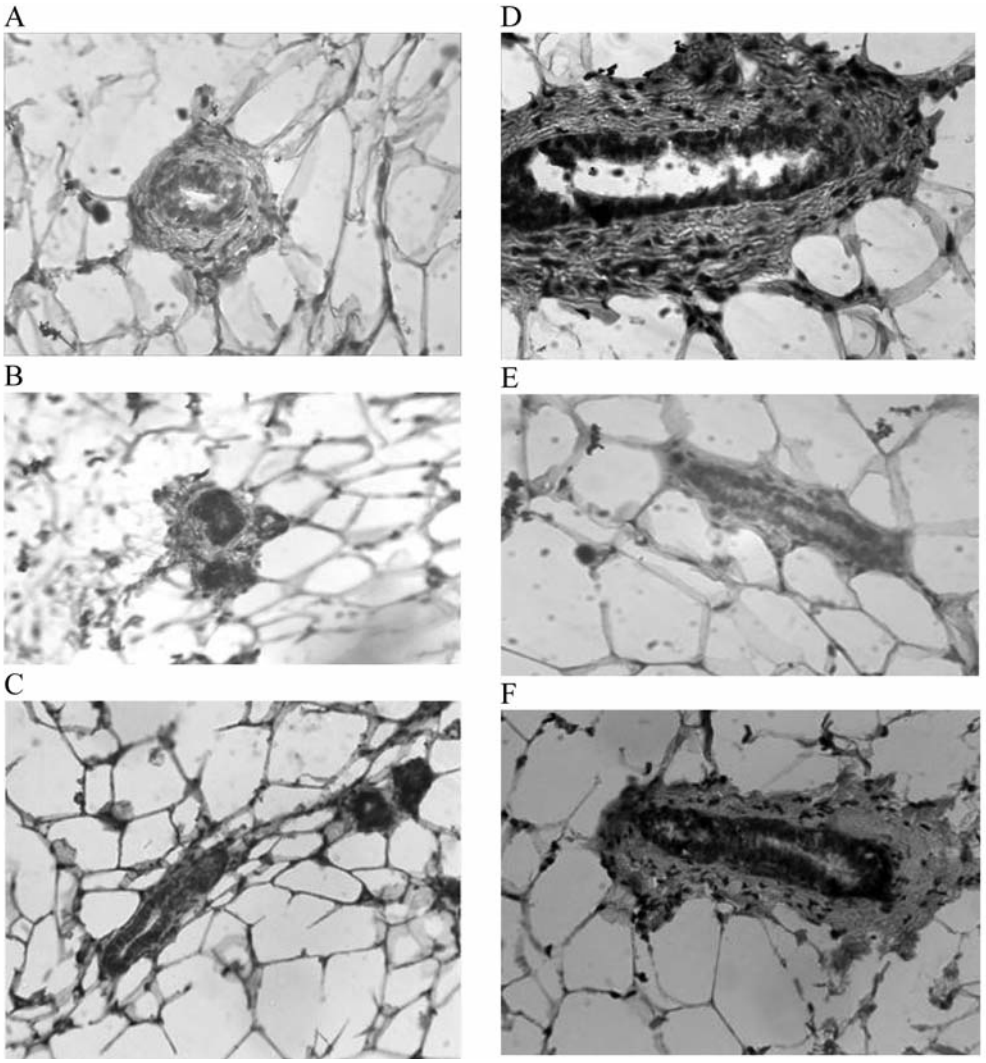

Figure 6. *Immunohistochemical staining of rat mammary glands on paraffin sections reflecting the extent of β-catenin and E-cadherin expression. Control mammary gland showed normal expression levels of β-catenin, mainly localized in the cytoplasm (brown stain) (A). (B) Untreated mammary gland showed high expression of β-catenin in cytoplasm and nucleus (B). Q2-treated mammary gland shows decrease in β-catenin expression in cytoplasm and nucleus. (C) Normal mammary gland showed normal levels of E-cadherin expression (D). Untreated mammary gland showed decrease in E-cadherin expression (E). Q2-treated mammary gland showed increase in of E-cadherin expression (F).*

led to strong staining in the cell membrane and cellular junctions compared to untreated groups. Biochemical and histopathological analyses revealed that our quinuclidinone derivative 2 was non-toxic in animals bearing breast cancer. In conclusion, our studies revealed that quinuclidinone derivative 2 does not induce any apparent toxicity in treated animals and seems to be promising in the management of breast cancer under the present experimental set up.

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