Second-generation Substituted Quinolines as Anticancer Drugs for Breast Cancer

BRIAN HEINIGER1, GUNJAN GAKHAR1, KESHAR PRASAIN2, DUY H. HUA2 and THU A. NGUYEN1

Departments of 1Diagnostic Medicine/Pathobiology and 2Chemistry, Kansas State University, Manhattan, KS 66506, U.S.A.

Abstract. Cancer cells have reduced capacity for gap junctional inter-cellular communication (GJIC). One feasible approach to reduce growth of cancer cells is to enhance GJIC. This report shows that a second-generation substituted quinoline, PQ7, has anti-tumor effect. Scrape load/dye transfer and colony growth assays were performed to measure GJIC and tumor formation of T47D breast cancer cells. PQ7 at 500 nM induced a 16-fold increase in the GJIC in T47D cells. In addition to an increase in GJIC, a 50% decrease of colony growth was observed with 100 nM of PQ7. PQ7-treated nu/nu mice showed a 100% regression of xenograft tumor growth of T47D cells. The results show that PQ7 has a promising role in exerting anti-tumor activity in human breast cancer cells.

Breast cancer is the most common cancer in women worldwide and mortality from breast cancer is consistently due to tumor metastasis (1). Cancer is a complex and evolving disease with the formation of defects at multiple genetic steps in a cell. Cancer was the first pathology associated with defects in gap junctions. In 1966, Lowenstein and Kanno associated gap junctional intercellular communication (GJIC) with cell growth control (2). They observed a decrease in electrical coupling in rat hepatomas compared to normal liver cells. A series of molecular mechanisms showed that the cancer phenotype is related to loss of coupling (3). Since the most obvious observation in tumor cell phenotype is deregulated growth, the assumption of all the studies is that gap junctions are involved in cell growth control (4). Gap junction deficiency has been defined as either lack of gap junctional plaques observed by ultrastructure approaches (electron microscopy and freeze-fracture) or by decrease in GJIC (5).

Gap junctions are transmembrane hydrophilic channels allowing the passage of molecules of less than 1200 Da between the cells through the intracytoplasmic space. The passage of small molecules through gap junctions suggests that the maximal functional pore size for the channel is about 1.5 nm in diameter in mammalian cells (6). Small molecules such as cAMP, inositol triphosphate, glucose, and calcium ions can pass while large molecules such as proteins or complex sugars cannot pass through the gap junctions (7). Gap junctions are the only specialization of the cell membranes which allow communication between the adjacent cells (8).

A deficiency of the connexin 43 (Cχ43) gap junction can be used as an independent marker for breast tumors (9). Early studies showed down-regulation of Cχ26 and Cχ43 in the primary cells derived from human breast tumor (10), rat mammary tumors (9), and breast cancer cell lines (9, 11). Since, the promoter of Cχ26 is located within a CpG (cytosine phosphate guanine) island, it is speculated that the methylation of these sites could lead to the repression of the gene (12). Investigational studies by Singal et al. found hypermethylation of Cχ26; however, inhibition of a DNA methyltransferase did not induce the expression of the gene (11). On the contrary, Tan et al. found that only one in eight breast cancer cell lines tested was hypermethylated in the Cχ26 promoter region but that in this case, it correlated with a complete loss of mRNA that was recovered after treatment with a DNA methyltransferase inhibitor (13). Furthermore, the Cχ26 promoter was found to be methylated in >50% of patient tissue samples tested, albeit heterogeneously. These conflicting results suggest that Cχ26 may indeed be a tumor suppressor that is inactivated by methylation, but this is likely not the only mechanism to down-regulate expression (12). Up-regulation of phosphorylated forms of Cχ43 was observed in both myoepithelial cells and transformed luminal cells of in situ carcinomas and all cells of invasive breast carcinomas (14). Connexins are mostly present on the cell membrane but in some tumors despite an increase in connexins, they are typically retained in the intracellular...
Several small organic molecules, such as caffeic acid phenethyl ester (CAPE) (16), sodium 4-phenylbutyrate (17), liarozole (18), lycopene (19), and lovastatin (20), have been reported for up-regulation or restoration of GJIC. It was previously reported that a new class of substituted quinolines (code name, PQ) can increase gap junction activity in T47D breast cancer cells (21). That study demonstrated that the first generation of substituted quinolines effectively induce apoptosis, decrease cell viability, and attenuate tumor growth. A second generation of PQ analogs was synthesized as described in the reported protocol (22). The goal of the current study was to examine a second generation substituted quinoline that specifically activates GJIC activity and subsequently inhibits tumor growth in xenograft T47D mice. The results demonstrated this second-generation small molecule provides a promising treatment for breast cancer.

Materials and Methods

Materials. Compound PQ7, 6-methoxy-8-[(2-furanylmethyl)amino]-4-methyl-5-(3-trifluoromethyl)phenoxoxy)quinoline, was prepared by following the reported procedure (22).

Cell line and cell culture. T47D human breast cancer cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in RPMI medium supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA), 10% antibiotic-actinomycotic at 37˚C with 5% CO2 in 75 cm2 flasks. Cells were grown in serum supplemented RPMI media until they were 90% confluent in 75 cm2 flasks.

Western blot analysis. Cells were grown in serum supplemented RPMI media until they were 90% confluent in 75 cm2 flasks. Cells were kept in starving media containing phenol red-free DMEM with 5% charcoal dextran stripped serum, overnight. Cells were dosed with 0, 10, 100, 200, 500, and 1000 nM of PQ7 for 24 hours. Cells were washed three times with cold PBS and then were harvested using lysis buffer (20 mM Tris pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, and 0.5 % Triton X-100) with 1:1000 dilution of protease inhibitors (Sigma-Aldrich, Saint Louis, MO, USA). Cell lysate was sonicated and centrifuged at 13,000 rpm for 30 minutes at 4˚C. Twenty-five μg of whole-cell extract was resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane (Midwest Scientific, Saint Louis, MO, USA). Nitrocellulose membrane was blocked in 5% milk for an hour at room temperature and then incubated with monoclonal antibody rabbit Cx43, 1:500 (Santa Cruz Biotehnhologies, Santa Cruz, CA, USA), rabbit caspase-9, 1:500 (BD Biosciences, San Jose, CA, USA), and rabbit actin, 1:1,000 (Sigma-Aldrich). Western blots were detected by enhanced chemiluminescence detection reagents (Pierce, Rockford, Illinois, USA).

Gap junction activity. For scrape load/dye transfer (SL/DT) assay, cells were grown to 90% confluency on coverslips, dosed with 0, 10, 100, 200, 500 and 1000 nM of PQ7 for 24 hours. After that, cells were washed three times with PBS then 2.5 μl of 1% (w/v) Lucifer yellow and 75% (w/v) of rhodamine dextran were mixed and added in the center of the coverslip. Two cuts crossing each other to the center of the coverslip were made. After three minutes, cells were washed three times with PBS and incubated at 37˚C in tissue culture media for 20 minutes. The cells were then washed with PBS three times and fixed in 2.5% paraformaldehyde for 10 minutes. Cells were mounted on a slide, sealed and visualized under a fluorescence microscope using x4 and x10 objectives.

Colony growth using soft agar. Cells were treated with 0, 10, 100, and 1000 nM PQ7 for 14 days. Base agar plates were prepared containing 0.8% agar and 0.4% agar in Ham’s F12. Cells (5×104 cells/33 mm2 well) were suspended in 100 μl of Ham’s F12 with 0.4% agar and plated. These plates were maintained at 37˚C for 14 days and examined for the presence of colonies. Individual colonies of 50 μm or greater were examined.

Trypan blue exclusion. Cell viability was measured using the trypan blue excision method. A total of 1×104 T47D breast cancer cells were treated with different concentrations of PQ7 for 48 hours. A cell suspension was mixed with trypan blue dye and then visually examined for viable cells by Cellometer Auto T4 (Nexcelom). The graphical presentation includes three independent samples.

Xenograft tumors of T47D cells in Nu/Nu mice. Nu/Nu mice were ordered from The Jackson Laboratory, (Bar Harbor, ME, USA). Mice were inoculated with estradiol-17β (1.7 mg/pellet) before an injection of 1×107 T47D breast cancer cells subcutaneously into inguinal region of mammary fat pad. Cell viability of T47D cells was performed prior to injection. The tumor size was measured in three dimensions with calipers every 2 days starting at day 7. Mice were observed for any change in behavior, appearance or weight. When tumors reached 30-50 mm3, three animals were randomly assigned to each treatment group. Animals were injected with 1 μM of PQ7 every two days and daily measurement of tumor size was recorded.

Statistical analysis. The level of significance was considered at p<0.05 using Student’s t-test. All data are presented as mean±SD of at least three independent experiments from different batches of cultures.

Results

Intercellular communication in many organs is maintained via GJIC. Several GJIC enhancers have been reported; however, an effective clinical drug targeting gap junction is not available at this time. The study goals were to synthesize small molecules that specifically activate GJIC activity and inhibit cancer cell growth. A second-generation substituted quinoline was designed and its enhancement of gap junction activities and killing of human breast cancer cells was demonstrated. The effect of PQ7 was tested on GJIC activity in T47D breast cancer cells. The results demonstrated that 500 nM of PQ7 showed a significant increase in gap junction activity compared to controls, without PQ7 treatment, using scrape load/dye transfer assay (Figure 1A). The distance of
dye transfer from section cut to the farthest cells with dye was measured. A graphical presentation of three experiments indicates that 500 nM PQ7 cause a 16-fold increase in distance of dye transfer compared to control (Figure 1B). Previously, it has been demonstrated that normal mammary epithelial cells (MECs) have uniform uptake of Lucifer yellow (21). This is due to the existing high level of gap junction activity of these normal cells. These results demonstrated that PQ7 is sufficient to cause an increase in GJIC activity in the SL/DT assay.

Various oncogenes (e.g. ras, raf, neu, src, mos) down-regulate GJIC, while several tumor suppressor genes can up-regulate GJIC. Thus, the effect of PQ7-upregulated gap junction activity on T47D colony growth formation was examined. Cells were grown in soft agar to assess their capacity for anchorage-independent growth, which is a key feature of cell transformation due to the importance of cell-cell and cell-matrix based tumor suppression. T47D cells were treated with 0, 10, 100 and 1000 nM PQ7 for 7 days. A graphical presentation of three experiment results is presented in log scale of PQ7 concentration. The effect of PQ7 on T47D cells showed a significant inhibition of T47D cell colony growth compared to control (Figure 2). PQ7 at 100 nM inhibited 50% of colony growth compared to controls without PQ7 treatment. Interestingly, the same concentration (100 nM PQ7) had no effect on MECs (data not shown). This suggests that 100 nM PQ7 can cause an increase in GJIC activity and subsequently can reduce colony growth of T47D cells. Furthermore, cell viability of T47D cells was also assayed (Figure 3). A 100 nM, PQ7 for 48 hours reduced T47D cell growth by 94%. This supports the notion that monolayer cells have more exposed surface area for the uptake of treatment.

Whole-cell extract of PQ7 treatment was analyzed for the changes in gap junctional proteins, connexins. Cells were treated with 0, 10, 100, 200, 500 and 1000 nM PQ7 for 24 hours. Western blot analysis was performed against Cx43...
Figure 4. Effect of PQ7 on the expression of connexin 43 in T47D cells. T47D cells were dosed with PQ7 for 24 hours. The Western blot analysis was performed as described in the Materials and Methods. Whole-cell extract was analysed for Cx43. Experiment was performed at least three times.

Figure 2. Effect of substituted quinoline on T47D cells. Base agar plates were prepared containing 0.8% agar and 0.4% agar in Ham’s F12. Cells (5×10⁴ cells/33 mm² well) were suspended in 100 μl of Ham’s F12 with 0.4% agar and plated. These plates were maintained at 37 °C for 14 days and examined for the presence of colonies. Individual colonies of 50 μm or greater were examined. T47D cells were treated with 10, 100, and 1000 nM PQ7 and SA (succinic acid) as a solvent control, indicated as ‘C’. Individual colonies of 50 μm or greater were examined. Statistical significance, *p<0.05, of at least three experiments. The results show that 100 nM of PQ7 is sufficient to cause a 50% decrease in colony growth compared to control at day 14.

Figure 3. Effect of PQ7 on cell viability. 1×10⁴ T47D breast cancer cells were treated with different concentrations of PQ7 for 48 hours. Cell suspension was mixed with trypan blue dye and then visually examined for viable cells. Statistical significance, *p<0.05, of at least three experiments.

Figure 4. Anti-actin was used as a loading control. The results show that PQ7 increases Cx43 expression in T47D breast cancer cells. This result was confirmed using confocal microscopy (data not shown). The effect of PQ7 on apoptosis was examined by performing Western blot analysis against caspase-9 (Figure 5). For the 500 and 1000 nM treatments, the results showed an increase in caspase-9 expression as well.

The anti-tumor effect of PQ7 was also observed in the animal model. Nu/Nu mice were implanted with estradiol-17β (1.7 mg/pellet) before the injection of 1×10⁷ T47D breast cancer cells subcutaneously into inguinal region of mammary fat pad. The results of xenograft tumors demonstrated a decrease in tumor size in the PQ7-treated group compared to control at day 2. The results showed a 100% decrease of tumor growth with PQ7 treatment after 7 injections compared to control (Figure 6). In previous in vivo studies (21), the results suggested that PQ compound exits the body of mice in ~2 days, hence, injections of the drug every 2 days appears to provide optimal results.

Discussion and Conclusion

The antitumor effects of the first generation of substituted quinolines have previously been demonstrated. These effects include an increase in GJIC activity, as well as attenuation of xenograft tumor growth in nude mice (21). This study demonstrated the efficacy of a second-generation molecule and the promising role of these compounds in the treatment of cancer.

Over forty years, the loss of GJIC has been described in cancer cells and led to the hypothesis that defects in GJIC is involved in carcinogenesis (23). Numerous reports have confirmed that gap junctions are frequently decreased in or absent from cancer cells. Co-culture of tumor cells with normal cells, or with connexin-overexpressing cells, results in growth retardation of the neoplastic component by the establishment of functional communication as observed with dye transfer (24). This retardation of tumor growth was prevented by co-culturing transformed cells with junctional competent normal cells transfected with a connexin-specific antisense reagent (25). Thus, these results indicate that signals from adjacent normal cells can reverse a malignant phenotype and that their failure to accomplish this is due to a lack of GJIC.

The relationship between cell communication and cell growth has been established such that the capability of cells to communicate through gap junctions is negatively related to their growth activity. In this study it was demonstrated...
that increased GJIC activity in T47D cells can cause a decrease in cell growth (Figures 1, 2, and 3). Saez et al. (26) also observed that an increase in GJIC is directly related to the anti-tumor effect in human mammary cancer cell line. In normal tissues, gap junctions are active and well regulated between the cytoplasm of contacting cells (27-29). Through the passage of signaling molecules, GJIC contributes to the regulation of cell proliferation, differentiation, cell death, and homeostatic maintenance. Numerous studies show clearly that altered GJIC is involved in cell cycle progression. In most cell types, GJIC is reduced in the late G1, S and M phases (30). The specific cell cycle state in which GJIC and/or connexin expression are modified, however, depends on both the cell type and the nature of the connexin being investigated. This study found evidence that PQ7 increases expression of Cx43 in T47D human breast cancer cells (Figure 4). This information may help to explain the subsequent increase in GJIC in PQ7-treated cells.

To study the effect of PQ7 in tumor-bearing mice, a xenograft tumor study of T47D cells was conducted. The results showed a 100% decrease of tumor growth with PQ7 treatment compared to control after seven injections (Figure 6), thus providing evidence that PQ7 has an anti-tumor effect in this animal model. The data clearly shows that PQ7 is an effective anticancer agent. When compared with the 70% decrease found in a similar study using first-generation substituted quinolines (21), it is clear that PQ7 is an improvement on the first generation of these compounds. Therefore, this second generation has an even more promising role in the treatment of breast cancer. The mechanism of enhancement of GJIC by PQ compounds is still being investigated.

Acknowledgements
We gratefully acknowledge financial support from the National Institutes of Health (COBRE-R20RR017686, K-INBRE-P20RR016475 and R01AG025500) and Kansas State University, Center for Basic Cancer Research.

References


