Abstract. Background: The tumor suppressor gene PTEN, mutated in 40-50% of patients with brain tumors, especially those with glioblastomas, maps to chromosome 10q23.3 and encodes a dual-specificity phosphatase. PTEN exerts its effects partly via inhibition of protein tyrosine kinase B (Akt/Protein Kinase B), which is involved in the phosphatidylinositol (PtdIns) 3-kinase (PI3K)-mediated cell-survival pathway. The naturally occurring bioflavonoid Quercetin (Qu) shares structural homology with the commercially available selective PI3K inhibitor, LY 294002 (LY). Here, the effects of Qu on the Akt/PKB pathway were evaluated.

Materials and Methods: The human breast carcinoma cell lines, HCC1937, with homozygous deletion of the PTEN gene, and T47D, with intact PTEN, were time-treated with Qu or LY and analyzed for activated levels of Akt by measuring phospho-Akt (p-Akt) levels using immunoblotting analysis. To detect p-Akt, the T47D cells were treated with EGF prior to treatment with or without Qu or LY. Cell proliferation after 24-h treatment with Qu or LY was quantified by the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Results: Treatment with Qu (25 ÌM) for 0.5, 1 and 3 h completely suppressed constitutively activated Akt/PKB phosphorylation at Ser-473 in HCC1937 cells. Pre-exposing T47D cells to Qu (25 ÌM) or LY (10 ÌM) abrogated EGF-induced Akt/PKB phosphorylation at Ser-473. Both Qu (100 ÌM) and LY (50 ÌM) treatments for 24 h significantly decreased cell proliferation, as shown by the MTT assay. Conclusion: Pharmacologically safe doses of the naturally occurring bioflavonoid Quercetin (Qu) inhibit the PI3K-Akt/PKB signaling pathway in PTEN-null cancer cells, and suggest that this compound may have therapeutic benefit against tumorigenesis and cancer progression.
no effect on the expression of other cell cycle and apoptosis regulators, such as p53, p21, p73, bcl-2 and bax (7). Qu is known to inhibit P13K, as well as mediating the inhibition directed towards the ATP-binding site of PI3K (8).

The BRCA1 mutant human breast carcinoma cell line HCC1937 displays constitutive activation of Akt/PKB due to homozygous deletion of the PTEN gene. The aim of this study was to suppress Akt/PKB activity in HCC1937 using the bioflavonoid Qu. In order to qualify the efficacy of Qu, T47D human breast cancer cells with intact PTEN and suppressed Akt/PKB pathways were treated with a growth factor so as to stimulate the cell survival pathway. LY and Qu were both found to significantly curb Akt/PKB activity.

Materials and Methods

Cell culture. The human breast cancer cell lines, HCC1937 with homozygous deletion of the PTEN gene, and T47D cells with intact PTEN, were obtained from the American Type Culture Collection (ATCC, VA, USA). Both cell lines were cultured in RPMI 1640, supplemented with 1% L-Glutamine, 7.5% fetal bovine serum, and 1% penicillin-streptomycin, and incubated in a humidified 5% CO2 atmosphere at 37°C. Serum-free RPMI (0.5% FBS) was given to 80-100% confluent cells 24 h prior to Qu or LY treatments to induce quiescence (G0 phase). The HCC1937 cells were treated with 25 μM Qu (Calbiochem, CA, USA) or 10 μM LY (Calbiochem) for 1 and 3 h. The T47D cells were treated with either Qu or LY prior to EGF (Calbiochem) or TPA (Sigma, MO, USA) treatment. Each experiment was repeated 3 times.

Western blot analysis. After treatment, the cells were harvested in a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA and 1% (V/V) Triton X-100 plus protease and phosphate inhibitors (Sigma). The protein content was measured by the Bradford procedure, with bovine serum albumin as a standard. The cell lysate (50 μg) was separated by SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to PVDF or nitrocellulose membranes. The sheets were pre-incubated in a mixture of TBS (20 mM Tris-HCl [pH 7.5], 150 mM NaCl), 0.05% Tween 20 and 5% de-fatted milk powder with the following antibodies, as appropriate: anti-phospho-Akt (Cell Signaling Technology, MA, USA), anti-Akt (Cell Signaling Technology). After being washed in TBS-0.05% Tween 20, the membranes were incubated in peroxide-coupled secondary antibodies (1:2000 dilution; Cell Signaling Technology) for 1 h at room temperature. Following incubation, the membranes were washed in TBS (20 mM Tris-HCl [pH 7.5], 150 mM NaCl), 0.05% Tween 20, 3 times for 5 min each. The membranes were incubated with secondary antibodies following the manufacturer’s instructions (Cell Signaling Technology). Following the immunoanalysis, the blots were stripped and re-probed with unphosphorylated Ab-Akt to ensure equal loading.

Cell proliferation assay. The HCC1937 cells were cultured and incubated as above. Approximately 15,000 HCC1937 cells/well were seeded in 96-well flat-bottomed microtiter culture plates (Sigma). The cells were incubated in a humidified 5% CO2 atmosphere at 37°C for 24 h. The cells were made quiescent by incubating with serum-free RPMI for 24 h. The quiescent cells were then treated with either the bioflavonoid Qu or LY in serum-free media for 24 h. At the end of the assay, all media was removed and replaced with 100 μl/well fresh serum-free RPMI. The MTT Cell Growth Assay Kit (Chemicon International, CA, USA) was utilized to evaluate cell proliferation. In brief, 10 μl AB solution (MTT, (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, 50 mg), and PBS pH 7.4, 15 mL) were added to each well. The culture plates were mixed gently and incubated at 37°C for 4 h in order for cleavage of MTT to occur. One hundred microliters of Solution C (isopropanol with 0.04 N HCl) were added to each well and mixed by pipetting. The absorbance was measured within 1 h on an ELISA plate reader at 595 nm.

Results

Structural similarity between LY and Qu. Chemical structural analysis revealed that both the naturally occurring bioflavonoid Qu and the commercially available selective P13K inhibitor LY are members of the benzopyranone class of compounds (Figure 1). LY was synthesized using Qu as a model (9). Studies have shown that LY has a greater potency than Qu, which is considered a broad-spectrum inhibitor. Binding of LY and Qu to P13K differ (8). The chromo moiety of Qu occupies the space filled by the morpholine ring of LY 294002. The hydrogen bond between the 3-OH of Qu and the side chain of LYS-833 mimics the interaction made by the ketone moiety of LY, indicating that the chromo moiety of LY is flipped 180 degrees with respect to the mode of binding in the Qu/P13K complex.

Qu and LY similarly inhibit Akt/PKB activation. HCC1937 cells with deletion of the PTEN gene and activated Akt/PKB, and T47D cells lacking constitutively activated Akt/PKB, were utilized in order to determine whether Qu inhibits Akt/PKB in a manner comparable to LY. As shown in Figures 2A and B, phosphorylated-Akt (p-Akt) levels were seen in vehicle-treated controls in HCC1937 cells. However,
treatment with LY (10 μM) and Qu (25 μM) for 0.5 h, 1 h and 3 h completely eliminated Akt/PKB activity, as evidenced by the lack of detectable p-Akt levels. Both LY and Qu were similarly effective. The same blot was stripped of the p-Akt antibody and then re-probed with anti-Akt antibody to ensure that the basic level of Akt/PKB was the same. As shown in Figures 3A and B, the T47D cell line displayed p-Akt only after EGF treatment. The control revealed no detectable p-Akt levels. However, EGF failed to induce Akt/PKB phosphorylation in cells pre-treated with LY (10 μM) or Qu (25 μM). Treatment with phorbol ester, TPA, insignificantly increased Akt/PKB activity, which was suppressed in the Qu- or LY-treated T47D cells.

Qu and LY and cell proliferation. In support of the proposed efficacy of Qu and LY, the results from the colorimetric MTT assay for cell survival and proliferation revealed significant decreases in cell growth in Qu- or LY-treated HCC1937 cells. The control values were set to 100. The LY-treated cells displayed cell growth value decreases of 57% at 24 h. The results of Qu on the HCC1937 cells also showed approximately 50% decreases in cell proliferation (Figures 4A and B).

Discussion

In the absence of the tumor suppressor gene PTEN, we expect a constitutive activation of the Akt/PKB pathway. In HCC1937 cells that carry homozygous deletions of PTEN, we found that Akt/PKB was constitutively activated. This activation was evidenced by increased p-Akt levels in the control cells. Furthermore, the results of this study demonstrated that Qu and LY were equally effective in suppressing Akt/PKB activity in PTEN-deficient cells within the same span of time. Qu may be more effective, since activation of ERK was seen 1 h after treatment, as compared to the 24 h that were required for activation with LY treatment (data not shown). The colorimetric MTT assay for cell proliferation and survival revealed significant decreases in cell growth in Qu- or LY-treated HCC1937 cells. This study clearly demonstrated that Qu suppresses cell proliferation and, thus, may have antimutagenic properties due to its ability to suppress the Akt/PKB pathway. Qu may be particularly useful in such cells where the PTEN gene is inactivated, and Akt/PKB pathways constitutively activated.

Phosphatidylinositol second messengers play an important role in the signal transduction pathways that regulate cell...
Figure 3. Western blot analysis revealing the levels of activated Akt/PKB (p-Akt) in T47D cells. The vehicle-treated control showed no activated Akt/PKB (p-Akt); the EGF-treated cells showed p-Akt/PKB expression (3A top panel; 3B). Pre-treatment with either Qu or LY prevented the activation of Akt/PKB induced by EGF. TPA treatment caused an insignificant increase in Akt/PKB activity, which was also abolished by pre-treatment with Qu or LY. The same blot was stripped of the p-Akt antibody and then re-probed with anti-Akt antibody to ensure that the basic level of Akt/PKB was the same (3A bottom panel; 3C).

Figure 4. Colorimetric MTT assay depicting LY and Qu inhibition of the Akt/PKB pathway in HCC1937 cells. The cells were exposed to either LY (50 μM) or Qu (100 μM) for 24 h, and cell proliferation measured by the MTT assay. Control wells contained untreated cells. Both Qu (100 μM) and LY (50 μM) treatments caused approximately 50% decreases in cell proliferation, compared to the control set to 100 (4A). Photomicrographs of Quercetin-induced apoptotic cells following 24-h treatment, and vehicle-treated control showing intact cells, are depicted in (4B).
growth, actin rearrangement and differentiation, as well as apoptosis. Abundant data have demonstrated that PI3K is required for the activation of Akt/PKB, a serine/threonine protein kinase that plays an important role in cell survival. PTEN antagonizes the PI3K-Akt/PKB pathway by de-phosphorylating PI3K, resulting in the translocation of Akt/PKB to the cellular membranes, and subsequent down-regulation of Akt/PKB activation. Furthermore, it has been established that expression of PTEN in cells leads to decreased levels of phospho-Akt, and to increased apoptosis (10). In addition, it has been shown that constitutively active Akt/PKB can rescue cells from PTEN-mediated G1 arrest and apoptosis. The selective PI3K inhibitor LY has been shown to mimic the effects of PTEN (11). The effect of PTEN on PI3K is phosphatase-dependent. Studies have shown that PTEN coordinates G1 arrest through the up-regulation of p27 and concomitant down-regulation of cyclin D1 (12, 13).

High Akt/PKB activity in breast cancer cells is associated with a poor patho-phenotype, as well as hormone and chemotherapy resistance. Moreover, the PTEN gene is commonly mutated or deleted in glioblastoma multiforme (GBM) and endometrial cancer. Therefore, a chemotherapeutic agent directed specifically towards poor prognosis tumors, especially those with high Akt/PKB activity, would be a worthy drug treatment model to explore. Furthermore, rapamycin, an inhibitor of the mammalian target of rapamycin (mTOR), downstream of Akt/PKB, has also been demonstrated to have an anti-proliferative effect on cancer cells (14). Future studies may demonstrate whether or not Qu inhibits mTOR in a similar manner and, thus, has potential as a therapeutic target for glioblastoma patients. It may be possible that long-term inhibition of the Akt/PKB pathway affects cell proliferation and growth, whereas short-term effects may modulate cell motility, leading to the invasive nature of cancer cells devoid of PTEN. Further investigation is needed to consider these differences in the response of cancer cells to agents such as Qu that influence Akt/PKB signaling pathways. For now, our results suggest that the inhibition of the constitutively activated Akt/PKB pathway in cancer cells, due to inactivation of the PTEN gene, can be suppressed by the naturally occurring compound Qu in a manner similar to the structurally related, commercially available compound LY.

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References


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