The Inhibition Kinetics and Potential Anti-Migration Activity of NQO1 Inhibitory Coumarins on Cholangiocarcinoma Cells

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Abstract
Altered expression of a cytosolic flavoenzyme NAD(P)H:quinone oxidoreductase-1 (NQO1) has been seen in many human tumors. Its remarkable overexpression in cholangiocarcinoma (CCA; an aggressive malignancy of the biliary duct system) was associated with poor prognosis and short survival of the patients. Inhibition of NQO1 has been proposed as a potential strategy to improve the efficacy of anticancer drugs in various cancers including CCA. This study investigated novel NQO1 inhibitors and verified the mechanisms of their enzyme inhibition. Among the different chemical classes of natural NQO1 inhibitors are coumarins, flavonoids, and triterpenoids. Coumarins are a group of particularly potent NQO1 inhibitors. The mechanisms and kinetics of enzyme inhibition of coumarin, aesculetin, umbelliferone, and scopoletin using the cell lysates as a source of NQO1 enzyme best fit with an uncompetitive inhibition model. Among the NOQ1 inhibitors tested in KKU-100 CCA cells, scopoletin and umbelliferone had the strongest inhibitory effect on this enzyme, while aesculetin and coumarin barely affected intracellular NQO1. All coumarins were further tested for cytotoxicity and anti-migration activity. At modest cytotoxic doses, scopoletin and umbelliferone greatly inhibited the migration of KKU-100 cells, whereas coumarin and aesculetin barely reduced cell migration. The anti-migration effect of scopoletin was associated with decreased ratio of matrix metalloproteinase 9/tissue inhibitors of metalloproteinases 1 (MMP9/TIMP1) mRNA. These findings suggest that natural compounds with potent inhibitory effect on intracellular NQO1 have useful anti-migration effects on CCA cells. In order to prove that the potent NQO1 inhibitor, scopoletin, is clinically useful in the enhancement of CCA treatment, additional in vivo studies to elucidate the mechanism of these effects are needed.

Keywords
NQO1 inhibitor, coumarins, scopoletin, cholangiocarcinoma, anti-migration

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Introduction
NAD(P)H:quinone oxidoreductase-1 (NQO1, EC 1.6.99.2) catalyzes the obligatory 2-electron reduction of a broad range of quinones, using NAD(P)H as an electron donor. Several important functions of NQO1 have been established, including xenobiotic detoxification, superoxide scavenging, modulation of p53 proteasomal degradation, and maintenance of endogenous antioxidants. In normal cells, NQO1 functions to protect against oxidative damage and electrophilic insult. A large number of studies report that NQO1 is expressed at high levels in many human cancers such as liver, pancreas, breast, colon, thyroid, uterine cervix, lung, melanoma as well as cholangiocarcinoma (CCA). In addition, high expression of NQO1 is associated with shorter survival time of cancer patients. Growing evidence suggests that altered redox status in cancer cells caused by an increase in antioxidant and cytoprotective enzymes as well as NQO1 is one of the most critical mechanisms responsible for tumor growth and acquired resistance to chemotherapy. Recent findings suggest that suppression of NQO1 is one potential strategy to improve the efficacy of anticancer drugs.

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Several studies have shown NQO1 to be a possible target for cancer treatment. Dicoumarol, a well-known inhibitor of NQO1, suppressed human urogenital and pancreatic cancer cell growth through accumulation of oxidative stress. In vivo data showed that depletion of NQO1 reduced cell proliferation and abated lung tumor xenograft growth. In addition, suppression of NQO1 by dicoumarol or NQO1 gene silencing potentiated a variety of anticancer cytotoxicity in cancer cells with high NQO1 activity. In CCA, NQO1 gene silencing caused a reduction in colony formation capacity, arrested cell cycle, and a decrease in cell migration. In view of this evidence, NQO1 clearly plays an essential role in CCA cell growth, metastasis, and chemoresistance. Suppression of NQO1 is a promising strategy to improve the treatment of CCA. It is, therefore, necessary to find effective NQO1 inhibitors with potential as anticancer drugs.

In recent decades, natural compounds with diverse mechanisms of action have been extensively explored for their cancer-preventive properties. The purpose of this work was to identify natural compounds that are potential inhibitors of NQO1 enzyme and to study their cytotoxicity and anti-migration properties. First, various classes of natural compounds including a group of coumarins, flavonoids, triterpenoids, and others were screened for their inhibitory effect on NQO1 using the cell lysates as a source of the enzyme. Coumarins were the most promising inhibitory compounds from the NQO1 screening assay, and consequently their mechanisms and inhibitory characteristics were further explored. The NQO1 inhibitory effect of coumarins was confirmed in KKU-100 cells, where their cytotoxic and anti-migration effects were also evaluated using sulforhodamine B (SRB), Transwell migration, and reverse transcription quantitative polymerase chain reaction (RT-qPCR) assays.

Materials and Methods

Reagents

Cell lysis buffer was purchased from Cell Signaling Technology, Inc (Danvers, MA). Menadione was acquired from Fluka Chemie GmbH (Buchs, Switzerland). Dithiothreitol and dimethyl sulfoxide were purchased from Bio Basic Inc (Markham, Ontario, Canada). NADPH and glucose-6-phosphate dehydrogenase grade II from yeast were obtained from Boehringer Mannheim GmbH (Mannheim, Germany). Phenylmethylsulfonyl fluoride, SRB, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), dicoumarol, ferulic acid, epigallocatechin-3-gallate, genistein, quercetin, ginsenoside Rg3, silymarin, coumarin, umbelliferosine, silibinin, taxifolin, luteolin, asiatic acid, tetrahydrocurcumin, ellagic acid, phenethyl isothiocyanate, piperine, and scopoletin were purchased from Sigma Chemical Co (St. Louis, MO). Kaempferol, caffeic acid, and curcumin were acquired from ICN Biochemical, Inc (Costa Mesa, CA). Aesculetin was purchased from Aldrich Chemical Co (Milwaukee, WI). Ham’s F-12 nutrient mixture, Dulbecco’s modified Eagle’s medium, fetal bovine serum (FBS), and trypsin-EDTA (0.25%) were purchased from Gibco (Grand Island, NY). Trizol LS reagent was acquired from Invitrogen (Life Technologies, Grand Island, NY). iScript Reverse Transcription Supermix for RT-qPCR was purchased from Bio-Rad Laboratories Inc (Hercules, CA). QPCR Green Master Mix LRox was obtained from Biotechrabbit (Hennigsdorf, Germany).

Cell Culture

Intrahepatic CCA KKU-100 and hepatocellular carcinoma HepG2 cell lines were used as sources of the NQO1 enzyme. KKU-100 cells were derived from poorly differentiated adenocarcinoma tissue from intrahepatic CCA patients at Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, kindly provided by Prof Dr Banchob Sripa. Cell culture techniques were carried out with aseptic techniques. Briefly, KKU-100 cells were cultured in Ham’s F12 media, while HepG2 and human embryonic kidney cells, HEK293T, were cultured in Dulbecco’s modified Eagle’s medium. All cell types were supplemented with 4 mmol/L L-glutamine, 12.5 mmol/L N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), 100 U/mL of penicillin G plus 50 µg/mL gentamicin sulfate, at pH 7.4 and 10% FBS (v/v), and maintained under an atmosphere of 5% CO2 at 37°C. The media was refreshed every 2 to 3 days. After the cells became 80% to 90% confluent, they were trypsinized with 0.25% trypsin-EDTA and subcultured in the same media.

Preparation of NQO1 Enzyme from KKU-100 and HepG2 Cell Lines

Both KKU-100 and HepG2 cells were cultured in 100-mm culture plates. At 80% to 90% confluence, cells were washed with ice-cold phosphate-buffered saline, scraped and lysed at 4°C with 1× cell lysis buffer containing 1 mmol/L DTT and 0.1 mmol/L PMSF with vigorous shaking. Following centrifugation at 12 000 g for 30 minutes, supernatant was collected and stored at −80°C until used. The protein concentration was determined by the Bradford protein assay and used for NQO1 screening assay.

NQO1 Activity Assay and Kinetic Analysis from Cell Lysates

NQO1 Screening Assay. The assay was performed according to a previously described method. Briefly, 10 µg of cell lysate protein, distilled water as control or the indicated concentrations of test compounds were mixed with the...
incubation mixture containing of menadione, Tris-HCl (pH 7.4), bovine serum albumin, Tween-20 solution, flavin adenine dinucleotide, glucose-6-phosphate, β-nicotinamide adenine dinucleotide phosphate sodium salt hydrate, yeast glucose-6-phosphate dehydrogenase, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). After a blue color developed, the plates were placed into a Sunrise microplate absorbance reader (TECAN Austria GmbH, Grödig, Austria) with a filter wavelength of 620 nm, and absorbance was measured at 30-second intervals for 9.5 minutes. The rate of amplification of the optical readings with times represents the activity of the reaction. Using the extinction coefficient of formazan of MTT of 11 300 M⁻¹ cm⁻¹ and a correction factor for the light path of the microplate, NQO1 activity was measured as nmol/min/mg protein. Percentage of NQO1 inhibition was calculated using the following formula:

\[
\%\text{NQO1 enzyme inhibition} = \left(\frac{\text{NQO1 activity without test compound}}{\text{NQO1 activity with test compound}}\right) \times 100
\]

**Kinetic Characterization.** The assay was determined using the same procedure. A typical incubation mixture consisted of various concentrations of menadione in final concentrations of 1, 2, 3, 4, and 5 µM. Then, various concentrations of coumarin (5, 10, 20, 40, and 80 µM), aesculetin (1.25, 2.5, 5, 10, and 20 µM), umbelliferone (0.625, 1.25, 2.5, 5, and 10 µM), or scopoletin (0.625, 1.25, 2.5, 5, and 10 µM) were added to determine the pattern and kinetics of NQO1 inhibition.

**NQO1 Activity Assays in KKU-100 Cells**

The assay was performed in KKU-100 cells. In brief, the cells were seeded at density of 7.5 × 10³ cells/well in flat-bottomed 96-well cultured plates and allowed to adhere overnight. Media was then renewed with serum-free Ham’s F12 medium containing one of compounds to be tested, at the following concentrations: coumarin, aesculetin, umbelliferone, or scopoletin at different concentrations for 24 and 48 hours. Cells were fixed with 10% trichloroacetic acid for at least 1 hour, then stained with 0.4% SRB solution for 30 minutes. The excess dye was removed by washing repeatedly with 1% acetic acid, then the protein-bound dye was dissolved in 10 mM Tris base solution. The optical density at 540 nm was then measured using an ELISA plate reader.

**NQO1 siRNA Transfection**

The NQO1 siRNA (siGENOME SMARTpool of siRNA M–005133-02-0010; Dharmacon Inc, Lafayette, CO) and the negative control siRNA (siGENOME non–targeting siRNA pool#2 D-001206-14-20) were transfected into KKU-100 cells according to a previously described method.\(^{21}\) The efficiency of the NQO1 knockdown by transient NQO1 siRNA transfection was evaluated using RT-qPCR. Afterwards, the transfected cells were used for the cell viability assay.

**Cell Migration Assay in KKU-100 Cells**

The Transwell migration assay was performed to assess cancer cell migration on treatments,\(^{22}\) and the assay was conducted as described previously.\(^{15}\) KKU-100 cells were plated into the upper compartment of the Transwell chamber (Corning Incorporated, Corning, NY) at a density of 2 × 10⁴ cells/well in serum-free Ham’s F-12 medium, while the lower compartment of the chamber were filled with Ham’s F12 medium supplemented with 10% FBS. After incubation at 37°C with 5% CO₂ for overnight, the media in the upper compartment of the migration chamber were renewed with serum-free Ham’s F-12 medium containing the compounds to be tested at different concentrations for 24 hours. The migrated cells on the bottom surface were fixed with cold absolute methanol for 20 minutes and stained with 0.5% crystal violet solution for 15 minutes at room temperature. The migrated cells on the lower surface of the filters were photographed under a light microscope (Eclipse Ni-U; Nikon Corporation, Tokyo, Japan). The numbers of migrated cells were counted in 6 randomly selected fields for each chamber.

\[
\%\text{inhibition} = \left(1 - \frac{\text{the migrated cells with test compound}}{\text{the migrated cells without test compound}}\right) \times 100
\]

**Real-Time Quantitative Polymerase Chain Reaction**

The RT-qPCR was performed to determine the mRNA expression levels of MMP9 and TIMP1. After 24
hours scopoletin treatment (0.33, 0.65 mM), total RNA of KKU-100 cells was extracted using Trizol LS reagent according to the manufacturer’s instructions. Then, the cDNA was synthesized from 2 µg of total RNA using 5x iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad). PCR amplification was performed using specific primers for MMP9, TIMP1, and the internal control β-actin, and QPCR Green Master Mix LRox as described previously. The real-time PCR was carried out using ABI/7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). To verify the purity of the products, a melting curve analysis was performed following each run. The concentration of PCR products was evaluated on the basis of an established standard curve derived from serial dilutions of the positive control for MMP9, TIMP1, and β-actin.

Data Analysis

The results are presented as mean ± SD. Statistical comparison between control and treatment group was performed using Student’s t test. Results were considered to be statistically significant at P < .05. IC₅₀ values calculation and statistical analysis were performed using SigmaStat Program. The kinetics of the inhibition of NQO1 enzyme activity was analyzed using SigmaPlot 12.0 program (SigmaPlot for Windows; Systat Software, Inc, San Jose, CA) and Prism 5.01 program (GraphPad Software Inc, San Diego, CA). Data were fitted to equations that describe enzyme kinetic in the presence of various type of inhibitors, including competitive, noncompetitive, uncompetitive, and mixed model inhibitors. The kinetic model that fit the data best was then used to estimate the inhibitory constant Ki.

Results

Inhibition of NQO1 Activity by Natural Compounds

Using NQO1 activity assay, 21 natural compounds (coumarin compounds: coumarin, aesculetin, and scopoletin; flavonoid compounds: epigallocatechin-3-gallate, genistein, kaempferol, luteolin, quercetin, silybin, silymarin, and taxifolin; the triterpenoids: ginsenoside Rg3 and asiatic acid; and miscellaneous compounds: tetrahydrocurcumin, curcumin, caffeic acid, ellagic acid, ferulic acid, phenethyl isothiocyanate, and piperine) were used to identify potent inhibitors of NQO1 enzyme. The chemical structures of dicoumarol, a well-characterized NQO1 inhibitor, and the other compounds investigated in this study are presented in Figure 1. Using the same concentration, all compounds were screened for their inhibitory effect on NQO1 enzyme activity. Among the tested compounds, those in the coumarin group (coumarin, aesculetin, umbelliferone, and scopoletin) were the strongest inhibitors of NQO1 enzyme (Figure 2). Scopoletin showed the greatest percentage of NQO1 inhibition at 77.38%, followed by umbelliferone at 63.45%, coumarin at 41.81%, and aesculetin at 34.88%. In the flavonoid group, the percentage of NQO1 inhibition ranged from 4.13% to 55.05%, with luteolin showing the highest NQO1 inhibition in this group. The triterpenoids showed NQO1 inhibition between 18.02% and 36.94%, with ginsenoside Rg3 showing the highest NQO1 inhibitory activity in the group. Tetrahydrocurcumin also inhibited NQO1 enzyme with 39.2% inhibition. Overall, coumarins were the most potent inhibitors of NQO1 enzyme, and therefore, the mechanisms and kinetics of enzyme inhibition of these compounds was further characterized.

Kinetics and Mode of NQO1 Inhibition by Coumarins

The mechanisms and kinetics of inhibition of human NQO1 enzyme were examined using menadione as a reaction substrate and 5 different concentrations of various inhibitors (coumarin, aesculetin, umbelliferone, and scopoletin). Each compound was tested at various concentrations of substrate. All of the reactions were examined assuming Michaelis-Menten kinetics, and the type of the inhibition of each compound was inferred from the goodness-of-fit to the equations of various models of enzyme kinetics. The Eadie-Hofstee plots, patterns of inhibition, and Ki values of each compound are shown in Figure 3. The data for all coumarins best fit with an uncompetitive inhibition model. Among the 4 coumarins, scopoletin exhibited the most potent inhibition of human NQO1 enzyme activity and the lowest Ki value, followed by umbelliferone and aesculetin. In contrast, coumarin had the highest Ki value, indicating the lowest NQO1 inhibition.

Inhibition of Intracellular NQO1 Enzyme Activity in KKU-100 Cells by Coumarins

To assess whether coumarins potently inhibit intracellular NQO1 enzyme activity, KKU-100 cells were incubated with 5 different concentrations of coumarins for 24 hours, after which the activity of intracellular NQO1 enzyme was measured. In order to achieve the highest inhibition of the intracellular NQO1 enzyme, the concentrations of the inhibitors had to be increased as follows: coumarin 0.4, 0.6, 0.8, 1.0, and 2.0 mM; aesculetin 0.1, 1, 10, 30, and 100 µM; umbelliferone 0.10, 0.19, 0.39, 0.77, and 1.54 mM; and scopoletin 0.08, 0.16, 0.33, 0.65, and 1.30 mM. The results showed that 80% of NQO1 inhibition in KKU-100 cells occurred at dose of 0.33 mM for scopoletin and 0.39 mM for umbelliferone. In contrast, coumarin and aesculetin had no inhibitory effect on intracellular NQO1 activity (Figure 4).
Effect of Coumarins on KKU-100 Cell Cytotoxicity

To test whether coumarins are cytotoxic in KKU-100 cells, the cells were treated with varied concentrations of coumarins for 24 and 48 hours. The SRB results showed that all coumarins exhibit concentration-dependent cytotoxicity (Figure 5). Considering the IC$_{50}$ values, which reflect sensitivity of coumarins against CCA cells, aesculetin showed the most cytotoxicity on KKU-100 cells. Scopoletin and umbelliferone, which are potent NQO1 inhibitors in KKU-100 cells, had only modest cytotoxicity (less than 30%) at a dose that inhibited 80% of intracellular NQO1 enzyme activity. These results indicate that coumarins have a cytotoxic effect in CCA cells. Meanwhile, the cytotoxic effect of scopoletin, the highest potent NQO1 inhibitor in the study, was less pronounced on human embryonic kidney cells, HEK293T cells (IC$_{50}$ at 48 hours was 1.75 mM; Supplementary Figure S1, available in the online version of the article), as compared with KKU-100 cells.

In order to investigate whether NQO1 is involved in scopoletin-mediated toxicity in KKU-100 cells, NQO1-knockdown cells using siRNA transfection were treated with scopoletin for 48 hours before determination of the cytotoxicity by SRB assay. Transfection with NQO1 siRNA was almost completely abolished NQO1 expression in KKU-100 cells (Figure 6). The cytotoxicity of scopoletin was less in NQO1-knockdown cells than that in nontargeting siRNA-transfected cells (IC$_{50}$ values were 0.88 mM and 0.42 mM for NQO1-knockdown and nontargeting siRNA-transfected cells, respectively). These results indicated that NQO1 expression was involved in the cytotoxicity of scopoletin and that its NQO1 inhibition may be one of the mechanisms that causes this cytotoxicity in KKU-100 cells.

Scopoletin Inhibited the Migration of KKU-100 Cells

A previous study by our group found that NQO1 siRNA-mediated knockdown repressed the migration of CCA cells.$^{15}$ Hence, to explore whether coumarins could
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suppress the migration of KKU-100 cells, the Transwell migration assay was performed (Figure 7a-d). Cells were incubated for 24 hours with 2 concentrations of coumarins that in previous experiments caused less than 30% cytotoxicity. The results showed that scopoletin (0.33 and 0.65 mM) exhibited the highest inhibition of the migration of KKU-100 cells at 50.19% to 82.55%, followed by umbelliferone (0.39 and 0.77 mM) with 10.11% to 31.89% inhibition. In contrast, coumarin (0.8 and 1.0 mM) and aesculetin (10 and 30 µM), which are poor NQO1 inhibitors in KKU-100 cells, barely altered the migrative activity of the cells. This result implies that NQO1 inhibition in CCA cells by scopoletin may be one of the anti-migrative mechanisms of the compound.

We further demonstrated the effect of scopoletin, which showed the highest inhibition of the migration of KKU-100 cells in the study, on the expression levels of migration-associated genes (MMP9, TIMP1) using RT-qPCR (Figure 7e). The results revealed that scopoletin decreased the MMP9/TIMP1 ratio compared with the control cells. Taken together, the finding implied that scopoletin impeded the migration of KKU-100 cells via regulating the migration-associated genes.

Discussion

NAD(P)H:quinone oxidoreductase-1 plays an important role in xenobiotic metabolism and cellular protection in normal cells. In several types of solid tumors, however, overexpression of NQO1 is related to tumor promotion, progression of cancer, and chemoresistance.4,5,15 In many solid tumors including CCA (an aggressive acquired malignancy of the biliary duct system), high expression of NQO1 is a predictor of poor prognosis and short survival time of patients. Accumulating evidence suggests that NQO1 inhibition together with anticancer agents can improve the efficacy of cancer treatment.13,21 Thus, effective NQO1 inhibitors are promising agents for the improvement of CCA treatment. In the current study, various classes of natural compounds were screened for their inhibitory effects on the NQO1 enzyme. The NQO1 screening assay showed the coumarins had potent inhibitory effects on this enzyme. All 4 coumarins (coumarin, aesculetin, umbelliferone, and scopoletin) were uncompetitive NQO1 inhibitors. Scopoletin and umbelliferone could effectively inhibit intracellular NQO1 enzyme in KKU-100 cells, barely altered the migrative activity of the cells. This result implies that NQO1 inhibition in CCA cells by scopoletin may be one of the anti-migrative mechanisms of the compound.

Dicoumarol (3,3’-methylene-bis(4-hydroxy coumarin)) has been known for several decades to be potent competitive inhibitor of NQO1 enzyme. Anticancer effects of dicoumarol have been reported in many types of solid cancers. However, clinical uses of dicoumarol are limited.
because of its unwanted side effects. To search for new effective NQO1 inhibitors, several classes of natural compounds were evaluated using the NQO1 inhibition-screening assay. In the current work, coumarin compounds (coumarin, aesculetin, umbelliferone, and scopoletin) showed potent inhibition of NQO1 enzyme activity. Considering the relationship between chemical structure and the activity of these compounds, it is worth noting that chemical structures with benzopyrone (a fusion of benzene and α-pyrone rings) confer a better NQO1 inhibitory effect compared with other structures. Therefore, the benzopyrone chemical structures may be essential for NQO1 inhibitory activity. Since natural compounds with 1-benzopyran-2-one structures examined in this study were good NQO1 inhibi-

**Table 1.**

| Compounds       | Ki (µM)       | Pattern of inhibition |
|-----------------|---------------|-----------------------|
| Coumarin        | 20.11 ± 11.95 | Uncompetitive         |
| Aesculetin      | 2.52 ± 0.06   | Uncompetitive         |
| Umbelliferone   | 1.51 ± 0.91   | Uncompetitive         |
| Scopoletin      | 0.55 ± 0.25   | Uncompetitive         |

**Figure 3.** Eadie-Hofstee plots derived from a kinetic analysis of NQO1 activity in the presence of the coumarin compounds. Assays were performed using cell lysates as a source of NQO1 enzyme. The incubation mixture consisted of cytosolic protein from KKU-100 cells (10 µg/well), menadione (1, 2, 3, 4, or 5 µM), and the indicated concentration of coumarin (a), aesculetin (b), umbelliferone (c), or scopoletin (d). Each plot depicts the mean of triplicate measurements in 1 typical experiment of 3 experiments. The kinetic parameters were analyzed using a nonlinear regression method (e). The data for all coumarin compounds best fit with an uncompetitive inhibition model. Each value is mean ± SD (3 individuals with triplicate determinations).
tors, the mechanism of enzyme inhibition of these compounds was further investigated.

The kinetic data from these analyses along with the Eadie-Hofstee plot revealed that coumarin, aesculetin, umbelliferone, and scopoletin were uncompetitive inhibitors of NQO1. As is characteristic of uncompetitive inhibitors, they work best when substrate concentration is elevated, suggesting that the substrates tend to potentiate the inhibition.23 In addition, the uncompetitive inhibitors also seemed to be more effective when there was overexpression of enzyme activity. The uncompetitive inhibitors in the coumarin group have only one benzopyrone in their structures, whereas a competitive NQO1 inhibitor dicoumarol has 2 benzopyrones. It is, therefore, possible that the number of benzopyrone chemical structures may be related to their pattern of inhibition.

In order to confirm the coumarins are effective NQO1 inhibitors, their effect on intracellular NQO1 enzyme activity in KKU-100 cells was examined. Higher concentrations of all compounds were required to inhibit NQO1 enzyme activity in the cells. To achieve 80% of inhibition on NQO1 enzyme activity in KKU-100 cells, the concentration of dicoumarol was 11-fold higher than those used in the NQO1 screening experiment. Likewise, scopoletin and umbelliferone required an 18-fold and 40-fold increase in dose, respectively, to achieve 80% of NQO1 inhibition, when compared with the screening assay.

In our previous study, NQO1 showed a role in cell growth, proliferation, and migration of CCA cells.15 To investigate whether coumarins, the most potent NQO1 inhibitors in this study, were able to inhibit the growth and migration of cancer cells, SRB, Transwell migration, and RT-qPCR assays were performed. Scopoletin and umbelliferone at concentrations that greatly inhibited NQO1 activity also moderately decreased the viability of KKU-100 cells.13 A previous study in CCA stated that scopoletin was relatively low cytotoxic against a human bile duct epithelial cell line (H69 cells).24 Herein, scopoletin exhibited higher cytotoxic effect against KKU-100 cells.

Figure 4. Effect of the coumarin compounds on NQO1 enzyme activity in KKU-100 cells. Cells were exposed to various doses of the coumarin compounds or 10 µM of dicoumarol (Dic) for 24 hours. Afterwards, NQO1 enzyme activity was analyzed by enzymatic method. Data are expressed as a percentage of inhibition compared with untreated control cells. Data represent mean ± SD, each from 3 separated experiments with triplicate determinations.
Figure 5. Cytotoxic effect of the coumarin compounds in KKU-100 cells as measured by SRB assay. Cells were treated with various concentrations of the coumarin compounds for 24 or 48 hours. The IC$_{50}$ value was computed using a nonlinear regression model. Results are presented as percentage of untreated control. The value for each concentration tested expressed as mean ± SD from 3 individual experiments with triplicate determinations.

Figure 6. NQO1-dependent cytotoxicity of scopoletin in KKU-100 cells. NQO1-knockdown cells using siRNA transfection were used to assess the cytotoxicity of scopoletin in comparison to cells with nontargeting siRNA transfection. Cells were treated with various concentrations of scopoletin for 48 hours. The SRB results are the mean ± SD from 2 individual experiments with triplicate determinations.

The study also found that the estimated IC$_{50}$ values of scopoletin on NQO1-knockdown cells was higher than that of nontargeting siRNA-transfected cells, which may indicate that NQO1 was responsible for the scopoletin-mediated toxicity in KKU-100 cells. An earlier study found that scopoletin mediated an anticancer effect in CCA by arresting cell cycle and inducing apoptosis in CCA cells. Also, scopoletin enhanced the cytotoxicity of chemotherapeutic cisplatin in CCA cells. Umbelliferone caused anticancer activity via the induction of apoptosis and cell cycle arrest in HepG2 hepatocellular carcinoma cells. Taken together, these data suggest that scopoletin and umbelliferone may cause cytotoxicity in cells partly through induction of apoptosis, and inhibition of NQO1 may be one mechanism of this cytotoxicity.

The ability of NQO1 to drive cell migration and invasion was previously documented, suggesting a role of NQO1 in promoting cancer metastasis. In this study, the potent intracellular NQO1 inhibitors scopoletin and umbelliferone effectively reduced migration of KKU-100 cells. In contrast, coumarin and aesculetin, which poorly inhibited NQO1 enzyme in the cells, did not appreciably alter CCA cell migration. Previous studies showed that dicoumarol or NQO1 siRNA decreased tumor necrosis factor α-induced human aortic smooth muscle cells migration by inhibiting...
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MMP9 protein and mRNA expression. In the present study, scopoletin suppressed the migration of KKU-100 cells, which was associated with decreased MMP9/TIMP1 mRNA ratio. These results suggest that inhibition of NQO1 may lead to diminished migratory ability of the cells via regulating the migration-associated genes. Overall, scopoletin is a promising compound for cancer treatment, since it is a potent NQO1 inhibitor that reduces cell viability and suppresses migration of the cancer cells. However, additional studies on the mechanism of NQO1 inhibitors in vivo as well as the effect of these inhibitors on the efficacy of chemotherapy are still needed.

Conclusions

The present work identified coumarin-based compounds including coumarin, aesculetin, umbelliferone, and scopoletin as uncompetitive inhibitors of the NQO1 enzyme. Two most promising NQO1 inhibitors in this study are scopoletin and umbelliferone, which strongly inhibited the NQO1 enzyme in KKU-100 CCA cells. Scopoletin could suppress the migration of KKU-100 cells through downregulating the MMP9/TIMP1 ratio. Because of its inhibitory effects on the NQO1 enzyme and cell migration, scopoletin is a promising agent for enhancing treatment of CCA. However, further in vivo study elucidating the mechanism underlying those effects are required.

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Supplemental Material

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