Acetyl derivative of quercetin 3-methyl ether-induced cell death in human leukemia cells is amplified by the inhibition of ERK

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Flavonoids are polyphenolic compounds that are ubiquitously in plants and display a vast array of biological activities. Here we have studied the effect of the phenylbenzo-γ-pyrene-derivative quercetin 3-methyl ether tetracetate (QD), obtained by acetylation of the natural product quercetin 3-methyl ether, on cell viability of human leukemia HL-60 and U937 cell lines. The results show that QD was cytotoxic and induced G2–M phase cell cycle arrest on both cell lines and it was a potent apoptotic inducer on HL-60 cells. QD-induced apoptosis is (i) mediated by caspase activation, since it was prevented by the non-specific caspase inhibitor z-VAD-fmk, (ii) associated with cytochrome c release and (iii) triggered in Bel-2 over-expressing U937 cells. The treatment of HL-60 and U937 cells with QD also induces the activation of the mitogen-activated protein kinases (MAPKs) pathway, including c-Jun N-terminal kinase, p38 mitogen-activated protein kinase and extracellular signal-regulated kinases (ERK) 1/2. Inhibition of c-Jun N-terminal kinase by SP600125 and of p38 mitogen-activated protein kinase by SB203580 had no influence on QD-mediated apoptosis. In contrast, inhibition of ERK1/2 with the pharmacologic inhibitors U0126 or PD98059, together with QD, resulted in an important enhancement of apoptosis. Cells are sensitized to QD-mediated apoptosis after blocking ERK1/2, which suggests that inhibition of this pathway is a valuable strategy to increase the sensitivity of human leukemia HL-60 cells toward QD.

Introduction

In recent years, the use of naturally occurring agents to prevent the development or recurrence of cancers has become widely accepted as a realistic option for fighting the disease. Flavonoids are phenylbenzo-γ-pyrones derivatives which comprise a very large class of naturally occurring polyphenol plant compounds (1). Flavonoids are ubiquitous in plant foods and drinks and therefore consumed in significant quantities in our daily diet (2). These polyphenolic compounds display a remarkable spectrum of biological activities and they are among the most promising anticaner agents (3). Quercetin (3,3',4',5,7-penta-hydroxyflavone) is one of the best-studied bioflavonoid and it is a remarkable spectrum of biological activities and they are among initiators or executioners, depending on their point of entry into the apoptotic cascade. The initiator caspases are the first to be activated in a particular death pathway, and they constitute the first step in a minimal two-step cascade by activating the executioner caspases. Two pathways of caspase activation during apoptosis have been described (10). The extrinsic pathway involves apoptosis mediated by death receptors, such as Fas or tumor necrosis factor receptors (11), involving caspase-8 activation, which can process effector caspases (caspase-3, -6 and -7), inducing a cascade of caspases. In the intrinsic pathway, diverse pro-apoptotic signals provoke the translocation of cytochrome c from mitochondria to cytoplasm and caspase-9 activation, which cleaves and activates pro-caspase-3. Caspase-3 is responsible for cleaving specific cellular proteins during apoptosis (12). However, nowadays, apoptosis is described as being mediated not only by the death receptor (or extrinsic pathway) and the mitochondrial (or intrinsic pathway) pathways but also by the endoplasmic reticulum signaling pathway (13). Moreover, apoptosis can also occur independently of caspase activation (14).

Cell responses to apoptotic-inducing drugs have been associated with the inactivation of survival kinases and the activation of apoptotic kinases. One of the most relevant aspects in the regulation of apoptosis is the involvement of mitogen-activated protein kinases (MAPKs), a family of proline-directed serine/threonine protein kinases that mediate intracellular signal transduction in response to various stimuli (15). The MAPKs are activated by phosphorylation on both a threonine and tyrosine in a TXY motif found in an activation loop proximal to the ATP- and substrate-binding sites (16). To date, three major MAPKs have been identified: the c-Jun N-terminal kinases/stress-activated protein kinases (JNKs/SAPKs), the p38 mitogen-activated protein kinases (p38MAPK) and the extracellular signal-regulated kinases (ERK) 1/2. The ERKs are mainly, but not exclusively, activated by growth factors and involved in the regulation of mitogenesis (17,18). On the other hand, JNK and p38MAPK are activated mainly by cytotoxic insults and are often associated with apoptosis. However, there are multiple exceptions to this rule. ERK activation can exert either anti-apoptotic or pro-apoptotic effects, depending upon the stimuli and cell type. JNK/SAPK is preferentially activated by stressfull stimuli including UV irradiation, hyperthermia, inflammatory cytokines, chemotherapeutic agents and the translational inhibitors such as anisomycin and cycloheximide. Activation of p38MAPK has been implicated in the cellular response to osmotic shock, physiological stress, UV radiation and treatment with chemotherapeutic drugs (19).

Here we have studied the effect of tetracetyl derivative of quercetin 3-methyl ether (QD), obtained by acetylation of the natural product quercetin 3-methyl ether, on cell viability of human leukemia cell lines. QD was relatively cytotoxic on both cell lines studied, human myeloid leukemia HL-60 and U937 cells. This compound induced G2–M arrest on both cells and it was a potent apoptotic inducer on HL-60 cells. QD-induced apoptosis is (i) mediated by caspase activation, since it was prevented by the non-specific caspase inhibitor z-VAD-fmk, (ii) associated with cytochrome c release and (iii) triggered in Bel-2 over-expressing U937 cells. The treatment of HL-60 and U937 cells with QD also induces the activation of the mitogen-activated protein kinases (MAPKs) pathway, including c-Jun N-terminal kinase, p38 mitogen-activated protein kinase and extracellular signal-regulated kinases (ERK) 1/2. Inhibition of c-Jun N-terminal kinase by SP600125 and of p38 mitogen-activated protein kinase by SB203580 had no influence on QD-mediated apoptosis. In contrast, inhibition of ERK1/2 with the pharmacologic inhibitors U0126 or PD98059, together with QD, resulted in an important enhancement of apoptosis. Cells are sensitized to QD-mediated apoptosis after blocking ERK1/2, which suggests that inhibition of this pathway is a valuable strategy to increase the sensitivity of human leukemia HL-60 cells toward QD.

Abbreviations: ara-C, 1-β-D-arabinofuranosylcytosine; ERK, extracellular signal-regulated kinase; IC50, 50% inhibition of cell growth; INK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated extracellular kinase; PARP, poly (ADP-ribose) polymerase; PBMC, peripheral blood mononuclear cell; p38 \(^{\text{MAPK}}\), p38 mitogen-activated protein kinase; ROS, reactive oxygen species; SAPK, stress-activated protein kinase; QD, tetracetyl derivative of quercetin 3-methyl ether.

Essential executioners of apoptosis are the caspases, a family of conserved cysteine aspartate-specific proteases (9). Caspases are generally synthesized as inactive proenzymes orzymogens, which are activated by proteolytic cleavage. The apoptotic caspases are classified as initiators or executioners, depending on their point of entry into the apoptotic cascade. The initiator caspases are the first to be activated in a particular death pathway, and they constitute the first step in a minimal two-step cascade by activating the executioner caspases. Two pathways of caspase activation during apoptosis have been described (10). The extrinsic pathway involves apoptosis mediated by death receptors, such as Fas or tumor necrosis factor receptor (11), involving caspase-8 activation, which can process effector caspases (caspase-3, -6 and -7), inducing a cascade of caspases. In the intrinsic pathway, diverse pro-apoptotic signals provoke the translocation of cytochrome c from mitochondria to cytoplasm and caspase-9 activation, which cleaves and activates pro-caspase-3. Caspase-3 is responsible for cleaving specific cellular proteins during apoptosis (12). However, nowadays, apoptosis is described as being mediated not only by the death receptor (or extrinsic pathway) and the mitochondrial (or intrinsic pathway) pathways but also by the endoplasmic reticulum signaling pathway (13). Moreover, apoptosis can also occur independently of caspase activation (14).

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Materials and methods

Reagents

Quercetin 3-methyl ether was isolated from Allagopappus viscosissimus as described (20). The QD was obtained as described previously (21). Antibodies for poly (ADP-ribose) polymerase (PARP), caspase-3, caspase-8 and caspase-9 were purchased from Stressgen (Victoria, British Columbia, Canada). Antibodies for cytochrome c and caspase-7 were purchased from BD Pharmingen (San Diego, CA, USA). Anti-caspase-6 monoclonal antibody was from Medical and Biological Laboratories, (Nagoya, Japan). Anti-JNK/SAPK, anti-p44/42 MAPK, anti-phospho-p44/42 MAPK (T202/Y204), anti-p38MAPK and a phosphorylated form (T180/Y182) of p38MAPK antibodies were purchased from New England BioLabs (Cell Signaling Technology, Beverly, MA, USA). JNK/SAPK (phosphor T183 + Y185) antibody was purchased from Abcam (Cambridge, UK). Secondary antibodies were from Amersham Biosciences (Freiburg, Germany). All other chemicals were obtained from Sigma (Saint Louis, MO, USA).

Cell culture

HL-60, U937 and U937 cells over-expressing human Bcl-2 protein (designated U937/Bcl-2, donated by Dr Jacqueline Bréard, Institut National de la Santé et de la Recherche Médicale U749, Faculté de Pharmacie Paris-Sud., Châte-nay-Malabry, France) were cultured in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. Human peripheral blood mononuclear cells (PBMCs) were isolated from heparin-anticoagulated blood of healthy volunteers by centrifugation with Ficoll-Paque Plus (Amersham Biosciences). PBMC were also stimulated with phytohemagglutinin (2 µg/ml) for 48 h before experimental treatment.

Cytotoxicity of QD on human myeloid leukemia cells

The cytotoxicity of QD on HL-60 and U937 cells was analyzed by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described (21). Concentrations inducing a 50% inhibition of cell growth (IC50) were determined graphically using the curve fitting algorithm of the computer software Prism™ 2.0 (GraphPad). Values are means ± SE from three independent experiments, each performed in triplicate.

Evaluation of apoptosis

The rate of apoptotic cell death was analyzed by fluorescent microscopy and by flow cytometric analysis of propidium iodide-stained nuclei as described (21).

Analysis of DNA fragmentation

DNA isolation and gel electrophoresis were performed as described previously (21).

Western blot analysis

HL-60 and U937 cells (1 × 10⁶/ml) were treated in the absence or presence of QD (3–10⁻¹⁰ M) for various time periods as indicated and harvested by centrifugation. Cell pellets were re-suspended in lysis buffer [20 mM Tris–HCl (pH 7.4), 2 mM ethylenediaminetetraacetic acid, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM tetrasodium pyrophosphate, 20 mM sodium β-glycerophosphate, 10 mM sodium fluoride, 2 mM sodium orthovanadate], supplemented with protease inhibitors phenylmethylsulfonyl fluoride (1 mM), leupeptin, aprotinin and pepstatin A (5 µg/ml each) for 15 min at 4°C. Lysates were homogenized by a sonifier (five cycles) and centrifuged at 11 000g for 10 min at 4°C. Protein concentration of supernatants was measured by the Bradford method (22) and samples containing equal amounts of proteins were boiled in sodium dodecyl sulfate sample buffer for 5 min before loading on an sodium dodecyl sulfate–polyacrylamide gel (7.5% for PARP, 10% for MAPKs and 12.5% for caspases). Proteins were electrotransferred to poly (vinylidene difluoride) membranes and detected as described (21).

Detection of cytochrome c release from mitochondria

Release of cytochrome c from mitochondria was detected by western blot analysis as described (21).

Assay of caspase activity

Caspase activity was performed as described previously (21).

Statistical analysis

Statistical significance of differences between control and treated samples were calculated using Student’s t-test. P values of <0.05 were considered significant.

Results

**QD inhibits the viability of human myeloid leukemia cells**

Recently, we have showed that human myeloid leukemia HL-60 cell line was highly sensitive to the anti-proliferative effect of QD (Figure 1A) (21). Here, we have examined the effect of this compound on the growth of human leukemia U937 cell line. The IC50 value in U937 cells (Figure 1B) was 5.4 ± 0.1 µM similar to the reported value for HL-60 cells (9.2 ± 3.4 µM) (21). The cytotoxic-inducing capacity of QD in human leukemia cells raised the question whether QD also affects healthy human PBMCs. Therefore, we also investigated whether QD was also cytotoxic for human PBMC. No cytotoxicity (up to 10 µM) to either fresh or proliferating PBMC growth was observed. However, there was an important reduction in the proliferation of HL-60 cells, which were included in the experiment as a positive control (Figure 1C).

**QD induces G2–M phase cell cycle arrest and apoptosis on human myeloid leukemia cells independently of RNA and protein biosynthesis**

When cells were incubated with this flavonoid, the DNA showed the typical fragmentation patterns formed by inter-nucleosomal
Hydrolysis of chromatin, thus confirming the apoptosis-inducing effects (Figure 2A). We also analyzed the morphological changes characteristic of apoptotic cells (condensed and fragmented chromatin) by fluorescent microscopy (Figure 2B). The quantification of apoptosis obtained by measurement of the number of hypodiploid cells by flow cytometry (Figure 2C) shows that the percentage of apoptotic cells increased 6-fold in QD-treated HL-60 cells, after 24 h exposure at a concentration as low as 10 μM (Figure 2D). Although U937 cells were growth suppressed as were HL-60 cells (Figure 1B), there was a lower induction of apoptosis in this cell line than HL-60 cells as demonstrated by flow cytometry (Figure 2D). We also investigated whether inhibition of macromolecule biosynthesis could affect the apoptotic action of QD. Pretreatment of HL-60 cells with actinomycin D or cycloheximide, used at concentrations that inhibited mRNA and protein biosynthesis (23), did not affect the apoptotic response induced by QD (Figure 2E). These results indicate that QD-induced apoptosis does not depend on protein biosynthesis.

To assess whether QD-induced cell growth inhibition is mediated via alterations in cell cycle progression, we evaluated the effect of this compound on cell cycle phase distribution by flow cytometric studies. As shown in Table I, consistent with growth inhibitory effects, QD (10 μM) caused a significant G2-M arrest at the expense of G1 phase cell population starting at 12 h of treatment on both cell lines. This decrease in the percentage of cells in G1 phase was also accompanied by an increase in G2-M phase cell population at 24 h of treatment, but this effect invariably started diminishing with the increase in treatment time (48 h) in HL-60 cells. The percentage of control-treated cells in G2-M was ~28%, which increased to ~46% after treatment with QD for 12 h.

QD showed a similar trend in G2-M arrest in U937 cells. QD showed a strong increase in G2-M arrest at the expense of G1 phase cell population and the effect on G2-M arrest was sustained after 24–48 h. In U937 cells, the percentage of sub-G1 cells increased until 11.3% (2-fold) and 15.7% (5-fold) by QD after 24 and 48 h of treatment, respectively. Taken together, these results indicate that QD induces cell cycle arrest in the G2-M phase and apoptosis of human myeloid leukemia HL-60 and U937 cells, although the HL-60 cell line was much more sensitive than the U937 cell line with respect to apoptosis induction.

**QD-induced cell death is mediated by a caspase-dependent pathway**

To determine whether caspases were involved in the response of the cells to QD, we examined whether this flavonoid induces PARP cleavage, a hallmark of apoptosis that indicates activation of caspase. Hydrolysis of the 116 kDa PARP protein to the 85 kDa fragment was detected in QD-treated cells after 12 h exposure at a concentration as low as 3 μM and increased in a dose-dependent manner (Figure 3A). These results indicate that PARP cleavage was involved in apoptosis induced by this compound.

Cytchrome c released from mitochondria binds to the adapter protein apoptotic protease activating factor-1 and pro-caspase-9 to stimulate the auto-catalytic cleavage of caspase-9 to its active form, thereby initiating the so-called intrinsic or mitochondrial pathway (24). Caspase-9 then cleaves caspase-3 which then cleaves multiple substrates including PARP. To determine whether QD-induced apoptosis on HL-60 cells involves the release of cytochrome c from mitochondria to cytosol, dose-response experiments were performed and cytosolic preparations were analyzed by immunoblotting. As demonstrated (Figure 3B), a significant increase in the amount of cytochrome c in the cytosol was detected after 12 h of treatment with QD. A low concentration of compound (3 μM) also induces cytochrome c release at the same proportion (results not shown).

We also examined the effect of this compound on proteolytic processing of caspases. To this end, HL-60 and U937 cells were treated with 3–10 μM QD for various time durations and initiators (caspase-9

### Table I. Effect of different durations of treatment with QD on cell cycle phase distribution of HL-60 and U937 cells

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>% Sub-G1</th>
<th>% G1</th>
<th>% S</th>
<th>G2-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HL-60</td>
<td>4.7 ± 0.1</td>
<td>52.8 ± 0.1</td>
<td>14.4 ± 0.5</td>
<td>28.4 ± 0.7</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>3.3 ± 0.2</td>
<td>54.8 ± 0.9</td>
<td>22.6 ± 0.0</td>
<td>20.8 ± 1.1</td>
</tr>
<tr>
<td>QD</td>
<td>30.6 ± 0.2</td>
<td>11.7 ± 0.1</td>
<td>11.6 ± 0.1</td>
<td>44.7 ± 0.1</td>
</tr>
<tr>
<td>48 h</td>
<td>3.9 ± 0.2</td>
<td>55.6 ± 0.8</td>
<td>17.3 ± 0.2</td>
<td>22.4 ± 0.4</td>
</tr>
<tr>
<td>QD</td>
<td>34.4 ± 2.8</td>
<td>21.2 ± 0.4</td>
<td>23.2 ± 1.1</td>
<td>12.4 ± 1.2</td>
</tr>
<tr>
<td>U937</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 h</td>
<td>3.3 ± 0.4</td>
<td>55.9 ± 1.4</td>
<td>15.3 ± 0.1</td>
<td>26.1 ± 2.4</td>
</tr>
<tr>
<td>QD</td>
<td>5.2 ± 0.2</td>
<td>30.8 ± 0.9</td>
<td>19.0 ± 0.4</td>
<td>46.1 ± 1.3</td>
</tr>
<tr>
<td>24 h</td>
<td>5.0 ± 0.5</td>
<td>59.9 ± 0.4</td>
<td>18.3 ± 0.7</td>
<td>47.8 ± 0.1</td>
</tr>
<tr>
<td>QD</td>
<td>11.3 ± 0.4</td>
<td>29.8 ± 0.2</td>
<td>16.4 ± 0.1</td>
<td>42.4 ± 0.5</td>
</tr>
<tr>
<td>48 h</td>
<td>3.0 ± 0.4</td>
<td>54.1 ± 1.3</td>
<td>21.1 ± 0.4</td>
<td>22.7 ± 0.8</td>
</tr>
<tr>
<td>QD</td>
<td>15.7 ± 0.7</td>
<td>31.2 ± 0.6</td>
<td>12.8 ± 1.0</td>
<td>38.9 ± 0.8</td>
</tr>
</tbody>
</table>

Cells were cultured with 10 μM QD for the indicated period of times and the cell cycle phase distribution was determined by flow cytometry. The values are means ± SD of two independent experiments with two determinations in each. Asterisks indicate a significant difference (P < 0.05) compared with the corresponding controls.
were determined after 24 h incubation of treatment. U937 cells were treated with indicated concentrations of QD and immunoblots of the different caspases -6 and -3 on HL-60 and U937 cells. HL-60 cells were treated with the tetrapeptide substrates DEVD-pNA, IETD-pNA and LEHD-pNA as specific substrates for caspase-3/7, caspase-8 and caspase-9, respectively. As shown (Figure 4B), induction of both caspase-9 and caspase-3 activities was significantly detectable after 12–14 h of treatment and increased with the incubation time. In contrast, caspase-8 was not detected until after 24 h of treatment (Figure 3C). In U937 cells, there was no cleavage of caspase-8 after 24 h of treatment. Proteolytic cleavage of the initiator caspase-8, which is demonstrated in response to QD, typically occurs after triggering cell surface death receptors like the CD95 receptor (25).

As shown in Figure 3C, QD also stimulates the proteolytic processing of caspase precursors (caspase-7, -6 and -3). In HL-60 cells, concentrations of QD as low as 3 μM significantly promotes pro-caspase-7, -6 and -3 hydrolysis at 12 h of treatment. In contrast, in U937 cells the hydrolysis of pro-caspases-7, -6 and -3 was only observed with the higher concentration tested (10 μM) at longer incubation time assayed (24 h). Protein loading was checked by reprobing the membranes with β-actin antibody, which did not show any change in the protein level.

To confirm that QD-triggered apoptosis requires the activation of caspases, HL-60 cells were pretreated with the broad-spectrum caspase inhibitor z-VAD-fmk. The almost complete inhibition by z-VAD-fmk of apoptosis induced by QD shows that this apoptosis is caspase-dependent (Figure 4A).

To identify which caspases were important in QD-induced apoptosis, the effect of cell-permeable caspase inhibitors was examined. None of the selective caspase inhibitors used, z-IETD-fmk (caspase-8-selective inhibitor), z-LEHD-fmk (caspase-9-selective inhibitor) and z-DEVD-fmk (caspase-3 and -7-selective inhibitor), had any significant effect on QD-triggered cell death (Figure 4A). However, the caspase-8 inhibitor suppressed cell death induced by tumor necrosis factor α and the caspase-9 inhibitor partially blocked apoptosis induced by etoposide (results not shown). It was surprising that the specific inhibitor of caspase-3, z-DEV Exposure (results not shown). It was surprising that the specific inhibitor of caspase-3, z-DEV-fmk, failed to show any inhibition of QD-induced apoptosis in HL-60 cells (Figure 4A). This result suggests that in vivo, a second caspase is probably required. It is possible that this caspase is caspase-6 (Figure 3C), which is z-VAD-fmk insensitive but not sensitive to z-DEV-fmk (26).

As shown (Figure 3C), QD also stimulates the proteolytic processing of caspase-9, -8, -7, -6 and -3 on HL-60 and U937 cells. HL-60 cells were treated with the indicated concentrations of QD and immunoblots of the different caspases were determined after 12 h incubation of treatment, except for caspase-8 which was determined after 24 h of treatment. U937 cells were treated with the indicated concentration of QD and immunoblots of the different caspases were determined after 24 h incubation of treatment. β-Actin was used as a loading control.

To clarify whether Bcl-2 (an anti-apoptotic protein known to prevent mitochondrial dysfunction) is involved in the activation of intrinsic pathway by QD treatment, we compared the effect on apoptosis induction in the U937 cell line over-expressing human Bcl-2 protein (U937/Bcl-2) and the parental U937 cell line. As shown in Figure 4C, there were no significant differences in apoptosis induction at different treatment times (12, 24 and 48 h). These results suggest that Bcl-2 is not involved in QD-induced apoptosis. To test the functioning of U937/Bcl-2 cells, we used the cytotoxic agent ara-C because the expression of the Bcl-2 protein blocks ara-C-induced apoptosis (28). As expected, ara-C (1 μM, 24 h) triggered apoptosis in U937 cells, whereas over-expression of Bcl-2 significantly protected cells from apoptotic cell death induced by this cytotoxic compound (Figure 4D).

**QD activates MAPKs**

In view of evidence that the MAPKs ERK, JNK/SAPK and p38MAPK play a critical role in cell fate, the effects of QD on the activation of apoptosis, HL-60 cells were treated with increasing doses of this compound and the cell lysates were then subjected to immunoblot analysis. The results clearly demonstrate that QD (10 μM) significantly promotes pro-caspase-8 hydrolysis, although this response was not detected until after 24 h of treatment (Figure 3C). In U937 cells, there was no cleavage of caspase-8 after 24 h of treatment.
MAPKs were examined. Incubation of HL-60 and U937 cells with QD leads to phosphorylation of JNK/SAPK, of p38MAPK and of ERK1/2 (Figure 5A and B). Phosphorylation of p38MAPK and ERK1/2 was detected 30 min and 1 h after QD addition, respectively, and both remained elevated for up to at least 240 min (Figure 5A). However, the activation of JNK/SAPK was not observed until 4 h under the same experimental conditions (Figure 5A). These results indicate that QD treatment of HL-60 and U937 cells leads to activation of JNK/SAPK, p38MAPK and ERK1/2 following different kinetics. To determine whether the phosphorylation of MAPKs plays a key role in QD apoptosis, we examined the effects of JNK/SAPK, p38MAPK and ERK1/2 inhibitors (Figure 5C–F). Pretreatment of HL-60 cells with the specific JNK/SAPK inhibitor SP600125 (10 μM) and with the specific p38MAPK inhibitor SB203580 (2 μM) did not alter the rate of QD-mediated apoptosis. SP600125 and SB203580 had no influence on basal levels of apoptosis (Figure 5D and E). These data suggest that activation of JNK/SAPK and p38MAPK are not involved in QD-induced apoptosis.

We also investigated a putative impact of ERK1/2 in the signal transduction pathway, leading to QD-mediated cell death. For this purpose, we used specific mitogen-activated extracellular kinase (MEK) 1/2 inhibitors (PD 98059 and U0126) in order to block the activation of ERK1/2. The results indicate that PD98059 and U0126 enhance QD-mediated apoptosis (Figure 5F). The percentage of apoptotic cells increased from 30% in QD-treated cells to 45 and 55% with PD98059 and U0126, respectively. To confirm the effect of the MEK1/2 inhibitors, the phosphorylation status of ERK1/2 in the combination treatment group was also determined. As shown in Figure 5C, PD98059 and U0126 completely abolished ERK1/2 phosphorylation by QD. In HL-60 cells, QD and U0126 combined caused almost two times more cell death than QD alone, and 10 times more cell death than U0126 alone (Figure 5F). The 2-fold (or 10-fold) increase in apoptosis was calculated by comparing the sub-G1 readings of treated samples with the value of the untreated control as 1.0. These results suggest that these specific inhibitors of ERK1/2 may serve as sensitizers towards QD-mediated apoptosis in human leukemia cells.

Discussion

In the area of cancer chemotherapy, some 67% of the effective drugs are of natural origin (29). Most modern medicines currently available for treating cancers are very expensive, toxic and less effective in treating the disease. Therefore, the agents derived from natural sources should be investigated further in detail for the prevention and treatment of cancer. In previous studies with naturally occurring and semi-synthetic phenylbenzo-γ-pyrones, we had shown that the cytotoxicity of quercetin was greatly improved by acetylation and methylation of the free hydroxyl groups. We have previously reported that quercetin 3-methyl ether and QD shows similar potency in HL-60 cells (IC50 = 14.3 ± 4.6 μM versus 9.2 ± 3.4 μM) (21). In agreement with these results, flow cytometry studies indicate that QD and the parental compound, quercetin 3-methyl ether, induce apoptosis and cell cycle arrest at G2–M to a similar extent (results not shown). Since the flavonoids are phenolic compounds and therefore prone to oxidation to quinones, we carried out the protection of the 3',4'-dihydroxy group to block the potential oxidation and to generate a more chemically stable compound. Therefore, the experiments were carried out with QD instead of the naturally occurring quercetin 3-methyl ether.

In the present study, we demonstrate that QD also displays strong anti-proliferative effect on a second leukemia cell line, U937, with an...
IC50 value close to that reported for HL-60 cells. The effects of QD against human PBMCs were also determined since selectivity toward cancer cells is an important criterion in the study of the agents used or developed for cancer treatment. Dose-response studies revealed that quiescent PBMC and proliferating PBMC were resistant toward QD.

Previous studies have documented that the phenylbenzo-c-pyrones derivatives wogonin and fisetin induce apoptotic cell death in the human myeloid leukemia HL-60 cells (30). These flavonoids seem to trigger apoptotic cell death through a caspase-3-mediated mechanism which involves a decrease of reactive oxygen species (ROS). However, the mechanism displayed for the quercetin derivative (QD) to decrease cell growth on HL-60 and U937 cells has not been assessed yet. Cell cycle phase distribution analysis indicated that QD promotes G2–M phase arrest and increases the percentage of cells in the sub-G1 region which is considered as a marker of cell death by apoptosis.

Several flavonoids have been reported to exert their anti-proliferative activity by targeting microtubules through tubulin binding (31). One of the structural requirements usually found in flavones for tubulin interaction is the presence of a methoxyl group at C-3. However, this does not represent an absolute requirement since that quercetin—which lacks of the 3-methyl group—binds to tubulin at the colchicine site and perturbs microtubule polymerization by inducing conformational changes in the monomer (32). Since QD is an analog of quercetin and also contains a methoxyl group at C-3, G2–M arrest induced by QD might be explained by the inhibition of microtubule formation. However, the changes in the expression and/or activity of G2–M cell cycle regulators might also be involved. Further studies are needed to determine the effect of this compound on these regulators such as B-type cyclin isoforms, cdk1, cdc25C and p21\textsuperscript{WAF/Cip1}.

Other apoptotic hallmarks such as DNA fragmentation and apoptotic bodies formation were also observed in QD-treated cells, in a dose- and time-dependent manner. Apoptosis induction was accompanied by PARP cleavage which indicates involvement of this protein as a key regulator of apoptosis in QD-treated cells (33). We have also carried out experiments to compare QD and quercetin. There was no evidence of either a DNA ladder or PARP cleavage in quercetin-treated HL-60 cells at times and doses assayed (up to 10 μM, 24 h). However, QD and quercetin 3-methyl ether induced DNA fragmentation and PARP cleavage even to 3 μM (results not shown).

**Fig. 5.** QD induces phosphorylation of MAPKs and impact of MAPKs inhibitors on QD-induced apoptosis. (A) Representative western blots show the time-dependent phosphorylation of p38\textsuperscript{MAPK} and ERK1/2 by QD. HL-60 or U937 cells were incubated with QD for the indicated time points. Protein extracts were prepared and analysed on western blots probed with specific antibodies to ascertain the phosphorylation of MAPKs. Membranes were stripped and reprobed with total p38 or total ERK antibodies as loading controls. (B) Representative western blots show the phosphorylation of JNK by QD and the expression levels of the respective total JNK. HL-60 cells were incubated with the indicated concentrations of QD for 4 h. U937 cells were incubated with QD for the indicated time points. Protein extracts were prepared and analysed as in (A). (C) Representative western blot showing ERK1/2 phosphorylation in response to QD in the absence or presence of MEK1/2 inhibitors PD98059 (PD, 10 μM) and U0126 (U0, 10 μM). HL-60 cells were pre-incubated with (D) SP600125 (SP, 10 μM), (E) SB203580 (SB, 2 μM) or (F) PD98059 (PD) and U0126 (U0) for 1 h and then treated with QD. Cells were also incubated with each inhibitor only. Apoptosis was quantified by flow cytometry as described in Materials and Methods. Bars represent the mean ± SE of three independent experiments each performed in triplicate. \( \cdot \)\( P < 0.05 \), significantly different from untreated control. \( \# \)\( P < 0.05 \), significantly different from QD treatment alone.
Previous work described by Nguyen et al. (34) indicates that the concentrations of quercetin and its metabolites in rats were 25.1, 43.3 and 54.3 μM after 10 days of treatment with 50, 100 and 150 μg of quercetin per kg body wt, respectively, suggesting the possibility that the concentrations of QD employed in the present study might be achieved in vivo. In this regard, we have used concentrations 5-fold lower (10 μM versus 54.3 μM) and even almost 20-fold lower (3 μM versus 54.3 μM) than that detected by Nguyen et al. In vitro studies have shown that quercetin is bound to human plasma proteins and albumin is the primary protein responsible for the binding. However, substitution of 3-OH in quercetin markedly weakens binding to albumin (35). This suggests that the introduction of a methyl group in C-3 might allow weak binding to albumin and therefore increase its delivery to cells.

It is interesting to note that a low concentration (3 μM) of QD was sufficient to induce cytochrome c release, hydrolysis of caspasases (caspases-3, -6, -7 and -9) and also PARP cleavage in HL-60 cells in similar extension to 10 μM concentration. Recent studies have also indicated that liposomal quercetin can significantly improve the solubility and bioavailability of quercetin and might enhance antioxidant efficacy (36) and these results could be applicable to other flavonoids and especially to QD. Further studies are needed to investigate the safety, the bioavailability and the bioefficacy of quercetin 3-methyl ether and QD.

Although QD-induced cell cycle perturbation showed a similar pattern in HL-60 and U937 cells, it is important to note that the U937 cell line demonstrated to be more resistant to entry in apoptosis which suggest that there is heterogeneity in the response to QD. The induction of apoptosis by QD does not involve protein biosynthesis, and therefore, the target tumor cells already contain the complete set of proteins required for QD-induced apoptosis.

The experiments shown here demonstrate proteolytic processing of the effector caspases-3, -6 and -7 and the initiator caspases-8 and -9, suggesting that QD activates these proteases in HL-60 cells. However, proteolytic cleavage of caspase-8 was not detected until after 24 h of treatment and then only with the higher concentration tested. Similar results were obtained in U937 cells, but at longer times (24 h), with the only exception of caspase-8 which was not activated.

The evidence indicates that caspase-independent cell death is usually initiated along with an apoptotic cascade, although the apoptotic mediators inhibit the caspase-independent cell death pathway (14). Our results indicate that the stress signal induced by QD was dependent on caspase activation since apoptosis was pharmacologically inhibited by the general caspase inhibitor z-VDAD-fmk.

Previous studies have already demonstrated that many cytotoxic agents induce the release of mitochondrial cytochrome c (37), which triggers a caspase-dependent assembly of the apoptosome (38). Here, we have shown that QD induces the release of cytochrome c from mitochondria into the cytosol, and thus the activation of caspase-9 and caspase-3. Although we have shown that QD-induced apoptosis was prevented by a broad-spectrum caspase inhibitor, the use of selective inhibitors against caspase-8 (z-IETD-fmk), caspase-3 (z-DEVD-fmk) and caspase-9 (z-LEHD-fmk) were unable to block the cell death.

We have previously demonstrated on HL-60 cells, an effective blockage of betelol 3-methyl ether-induced apoptosis by z-IETD-fmk but not by z-LEHD-fmk, supporting a caspase-8-mediated mechanism (21). Therefore, different apoptotic pathways are activated in this cell line in response to the phenylbenzopyrones QD and betelol 3-methyl ether. A possible role of Bid can be ruled out in QD-induced apoptosis since Bid requires its previous proteolytic activation by caspase-8, and inhibition of this caspase with z-IETD-fmk did not prevent cell death.

Our data clearly demonstrate that caspases-3 and -7 are activated in HL-60 cells in response to QD. However, the lack of any effect of z-DEVD-fmk, a caspases-3 and -7-selective inhibitor, on QD-induced apoptosis indicates that cell death can occur in absence of caspase activity. Consequently, it appears that other factors may be able to compensate for a lack of caspase-3 and caspase-7 activity. Since z-DEVD-fmk is a more potent inhibitor for caspase-3 than for caspase-7 (39), one possibility is that other executioner caspasases, such as caspase-7 or -6, also contribute to apoptosis caused by QD.

The lack of a significant effect of z-LEHD-fmk on QD-induced cell death was also surprising, given the presence of proteolytic processing of caspase-9 and suggests that caspase-9 may not be involved in QD-mediated cell death. Since caspase-9 activation does not require proteolytic processing (40,41), we also performed kinetic studies. The results clearly demonstrate that caspase-9 is activated by QD, suggesting that QD-induced apoptosis is linked with cytochrome c but not with a receptor-mediated mechanism. The improved protection offered by z-VAD-fmk versus z-LEHD-fmk is probably caused by its better accessibility to cellular caspase-9 (42). Although it is often extremely difficult to determine the order of caspase activation, kinetic studies shown in this paper indicate inductions of both caspases-9 and -3 activities were significantly detectable at 12–14 h of treatment. In contrast, the increase in caspase-8 activity was not detectable until 24 h of treatment.

Increased expression of Bcl-2 has been associated with chemoresistance, especially in the case of hematologic malignancies (43). Therefore, the finding and development of compounds capable of inducing cell death in tumor cells over-expressing Bcl-2 is of great therapeutic importance. The present results suggest that QD treatment effectively induces cell death in leukemic cells over-expressing Bcl-2. In this regard, it has been shown that flavopiridol induces mitochondrial injury in various neoplastic cells, including those over-expressing Bcl-2 (44). Thus, although it is tempting to speculate that the activation of the extrinsic apoptotic pathway might be responsible for QD cell death in Bcl-2 over-expressing cells, another possibility could be that QD would be able to overcome blockade of cytochrome c release and mitochondrial injury conferred by anti-apoptotic Bcl-2. In this regard, previous works have described how the inactivation of Bcl-2 can be achieved by different mechanisms including cleavage by activated caspasases and hyperphosphorylation (45,46).

MAPKs are essential parts of the signal transduction machinery and play central roles in cell growth, differentiation and programmed cell death. The JNK/SAPK and p38MAPK have generally been associated with pro-apoptotic actions, whereas ERK1/2 in most cases exerts cytoprotective effects (47). The ability of ERK1/2 inhibitors has been reported to potentiate the antitumour effects of cytotoxic agents such as ara-C in a substantial increase in release of cytochrome c (48). Another hierarchical model of activation of JNK/SAPK by endoplasmic reticulum stress suggests induction of this pathway in Jurkat cells downstream of cytochrome c release and caspase-3 (49). Although most reports support the idea that JNK/SAPK contributes to stress-induced apoptosis, its effect appears to depend on cell type and the context of other signals received by the cell (49). Here, we show that QD induces JNK/SAPK activation in HL-60 and U937 cells. However, the selective JNK/SAPK inhibitor SP600125 did not influence QD-induced apoptosis, indicating that JNK/SAPK is not required for cell death.

The p38MAPK signaling has been shown not only to promote cell death in some cell lines but also to enhance survival, cell growth and differentiation. Therefore, the role of p38MAPK in apoptosis is dependent on cell types and stimuli (50). In the present study, we show that p38MAPK phosphorylation occurred prior to caspase activation in terms of time (30 min). However, the p38MAPK inhibitor SB203580 did not attenuate apoptosis, suggesting that activation of p38MAPK is not required for QD-induced apoptosis. Activation of p38MAPK has been reported to be involved in apoptosis in a variety of cell types. For example, the activation of p38MAPK is involved in cadmium-induced apoptosis on U937 cells (51) and resveratrol-induced apoptosis of human malignant B cells (52); however, this activation is not involved in apoptosis in UV-treated U937 cells (53) and/or in Fas- and UV-treated Jurkat T cells (54).

Previous studies have shown that quercetin treatment results in high and sustained activation of ERK in A549 lung cancer epithelial cells (34). However, the inhibition of MEK–ERK activation utilizing U0126 or PD98059 abolishes quercetin-induced apoptosis (34). Treatment of ovarian carcinoma cells (55,56) and the CB161 melanoma cell...
line (57) with cisplatin caused ERK activation and cell death, and this latter effect was potentiated by ERK inhibitors, indicating that ERKs behave as survival-inducing kinases in these cells.

One of the findings described here is that QD also enhances the activation of the MEK/ERK pathway, which is expected to increase cell proliferation and survival, and may compromise the efficacy of QD in potential cancer treatment. Our data indicated that ERK inhibitors PD98059 and U0126 potentiated the apoptotic effects of QD. The combination of QD plus PD98059 or QD plus U0126 enhanced cell death. The potential use of low-dose chemotherapy is important because lower dosages are more attainable during cancer therapy and probably to be less toxic to patients. These results have important clinical implications for the use of QD in combination of ERK inhibitors as potential therapeutic agents. Although it has been shown that increased ROS production in leukemic cells leads to the activation of MAPKs and cell death (58–61), however, we were unable to demonstrate intracellular ROS generation after exposure to QD (results not shown). So, the mechanism of cell death triggered by QD seems to be different to wogonin and fisetin which involves a decrease in ROS (30).

In summary, our results show that QD displays cytotoxic properties, induces G2–M phase cell cycle arrest and apoptosis on human myeloid leukemia HL-60 and U937 cells. Since these cells are p53 null, our results clearly demonstrate that apoptosis induced by QD could occur independently of p53-mediated cellular events. Given the fact that ~50% of human cancers harbor p53 mutations (62,63), these results have important implications for developing QD as a chemo-preventive or chemotherapeutic agent. QD-induced apoptosis is accompanied by the activation of multiple caspases, mitochondrial release of cytochrome c, PARP cleavage and DNA fragmentation. Although cleavage of pro-caspase-8, -9, -3 and -7 is detected in QD-treated cells by immunobloting, the inhibition data indicate that these caspases do not fully account for cell death as a result of exposure to QD. These results, when considered together with the complete blockage of apoptosis by z-VAD-fmk, suggest that additional caspases may contribute to QD-induced apoptosis. One possibility is that other executioner caspases, such as caspase-6, are involved in this process as it is shown in this paper. The mitochondria-protecting protein Bcl-2 was unable to prevent QD-induced apoptotic cell death. This result suggests that QD might trigger an alternative pathway that bypasses mitochondria and/or that QD might be able to inactivate the mitochondrial protection conferred by Bcl-2 protein. QD enhances the activation of the MEK/ERK pathway, and the combined treatment of QD and inhibitors of MEK1/2 kinase leads to enhanced cell death. The findings of this study suggest that QD could be useful in the development of novel anticancer agents.

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References

Induced cell death in human leukemia cells


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