Cytotoxic action of methylquercetins in human lung adenocarcinoma cells

KATRIN SAK¹, HELEN LUST¹, MARJU KASE² and JANA JAAL¹,²

¹Clinic of Hematology and Oncology, Institute of Clinical Medicine, University of Tartu; ²Department of Radiotherapy and Oncological Therapy, Tartu University Hospital, 51014 Tartu, Estonia

Received July 10, 2017; Accepted November 6, 2017

DOI: 10.3892/ol.2017.7466

Abstract. Lung cancer is the malignant disorder associated with a high number of fatalities in women and men worldwide. Despite continuous improvements in diagnostic strategies and therapeutic modalities over the past decades, the prognosis and survival rate of patients suffering from lung cancer are still unsatisfactory and suggest the requirement for further molecular studies with different lung cancer models. In the present study, the anticancer action of two methylated metabolites of quercetin, isorhamnetin and tamarixetin, was assessed by studying their antiproliferative and apoptosis-inducing potential in human lung adenocarcinoma cell lines, A549 and HCC-44. Both methylquercetins decreased the viability of lung cancer cells at doses significantly lower than those effective for parent quercetin. The IC₅₀ values measured for isorhamnetin were 26.6 and 15.9 µM in A549 and HCC-44 cells, respectively. For tamarixetin, the IC₅₀ values were 19.6 and 20.3 µM in A549 and HCC-44 cells, respectively. These results were many-fold lower than the respective values for quercetin (72.2 and 107.6 µM for A549 and HCC-44 cells, respectively). Based on the activation of caspase family members, both metabolites induced apoptotic cell death in the tested cell lines, predominantly via the extrinsic pathway in A549 cells and in both intrinsic and extrinsic pathways in HCC-44 cells. As A549 and HCC-44 lines were originally established from a male and female patient, current data may suggest some gender differences in the action of quercetin derivatives. Addition of a methyl group in the 3'- or 4'-position of the B-ring of quercetin significantly increased the anticancer activity of this flavonol towards lung adenocarcinoma cells, which demonstrated that these compounds may be considered as potential novel candidates for the development of future chemotherapeutics in the fight against lung cancer.

Introduction

Lung cancer has remained a global leading cause of cancer mortality in both men and women (1-3). In 2013, this malignant disorder constituted approximately 1.8 million new cancer cases and accounted for 1.6 million cancer deaths worldwide (2). Moreover, the number of lung cancer deaths is expected to grow up to 3 million for the year 2035 (3). Lung cancer is generally divided into two differently growing histological types, i.e., small-cell lung cancer and non-small cell lung cancer that accounts for the most of lung cancer cases (around 85%) and includes adenocarcinoma, squamous cell carcinoma and large cell carcinoma (1,3,4). Adenocarcinoma is the most common histological subtype that represents about 40% of all lung cancer cases (1,4). Although men are more likely to be affected by this malignancy than women, with 1 in 18 men and 1 in 51 women diagnosed with lung cancer at some point in their lives, its incidence in women is globally increasing (3). Potential male-female differences have been demonstrated also in other aspects of lung cancer, including better prognosis, higher treatment responses and survival in women as compared to men (5-8). Nevertheless, the mean 5-year survival rate of lung cancer is estimated to be less than 18%, showing an urgent need for more effective treatment choices (2,4). Recent bibliometric analysis revealed that international research level of lung cancer lags substantially behind the publication outputs for other malignancies. Despite the poor prognosis, high mortality rate and huge economic costs, in 2013 the research in lung cancer accounted only a small proportion, i.e., 5.6% of all oncology research (2). Consequently, further in vitro and in vivo investigations using different lung cancer models are highly needed to develop novel treatment strategies and improve the survival rate of lung cancer patients.

In the recent years, several epidemiological studies have demonstrated that higher intake of fruits and vegetables can be beneficial for prevention of different types of human cancers, including lung tumors (9-13). As anticarcinogenic components of these plant products, polyphenolic flavonoids have been proposed with numerous experimental investigations to display various antitumoral activities (14-16). Indeed,
these polyphenols can express antiproliferative, cytotoxic, proapoptotic, antiinvasive, antimetastatic, angiogenic, and antiinflammatory properties in different cancer cell lines or animal models (17). However, it is well known that in the human body, flavonoids undergo an extensive metabolism and as a result of this conversion only different metabolic conjugates enter the circulatory system and can reach target malignant tissue (18,19). Differently from parent flavonoids, current knowledge about the possible anticancer action of their metabolites is still rather limited making the prediction of bioactive behavior of flavonoids in the human body complicated.

One of the most important metabolic pathways that flavonoids undergo in the small intestine and liver is their methylation catalyzed by catechol-O-methyltransferase (COMT) (20). This phase II enzyme catalyzes the transfer of a methyl moiety from S-adenosylmethionine donor substance to a catecholic substrate, such as flavonoids (21). As the methylated flavonoids formed in this way may potentially reveal substantially different biological properties than the parent compounds, we focus in this study on the anticancer effects and mechanisms of two methylated quercetin molecules, i.e., 3′-O-methylquercetin or isorhamnetin and 4′-O-methylquercetin or tamarixetin in human non-small cell lung cancer (adenocarcinoma) lines, A549 and HCC-44, and investigate their antiproliferative activities compared to the parent quercetin. In addition to studying the cell growth inhibitory effects of these methylated flavonoids by the MTT assay, their ability to trigger apoptotic pathways (i.e., intrinsic vs extrinsic routes) is also under examination by determining caspase-9 and -8 activities. This investigation shows that flavonoid metabolites can be considered as leading compounds for further development of lung cancer chemotherapeutics possibly supplementing the available treatment arsenal in the future.

Materials and methods

Reagents. All flavonoids (genistein, daidzein, fisetin, quercetin, hesperetin, luteolin, chrysins, baicalein) and methylated derivatives of quercetin (isorhamnetin, tamarixetin) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and L-glutamine were the products of Sigma-Aldrich (St. Louis, MO, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and L-glutamine were the products of Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was from Mediatech, Inc. (Manassas, VA, USA). Dimethyl sulfoxide (DMSO) was from Mediatech, Inc. (Manassas, VA, USA). Phosphate-buffered saline (PBS) was obtained from Lonza (Verviers, Belgium).

Cell lines and culture conditions. A549 and HCC-44 human lung adenocarcinoma cell lines were obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany).

A549 and HCC-44 are cell lines derived from the adenocarcinoma of a 58-year-old Caucasian man (22) and a 54-year-old woman, respectively (23). To exclude clinically distinct disease entity—oncogene addicted lung cancer, these cell lines were further tested in our laboratory for epidermal growth factor receptor (EGFR) mutation and anaplastic lymphoma kinase (ALK) translocation. Both cell lines were wild type and did not have these genetic alterations (data not shown).

A549 cells were cultivated in Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies Corporation, Grand Island, NY, USA) supplemented with 10% of heat-inactivated fetal bovine serum (FBS; Invitrogen™, Auckland, NZ, USA). HCC-44 cells were cultivated in RPMI 1640 medium (Life Technologies Corporation) supplemented with 10% of heat-inactivated FBS. Cells were maintained in a 5% CO₂ incubator at 37°C with and passed 1:2 times per week.

Measurement of cell viability by MTT assay. The cell growth inhibitory effects of flavonoids against human lung cancer cell lines were tested by the MTT colorimetric assay first described by Mosmann in 1983 (24). In detail, the cells were plated on to 96-well U shaped bottom plates at concentration of 1x10⁵ cells/ml of medium, putting 100 µl of suspension to each well. Cells were counted in Bürker counting chamber. As phenol red can interfere with the reading of absorbance (25), the cells were seeded in the phenol red-free RPMI-1640 medium (Mediatech, Inc.). After overnight culturing, cells were treated with varying doses of flavonoids (10 nM-500 µM) for 48 h at 37°C and 5% CO₂. At the end of the incubation, 50 µl of MTT solution in PBS was added to the wells with the final concentration of MTT of 5 mg/mL. Plates were further incubated for 4 h followed by centrifugation at 1,000 rpm for 10 min and removing of the supernatant. To dissolve the purple formazan crystals 150 µl of DMSO was added and the plates were shaken for 30 min. Absorbance was measured at 540 nm using a LED based microplate reader (Ledetect 96; Labexim Products, Lengau, Austria). To calculate the proportion of surviving cells, the following formula was used: (OD of drug-treated sample - OD of blank)/(OD of control - OD of blank) x100%, where OD of blank represents the absorbance reading of wells containing the buffer only (without cells) and OD of control represents the reading value of wells without any added test compounds. Dose-response curves were constructed to evaluate the half-maximal inhibitory concentrations (IC₅₀ values). All separate tests were carried out 2-3 times in different days, performing the experiments in triplicates.

Measurement of caspase activities. To study the cell death mechanisms triggered by the treatment of human lung cancer cell lines with methylated quercetin derivatives, the Caspase Colorimetric Protease Assay Sampler kit (Invitrogen Corporation, Frederick, MD, USA) was used according to the protocol provided by the manufacturer. As this kit uses para-nitroaniline-labeled synthetic peptides as substrates of different caspases, absorption of cleaved para-nitroaniline was spectrophotometrically quantified at 405 nm.

Statistics. Data were treated using the GraphPad Prism statistical software (version 4.0; GraphPad Software, Inc., La Jolla, CA, USA). The Kolmogorov-Smirnov test for normality was applied and the one-way analysis of variance (ANOVA) was performed to determine whether the differences between means were statistically significant. P-value <0.05 were considered as statistically significant and all values were expressed as mean ± standard deviation (SD).
Results

Cytotoxicity profiles of flavonoids in human lung adenocarcinoma cells A549 and HCC-44. Among the tested panel of flavonoids, isoflavones genistein and daidzein and flavanone hesperetin had no growth inhibitory effects on both lung cancer cell lines up to 100 µM concentration. Flavonols fisetin and quercetin as well as flavones luteolin, chrysin and baicalein revealed low antiproliferative efficiency with half maximal inhibitory constants (IC₅₀) more than 100 µM, except quercetin in A549 cells with IC₅₀ of 72.2 µM, and fisetin and chrysin in HCC-44 cells with IC₅₀ values of 78.7 and 79.6 µM, respectively (Table I).

Growth inhibition of human lung adenocarcinoma cells A549 and HCC-44 by methylated metabolites of quercetin. Differently from the parent quercetin, its metabolites with one methyl group in the different positions of B-ring revealed much higher efficiency in inhibition of growth of lung cancer cells. In detail, the derivative containing a methyl moiety in the 3′-position, i.e., 3′-O-methylquercetin or isorhamnetin, displayed the inhibitory constants of 2.7- and 6.8-fold lower compared to the parent quercetin in A549 (IC₅₀, 26.6 µM) and HCC-44 cells (IC₅₀, 15.9 µM). The respective increases in cytotoxic potencies were 3.7- and 5.3-fold for 4′-O-methylquercetin or tamarixin in A549 (IC₅₀, 19.6 µM) and HCC-44 cells (IC₅₀, 20.3 µM) (Table I). Chemical structures of these methylquercetins with the dose-response curves in both lung adenocarcinoma cell lines are presented in Fig. 1.

Effect of methylquercetins on activation of caspase family members. The effect of methylated quercetins on lung adenocarcinoma cell lines was further estimated by analysis of activity of caspase family members. In both A549 and HCC-44 cells, isorhamnetin increased caspase-3 activity by 2.5- and 3.5-fold, respectively, indicating induction of apoptosis. Similar results were measured for tamarinixin in both cell lines pointing to the occurrence of apoptosis via extrinsic pathway. In HCC-44, but not in A549 cells, also the activity of caspase-9 was increased demonstrating that in these cells the apoptosis is induced through both intrinsic and extrinsic routes (Fig. 2).

Discussion

A549 and HCC-44 cell lines are two non-small cell lung cancer lines derived from the adenocarcinoma of a 58-year-old Caucasian man (22) and a 54-year-old woman, respectively (23). The current work is the first study to describe the inhibitory effects of flavonoids on the viability of HCC-44 cells. As A549 line has been widely used as a model system to study human alveolar carcinoma, cytotoxic profile of flavonoids in these cells was previously characterized with the results very similar to those measured in our work. Indeed, the IC₅₀ value of 72.2 µM for quercetin is close to the inhibitory constants published by Loizzo et al (26), Tan et al (27-29), Robaszkiewicz et al (30), and Chan et al (31); and IC₅₀ values more than 100 µM were previously reported for fisetin (32), hesperetin (33-35), luteolin (32,36), chrysin (35), baicalein (34), daidzein (37), and genistein (38). Based on our results presented in this article, cytotoxic activity profiles of flavonoids were rather similar for both A549 and HCC-44 lung cancer lines, despite the gender difference of initial origin of these cells.

However, the data clearly show that the growth inhibitory effects of tested flavonoids on lung cancer cells revealed only at very high micromolar doses that are physiologically unachievable. Indeed, the maximum serum concentrations of daidzein and genistein were measured to be less than 0.5 µM following to consumption of 100 ml of untreated soymilk (39). The baseline plasma concentration of quercetin was reported to be generally about 50-80 nM reaching 0.63 µM after supplementation with 80 mg quercetin per day for one week or 1.5 µM after supplementation with >1 g quercetin per day for 4 weeks (40). Although bioavailability of flavonoids depends on food sources (for instance, quercetin is somewhat better bioavailable from onions than apples) and there is also high interindividual variability (40,41), it is rather impossible to achieve plasma doses exceeding some micromolar level by oral ingestion of flavonoids-rich food items or dietary supplements.

Despite numerous experimental works performed with bioactivity of parent flavonoids, the knowledge about possible antiproliferative effects of their metabolites is still rather sparse today. One reason for this is the very limited commercial availability of metabolites for experimental testing. There are three major types of metabolic derivatives of flavonoids formed as a consequence of enzymatic conjugation with methyl-, sulfate- or glucuronyl groups in the small intestine and liver catalyzed by COMT, sulfotransferase (SULT) or UDP-glucuronosyltransferase (UGT), respectively (19,20,42). Considering some structural modification

<table>
<thead>
<tr>
<th>Variable</th>
<th>A549</th>
<th>HCC-44</th>
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<tbody>
<tr>
<td>Isoflavones</td>
<td>&gt;500</td>
<td>&gt;500</td>
</tr>
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<td>Genistin</td>
<td>&gt;500</td>
<td>&gt;500</td>
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<tr>
<td>Daidzein</td>
<td>&gt;500</td>
<td>&gt;500</td>
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<tr>
<td>Flavonols and their methylated metabolites</td>
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<tr>
<td>Fisetin</td>
<td>127.9±1.9</td>
<td>78.7±2.0</td>
</tr>
<tr>
<td>Quercetin</td>
<td>72.2±2.3</td>
<td>107.6±2.2</td>
</tr>
<tr>
<td>3′-O-Methylquercetin or isorhamnetin</td>
<td>26.6±1.7</td>
<td>15.9±1.7</td>
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<tr>
<td>4′-O-Methylquercetin or tamarixin in</td>
<td>19.6±1.3</td>
<td>20.3±1.4</td>
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<tr>
<td>Flavanones</td>
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<tr>
<td>Hesperetin</td>
<td>&gt;500</td>
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<tr>
<td>Flavones</td>
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<tr>
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<td>Chrysin</td>
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<tr>
<td>Baicalein</td>
<td>307.6±3.1</td>
<td>194.5±1.9</td>
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of initial compounds changes in their bioactivities could also be expected. Our results with two methyl conjugates of quercetin indeed confirm this standpoint indicating significantly higher antiproliferative potencies of both isorhamnetin and tamarixetin in HCC-44 and A549 lung adenocarcinoma cells compared to the parent flavonol. As the growth inhibitory effects of isorhamnetin have been previously reported in A549 cells (43–45), to the best knowledge of the authors this is the first study at all to describe the action of tamarixetin in human lung cancer cells. The results demonstrate that methylated metabolites of quercetin have considerably stronger anticancer activity than quercetin itself, whereas the potency does not depend on whether the methyl group is located in 3’-(isorhamnetin) or 4’-position (tamarixetin) of the B-ring in quercetin skeleton. In the future, it would be interesting to test the cytotoxic activity of other methylated derivatives of quercetin in lung cancer cell lines to study the possible structure-activity relationships. Furthermore, testing the potential antiproliferative action of other types of quercetin conjugates, i.e., sulfates and glucuronidates, would be equally important to better understand the anticarcinogenic role of flavonoids in the human body.

The two main mechanisms of cytotoxic action of flavonoids in malignant cells involve cell cycle arrest and induction of apoptosis. At that, apoptosis is largely mediated by two major routes: The intrinsic or mitochondrial signaling and extrinsic death receptor pathway. The former way is triggered by the release of cytochrome c to the cytoplasm, cleavage of caspase-9 and activation of caspase-3. The latter pathway includes the interaction with death receptor and sequential activation of caspase-8 and caspase-3. In this study, we demonstrated that both methyl conjugates of quercetin, isorhamnetin and tamarixetin, induced apoptotic cell death in A549 and HCC-44 cells, characterized by the activation of effector caspase-3. However, as the cell death was predominantly mediated by extrinsic pathway in A549 cells, both extrinsic and intrinsic pathways were activated in HCC-44 cells by both methylated quercetin metabolites. The differences in activated caspase cascades in the tested cellular models can probably be caused by the different cellular signaling pathways triggered by quercetin.
derivatives. However, as A549 and HCC-44 lines were initially derived from a male and female lung adenocarcinoma patient, respectively, it is also possible that differences in the induced apoptotic pathways might involve some gender-specific aspects. Interestingly, the increase in cytotoxicity of methylquercetins compared to the parent quercetin molecule was significantly stronger (6.8-fold for isorhamnetin and 5.3-fold for tamarixetin) in female origin line HCC-44 than in male origin line A549 (2.7- and 3.7-fold increases, respectively). Therefore, it is possible that lung adenocarcinoma cells derived from men and women can behave differently to the treatment with methylated quercetins, a situation similar to the higher responses of female patients to the current therapeutic modalities in clinical use (7). These aspects clearly need further exploration. Moreover, considering that apoptosis has emerged as an important molecular mechanism for the anticancer action of standard chemotherapeutic drugs and novel candidate agents, further experiments for investigation of signaling pathways activated by methylated quercetins in different lung cancer cell lines are highly needed. In addition, the potential effects as well as possible toxicity issues of these compounds in xenograft rodent models also wait for testing.

Although the main aim of this work was to study the role of structural modification with adding a methyl group to quercetin molecule on its cytotoxic activity, this study has also several limitations. Among these, rate of apoptosis was not evaluated by flow cytometry and expression of pro- and anti-apoptotic proteins, such as caspase-3 and cleaved caspase-3, were not detected. Moreover, the effect of quercetin, isorhamnetin and tamarixetin on the cell cycle progression of A549 and HCC-44 lung adenocarcinoma cells as well as potential triggering of necrosis needs further unraveling in the future studies.

In conclusion, we showed that two methylated quercetin metabolites, isorhamnetin and tamarixetin, dose-dependently decreased the viability of A549 and HCC-44 lung adenocarcinoma cells at doses many-fold lower than those cytotoxically active for parent quercetin. Both metabolites also induced the apoptotic cell death in tested lung cancer experimental models revealing methylated quercetins as potential novel drug candidates for future treatment of non-small cell lung cancer.

Acknowledgements

This study was supported by the Estonian Society of Clinical Oncologists.

References


