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The antioxidant quercetin protects HL-60 cells with high myeloperoxidase activity against pro-oxidative and apoptotic effects of etoposide

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The protective action of quercetin against the pro-oxidant and apoptotic effect of etoposide was investigated in HL-60 cells with a high level of myeloperoxidase (MPO) activity and in cells treated with MPO inhibitor, 4-aminobenzoic acid hydrazide (ABAH). Quercetin significantly protected MPO-rich cells against the pro-oxidative ($p<0.05$) and apoptotic ($p<0.05$) effects of etoposide. Pretreatment with ABAH abolished this protective influence of quercetin on apoptosis induced by etoposide but actually enhanced the action effect of quercetin against etoposide-generated reactive oxygen species (ROS) level by this cytostatic drug. Thus quercetin can protect HL-60 cells against the pro-oxidative activity of etoposide regardless of MPO activity.

Key words: apoptosis, etoposide, quercetin, myeloperoxidase, reactive oxygen species, 4-aminobenzoic acid hydrazide

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INTRODUCTION

Etoposide is a first-line anticancer drug used in the treatment of certain solid tumours, lymphomas and leukemias (Slevin, 1991). In normal bone marrow cells, etoposide can initiate carcinogenesis leading to the development of myeloid leukemia, which occurs in a few percentage of patients (Whitlock *et al.*, 1991). On the other hand, the most common side effect induced by etoposide is myelosuppression, which limits its use (Kobayashi & Ratain, 1994).

Currently, a major role in the cancerogenic action of etoposide is assigned to its radicals formed in the precursor myeloid cells, probably mainly owing to the action of myeloperoxidase (MPO) (Kagan *et al.*, 1999; Fan *et al.*, 2006). Studies with the use of cyanide and azide, which are inhibitors of MPO, have demonstrated that etoposide undergoes one-electron oxidation to phenoxyl radicals by MPO in leukemic promyelocytic HL-60 cells and human myeloid progenitor $CD34^+$ cells (Kagan *et al.*, 1999; Vlasova *et al.*, 2011). Studies on HL-60 cells have also confirmed that the etoposide redox-cycling catalyzed by MPO involves intracellular reducing agents, mainly reduced glutathione (GSH), leading to the oxidation of thiols and the formation of thiyl radicals (Kagan *et al.*, 1999). Because of further reactions involving thiyl radicals, oxygen radicals may arise, including the highly toxic hydroxyl radical. In addition, the action of genotoxic etoposide radicals may include the oxidation of topoisomerase II, because incubation of HL-60 cells with ascorbate leads to a decrease in the level of these radicals and also reduces the amount of etoposide-topo II-DNA adducts (Kagan *et al.*, 1999).

The oxidative stress caused by toxic etoposide radicals may enhance oxidative DNA damage and topoisomerase II-dependent recombination (Vlasova *et al.*, 2011). In addition, DNA damage leads to apoptosis and the inhibition of proliferation, and is partly responsible for myelosuppression.

Thus, reduction of the radicals produced during the oxidation of etoposide in precursor cells of the myeloid lineage could contribute to both an increase in the applicable dosage of etoposide and enhanced therapeutic results while minimizing the side effects of treatment. It is known that treatment of HL-60 cell homogenates with an MPO inhibitor or antioxidants such as ascorbate reduces the formation of etoposide phenoxyl radicals (Kagan *et al.*, 1999). However, ascorbate is rapidly consumed in the reaction and not adequately protects against the pro-oxidative action of etoposide (Kagan *et al.*, 1999).

Polyphenols, such as quercetin can limit the pro-oxidant and cytogenotoxic action of etoposide more effectively than classical antioxidants. Quercetin exerts antioxidant activity via a variety of mechanisms, such as scavenging free radicals, chelating transition metal ions and inducing the antioxidant defence system regulated by nuclear factor erythroid 2-related factor 2 (Nrf2) (Hu *et al.*, 1995; Cheng & Breen, 2000; Ramyaa *et al.*, 2014). Given that quercetin can also inhibit the activity of MPO and CYP3A4 (Pincemail *et al.*, 1988; Kagan *et al.*, 1999; Choi *et al.*, 2011; Jacob *et al.*, 2011) an even more effective action of this flavonoid in reducing the side effects of etoposide could be expected than is the case with conventional antioxidants.

Previously, it has been demonstrated that the antioxidant quercetin can protect mature granulocytes against the formation of phenoxyl etoposide radicals and apoptosis induced by this cytostatic drug (Kapiszewska *et al.*, 2007). So far, there has been no research into whether quercetin can modify the action of etoposide in myeloid cells in a manner dependent on the activity of MPO.

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Abbreviations: ABAH, 4-aminobenzoic acid hydrazide; APC, allophycocyanin; CD, cluster of differentiation; CYP3A4, cytochrome P450 3A4; DSBs, double-strand breaks; FSC, forward scatter; GSH, reduced glutathione; HBSS, hank's balanced salt solution; MPO, myeloperoxidase; PI, propidium iodide; RFU, relative fluorescence units; ROS, reactive oxygen species; SSC, side scatter

The aim of this study was to determine the effect of quercetin on the apoptotic and pro-oxidant action of etoposide in HL-60 cells with a high MPO activity and in cells treated with an MPO inhibitor.

MATERIAL AND METHODS

HL-60 cell culture conditions. HL-60 cell line was purchased from ATCC (American Type Cell Culture, UK). The cells were cultured in RPMI 1640 (ATCC, UK) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA), without antibiotics, and in standard conditions at 37°C in a 5% CO_2 and 95% humidified atmosphere. The cells in the logarithmic growth phase were seeded in 24-well culture plates at a density of 0.5×10^6 cells/well.

Treatment of the cells. The cells were preincubated with 100 μM 4-aminobenzoic acid hydrazide (ABAH) for 24 hours followed by incubation in the presence of different concentrations of quercetin (0.5–50 μM) and/or etoposide (1–10 μM) for 24 hours. ABAH was dissolved in RPMI and other compounds in dimethyl sulfoxide (DMSO) and subsequently diluted in PBS. Final concentration of DMSO did not exceed 1% in the culture medium. Reagents were purchased from Sigma-Aldrich, St. Louis, Missouri, USA. Control cells were incubated in the presence of DMSO. The experiment was repeated three times.

Cytochemical staining for MPO. MPO activity in the cells was detected using 4-chloro-1-naphthol kit (Merck KgaA, Darmstadt, Germany) according to manufacturer's instruction. The cytochemical reaction is based on the oxidation of 4-chloro-1-naphthol which is converted to a black brownish insoluble dye. The intensity of staining is proportional to MPO activity.

ROS measurements. Reactive oxygen species (ROS) were detected in the cells using 5-(and-6')-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCF-DA, Life Science Technology) staining. Acetate groups of CM-H_2DCFDA are cleaved by intracellular esterases to non fluorescent dichlorofluorescein (DCFH) which reacts with intracellular antioxidants like glutathione and other thiols. DCFH undergoes rapid oxidation under the influence of ROS to a fluorescent dye with Ex/Em of 502/523 nm. Briefly, HL-60 cells were incubated with quercetin (0.5–100 μM) and/or etoposide (1–10 μM) for 1 hour. After incubation, the cells were washed with Hank's balanced salt solution (HBSS, Life Science Technology) and incubated with 10 μM CM-H_2DCF-DA in 1 ml HBSS for 30 min in the dark. Then, they were washed with cold HBSS and suspended with 400 μl of this buffer and quickly analyzed. The intensity of fluorescence was measured in an LSR II flow cytometer (Becton Dickinson, BD Biosciences Immunocytometry Systems, San Jose, CA, USA) equipped with a blue laser (488 nm excitation, 575/26 BP filter). Histograms were analyzed using FACSDiva software. Doublets were excluded from the analysis using gating on width versus area of the forward scatter (FSC) and side scatter (SSC). The analysis was performed on at least 20 000 events.

Analysis of apoptosis. The cells were incubated with quercetin (0.5–1 μM) and/or etoposide (10 μM) for 24 hours. After incubation, the cells were washed ($230 \times g$, 10 min) and resuspended in hydroxyethyl piperazineethanesulfonic acid (HEPES) binding buffer (Becton Dickinson). The cells were incubated with 2 μl of Annexin V-allophycocyanin (APC) (Becton Dickinson) and 2 μl of propidium iodide (PI, 50 μg/ml, Sigma-Aldrich) per 50 μl of HEPES for 15 min in the dark. Then, they were suspended in 400 μL HEPES (Sigma-Aldrich) and analyzed in an LSR II flow cytometer equipped with red and blue lasers, using 633 nm excitation for APC (660/20 BP filter) and 488 nm excitation for PI (575/26 BP filter). The percentages of early apoptotic (annexin V-APC-positive/PI-negative), necrotic (annexin-APC-negative/PI-positive) and late apoptotic cells (annexin-APC-positive/PI-positive) were analyzed using FACSDiva software (Becton Dickinson). Doublets were excluded from analysis using gating on width versus area of the forward scatter (FSC) and side scatter (SSC). Analysis was performed on at least 20 000 events.

Cell cycle analysis. The cells were incubated with quercetin (0.5 or 1 μM) and/or etoposide (10 μM) for 24 hours. After incubation, the cells (1×10^6 cells) were washed with PBS and fixed in 70% ethanol at –20°C for 24 hours. After fixation, the cells were washed in PBS and incubated for 30 min in 0.5 ml of solution containing 0.1% Triton X-100, 40 mg/ml RNAse A and 2% PI. The cell cycle was analyzed in an LSR II flow cytometer. Analysis was performed on at least 20 000 events.

Statistical analysis. Values are expressed as means ± SD. Student's *t*-test and one-way analysis of variance followed by Newman-Keuls and Tukey post hoc tests were used to determine significant differences ($p<0.05$) between samples.

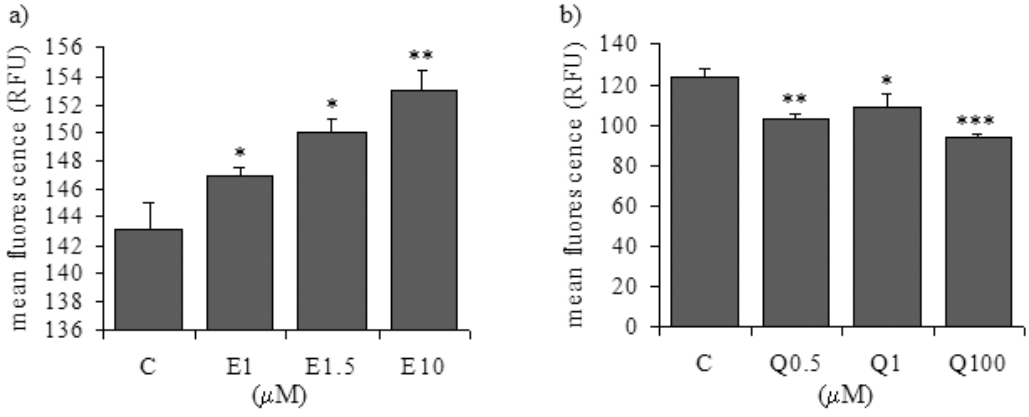

Figure 1. ROS accumulation in HL-60 cells treated with etoposide (a) and quercetin (b).
The cells were incubated with increasing concentrations of quercetin and etoposide. ROS levels were evaluated using staining with CM-H_2DCFDA. The analysis was performed by flow cytometry. RFU — relative fluorescence units. C — control, Q — quercetin, E — etoposide. Data are presented as mean ± S.D. of the mean. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

RESULTS AND DISCUSSION

Quercetin, as an antioxidant, can interrupt the redox cycle of etoposide by reducing the amount of phenoxyl radicals in cells. The present results indicate that quercetin can protect human myeloid cells with high MPO activity against the pro-oxidant and apoptotic effects of etoposide.

High concentrations of quercetin (50–200 μM) potentiate the cytotoxic effects of chemotherapy *in vitro* (Samuel *et al.*, 2012). However, less is known about the effect of low concentrations of this flavonoid on the action of chemotherapeutic agents. In this study quercetin concentrations of 0.5 and 1 μM were selected based on reports that the use of this flavonoid in concentrations below 6 μM can protect HCT116 p53-null cells against the effects of 5-fluorouracil *in vitro* (Samuel *et al.*, 2012). In addition, low concentrations of quercetin (0.5–5 μM) are achievable in blood plasma (Wiczkowski *et al.*, 2008).

The present study proved that etoposide can act as a pro-oxidant in dose dependent manner toward different intracellular constituents in HL-60 cells (Fig. 1a). However, cell pre-treatment with ABAH a selective inhibitor of MPO significantly reduced the level of ROS generated by etoposide (10 μM) (Fig. 2a, b). Quercetin protected HL-60 cells even more effectively than ABAH against the pro-oxidant action of etoposide (Fig. 2b). This fact indicates the different contribution of the pro-oxidative metabolism of etoposide than that of the oxidation of etoposide by MPO. ABAH, significantly reduced the activity of this enzyme in HL-60 cells, when used at a concentration of 100 μM (Fig. 2c).

Furthermore, it can be seen that quercetin alone reduces the amount of free radicals in HL-60 cells compared to the control (Fig. 1b). Quercetin may therefore reduce endogenous levels of free radicals produced as a result of various intracellular redox-cycling reactions, which are usually elevated in neoplastic cells. This flavonoid can inhibit both prostaglandin synthase and CYP3A4 cytochrome, contributing to reduced levels of etoposide catechol metabolites which can be oxidized by MPO and other enzymes capable of producing superoxide (Bender *et al.*, 2004; Moon *et al.*, 2006; Choi *et al.*, 2011).

It has been demonstrated in a cell-free system that etoposide quinones induce far more DNA strand breaks associated with the activity of topoisomerase II than their parent drug. Also, etoposide catechols are stronger topoisomerase II poisons (Gantchev & Hunting, 1998; Lovett *et al.*, 2001). Quinone and catechols of etoposide may increase the genotoxic action of the parent drug leading to an increase in the DNA double-strand breaks (DSBs), which are one of the most deleterious forms of DNA damage, resulting in further consequences such as cell cycle arrest and apoptosis (Karanjawala *et al.*, 2002). The cell cycle suppression and apoptosis of normal proliferating hematopoietic precursors can result in myelosuppression.

Protective effect of quercetin may also consist in quercetin competing with etoposide to be a substrate of MPO and thereby preventing it from entering the redox-cycling reactions. Importantly, quercetin exerted its protective effect already at a concentration of 0.5 μM, a level which may occur in the blood plasma following

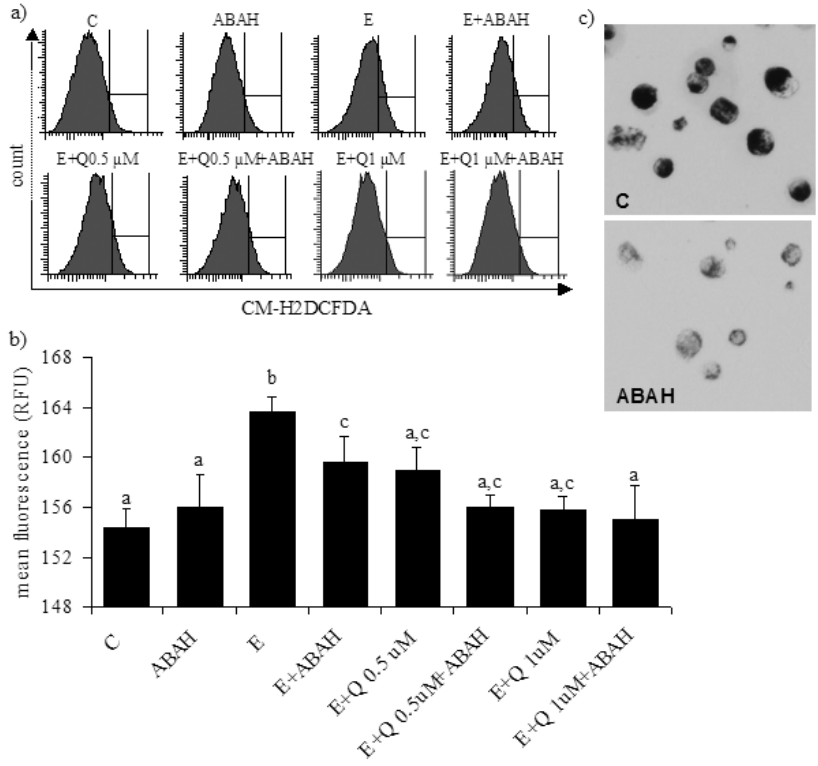

Figure 2. The influence of quercetin on ROS level generated under the action of etoposide in HL-60 cells with normal or decreased activity of MPO.

The cells were pretreated or not with 100 μM ABAH followed by incubation with quercetin (0.5 or 1 μM) and/or etoposide (10 μM). ROS accumulation was detected using staining with CM-H_2DCFDA. The analysis was performed by flow cytometry. (**a**) Representative histograms of fluorescence (**b**) RFU — relative fluorescence units. Data are presented as mean ±S.D. of the mean. Means having different signs (**a, b, c**) differ significantly, $p<0.05$. (**c**) Cytochemical reaction for MPO activity in control and ABAH-treated cells. C — control, Q — quercetin, E — etoposide.

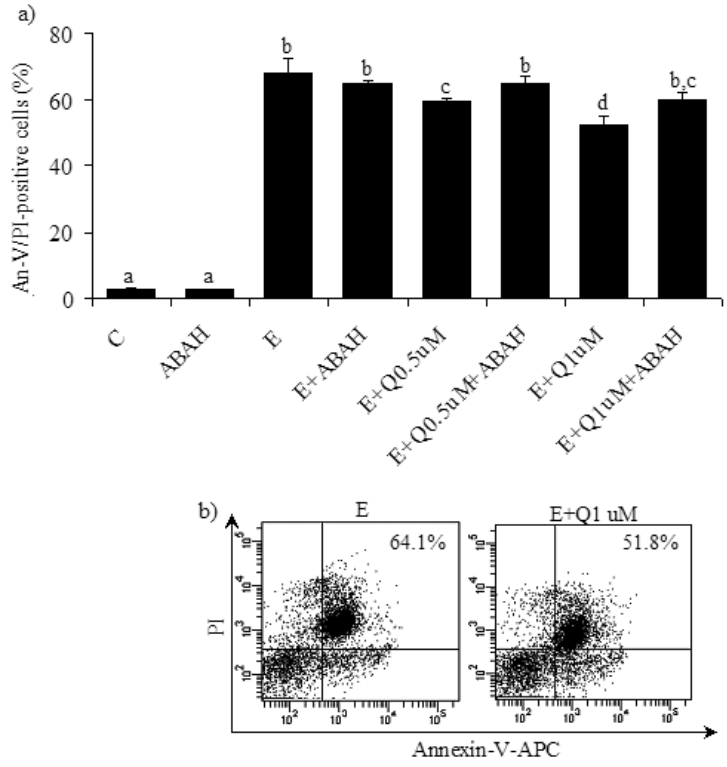

Figure 3. The influence of quercetin on the fraction of late apoptotic cells induced by etoposide in HL-60 cells with normal or decreased activity of MPO.
The cells were pretreated or not with 100 μM ABAH followed by incubations with quercetin (0.5 or 1 μM) and/or etoposide (10 μM). Apoptosis was analyzed by flow cytometry. (**a**) Data are presented as mean ±S.D. of the mean. Means having different signs (**a, b, c , d**) differ significantly, $p<0.05$. (**b**) Representative dot plots. C — control, Q — quercetin, E — etoposide.

ingestion of this flavonoid. It is worth nothing that in other studies quercetin at the same concentration of 0.5 μM induced a significant increase in the proliferation of HeLa cells (Spagnuolo *et al.*, 2012).

In comparison to the control, etoposide at a concentration of 10 μM significantly increased the percentage of late apoptotic cells (annexin V+/PI+) and the sub-G1 fraction corresponding to apoptotic cells (Fig. 3a, Table 1) but did not affect the percentages of early apoptotic or necrotic cells (data not shown). Pretreatment with ABAH decreased the level of etoposide-induced late apoptosis non-significantly. In contrast, quercetin significantly decreased the fraction of late apoptotic cells and sub-G1 fraction induced by etoposide. However, additional pretreatment with ABAH did not enhance the protective effect of quercetin against apoptosis induced by etoposide (Fig. 3a, b, Table 1).

Etoposide arrested the cell cycle in the S phase, and quercetin and ABAH failed to significantly modify the action of this chemotherapeutic agent (Table 1).

In the presence of MPO, when etoposide radicals are formed, quercetin acts as an antioxidant, reducing the level of free radicals generated by etoposide and decreasing the extent of apoptosis induced by this chemotherapeutic agent. In this manner, quercetin can contribute to reducing the cytotoxic effects of etoposide in bone marrow cells. After inhibiting the pro-oxidative effect of MPO using ABAH, quercetin did not protect cells against the cytotoxic action of etoposide. It is known from other studies that quercetin can suppress the cell cycle and induce the apoptosis of leukemic cells. Also, it can induce apoptosis by reducing AKT, BCL-2, BAX and increasing the level of caspase activation (Yuan *et al.*, 2012).

Reactive intermediates produced during the metabolism of chemotherapeutic drugs can lead directly to DSBs (Kagan *et al.*, 1999; Sallmyr *et al.*, 2008). Unrepaired DSBs can cause apoptosis or, in the case of disrupted apoptotic pathways, promote mutagenesis. Our earlier study has demonstrated that quercetin can protect myeloid cells against etoposide-induced DSBs *in vitro* and *in vivo* (Papież, 2014). In the present study, the protective an-

Table 1. Cell cycle in HL-60 cells treated with quercetin and/or etoposide.
The cells were pretreated or not with 100 μM ABAH followed by incubations with quercetin (0.5 or 1 μM) and and/or etoposide (10 μM). Data are presented as mean ±S.D. of the mean. Values marked with different letters (a, b, c) in the same column differ significantly, $p<0.05$. Q — quercetin, E — etoposide.

Treatment of cells	sub-G1	G1	S	G2/M
Control	3.3±0.6^{a}	58.0±0.5^{a}	23.8±0.2^{a}	14.7±1.1^{a}
ABAH	3.1±0.4^{a}	54.7±2.6^{a}	24.7±0.7^{a}	17.3±2.2^{a}
E	21.4±1.6^{b}	44.2±1.1^{b}	28.4±1.1^{b}	5.7±0.6^{b}
E + ABAH	19.7±1.2^{b}	45.8±3.1^{b}	28.3±2.6^{b}	6.4±1.3^{b}
E + Q 0.5 μM	18.3±0.5^{c}	45.2±1.5^{b}	30.0±0.6^{b}	6.3±1.0^{b}
E + Q 0.5 μM + ABAH	18.5±1.4^{c}	47.2±2.9^{b}	28.7±1.7^{b}	6.1±0.9^{b}
E + Q 1 μM	19.4±1.0^{b,c}	44.4±2.1^{b}	29.1±1.5^{b}	6.9±0.8^{b}
E + Q 1 μM + ABAH	19.6±1.0^{b,c}	45.6±4.7^{b}	27.9±1.9^{b}	6.7±1.9^{b}

tioxidant effect of low concentrations of quercetin correlated with its anti-apoptotic action. It can be assumed that the antioxidant quercetin should prevent apoptosis and DNA damage in myeloid precursor cells during etoposide therapy.

The present study demonstrates for the first time that quercetin can limit the pro-oxidant and apoptotic action of etoposide in human myeloid precursor cells with high levels of MPO activity and, at concentrations of 0.5 or 1 μM, it may act more strongly than an inhibitor of this enzyme. On the other hand, if quercetin can protect leukemia cells against etoposide action, it would be necessary to conduct further research in order to clarify whether the dosages of this flavonoid achievable in blood plasma will not limit the antileukemic activity of this cytostatic drug. The antagonistic effect on chemotherapeutic agents of another antioxidant, vitamin C, is known from *in vitro* studies (Samuel *et al*., 2012).

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