Quercetin and its microbial degradation product 3,4-dihydroxyphenylacetic acid generate hydrogen peroxide modulating their stability under in vitro conditions

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Summary

In contrast to the microbial degradation of quercetin (QUE), little is known about its degradation under cell culture conditions and the contribution of potential degradation products to the observed cellular effects of QUE. In a cell culture medium, the concentration of QUE was found to decrease rapidly in a time-dependent manner. The presence of catalase substantially affected the stability of QUE but not of the microbial degradation product, 3,4-dihydroxyphenylacetic acid (HPA). Within human colon carcinoma cells (HT29), formation of a QUE glucuronide, the only metabolite, was observed with a maximum at 3 h, whereas no free QUE was detected under the chosen experimental conditions. From the putative degradation products, only the microbial QUE-metabolite HPA, which was not generated under cell culture conditions, exhibited substantial growth-inhibitory properties in HT29 cells. Both QUE and HPA led to the formation of hydrogen peroxide in the cell culture medium. The presence of catalase or ascorbic acid was found to diminish the growth-inhibitory effect of HPA in cell culture, but also under these conditions, still substantial growth inhibition was observed. However, HPA and phloroglucinol lacked the effectiveness of QUE against the epidermal growth factor receptor and cAMP-hydrolysing phosphodiesterases, indicating a different pattern of cellular activities.

Keywords

microbial degradation products; quercetin; 3,4-dihydroxyphenylacetic acid; phloroglucinol; epidermal growth factor receptor; 3',5'-cAMP- phosphodiesterase; HT29 cells

Quercetin (QUE) glycosides represent the most abundant flavonoids in western-style diet [1–3]. The aglycone QUE has been associated with a multitude of biological activities in vitro including antioxidative [4-6] and antiproliferative [7, 8] effects. Several studies have shown that QUE inhibits the growth of human cancer cells [9–11], induces apoptosis in vitro [12–15] and affects cellular signalling elements involved in carcinogenesis, e.g. the epidermal growth factor receptor (EGFR) and its subsequent mitogen activated protein kinase (MAPK) cascade [11, 12, 15–17], phosphatidylinositol 3-kinase [9, 18, 19] or the Wnt-pathway [20–22].

However, QUE has also been reported to represent a substrate for microbial degradation by human intestinal bacteria [23–30]. This degradation of QUE comprises an initial reduction of the double bond in 2,3-position, generating taxifolin as an intermediate. Subsequently, 3,4-dihydroxyphenylacetic acid (HPA) and phloroglucinol (PHG) are formed, probably by oxidative decarboxylation (Fig. 1) [23–25].

Whereas the cellular effects of quercetin are subject of numerous studies, little is known so far about the biological relevance of the microbial degradation products and whether degradation products might at least contribute to the observed biological effects of QUE. Also the formation of H_2O_2 under cell culture conditions, resulting from the reaction of polyphenols with yet unknown culture media constituents, is discussed to generate

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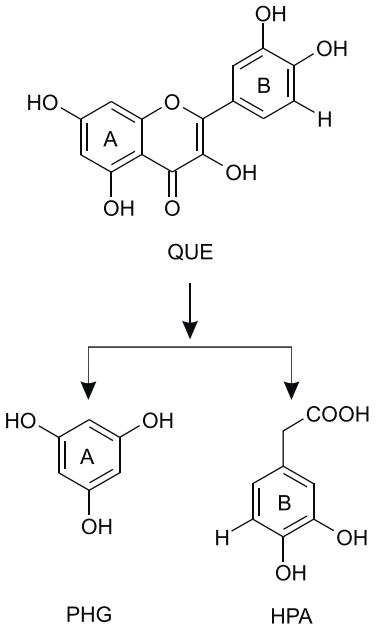

Fig. 1. Postulated microbial degradation pathway of quercetin (QUE) [23–25].

PHG – phloroglucinol, HPA – 3,4-dihydroxyphenylacetic acid.

in vitro artefacts with respect to the cellular effectiveness of the respective polyphenols [31–37]. We previously reported that QUE potently inhibits the protein tyrosine kinase activity of the EGFR and elements of the subsequent MAPK cascade like the extra-cellular regulated kinases and the transcription factor E twenty-six like kinase [11, 12]. The MAPK cascade is connected with several other signalling pathways in a complex network pattern. One important regulative factor is the deactivating phosphorylation of the serine-threonine kinase Raf-1 by protein kinase A, a key enzyme in the cAMP-pathway [38–40]. A central element in the regulation of cAMP homeostasis represents the superfamily of cAMP-hydrolysing phosphodiesterases (PDE). Several flavonoids, including QUE, have been reported to target PDE activity [41–43].

In the present study, we investigated the stability of QUE and its microbial degradation product HPA under cell culture conditions and the potential role of H_2O_2 formation for the growth inhibitory efficiency and its potential degradation products. Furthermore, we addressed the question whether degradation products contribute to the growth inhibitory properties of QUE on human colon carcinoma cells (HT29) and we studied their impact on cell signalling targets such as the EGFR and cAMP-hydrolysing PDE compared to QUE.

MATERIALS AND METHODS

Chemicals

Phloroglucinol was purchased from Extrasynthèse (Genay, France). Quercetin (QUE), 3,4-dihydroxyphenylacetic acid (HPA) and catalase were obtained from Sigma (Taufkirchen, Germany). For all assays, the compound solutions were freshly prepared prior to the start of the experiment, without the use of stored stock solutions. All compounds and mixtures were dissolved in dimethyl sulphoxide (DMSO) with a final concentration of a maximum of 1% in the respective test systems.

Cell culture

The human colon carcinoma cell line HT29 was purchased from the German Collection of Microorganism and Cell Culture in Braunschweig, Germany. HT29 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) (with 4 500 mg·l^{-1} glucose, without sodium pyruvate), supplemented with 10% fetal calf serum and 1% penicillin + streptomycin. DMEM and the supplements were obtained from Invitrogen Life Technologies (Karlsruhe, Germany).

Stability and cellular uptake in HT29 cells of quercetin and 3,4-dihydroxyphenylacetic acid

Cellular uptake

QUE and HPA were dissolved in DMSO to yield 2.5 mM stock solutions. HT29 cells were seeded onto 6-well culture plates at a density of 100 000 cells per well. In 72 h after cell plating, cell culture medium was completely removed and cells were treated with DMSO (0.1%, v/v), QUE or HPA at concentrations of 25 μM (stock solutions in DMSO, final DMSO concentration 0.1% (v/v)) as well as in the absence or presence of catalase (100 U·ml^{-1}). After different incubation periods (0, 0.5, 1, 3, 24, 72 h), supernatants of cell culture media were removed and collected for further analysis. Cells were washed three-times with phosphate buffered saline (PBS) containing 0.1% bovine serum albumin to remove QUE and HPA, respectively, sticking to the cell surface. Attached cells were lysed with 500 μl distilled water, and wells were washed with 500 μl distilled water.

Sample clean-up

To the cell culture media, 10% (v/v) HCl (10 M) was added. The samples were subjected directly to HPLC-diode array detector (DAD)-MS analysis.

The cell lysates were homogenized for 15 min in a bath sonicator after one cycle of freezing and thawing. After centrifugation, an aliquot of

the supernatant was acidified using sodium acetate buffer (0.15 M; pH 5). For extraction, methanol was added and the samples were sonicated for 15 min. The organic extracts were evaporated to dryness under a stream of nitrogen gas and reconstituted with 25% methanol for HPLC-DAD-MS analysis. Protein concentration in the cell lysates was analysed using the DC protein assay according to the method of Lowry (Bio-Rad, Munich, Germany) with BSA as standard.

HPLC analysis

Samples were analysed by HPLC-DAD using a Prontosil C18 (250 mm × 4.6 mm i. d.; particle size 3 μm) reversed-phase column (Bischoff Analysentechnik, Leonberg, Germany). The solvent system consisted of 0.1% formic acid in water as solvent A and acetonitrile as solvent B. The following linear gradient was used: from 85% to 70% A in 50 min, from 70% to 50% A in 10 min, and from 50% to 43.3% A in 20 min. The flow rate was 0.8 ml·min^{-1} and the eluent was recorded at 370 nm for QUE and 280 nm for HPA. Observed peaks were scanned between 200 nm and 600 nm. Quantification of the formed QUE-glucuronide (QUE-gluc) was done on the basis of the peak area in the HPLC chromatogram at 370 nm assuming similar ε values of the aglycone and its conjugate. The UV-vis absorption spectra showed that this was a reasonable assumption since the effects of conjugation on the UV-vis spectra were of minor importance.

For identification of the formed metabolite, samples were analysed by HPLC-MS analysis. HPLC-MS analysis was performed on a HP 1100 series instrument (Agilent Technologies, Waldbronn, Germany) equipped with an autoinjector, binary HPLC pump, column heater, UV detector and HP Chem Station (Agilent Technologies) for data collection and handling. The HPLC was interfaced to an HP series 1100 mass selective detector (Agilent Technologies) equipped with an atmospheric pressure ionization-electrospray chamber. Conditions in the negative mode were as follows: capillary voltage 3.5 kV; fragmentor voltage 90 V; nebulizing pressure 60 psi; drying gas temperature 350 °C; drying gas flow 11 l·min^{-1}. Data were collected using the scan mode. Spectra were scanned over a mass range of m/z 100–700 at 1.08 s per cycle. HPLC conditions were identical to those used for HPLC-DAD analysis.

Sulforhodamine B (SRB) assay

Effects on cell growth were determined according to the method of Skehan et al. with slight modifications [44]. Briefly, 4500 HT29 cells were seeded per well into 24-well plates and allowed to grow for 48 h before treatment. Thereafter, cells were incubated with the respective drug in the absence or presence of catalase (100 U·ml^{-1}) or ascorbic acid (250 μM) for 72 h in serum containing medium. Incubation was stopped by addition of trichloroacetic acid (50% solution). The fixed cells were stained with a 0.4% solution of sulforhodamine B. The dye was eluted with Tris-buffer (10 mM, pH 10.0) and quantified photometrically at 570 nm. Cell growth inhibition was determined as percent survival, determined by the number of treated over control cells × 100 (% T/C).

Hydrogen peroxide formation

Briefly, 40000 HT29 cells were seeded per well into 24-well plates and allowed to grow for 48 h before treatment. The incubation conditions were adjusted to the SRB-assay (see above). The formation of hydrogen peroxide was measured after several periods (15 min, 45 min, 24 h and 72 h) using the Amplex Red hydrogen peroxide assay kit (Sigma) following the manufacturer's protocol. Final hydrogen peroxide concentrations of 0, 0.5, 0.75, 1, 2 and 3 μM were used for a standard curve.

Tyrosine kinase assay

EGFR was isolated from the human vulva carcinoma cell line A431 and purified by affinity chromatography using wheat germ lectin agarose (Pharmacia Biotech, Uppsala, Sweden) according to [11]. Effects of the test compounds on the protein tyrosine kinase activity of EGFR were determined by enzyme-linked immunosorbent assay as described previously [11, 42].

Inhibition of phosphodiesterase activity

A number of 1.5 × 10^6 HT29 cells were spread in Petri dishes (d = 9 cm) and cultured for 48 h. Before harvesting, medium was removed and cells were washed with 3 ml of PBS. Harvesting and lysate preparation were performed at 4 °C. Cells were scraped in buffer A (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM EGTA, 5 mM benzamidine hydrochloride, 0.5 μM trypsin inhibitor from soya, 0.5 mM PMSF, 0.5 mM β-mercaptoethanol, 1 μM pepstatin, 1 μM leupeptin) and homogenized with 40 strokes in a Wheaton homogenizer (tight pestle). The 100000 g supernatant (50 min, 4 °C) was directly subjected to the PDE assay. PDE activity was determined according to a modified method of Poech [45] as reported previously [42].

RESULTS

Stability of test compounds in cell culture medium

To study the stability of quercetin (QUE) and 3,4-dihydroxyphenylacetic acid (HPA) under serum-containing medium, HT29 cells were incubated with 25 μM of the test compounds (final concentration of the solvent DMSO 0.1%). In the absence of catalase, the concentration of QUE in the cell culture medium decreased rapidly, with a loss of nearly 50% in the first 30 min (Fig. 2A). After 3 h of incubation, only (20 ± 4) % of the applied QUE concentration was still detectable. In the presence of catalase, the stability of QUE was significantly higher within the first 3 h of incubation. After 3 h, (66 ± 7) % of the originally applied QUE concentration was found in the cell culture medium. However, after 24 h of incubation QUE was not longer detectable irrespective of the presence or absence of catalase (Fig. 2A).

We previously reported that the degradation products of anthocyanidins, the benzoic acids, accumulate in the medium after incubation with the parental compounds under in vitro conditions [46]. However, the incubation of HT29 cells with QUE did not result in the accumulation of the respective phenolic acids, indicating that the formation of HPA and PHG only occurs under microbial degradation previously described by [23–25]. But after 1 h, a so far unknown metabolite was clearly detectable by HPLC-DAD analysis, which exhibited an absorbance maximum at λ = 370 nm and was not present directly after the addition of QUE to the cell culture medium of HT29 cells (Fig. 3). This metabolite was analysed by HPLC-MS in the negative mode and was identified as QUE glucuronide (QUE-gluc) due to the loss of the glucuronic acid moiety of 176 atomic mass units. However, it was not possible to identify the position of the glucuronidation with the single quadrupol HPLC-MS-system used in this study. In the cell culture medium, only minor amounts of QUE-gluc were

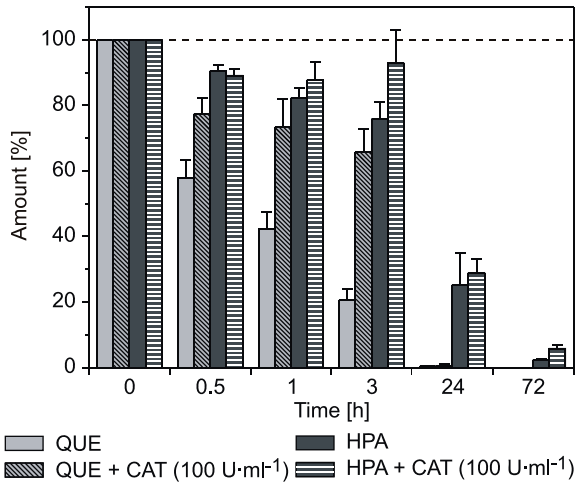

Fig. 2A. Degradation of quercetin (QUE) and 3,4-dihydroxyphenylacetic acid (HPA) in HT29 cells cultured in a medium containing 10% FCS.

Values are expressed as relative peak area of the original applied QUE concentration (in %) (mean ± SD of at least four independent experiments).

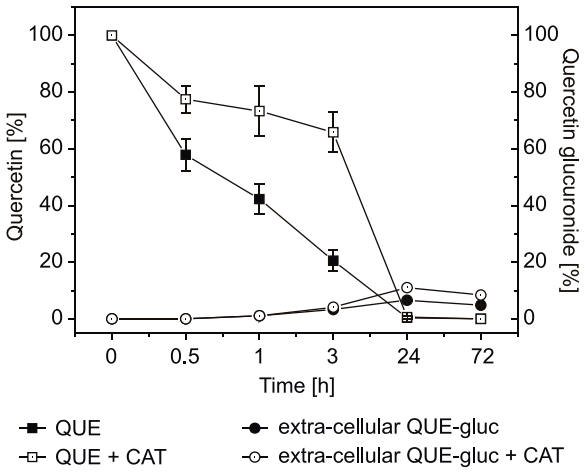

Fig. 2B. Degradation of QUE and the formation of the respective extracellular QUE-glucuronide in the presence or absence of catalase (CAT, 100 U·ml^{-1}) in the cell culture medium.

Values are expressed as relative peak area based on the original amount of QUE applied to the cell culture medium (in %) (mean ± SD of at least two independent experiments).

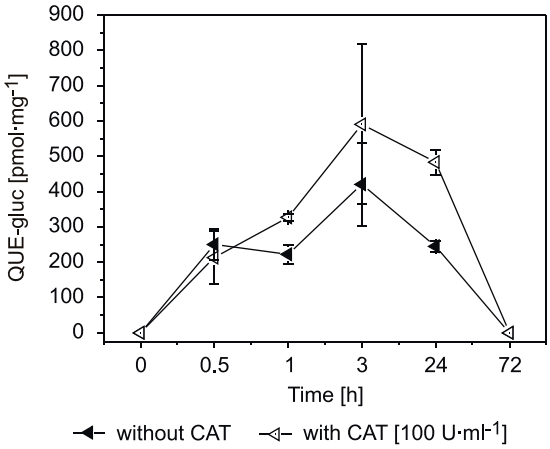

Fig. 2C. Time-dependent accumulation of QUE glucuronide by the incubation of HT29 cells with QUE.

Values are expressed as the ratio of amount taken up per mg protein of cells [pmol/mg protein] (mean ± SD of at least two independent experiments).

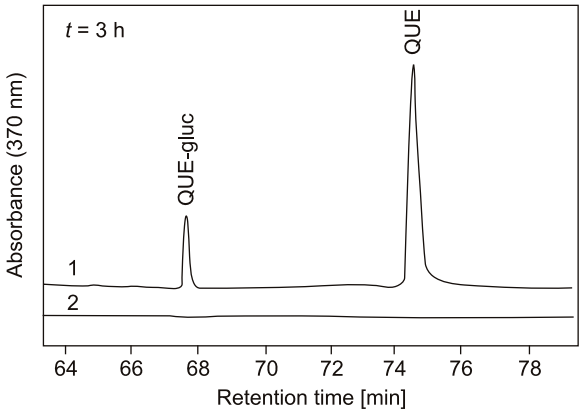

Fig. 3. Representative HPLC chromatograms of the cell culture medium after 3 h of incubation with quercetin (QUE) (1) and without the test substance (2) at λ = 370 nm.

found after 1, 3, 24 and 72 h of incubation time irrespective of the absence or presence of catalase compared to the mother substance QUE. Maximum amounts were detected after 24 h (Fig. 2B).

The amount of HPA applied to the cell culture medium of HT29 cells decreased in a time-dependent manner in accordance to the results for QUE. However, HPA was found to be more stable than QUE, showing a reduction of (25 ± 5) % within 3 h of incubation (Fig. 2A). In contrast to QUE, the presence of catalase did not significantly enhance the stability of HPA. After 24 h of incubation, the concentration of HPA was diminished to (25 ± 10) % without catalase and to (29 ± 4) % with catalase, respectively. After 72 h, only minor amounts were still detectable in the medium irrespective of the presence or absence of catalase (Fig. 2A). No metabolites were detected in the cell culture medium (data not shown).

Uptake of test compounds in HT29 cells

Besides the cell culture medium, we analysed the remaining cell lysates for the uptake of QUE and HPA in the cells and for the ability of HT29 cells to form metabolites. Whereas no QUE and its degradation products, HPA or PHG, were detected in HT29 cells exposed to QUE, the HPLC profile of the incubated cells revealed the presence of QUE-gluc. With and without catalase, the highest amounts of QUE-gluc, (590 ± 227) pmol·mg^{-1}

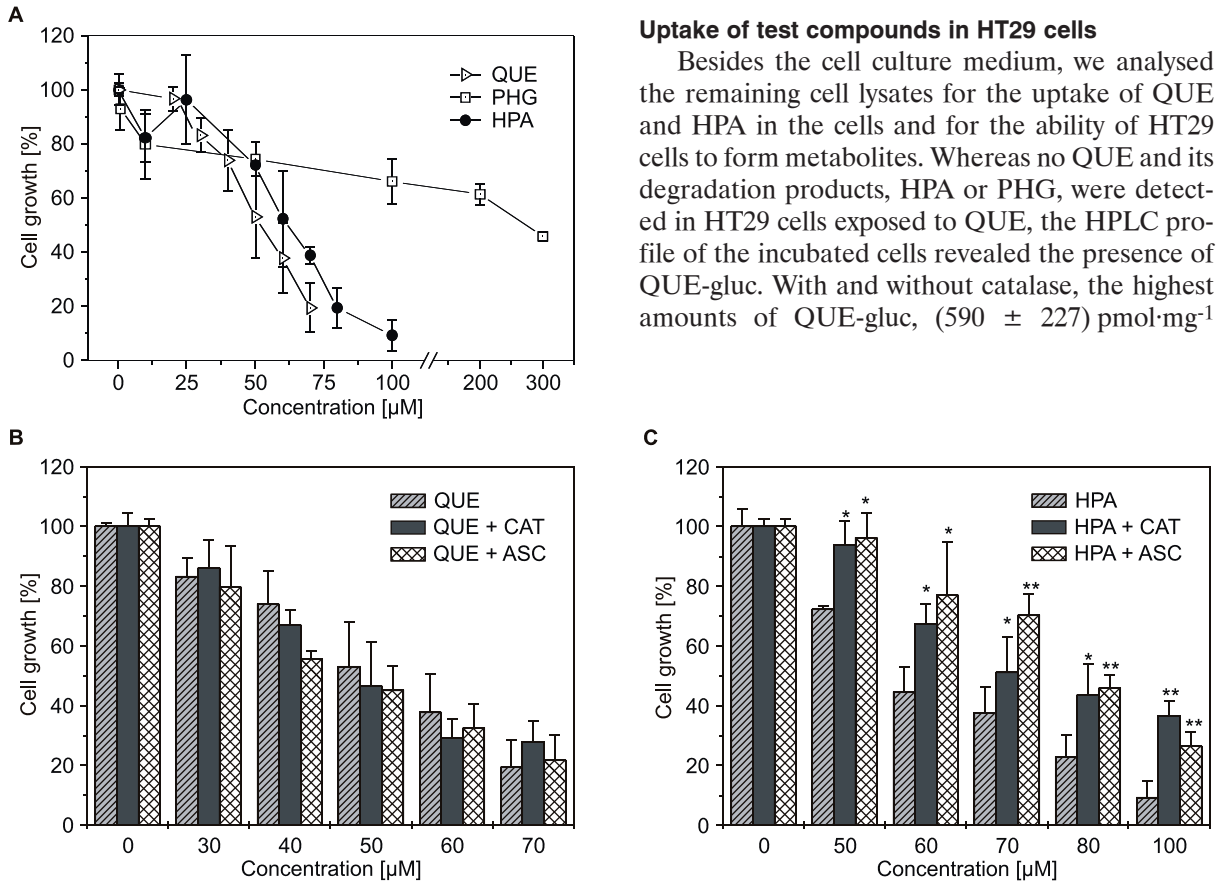

Fig. 4. Inhibition of tumour cell growth determined in the sulforhodamine B assay.

A – HT29 cells were incubated for 72 h with quercetin (QUE) in comparison to phloroglucinol (PHG) and 3,4-dihydroxyphenylacetic acid (HPA).
B – Impact of catalase (CAT, 100 U·ml^{-1}) or ascorbic acid (ASC, 250 μM) on the growth-inhibitory properties of quercetin QUE.
C – Impact of catalase (CAT, 100 U·ml^{-1}) or ascorbic acid (ASC, 250 μM) on the growth-inhibitory properties of 3,4-dihydroxyphenylacetic acid (HPA).
Growth inhibition was calculated as percent survival of treated cells compared to control cells (treated with the solvent 1% DMSO) × 100 [T/C %]. The values given are the mean ± SD of at least three independent experiments, each performed in quadruplicate. Significances indicated refer the significance level compared to the respective concentration of HPA without CAT or ASC (Student's t-test, * = $p < 0.05$; ** = $p < 0.01$).

protein and (420 ± 116) pmol·mg^{-1} protein respectively, were found after 3 h of incubation, followed by a decrease after 24 h. QUE-gluc was no longer detectable after 72 h (Fig. 2C). The incubation of HT29 cells with the microbial degradation product HPA did neither result in an uptake of HPA nor in a metabolite formation up to 72 h (data not shown).

Inhibition of tumour cell growth in vitro

Phloroglucinol (PHG), resulting from the microbial degradation of QUE, only marginally affected tumour cell growth with a half-maximal (50%) inhibitory concentration (IC_{50}) of (273 ± 33) μM, whereas HPA showed a substantial growth-inhibitory effect (IC_{50} value of 61 ± 7 μM) within the range of growth inhibition achieved by incubation with QUE (Fig. 4A). The presence of catalase or ascorbic acid did not diminish the growth inhibitory effect of QUE on HT29 cells up to 70 μM (Fig. 4B). In contrast, growth inhibition mediated by HPA was significantly decreased in the presence of both catalase or ascorbic acid (Fig. 4C).

Hydrogen peroxide formation

HT29 cells were incubated with QUE or HPA for 72 h. The hydrogen peroxide concentration in the cell culture medium was measured after 15 min, 45 min, 24 h and 72 h. After 15 min incubation with QUE, a concentration-dependent formation of hydrogen peroxide in the low micromolar range was observed (Fig. 5A), which was slightly enhanced after 45 min (Fig. 5B). However, after 24 h or 72 h, the hydrogen peroxide concentration in the medium of HT29 cells, incubated with QUE, was equal to the solvent control (data not shown). Coincubation with catalase or sodium ascorbate reduced the hydrogen peroxide concentration in the cell culture medium to the level of

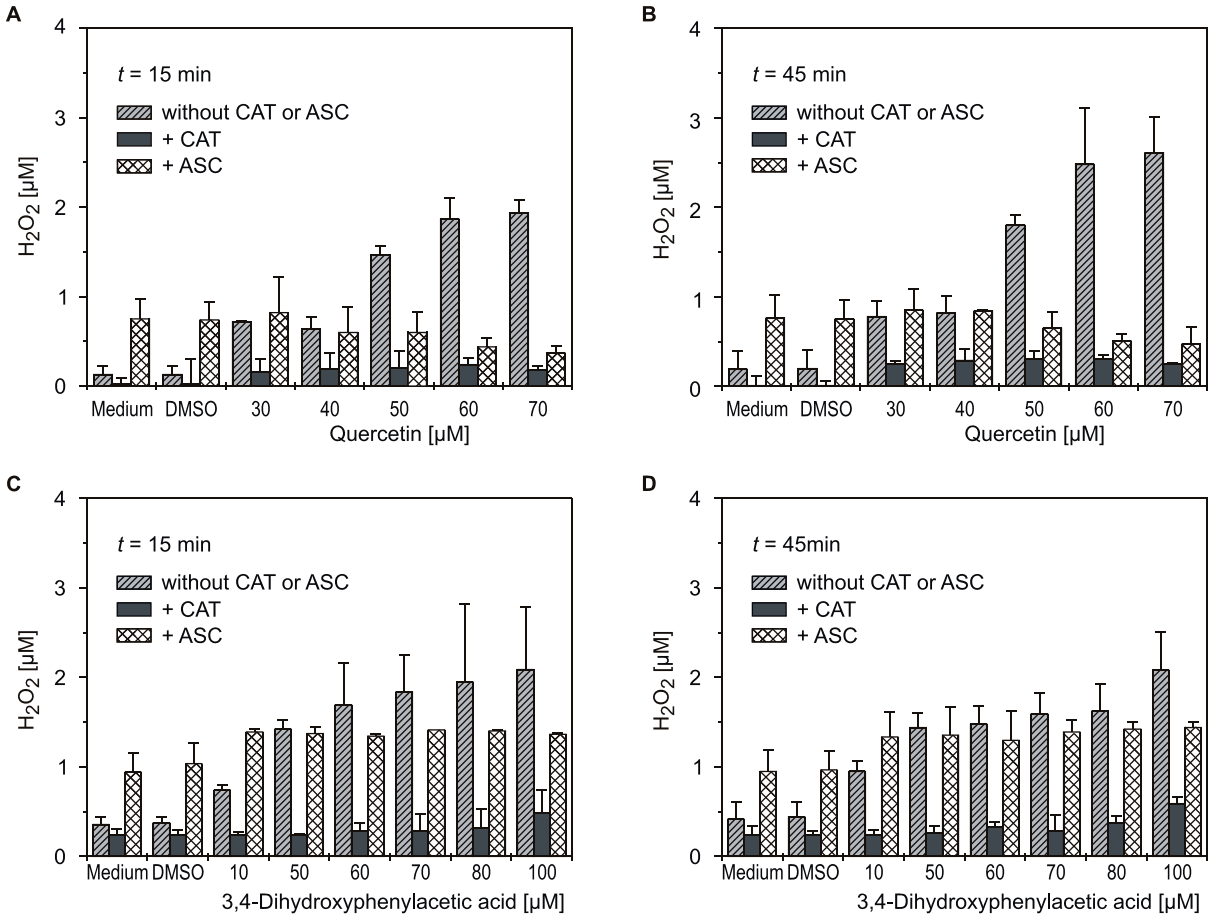

Fig. 5. Hydrogen peroxide formation in cell culture medium under serum-containing conditions in the presence and absence of catalase (CAT, 100 U·ml^{-1}) or sodium ascorbate (ASC, 250 μM).

A – incubation of HT29 cells with quercetin (QUE) for 15 min, **B** – incubation of HT29 cells with QUE for 45 min, **C** – incubation of HT29 cells with 3,4-dihydroxyphenylacetic acid (HPA) for 15 min, D – incubation of HT29 cells with HPA for 45 min.
The data presented are the mean ± SD of at least two independent experiments, each performed in duplicate.

the solvent control (Fig. 5A + B). Coincubation with sodium ascorbate showed an overall increased level of hydrogen peroxide compared to the effects of catalase (Fig. 5A + B).

HPA generated equal concentrations of hydrogen peroxide to the flavonol QUE after 15 min of incubation (Fig. 5C). In contrast to QUE, the amount of hydrogen peroxide in the medium was not enhanced after 45 min of incubation with HPA (Fig. 5D). The hydrogen peroxide formation was almost completely suppressed by coincubation with catalase. With sodium ascorbate, increased hydrogen peroxide concentrations were determined, but in the same concentration range as the respective solvent control (Fig. 5C + D). In HT29 cells incubated with HPA for 24 or 72 h, the observed hydrogen peroxide concentrations were comparable to the solvent control, independent of the presence of catalase, similar to the results with QUE (data not shown).

Interference with potential cellular targets

Furthermore, we investigated the potential contribution of microbial degradation products of QUE to the cellular effects of the parent compound. QUE has been reported to act on multiple cellular targets. As examples, the effect on the PTK activity of the EGFR and the inhibitory effect on PDE-dependent cAMP-hydrolysis were investigated. PHG and HPA exhibited only weak inhibitory effects on the PTK activity of the EGFR, several orders of magnitude weaker than the respective flavonol QUE (Fig. 6). Furthermore, QUE was found to effectively inhibit the cAMP-hydrolysing PDE-activity from cytosolic preparations of HT29 cells (Fig. 7). In contrast, the respective degradation products PHG and HPA were inactive up to 300 μM (Fig. 7).

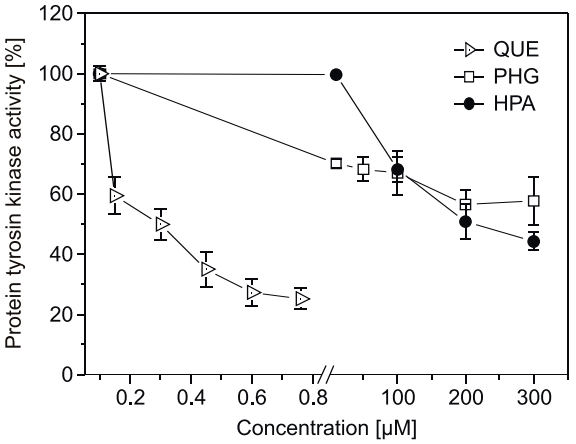

Fig. 6. Inhibition of the tyrosine kinase activity of the EGF-receptor by quercetin (QUE) [11], 3,4-dihydroxyphenylacetic acid (HPA) and phloroglucinol (PHG).

The phosphorylation of tyrosine residues of a poly (Glu-Tyr) peptide was determined by ELISA. The data presented are the mean ± SD of three independent experiments, each performed in triplicate.

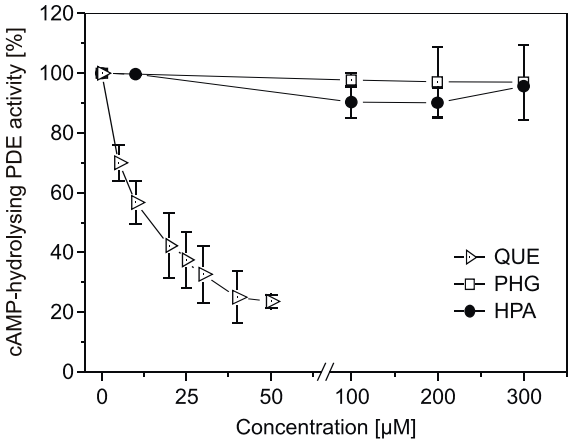

Fig. 7. Inhibition of cAMP-hydrolysing phosphodiesterase (PDE) activity by quercetin (QUE) in comparison to the microbial degradation products 3,4-dihydroxyphenylacetic acid (HPA) and phloroglucinol (PHG).

The data presented are the mean ± SD of four independent experiments, each performed in triplicate.

DISCUSSION

Whereas the microbial degradation of QUE (Fig. 1) has been intensively studied [23–30], little is known about the stability of QUE and its microbial degradation product HPA under cell culture conditions. In accordance with earlier reports [4], substantial decrease was observed for QUE in the cell culture medium, whereas HPA was found to be more stable under these conditions (Fig. 2A).

Under cell culture conditions, the presence of catalase substantially affected the stability of QUE (Fig. 2A). These results are in accordance with previous studies showing that the presence of ascorbate enhances the stability of QUE under in vitro conditions, arguing for a contribution of hydrogen peroxide to the degradation of QUE [47, 48]. In contrast to the microbial degradation of QUE, the formation of HPA and/or PHG was not observed under the chosen in vitro conditions. However, already after 1 h of incubation, HPLC-DAD analysis revealed an additional peak at 370 nm in cell culture medium (Fig. 3) with the characteristic mass spectrum of a QUE-glucuronide (QUE-gluc), as determined by HPLC-MS. In HT29 cells, the formation of QUE-3-gluc (29%), QUE-4'-gluc (40%) and, to a minor extent, QUE-3'-gluc (7%)

has been reported previously [49]. QUE-3-gluc is also a major metabolite in human plasma [50]. However, in this study it was not possible to identify the position of the glucuronidation with the single quadrupol HPLC-MS-system used. We were able to demonstrate that within the first 3 h of incubation the amount of QUE-gluc in the cell culture medium was in line with the extent of the loss of QUE (Fig. 2B).

Furthermore, formation of QUE-gluc within the cells was observed earlier compared to the extracellular compartment. De Boer et al. [48] reported that QUE-gluc was immediately transported out of the cell, which can explain the delayed appearance of QUE-gluc in the cell culture medium. In the presence of catalase, QUE-gluc was observed up to 24 h in HT29 cells, whereas QUE was no longer detectable in the medium (Fig. 2B and 2C). These results implicate that catalase affects not only the stability of QUE but also of QUE-glucuronides. HT29 cells are known as a cell line with substantial uridine-5'-diphosphoglucuronosyl-transferase activity [51]. Furthermore, previous studies have demonstrated that QUE increased the activity of various uridine-5'-diphosphoglucuronosyl-transferase isoforms in colon cells in vitro [52] and in vivo [53]. Thus it is tempting to speculate that the glucuronides might contribute to the observed multiple biological effects of QUE. Yang et al. characterized QUE-gluc as inhibitors of the growth of human lung cancer cell via cell cycle arrest and the induction of apoptosis [54], whereas the physiological relevant metabolites QUE-3'-sulfate and QUE-3-gluc did not influence the proliferation of human vascular smooth muscle cells [55].

However, so far little is known about cellular effects of QUE degradation products. In the present study, we showed that one of the microbial degradation products of QUE, HPA (Fig. 1) [23–30], substantially affected the growth of human colon carcinoma cells (Fig. 4A), whereas PHG arising from the A-ring, only marginally inhibited the growth of HT29 cells. HPA was found to possess effective growth-inhibitory properties, comparable in its potency to the parent flavonol, at least in the absence of catalase (Fig. 4A). HPA was previously described to exhibit growth-inhibitory properties in the human colon carcinoma cell line HCT116, whereas the growth of the colon cell line IEC6 was not significantly affected by HPA up to 400 μM [56]. In the present study, the calculated sum of the growth-inhibitory effects of PHG and HPA corresponds to the extent of growth inhibition mediated by incubation with QUE alone. These results suggest that PHG and HPA might be responsible for, or at least contribute to, the growth-inhibitory properties of QUE under microbial culture conditions. In line with our results, a limited stability (half-life time 2 h) of QUE in aqueous McCoy's 5A medium was described by van der Woude et al. [37]. Furthermore, the authors found an enhanced stability up to 24 h at the addition of 1 mM ascorbic acid. On the contrary, van der Woude et al. [37] reported that QUE acts in a biphasic effect on the proliferation of HT29 cells in the presence of ascorbic acid. In lower concentrations ($\leq$ 40 μM), a significant increase in cell proliferation was observed, whereas in concentrations $\geq$ 80 μM, QUE had antiproliferative effects. In our study, no stimulating effect of QUE on HT29 cell proliferation in the presence or absence of catalase or ascorbic was observed (Fig. 4B), which might be explained by the differences in the chosen cell culture medium, test system or incubation duration.

Furthermore, in the present study we found that in the presence of catalase or ascorbic acid, the growth-inhibitory effect of HPA was substantially diminished, whereas QUE remained at least equipotent (Fig. 4B, 4C). These results indicate that artefacts by hydrogen peroxide formation, previously described for several flavonoids [31–37, 46], might be involved in the growth-inhibitory effect of HPA but not of QUE in the respective concentration range.

Nevertheless, also in the presence of catalase or ascorbic acid, HPA was found to represent a potential degradation product of QUE with substantial growth-inhibitory properties. However, under conditions suppressing hydrogen peroxide formation, QUE was clearly more effective than HPA. For clarifying the impact of hydrogen peroxide formation to the growth-inhibitory properties of QUE and HPA, we investigated hydrogen peroxide concentrations in cell culture medium after several incubation periods with the respective compounds (Fig. 5). In line with reported data [31, 37] we determined hydrogen peroxide concentration of about 2 μM already after 15 min of incubation with the highest tested concentration of QUE (70 μM), which slightly increased up to 3 μM after 45 min. Incubation with HPA led to a concentration-dependent hydrogen peroxide formation with a maximum of 2 μM after 45 min. In the presence of catalase or sodium ascorbate, the accumulation of hydrogen peroxide was effectively suppressed to the level of the solvent control (Fig. 5). In comparison to other flavonoids or phenolic acids, e. g. delphinidin [46] or gallic acid [37, 46], QUE and HPA generate rather low concentrations of hydrogen peroxide in the medium of HT29 cells. How-

ever, as observed in the sulforhodamine B assay, 2 μM of hydrogen peroxide seems to contribute to the growth-inhibitory properties of HPA. The extent of hydrogen peroxide formation corresponds to the levels previously reported for polyphenol rich apple extracts, leading to changes in the cellular effectiveness [57].

With respect to the in vivo situation, it is noteworthy that not only the mother substance QUE has growth-inhibitory properties but that also the microbial degradation of QUE leads to the formation of compounds with these activities, namely HPA and, to a minor extent, PHG. It should be noted that plasma serum levels in humans after ingestion of QUE-rich foods are 30- to 100-times lower than the concentrations with growth-inhibitory properties in vitro [58–60]. However, high local gastrointestinal QUE concentrations after the consumption of QUE-rich food or food supplements cannot be excluded [37, 61]. Considering the intestinal uptake, the growth-inhibitory properties of QUE and its microbial degradation products in human colon carcinoma cells might contribute to the reported potential health effects of QUE [2, 8, 62].

However, irrespective of the maintenance of growth-inhibitory properties, a change in the cellular activity profile appears to be implicated, as exemplified for the inhibition of the EGFR and the cAMP-degrading PDE activity. Whereas QUE effectively inhibits both enzymes, HPA was found to only marginally affect the PTK activity of EGFR (Fig. 6) and to be even inactive versus cAMP-hydrolysing PDE (Fig. 7). So far, the mechanism of action of HPA is not known and its further study is required. EGFR and its subsequent signalling cascade are known to play a role in the regulation of cell proliferation. Several flavonoids of different classes have been shown to inhibit the protein tyrosine kinase activity of EGFR [11, 12, 15–17, 19]. We previously reported that the flavonol QUE represents a potent inhibitor of this receptor tyrosine kinase [11, 12]. Vicinal hydroxy groups at the B-ring were identified as crucial structural features for effective EGFR inhibition [11, 38]. In contrast to the native flavonoid QUE, the reported microbial degradation product HPA was a weak inhibitor and PHG was even inactive. These data show that, in addition to the substituted phenyl ring, the respective flavonoid structure plays a crucial role for effective inhibition of EGFR activity. These results are in line with previous reports on the loss of EGFR-inhibitory properties by the formation of gallic acid as a degradation product of delphinidin, an effective EGFR inhibitor [46].

CONCLUSION

Our results indicate that the degradation of QUE is of relevance under microbial culture conditions, whereas the glucuronidation of QUE seems to be the prior metabolism under the applied in vitro conditions. However, particularly with regard to the in vivo situation, the results show that QUE itself and not only microbial degradation products induce growth inhibition in vitro. The microbial degradation products HPA and PHG are, if at all, weak inhibitors of EGFR and are inactive towards cAMP-hydrolysing PDE. Thus, inhibitory effects on these potential target enzymes, measured by application of the parent flavonoid, can be attributed to QUE and are not merely the effect of degradation products. Coincubation experiments with catalase or ascorbic acid indicate that the formation of hydrogen peroxide is of relevance for cellular responses in cell culture experiments. Under in vitro conditions, it has to be considered that the aglycone QUE but not its microbial degradation product HPA, is a substrate for phase II enzymes, leading to the formation of QUE-gluc, which is secreted into the cell culture medium. Furthermore, we showed that HPA has substantial growth-inhibitory properties, which might be of relevance for biological responses after QUE consumption in vivo. However, HPA appears to differ from the parent flavonoid with respect to its impact on signalling cascades crucial for cell proliferation, as exemplified for EGFR and PDE4. The mechanism of action responsible for the inhibition of tumour cell growth by HPA is not known so far and demands further investigation.

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