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ORIGINAL PAPER

3′-*O*-(3-Chloropivaloyl)quercetin, α-glucosidase inhibitor with multi-targeted therapeutic potential in relation to diabetic complications

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The novel derivative of quercetin 3′-*O*-(3-chloropivaloyl)quercetin (CPQ) inhibited α-glucosidase in a non-competitive manner with an efficacy exceeding that of the parent quercetin. In addition, it inhibited aldose reductase isolated from rat lenses with an IC_{50} in the low micromolar range and attenuated sorbitol accumulation in isolated rat eye lenses with an activity comparable with that of quercetin. Moreover, it scavenged stable free-radicals of DPPH more efficiently than did quercetin. By inhibiting α-glucosidase and affecting both the polyol pathway and oxidative stress, CPQ represents a promising agent for the multi-targeted pharmacology of diabetic complications.

Keywords: 3′-*O*-(3-chloropivaloyl)quercetin, α-glucosidase inhibitor, aldose reductase inhibitor, antioxidant, diabetic complications

Introduction

In type 2 diabetes, the inhibition of α-glucosidase reduces the rate of glucose absorption via delayed carbohydrate digestion and extended digestion time, resulting in a decrease in postprandial blood glucose levels. Accordingly, α-glucosidase represents a significant therapeutic target in diabetes and other carbohydrate-mediated diseases. Much attention has been devoted to the design and development of pharmacologically applicable α-glucosidase inhibitors (Park et al., 2008; Hakamata et al., 2009). Among them, flavonoids have been widely studied as α-glucosidase inhibitors (Cao & Chen, 2012; Xiao et al., 2013). In addition to inhibiting α-glucosidase, flavonoids have been extensively reported as affecting other multiple key molecular mechanisms involved in the development of diabetic complications, including oxidative stress, non-enzymatic glycation, and polyol pathway (reviewed in: Nijveldt et al., 2001; Bors & Michel, 2002; Matsuda et al., 2002, 2003; Williams et al., 2004; Amic et al., 2007; Boots et al., 2008; Kelsey et al., 2010; Majumdar & Srirangam, 2010; Stefek, 2011; Singh et al., 2013; Chen et al., 2015).

Recently, 21 novel semi-synthetic derivatives of quercetin have been prepared and their inhibition of aldose reductase, the first enzyme of the polyol pathway, and their antioxidant action were studied (Veverka et al., 2013; Milackova et al., 2013). Among the compounds studied, 2-chloro-1,4-naphthoquinone derivative (CHNQ), 3-chloropivaloyl derivative (CPQ) and monoacetylferuloyl derivative (MAFQ), shown in Fig. 1, exhibited the highest biological activities. CHNQ was reported as an efficient aldose reductase inhibitor and anti-inflammatory agent (Milackova et al., 2015). The anti-inflammatory action of CPQ was reported in cellular models of immortalised mouse microglial cell lines BV-2 (Mrvová et al., 2015)

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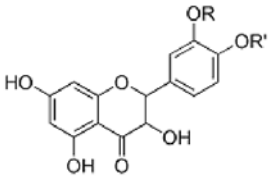

Compound	R	R'
QC	H	H
CPQ	3-chloropivaloyl	H
CHNQ	H	2-chloro-1,4-naphtoquinone-3-yl
MAFQ	H	4-*O*-acetyl-feruloyl

Fig. 1. Structures of compounds under study.

and rat primary microglia (Kuniaková et al., 2015). A protective effect of CPQ against injury to the sarco/endoplasmic reticulum calcium-ATPase by peroxynitrite was recorded (Žižková et al., 2014).

In the present study, the compounds were screened for the inhibition of α-glucosidase. For CPQ, the most efficient α-glucosidase inhibitor, the enzyme kinetics was studied. A marked inhibition of aldose reductase and free-radical scavenging activity were recorded for CPQ, which is thus reported as a promising multitarget agent of therapeutic potential.

Experimental

CPQ, CHNQ, MAFQ (purity > 99.5 mass %) were synthesised, purified and verified by BEL/NOVAMANN (Bratislava, Slovakia) as described previously (Veverka et al., 2013). Quercetin, α-glucosidase from *Saccharomyces cerevisiae*, *p*-nitrophenyl-α-D-glucopyranoside (PNPG), 1,1′-diphenyl-2-picrylhydrazyl (DPPH), trolox, acarbose, diaphorase, resazurin, M-199 medium (M 3769), D,L-glyceraldehyde, NADPH, D-glucose and epalrestat were obtained from Sigma–Aldrich (St. Louis, MO, USA). Other chemicals were purchased from local commercial sources and were of analytical grade quality.

Male Wistar rats, 8–9 weeks old, weighing 200–250 g, were used as organ donors. The animals came from the Breeding Facility of the Institute of Experimental Pharmacology and Toxicology, Dobrá Voda (Slovak Republic). The study was approved by the Ethics Committee of the Institute and performed in accordance with the Principles of Laboratory Animal Care (NIH publication 83–25, revised 1985) and the Slovak law regulating animal experiments (Decree 289, Part 139, July 9th 2003).

α-Glucosidase inhibition assay

The α-glucosidase inhibition activity was determined by partial modification of the procedure reported by Kwon et al. (2006): 50 μL of 0.067 M phosphate buffer (pH 6.9), 50 μL of the sample solution or vehicle and 50 μL of the enzyme solution (1 U mL^{-1} of 0.067 M phosphate buffer pH 6.9) were pre-incubated in 96 well plates at 25 ℃ for 10 minutes. The reaction was initiated by the addition of 50 μL of substrate PNPG (5 mM solution in 0.067 M phosphate buffer; pH 6.9) to each well at timed intervals. The reaction was monitored at 25 ℃ for 5 min. The absorbance of the resulting *p*-nitrophenol was determined at 405 nm using a spectrofluorimeter Infinite M200 TECAN analyser. The control reaction contained only the vehicle (dimethylsulphoxide (DMSO) or water for acarbose), enzyme and substrate. The mixtures without enzyme and sample served as blanks. IC_{50} values (concentration of the inhibitor required to produce 50 % inhibition of the enzyme reaction) were determined both by the least-squares analysis of the linear portion of the semilogarithmic inhibition curves and by non-linear regression analysis. Each curve was generated using at least four concentrations of the inhibitor, causing an inhibition in the range from 25–75 %.

The most efficient inhibitor CPQ was tested in experiments to determine the type of inhibition exerted. The reaction mixture was as described above, except that the concentration of the substrate varied from 0.1 mM to 1 mM and the concentration of the CPQ was kept constant at 7.5 μM, 15 μM and 30 μM. The results were used to construct Lineweaver–Burk plots and for the determination of K_m and K_i values by using the Graf Pad Prism 5 program.

Aldose reductase inhibition assay

ALR2 from rat lenses was partially purified using a procedure adapted from Hayman and Kinoshita (1965) as described previously (Veverka et al., 2013).

ALR2 activities were assayed spectrophotometrically as described previously (Veverka et al., 2013) by determining NADPH consumption at 340 nm and were expressed as a decrease in the optical density (O.D.) per second per mg of the protein.

Eye lens sorbitol assay

The freshly dissected eye lenses (1 lens per tube) were incubated in M-199 medium at pH 7.4, bubbled at 37 ℃ with pneumoxide (5 vol. % CO_2, 95 vol. % O_2) in the presence of the compound studied dissolved in DMSO. The final concentration of DMSO in all incubations was 1 %. After 30 min of pre-incubation, the reaction was initiated by adding glucose to the final concentration of 50 mM and incubation then continued at 37 ℃. The incubations proceeded for 3 hours and were terminated by cooling the mixtures in an ice bath, followed by triple washing of the lenses with ice-cold phosphate buffered saline (1 mL). The washed lenses were kept deep-frozen for the sorbitol assay, which was performed according to Mylari et al. (2003) as previously described by Stefek et al. (2011).

DPPH test

To examine the anti-radical activity of the compounds studied, the ethanolic solution of DPPH (50 μM) was incubated in the presence of the compounds tested (50 μM) at ambient temperature, as described previously (Stefek et al., 2008). The absorbance drop, monitored at $\lambda_{max} = 518$ nm, during the first 75-s interval was taken as a measure of the anti-radical activity. During the 75-s interval used, an approximately linear decrease in DPPH absorbance was observed, which was considered to be a good estimate of the initial rate of the radical reaction. The radical studies were performed at ambient temperature.

Results and discussion

The synthetic pathways for CPQ, CHNQ and MAFQ were previously reported by the present study group (Veverka et al., 2013). CHNQ and MAFQ were synthesised by the direct acylation of quercetin with 3-chloro-2,2-dimethylpropanoyl chloride and 4-*O*-acetylferulic acid chloride, respectively, in the mixture of *N*,*N*-diisopropylethylamine and toluene. The final products were isolated by silica gel chromatography and crystallised from toluene–methanol (3 : 1, vol.).

CPQ was synthesised in a two-step procedure: 3,7-bis-benzyloxy-2-(4′-benzyloxy-3′-hydroxyfenyl)-5-hydroxy-chromen-4-one was treated with chloropivaloyl chloride in dry acetone under a nitrogen atmosphere. The intermediate product, 3,7-bis-benzyloxy-2-(4-benzyloxy-3-(chlorpivaloyloxy)fenyl)-5-hydroxy-chromen-4-one (A), was purified by silica gel chromatography and crystallised from ethylacetate–hexane (2 : 3, vol.). In the second step, the final product CPQ was obtained by catalytic hydrogenation of the compound A in methanol catalysed by 10 % Pd/carbon under a hydrogen atmosphere (415 kPa) at 25 °C. The reaction mixture was filtered through celite and the product was isolated by silica gel chromatography using methylene chloride–methanol (18 : 1, vol.).

α-Glucosidase inhibition

α-Glucosidase inhibitors decrease the rate of glucose absorption, hence have been suggested as means of adjunct therapy, in addition to insulin and/or antihyperglycemic drug treatment, to control blood glucose in diabetic patients. Innovative strategies in the treatment of disorders of multifactorial origin, including diabetic complications, are focused on the rational design of chemical entities able to simultaneously affect multiple key mechanisms. This approach increases the possibility of successful therapeutic intervention, decreases the risk of side effects and is economical.

Table 1. Inhibition of α-glucosidase

Compound	IC_{50}/μM
CPQ	14.86 ± 4.35
CHNQ	49.28 ± 4.86
MAFQ	74.83 ± 12.79
QC	39.78 ± 2.46
Acarbose	303.30 ± 5.22

Experimental results are mean values ± SD from at least three experiments.

In the present study, the ability of three novel derivatives of quercetin to inhibit α-glucosidase activity, using quercetin and acarbose as references, was evaluated. The IC_{50} values shown in Table 1 point to CPQ, the 3′-substituted derivative of quercetin, as being the most efficient inhibitor of α-glucosidase, exceeding the inhibition activity of unsubstituted quercetin. Structural modifications of quercetin at position 4′ resulted in the less active derivatives CHNQ and MAFQ.

In the next step, the enzyme kinetics for CPQ, the most efficient inhibitor of α-glucosidase, was analysed. Non-competitive inhibition was observed in relation to the substrate 4-nitro-phenyl-α-D-glucopyranoside with an inhibition constant of $K_i = (15.6 \pm 3.5)$ μM (Fig. 2).

The non-competitive type of α-glucosidase inhibition indicates that carbohydrate substrates of meal origin would not compete with the inhibitor for the enzyme.

Aldose reductase inhibition

In diabetic patients, some of the excessive glucose is metabolised in the polyol pathway. Aldose reductase, the first enzyme of this pathway, reduces glucose to the organic osmolyte sorbitol in an NADPH-dependent manner. Due to its poor membrane penetration, sorbitol accumulates in the cells, resulting in disruption of the osmotic homeostasis of the cells. Depletion of NADPH due to aldose reductase activity reduces intracellular GSH, an endogenous antioxidant, thereby inducing oxidative stress. In this way, the polyol pathway in cells is thought to contribute to the aetiology of chronic diabetic complications (Yabe-Nishimura, 1998; Srivastava et al., 2005). Aldose reductase thus became a therapeutic target to be inhibited and the search for inhibitors of the enzyme has become an important pharmacological goal (Miyamoto, 2002; Alexiou et al., 2009; Chatzopoulou et al., 2012).

Aldose reductase, apart from its involvement in diabetic complications via reducing glucose, was found to efficiently reduce lipid peroxidation-derived aldehydes and their glutathione conjugates. Lipid peroxidation-derived lipid aldehydes, such as 4-hydr-

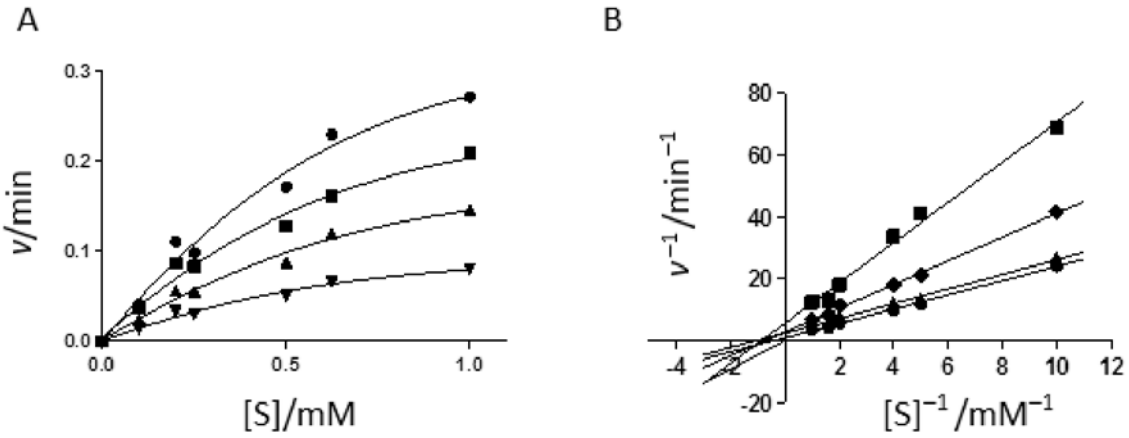

Fig. 2. Inhibitory effect of CPQ on α-glucosidase. Michaelis–Menten plot (A); Lineweaver–Burk transformation (B). Initial enzyme velocity versus concentration of substrate 4-nitrophenyl-α-D-glucopyranoside in the absence (●) or presence of inhibitor: 7.5 μM (▲),15 μM (◆), 30 μM (■). Non-competitive inhibition, K_i = (15.6 ± 3.5) μM.

Table 2. Inhibition of rat lens aldose reductase

Compound	IC_{50}/μM
CPQ	14.09 ± 0.02
QC	13.60 ± 3.60
Epalrestat	0.25 ± 0.05

Experimental results are mean values ± SD from at least three experiments.

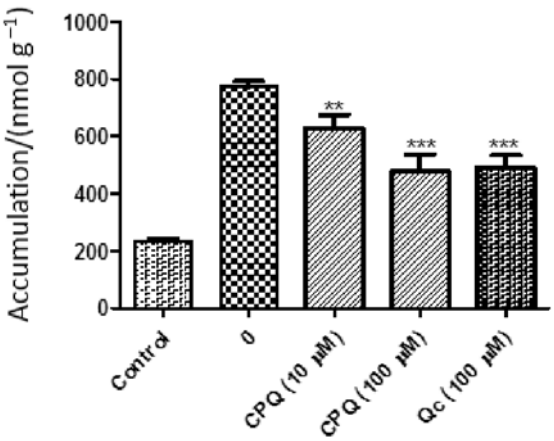

Fig. 3. Effect of CPQ on sorbitol accumulation in isolated rat lenses cultivated with high glucose in comparison with quercetin. Glucose, 50 mM; time of incubation, 3 h; 37 °C. Control samples were incubated in the absence of glucose. Results are mean values ± SEM from $n \geq 3$ independent incubations. $^{**}p < 0.01$ vs. 0, $^{***}p < 0.001$ vs. 0, parametric Student's t-test for independent samples.

oxy-*trans*-2-nonenal (HNE), and their glutathione conjugates (e.g. GS-HNE) were found to be efficiently reduced by aldose reductase to the corresponding alcohols DHN (1,4-dihydroxy-nonene) and GS-DHN (glutathionyl-1,4-dihydroxynonene), which mediate inflammatory signals (Chatzopoulou et al., 2013).

In a previous study (Veverka et al., 2013), screening of the novel derivatives of quercetin revealed the aldose reductase inhibitory activity of CPQ. In the present study, the interaction of CPQ with aldose reductase was studied in greater detail. The CPQ inhibition of the in vitro reduction of glyceraldehyde by rat lens aldose reductase in comparison with quercetin and standard epalrestat was determined and characterised by the IC_{50} value shown in Table 2. 3′-*O*-(3-chloropivaloyl) substitution of quercetin did not significantly affect the IC_{50}.

Next, the inhibitory effect of CPQ was determined at the organ level in isolated rat eye lenses. As shown in Fig. 3, increased sorbitol levels were recorded in the isolated rat eye lenses incubated with glucose, in comparison with control incubations without glucose, reflecting the increased flux of glucose through the cytosolic lens aldose reductase. Sorbitol accumulation was significantly, and in a concentration-dependent way, inhibited by CPQ starting at a concentration as low as 10 μM. This finding indicates the ready uptake of the compound by eye lens tissue, followed by inhibition of the cytosolic aldose reductase.

Free-radical scavenging activity

As a weak hydrogen atom abstractor, DPPH is considered to be a good kinetic model for peroxyl ROO$^{\bullet}$ radicals (Blois, 1958; Ratty et al., 1988). The DPPH assay is routinely used as a primary screening test of anti-radical efficacy. Fig. 4 shows the time-dependent decrease in the characteristic absorbance of the ethanolic solution of DPPH at 518 nm in the presence of CPQ and QC. The initial fast absorbance decrease, corresponding to the transfer of the most labile H atoms, is followed by a slow absorbance de-

Table 3. Anti-radical activities of CPQ and QC in DPPH test in comparison with standard trolox

Compound	Absorbance decrease	
	$-\Delta A$ at 75 s	$-\Delta A$ at 135 s
CPQ	0.446 ± 0.024	0.466 ± 0.030
QC	0.376 ± 0.010	0.442 ± 0.033
Trolox	0.520 ± 0.031	0.533 ± 0.029

The ethanol solution of the DPPH radical (50 μM) was incubated in the presence of the compound tested (50 μM). The absorbance decrease at 518 nm in the first 75 s and 135 s interval was determined. The results are the mean values ± SD from at least three measurements.

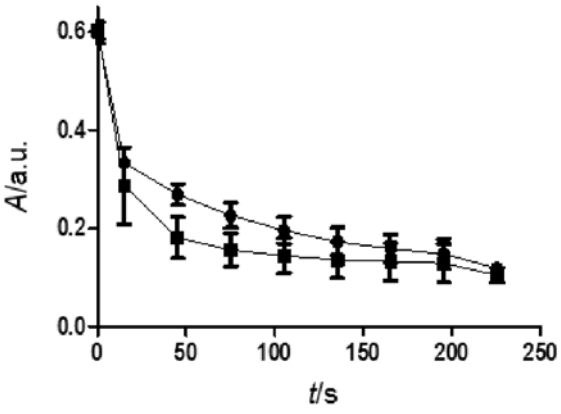

Fig. 4. Continual absorbance decrease in ethanol solution of DPPH radical (50 μM) in the presence of equimolar concentrations of compounds tested at $\lambda_{max} = 518$ nm, quercetin (●), CPQ (■). Each point represents mean value ± SD from three experiments.

crease representing the residual anti-radical activity of the antioxidant degradation products. The initial velocity of DPPH decolorisation determined for the first 75 s and 135 s intervals was used as a marker of the anti-radical activity shown in Table 3. Based on this kinetic parameter, CPQ appears a more efficient scavenger of DPPH free radical than the parent quercetin, yet less efficient than the standard trolox. The initial velocity of DPPH decolorisation as a kinetic parameter is considered to be of primary importance in antioxidant evaluation, since a rapid reaction with low concentrations of short-lived damaging radicals is of the utmost importance for antioxidant protection. Other authors applied the kinetic approach to rank flavonoids according to their antioxidant efficacy (Goupy et al., 2003; Butković et al., 2004; Villaño et al., 2007).

Conclusions

The novel derivative of quercetin CPQ: *i*) inhibited α-glucosidase in a non-competitive manner with an efficacy exceeding that of the parent quercetin; *ii*) inhibited ALR2 isolated from rat lenses and attenuated sorbitol accumulation in isolated rat eye lenses with an activity comparable with that of quercetin; *iii*) scavenged stable free radical of DPPH more efficiently than did quercetin. By inhibiting α-glucosidase and affecting both the polyol pathway and oxidative stress, CPQ represents an example of a promising agent for the multi-targeted pharmacology of diabetic complications.

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