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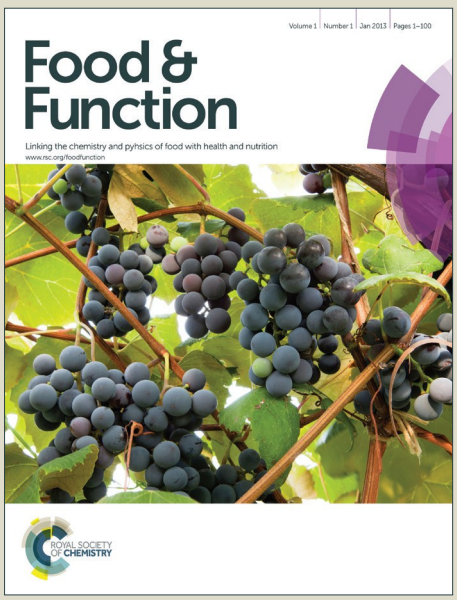

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The effect of quercetin and kaempferol aglycones and glucuronides on peroxisome proliferator-activated receptor-gamma (PPAR-γ)

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Abstract

The consumption of dietary flavonoids has been associated with a variety of health benefits, including effects mediated by the activation of peroxisome proliferator-activated receptor-gamma (PPAR-γ). Flavonoids are extensively metabolized during and after uptake and there is little known on the biological effects of these conjugated metabolites of flavonoids that are found in plasma. To investigate the effect of glucuronidation on the ability of flavonoids to activate PPAR-γ we studied and compared the activity of quercetin, kaempferol and their relevant plasma conjugates quercetin-3-O-glucuronide (Q3G) and kaempferol-3-O-glucuronide (K3G) on different PPAR-γ related endpoints. The flavonoid aglycones increased PPAR-γ mediated gene expression in a stably transfected reporter gene cell line and glucuronidation diminished their effect. To study the intrinsic activity of the test compounds to activate PPAR-γ we used a novel microarray technique to study ligand induced ligand binding domain (LBD) – nuclear receptor coregulator interactions. In this cell-free system we demonstrate that, unlike the known PPAR-γ agonist rosiglitazone, neither the flavonoid aglycones nor the conjugates are agonistic ligands of the receptor. The increases in reporter gene expression in the reporter cells were accompanied by increased PPAR-γ receptor-mRNA expression and quercetin synergistically increased the effect of rosiglitazone in the reporter gene assay.

It is concluded that flavonoids affect PPAR-γ mediated gene transcription by a mode of action different from agonist binding. Increases in PPAR-γ receptor mRNA expression and synergistic effects with endogenous PPAR-γ agonists may play a role in this alternative mode of action. Glucuronidation reduced the activity of the flavonoid aglycones.

Abbreviations

K3G = kaempferol-3-O-glucuronide

LBD = ligand binding domain

PPAR-γ = peroxisome proliferator-activated receptor-gamma

PPRE = peroxisome proliferator-responsive element

Q3G = quercetin-3-O-glucuronide

VitC = vitamin C (L-ascorbic acid)

1. Introduction

Flavonoids are plant secondary metabolites and ubiquitously present in many plant-derived foodstuffs. As a result, flavonoids are generally consumed on a regular basis via fruits, vegetables and their juices, as well as via wine, tea and cocoa-derived products [1, 2]. Dietary intake of flavonoids has been correlated with the prevention of various degenerative diseases and improvement of disease states [3, 4]. One possible mode of action behind beneficial health effects of flavonoids has been suggested to be the activation of PPAR-γ [5]. PPARs are ligand-activated transcription factors which form obligate heterodimer partners with the retinoid X receptor. The heterodimers bind to peroxisome proliferator-responsive elements (PPREs) in the regulatory region of target genes and upon activation recruit nuclear co-activators required for gene transcription, while dismissing co-repressors that are bound in the unliganded state [6]. Three PPAR isoforms are currently known, i.e. PPARα (NR1C1), PPARβ/δ (NR1C2) and PPAR-γ (NR1C3). Apart from certain overlaps, these isoforms are activated by different ligands and regulate specific target genes [7]. Various health promoting effects are ascribed to PPAR activation and especially PPAR-γ is highlighted for its effects on for example adipogenesis, insulin resistance and inflammation [8]. There are two PPAR-γ splice variants, i.e. PPAR-γ1 and PPAR-γ2 which have different expression levels in tissues [7]. The functional differences between these two are not fully elucidated but there are indications that PPAR-γ2 is of higher importance in adipogenesis and insulin sensitivity [9-11]. Various preferably unsaturated fatty acids serve as endogenous receptor agonists [12], and the receptor is target of a variety of drugs to treat reduced insulin sensitivity and hyperlipidemia such as the well-known class of thiazoledinediones [13]. Several flavonoids are reported to activate PPAR-γ mediated gene transcription and other related endpoints (see Table 1).

With only few exceptions, flavonoids occur in nature in their glycosidic form. Upon ingestion, these flavonoid glycosides have to be deconjugated to their respective aglycones before or during uptake in the gastrointestinal tract. During uptake the aglycones are extensively metabolized to sulfated, methylated and/or glucuronidated conjugates in intestinal tissue or the liver before they enter the systemic circulation [14]. As a result, under physiological conditions flavonoids usually do not occur as aglycones in biological fluids. It is widely accepted that conjugation and deconjugation can significantly influence the biological activity of flavonoids [15, 16]. The aim of the present study was to investigate the effect of flavonoid conjugation on the reported activity of flavonoids to induce PPAR-γ mediated gene expression. To this end we selected the dietary flavonoids quercetin and kaempferol as model flavonoids to compare their activity with their respective 3-O-glucuronidated conjugates. Q3G and K3G belong to the most abundant conjugates of quercetin and kaempferol found in plasma and urine [17-22]. In this study we describe the effect of these flavonoid aglycones and conjugates on PPAR-γ mediated gene expression, receptor mRNA expression and PPAR-γ LBD-coregulator interaction.

2 Materials and Methods

2.1 Chemicals

Rosiglitazone (CAS no: 122320-73-4) was obtained from Cayman Chemical (Ann Arbor, USA). Kaempferol (CAS no: 520-18-3), K3G (CAS no: 22688-78-4), quercetin (CAS no: 117-39-5), Q3G (CAS no: 22688-79-5), DL-dithiothreitol (DTT, CAS no: 3483-12-3) and L-ascorbic acid (VitC, CAS no:50-81-7) were purchased from Sigma Aldrich (Missouri, USA). Stock solutions of the flavonoids were prepared in dimethylsulphoxide (DMSO, 99.9% purity) obtained from Acros (Geel, Belgium) and

stored at –20 °C. G418 solution and fetal bovine serum (FBS) were purchased from PAA (Pasching, Austria). Acetic acid was purchased from VWR International (Darmstadt, Germany). Acetonitrile (ULC/MS grade) and methanol (HPLC supra-gradient) were purchased from Biosolve BV (Valkenswaard, the Netherlands). Phosphate buffered saline (PBS), Dulbecco's Modified Eagle Medium with Ham´s Nutrient Mixture F-12 (1:1) (DMEM/F12), DMEM/F12 without phenol red, nonessential amino acids (NEAA) and trypsin were purchased from Invitrogen (Breda, The Netherlands).

Dextran-coated charcoal-stripped fetal calf serum (DCC-FCS) was purchased from Thermo Scientific (Waltham, Missouri, USA). Nanopure water was prepared with a Barnstead Nanopure Type I ultrapure water system.

2.2 Cell cultures

The PPAR-γ2 CALUX cells (provided by BioDetection Systems BV, Amsterdam, the Netherlands) are human osteosarcoma U2OS cells stably transfected with an expression vector for PPAR-γ2 and a firefly luciferase reporter construct under control of the peroxisome proliferator responsive element [23]. The cells were cultured in DMEM/F12 GlutaMAX supplemented with 7.5% FBS and NEAA. To maintain selection pressure 200 µg/mL G418 was added once per week. The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

All compounds were tested for cytotoxicity and potential effects on luciferase stability using the Cytotox CALUX cell line (provided by BioDetection Systems BV) as described before [24]. The Cytotox CALUX cells show an invariant luciferase expression and a decrease in luciferase activity therefore indicates a cytotoxic effect.

Moreover, an increase in luciferase activity in the Cytotox CALUX cells may indicate stabilization of the luciferase enzyme and possible false positives for reporter gene expression in the PPAR-γ2 CALUX assay [25]. Only non-cytotoxic concentrations of the test compounds were used in the PPAR-γ2 CALUX assay.

The Cytotox CALUX cells were cultured in DMEM/F12 supplemented with 7.5% FBS and NEAA. Once per week 200 µg/ml G418 was added to the culture medium in order to maintain the selection pressure.

2.3 PPAR-γ2 CALUX and cytotox CALUX assay

The ability of the tested flavonoids to induce PPAR-γ2 mediated luciferase expression at protein level in an intact cell system was tested by measuring luciferase activity in the PPAR-γ2 CALUX reporter cells. To this end PPAR-γ2 CALUX cells were seeded in a white 96-wells microtiter plate with clear bottom (View Plate-96 TC, PerkinElmer) at a density of 10,000 cells per well in 100 µl exposure medium (DMEM/F12 without phenol red +5 % (v/v) DCC- FCS +1% (v/v) NEAA). The seeded cells were incubated for 24 h to allow them to attach and form a confluent monolayer. Subsequently, the 60 inside wells of the plate were exposed for 24 h to the test compounds in exposure medium at the concentrations indicated. The final DMSO concentration in the exposure medium was 0.5%. On each plate, 100 nM rosiglitazone, a known PPAR-γ agonist [26] was included as positive control. Quercetin was co-incubated with 500 µM VitC to prevent auto oxidation; this concentration of VitC was determined not to interfere with cell viability, luciferase expression or luciferase stabilization.

After 24 h of exposure, medium was removed and the cells were washed twice with 100 µl 0.5x PBS. Subsequently, cells were lysed by addition of 30 µl low salt lysis

buffer [27] and stored overnight at −80 °C. Luciferase activity in the lysate was measured using a luminometer (Luminoscan Ascent, Thermo Scientific, Waltham, MA) and flash mix as described previously [27]. Background light emission and luciferase activity was measured per well and expressed in relative light units (RLU). Background values were subtracted prior to data analysis. Data and statistical analyses were conducted using Microsoft Excel (Version 14.0.7106.5003; Microsoft Corporation) and GraphPad Prism software (version 5.00 for windows, GraphPad software, San Diego, USA). The depicted graphs are representative curves giving mean and standard deviations of sextuplicate measurements. The Cytotox CALUX cells were cultured, exposed, lysed and measured in the same manner as the PPAR-γ2 CALUX cells.

2.4 Quantitative polymerase chain reaction (qPCR)

For qPCR the PPAR-γ2 CALUX cells were propagated as described above with some minor modifications. Cells were seeded in 12 well plates, at 100,000 cells in 1 ml of exposure medium per well. After 24h of incubation, cell culture medium was removed and 750 µl of exposure medium were added containing the test compounds (added from a 200 times concentrated stock solution in DMSO). Each test compound was tested in two independent experiments in triplicates giving a total of six replicates.

RNA isolation

For the isolation and purification of mRNA QIAshredder spin columns and the RNeasy mini kit from QIAGEN (Venlo, the Netherlands) were used. After 24h of exposure of the PPAR-γ2 CALUX cells medium was aspirated and the cells were

washed with 600 µl PBS. Subsequently, 300 µl of RLT lysis buffer (RNeasy Mini Kit, Qiagen, Venlo, the Netherlands) were added and the plates were placed on an orbital shaker. The lysate was added to QIAshredder spin columns and centrifuged at 8,000x g for 15 seconds. Then 350 µl of 70% ethanol were added to the flow through of the spin columns and the samples were mixed thoroughly. These mixtures were transferred to RNeasy spin columns and centrifuged at 8,000 rcf for 20 seconds. The flow through was discarded. Then 700 µl RW1 buffer (RNeasy Mini Kit) were added to the columns and the columns were centrifuged at 8,000 rcf for 20 seconds. The flow through was discarded. Next, 500 µl of RPE buffer (RNeasy Mini Kit) were added to the columns and the columns were centrifuged at 8,000 rcf for 20 s. The flow through was discarded. The previous step was repeated and followed by 2 min of centrifugation. Subsequently, the columns were placed in new tubes and centrifuged at 14,000 rcf for 1 minute to dry the columns. Next, the columns were transferred to new tubes and 30 µl RNase-free water were added. The columns were kept at room temperature for 5 minutes and subsequently centrifuged at 8,000 rcf for 1 minute to elute total RNA. The concentration of total RNA in the flow through was determined spectrophotometically at 260 nm using a Nanodrop (ND-1000, Thermo scientific, Wilmington, Delaware).

Reverse transcriptase reaction and real-time PCR with SYBR green

To obtain cDNA, a QuantiTect Reverse Transcription Kit (Qiagen) was used according to the manufacturer's protocol. Total RNA samples were diluted to 50 ng/µl in RNase-free water. To eliminate genomic DNA, 2 µl of gDNA Wipeout Buffer (7x) were added to 8 µl of sample and 4 µl of RNase-free water per reaction. Mixtures were incubated for 2 minutes at 42 °C and subsequently put on ice. Per reaction, 1 µl

of Quantiscript Reverse Transcriptase, 4 µl of Quantiscript ER Buffer (5x) and 1 µl RT Primer mix were added. These mixtures were incubated for 15 minutes at 42 °C and subsequently for 3 minutes at 95 °C to inactivate the reverse transcriptase. After incubation the samples were immediately cooled to 4°C and used for gene expression analysis.

The expression of the reporter gene pGL4 mRNA (FW: ATCAGCCAGCCCACCGTCGTATTC, RV: ACAAGCGGTGCGGTGCGGTAGG) and PPAR-γ2 mRNA (FV: GCGATTCCTTCACTGATAC, RV: CTTCCATTACGGAGAGATCC; from [28]) was measured by real-time quantitative chain polymerase reaction (RT-qPCR) using Rotor-Gene Q (Qiagen) and normalized against the expression of beta-actin (FW: GCAAAGACCTGTACGCCAACAC, RV: TCATACTCCTGCTTGCTGATCCCAC) and GAPDH (FW: TGATGACATCAAGAAGGTGGTGAAG, RV: TCCTTGGAGGCCATGTGGGCCAT). For every reaction 5 µl of 20 times diluted sample cDNA, 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 12.5 µl of Rotor-Gene SYBR Green PCR Master Mix (Qiagen) and 5.5 µl of RNase free water were used. The plate was incubated at 95 °C for 10 min, and then for 40 cycles each consisting of incubation at 95 °C for 10 s, at 60 °C for 15 s, at 72 °C for 20 s. This was followed by pre-melt conditioning at 72 °C for 90 s, increasing by 1°C every 5 seconds to 95°C for the melting curve. Every reaction was carried out in technical duplicates.

qPCR data analysis

Threshold cycle (Ct)-values were derived using Rotor-Gene 6000 Series Software (Qiagen). For the data and statistical analyses Microsoft Excel and GraphPad Prism software were used. The formulas used are adapted from literature [29].

The efficiencies (E) of the primer pairs were calculated using the formula

$E = 10\text{^}(-\frac{1}{slope})$

where slope is the slope of the standard curve (crossing threshold (Ct) versus cDNA input). An E value of 2 is reached when there is exact doubling of the cDNA every cycle.

The relative quantity of a given sample (RQ_{sample}) and gene of interest or reference gene was calculated using the formula

$RQ_{sample} = E\text{^}(Ct_{(control)}\text{-}Ct_{(sample)})$

where $Ct_{(control)}$ is the average of the Ct values of the solvent control reactions of a certain gene of interest and $Ct_{(sample)}$ the Ct value of the sample to be quantified.

The relative normalized expression (RNE) or fold change for a specific sample and gene of interest (GOI) against the two reference genes (REF) used is calculated using the following formula

$RNE = RQs_{ample(GOI)} / (RQ_{sample(REF1)} \times RQ_{sample(REF2)})\text{^}1/n$

where n is the number of reference genes.

2.5 PamGene Nuclear Receptor-Coregulator Interaction Profiling

Ligand-modulated interaction of the PPAR-γ ligand binding domain (LBD) with coregulators (154 different binding motifs of 66 different coregulators) was assessed using PamChip 4 microarray chips for nuclear hormone receptors (PamGene International B.V., 's-Hertogenbosch, The Netherlands) as described previously [30].

Briefly, the PPAR-γ LBD (His-tagged #P1065; Protein One, Rockville, MD, USA) was mixed with an anti-His antibody conjugated to Alexa Fluor 647 (Penta·His Alexa Fluor 647 Conjugate #35370; Qiagen, Venlo, the Netherlands) in the absence and presence of the potential ligands added from a stock solution in DMSO (2% final concentration) to the reaction buffer (Nuclear Receptor Buffer F #PV4547; Invitrogen, Breda, the Netherlands) containing 5 mM DTT. Ligand concentrations used were EC_{90} concentrations obtained in the PPAR-γ reporter gene assays. All assays were performed in a fully automated microarray processing platform (PamStation12, PamGene International B.V.) at 20 °C. After incubation, excess incubation mix was removed and the arrays washed prior to acquisition of images.

Image analysis was performed using BioNavigator software (PamGene International B.V.) which performs automated array grid finding and subsequent quantification of signal and local background for each individual peptide. The median signal-minus-background values were used as the quantitative parameter of binding. For data and statistical analyses Microsoft Excel was used. Experiments were performed in triplicate and the graphs are corrected for binding levels obtained in the solvent control; coregulators for which none of the tested compounds induced an effect that was statistically significantly different from the solvent control ($p \leq 0.05$) are excluded from the figure.

3 Results

PPAR-γ2 CALUX reporter gene expression

The effect of quercetin and kaempferol on PPAR-γ2 mediated gene expression was measured in U2OS cells stably transfected with the PPAR-γ2 receptor and the firefly luciferase gene regulated by the PPRE. Quercetin and kaempferol, as well as the

known PPAR-γ agonist rosiglitazone increase luciferase activity in a concentration-dependent way (Figure 1). In addition, the compounds were tested in a control cell line that invariably expresses firefly luciferase to measure effects on cell viability and post-transcriptional stabilization of luciferase. Quercetin and rosiglitazone did not affect the luciferase signal in the control cell line at the concentrations tested. Kaempferol increased the luminescence signal indicating stabilization of the luciferase enzyme – an effect that is likely to cause artificially increased luciferase activity in the PPAR-γ2 reporter gene assay. To avoid false positive results through post-translational stabilization of the luciferase reporter-protein [31] the effect of glucuronidation on the induction of PPAR-γ mediated gene expression was studied on mRNA-expression level by qPCR. The results of these experiments are expressed in Figure 2. Rosiglitazone, quercetin and kaempferol significantly increased pGL4 reporter gene expression also at the mRNA level in the PPAR-γ reporter gene assay. The glucuronidated conjugates of quercetin and kaempferol, i.e. Q3G and K3G did not significantly affect pGL4 reporter gene expression (Figure 2). The stability of all tested compounds during the 24h of incubation was determined by UPLC and the UPLC chromatograms obtained revealed that all tested compounds remained stable in the exposure medium during incubation (data not shown).

PPAR-γ coregulator binding

Given that the lower activity of the flavonoid glucuronides to activate PPAR-γ in the cell based reporter gene assay and the cell based qPCR assay might be due to their lower cellular bioavailability, additional studies were performed to investigate the intrinsic ability of the tested flavonoid aglycones and their glucuronidated conjugates to activate PPAR-γ. To that end subsequent experiments were performed in a cell-

free assay system characterizing PPAR-γ LBD activation using a microarray technique to analyze nuclear receptor - coregulator interactions. The assay employs microarrays containing a total of 154 distinct binding motifs of 66 different nuclear receptor-coregulators that are immobilized on a porous membrane. Figure 3 shows the binding patterns of the ligand binding domain of PPAR-γ to these coregulator binding motifs in the presence of quercetin, kaempferol, Q3G, K3G and the positive control rosiglitazone for comparison. Quercetin, kaempferol and rosiglitazone were tested at their EC_{90} concentrations derived from the reporter gene assay and the glucuronides were tested at equimolar concentration as the respective aglycones. The results presented reveal that incubation with rosiglitazone increases LBD binding to specific coactivator binding motifs (e.g. CREP-binding protein (CBP), E1A binding protein p300 (EP300), nuclear receptor coactivators 1 and 2 (NCOA1, NCOA2) etc.) and decreases binding to corepressor motifs (nuclear receptor corepressors 1 and 2 (NCOR1, NCOR2)). Incubation with quercetin, kaempferol, Q3G and K3G does not affect LBD binding to coregulators in a comparable manner and resulted in binding patterns similar to the solvent control. These results indicate that the observed effects of the flavonoids on PPAR-γ mediated gene expression cannot be ascribed to an agonistic effect of the flavonoids on the PPAR-γ LBD.

PPAR-γ receptor-mRNA expression

As the tested flavonoids were active in the PPAR-γ reporter gene assay but did not activate the LBD of PPAR-γ we investigated other endpoints that could affect the observed activity. To this end the effect of the compounds on PPAR-γ2 receptor-mRNA expression in the reporter gene cell line by qPCR was quantified. Figure 4 shows that quercetin and kaempferol significantly increase the expression of PPAR-

γ2 receptor mRNA, Q3G increases gene expression to a lesser extent than the aglycone, and rosiglitazone and K3G do not significantly affect receptor mRNA expression. These results show that the effects of quercetin and kaempferol on reporter gene expression in the PPAR-γ2 CALUX cell line are accompanied by an increase in PPAR-γ2 receptor mRNA transcription.

In additional experiments the PPAR-γ2 reporter gene cells were exposed to rosiglitazone in the presence of quercetin (Figure 5). Figure 5 presents a full concentration response curve of rosiglitazone in the presence of a low concentration of quercetin that by itself causes only a low increase in reporter gene expression (i.e. 10 µM). The results obtained reveal that quercetin synergistically increased the effect of rosiglitazone by about 3-fold over the complete range of concentrations tested. This further supports that quercetin has a different mode of action from that of rosiglitazone, and reveals that quercetin can synergistically increase the response of a regular PPAR-γ2 agonist.

4 Discussion

The objective of this study was to investigate and compare the effect of the dietary flavonoids quercetin and kaempferol and their relevant glucuronidated conjugates Q3G and K3G on PPAR-γ mediated gene expression.

We observed increased luciferase activity and pGL4 reporter gene expression in the PPAR-γ2 reporter gene assay upon exposure to quercetin and kaempferol. Other studies reported that quercetin does not activate PPAR-γ mediated gene expression in reporter gene assays at concentrations reaching up to 300 µM [32-35]. This difference compared to our results can be explained by the instability of quercetin in vitro where

it is known to oxidize rapidly [36]. As already described earlier [27, 37], the addition of ascorbic acid can prevent the auto-oxidation of quercetin.

Of the tested glucuronides, Q3G increased gene expression to a lesser extent than the aglycone, while K3G did not significantly affect reporter gene expression. Based on these results it can be concluded that glucuronidation reduces the ability of the flavonoids to activate PPAR-γ mediated gene expression. Given that this effect was observed in a reporter gene assay with intact cells this can be due either to a lower intrinsic activity to induce PPAR-γ mediated gene expression or a reduced uptake of the conjugates into the cells. It has been well recognised that flavonoid conjugates may have to be deconjugated to enter cells and exert their biological activities [37, 38], although there are cell types that appear to be able to take up flavonoid glucuronides [39].

To investigate the potential inherent activity of the tested flavonoids to activate PPAR-γ the possible effect of the flavonoids on the interaction of the LBD of PPAR-γ with nuclear receptor coregulators was studied in a cell free model system. Our results show that, surprisingly, none of tested flavonoids interacts with the LBD inducing conformational changes of the LBD comparable to the well-known PPAR-γ agonist rosiglitazone. The observed effects of the flavonoids on PPAR-γ mediated reporter gene expression are therefore likely due to another mode of action. While LBD agonism is the key step to receptor activation, there are other ways to interfere with PPAR-γ activity, for example PPAR-γ modification through receptor phosphorylation, deacetylation, and sumoylation can modulate its activity [40-42]. In addition, the expression of PPAR-γ itself can be regulated by kinase activities [41, 43] and flavonoids are reported to directly and indirectly affect protein kinase activities [44].

Thus the results of the present study lead to the conclusion that flavonoids activate PPAR-γ mediated gene expression by a mode of action different from that of regular PPAR-γ agonists.

We observed increased PPAR-γ2 mRNA expression upon flavonoid exposure, an effect that is not exerted by the known agonist rosiglitazone. Various flavonoids are reported to affect PPAR-γ expression in a variety of in vitro and vivo systems. Quercetin has been reported to increase PPAR-γ mRNA and protein level in spontaneously hypertensive rats [45], as well as in primary human adipocytes [46], H9C2 cells [45] and THP-1 macrophages [47]. Interestingly, quercetin downregulates PPAR-γ in 3T3-L1 cells [48, 49]; this is also in line with the general observation that flavonoids can inhibit PPAR-γ dependent adipocyte differentiation in vitro in 3T3-L1 cells (see Table 1). Treatment with quercetin can also prevent up-regulated PPAR-γ levels in liver [50] and adipose tissue [51] in laboratory animals fed a high fat diet. One study reports the effect of quercetin conjugates on PPAR-γ expression [52]. In A549 cells, quercetin-3-glucuronide and quercetin-3'-sulfate slightly but significantly increased PPAR-γ expression; the aglycone however did not affect PPAR-γ expression [52]. The inactivity of the aglycone in this study is likely to be due to the instability of quercetin, as discussed above.

Flavonoid-induced increases in PPAR-γ receptor levels combined with receptor activation by endogenous agonists is a likely mechanism behind the observed activity of the flavonoids in the reporter gene assay. It is of interest to note that while kaempferol significantly affects both PPAR-γ mediated PGL4 mRNA expression (Figure 2) and PPAR-γ receptor mRNA expression (Figure 4), for Q3G only the latter endpoint is significantly modulated. Such differences may be due to as yet undefined additional modulatory effects of the flavonoids on for example endogenous PPAR-γ

ligands (i.e. fatty acids) and/or the aforementioned modulation of receptor activities by phosphorylation, deacetylation, and/or sumoylation which could altogether further contribute to the flavonoids' effects on PPAR-γ. Further, we also show that quercetin synergistically enhances the effect of rosiglitazone in the PPAR-γ reporter gene assay which may also be due to increased cellular receptor levels. The observed synergistic effects underline that the tested flavonoids have a different mode of action compared to the agonist rosiglitazone and that flavonoids can potentially increase the effect of PPAR-γ ligands.

5 Conclusion

Our results show that glucuronidation reduces the activity of quercetin and kaempferol on cellular PPAR-γ mediated gene expression. These differences in activity between the aglycone and the conjugated forms that are present in biological fluids highlight the importance of using relevant flavonoid conjugates in in vitro studies. We further observed that none of the tested flavonoid compounds act as agonists on PPAR-γ LBD. It is concluded that flavonoids affect PPAR-γ mediated gene transcription by a mode of action different from agonist binding. Increased PPAR-γ receptor mRNA expression and synergistic effects with endogenous PPAR-γ agonists are likely to play a role in this alternative mode of action.

References

1. P. M. Kris-Etherton, K. D. Hecker, A. Bonanome, S. M. Coval, A. E. Binkoski, K. F. Hilpert, A. E. Griel and T. D. Etherton, *American Journal of Medicine*, 2002, 113, 71S-88S.
2. C. Manach, A. Scalbert, C. Morand, C. Rémésy and L. Jiménez, *American Journal of Clinical Nutrition*, 2004, 79, 727-747.
3. I. C. Arts and P. C. Hollman, *The American journal of clinical nutrition*, 2005, 81, 317S-325S.
4. A. Scalbert, C. Manach, C. Morand, C. Rémésy and L. Jiménez, *Critical Reviews in Food Science and Nutrition*, 2005, 45, 287-306.
5. M. H. Pan, C. S. Lai and C. T. Ho, 2010, 1, 15-31.
6. N. Viswakarma, Y. Jia, L. Bai, A. Vluggens, J. Borensztajn, J. Xu and J. K. Reddy, *PPAR Research*, 2010, 2010, 21.
7. S. Mandrup and A. Bugge, *PPAR Research*, 2010.
8. P. Tontonoz and B. M. Spiegelman, in *Annual Review of Biochemistry*, 2008, vol. 77, pp. 289-312.
9. G. Medina-Gomez, S. Virtue, C. Lelliott, R. Boiani, M. Campbell, C. Christodoulides, C. Perrin, M. Jimenez-Linan, M. Blount, J. Dixon, D. Zahn, R. R. Thresher, S. Aparicio, M. Carlton, W. H. Colledge, M. I. Kettunen, T. Seppänen-Laakso, J. K. Sethi, S. O'Rahilly, K. Brindle, S. Cinti, M. Orešič, R. Burcelin and A. Vidal-Puig, *Diabetes*, 2005, 54, 1706-1716.
10. E. Mueller, S. Dr.ori, A. Aiyer, J. Yie, P. Sarraf, H. Chen, S. Hauser, E. D. Rosen, K. Ge, R. G. Roeder and B. M. Spiegelman, *Journal of Biological Chemistry*, 2002, 277, 41925-41930.

11. D. Ren, T. N. Collingwood, E. J. Rebar, A. P. Wolffe and H. S. Camp, *Genes and Development*, 2002, 16, 27-32.
12. B. Desvergne and W. Wahli, *Endocr Rev*, 1999, 20, 649-688.
13. T. M. Larsen, S. Toubro and A. Astrup, 2003, 27, 147-161.
14. P. C. H. Hollman, *Pharmaceutical Biology*, 2004, 42, 74-83.
15. K. Beekmann, L. Actis-Goretta, P. J. Van Bladeren, F. Dionisi, F. Destaillats and I. M. C. M. Rietjens, *Food and Function*, 2012, 3, 1008-1018.
16. G. Williamson, D. Barron, K. Shimoi and J. Terao, *Free Radical Research*, 2005, 39, 457-469.
17. A. J. Day, F. Mellon, D. Barron, G. Sarrazin, M. R. A. Morgan and G. Williamson, *Free Radical Research*, 2001, 35, 941-952.
18. M. S. DuPont, A. J. Day, R. N. Bennett, F. A. Mellon and P. A. Kroon, *European Journal of Clinical Nutrition*, 2004, 58, 947-954.
19. I. B. Jaganath, W. Mullen, C. A. Edwards and A. Crozier, *Free Radical Research*, 2006, 40, 1035-1046.
20. Y. Kawai, Y. S. Saito, T. Nishikawa, A. Ishisaka, K. Murota and J. Terao, *Bioscience, Biotechnology and Biochemistry*, 2009, 73, 517-523.
21. W. Mullen, A. Boitier, A. J. Stewart and A. Crozier, *Journal of Chromatography A*, 2004, 1058, 163-168.
22. W. Mullen, C. A. Edwards and A. Crozier, *British Journal of Nutrition*, 2006, 96, 107-116.
23. L. Gijsbers, H. Y. Man, S. K. Kloet, L. H. J. De Haan, J. Keijer, I. M. C. M. Rietjens, B. Van Der Burg and J. M. M. J. G. Aarts, *Analytical Biochemistry*, 2011, 414, 77-83.

24. S. C. v. B. Van der Linden, A. R. M.; Van Vugt-Lussenburg, B.; Jonker, L.; Brouwer, A.; Teunis, M.; Krul, C. A. M.; Van der Burg, B., *Submitted for publication.*

25. L. Gijsbers, H. D. L. M. Van Eekelen, T. H. Nguyen, L. H. J. De Haan, B. Van Der Burg, J. M. M. J. G. Aarts, I. M. C. M. Rietjens and A. G. Bovy, *Food Chemistry*, 2012, 135, 1166-1172.

26. J. M. Lehmann, L. B. Moore, T. A. Smith-Oliver, W. O. Wilkison, T. M. Willson and S. A. Kliewer, *Journal of Biological Chemistry*, 1995, 270, 12953-12956.

27. A. M. J. F. Boerboom, M. Vermeulen, H. van der Woude, B. I. Bremer, Y. Y. Lee-Hilz, E. Kampman, P. J. van Bladeren, I. M. C. M. Rietjens and J. M. M. J. G. Aarts, *Biochemical Pharmacology*, 2006, 72, 217-226.

28. V. Giusti, C. Verdumo, M. Suter, R. C. Gaillard, P. Burckhardt and F. Pralong, 2003, 52, 1673-1676.

29. M. W. Pfaffl, *Nucleic acids research*, 2001, 29.

30. A. Koppen, R. Houtman, D. Pijnenburg, E. H. Jeninga, R. Ruijtenbeek and E. Kalkhoven, *Molecular and Cellular Proteomics*, 2009, 8, 2212-2226.

31. A. M. Sotoca, T. F. H. Bovee, W. Brand, N. Velikova, S. Boeren, A. J. Murk, J. Vervoort and I. M. C. M. Rietjens, *The Journal of Steroid Biochemistry and Molecular Biology*, 2010, 122, 204-211.

32. K. B. Christensen, R. K. Petersen, K. Kristiansen and L. P. Christensen, *Phytotherapy Research*, 2010, 24, S129-S132.

33. L. Gijsbers, H. D. L. M. Van Eekelen, L. H. J. De Haan, J. M. Swier, N. L. Heijink, S. K. Kloet, H. Y. Man, A. G. Bovy, J. Keijer, J. M. M. J. G. Aarts, B. Van Der Burg and I. M. C. M. Rietjens, *Journal of Agricultural and Food Chemistry*, 2013, 61, 3419-3427.

34. Y. C. Liang, S. H. Tsai, D. C. Tsai, S. Y. Lin-Shiau and J. K. Lin, *FEBS Letters*, 2001, 496, 12-18.
35. M. Mueller, B. Lukas, J. Novak, T. Simoncini, A. R. Genazzani and A. Jungbauer, *Journal of Agricultural and Food Chemistry*, 2008, 56, 11621-11630.
36. D. W. Boulton, U. K. Walle and T. Walle, *Journal of Pharmacy and Pharmacology*, 1999, 51, 353-359.
37. Y. Y. Lee-Hilz, M. Stolaki, W. J. H. van Berkel, J. M. M. J. G. Aarts and I. M. C. M. Rietjens, *Food and Chemical Toxicology*, 2008, 46, 2128-2134.
38. P. Galindo, I. Rodriguez-Gómez, S. González-Manzano, M. Dueñas, R. Jiménez, C. Menéndez, F. Vargas, J. Tamargo, C. Santos-Buelga, F. Pérez-Vizcaíno and J. Duarte, *PLoS ONE*, 2012, 7.
39. J. Wittig, Universitätsbibliothek der Universität Würzburg, 2002.
40. M. Ahmadian, J. M. Suh, N. Hah, C. Liddle, A. R. Atkins, M. Downes and R. M. Evans, 2013, 99, 557-566.
41. M. Moreno, A. Lombardi, E. Silvestri, R. Senese, F. Cioffi, F. Goglia, A. Lanni and P. de Lange, *PPAR Research*, 2010, 2010.
42. K. A. Burns and J. P. Vanden Heuvel, *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, 2007, 1771, 952-960.
43. S. R. Farmer, 2005, 29, S13-S16.
44. R. J. Williams, J. P. E. Spencer and C. Rice-Evans, *Free Radical Biology and Medicine*, 2004, 36, 838-849.
45. L. Yan, J. D. Zhang, B. Wang, Y. J. Lv, H. Jiang, G. L. Liu, Y. Qiao, M. Ren and X. F. Guo, 2013, 8.

46. C. C. Chuang, K. Martinez, G. Xie, A. Kennedy, A. Bumrungpert, A. Overman, W. Jia and M. K. McIntosh, 2010, 92, 1511-1521.
47. S. M. Lee, J. Moon, Y. Cho, J. H. Chung and M. J. Shin, 2013, 33, 136-143.
48. P. J. Chien, Y. C. Chen, S. C. Lu and F. Sheu, 2005, 13, 168-175+195.
49. S. G. Kim, D. Y. Rhyu, D. K. Kim, D. H. Ko, Y. K. Kim, Y. M. Lee and H. J. Jung, 2010, 41, 21-25.
50. M. Kobori, S. Masumoto, Y. Akimoto and H. Oike, 2011, 55, 530-540.
51. O. Y. Kim, S. M. Lee, H. Do, J. Moon, K. H. Lee, Y. J. Cha and M. J. Shin, 2012, 26, 432-437.
52. S. L. Yeh, C. L. Yeh, S. T. Chan and C. H. Chuang, 2011, 77, 992-998.
53. K. Hu, Y. Yang, Q. Tu, Y. Luo and R. Ma, 2013, 721, 96-102.
54. M. A. Kim, K. Kang, H. J. Lee, M. Kim, C. Y. Kim and C. W. Nho, 2014, 101, 64-72.
55. N. K. Salam, T. H. W. Huang, B. P. Kota, M. S. Kim, Y. Li and D. E. Hibbs, *Chemical Biology and Drug Design*, 2008, 71, 57-70.
56. H. A. Lim, E. K. Lee, J. M. Kim, M. H. Park, D. H. Kim, Y. J. Choi, Y. M. Ha, J. H. Yoon, J. S. Choi, B. P. Yu and H. Y. Chung, *Biogerontology*, 2012, 13, 133-145.
57. D. H. Kwak, J. H. Lee, K. H. Song and J. Y. Ma, 2014, 385, 257-264.
58. P. Shen, M. H. Liu, T. Y. Ng, Y. H. Chan and E. L. Yong, *Journal of Nutrition*, 2006, 136, 899-905.
59. B. K. Chacko, R. T. Chandler, T. L. D'Alessandro, A. Mundhekar, N. K. H. Khoo, N. Botting, S. Barnes and R. P. Patel, *Journal of Nutrition*, 2007, 137, 351-356.

60. X. Feng, H. Qin, Q. Shi, Y. Zhang, F. Zhou, H. Wu, S. Ding, Z. Niu, Y. Lu and P. Shen, 2014, 89, 503-514.
61. Y. Jia, J. Y. Kim, H. J. Jun, S. J. Kim, J. H. Lee, M. H. Hoang, H. S. Kim, H. I. Chang, K. Y. Hwang, S. J. Um and S. J. Lee, 2013, 1831, 698-708.
62. Z.-C. Dang, V. Audinot, S. E. Papapoulos, J. A. Boutin and C. W. G. M. Löwik, *Journal of Biological Chemistry*, 2003, 278, 962-967.
63. K. W. Cho, O. H. Lee, W. J. Banz, N. Moustaid-Moussa, N. F. Shay and Y. C. Kim, *Journal of Nutritional Biochemistry*, 2010, 21, 841-847.
64. Y. Nishide, Y. Tousen, M. Inada, C. Miyaura and Y. Ishimi, 2013, 77, 201-204.
65. Y. Lee and E. J. Bae, 2013, 36, 1377-1384.
66. C. H. Jung, H. Kim, J. Ahn, T. I. Jeon, D. H. Lee and T. Y. Ha, 2013, 24, 1547-1554.
67. A. Ghorbani, M. Nazari, M. Jeddi-Tehrani and H. Zand, *European Journal of Nutrition*, 2012, 51, 39-46.
68. T. H. Quang, N. T. T. Ngan, C. V. Minh, P. V. Kiem, B. H. Tai, N. X. Nhiem, N. P. Thao, B. T. T. Luyen, S. Y. Yang and Y. H. Kim, 2013, 27, 1300-1307.
69. J. H. Chen, C. W. Tsai, C. P. Wang and H. H. Lin, 2013, 272, 313-324.
70. A. Iio, K. Ohguchi, M. Iinuma, Y. Nozawa and M. Ito, *Journal of Natural Products*, 2012, 75, 563-566.
71. L. Liu, S. Shan, K. Zhang, Z. Q. Ning, X. P. Lu and Y. Y. Cheng, *Phytotherapy Research*, 2008, 22, 1400-1403.
72. D. Halder, N. D. Das, K. H. Jung, M. R. Choi, M. S. Kim, S. R. Lee and Y. G. Chai, 2014, 38, 216-226.
73. M. Nazari, A. Ghorbani, A. Hekmat-Doost, M. Jeddi-Tehrani and H. Zand, *European Journal of Pharmacology*, 2011, 650, 526-533.

74. M. R. Byun, H. Jeong, S. J. Bae, A. R. Kim, E. S. Hwang and J. H. Hong, 2012, 50, 364-372.
75. L. Ding, D. Jin and X. Chen, *Journal of Nutritional Biochemistry*, 2010, 21, 941-947.
76. P. Thuillier, A. R. Brash, J. P. Kehrer, J. B. Stimmel, L. M. Leesnitzer, P. Yang, R. A. Newman and S. M. Fischer, *Biochemical Journal*, 2002, 366, 901-910.
77. J. Goldwasser, P. Y. Cohen, E. Yang, P. Balaguer, M. L. Yarmush and Y. Nahmias, *PLoS ONE*, 2010, 5.
78. M. L. Zhang, D. Irwin, X. N. Li, F. Sauriol, X. W. Shi, Y. F. Wang, C. H. Huo, L. G. Li, Y. C. Gu and Q. W. Shi, *Journal of Natural Products*, 2012, 75, 2076-2081.
79. H. Hui, Y. Chen, H. Yang, K. Zhao, Q. Wang, L. Zhao, X. Wang, Z. Li, N. Lu and Q. Guo, 2014, 134, 1195-1206.
80. U. H. Park, J. C. Jeong, J. S. Jang, M. R. Sung, H. Youn, S. J. Lee, E. J. Kim and S. J. Um, 2012, 35, 1525-1533.
81. S. Ulrich, S. M. Loitsch, O. Rau, A. Von Knethen, B. Brüne, M. Schubert-Zsilavecz and J. M. Stein, *Cancer Research*, 2006, 66, 7348-7354.
82. T. Saito, D. Abe and K. Sekiya, *Biochemical and Biophysical Research Communications*, 2008, 372, 835-839.
83. S. H. Yang, P. H. Liao, Y. F. Pan, S. L. Chen, S. S. Chou and M. Y. Chou, 2013, 27, 1154-1161.
84. E. J. Bak, J. Kim, Y. H. Choi, J. H. Kim, D. E. Lee, G. H. Woo, J. H. Cha and Y. J. Yoo, *Clinical Nutrition*, 2013.
85. E. J. Bak, J. Kim, Y. H. Choi, J. H. Kim, D. E. Lee, G. H. Woo, J. H. Cha and Y. J. Yoo, 2014, 33, 156-163.

86. J. Y. Lee, J. K. Kim, M. C. Cho, S. Shin, D. Y. Yoon, Y. S. Heo and Y. Kim, *Journal of Natural Products*, 2010, 73, 1261-1265.

Table 1 Effects of flavonoids on common PPAR-γ related endpoints. Regular print: positive association; italic print: negative association; underlined print: inactive.

	Reporter gene assays	Competitive binding / coregulator binding	Target gene expression	PPAR-γ expression	Adipocyte differentiation	PPRE binding/ activation
Alpinetin		53		53		
Apigenin	34,35	34,35		34	34,*54*	55
Baicalin	56		*57*		*57*	
Biochanin A	34,35,58,59	35,35				55
Calcycosin	58					
Chrysin	34,60	34	60	34,60	34	55
Cyanidin		61	61	61		
Daidzein	58,59,62,63					
Diosmetin	35	35				
Equol	59,63			64,*64*	*64*	
Eriodictyol	34,35	35				
(-)-Epigallocatechin -3-gallate	34					
Fisetin	34			*65*	*65,66*	67
Flavone	34					
Formononetin	58					
Formononetin	68					
Galangin	34					
Genistein	34,58,59,62	62				55
Glycitein	62					
Gossypetin				*69*		
Hesperetin	34,70,71		71	*72*		
Hesperidin						55,67,73
Isoquercetrin	35	35				
Isosakuranetin	34					
Kaempferol	34,35,33,32	34,35		34	34,74	
Luteolin	34,35	35	75			
Morin	34	76				67, 76
Myricetin	34					67
Naringenin	34,35,71,33, 32,77	35	71,77	*72*	*72*	77
Naringenin chalcone	33					
Naringin	34					
Odoratin	78					
Oroxylin A				79		*79*
Pinocembrin	34					
Quercetin	34,35,33,32	35,76		45	80	76
Resveratrol	81		81			
Rutin	34					
Sakuranetin	82					
Tangeretin	34					
Taxifolin		35				
Theaflavin-3,3'-digallate	34					
Vitexin	35	35		83		
Wogonin	84,85				85	
3,6-dihydroxyflavone	86					
3-hydroxyflavone	34					
5,7-dimethoxyflavone	34					
5-methoxyflavone	34					
7,8-dihydroxyflavone	34					

Figure Captions

Figure 1 PPAR-γ2 CALUX luciferase activity: Concentration-response curves of rosiglitazone, quercetin and kaempferol in the PPAR-γ2 CALUX assay determined by luciferase activity measurement. VitC (0.5 mM) is added to quercetin incubations to prevent auto-oxidation. Values are means ± standard deviations; concentrations of 0.7 log µM (flavonoids) and -2 log µM (rosiglitazone) are significantly different from solvent control ($p<0.05$). EC_{90} concentrations are indicated in the figure.

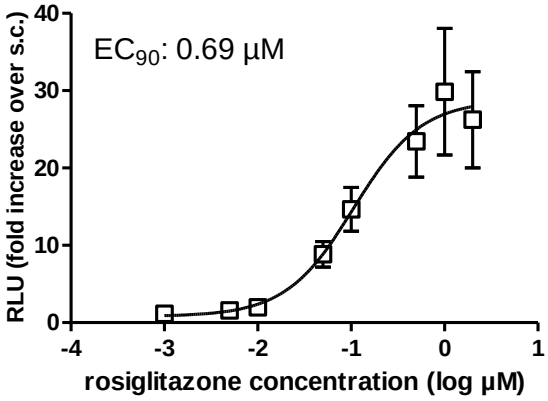
Figure 2 PPAR-γ2 reporter gene expression: Induction of the reporter gene expression (i.e. pGL4) by rosiglitazone (0.5 µM) and flavonoids (30 µM). VitC (0.5 mM) is added to quercetin and Q3G incubations to prevent auto-oxidation. Rosiglitazine, quercetin and kaempferol increase pGL4 expression. Values are means ± standard deviations. Statistically significant differences from solvent control: ** $p<0.01$, *** $p<0.001$.

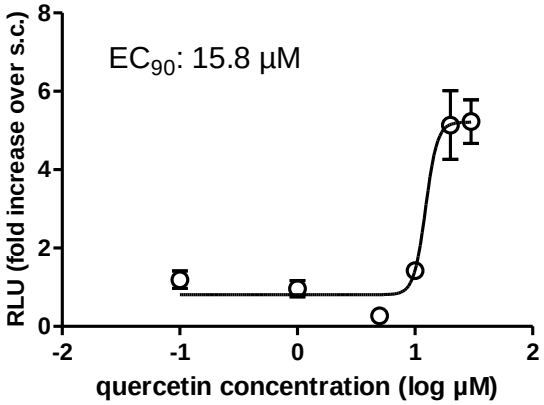
Figure 3 PPAR-γ LBD – coregulator interactions: Binding patterns of PPAR-γ LBD to coregulator-derived binding peptides exposed to rosiglitazone (red), quercetin (dark green), kaempferol (dark purple), Q3G (light green) and K3G (light purple) at EC_{90} concentrations derived from the reporter gene assay. Coregulator-derived binding peptides are plotted on the x-axis, the fluorescence signal indicating coregulator peptide binding is given on the y-axis. Rosiglitazone induces changes in binding to coregulator-derived peptides; quercetin, kaempferol, Q3G and K3G do not induce comparable changes. Values are means ± standard deviations.

Figure 4 PPAR-γ2 receptor-mRNA expression: Induction of PPAR-γ2-mRNA expression by rosiglitazone (0.5 µM) and flavonoids (30 µM). VitC (0.5 mM) is added to quercetin and Q3G incubations to prevent auto-oxidation. Quercetin, kaempferol and Q3G increase PPAR-γ2 receptor-mRNA expression. Values are means ± standard deviations. Statistically significant differences from solvent control: ** $p<0.01$, *** $p<0.001$.

Figure 5 PPAR-γ2 CALUX co-incubation of quercetin and rosiglitazone: Concentration-response curves of rosiglitazone in the absence and presence of 10 µM quercetin in the PPAR-γ2 CALUX determined by luciferase activity measurement; luciferase activity is expressed as percentage of maximum response by rosiglitazone alone. Data points on the y-axis are solvent control values in the absence of rosiglitazone; all concentrations of -1.3 log µM and higher are significantly different from solvent control ($p<0.01$). Quercetin synergistically increases reporter activity about 3-fold ($p<0.05$ at all concentrations). VitC (0.5 mM) is added to incubations to prevent auto-oxidation of quercetin. Values are means ± standard deviations.

Figure 1





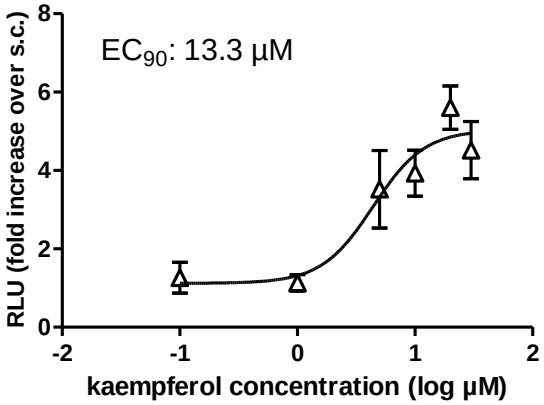


Figure 2

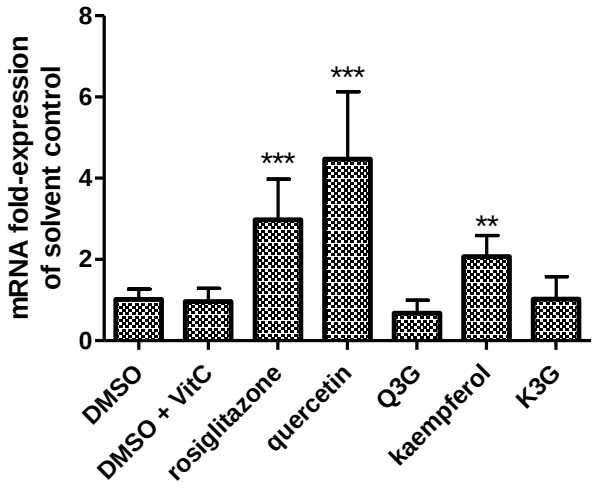


Figure 3

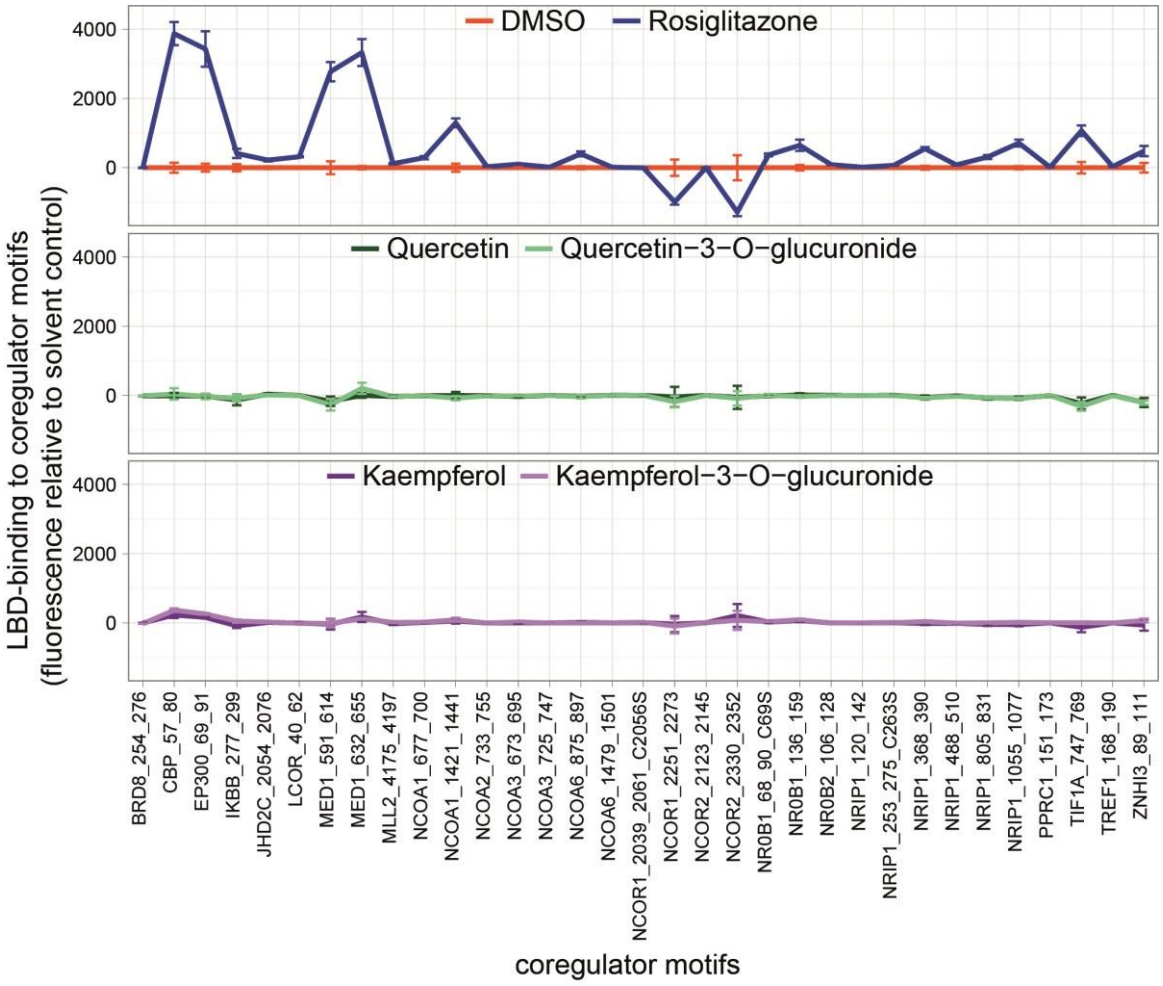


Figure 4

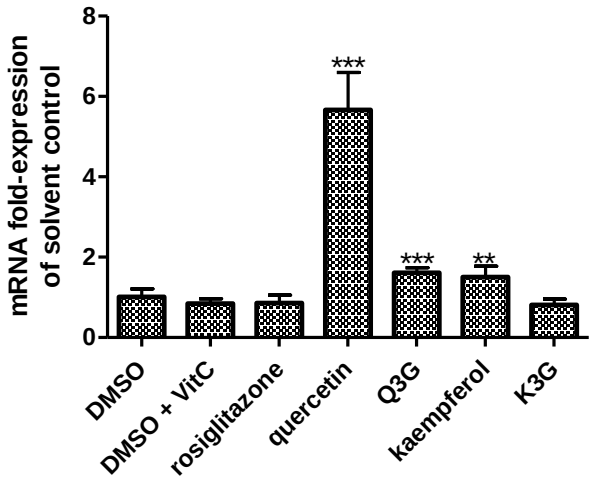


Figure 5