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The *O*-methylation of chrysin markedly improves its intestinal anti-inflammatory properties: Structure–activity relationships of flavones☆

Alexandrine During*, Yvan Larondelle

Institut des Sciences de la Vie, UCLouvain, B 1348 Louvain-la-Neuve, Belgium

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3′,4′,5,7-tetramethoxyflavone (PubChem CID 631170)
quercetin (PubChem CID 5280343)
3′,4′,3,5,7-pentamethoxyflavone (PubChem CID 97332)

ABSTRACT

The aim of this study was to investigate whether methoxylated flavones versus their unmethylated analogs can modulate the intestinal inflammatory response.

Flavone effects were assessed on soluble pro-inflammatory mediator (IL-8, IL-6, macrophage chemoattractant protein-1 (MCP-1), and cyclooxygenase-2 (COX-2)-derived PGE_2) production and on nuclear factor (NF)-κB activation in 3d-confluent and 21d-differentiated Caco-2 cells stimulated with interleukin (IL)-1β.

Chrysin (CHRY) showed anti-inflammatory properties by decreasing COX-2-derived PGE_2 and reducing NF-κB activation. Compared to CHRY, the dimethoxylated form (CHRY-DM) significantly reduced the secretion of all pro-inflammatory mediators, except IL-8, at both cellular stages ($P < 0.05$); these effects being dose-dependent in 3d-cells. The reduction of NF-κB activation was significantly more pronounced with CHRY-DM. By evaluating other flavones, it was established that several structural dispositions of flavones seemed to be determinant in order to attenuate the intestinal inflammatory response, such as methoxylation of the 5- and 7-hydroxyl groups on the A-ring, non-methoxylation of the 3′-hydroxyl groups on the B-ring, and methoxylation of the 3-hydroxyl group on the C-ring. Of all flavones examined, CHRY-DM exhibited the strongest anti-inflammatory activity.

These data indicate that, in the Caco-2 cell model, methoxylation of CHRY greatly improves its anti-inflammatory properties, probably through a more pronounced inhibition of the NF-κB signaling pathway. Nevertheless, methoxylation of other flavones was not systematically beneficial.

1. Introduction

Flavonoids are phenolic derivatives from natural origin that have shown beneficial health effects by displaying antioxidant, chelating, anti-inflammatory and/or anti-thrombotic activities, suggesting that they can protect against cardiovascular and neurodegenerative diseases, cancers, and diabetes [1,2]. They are classified into six structural groups according to the hydroxylation pattern and the degree of unsaturation of their backbone. Flavones and flavonols (or 3-hydroxy-flavones) are characterized by an unsaturated benzo-γ-pyrone (A, C-rings) substituted to a phenyl (B-ring) and up to seven hydroxyl groups surrounding their entire skeleton (Fig. 1). In that regard, in vitro structure–activity relationship studies have indicated that the

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Abbreviations: API, apigenin or 4′,5,7-trihydroxyflavone; API-TM, 4′,5,7-trimethoxyflavone; CHRY, chrysin or 5,7-dihydroxyflavone; CHRY-DM, dimethylated chrysin or 5,7-dimethoxyflavone; COMT, catechol-*O*-methyltransferase; COX, cyclooxygenase; CTL, control; 3′,4′-DHF, 3′,4′-dihydroxyflavone; 3′,4′-DMF, 3′,4′-dimethoxyflavone; DSS, dextran sodium sulfate; FBS, fetal bovine serum; IBDs, intestinal bowel diseases; IL, interleukin; LDH, lactate dehydrogenase; IECs, intestinal epithelial cells; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; LUT, luteolin or 3′,4′,5,7-tetrahydroxyflavone; LUT-QM, 3′,4′,5,7-tetramethoxyflavone; MCP-1, monocyte chemotactic protein-1; NEAA, nonessential amino acids; NF-κB, nuclear factor κB; QUER, quercetin or 3′,4′,3,5,7-pentahydroxyflavone; QUER-PM, 3′,4′,3,5,7-pentamethoxyflavone.

* Corresponding author.
E-mail address: alexandrine.during@univ-lille2.fr (A. During).

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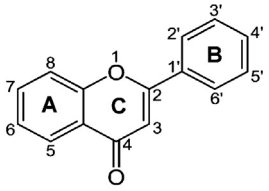

Positions	3	5	7	3'	4'	6, 8, 2', 5', 6'
CHRY	H	OH	OH	H	H	H
CHRY-DM		OCH_3	OCH_3			
3',4'-DHF	H	H	H	OH	OH	H
3',4'-DMF				OCH_3	OCH_3	
API	H	OH	OH	H	OH	H
API-TM		OCH_3	OCH_3		OCH_3	
LUT	H	OH	OH	OH	OH	H
LUT-QM		OCH_3	OCH_3	OCH_3	OCH_3	
QUER	OH	OH	OH	OH	OH	H
QUER-PM	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	

Fig. 1. Chemical structures of flavones and their methoxylated forms used in the present study. Abbreviations: CHRY, chrysin or 5,7-dihydroxyflavone; CHRY-DM, dimethylated chrysin or 5,7-dimethoxyflavone; 3′,4′-DHF, 3′,4′-dihydroxyflavone; 3′,4′-DMF, 3′,4′-dimethoxyflavone; API, apigenin or 4′,5,7-trihydroxyflavone; API-TM, 4′,5,7-trimethoxyflavone; LUT, luteolin or 3′,4′,5,7-tetrahydroxyflavone; LUT-QM, 3′,4′,5,7-tetramethoxyflavone; QUER, quercetin or 3′,4′,3,5,7-pentahydroxyflavone; QUER-PM, 3′,4′,3,5,7-pentamethoxyflavone.

number and position of hydroxyl groups on the flavonoid structure were one of the major features. For instance, the hydroxyl configuration on the B-ring of flavonoids appears to be determinant for their antioxidant activity as well as their inhibitory effect on cytochrome P450 1A [3,4], while hydroxyl groups in positions 5 and 7 on the A-ring were identified as one of the main structural characteristics allowing to lower the endothelial adhesion molecule expression [5]. However, hydroxyl groups are rarely found free on the flavonoid structure, thus questioning the validity of these structure–activity relationships in vivo.

As a result of the methylation of their free hydroxyl groups, flavones can be found *O*-methylated (or methoxylated) in both plant and animal kingdoms [6,7]. That reaction is catalyzed by several *O*-methyltransferases in plants [8], and by the catechol-*O*-methyltransferase (COMT, EC 2.1.1.6) in animals [9]. COMT activity is expressed in all animal tissues, including the intestine [10]. As such, methoxylated flavones present in the enterocytes can thus originate both from ingested plant-derived foods and from in situ biotransformation. The *O*-methylation of flavones not only generates more lipophylic molecules, but also improves their intestinal absorption and metabolic stability [11,12]. Due to their increased lifespan in our body [11,12], methoxylated flavones are more able to induce potential health effects in vivo as compared to their parent unmethylated analogs.

In terms of biological activities, only a few studies have examined the *O*-methylation impact on flavone properties. The most studied (poly)methoxylated flavones, which have more than 5 methoxyl groups and are mostly originating from citrus (i.e., tangeretin and nobiletin), were reported to exhibit anti-proliferative and anti-inflammatory properties [13–15], but these effects were not compared to those of the respective unmethylated analogs. Methoxylated flavones with lesser methoxyl groups (i.e., 5,7-dimethoxyflavone, 4′,5,7-trimethoxyflavone) showed more potent cancer chemopreventive activities than their unmethylated analogs (chrysin and apigenin, respectively) in human oral SCC-9 cancer cells and hepatoma HepG2 cells [12,16], but not in human leukemia HL60 cells [17].

In regard to our research interest [18], the aim of the present study was to further assess the effects of methoxylated flavones versus their unmethylated analogs on the production of inflammatory mediators in an in vitro intestinal inflammation model (e.g., the human Caco-2 cells stimulated by IL-1β). A particular attention was paid to the natural flavone chrysin (CHRY) that was reported to improve murine inflammatory bowel diseases (IBDs) by reducing the production of inflammatory markers in the dextran sodium sulfate (DSS)-administrated mice model of intestinal inflammation [19]. Thus, we were interested to see if *O*-methylation of CHRY might affect its intestinal anti-inflammatory properties.

2. Materials and methods

2.1. Chemicals

Chrysin (CHRY) and quercetin (QUER) were purchased from Sigma–Aldrich (Schnelldorf, DE). Chrysin dimethylether (CHRY-DM or 5,7-DMF), 3′,4′-dihydroxyflavone (3′,4′-DHF), 3′,4′-dimethoxyflavone (3′,4′-DMF), apigenin (API), apigenin-4′,5,7-trimethylether (API-TM), luteolin (LUT), luteolin tetramethylether (LUT-TM), and quercetin-3′,4′,3,5,7-pentamethylether (QUER-PM) were from Extrasynthese (Genay, FR). All these compounds presented a purity ≥95%. DMSO (99.8+%) was obtained from ACROS Organics (Thermo Fisher Scientific, Geel, BE). Human IL-1β recombinant (>98%), arachidonic acid from porcine liver (≥99%), and taurocholic acid (≥95%) were from Sigma–Aldrich.

2.2. Cell culture

Caco-2 cells (passage 18) derived from a human colon adenocarcinoma and obtained from the American Type Culture Collection (ATCC# HTB-37, LGC Promochem, Molsheim, FR) were grown in the presence of Dubelcco's modified Eagles medium (DMEM) containing 25 mM glucose and 4 mM glutamine and supplemented with 1% (v/v) of a non-essential amino acid (NEAA) solution at 10 mM, 1% (v/v) of an L-glutamine solution at 200 mM (Invitrogen, Merelbeke, BE) and 10% (v/v) of heat-inactivated fetal bovine serum (FBS) (Perbio Science, Erembodegem, BE), in previously described cell culture conditions [20]. Cells were seeded at a density of 4×10^4 and 2×10^4 cells/cm^2, respectively to reach confluence (3d) or full differentiation (21d).

2.3. Cytotoxicity assay

Flavonoid cytotoxicity was determined by using a colorimetric lactate dehydrogenase (LDH) assay (Cytotoxicity detection kit (LDH), Roche Diagnostics, Mannheim, DE).

2.4. Preparation of polyphenol solutions

Flavone standards were solubilized in DMSO at stock concentrations of 100–200 mM and stored at −20 °C.

2.5. Induction of inflammation

Cells were grown on 12 well-plates for 3d or 21d. Then, the growth medium was replaced by 1 mL of DMEM supplemented with 1% of a 10 mM NEAA and 0.5% FBS (without phenol red) and the cells from two wells were exposed to either the medium alone (control cells), or the vehicle DMSO (vehicle control), or one flavonoid at a concentration up to 50 μM. After 1 h pre-incubation, inflammation was induced in one of the two pre-treated wells by adding IL-1β (25 μg/L). Cells were further incubated for 24 h. Flavonoid effects were thus examined under unstimulated and IL-1β-stimulated conditions.

2.6. IL-8, IL-6 and MCP-1 levels in the cell culture medium

Following a treatment (medium only or DMSO or polyphenol, with or without IL-1β), cell culture media were harvested and centrifuged at 3000 × *g* for 10 min, and the supernatants were analyzed for their contents in IL-8, IL-6 and monocyte chemotactic protein-1 (MCP-1) by using, respectively, the Human IL-8, IL-6 and MCP-1 ELISA Kits, according to the manufacturer's instructions (BD Biosciences, Erembodegem, BE). Values are expressed as pg of IL-8, IL-6 or MCP-1 secreted per mg of cellular protein.

2.7. COX-2-derived PGE_2 production by cells

Following a treatment (medium only or DMSO or polyphenol, with or without IL-1β), the cells were assayed for cyclooxygenase-2 (COX-2)-derived PGE_2 production as described previously [20]. Briefly, cells were incubated with HBSS supplemented with arachidonic acid (10 μM) and taurocholate (250 μM) for 10 min at 37 °C. PGE_2 levels in cell culture media were determined using the PGE_2 Enzyme Immunoassay ACE™ Competitive EIA Kit (Cayman Chemical, Montigny le Bretonneux, FR). COX-2-derived PGE_2 data were calculated by difference between the PGE_2 quantity produced by IL-1β-stimulated cells and the basal PGE_2 level produced by unstimulated cells, and were expressed as pg of PGE_2 produced per mg of cellular protein.

2.8. NF-κB signaling pathway reporter luciferase assay

CHRY and CHRY-DM effects on NF-κB activation by IL-1β were examined using the Cignal NF-κB Reporter Luciferase Kit (CCS-013L, SABiosciences, Boechout, BE). Caco-2 cells (passage <34, viability ≥94%) were seeded into 96-well microplates and allowed to grow to reach ~80% confluence. Cells were then transfected with 100 ng DNA reporter/well using Lipofectamine LTX as transfection reagent (Invitrogen). The DNA reporter consisted of a mixture of an NF-κB-responsive firefly luciferase construct and a constitutively expressing Renilla luciferase construct (40:1). Transfection was carried out for 24 h at 37 °C. Cells were then treated with or without IL-1β and a flavonoid. After 18 h of incubation, firefly and Renilla luciferase activities were sequentially measured with the Dual-Glo™ Luciferase Assay system according to the manufacturer's instructions (E2920, Promega Benelux, Leiden, NL) and using a Fluoroskan Ascent Microplate Fluorometer (Thermo Fisher Scientific). For each experimental condition, the signal (*S*) firefly/Renilla was determined as described previously [21].

2.9. Cellular protein concentration assay

Cellular protein concentration was determined by the bicinchoninic acid assay (Sigma–Aldrich) using bovine serum albumin as standard.

2.10. Statistical analysis

Data are means ± SEM. All data were tested for homogeneous variances by the Bartlett test. If variances were homogeneous, data were further analyzed by one-way ANOVA, followed by the post hoc Tukey–Kramer HSD test for multiple comparisons of means or the Dunnett test for comparison with a control. When unequal variances were detected, the non-parametric multiple comparison Steel–Dwass test was used. For NF-κB signaling pathway data, differences between two groups were assessed using the unpaired Student's *t* test. Statistical analyses were performed using the version 10 of JMP software (SAS Institute, Cary, NC). Differences were considered significant at $P < 0.05$.

3. Results

3.1. Cytotoxic effects of unmethylated and methoxylated flavones

Following 24 h of incubation with unmethylated and methoxylated flavone standards at 50 μM, both 3d-confluent and 21d-fully differentiated Caco-2 cells did not exhibit any significant cytotoxicity (less than 5%), compared to the positive control Triton X-100 at 1%. Globally, 3d-cells were less sensitive to flavone treatments than 21d-cells. For instance, at 50 μM, CHRY and CHRY-DM treatments resulted in cytotoxicity of 0.7 and 0.5% in 3d-cells and 4.1 and 3.0% in 21d-cells, respectively (data not shown).

3.2. Effects of CHRY and CHRY-DM on IL-1β-induced secretions of IL-8, IL-6 and MCP-1

3.2.1. Secretion of IL-8

Basal secretions of IL-8 were ~90 and 50 pg/mg protein, respectively in 3d- and 21d-Caco-2 cells. These values were unchanged when the cells were incubated with the vehicle DMSO or with CHRY or CHRY-DM (Fig. 2A and B). Under IL-1β induction, IL-8 secretion was increased by ~70 to 90-fold, respectively in 3d- and 21d-cells (8300 and 3300 pg of IL-8/mg protein). These values were not changed by DMSO or CHRY. In comparison to CHRY, CHRY-DM significantly reduced IL-8 by 2.2-fold in 3d-cells ($P < 0.001$), but did not show any significant effect in 21d-cells.

3.2.2. Secretion of IL-6

Basal levels of IL-6 were ~17 and 4 pg/mg protein, respectively for 3d- and 21d-Caco-2 cells. These basal values were unchanged when the cells were incubated with the vehicle DMSO or with CHRY or CHRY-DM (Fig. 2C and D). Under IL-1β stimulation, IL-6 secretion was increased by ~14- and 26-fold, respectively in untreated 3d- and 21d-cells (200 and 104 pg of IL-6/mg protein). These values were not affected by DMSO and CHRY. In contrast, CHRY-DM significantly reduced IL-1β-induced IL-6 secretion by 8- and 9-fold compared to CHRY, respectively in 3d- and 21d-cells ($P < 0.05$).

3.2.3. Secretion of MCP-1

Basal secretions of MCP-1 were ~300 and 50 pg/mg protein, respectively in untreated 3d- and 21d-Caco-2 cells. These values were unchanged when the cells were incubated with the vehicle DMSO or with CHRY or CHRY-DM (Fig. 2E and F). Under IL-1β stimulation, levels of MCP-1 were increased by ~8- and 13-fold, respectively in control 3d- and 21d-cells (2430 and 630 pg of MCP-1/mg protein). These values were not significantly changed by the vehicle DMSO or by CHRY. Again here, CHRY-DM significantly reduced IL-1β-induced MCP-1 secretion by ~9- and 6-fold compared to DMSO, and by ~10- and 6-fold compared to CHRY, respectively in 3d- and 21d-cells ($P < 0.05$).

3.3. Effect of CHRY and CHRY-DM on COX-2-derived PGE_2 production

In the absence of IL-1β, basal levels of PGE_2 produced by Caco-2 cells were ~980 and 95 pg PGE_2/mg of protein, respectively for 3d- and 21d-cells. These basal PGE_2 levels were unchanged in the presence of the vehicle DMSO or of CHRY or CHRY-DM (data not shown). After stimulation with IL-1β, PGE_2 production was increased by 2.3- and 1.6-fold, respectively in 3d- and 21d-cells (data not shown). The difference between the IL-1β-induced PGE_2 level and the basal PGE_2 level is assumed to be associated to the COX-2 activity (i.e. COX-2-derived PGE_2) and is presented in Fig. 3A and B. CHRY significantly reduced the COX-2-derived PGE_2 level in 3d-cells (2.5-fold, $P < 0.05$) and tended to do it in 21d-cells ($P = 0.076$) in comparison to DMSO. Interestingly enough, these

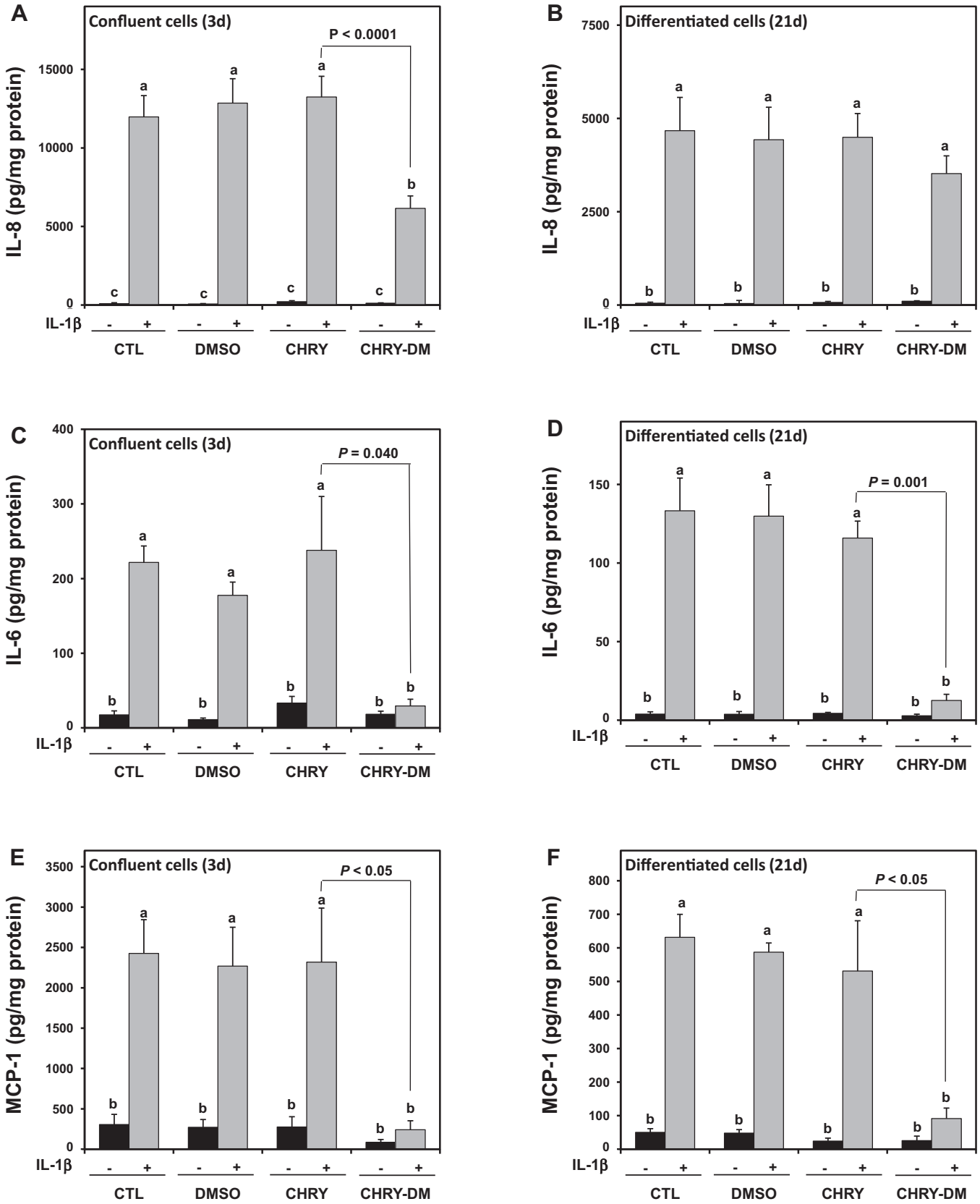

Fig. 2. Effects of chrysin (CHRY) and its dimethoxylated form (CHRY-DM) (50 μM) on IL-8 secretion (graphs A and B), IL-6 secretion (graphs C and D) and MCP-1 secretion (graphs E and F) by 3d-confluent and 21d-differentiated Caco-2 cells treated with or without IL-1β. Values are means ± SEM, n = 5–6 independent experiments. Means without a common letter differ ($P < 0.05$). CTL, cells exposed to the medium alone; DMSO, cells treated with the vehicle DMSO.

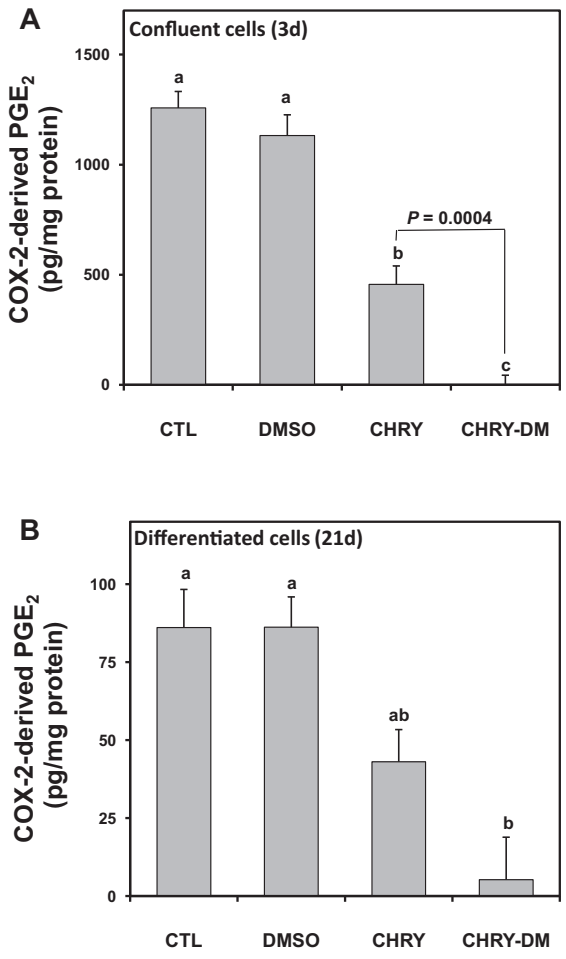

Fig. 3. Effects of chrysin (CHRY) and its dimethoxylated form (CHRY-DM) (50 μM) on COX-2-related PGE_2 production by 3d-confluent (graph A) and 21d-differentiated (graph B) Caco-2 cells treated with IL-1β. Values are means ± SEM, $n = 4$ independent experiments. Means without a common letter differ ($P < 0.05$). CTL, cells exposed to the medium alone; DMSO, cells treated with the vehicle DMSO.

effects were significantly more pronounced (3d) or became significant (21d) with CHRY-DM.

3.4. Dose effects of CHRY-DM on IL-8, IL-6 and MCP-1 secretions and COX-2-derived PGE_2 production

There was no significant dose effect of CHRY on IL-8, IL-6, and MCP-1 secretions and on COX-2-derived PGE_2 production (data not shown). In contrast, CHRY-DM reduced IL-8, IL-6 and MCP-1 secretions, as well as COX-2-derived PGE_2 production, in a dose-dependent manner (Fig. 4A–D). IC_{50} were 12.5, 10 and 3 μM, respectively for IL-6, MCP-1 and PGE_2, while no IC_{50} was determined for IL-8 since none of the CHRY-DM concentrations used was able to induce at least 50% inhibition.

3.5. Effect of CHRY and CHRY-DM on NF-κB activation by IL-1β in Caco-2 cells

CHRY and CHRY-DM dose-dependently decreased IL-1β-induced NF-κB activation and, for a same concentration, that inhibitory effect was more pronounced for CHRY-DM (Fig. 5). For instance, IL-1β-induced NF-κB activation was reduced by 23% and 41% at 50 μM and by 38% and 62% at 100 μM, respectively for CHRY and CHRY-DM treatments.

3.6. Relationships between structure and anti-inflammatory activity of methoxylated derivatives

Table 1 summarizes the effects of different unmethylated flavones (CHRY, 3′,4′-DHF, API, LUT and QUER) and their respective methoxylated derivatives (CHRY-DM, 3′,4′-DMF, API-TM, LUT-QM, and QUER-PM) on IL-8, IL-6, and MCP-1 secretions and on COX-2-derived PGE_2 production by Caco-2 cells under IL-1β stimulation.

Among all the unmethylated flavones and in comparison to the vehicle DMSO, LUT showed the strongest anti-inflammatory properties by reducing IL-1β-induced IL-6, MCP-1 and PGE_2 secretions, followed by 3′,4′-DHF and API that decreased IL-6 and MCP-1, QUER that reduced IL-6 and PGE_2, and finally CHRY that diminished PGE_2, at least in 3d-cells. Looking at these results in terms of hydroxyl positions, we can observe that reduction of IL-6 and MCP-1 is mostly favored by the 3′,4′-dihydroxyl substitution of the flavone B-ring, whereas the 5,7-dihydroxyl substitution of the flavone A-ring has a more pronounced reducing effect on PGE_2. In contrast, the 3-hydroxyl substitution of the flavone C-ring appears not favorable for the reduction of MCP-1.

Among the methoxylated flavones and in comparison to DMSO, CHRY-DM showed the strongest anti-inflammatory properties by reducing IL-6, MCP-1 and PGE_2 secretions/production in both 3d- and 21d-cells and IL-8 secretion in 3d-cells, followed by API-TM and LUT-QM that decreased IL-6, MCP-1 and PGE_2 at least at one cellular stage, 3′,4′-DMF that reduced IL-6 and PGE_2, and finally QUER-PM that tended to diminish PGE_2, in 3d-cells.

The reducing effects of CHRY-DM on all pro-inflammatory markers remained significant when compared to its unmethylated analog CHRY, indicating that methoxylation of the 5- and 7-hydroxyl groups on the A-ring enhanced its anti-inflammatory properties. Interestingly, 3′,4′-DMF and LUT-QM, but not API-TM, resulted in a marked increase of MCP-1 secretion (>190%), in comparison to their respective analogs 3′,4′-DHF and LUT, suggesting that methoxylation of the 3′-hydroxyl group, but not that of 4′-hydroxyl group, on the B-ring stimulated pro-inflammatory properties of the flavones. Finally, QUER-PM did not show any effect on MCP-1 secretion compared to its parent molecule QUER, suggesting that the presence of a 3-methoxyl group on the C-ring canceled the pro-inflammatory effect associated to the 3′-methoxy group on the B-ring.

4. Discussion

The *O*-methylation of flavonoids was reported to increase their bioavailability as well as to improve their chemopreventive properties [11,12]. In addition, accumulating evidence indicate that polyphenols can modulate the intestinal inflammatory response and thus could be used as natural preventive agents against inflammatory bowel diseases (IBDs) [18]. Therefore, we were interested to see if methoxylated vs. unmethylated flavones presented different intestinal anti-inflammatory activities by using the IL-1β-stimulated Caco-2 cell model. The flavone concentration commonly used in the present study (50 μM) was not cytotoxic for the cells and was assumed to be a physiological one, since dietary polyphenols can reach concentrations up to several hundred μM in the gut lumen [7]. Anti-inflammatory properties of flavones were examined at two cellular stages (confluency and full differentiation), because the intestinal barrier is in perpetual renewal and bears cells at different stages of maturity. After 24 h-exposure with IL-1β, Caco-2 cells responded by increasing the secretion/production of the pro-inflammatory mediators IL-8, IL-6, MCP-1, and COX-2-derived PGE_2 and that inflammatory response was more pronounced for confluent cells than for differentiated cells, as previously noticed [20,21]. Under IBDs conditions, there is an uncontrolled intestinal inflammatory

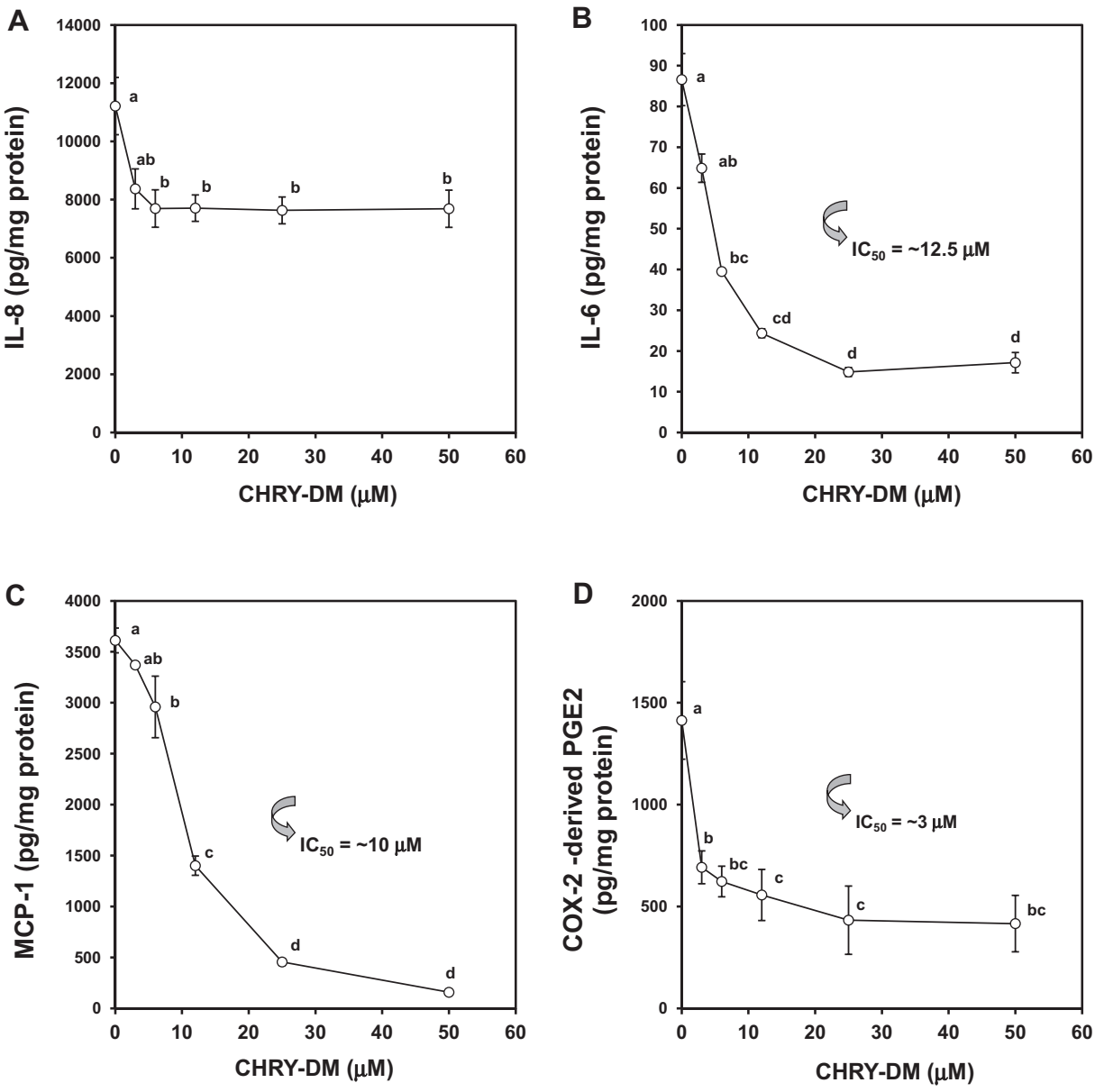

Fig. 4. Dose effect of dimethoxylated chrysin (CHRY-DM) (up to 50 μM) on the secretion of IL-8 (A), IL-6 (B), MCP-1 (C) and COX-2-related PGE_2 production (D) in IL-1β-stimulated 3d-cells. For each marker secretion, values are means ± SEM of 3 replicate wells. Means without a common letter differ ($P < 0.05$).

response toward the overproduction of pro-inflammatory mediators and in which intestinal epithelial cells (IECs) participate [22]. As such, freshly isolated IECs, issued from IBD patients or from normal individuals and exposed to IL-1β, showed a significant increased expression and/or secretion of IL-8, IL-6, MCP-1 and COX-2 compared to unstimulated IECs from normal individuals [23–25], pointing out the relevance to study these soluble markers in our in vitro intestinal inflammation model.

CHRY was recently reported to improve murine IBDs by reducing PGE_2, IL-1β, IL-6, IL-8, MCP-1 and TNF-α production in a DSS-induced colitis mouse model [19]. In IL-1β-stimulated Caco-2 cells, CHRY also reduced COX-2-derived PGE_2 production, but was unable to decrease IL-6, IL-8 and MCP-1 secretions, thus contrasting with the previous in vivo data [19]. Discrepancies between the two studies could be attributed to the model and/or to the chemical molecule employed to induce inflammation. Furthermore, along with other in vitro data using stimulated intestinal cells [19,26], we reported that CHRY inhibited NF-κB activation, which is a key transcription factor in the intestinal inflammatory response by controlling the expression of multiple pro-inflammatory genes, e.g., genes encoding for COX-2, IL-8, IL-6 and MCP-1 [27]. CHRY could thus attenuate the PGE_2-COX-2 pathway via the inhibition of the NF-κB activation cascade.

CHRY-DM has shown remarkable properties by being a chemopreventive, antiviral, anticholinesterase, vasodilatator, and pigment stimulator agent [16,28–32]. A few studies have also suggested some anti-inflammatory effects for CHRY-DM. For instance, CHRY-DM reduced carrageenan-induced paw edema in rats [33], inhibited inducible nitric oxide synthase (iNOS) expression in lipopolysaccharide (LPS)-treated macrophages (RAW 267.4 cells) [34], and attenuated NF-κB signaling pathway in an early hepatocarcinogenesis rat model [28]. CHRY-DM also inhibited NF-κB and mitogen-activated protein kinase/activator protein-1 pathways and strongly suppressed IL-6 and IL-8 syntheses in human skin fibroblast cells [35]. However, to the best of our knowledge, nor such study was performed in an intestinal model, neither CHRY-DM effect was compared to its unmethylated analog. In IL-1β-stimulated Caco-2 cells, CHRY-DM

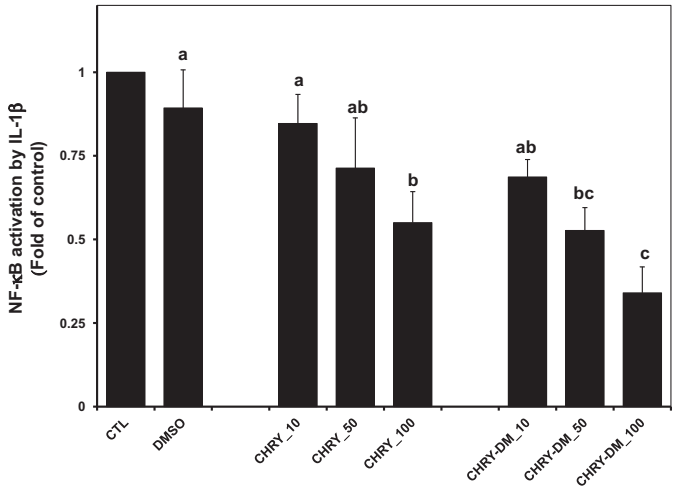

Fig. 5. Effects of phenolic compounds on NF-κB activation by IL-1β. Cells were transfected with a DNA reporter (mixture of NF-κB-responsive firefly luciferase construct and a constitutively expressing Renilla luciferase construct (40:1)). Transfected cells were then treated with either chrysin (CHRY) or dimethoxylated chrysin (CHRY-DM) at 10, 50 or 100 μM in the presence or not of IL-1β. Values are means ± SEM, $n = 3$ independent experiments. Data without a common letter differ ($P < 0.05$). CTL, cells exposed to the medium alone; DMSO, cells treated with the vehicle DMSO.

markedly reduced IL-8, IL-6, MCP-1 and COX-2-related PGE_2 secretion/production and inhibited more profoundly NF-κB activation than CHRY.

These improved anti-inflammatory properties of CHRY-DM could be due to the fact that CHRY-DM was more efficiently transported through Caco-2 cell monolayers and showed a greater metabolic stability than CHRY [11]. On another hand, it was suggested that CHRY bioactivity could be seriously compromise due to its extensive glucuronidation and sulfation in the enterocyte associated with a quantitative apical efflux of the metabolites formed [36]. Thus, the *O*-methylation of CHRY could protect against that important intestinal metabolism. As a result, CHRY-DM might see its intracellular concentration increased and thus could be more efficient to target intracellular molecules/reactions involved in the NF-κB activation cascade, e.g., inhibitor κBα (IκBα) phosphorylation/degradation, IκB kinase activation, NF-κB binding to DNA, as seen for other polyphenols [37].

In regard to the CHRY data and since methylation improves intestinal flavonoid absorption in general [11], we were then interested to see if other methoxylated flavones also presented beneficial effects. The unmethylated flavones exhibited anti-inflammatory activities according to the decreasing order of efficiency: LUT > API and 3′,4′-DHF > QUER > CHRY. In an induced ear edema mouse model, flavones showed a similar order of efficiency, namely LUT > API > CHRY, for reducing the systemic TNF-α production [38]. Of numerous flavone derivatives examined, LUT and 2′,3′,5,7-tetrahydroxyflavone showed the strongest inhibitory effects on iNOS in LPS-treated RAW 267.4 cells [39]. Taken together, the data suggest that the presence of hydroxyl groups on the B-ring of unmethylated flavones determines the degree of their anti-inflammatory activity, possibly in relation to their conjugative metabolism. In that regard, it would be interesting to examine if the conjugation rate of unmethylated flavones followed an inverse order to the one observed for their anti-inflammatory properties in the intestinal cells.

The *O*-methylation of flavones altered their anti-inflammatory capacity on one or another way, depending on the type of pro-inflammatory marker studied. For instance, *O*-methylation of both 5- and 7-hydroxyl groups on the A-ring accentuated COX-2-derived PGE_2 inhibition. Of all the methoxylated and unmethylated flavones evaluated, CHRY-DM exhibited the strongest anti-inflammatory activity, suggesting that 5- and 7-methoxyl groups are determinant for attenuating the intestinal inflammatory response. Similarly, only flavonoids with methoxyl groups in 5- and 7-positions on the A-ring potently inhibited edema formation in rat paws [40]. In contrast, methoxylation of the 3′-hydroxyl group on the B-ring resulted in a marked increase of MCP-1, suggesting that free 3′-hydroxyl group is required to lower MCP-1

Table 1
Percent changes of pro-inflammatory marker secretion by IL-1β-stimulated Caco-2 cells in relation to the treatment with different flavones at 50 μM in their unmethylated and methoxylated forms.

Stages of cells	IL-8		IL-6		MCP-1		PGE_2	
	3d[a]	21d	3d	21d	3d	21d	3d	21d
Unmethylated flavone vs. DMSO[b]								
CHRY	+3%	+2%	+34%	−11%	+2%	−10%	−60%**	−50%†
3′,4′-DHF	+27%	+33%	−76%**	−77%**	−81%**	−67%*	−59%	−32%
API	+53%	+30%	−73%**	−61%*	−72%**	−50%	−57%	−82%
LUT	+18%	+24%	−64%*	−56%*	−92%***	−88%***	−116%*	+26%
QUER	−33%	−5%	−47%*	−45%†	+10%	−34%	−110%*	−112%*
Methoxylated flavone vs. DMSO								
CHRY-DM	−52%**	−20%	−83%*	−88%**	−89%*	−84%*	−128%***	−93%**
3′,4′-DMF	+7%	−21%	−75%**	−80%**	−19%	−2%	−77%*	−59%
API-TM	+41%	−6%	−63%	−86%**	−85%**	−88%†	−91%†	−103%†
LUT-QM	+4%	−29%	−81%*	−76%*	−46%*	−6%	−112%*	−23%
QUER-PM	−4%	+70%	−31%	−43%	+1%	+3%	−100%†	−58%
Methoxylated vs. unmethylated flavone								
CHRY-DM vs. CHRY	−59%***	−23%	−91%*	−89%**	−90%*	−83%*	−75%**	−88%**
3′,4′-DMF vs. 3′,4′-DHF	−16%	−29%	+2%	−14%	+465%*	+193%*	−51%	−38%
API-TM vs. API	−10%	−28%	+35%	−64%	−52%	−77%	−40%	−115%
LUT-QM vs. LUT	−11%	−43%	−45%	−51%	+601%**	+693%***	+28%	−39%
QUER-PM vs. QUER	+15%	+79%**	+11%	+4%	−8%	+57%	+98%	+44%

[a] Statistical analyses were realized by multiple comparison tests between the four groups: CTL, DMSO, unmethylated flavone, and methylated flavone.
[b] DMSO: dimethylsulfoxide; CHRY, chrysin or 5,7-dihydroxyflavone; CHRY-DM, dimethylated chrysin or 5,7-dimethoxyflavone; 3′,4′-DHF, 3′,4′-dihydroxyflavone; 3′,4′-DMF, 3′,4′-dimethoxyflavone; API, apigenin or 4′,5,7-trihydroxyflavone; API-TM, 4′,5,7-trimethoxyflavone; LUT, luteolin or 3′,4′,5,7-tetrahydroxyflavone, LUT-QM, 3′,4′,5,7-tetramethoxyflavone; QUER, quercetin or 3′,4′,3,5,7-pentahydroxyflavone; QUER-PM, 3′,4′,3,5,7-pentamethoxyflavone.
* $P < 0.05$.
** $P < 0.005$.
*** $P < 0.0005$.
† Tendency with $0.05 < P < 0.1$.

secretion. Furthermore, the presence of either a 3-hydroxyl or 3-methoxyl group on the C-ring canceled either MCP-1 inhibition or MCP-1 activation, respectively associated to the presence of 3′- and 4′-hydroxyl groups or of 3′-methoxyl group. These data suggested a "spatial" interaction between the 3-position of the C-ring and the 3′- and 4′-positions of the B-ring, and also highlight the importance of the conformation of the flavone molecule for its anti-inflammatory capability. Note that the 3-hydroxyl group on the C-ring can form intramolecular hydrogen bonds with the 3′- and 4′-hydroxyl groups on the B-ring, leading to a planar structure of the flavonoid [41].

In summary, the present study indicates that *O*-methylation of CHRY greatly improves its anti-inflammatory properties. Of all flavones, CHRY-DM exhibited the highest anti-inflammatory activity by reducing IL-8, IL-6 and MCP-1 secretions and COX-2-related PGE_2 production. In terms of structure–activity relationship, several structural dispositions of flavones seemed to be determinant in order to attenuate the intestinal inflammatory response, such as methoxylation of the 5- and 7-hydroxyl groups on the A-ring, non-methoxylation of the 3′-hydroxyl group on the B-ring, and methoxylation of the 3-hydroxyl group on the C-ring. However, more flavonoid molecules need to be evaluated and other intestinal inflammatory models than the present in vitro model should be used in order to draw definitive conclusions about those structural requirements in regard to methoxylation and anti-inflammatory capability. Nevertheless, these preliminary findings can be useful to direct syntheses of new flavonoid *O*-methyl analogs toward prevention and/or treatment of IBDs.

Conflict of interest

The authors have no conflicts of interest.

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