Original Article

Effect of Diplacone on LPS-Induced Inflammatory Gene Expression in Macrophages

(geranyl flavanone / inflammation / MCP-1 / mRNA / TNF-α / ZFP36)

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Abstract. Flavonoids are commonly studied for their anti-inflammatory effects; however, this is the first paper describing the possible antiphlogistic activity of a geranylated flavanone. This study focused on the ability of diplacone to modulate the gene expression of pro-inflammatory tumour necrosis factor α and monocyte chemoattractant protein 1, and of anti-inflammatory zinc finger protein 36. The action of diplacone was also compared with that of conventional drug indomethacin. Human monocyte-derived macrophages of the human monocytic leukaemia cell line were pretreated with diplacone or indomethacin. Subsequently, inflammatory reaction was induced by lipopolysaccharide, and changes of tumour necrosis factor α, monocyte chemoattractant protein 1 and zinc finger protein 36 gene expression at the transcriptional level were measured. In this model, diplacone significantly down-regulated the expression of tumour necrosis factor α and monocyte chemoattractant protein 1 and up-regulated the zinc finger protein 36 expression. This makes diplacone a promising molecule for treatment of the inflammatory stage of diseases. The effect of diplacone in decreasing lipopolysaccharide-induced inflammatory gene expression is in many ways similar to that of the conventional drug indomethacin.

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Abbreviations: AU – arbitrary unit, AUC – area under curve, DMSO – dimethylsulphoxide, FBS – foetal bovine serum, ERK – extracellular receptor kinase, iNOS – inducible isoform of nitric oxide synthase, LPS – lipopolysaccharide, MCP-1 – monocyte chemoattractant protein 1, PBS – phosphate-buffered saline, PCR – polymerase chain reaction, PMA – phorbol myristate acetate, RT-qPCR – reverse transcription quantitative polymerase chain reaction, THP-1 – human monocytic leukaemia cell line, TNF-α – tumour necrosis factor α, TTP – tristetraprolin, ZFP36 – zinc finger protein 36.

Introduction

Diplacone (also known as propoline C or nymphaeol A) is a naturally occurring geranyl flavanone that belongs to plant polyphenols (Fig. 1). Wu et al. (2008) demonstrated that adding an alkyl side chain can markedly change the features of the original natural compound. Plant polyphenols, especially flavonoids, have been studied intensively for their potential therapeutic applications, as was described in two excellent reviews (Havsteen, 2002; Dixon, 2004). Diplacone has been found in extracts of *Paulownia tomentosa* Steud. (*Scrophulariaceae*) (Smejkal et al., 2007), *Macaranga tanarius* (L.) Muell. Arg. (*Euphorbiaceae*) (Phommart et al., 2005), *Macaranga alnifolia* Baker (*Euphorbiaceae*) (Yoder et al., 2007), *Schizolaena hystrix* Capuron (*Sarcolaenaceae*) (Murphy et al., 2005), and *Mimulus clevelandii* Brandegee (*Scrophulariaceae*) (Phillips et al., 1996) and also in some kinds of propolis (Chen et al., 2004). Several biological activities of diplacone have been described. For example, cytotoxic and anti-cancer or anti-proliferation effects on various human carcinoma cells (Yoder et al., 2007; Smejkal et al., 2008a), probably caused by the ability of diplacone to induce apopto-

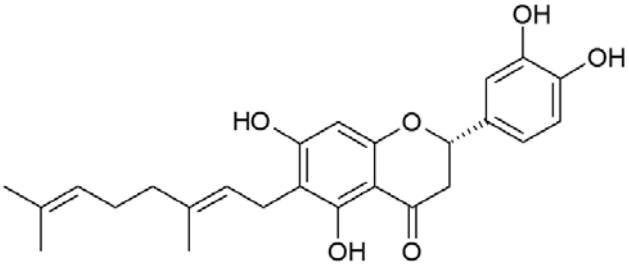

Fig. 1. Molecular structure of diplacone

sis (Chen et al., 2004), are well established. Diplacone also exhibits a strong anti-oxidative capacity (Chen et al., 2004; Smejkal et al., 2007). Antibacterial activity against Gram negative bacteria has also been described (Smejkal et al., 2008b).

The flavonoids, among which diplacone is classified, are commonly studied for their anti-inflammatory effects (Guardia et al., 2001). The favourite model used to study induced inflammation both *in vitro* and *in vivo* is stimulation of macrophages (or other cell types) by lipopolysaccharides (LPS) obtained from Gram-negative bacteria. Some previous papers have reported decreased production of pro-inflammatory cytokines (e.g., tumour necrosis factor α (TNF-α) or IL-1β) and enzymes (e.g., inducible isoform of nitric oxide synthase (iNOS)) after treatment of inflamed cells with a flavonoid (Hämäläinen et al., 2007; Bodet et al., 2008). This result is probably due to the modulating effects of NF-κB, ERK, STAT-1 or combination of any two or all three of them (Hämäläinen et al., 2007; Park et al., 2007). It has been suggested that the imbalance between pro-inflammatory and anti-inflammatory cytokines may contribute to the pathogenesis of autoimmune diseases (O'Shea et al., 2002), which are usually characterized by chronic inflammation. The effects of cytokines are clearly evident, especially for this kind of inflammation.

Therapeutic uses of different plant preparations as antiphlogistics are currently being resurrected and research on antiphlogistics is increasing in the global world (Plaeger, 2003). Many plant flavones and flavanones have been studied for potential application in the therapy of such chronic inflammatory diseases as chronic obstructive pulmonary disease (Weseler et al., 2009), type 2 diabetes (Weseler et al., 2009), and inflammatory bowel disease (Shin et al., 2009).

In this paper, we focused on the study how diplacone affects gene expression of proinflammatory cytokines tumour necrosis factor α (TNF-α) and monocyte chemoattractant protein 1 (MCP-1), also known as CCL2, and regulatory protein zinc finger protein 36 (ZFP36), also known as tristetraprolin (TTP) at the transcription level. TNF-α is a typical hallmark of inflammation, MCP-1 chemotactically regulates movement of monocytes to the site of inflammation, and ZFP36 is an anti-inflammatory protein, which binds to AU-rich regions and destabilizes pro-inflammatory mRNA. The action of diplacone was compared with that of conventional drug indomethacin.

Material and Methods

Material

The RPMI 1640 medium, penicillin-streptomycin mixture, and trypsin 170 U/ml supplemented with EDTA 200 μg/ml were purchased from Lonza (Verviers, Belgium). Phosphate-buffered saline (PBS), foetal bovine serum (FBS), phorbol myristate acetate (PMA), indomethacin, Erythrosin B, and the lipopolysaccharide (LPS) obtained from *Escherichia coli* 0111:B4 were purchased from Sigma-Aldrich (Steinheim, Germany). Monoclonal antibody against F4/80-like receptor was obtained from BD Biosciences (San Jose, CA). Diplacone was isolated from *Paulownia tomentosa* fruits according to the procedure of Smejkal et al. (2007). A QuickGene RNA cultured cell HC kit S from FujiFilm (Tokyo, Japan) and an RNase-free DNase Set from Qiagen (Hilden, Germany) were used for isolation of RNA. Reverse transcription quantitative PCR (RT-qPCR) was accomplished with a TaqMan RNA-to-C_T 1-Step Kit from Applied Biosystems (Cheshire, UK) and TaqMan Gene Expression Assays from Applied Biosystems (Foster City, CA) were used for these reactions.

Maintenance of cell culture and differentiation to macrophages

Human monocytic leukaemia cell line THP-1 was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). This cell line was used because it is the most similar to native immune cells (Auwerx, 1991). The cells were cultivated at 37 °C in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% FBS, 100 U/ml of penicillin and 100 μg/ml of streptomycin in a humidified atmosphere containing 5% CO_2. The medium was changed twice a week when the cell concentration was 5–7 × 10^5 cells/ml. The cell number and viability were determined by staining with Erythrosin B. Cells were counted manually using a haemocytometer and a light microscope. Cells that remained unstained were considered viable, light red cells as non-viable.

Stabilized cells were split into 6-well plates to get a concentration of 150,000 cells/ml and cultivated for 72 h. To promote differentiation of monocytes to macrophages, PMA was added to make the final concentration 50 ng/ml and the cells were incubated for 24 h. In comparison with monocytes, differentiated macrophages tend to adhere to the bottoms of the cultivation plates. Maturation of macrophages was also confirmed by the immunohistochemical detection of surface glycoprotein marker F4/80. The F4/80 antigen is expressed on a wide range of mature macrophages. For the next 24 h the cells were incubated with fresh complete RPMI medium, i.e. containing antibiotics and FBS, without PMA. The medium was then aspirated, and the cells were washed with PBS and cultivated for another 24 hours in serum-free RPMI 1640 medium. These prepared macrophages were used for the follow-up experiments.

Drug treatment and induction of inflammation

Differentiated macrophages were pretreated for 1 h with 10 μM or 20 μM diplacone dissolved in dimethylsulphoxide (DMSO). Our previous study showed that these concentrations lack cytotoxic effect (data not showed). For comparison with a conventional drug, 10 μM indomethacin dissolved in DMSO was used. This concentration is commonly used for *in vitro* tests (Assreuy et al., 2003). Control cells contained a vehicle

(DMSO) only. The concentration of DMSO was 0.1 % in each well.

The effect of diplacone on the modulation of inflammatory gene expression was tested by adding 1 μg/ml LPS dissolved in water to drug-pretreated macrophages. LPS is able to trigger an inflammatory reaction through binding on TLR-4 and subsequently activates the NF-κB signalling pathway (Sharif et al., 2007).

Cell samples were harvested by trypsinization and scraping 1, 2, 4, 6, 10, and 24 h after the LPS treatment. Cells were spun down, frozen in liquid nitrogen, and stored at -80 °C for further processing.

RNA isolation and quantification of gene expression

In order to evaluate the expression of *TNF-α*, *MCP-1* and *ZFP36* mRNA, the total RNA was isolated from frozen samples using QuickGene RNA cultured cell HC kit S (FujiFilm) according to the manufacturer's instructions, and supplementing this with DNase treatment. The concentration and purity of the RNA was determined by using UV spectrophotometry.

The gene expression was quantified by using a one-step reverse-transcription quantitative (real-time) polymerase chain reaction (PCR) (RT-qPCR) with TaqMan Gene Expression Assays, which contain specific primers and a TaqMan probe that binds to an exon-exon junction to avoid DNA contamination. Assay number Hs00174128_m1 was used for *TNFα*, Hs00234140_m1 for *MCP-1* and Hs00185658_m1 for *ZFP36* gene expression quantification. β-Actin, assay number 4326315E, served as an internal control for gene expression. A total of 1 μg of isolated RNA was added to 25 μl of the PCR reaction mixture containing both reverse transcriptase and DNA polymerase. The parameters for the qPCR work with the TaqMan RNA-to-C_T 1-Step Kit were set up according to the manufacturer's recommendations: 48 °C for 15 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. RT-qPCR reactions were designed to be duplex, expressions of both β-actin and the gene of interest were evaluated in one tube. Results were normalized to the amount of β-actin and the change in gene expression was determined by the $\Delta\Delta C_T$ method using StepOne Software, version 2.1 (Applied Biosystems).

Statistical analysis

All experiments were performed in triplicate and the results are presented as the mean values with error bars representing the standard error (SE) of the mean. A one-way ANOVA test was used for statistical analysis, followed by Tukey's test for multiple comparisons. A value of $P < 0.05$ was considered to be statistically significant. Unistat 5.1 (Unistat Ltd., London, UK) was used to perform the analysis.

Results

The expression peaks of *TNF-α* and *ZFP36* were observed between 1 and 2 h after LPS stimulation, and the mRNA level then rapidly decreased, whereas the peak of *MCP-1* was achieved 10 hours after LPS stimulation and the decrease was much slower (Figs. 2–4).

In Table 1, the relative changes in the gene expression of *TNF-α* for LPS-stimulated cells are compared to those for vehicle-treated cells. Two hours after the LPS-induced inflammation, both concentrations of diplacone had significantly decreased the *TNF-α* expression by a factor of ~1.7 ($P < 0.001$). A similar effect was observed for the indomethacin-treated cells (Fig. 2). The *TNF-α* expression rapidly decreased after reaching a maximum, when cells were stimulated with LPS alone. Pre-treatment with diplacone led to a more moderate decline of the *TNF-α* expression. Ten hours after LPS induction,

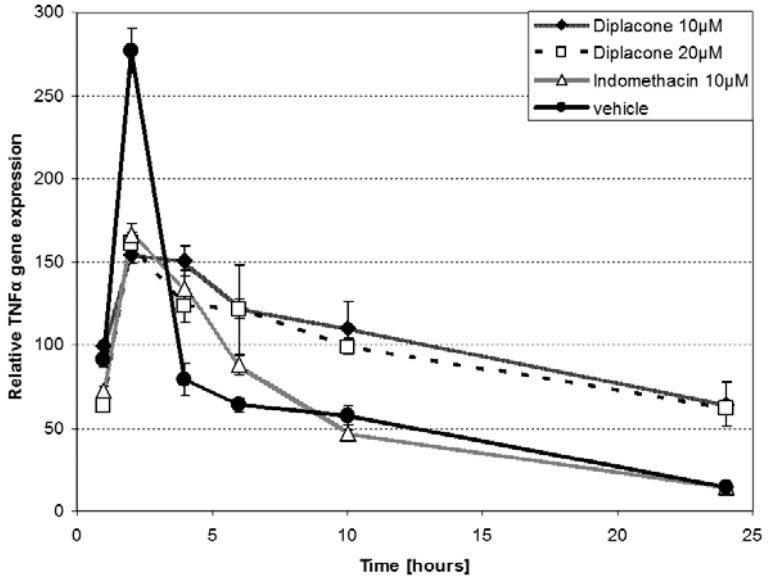

Fig. 2. Effect of diplacone and indomethacin on LPS-induced TNF-α gene expression. Cells were pre-treated with diplacone, indomethacin or vehicle only. After one hour of incubation, inflammation was induced by LPS.

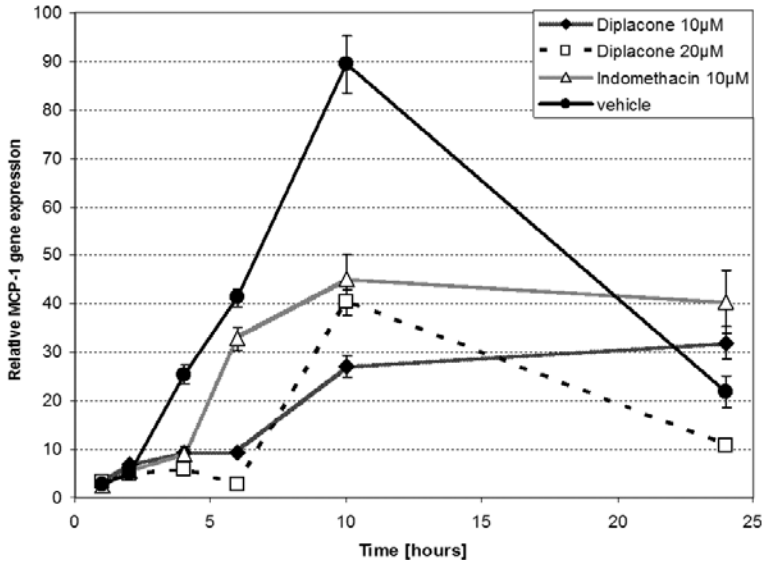

Fig. 3. Effect of diplacone and indomethacin on LPS-induced MCP-1 gene expression. Cells were pre-treated with diplacone, indomethacin or vehicle only. After one hour of incubation, inflammation was induced by LPS.

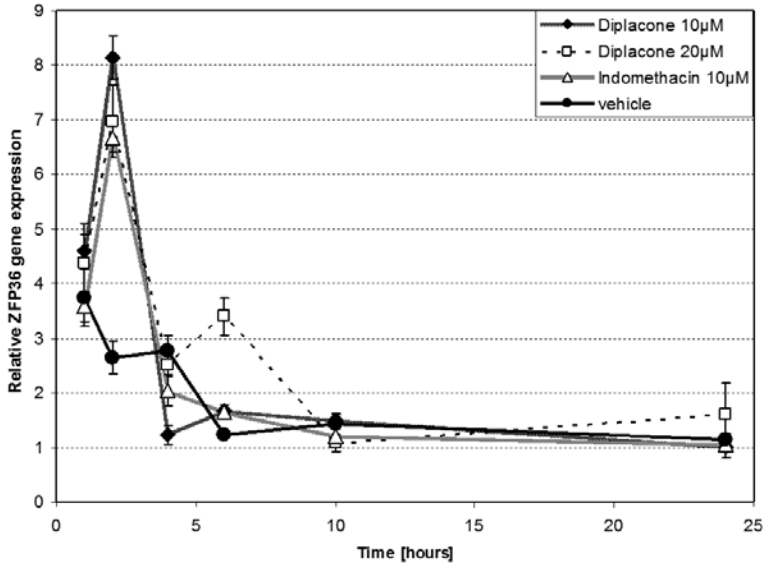

Fig. 4. Effect of diplacone and indomethacin on LPS-induced ZFP36 gene expression. Cells were pre-treated with diplacone, indomethacin or vehicle only. After one hour of incubation, inflammation was induced by LPS.

1.8 times as much mRNA for *TNF-α* was presented in pre-treated cells as in cells without pre-treatment. After 24 h there was 4.4 times as much mRNA in the pre-treated as in the untreated cells. Indomethacin also showed slower decrease of *TNF-α* mRNA, but in this case the *TNF-α* mRNA reached a level similar to that of the mRNA treated by the vehicle 10 hours after LPS induction (Fig. 2).

The expression of *MCP-1*, another pro-inflammatory gene, was also studied. The influence of diplacone on this expression is summarized in Table 2. Four hours after LPS stimulation, cells influenced by 10 μM diplacone and by indomethacin showed expression of *MCP-1* lower by a factor of 2.8 ($P = 0.0005$) than was found for cells treated with vehicle alone; for those treated with 20 μM diplacone the factor was 4.3 ($P = 0.0001$) (Fig. 3). Six hours after LPS stimulation, statistically significant lower expressions of *MCP-1* mRNA were detected only for the diplacone treatments at both concentrations ($P < 0.0001$); indomethacin decreased the level of *MCP-1* mRNA by only a factor of 1.3, which was not statistically significant ($P = 0.063$). However, when the peak of expression was observed, 10 hours after LPS induction, both diplacone and indomethacin significantly diminished the *MCP-1* expression; the lowest expression was then found for cells treated with 10 μM diplacone (lower by a factor of 3.3 than for cells treated with the vehicle ($P = 0.0001$)).

The only anti-inflammatory gene for which the expression was studied was *ZFP36*. Diplacone and indomethacin significantly increased the expression of mRNA for *ZFP36* two hours after LPS stimulation (Ta-

Table 1. Relative changes in LPS-induced TNF-α expression

	Time after LPS stimulation [hours]					
	1	2	4	6	10	24
Control			1.12 ± 0.20			
Vehicle	90.97 ± 4.64	276.67 ± 13.83	78.84 ± 9.95	63.78 ± 4.23	57.23 ± 5.72	14.13 ± 1.18
Diplacone 10 μM	99.26 ± 3.02	153.83 ± 5.08**	150.07 ± 9.04‡	121.74 ± 6.03	109.92 ± 16.16‡	64.07 ± 13.08‡
Diplacone 20 μM	63.14 ± 3.35*	160.83 ± 7.16**	123.53 ± 10.05	120.70 ± 27.73	98.63 ± 4.84	61.54 ± 2.87‡
Indomethacin 10 μM	72.34 ± 3.09	165.85 ± 6.94**	133.52 ± 11.83	87.72 ± 6.14	46.36 ± 2.64	13.91 ± 1.96

Results are means ± SE for three independent experiments. * indicates significant decrease in TNF-α expression relatively to vehicle-treated cells ($P < 0.05$), ** indicates significant decrease in TNF-α expression relatively to vehicle-treated cells ($P < 0.005$), ‡ indicates significant increase in TNF-α expression relatively to vehicle-treated cells ($P < 0.05$).

ble 3). The expression of *ZFP36* was otherwise comparable for all samples at all other times (Fig. 4). For the same period, 2 h after LPS induction, a significant decrease of mRNA was detected for TNF-α (Fig. 2). Cells pretreated with 20 μM diplacone exhibited significantly higher (a factor of 2.7, $P = 0.0006$) expression of *ZFP36* 6 h after LPS stimulation than cells without diplacone treatment. However, this value does not differ to a statistically significant degree from values obtained 4 h ($P = 0.91$) and 10 h ($P = 0.18$) after LPS induction.

From Fig. 2–4 it is apparent that diplacone has almost the same effect on the expression of selected genes at a concentration of 10 μM as at a concentration of 20 μM. This suggests that the maximal biological effect might be achieved with concentrations lower than 10 μM.

To obtain the overall picture for the total production of mRNA, the area under curve (AUC) of the mean values was calculated. Both concentrations of diplacone had almost twice higher AUC (2384 AU for 10 μM and 2200 AU for 20 μM diplacone) than indomethacin and vehicle-treated cells (1330 AU for indomethacin and 1423 AU for vehicle) in the case of the *TNF-α* gene expression. The opposite effect was observed for the *MCP-1* gene expression; diplacone decreased the total production of this cytokine mRNA to one half compared to the vehicle (525.3 AU for 10 μM and 466 AU for 20 μM diplacone vs. 1140 AU for the vehicle). Indomethacin had a moderate effect – 814.9 AU. In the case of the *ZFP36* gene expression, the differences were not so noticeable. Diplacone and indomethacin slightly increased total production of mRNA for ZFP36 compared to the vehicle (42.49 AU for 10 μM, 48.69 AU for 20 μM diplacone and 39.17 AU for indomethacin vs. 35.94 AU for the vehicle).

Discussion

The anti-inflammatory effects of various flavonoids have been studied using different models. For example, sigmoidins A and B attenuate 12-*O*-tetradecanoylphorbol 13-acetate and phospholipase-A_2-induced mouse paw oedema (Njamen et al., 2004), naringenin inhibits inflammatory neuronal injury by reducing the LPS/INF-γ-induced glial cell activation (Vafeiadou et al., 2009), and fisetin eliminates pulmonary LPS-induced inflammation (Geraets et al., 2009). *In vitro* studies on cell cultures have also been carried out (Manna et al., 2007; Matsuda et al., 2008). It is difficult to base comparison of

Table 2. Relative changes in LPS-induced MCP-1 expression

	Time after LPS stimulation [hours]					
	1	2	4	6	10	24
Control			0.99 ± 0.24			
Vehicle	2.85 ± 0.26	4.92 ± 0.55	25.36 ± 1.85	41.24 ± 1.91	89.39 ± 5.81	21.75 ± 3.32
Diplacone 10 μM	2.99 ± 0.19	6.87 ± 0.46	9.22 ± 0.58**	9.23 ± 0.25**	27.08 ± 2.33**	31.95 ± 3.32
Diplacone 20 μM	3.34 ± 0.24	4.66 ± 0.13	5.86 ± 1.03**	2.87 ± 0.94**	40.26 ± 2.55**	10.67 ± 1.05
Indomethacin 10 μM	2.53 ± 0.48	5.61 ± 0.48	9.10 ± 1.49**	32.77 ± 2.40	45.16 ± 5.05**	40.33 ± 6.45

Results are means ± SE for three independent experiments. ** indicates significant decrease in MCP-1 expression relatively to vehicle-treated cells ($P < 0.005$).

Table 3. Relative changes in LPS-induced ZFP36 expression

	Time after LPS stimulation [hours]					
	1	2	4	6	10	24
Control			1.09 ± 0.08			
Vehicle	3.74 ± 0.53	2.65 ± 0.30	2.77 ± 0.29	1.24 ± 0.08	1.42 ±0.19	1.15 ± 0.11
Diplacone 10 μM	4.61 ± 0.31	8.14 ± 0.39 ‡‡	1.24 ±0.18 *	1.67 ± 0.11	1.49 ± 0.14	1.01 ± 0.19
Diplacone 20 μM	4.37 ± 0.72	6.97 ± 0.66 ‡‡	2.51 ± 0.21	3.40 ± 0.34 ‡‡	1.07 ± 0.14	1.60 ± 0.59
Indomethacin 10 μM	3.58 ± 0.27	6.66 ± 0.26 ‡‡	2.05 ± 0.28	1.66 ± 0.04	1.21 ± 0.02	1.06 ± 0.14

Results are means ± SE for three independent experiments. * indicates significant decrease in ZFP36 expression relatively to vehicle-treated cells ($P < 0.05$), ‡‡ indicates significant increase in ZFP36 expression relatively to vehicle-treated cells ($P < 0.005$).

the anti-inflammatory potentials of flavonoids directly on previous evaluations. In this paper we focused on the human macrophages derived from monocytic leukaemia cell line THP-1, which behave very similarly to native monocyte-derived macrophages (Auwerx, 1991), to test inflammatory gene expression modulation *in vitro*.

Diminished expression and production of TNF-α have been described for various flavones (Lin et al., 2003; Geraets et al., 2009; Vafeiadou et al., 2009) and are ascribed to the inhibition of NF-κB activity (Manna et al., 2007), which controls the transcription of this cytokine (Pahl, 1999). We have not been able to explain the slower decrease of *TNF-α* mRNA after diplacone treatment. The *MCP-1* gene is also under the transcriptional control of NF-κB (Pahl, 1999), so the inhibition of this transcription factor, likely caused by diplacone, has a similar influence on the expression of *MCP-1* and *TNF-α*.

Deleault et al. (2008) found that the function of ZFP36 is controlled by the activity of ERK and p38 kinases. LPS activates these kinases, which subsequently inhibit the function of ZFP36. Inhibition of ERK by isoflavones, which can display activities such as antioxidant activity or oestrogen receptor-binding ability, comparable to flavones in some cases (Dixon, 2004), has been observed (Park et al., 2007). Diplacone could have a similar effect – decreasing the activity of extracellular receptor kinase (ERK), and thereby stabilizing ZFP36 protein and subsequently reducing the mRNA level of TNF-α and elevating the level of ZFP36. Significantly higher expression of *ZFP36* was observed six hours after LPS induction, when 20 μM diplacone was used. On the other hand, the measured value did not statically differ from values obtained 4 and 10 h after LPS induction. However, the effect of 20 μM diplacone on *ZFP36* gene expression is opposite to the effect of 10 μM diplacone or indomethacin, which did not affect this expression at that time point. We have yet to satisfactorily explain the biological relevance of this observation.

Total production of selected mRNAs was calculated from AUC of the mean values. Diplacone increased almost twice the total production of TNF-α mRNA, but on the other hand decreased twice production of mRNA of another pro-inflammatory cytokine, MCP-1. The total production of mRNA of anti-inflammatory protein ZFP36 was slightly higher when the cells were pretreated with diplacone. Both TNF-α and MCP-1 are target molecules of ZFP36 and their production is modulated by this protein (Sauer et al., 2006). It is possible that ZPF36 is more active in down-regulation of MCP-1 than TNF-α in this case. There may also be some unknown mechanism of diplacone that prolongs the half-life of TNF-α mRNA in the cells. In any case, the diplacone's biological effect of higher total production of TNF-α mRNA and lower production of MCP-1 mRNA should be elucidated on an *in vivo* model. Although indomethacin is primarily used to inhibit cyclooxygenase activity, in our model it was able to moderately decrease the total production of TNF-α and MCP-1 mRNA and slightly increase the total production of ZFP36 mRNA.

Conclusion

This paper is the first to describe the effects of geranylated flavanone, diplacone, on transcription of pro-inflammatory and anti-inflammatory genes. We have found that diplacone is able to down-regulate the expression of pro-inflammatory genes for TNF-α and MCP-1 and up-regulate that of anti-inflammatory genes for ZFP36 at the transcriptional level. It thus represents a promising drug candidate for the treatment of inflammation. The exact mechanisms of its action should be elucidated in detail in order to better understand its biological function *in vivo*. The effect of diplacone in decreasing LPS-induced inflammatory gene expression is in many ways similar to that of the conventional drug indomethacin in this model of inflammation.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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