Cyclolinteinone, a sesterterpene from sponge *Cacospongia linteiformis*, prevents inducible nitric oxide synthase and inducible cyclo-oxygenase protein expression by blocking nuclear factor- κ B activation in J774 macrophages

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We investigated the effect of cyclolinteinone, a sesterterpene from Caribbean sponge *Cacospongia linteiformis*, on inducible NO synthase (iNOS) and cyclo-oxygenase-2 (COX-2) protein expression in lipopolysaccharide (LPS)-stimulated J774 macrophages. Incubation of J774 cells with LPS (1 μ g/ml) caused an increase of both iNOS and COX-2 protein expression, which was prevented in a concentration-dependent fashion by cyclolinteinone (12.5, 25 and 50 μ M). Electrophoretic mobility-shift assay indicated that cyclolinteinone blocked the activation of nuclear factor- κ B (NF- κ B), a transcription factor necessary for

either iNOS or COX-2 induction. Cyclolinteinone also blocked disappearance of I κ B- α from cytosolic fraction and nuclear translocation of NF- κ B subunits p50 and p65. These results show that cyclolinteinone down-regulates iNOS and COX-2 protein expression by inhibiting NF- κ B activation and suggest that it may represent a novel anti-inflammatory compound capable of controlling the excessive production of prostaglandins and nitric oxide occurring in several inflammatory diseases.

Key words: nitric oxide, prostaglandins, Thorectidae family.

INTRODUCTION

Cyclo-oxygenase (COX) is the key enzyme for the generation of prostaglandins (PGs) and other eicosanoids from arachidonic acid [1]. Nitric oxide synthase (NOS) is the enzyme that catalyses the formation of NO from the guanidino nitrogen atom of L-arginine [2]. Two isoforms of both enzymes occur: a constitutive one, COX-1, and the inducible counterpart, COX-2 [3]; also NOS has constitutive counterparts (endothelial NOS and neuronal NOS) and an inducible isoform, called iNOS [2]. After stimulation with lipopolysaccharide (LPS) or interleukin-1, many cell types, including endothelial cells and macrophages, express the inducible isoforms of either COX or NOS, which are responsible for the production of large amounts of PGs and NO respectively [3–5]. These inducible enzymes are essential components of the inflammatory response and implicated in the pathogenesis of several inflammatory diseases [6–9]. The promoter regions of *COX-2* [10–12] and *iNOS* genes [13] have been cloned and sequenced. These promoter regions contain at least one putative nuclear factor- κ B (NF- κ B) consensus sequence that has been shown to act as a positive regulatory element for either COX-2 [14] or iNOS [15] transcription. NF- κ B is a ubiquitous transcription factor composed of a group of nuclear proteins: c-Rel, p50/p105 (NF- κ B1), p65 (Rel A), p52/p100 (NF- κ B2) and Rel B. NF- κ B complexes are sequestered in the cytoplasm of most resting cells by inhibitory proteins belonging to the inhibitor of NF- κ B activation (I κ B) family. In response to various stimuli, I κ B- α is first phosphorylated and then degraded rapidly by the

proteasome, allowing NF- κ B nuclear translocation and gene activation [16,17]. We have shown previously that cyclolinteinone (Figure 1), a sesterterpene from the Caribbean sponge of the family Thorectidae, *Cacospongia linteiformis*, inhibits NO generation in LPS-stimulated J774 macrophages [18]. In the present study we investigated the effect of cyclolinteinone on the expression of either COX-2 or iNOS in LPS-stimulated J774 macrophages. Moreover, since the activation of NF- κ B has been shown to be an essential step in the induction of COX-2 [19] and iNOS [20] expression, we investigated whether cyclolinteinone was able to down-regulate COX-2 and iNOS protein expression by inhibiting NF- κ B activation in LPS-stimulated J774 macrophages.

MATERIALS AND METHODS

Sponge characterization and chemistry

A sample of *C. linteiformis* was collected by hand using scuba (–9 m) along the coast of Grand Bahama Island (Bahamas). The purification, structure and absolute stereochemistry of cyclolinteinone and related metabolites have been described previously [18].

Cell culture

The murine monocyte/macrophage cell line J774 (ATCC T1B p. 231) was cultured at 37 °C in humidified 5% CO₂/95% air in Dulbecco's modified Eagle's medium (DMEM) containing 10%

Abbreviations used: PG, prostaglandin; PGE₂, prostaglandin E₂; COX, cyclo-oxygenase; NOS, nitric oxide synthase; iNOS, inducible NOS; NF- κ B, nuclear factor- κ B; LPS, lipopolysaccharide; PDTC, pyrrolidinedithiocarbamate; I κ B, inhibitor of NF- κ B activation; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility-shift assay.

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Figure 1 Chemical structure of cyclolinteinone

foetal bovine serum, 2 mM glutamine, 100 I. U./ml penicillin and 100 µg/ml streptomycin. The cells were plated in 24 culture wells at a density of 2.5×10^5 cells/ml per well or 10 cm diameter culture dishes at a density of 1×10^7 cells/ml per dish and allowed to adhere for 2 h. Thereafter the medium was replaced with fresh medium and cells were stimulated with LPS (1 µg/ml). Cyclolinteinone (12.5, 25 and 50 µM) or pyrrolidinedithiocarbamate (PDTC; 10 µM), a well-known inhibitor of NF-κB activation [21], were added to the cells 10 min before LPS challenge. In some experiments cyclolinteinone (50 µM) or PDTC (10 µM) were added 12 h after LPS challenge. The cell viability was assessed through MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] conversion assay. Briefly, 100 µl of MTT (5 mg/ml in complete DMEM) was added and the cells were incubated for an additional 3 h. Following this time the cells were lysed and the dark blue crystals solubilized with 500 µl of a solution containing 50% (v/v) *N,N*-dimethyl-formamide and 20% (w/v) SDS with an adjusted pH of 4.5. The absorbance (*A*) of each well was measured with a microplate spectrophotometer (Titertek Multiskan MCCC/340) equipped with a 620-nm filter. The cell viability in response to treatment with test compounds was calculated as: dead cells (%) = $100 - (A_{\text{treated}}/A_{\text{control}}) \times 100$.

Prostaglandin E₂ (PGE₂) and nitrite determination

The accumulation of PGE₂ in the culture medium was measured by ELISA kit according to the manufacturer's instructions (TEMA Ricerche, Milan, Italy). NO generation was measured as nitrite (NO₂⁻, nmol/10⁶ cells) accumulated in the incubation medium (24 h after LPS challenge) using a spectrophotometric assay based on the Griess reaction.

Preparation of cytosolic and nuclear extracts

Cytosolic and nuclear extracts of macrophages stimulated with LPS (1 µg/ml) in the presence or absence of cyclolinteinone (12.5, 25 and 50 µM) or PDTC (10 µM) were prepared as described previously [20]. Briefly, harvested cells (2×10^7) were washed twice with ice-cold PBS and centrifuged at 180 *g* for 10 min at 4 °C. The cell pellet was resuspended in 100 µl of ice-cold hypotonic lysis buffer (10 mM Hepes, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM PMSF, 1.5 µg/ml soya bean trypsin inhibitor, 7 µg/ml pepstatin A, 5 µg/ml leupeptin, 0.1 mM benzamidine and 0.5 mM dithiothreitol) and incubated on ice for 15 min. The cells were lysed by rapid passage through a syringe needle five or six times and the cytosolic fraction was then obtained by centrifugation for 1 min at 13000 *g* for 1 min. The nuclear pellet was resuspended in 60 µl of high-salt extraction buffer (20 mM Hepes, pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂,

0.2 mM EDTA, 25% (v/v) glycerol, 0.5 mM PMSF, 1.5 µg/ml soya bean trypsin inhibitor, 7 µg/ml pepstatin A, 5 µg/ml leupeptin, 0.1 mM benzamidine and 0.5 mM dithiothreitol) and incubated with shaking at 4 °C for 30 min. The nuclear extract was then centrifuged for 15 min at 13000 *g* and supernatant was aliquoted and stored at -80 °C. Protein concentration was determined by the Bio-Rad (Milan, Italy) protein-assay kit.

Electrophoretic mobility-shift assay (EMSA)

Double-stranded oligonucleotides containing the NF-κB recognition sequence (5'-AGT TGA GGG GAC TTT CCC AGG-3') were end-labelled with [γ -³²P]ATP. Nuclear extracts containing 5 µg of proteins were incubated for 30 min with radiolabelled oligonucleotides (2.5×10^4 – 5.0×10^4 c.p.m.) in 20 µl of reaction buffer containing 2 µg of poly(dI-dC), 10 mM Tris/HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 µg/ml BSA and 10% (v/v) glycerol. The specificity of the DNA-protein binding was determined by competition reactions in which a 50-fold molar excess of unlabelled wild-type, mutant or Sp-1 oligonucleotide was added to the binding reaction 10 min before addition of radiolabelled probe. In supershift assay, antibodies reactive to p50, p65 or c-Rel proteins were added to the reaction mixture 30 min before the addition of radiolabelled NF-κB probe. Nuclear protein-oligonucleotide complexes were resolved by electrophoresis on a 6% non-denaturing polyacrylamide gel in 1× Tris/borate/EDTA buffer at 150 V for 2 h at 4 °C. The gel was dried and autoradiographed with intensifying screen at -80 °C for 20 h. Subsequently, the relative bands in nuclear fractions were quantified by densitometric scanning of the X-ray films with a GS 700 Imaging Densitometer (Bio-Rad) and a computer program (Molecular Analyst, IBM).

Western-blot analysis

Immunoblotting analysis of COX-2, iNOS, IκB-α, p50 and p65 proteins was performed on J774 cells incubated with LPS (1 µg/ml) in the presence or absence of cyclolinteinone or PDTC at different time points. Cytosolic and nuclear-fraction proteins were mixed with gel loading buffer (50 mM Tris/10% SDS/10% glycerol/10% 2-mercaptoethanol/2 mg/ml bromophenol) in a ratio of 1:1, boiled for 3 min and centrifuged at 10000 *g* for 10 min. Protein concentration was determined and equivalent amounts (75 µg) of each sample were electrophoresed in a 12% discontinuous polyacrylamide mini-gel. The proteins were transferred on to nitrocellulose membranes, according to the manufacturer's instructions (Bio-Rad). The membranes were saturated by incubation at 4 °C overnight with 10% non-fat dry milk in PBS and then incubated with (1:1000) anti-COX-2, anti-iNOS, anti-IκB-α, anti-p50 and anti-p65 antibodies for 1 h at room temperature. The membranes were washed three times with 1% Triton X-100 in PBS and then incubated with anti-rabbit or anti-goat immunoglobulins coupled to peroxidase (1:1000). The immunocomplexes were visualized by the ECL[®] enhanced chemiluminescence method (Amersham, Milan, Italy). Subsequently, the relative expression of COX-2, iNOS, IκB-α, p50 and p65 proteins in cytosolic and nuclear fractions was quantified by densitometric scanning of the X-ray films with a GS 700 Imaging Densitometer and the Molecular Analyst computer program.

Statistics

Results are expressed as the means ± S.E.M. of *n* experiments. Statistical analysis was determined by Student's unpaired *t* test with *P* < 0.05 considered significant.

Reagents

LPS (from *Salmonella typhosa*) was from Difco (Detroit, MI, U.S.A.). DMEM and PBS were from Celbio (Milan, Italy). D,L-Dithiothreitol, PMSF, soya bean trypsin inhibitor, pepstatin A, leupeptin and benzamide were from Calbiochem (Milan, Italy). [γ - 32 P]ATP was from Amersham (Milan, Italy). Poly(dI-dC) was from Boehringer Mannheim (Milan, Italy). Anti-COX-2, anti-iNOS, anti-I κ B- α , anti-p50, anti-p65 and anti-c-Rel antibodies were from Santa Cruz (Milan, Italy). Non-fat dry milk was from Bio-Rad. All the other reagents were from Sigma (Milan, Italy).

RESULTS

Effect of cyclolinteinone on LPS-induced COX-2 and iNOS protein expression

The production of PGE₂ and nitrite by unstimulated J774 macrophages was undetectable (< 3 nmol/10⁶ cells; $n = 6$). The stimulation of cells with LPS (1 μ g/ml) for 24 h resulted in an accumulation of PGE₂ and nitrite in the medium (25.7 ± 1.4 and 35.5 ± 1.3 nmol/10⁶ cells, respectively; $n = 9$). Treatment of cells with cyclolinteinone (12.5, 25 and 50 μ M) or PDTC (10 μ M) reduced, in a concentration-dependent manner, PGE₂ (by 11.5 ± 0.71 , 22.3 ± 1.4 , 44.3 ± 1.6 and 52.1 ± 1.6 %, respectively; $n = 6$; Figure 2, bottom panel) and nitrite (by 15.1 ± 0.6 , 25.3 ± 1.1 ,

Figure 2 Effect of cyclolinteinone and PDTC on PGE₂ and nitrite production in LPS-stimulated J774 macrophages

Effect of cyclolinteinone and PDTC on PGE₂ (bottom panel) and nitrite (top panel) production by J774 macrophages stimulated with LPS (1 μ g/ml) for 24 h. The values are expressed as means $\pm$ S.E.M. ($n = 4$). * $P < 0.05$ and *** $P < 0.0001$ versus control (LPS alone).

Figure 3 Effect of cyclolinteinone and PDTC on COX-2 expression in LPS-stimulated J774 macrophages

Representative Western blot of COX-2 protein (A) as well as densitometric analysis (B) show the effect of cyclolinteinone and PDTC on COX-2 protein expression in J774 macrophages stimulated with LPS (1 μ g/ml) for 24 h. Data are expressed as means $\pm$ S.E.M. of three separate experiments. ** $P < 0.001$ versus control (LPS alone).

Figure 4 Effect of cyclolinteinone and PDTC on iNOS expression in LPS-stimulated J774 macrophages

Representative Western blot of iNOS protein (A) as well as densitometric analysis (B) show the effect of cyclolinteinone and PDTC on iNOS protein expression in J774 macrophages stimulated with LPS (1 μ g/ml) for 24 h. Data are expressed as means $\pm$ S.E.M. of three separate experiments. * $P < 0.05$ and ** $P < 0.001$ versus control (LPS alone).

49.32 ± 2.1 and 41.6 ± 1.3 %, respectively; $n = 6$; (Figure 2, top panel) production at 24 h. Moreover, upon stimulation with LPS (1 μ g/ml) for 24 h, cells expressed a significantly high level of

Figure 5 Effect of delayed addition of cyclolinteinone and PDTC on COX-2 and iNOS expression in LPS-stimulated J774 macrophages

Representative Western blots of iNOS (A) and COX-2 protein (C) as well as densitometric analyses (B and D respectively) show the effect of cyclolinteinone or PDTC added to the cells at the same time as LPS (time 0) or 12 h after LPS challenge on COX-2 and iNOS protein expression in J774 macrophages stimulated with LPS (1 µg/ml) for 24 h. Data are expressed as means ± S.E.M. of three separate experiments. ** $P < 0.001$ versus control (LPS alone at time 0).

either COX-2 (Figure 3) or iNOS (Figure 4) protein expression when compared with control, untreated cells. The reduction of PGE₂ and nitrite accumulation by cyclolinteinone and PDTC was comparable with the inhibition of COX-2 and iNOS protein expression (Figures 3 and 4, respectively). The addition of cyclolinteinone (50 µM) or PDTC (10 µM) to the cells 12 h after LPS challenge did not affect either iNOS or COX-2 protein expression (Figures 5A and 5C, respectively) or PGE₂ production (26.9 ± 0.8 and 23.4 ± 1.3 nmol/10⁶ cells, respectively; $n = 3$) or nitrite accumulation (44.9 ± 1.6 and 41.2 ± 1.8 nmol/10⁶ cells, respectively; $n = 3$) at 24 h, indicating that both compounds did not cause direct inhibition of enzyme activity. Cyclolinteinone and PDTC did not affect cell viability.

Effect of cyclolinteinone on NF-κB activation

The effects of different concentrations of cyclolinteinone on NF-κB-DNA binding activity in J774 macrophages stimulated for 2 h with LPS (1 µg/ml) were tested by EMSA. PDTC (10 µM), a well-known inhibitor of NF-κB activation, was used as reference drug. A low basal level of NF-κB-DNA binding activity was detected in nuclear proteins from unstimulated macrophages.

Figure 6 Effect of cyclolinteinone and PDTC on NF-κB-DNA binding in LPS-stimulated J774 macrophages

Representative EMSA of NF-κB (A) as well as densitometric analysis (B) show the effect of cyclolinteinone and PDTC on NF-κB-DNA binding activity in J774 macrophages stimulated with LPS (1 µg/ml) for 2 h. Data are expressed as means ± S.E.M. of three separate experiments. * $P < 0.05$ and ** $P < 0.001$ versus control (LPS alone).

Figure 7 Identification of LPS-induced NF-κB-DNA binding proteins in J774 macrophages

Nuclear extracts from LPS-treated J774 macrophages were prepared as described in the Materials and methods section and incubated with ³²P-labelled NF-κB probe. In competition reactions, nuclear extracts were incubated with radiolabelled NF-κB probe in the absence or presence of identical but unlabelled oligonucleotides (W.T. 50 ×), mutated NF-κB probe (Mut. 50 ×) or unlabelled oligonucleotide containing the consensus sequence for Sp-1 (Sp-1 50 ×). In supershift experiments nuclear extracts were incubated with antibodies against p50, p65 or c-Rel 30 min before incubation with radiolabelled NF-κB probe. Data are from a single experiment and are representative of three separate experiments.

Conversely, a retarded band was clearly detected following stimulation with LPS (1 µg/ml). Treatment of cells with cyclolinteinone (12.5, 25 and 50 µM) caused a concentration-dependent inhibition of NF-κB-DNA binding activity (Figure 6). The specificity of the NF-κB-DNA binding complex was evident by the complete displacement of NF-κB-DNA binding in the presence of a 50-fold molar excess of unlabelled NF-κB probe (W.T. 50 × in Figure 7) in the competition reaction. In contrast, a 50-fold molar excess of unlabelled mutated NF-κB probe (Mut. 50 × in Figure 7) or Sp-1 oligonucleotide (Sp-1 50 × in Figure 7) had no effect on this DNA-binding activity. The composition of the NF-κB complex activated by LPS was determined by using specific antibody against p50, p65 and c-Rel subunits of NF-κB

Figure 8 Effect of cyclolinteinone and PDTC on LPS-induced degradation of I κ B- α in J774 macrophages

Representative Western blot of I κ B- α (A) as well as densitometric analysis (B) show the effect of cyclolinteinone or PDTC on degradation of I κ B- α in J774 macrophages collected at 30 min after LPS (1 μg/ml) challenge. Data are expressed as means $\pm$ S.E.M. of three separate experiments. ** P < 0.001 versus control (unstimulated cells); ^{oo} P < 0.001 versus LPS-stimulated cells.

proteins. Addition of either anti-p50 or anti-p65 but not anti-c-Rel to the binding reaction resulted in a marked reduction of NF- κ B band intensity (Figure 7).

Effects of PGs on the degradation of I κ B- α and nuclear translocation of NF- κ B subunits

The effects of cyclolinteinone or PDTC on the presence of I κ B- α in the cytosolic fraction or p50 and p65 subunits in nuclear fraction were examined by immunoblotting analysis. Unstimulated cells expressed a basal level of either I κ B- α in the cytosolic fraction or p50 and p65 in the nuclear fraction. When the cells were activated with LPS (1 μg/ml), I κ B- α disappeared from the cytosolic fraction whereas p50 and p65 levels increased in the nuclear fraction. Treatment of cells with cyclolinteinone (50 μM) or PDTC (10 μM) prevented either I κ B- α degradation (Figure 8) or p50 and p65 nuclear translocation (Figures 9A and 9C, respectively).

DISCUSSION

The identification of selective inhibitors of COX-2 and iNOS may lead to advances for the control of inflammation [22,23]. We have shown previously that cyclolinteinone, a sesterterpene from the Caribbean sponge *C. linteiformis*, inhibits NO generation in LPS-stimulated J774 macrophages [18]. The results of the present study show that cyclolinteinone suppresses LPS-induced PGE₂ and nitrite production in a concentration-dependent manner, an effect related to a reduction of either COX-2 or iNOS protein expression, as demonstrated by immunoblotting analysis. Moreover, cyclolinteinone, when added to the cells 12 h after LPS challenge, failed to inhibit either PGE₂ or nitrite production, suggesting that it did not affect the enzyme catalytic activity. Several studies *in vitro* have defined LPS- and cytokine-responsive elements on COX-2 [14] and iNOS [15] gene promoters. We and other authors have demonstrated that NF- κ B is a transcription factor essential in the regulation of COX-2 [14,19] and iNOS [15,20] expression after LPS challenge. In the light of these findings, we investigated the effect of cyclolinteinone on LPS-induced NF- κ B activation. Our data show that cyclolinteinone

Figure 9 Effect of cyclolinteinone and PDTC on p65 and p50 nuclear translocation in LPS-stimulated J774 macrophages

Representative Western blots of p65 (A) and p50 (C) and densitometric analyses (B and D respectively) show the effect of cyclolinteinone or PDTC on LPS-induced p65 and p50 nuclear translocation in J774 macrophages. Data are expressed as means $\pm$ S.E.M. of three separate experiments. ** P < 0.001 versus control (LPS alone).

inhibited in a concentration-dependent manner the LPS-induced NF- κ B-DNA-binding activity. These results obtained with EMSA analysis were very similar to those observed with Western blotting. Cyclolinteinone also inhibited the disappearance of I κ B- α from the cytosolic fraction and the translocation of NF- κ B subunits p50 and p65 into the nuclear fraction. Furthermore, the nuclear translocation of p50 and p65 subunits was prevented by cyclolinteinone at the same concentrations that blocked proteolytic degradation of I κ B- α , suggesting that this compound inhibits NF- κ B activation by stabilizing I κ B- α . Therefore, the mechanism by which cyclolinteinone blocks NF- κ B activation may be related to an inhibitory effect on I κ B- α degradation. Finally, PDTC, a well-known inhibitor of NF- κ B activation [15] and reference drug used in this study, showed the same profile of activity as cyclolinteinone. A growing body of evidence suggests that COX-2 and iNOS induction contribute to oedema formation and leukocyte migration at inflammation sites [6–9,23,24]. Recently, several studies have been devoted to the development of new molecules that are selective inhibitors of the enzymic activity of either iNOS or COX-2. However, an alternative approach is to find new agents that can prevent expression of the respective genes coding for these enzymes. Furthermore, it is now becoming clear that many of the important anti-inflammatory agents, including salicylates [25] and glucocorticoids [26], share the ability to inhibit NF- κ B activation and thus a large variety of pro-inflammatory genes. Some medicinal plants have been shown to be a potential source of inhibitors of NF- κ B activation [27,28]. Other sesterterpenes isolated from a sponge of family Thorectidae exhibit analgesic and anti-inflammatory activities [29,30]. Moreover, a recent report demonstrated that cacospongionolide B, a marine metabolite isolated from another member of the family Thorectidae, the Mediterranean sponge *Faciospongia cavernosa*, is a potent inhibitor of soluble phospholipase A₂ [31], an inflammatory enzyme controlled at the transcriptional level by NF- κ B [32]. Our findings indicate that cyclolinteinone inhibits COX-2 and iNOS protein expression by preventing NF- κ B activation. Although the effect of this compound on other genes involved in inflammation and controlled at the transcriptional level by NF- κ B remains to be investigated, our results suggest that cyclolinteinone, by preventing NF- κ B activation, may represent a novel agent for the pharmacological control of inflammation. In order to develop this hypothesis, further studies on pharmacokinetic properties and anti-inflammatory activity *in vivo* are necessary.

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REFERENCES

- Xie, W., Robertson, D. L. and Simmons, D. L. (1992) *Drug Dev. Res.* **25**, 249–265
- Nathan, C. (1992) *FASEB J.* **6**, 3051–3064
- Lee, S. H., Soyoola, E., Chanmugarn, P., Hart, S., Sun, W., Zhong, H., Liou, S., Simmons, D. and Hwang, D. (1992) *J. Biol. Chem.* **267**, 25934–25939
- Maier, J. A. M., Hla, T. and Machuga, T. (1990) *J. Biol. Chem.* **265**, 10805–10809
- Schmidt, H. H. H. W. and Ulrich, W. (1994) *Cell* **78**, 919–925
- Ialenti, A., Iannaro, A., Moncada, S. and Di Rosa, M. (1992) *Eur. J. Pharmacol.* **211**, 177–182
- Ialenti, A., Moncada, S. and Di Rosa, M. (1993) *Br. J. Pharmacol.* **110**, 701–706
- Iuvone, T., Carnuccio, R. and Di Rosa, M. (1994) *Eur. J. Pharmacol.* **256**, 89–92
- Mitchell, J. A., Akarasereenont, P., Thiemermann, C., Flower, R. G. and Vane, J. R. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 11693–11697
- Fletcher, B. S., Kujubu, D. A., Perrin, D. M. and Herschman, H. R. (1992) *J. Biol. Chem.* **267**, 4338–4343
- Sirois, J., Levy, L. O., Simmons, D. L. and Richards, J. S. (1993) *J. Biol. Chem.* **268**, 12199–12206
- Kosaka, T., Miyata, A., Ihara, H., Hara, S., Sugimoto, T., Takeda, O., Takashi, E. and Tanabe, T. (1994) *Eur. J. Biochem.* **221**, 889–897
- Weisz, A., Oguchi, S., Cicatiello, L. and Esumi, H. (1994) *J. Biol. Chem.* **269**, 8324–8333
- Yamamoto, K., Arakawa, T., Ueda, N. and Yamamoto, S. (1995) *J. Biol. Chem.* **270**, 31315–31320
- Xie, Q., Kashiwabara, Y. and Nathan, C. (1994) *J. Biol. Chem.* **269**, 4705–4708
- Baeuerle, P. A. and Henkel, T. (1994) *Annu. Rev. Immunol.* **12**, 141–179
- Thanos, D. and Maniatis, T. (1995) *Cell* **80**, 529–532
- Carotenuto, A., Fattorusso, E., Lanzotti, V., Magno, S., Carnuccio, R. and D'Acquisto, F. (1997) *Tetrahedron* **53**, 7305–7310
- D'Acquisto, F., Iuvone, T., Rombolà, L., Sautebin, L., Di Rosa, M. and Carnuccio, R. (1997) *FEBS Lett.* **418**, 175–178
- D'Acquisto, F., Sautebin, L., Iuvone, T., Di Rosa, M. and Carnuccio, R. (1998) *FEBS Lett.* **440**, 76–80
- Schreck, R., Meier, B., Mannel, D. N., Droge, W. and Baeuerle, P. A. (1992) *J. Exp. Med.* **175**, 1181–1194
- Vane, J. (1994) *Nature (London)* **367**, 215–216
- Salvemini, D., Manning, P. T., Zweifel, B. S., Seibert, K., Connor, J., Currie, M. G., Needleman, P. and Masferrer, J. L. (1995) *J. Clin. Invest.* **96**, 301–308
- Tomlinson, A., Appleton, I., Moore, A. R., Gilroy, D. W., Willis, D., Mitchell, J. A. and Willoughby, D. A. (1994) *Br. J. Pharmacol.* **113**, 693–698
- Kopp, E. and Ghosh, S. (1994) *Science* **265**, 956–959
- Auphan, N., Di Donato, J. A., Rosette, C., Helmberg, A. and Karin, M. (1995) *Science* **270**, 286–290
- Riehemann, K., Behnke, B. and Schulze-Osthoff, K. (1999) *FEBS Lett.* **442**, 89–94
- Rungeler, P., Lyss, G., Castro, V., Mora, V., Pahl, H. L. and Merfort, I. (1998) *Planta Med.* **64**, 588–593
- Faulkner, D. J. (1996) *Nat. Prod. Rep.* **13**, 75–125
- Jacobs, R. S., Culver, P., Langdon, R., O'Brian, T. and White, S. (1985) *Tetrahedron* **41**, 981–984
- Pastor, P. G., De Rosa, S., De Giulio, A., Payà, M. and Alcaraz, M. J. (1999) *Br. J. Pharmacol.* **126**, 301–311
- Walker, G., Kunz, D., Pignat, W., van den Bosch, H. and Pfeilschifter, J. (1995) *FEBS Lett.* **364**, 218–222

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