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Fluridone as a new anti-inflammatory drug

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ABSTRACT

Fluridone is a herbicide extensively utilized in agriculture for its documented safety in animals. Fluridone contains a 4(1*H*)-pyridone and a trifluoromethyl-benzene moiety, which are also present in molecules with analgesic and anti-inflammatory properties. The established absence of adverse effects of Fluridone on animals prompted us to investigate whether it could represent a new anti-inflammatory compound targeting human cells. In stimulated human monocytes, micromolar Fluridone inhibited cyclooxygenase-2 expression and the release of monocyte chemoattractant protein-1 and prostaglandin-E_2, to a similar extent as Acetylsalicylic acid. Fluridone also inhibited the proliferation of aortic smooth muscle cells and reduced proliferation and cytokine release by human activated lymphocytes. The mechanism of Fluridone seems to rely on the dose-dependent inhibition of the nuclear translocation of nuclear factor-κB, a transcription factor playing a pivotal role in inflammation. Fluridone also inhibited the release from stimulated human monocytes of abscisic acid, a plant stress hormone recently discovered also in mammalian cells, where it stimulates pro-inflammatory responses. Interestingly, the mechanism of Fluridone's toxicity in plants relies on the inhibition of the enzyme phytoene desaturase, involved in the biosynthetic pathway of ß-carotene, the precursor of absciscic acid in plants. Finally, administration of Fluridone reduced peritoneal inflammation in Zymosan-treated mice. These results suggest that Fluridone could represent a new prototype of anti-inflammatory drug, also active on abscisic acid pro-inflammatory pathway.

1. Introduction

Acetylsalycilic acid (ASA) and non-steroidal anti-inflammatory drugs (NSAIDs) in general are widely used for the treatment of inflammatory diseases. However, chronic use of these drugs may lead to gastrointestinal complications (Scheiman and Hindley, 2010), including esophagitis and gastric and duodenal ulcers (Lanza et al., 2009; Singh and Triadafilopoulos 1999; Lanas and Hirschowitz, 1991). For this reason, the development of new "safe NSAIDs", able to overcome or to reduce the risk associated with commercially available NSAIDs in the treatment of chronic inflammation is a much coveted result for the pharmaceutical industry.

Fluridone (1-methyl-3-phenyl-5-(α,α,α-trifluoro-*m*-tolyl)-4-pyridone) has been largely utilized since the second half of the Eighties as a herbicide, particularly to eliminate aquatic plant growth in water reservoirs and irrigation channels. The documented absence of adverse effects on animals has enabled the American Environmental Protection Agency (EPA) to authorize its use in agriculture. Indeed, studies on animals, including mice, rats, dogs, fish and birds, demonstrated absence of toxic, mutagenic, teratogenic and cancerogenic effects of Fluridone, even at high concentrations. Experiments of toxicity in mice fed with Fluridone showed a $LD_{50} > 10$ g/kg and a no observed adverse effect level of 15 mg/kg/die in a 2-year feeding study (Chin and Whang, 2004).

Presence of a pyridone and/or of a trifluoromethyl-benzene moiety is a feature of several new NSAIDs (Hamdy and Gamal-Eldeen, 2009; Lee et al., 2012; Morshed et al., 2009; Oztürk et al., 2002; Yu et al., 2010). Since these moieties are present in the Fluridone molecule (Fig. 1), we surmised that Fluridone might have anti-inflammatory properties. The fact that in-depth toxicity studies already exist for this molecule should allow a faster transition to the clinical phase, if its efficacy were demonstrated *in vitro* and *in vivo* in animal models of inflammation.

Results obtained suggest that Fluridone indeed exerts broad-spectrum anti-inflammatory effects both *in vitro* on human inflammatory cells and *in vivo*, in a mouse model of peritoneal

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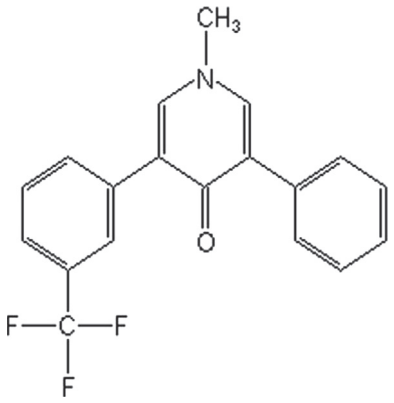

Fig. 1. Structure of Fluridone.

inflammation, possibly representing the prototype of a new class of anti-inflammatory agents.

2. Materials and methods

2.1. Materials

Fetal bovine serum (FBS) was purchased from HyClone (Logan, Utah, USA). D-MEM and RPMI were obtained from Lonza (Milan, Italy). The protein detection assays, PVDF membranes and Chemi-Doc System were purchased from Bio-Rad (Milan, Italy). The ECL-PLUS kit was obtained from GE-Healthcare (Little Chalfont, UK). All primary and secondary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). PGE_2 Monoclonal EIA kit was from Cayman Chemical Company (Ann Arbor, MI, USA); the MCP-1 Human ELISA kit was purchased from Ge-Healthcare. The Ray Bio Human Cytokine Antibody Array was from RayBiotech, Inc. (Norcross, GA). All other chemicals were obtained from Sigma (Milan, Italy).

2.2. Isolation of human lymphocytes and monocytes

Lymphocytes and monocytes were isolated by density gradient centrifugation on Ficoll-Paque of blood enriched in leukocytes (buffy coat), obtained from healthy donors. Low-density mononuclear cells (including lymphocytes and monocytes) were collected at the plasma-Ficoll interphase and monocytes were further purified from lymphocytes by adherence to plastic cell culture flasks. Both cell types were then cultured in RPMI supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml, complete medium).

2.3. Cell cytotoxicity

Lymphocytes or monocytes (1×10^5/well) were seeded in 96-well plates in complete medium and 24 h after seeding cells were treated for 24, 48 or 96 h without (control) or with 0.5, 5, or 50 μM Fluridone. AoSMC (3×10^3/well) were seeded in 96-well plates in SMC medium; after 24 h, the medium was replaced with DMEM without serum, and cells were cultured for further 24 h to induce growth arrest. Cells were then treated for 24 h without (control) or with the same concentrations of Fluridone used for lymphocytes. At the end of the incubations, cell viability was determined on lymphocytes and on AoSMC with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, the wells were washed once with fresh medium, then 200 μl of MTT solution (0.5 mg/ml in complete medium) was added to each well. After 2 h incubation at 37 °C and 5% CO_2, the medium was removed and formazan crystals were solubilized with 100 μl DMSO. Absorbance at 570 nm was read on a FLUOstar OPTIMA fluorimeter (BMG Labtechnologies, Germany). Cell number for each cell type was calculated from a standard curve, obtained in parallel by seeding known cell numbers.

2.4. Prostaglandin-E_2 (PGE_2) and monocyte chemoattractant protein-1 (MCP-1) release by human monocytes

Monocytes (3×10^6/dish) were incubated for 6 h with 100 ng/ml lipopolysaccharide (LPS) from *E. coli* without or with pre-incubation for 2 h with 0.5, 5 or 50 μM Fluridone. After incubation with LPS, the culture medium was recovered for quantification of PGE_2 and MCP-1 release using specific EIA or ELISA kits, respectively.

2.5. Proliferation assay on human lymphocytes

Lymphocytes were seeded in 96-well plates at a concentration of 1×10^5/well and stimulated with human anti-CD3 (2 μg/ml) and anti-CD28 (1 μg/ml) antibodies in the presence or absence of increasing concentrations of Fluridone (ranging between 0.5 and 50 μM) for 4 days. Cells were pre-incubated with Fluridone for 2 h before stimulation and the compound was added again after 48 h. At the end of the incubation, cells were pulsed with 0.5 μCi of [^{3}H] thymidine for 8 h (5 Ci/mmole specific activity; GE-Healthcare) and harvested on Multiscreen Harvest Plates (Millipore, Milan, Italy) using a cell harvester 96 (TomTec, Unterschleissheim, Germany). Multiscreen Harvest plates were quantified in the presence of Maxilight scintillation liquid (Fischer Chemicals, Milan, Italy) using a β-counter (Chameleon TM 425-104 Multilabel Counter, Bioscan, Washington DC).

2.6. Cytokine array on human lymphocytes

Lymphocytes (5×10^6/dish) were stimulated for 24 h with 20 ng/ml anti-CD3 in the presence or absence of 50 μM Fluridone. At the end of the incubation, the supernatants were collected and analyzed by using Human Cytokine Antibody Array III (RayBiotech Inc.) according to manufacturer's instructions. Medium from unstimulated lymphocytes was used as control. Positive spot detection on the membranes and densitometry were performed using Chemi-Doc System (Bio-Rad).

2.7. Histamine release

Heparinized fresh blood samples obtained from healthy volunteers were pre-treated or not with Fluridone at different concentrations for 2 h, and then stimulated with 100 ng/ml LPS for further 3 h at 37 °C. At the end of the incubation samples were centrifuged at $1600 \times g$ for 20 min, the plasma fraction was recovered and re-centrifuged at $1600 \times g$ for 20 min to remove fibrin. Histamine detection was performed using a specific immunoenzymatic assay kit (SPI-Bio, Montigny le Bretonneux, France).

2.8. Aortic smooth muscle cell (AoSMC) proliferation

Human AoSMC (Lonza, Milan, Italy) were seeded in 96-well plates (3×10^3/well) in Smooth Muscle Cells Medium (SMC medium) containing smooth muscle cells bullet kit (Lonza), 100 units/ml penicillin and 100 μg/ml streptomycin. After 24 h the medium was replaced with D-MEM without serum, and cells were cultured for a further 24 h to arrest growth. Cells were then exposed for 24 h to the following media: 100 μl of D-MEM without addition

(control); D-MEM containing 10 ng/ml Platelet-derived growth factor-BB (PDGF-BB) with or without 0.5, 5 and 50 μM Fluridone. After 24 h, the medium was removed; cells were washed with phosphate-buffered saline and incubated 20 min at 37 °C in HBSS containing 2.5 μM calcein-AM and then washed with HBSS; florescence was quantified on FLUOstar OPTIMA fluorimeter (BMG Labtechnologies, Germany) with ex/em set at 488/520 nm. Cell numbers were calculated from a standard curve, obtained in parallel by seeding known cell numbers.

2.9. Abscisic acid (ABA) release from monocytes

Monocytes (2×10^6/dish) were stimulated for 1 h with 50 ng/ml MCP-1 at 39 °C without or with 0.5, 5 or 50 μM Fluridone, preincubated for 2 h. The culture supernatant was recovered, centrifuged ($2200 \times g$ for 10 min) and extracted with 4 volumes of distilled methanol. The monocyte layer was washed with 2 ml Hank's Balanced Salt Solution (HBSS), harvested in 0.5 ml of HBSS and protein content was evaluated by Bradford assay. Acid extraction of ABA and measurement of ABA content by a sensitive (lower limit of detectability 3 fmoles ABA) and specific ELISA kit were performed as described (Bruzzone et al., 2007).

2.10. Nuclear factor-κB (NF-κB) nuclear translocation

Monocytes (3×10^6/dish) and lymphocytes (10×10^6/dish) pretreated for 2 h without or with Fluridone at different concentrations (ranging between 0.5 and 50 μM) were stimulated for 30 min with 100 ng/ml LPS or 20 ng/ml anti-CD3 antibody, respectively. After incubation, cells were washed three times with ice-cold phosphate buffered saline, harvested and resuspended in 400 μl of ice-cold buffer A (20 mM Tris–HCl, pH 7.8; 50 mM KCl; 10 μg/ml Leupeptin; 0.1 M DTT; 1 mM PMSF) and 400 μl buffer B (buffer A containing 1.2% Nonindet P40), vortexed for 10 s, centrifuged at $14{,}000 \times g$ for 30 s at 4 °C and supernatants were discarded. Pelleted nuclei were washed once with buffer A, resuspended in 100 μl buffer B, sonicated (10 s at 3 W) and centrifuged at $14{,}000 \times g$ for 20 min at 4 °C. Supernatants were recovered and 40 μg nuclear proteins per sample were subjected to 10% SDS-PAGE and Western blot. Densitometric analysis was performed using the Chemi-Doc System (Bio-Rad). Values are expressed as luminescence increase relative to control, untreated cells, and normalized on RNA polymerase II.

2.11. Real-time PCR (qPCR) of IκB-α, cyclooxygenase-1 and -2 (COX-1 and -2) in Fluridone-treated human monocytes and lymphocytes

To quantify IκB-α mRNA levels, monocytes (3×10^6/dish) and lymphocytes (5×10^6/dish) were incubated for 2 h without or with 0.5, 5 or 50 μM Fluridone. To quantify COX-1 and -2 mRNA levels, monocytes (3×10^6/dish) were pre-incubated or not with 50 μM Fluridone and then stimulated for further 6 h with or without 100 ng/ml LPS. Total RNA was extracted from monocytes and lymphocytes using the RNeasy micro kit (Qiagen, Milan, Italy), according to the manufacturer's instructions, and reverse transcribed into cDNA using the iScript™ cDNA Synthesis Kit (Bio-Rad). The cDNA was used as template for real-time PCR analysis: reactions were performed in an iQ5 PCR detection system (Bio-Rad). The specific primers for IκB-α, COX-1, COX-2 and GAPDH were designed using Beacon Designer 2.0 software (Bio-Rad) and their sequences were as follows: 5′-CCACTCCATCCTGAAGGCTACC-3′ (forward) 5′-GGTCCACTGCGAGGTGAAGG-3′(reverse) for human IKB-α; 5′-ATCCAGAACAGTGGCTCGTATCC-3′ (forward) 5′-TCAGTGCAGGCACAGATTCAGG-3′ (reverse) for human COX-1; 5′-CACAGGCTTCCATTGACCAGAG-3′ (forward) 5′-CGATGTCACCATAGAGTGCTTCC-3′ (reverse) for human COX-2 and 5′-CCTGTTCGACAGTCAGCCG-3′ (forward) 5′-CGACCAAATCCGTTGACTCC-3′ (reverse) for human GAPDH (reference gene).

Each sample was assayed in triplicate in a 20-μl amplification reaction, containing 25 ng of cDNA, primers mixture (0.2 μM each of sense and antisense primers), and 12.5 μl of 2x iQ SYBR Green Supermix Sample (Bio-Rad). The amplification program included 40 cycles of two steps, each comprising heating to 95 °C and to 62 °C, respectively. Fluorescent products were detected at the last step of each cycle. To verify the purity of the products, a melting curve was produced after each run. Values for human genes were normalized to GAPDH mRNA expression. Statistical analysis of the quantitative real-time PCR was obtained using the iQ5 Optical System Software version 1.0 (Bio-Rad) based on the $2-^{\Delta\Delta}$Ct method, which calculated relative changes in gene expression of the target normalized to GAPDH.

2.12. Zymosan-induced peritonitis in mice

Male CD mice (20–22 g; Charles River, Milan, Italy) were housed in a controlled environment and provided with standard rodent chow and water. The study was approved by the University of Messina Review Board for the care of animals. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other specific purpose (D.M: 116192) as well as with the EEC regulations (O.J. of E.C. L358/1 12/18/1986).

Mice (10 mice/group) were randomly allocated into the following groups: (1) mice treated intraperitoneally (i.p.) with 500 mg/kg Zymosan (Zymosan+vehicle group); (2) mice administered with Fluridone (8.25 mg/kg) at 30 min before Zymosan treatment (Zymosan+Fluridone group) and (3) mice administered with vehicle alone (vehicle group). Eigtheen hours after administration of Zymosan, animals were assessed for shock and peritoneal fluids were collected.

2.13. Statistical analysis

All parameters were tested by paired *t*-test. *P* values < 0.05 were considered significant.

3. Results

3.1. Anti-inflammatory effect of Fluridone on activated human monocytes and lymphocytes

Monocytes and lymphocytes represent the main cell types involved in the immune response against environmental stimuli. Indeed, both cell types play a key role in both inflammation and immunity, by performing antigen presentation, phagocytosis, and immunomodulation through the production of various cytokines and growth factors (Ingersoll et al., 2011; Medzhitov, 2007; Shi and Pamer, 2011). Preliminary experiments were performed to evaluate the possible cytotoxic effect of Fluridone on cultured human monocytes and lymphocytes. As determined by MTT assay, Fluridone was not toxic to these cell types cultured in the presence of 0.5 or 5 μM of the compound for up to 4 days. A slight cytotoxicity was observed only at 50 μM Fluridone after 4 days treatment in lymphocytes (15% mortality).

Preincubation of monocytes for 2 h with 0.5, 5 or 50 μM Fluridone followed by stimulation with 100 ng/ml LPS for 6 h, dose-dependently inhibited PGE_2 and MCP-1 release (Fig. 2A and B). Similar results were obtained when Fluridone was administered together with LPS (not shown). As PGE_2 is one of the main product of COX-2 activity, qPCR experiments were performed to evaluate COX-1 and COX-2 mRNA expression in LPS-treated monocytes, pre-incubated or not with 50 μM Fluridone. COX-2

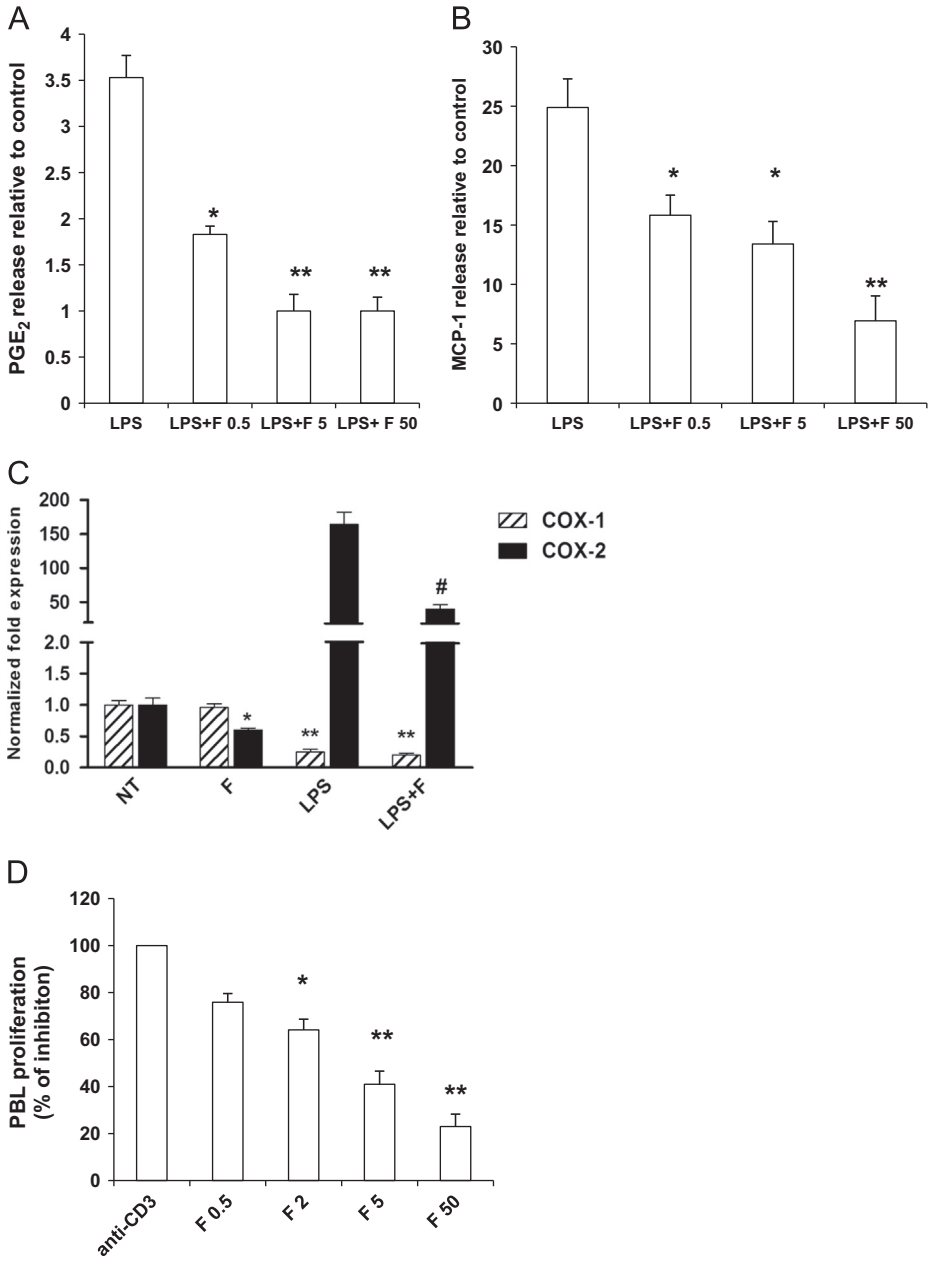

Fig. 2. Effects of Fluridone on human monocytes and lymphocytes activated by inflammatory compounds. (A) and (B) Monocytes (3×10^6/dish) were stimulated for 6 h with 100 ng/ml LPS in the presence or absence of 0.5, 5 or 50 μM Fluridone (F). PGE_2 (A) and MCP-1 (B) production in the medium were quantified as described in Section 2. Results are expressed as increase relative to control levels, present in the medium of untreated cells, and are the mean ± S.D. of four different experiments. $^*P < 0.05$ and $^{**}P < 0.01$ compared to LPS-treated monocytes. (C) COX-1 and COX-2 mRNA levels, as detected by qPCR, in monocytes pre-incubated or not with 50 μM Fluridone (F) and then stimulated for 6 h with or without 100 ng/ml LPS. Results are expressed as normalized fold expression relative to untreated controls (NT), and are the mean ± S.D. of three different experiments. $^*P < 0.05$ and $^{**}P < 0.01$ compared to NT; $\#P < 0.005$ relative to LPS-treated monocytes. (D) Lymphocytes (1×10^5/well) were seeded in 96-well plates and stimulated with 2 μg/ml anti-CD3 and 1 μg/ml anti-CD28 antibodies in the presence or absence of increasing concentrations of Fluridone (F) for 4 days. Proliferation was evaluated as described in Section 2. Results are the mean ± S.D. of three different experiments. $^*P < 0.05$ and $^{**}P < 0.01$ compared to anti-CD3-treated lymphocytes.

was strongly up-regulated after cell stimulation with LPS, while COX-1 levels were significantly reduced (Fig. 2C), in line with published observations (Font-Nieves et al., 2012). COX-2, but not COX-1, mRNA levels in LPS-stimulated monocytes were strongly reduced (by approximately 75%) in Fluridone-treated compared to -untreated cells (Fig. 2C). COX-2 protein expression, as evaluated by Western blot, confirmed a similar extent of inhibition as observed in qPCR (not shown). Lymphocyte proliferation induced by co-stimulation with anti-CD3 and anti-CD28 antibodies (Taub et al., 1996) was inhibited by Fluridone in a dose-dependent fashion, with maximal inhibition (approximately 80%) in the presence of 50 μM Fluridone (Fig. 2D).

To investigate the effect of Fluridone on cytokine release by lymphocytes, the supernatants from lymphocytes pre-incubated or not with 50 μM Fluridone for 2 h and then stimulated with anti-CD3 antibody for further 24 h were analyzed with a human cytokine array detecting several different inflammation- and proliferation-related cytokines. Fluridone almost completely inhibited the release of ENA-78 and of TNF-α and reduced by approximately 50% the release of the IL-1 β, RANTES, GRO-α,

MCP-1/3 and PDGF-BB (Table 1). A lower extent of inhibition was observed on the release of MCP-2 (approx. 30%) (Table 1).

3.2. Comparison between the anti-inflammatory effect of Fluridone and ASA on activated human monocytes

ASA is one of the most widely used medications for its analgesic, antipyretic, and anti-inflammatory effects. At a lower dosage, ASA is also used in the prevention of cardiovascular disease due to its anti-aggregant action (Lu et al., 2010). To compare the anti-inflammatory efficacy of ASA *versus* Fluridone, PGE_2 and MCP-1 release were evaluated in monocytes stimulated with 100 ng/ml LPS. PGE_2 release from monocytes incubated with LPS for 6 h was abrogated by Fluridone (at both 5 and 50 μM) and by 100 μM ASA (Fig. 3A), the lowest concentration experimentally found capable of producing a complete inhibition of PGE_2 and MCP-1 release. A similar extent of inhibition (approximately 90%) was also observed on MCP-1 release by LPS-stimulated monocytes in the presence of 50 μM Fluridone or 100 μM ASA, whereas 5 μM Fluridone was less effective (approximately 45% inhibition) (Fig. 3B), in line with previous results in the same cells (Fig. 2B).

Table 1
Effect of Fluridone on cytokine release from anti-CD3 stimulated lymphocytes.

Cytokine/growth factor	*% Of inhibition*
IL-1β	44.8 ± 6.1
RANTES	43.6 ± 4.8
GRO-α	52.0 ± 7.2
ENA-78	93.7 ± 3.7
MCP-1	50.0 ± 6.4
MCP-2	26.0 ± 4.4
MCP-3	45.4 ± 5.3
TNF-α	85.0 ± 6.5
PDGF-BB	46.6 ± 3.3

Lymphocytes (2.5×10^6/dish) were stimulated for 24 h with or without an anti-CD3 antibody in the presence or absence of 50 μM Fluridone. Media were recovered and cytokine array assays were performed according to the manufacturer's instructions. Results are the mean ± S.D. of three different experiments.

3.3. Fluridone reduces histamine release

Histamine is one of the most important inflammatory mediators, involved in several pathological conditions (Leurs et al., 1995). The cell types responsible for regulation of the immune response (*i.e.* T cells, monocytes and dendritic cells) all possess histamine receptors on their surface and are capable of producing and secreting histamine (Jutel et al., 2002). Histamine release in freshly drawn blood samples incubated for 2 h with 100 ng/ml of LPS, without (control) or with increasing concentrations of Fluridone, was already inhibited by 74% at the lowest concentration tested (0.5 μM) (Fig. 4).

3.4. Fluridone inhibits PDGF-BB-induced AoSMC proliferation

Atherosclerosis is an inflammatory disease (Libby, 2002) and several lines of evidence suggest that COX-2, and its product PGE_2, contribute to the instability of atherosclerotic plaques (Cipollone et al., 2001; Gómez-Hernández et al., 2006). The inhibitory effect of Fluridone on the production and release of different cytokines potentially involved in atherosclerosis (*i.e.* MCP-1, PGE_2, PDGF-BB, MCP-1, MCP-2, MCP-3) prompted us to investigate the effect of Fluridone on human AoSMC proliferation, a critical event in

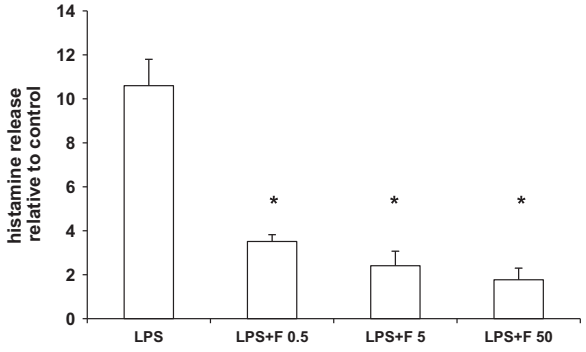

Fig. 4. Fluridone inhibits histamine release. Fresh blood samples from healthy donors were pre-treated or not with Fluridone (F) at different concentrations for 2 h, and then stimulated with 100 ng/ml LPS for further 3 h at 37 °C. At the end of the incubation all samples were centrifuged and histamine detection was performed using a specific immunoenzymatic assay. Results are the mean ± S.D. of three different experiments. $^*P < 0.01$ compared to LPS-treated blood.

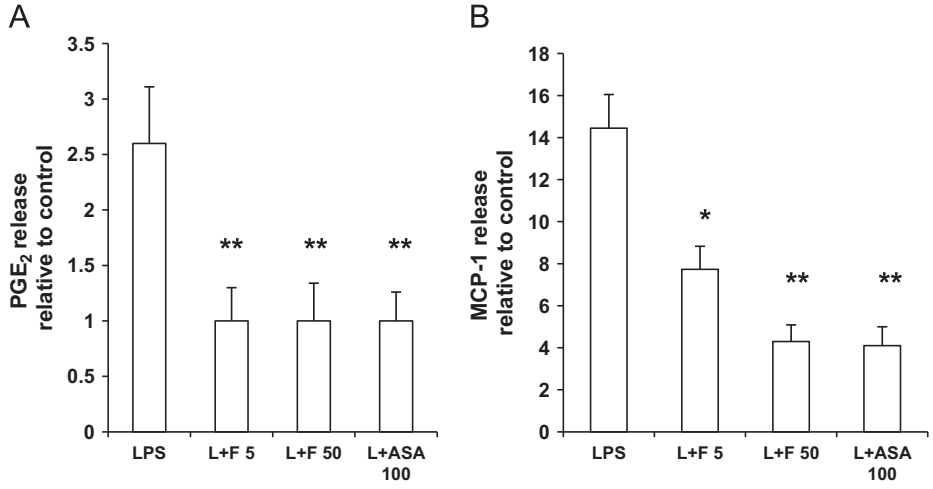

Fig. 3. Comparison between Fluridone and Acetylsalicylic acid in anti-inflammatory activity on human monocytes. Monocytes (3×10^6/dish) were stimulated for 6 h with 100 ng/ml LPS in the presence or absence of 5 or 50 μM Fluridone (F) or 100 μM Acetylsalicylic acid (ASA). PGE_2 (A) or MCP-1 (B) production in the medium were quantified as described in Section 2. Results are expressed as increase relative to control levels, present in the medium of untreated cells, and are the mean ± S.D. of three different experiments. $^*P < 0.05$ and $^{**}P < 0.01$ compared to LPS-treated monocytes.

atherogenesis (Cifarelli et al., 2008;Perez et al., 2011). Incubation of AoSMC for 24 h with 10 ng/ml PDGF-BB, a known stimulator of AoSMC proliferation (Sachinidis et al., 1990; Yoo et al., 2012), increased cell number by approximately 1.5-fold over control, untreated cells (Fig. 5). Pre-incubation of AoSMC with Fluridone abrogated the stimulatory effect of PDGF-BB on AoSMC proliferation at all concentrations tested (Fig. 5). Preliminary experiments were performed to evaluate the possible cytotoxic effect of Fluridone on cultured AoSMC and, as determined by MTT assay, the molecule was not toxic within the range of concentrations tested for 24 h.

3.5. *Fluridone inhibits release of abscisic acid from human activated monocytes*

As it has been recently observed that abscisic acid (ABA) is released from several mammalian cells where it acts as a pro-inflammatory hormone (Bruzzone et al., 2007; Magnone et al., 2012, 2009), we investigated whether Fluridone could inhibit ABA release from human monocytes activated by pro-inflammatory stimuli. Stimulation of human monocytes for 1 h with 50 ng/ml MCP-1 at 39 °C induced a 6-fold higher ABA release into the culture medium compared to control, unstimulated monocytes (Fig. 6). Pre-incubation of the cells for 2 h with increasing concentrations of Fluridone (ranging between 0.5 and 50 μM) prior to stimulation reduced ABA release in a dose dependent manner, down to the levels of unstimulated cells with 50 μM Fluridone (Fig. 6).

3.6. *Fluridone inhibits NF-κB nuclear translocation in human monocytes and lymphocytes*

Several functional activities of monocytes and lymphocytes inhibited by Fluridone, such as monocyte release of PGE_2 and MCP-1 (Fig. 2A and B), release of TNF-α, IL-1β, RANTES, GRO-α, MCP-1/3 and PDGF-BB by activated lymphocytes (Table 1) and lymphocyte and AoSMC proliferation in response to anti-CD3/CD28 or PDGF-BB stimulation, respectively (Fig. 2C, 5), are known to be controlled by the transcription factor NF-κB (Jeong et al., 2011; Kane et al., 2002; Ueda et al., 1994). This prompted us to investigate whether Fluridone could inhibit the nuclear translocation of NF-κB. A key step in the signal transduction triggered by pro-inflammatory agonists is the activation of a kinase complex (IKK) responsible for the phosphorylation and inactivation of NF-κB inhibitors (IκB-α, IκB-β and IκB-ε) (Baldwin 1996), thus

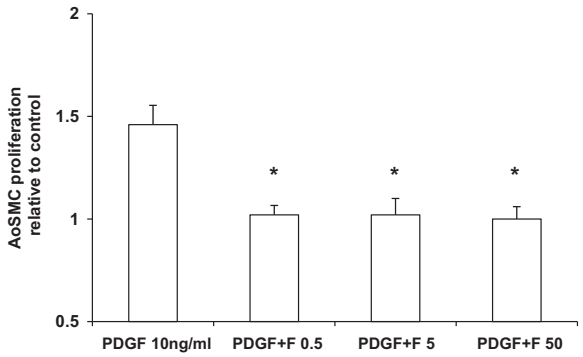

Fig. 5. Effect of Fluridone on AoSMC proliferation. Proliferation of AoSMC was evaluated after the following treatments: incubation for 24 h with D-MEM without serum, in the absence (control cells) or in the presence of PDGF-BB 10 ng/ml without or with increasing concentration of Fluridone (F). No cytotoxicity of Fluridone on AoSMC was detected at the concentrations tested, by means of a parallel MTT assay (see Section 2). Results are the mean ± S.D. of three different experiments. $^{*}P < 0.05$ compared to PDGF-BB-treated AoSMC.

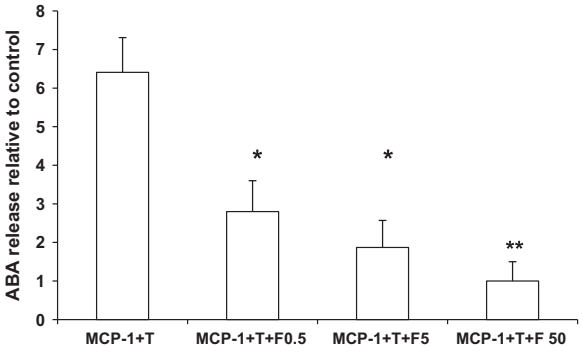

Fig. 6. Fluridone inhibits ABA release from human monocytes. Monocytes (3×10^6/assay) cultured in 1.5 ml D-MEM without serum and phenol red were stimulated or not at 39 °C for 1 h with 50 ng/ml of MCP-1 (MCP-1+T) in the presence or absence of 0.5, 5 or 50 μM Fluridone (F). At the end of the incubation, media were recovered and the amount of ABA released into the culture medium was determined by ELISA. Results are expressed as ABA release relative to untreated cultures and are the mean ± S.D. ($n \geq 4$). Fluridone did not modify basal ABA release in the absence of MCP-1. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to MCP-1+T-treated monocytes.

allowing translocation of NF-κB to the nucleus. It has been demonstrated that the β subunit of IKK is primarily responsible for LPS-induced phosphorylation of IκB-α, IκB-β and IκB-ε in human monocytes (Hawiger et al., 1999; O'Connell et al., 1998); moreover, in the adaptive immune system, activation of the T-cell receptor/CD3 complex together with CD28 initiates several distinct signalling cascades, including NF-κB activation required for antigen-induced proliferation, cytokine production and T cell survival (Kane et al., 2002). Pre-treatment of monocytes with increasing concentrations of Fluridone before LPS stimulation induced a dose-dependent inhibition of NF-κB nuclear translocation, with maximal effect observed at 50 μM Fluridone (Fig. 7A). Similarly, pre-incubation of lymphocytes with Fluridone, followed by stimulation with anti-CD3, resulted in a 65% inhibition of NF-κB translocation at 0.5 μM and in its abrogation at 5 μM (Fig. 7C). The observed inhibition by Fluridone of NF-κB translocation suggested to explore the possible role of NF-κB inhibitors in sequestrating the transcription factor in the cytosolic compartment (Baeuerle and Baltimore, 1988). Incubation of monocytes and lymphocytes for 2 h with increasing concentrations of Fluridone induced a dose-dependent rise of the IKB-α mRNA level, with maximal levels (approximately 70% increase over control values) being observed in both cell types with 50 μM Fluridone (Fig. 7B and D). Moreover, Fluridone increased IKB-α transcription also in stimulated cells: upon exposure to LPS or to anti-CD3, IKB-α mRNA levels increased rapidly in monocytes and lymphocytes, 12- and 3- fold respectively, over unstimulated control cells after 30 min (not shown). This increase was 5-fold higher in monocytes and 1.5-fold higher in lymphocytes, when stimulation with the respective activator occurred after pre-incubation for 2 h with 50 μM Fluridone (not shown).

3.7. *Anti-inflammatory effect of Fluridone in Zymosan-induced peritonitis in mice*

To confirm the anti-inflammatory properties of Fluridone observed *in vitro* on human monocytes and lymphocytes, we investigated whether similar results could be obtained in an *in vivo* murine model of inflammation. Two animal groups were subjected to i.p. injection of 500 mg/kg Zymosan, in the presence or absence of Fluridone (8.25 mg/kg dissolved in DMSO). As a control, i.p injection of DMSO alone was performed in a third group. The concentration of ABA, MCP-1 and PGE_2 in the peritoneal fluid recovered 18 h after mice treatment was strongly

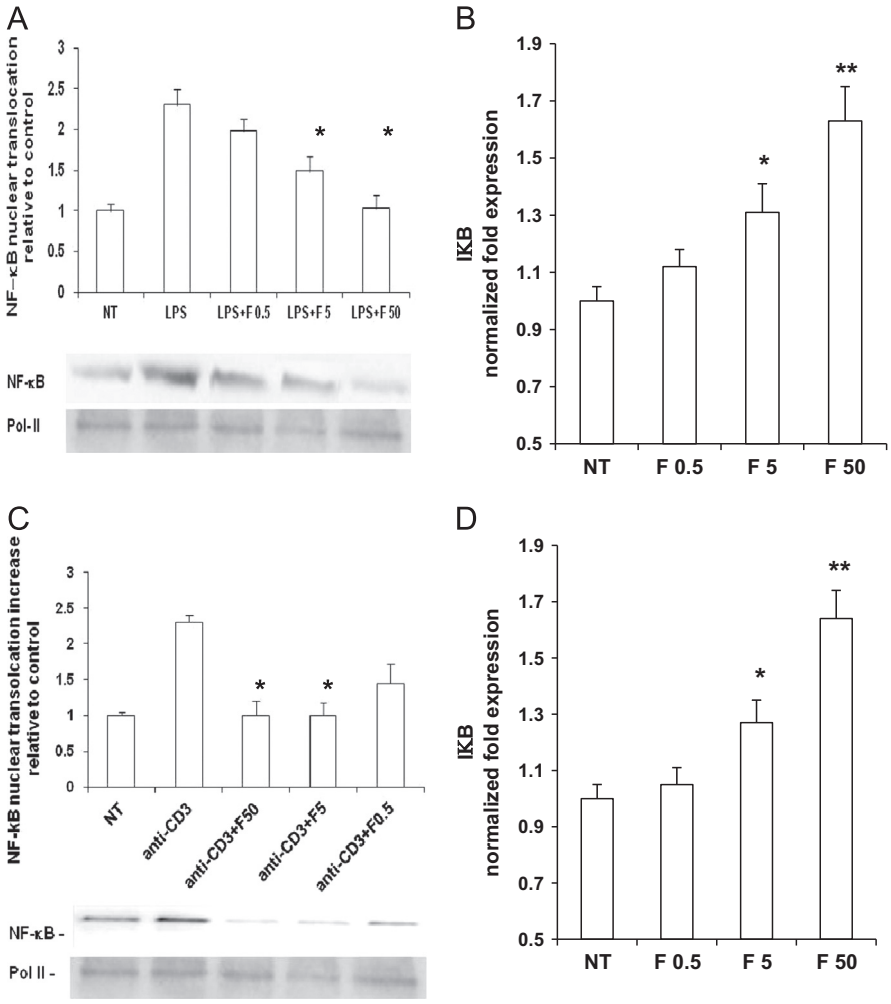

Fig. 7. Fluridone inhibits the nuclear translocation of NF-κB and increases IκB transcription. NF-κB nuclear translocation was quantified on Western blots of nuclear extracts of monocytes (A) and of lymphocytes (C), untreated (NT) or incubated for 30 min with LPS or anti-CD3 antibody, respectively, in the absence or in the presence of increasing concentrations of Fluridone (F). Results shown are the mean ± S.D. of three different experiments. Representative Western blots are shown under each histogram. $^{*}P < 0.05$ compared to LPS-treated monocytes; $^{*}P < 0.05$ compared to anti-CD3-treated lymphocytes. Monocytes (B) and lymphocytes (D) were incubated for 2 h in the absence, untreated cells (NT), or in the presence of 0.5, 5 or 50 μM Fluridone (F). At the end of the incubation cells were subjected to total RNA extraction and real-time PCR were performed to evaluate IκB expression. Results are expressed as mean ± S.D. of three experiments performed in triplicate. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to untreated cells (NT).

reduced in the Fluridone-treated animals (by 44%, 100% and 75%, respectively) compared to the corresponding values detected in mice injected with Zymosan alone (Fig. 8A–C).

4. Discussion

Inhibition by Fluridone of the nuclear translocation of NF-κB (Fig. 7A and C) could account for most of the anti-inflammatory effects reported in the present study. Micromolar Fluridone down-regulates COX-2 expression, PGE_2 and MCP-1 release from LPS-activated human monocytes (Fig. 2A and B) as well as cytokine release and cell proliferation in anti-CD3-stimulated human lymphocytes (Table 1 and Fig. 2D). All these functional activities lie downstream of NF-κB activation (Kane et al., 2002; Ueda et al., 1994). Similarly, AoSMC proliferation in response to PDGF-BB and histamine release are also controlled by NF-κB activation (Miyake et al., 2008; Park and Lim, 2010) and both processes are inhibited by micromolar Fluridone (Figs. 4 and 5). Interestingly, Fluridone also inhibits ABA release from activated human monocytes. ABA is a plant hormone regulating response to abiotic stress that has recently been discovered also in mammalian inflammatory cells, where it acts as an autocrine hormone stimulating cell responses to chemical or physical stimuli. In human monocytes and in murine macrophages, ABA stimulates the release of inflammatory mediators such as MCP-1, PGE_2, matrix metalloproteinase-9 (MMP-9) and TNF-α in response to different types of inflammatory stimuli (Magnone et al., 2012, 2009). ABA also activates human granulocytes, stimulating release of reactive oxygen species and nitric oxide, phagocytosis and migration (Bruzzone et al., 2007). The pro-inflammatory effects of ABA on human monocytes/macrophages are mediated by activation of NF-κB (Magnone et al., 2012, 2009). Thus, inhibition by Fluridone of ABA release from human monocytes (Fig. 6) could contribute to the impairment of NF-κB-mediated signaling in Fluridone-treated cells. This conclusion is strengthened by the observation that excess of ABA (50 μM) partly restored PGE_2 and MCP-1 release from human monocytes stimulated with LPS in the presence of 5 μM Fluridone (60% restoration, not shown).

Results obtained *in vivo*, on a murine model of peritoneal inflammation induced by Zymosan (Hung et al., 2011), confirm the anti-inflammatory effect of Fluridone, which, at a dosage of 8.25 mg/kg, strongly reduces the concentration of PGE_2, MCP-1 and ABA in the peritoneal fluid (Fig. 8). The LD_{50} of Fluridone in

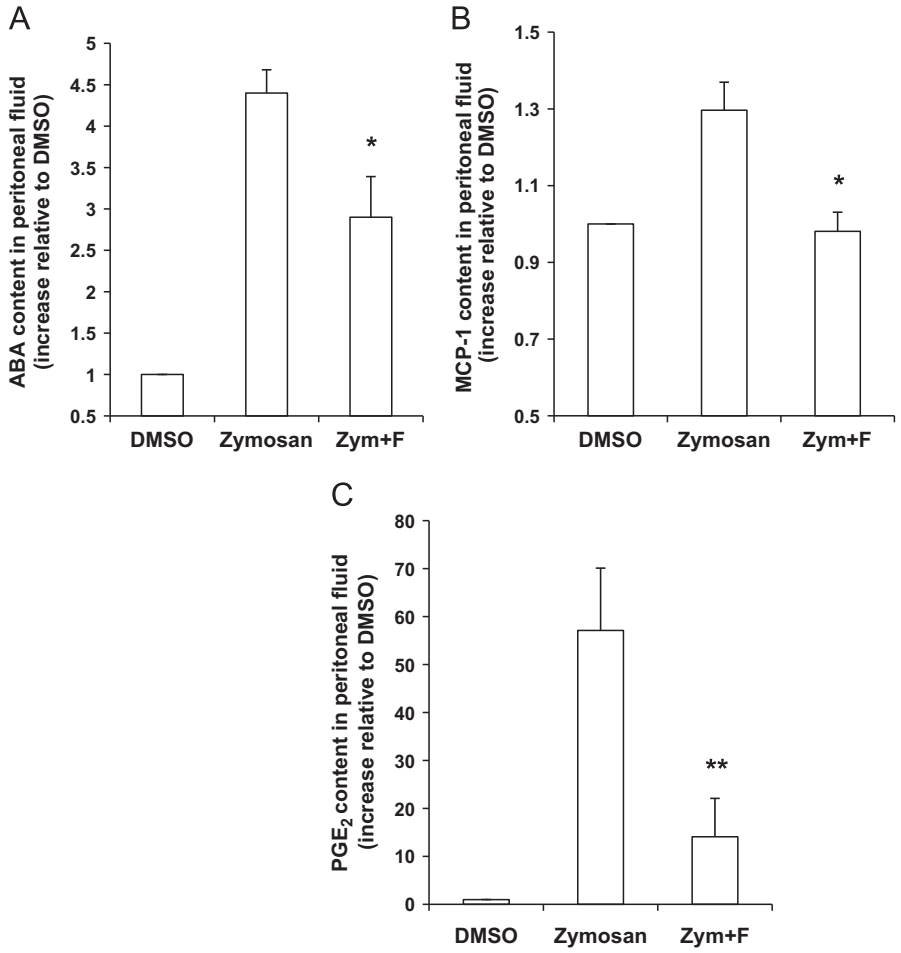

Fig. 8. Inhibitory effect of Fluridone on the production of inflammatory cytokines in Zymosan-induced peritonitis in mice. Mice (10 per group) were injected intraperitoneally with 500 mg/kg Zymosan, or with 500 mg/kg Zymosan and 8.25 mg/kg of Fluridone dissolved in DMSO or with DMSO alone. Eighteen h after injection, peritoneal fluid from each mouse was recovered and ABA (A), MCP-1 (B) and PGE_2 (C) content was evaluated by ELISA. Results are expressed as mean ± S.D. of three experiments performed in triplicate. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to Zymosan-treated mice.

mice is > 10 g/kg by oral administration (Chin and Whang, 2004). Thus, the therapeutic ratio of Fluridone in mice appears to be at least 1000:1. Indeed, no adverse effects were observed after administration of 15 mg/kg/day in mice for 2 years (Chin and Whang, 2004).

Assuming that Fluridone was evenly distributed in the mouse tissues (20 g/animal) after i.p. administration of 8.25 mg/kg, the approximate blood concentration of Fluridone would be 25 μM, *i.e.* within the range of concentrations (5–50 μM) exerting anti-inflammatory effects on human cells *in vitro*.

Based on a developmental no observed adverse effect level of 125 mg/kg/day in rabbits (Chin and Whang, 2004), and applying a dose reduction of two logarithms as an accepted measure of safety, an acute human intake of 1.25 mg/kg/day would allow to reach blood concentrations of Fluridone between 4 and 50 μM, considering a distribution volume of the compound of 60 kg (whole body) or of 5 kg (blood only), respectively. These values are in the range of the effective concentrations eliciting anti-inflammatory effects on human cells *in vitro*.

Finally, the following observations suggest that the anti-inflammatory action of Fluridone could be particularly well suited to delay or inhibit the initial micro-inflammatory events leading to the development of the atherosclerotic lesion: (i) at nanomolar concentrations, Fluridone directly inhibits the proliferation of AoSMC (Fig. 5) and also inhibits the release by activated lymphocytes of PDGF-BB (Table 1), the cytokine principally responsible for stimulating the migration of SMC into the neointima (Raines, 2004); (ii) Fluridone inhibits COX-2 transcription in activated monocytes and release of PGE_2 (Fig. 2A), both processes being involved in atherosclerotic lesion progression (Páramo et al., 2005); (iii) Fluridone inhibits the release by activated monocytes of MCP-1 (Fig. 2B), a key mediator in vascular inflammation, acting as one of the most potent chemoattractants to monocytes; indeed, anti-MCP-1 genetic strategies are being proposed as new approaches to the prevention of atherosclerosis (Kitamoto and Egashira, 2002). The fact that Fluridone inhibits COX-2, but not COX-1 expression (Fig. 2C), might prove advantageous to reduce gastrointestinal injury upon chronic administration, as recent evidence points to the causative role of the simultaneous inhibition of both COX isoforms by several NSAIDs in inducing gastric ulcers (Imanishi et al., 2011).

5. Conclusions

Glucocorticoids and NSAIDs are the most commonly prescribed drug categories worldwide for the treatment of inflammation. Although both classes of drugs can be highly effective in treating inflammation, their administration, mainly in long-term treatment, is often affected by serious adverse effects. Therefore, the identification of new anti-inflammatory compounds is an ongoing effort of the pharmaceutical industry. Here we show that Fluridone, a pyridone compound widely used in agriculture and devoid of toxic, mutagenic, teratogenic and cancerogenic effects

on animals (Chin and Whang, 2004), has anti-inflammatory effects. Inhibition by Fluridone of the production of several inflammatory mediators (including PGE_2, MCP-1, histamine) through a negative regulation of the transcriptional activity of NF-κB suggest that Fluridone could represent the prototype of a new class of anti-inflammatory compounds.

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