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# Adenosine signalling mediates the anti-inflammatory effects of the COX-2 inhibitor nimesulide

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-(2-[7-amino-2-[2-furyl][1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-yl-amino]ethyl)phenol (ZM241385, PubMed CID: 176407)
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ABSTRACT

Extracellular adenosine formation from ATP is controlled by ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase/CD39) and ecto-5′-nucleotidase (e-5NT/CD73); the latter converts AMP to adenosine and inorganic phosphate, representing the rate limiting step controlling the ratio between extracellular ATP and adenosine. Evidence that cellular expression and activity of CD39 and CD73 may be subject to changes under pathophysiological conditions has identified this pathway as an endogenous modulator in several diseases and was shown to be involved in the molecular mechanism of drugs, such as methotrexate, salicylates , interferon-β. We evaluated whether CD73/adenosine/$A_{2A}$ signalling pathway is involved in nimesulide anti-inflammatory effect, in vivo and in vitro. We found that the adenosine $A_{2A}$ agonist, 4-[2-[[6-amino-9-(*N*-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzenepropanoic acid hydrochloride (CGS21680, 2 mg/kg ip.), inhibited carrageenan-induced rat paw oedema and the effect was reversed by co-administration of the A2A antagonist -(2-[7-amino-2-[2-furyl][1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-yl-amino]ethyl)phenol (ZM241385; 3 mg/kg i.p.). Nimesulide (5 mg/kg i.p.) anti-inflammatory effect was inhibited by pre-treatment with ZM241385 (3 mg/kg i.p.) and by local administration of the CD73 inhibitor, adenosine 5′-(α,β-methylene)diphosphate (APCP; 400 μg/paw). Furthermore, we found increased activity of 5′-nucleotidase/CD73 in paws and plasma of nimesulide treated rats, 4 h following oedema induction. In vitro, the inhibitory effect of nimesulide on nitrite and prostaglandin $E_2$ production by lipopolysaccharide-activated J774 cell line was reversed by ZM241385 and APCP. Furthermore, nimesulide increased CD73 activity in J774 macrophages while it did not inhibit nitrite accumulation by lipopolysaccharide-activated SiRNA CD73 silenced J774 macrophages. Our data demonstrate that the anti-inflammatory effect of nimesulide in part is mediated by CD73-derived adenosine acting on $A_{2A}$ receptors.



## 1. Introduction

Adenosine is the final product of ATP degradation, and is released into the extracellular space following metabolic disturbances. Elevated levels of extracellular adenosine have been found in inflamed tissues, ischaemic tissues and under hypoxia, where ATP is released from damaged cells [1]. Levels of extracellular adenosine from ATP are controlled by ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase/CD39) and ecto-5′-nucleotidase (e-5′NT/CD73); the latter converting AMP to adenosine and inorganic phosphate (P*i*) and represents the rate limiting step reaction controlling the ratio between extracellular pro-inflammatory ATP and anti-inflammatory adenosine [2]. Evidence that cellular expression and activity of CD39 and CD73 may be subject to changes under pathophysiological conditions has led to identify this pathway as an endogenous modulator in several diseases [3]. In particular, CD73 was shown to play an important role in regulating vascular permeability and leucocyte trafficking in inflammatory diseases, as evidenced also by studies in CD73 deficient mice [4,5]. Its function would be to regulate the accumulation of adenosine into the extra cellular milieu that, in turn, would limit tissue

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damage through $A_{2A}$ receptor activation [6]. Nonetheless, CD73 has been shown to play a crucial role in the regulation of immune/inflammatory cell function [3,7,8] and several well known anti-inflammatory drugs seem to act through adenosine signalling. Indeed, there is evidence that CD73 is required for the anti-inflammatory effect of methotrexate [9]; furthermore, the beneficial effect of interferon (IFN)-β in patients affected by multiple sclerosis has been associated to CD73 upregulation at the blood–brain barrier [10]; it has been demonstrated that the anti-inflammatory effect of aspirin involves also adenosine accumulation, independently from cyclooxygenase (COX) inhibition [11]. Very recently, it has been shown that in mice the anti-arthritic effect of exogenously administered fructose 1,6-biphosphate, an endogenous intermediate of glycolysis, involves CD39/CD73/adenosine signalling pathway [12].

Nimesulide (*N*-(4-nitro-2-phenoxyphenyl)-methanesulfonamide) is a selective cyclooxygenase 2 (COX-2) inhibitor with a unique chemical structure, belonging to the sulfonanilide class. Nimesulide has been the primary treatment choice for a rapid anti-inflammatory and analgesic effect [13,14]. In addition, nimesulide has been shown to inhibit cancer cell proliferation [15,16], to protect from cerebral ischaemia [17] and from non steroidal anti-inflammatory drug-induced ulcers [18]. Nimesulide effects are unique and not shared with all selective COX-2 inhibitors. On the inflammatory process, nimesulide shows a wide spectrum of actions, with a biochemical mechanism that has not been elucidated at all, but it goes beyond COX-2-dependent prostaglandin synthesis inhibition and implies inhibition of cytokine production and of cell activation, but also reactive oxygen species (ROS) scavenging activity [13,14]. In an early in vitro study, it was suggested that nimesulide inhibited neutrophil function through a direct interaction with adenosine receptors [19]. More recently, nimesulide has been demonstrated to improve the antirheumatic profile of methotrexate in a murine model of collagen-induced arthritis, with a mechanism that might involve adenosine [20]. Although nimesulide usage has been limited since its toxicity, nevertheless, compared to other COX-2 selective inhibitors, it presents the advantage of no cardiotoxicity [14]. To unravel the mechanism of action of nimesulide may help to find target(s) for an anti-inflammatory therapy devoid of gastrolesivity and cardiotoxicity; nonetheless in this context nimesulide, being a peculiar COX-2 inhibitor, can be a useful tool to explore molecular mechanisms involved in the control of inflammation.

In the present study we investigated the possible involvement of the CD73/adenosine pathway on the anti-inflammatory effect of nimesulide.

## 2. Materials and methods

### 2.1. Carrageenan-induced paw oedema

All in vivo experiments were performed on male Wistar rats (Harlan Nossan, 200–250 g; 7–8 weeks old). Rats, slightly anaesthetised with enflurane, received in the left hind paw 100 μl of carrageenan (Sigma–Aldrich S.r.l., Milan, Italy) suspension (1% w/v). Paw volume was measured at the time zero and each hour for 6 h by a hydropletismometer (Ugo Basile, Comerio, VA, Italy).

### 2.2. Drug treatments

Rats were assigned to groups of 5 and treated intraperitoneally (i.p.), just before oedema induction, with the $A_{2A}$ agonist, CGS21680 (2 mg/kg; Tocris Bioscience, Bristol, U.K.), or with the $A_{2A}$ antagonist, ZM241385 (3 mg/kg; Tocris Bioscience, Bristol, U.K.), given alone or in combination. In another series of experiments, animals were treated with nimesulide (5 mg/kg i.p.; Sigma–Aldrich S.r.l., Milan, Italy) given alone or co-administered with ZM241385 (3 mg/kg i.p.), just before carrageenan injection. Control groups received the vehicle, DMSO (0.5 ml/kg; Sigma–Aldrich S.r.l., Milan, Italy). The effect of CD73 inhibitor, adenosine 5′-(α,β-methylene) diphosphate (APCP; Tocris Bioscience, Bristol, UK), injected into the rat paw (400 μg/paw) 1 h following carrageenan injection, was also evaluated in both controls and nimesulide treated animals. In some experiments, blood was withdrawn 4 h following oedema induction by cardiac puncture and anticoagulated with trisodium citrate (3.8% w/v; Carlo Erba Reagents S.r.l., Cornaredo, Milan, Italy). Plasma was then obtained by centrifugation at 3000 rpm for 15 min. Animals were then sacrificed and inflamed tissues were excised and immediately frozen in liquid nitrogen and stored until further analysis. All procedures were performed according to the Italian and European regulations (DL 26/2014) on the protection of animals used for experimental and other scientific purposes and were approved by Italian Ministry of Health.

### 2.3. Cell culture

The murine monocyte/macrophage cell line J774A.1, (American Type Culture Collection, Rockville, MD) was grown at 37 °C, in humidified 5% $CO_2$/95% air in Dulbecco's modified Eagle's medium (DMEM, Gibco; Thermo Fisher Scientific, Waltham, MA,USA) supplemented with 10% foetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA), 25 mM HEPES, 100 U/ml penicillin, 100 mg/ml streptomycin and 1% sodium pyruvate (Thermo Fisher Scientific, Waltham, MA,USA). The cells were maintained in 10 $cm^2$ dishes and for experiments were plated in 24 or 96 culture wells at a density of $2.5 \times 10^5$ cells/ml/well for 18 h. Non-adherent cells were removed by washing with sterile PBS (Gibco; Thermo Fisher Scientific, Waltham, MA,USA) and, immediately before the experiments, the culture medium was replaced by fresh medium. Cells were pre-incubated with the following compounds: the $A_{2A}$ agonist, CGS21680 (1 μM) alone or in combination with the ZM241385 (10 μM); nimesulide (100 μM) alone or in combination with the $A_{2A}$ antagonist, ZM241385 (10 μM), or with the CD73 inhibitor, APCP (5 μM), celecoxib (10 μM; Sigma–Aldrich S.r.l., Milan, Itlay) alone or in combination with APCP (5 μM) or with ZM241385 (10 μM). Drug concentrations to be used throughout the whole study were established following preliminary experiments. All incubations were performed 1 h before cell activation with lipopolysaccharide (LPS) from *Escherichia Coli* (serotype 011: B4; 1 μg/ml, Sigma–Aldrich S.r.l., Milan, Italy) except for ZM241385 that in some experiments was added 6 h following cell incubation with nimesulide or with celecoxib. Controls were performed by cell incubation with DMSO (final concentration 0.05%) that was the vehicle for all compounds, apart from APCP that was dissolved in the cell medium. Following LPS activation, cells were then maintained for 24 h in a humidified incubator at 37 °C under 5% $CO_2$ atmosphere.

Cell viability (>95%) was determined with the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (Sigma–Aldrich S.r.l., Milan, Italy) [21].

### 2.4. Nitrite assay

J774 were cultured and treated as described above. Nitrite production, expressed as nitrite (μM) accumulated in the culture medium 24 h following challenge with LPS, was measured by a spectrophotometric assay, based on Griess reaction, as previously described [22].

### 2.5. Prostaglandin $E_2$ assay

Levels of prostaglandin $E_2$ ($PGE_2$) were assayed in plasma obtained from inflamed rats and in the cell culture medium by an Enzymatic Immune Assay (EIA; Cayman Chemical, Michigan, USA) according to the manufacturer's instructions, and expressed as pg/ml.

### 2.6. Enzymatic assay in rat inflamed tissues and plasma

AMP hydrolysis was evaluated in samples of inflamed tissues and of plasma collected from rats 4 h following oedema induction, as a measure of ecto-5NT and soluble-5NT activity, by colorimetric measurement of the inorganic phosphate (P*i*) released following incubation with the substrate, as described by Nedeljkovic et al. [23]. Briefly, on the day of analysis, inflamed tissues were homogenised using liquid nitrogen in the following lysis buffer: Tris–HCl (50 mM) pH 7.5; NaCl (150 mM); sodium orthovanadate (1 mM); β-glycerophosphate (20 mM); EDTA (2 mM); phenylmethanesulfonyl fluoride (PMSF, 1 mM); leupeptin (5 μg/ml;); aprotinin (5 μg/ml); pepstatin (5 μg/ml). Protein concentration was determined by the Bio-Rad protein assay kit (Bio-Rad Laboratories S.r.l., Segrate, Milan, Italy). To initiate the enzymatic reaction, samples of tissue homogenates or plasma (50 μg of proteins) were incubated with 200 μl of medium containing $MgCl_2$ (10 Mm; Carlo Erba Reagents S.r.l., Cornaredo, Milan, Italy), NaCl (120 mM), KCl (5 mM), glucose (60 mM), Tris–HCl (50 mM), pH 7.4. After 10 min, AMP (2 mM) was added as substrate and samples kept at 37 °C for 40 min. The reaction was then stopped by the addition of trichloroacetic acid (TCA, final concentration 5% w/v). Following sample centrifugation at 3000 rpm for 10 min, at 37 °C, P*i* released was quantified using malachite green as a colorimetric reagent and $KH_2PO_4$ as standard (SensoLyte® MG Phosphate Assay Kit; AnaSpec, Inc., Fremont, CA, USA) [24]. To have the net value of P*i* produced following enzymatic reaction, aspecific P*i* released in absence of AMP in each sample was evaluated and the value obtained was subtracted from the value obtained following incubation with AMP. All samples were run in triplicate; results were expressed as P*i* released (pmol/min/μg protein). All reagents, as not indicated otherwise, were from Sigma–Aldrich S.r.l. (Milan, Italy).

### 2.7. Enzymatic assay in J774

AMPase activity was also evaluated in 24 well plates containing J774 macrophage cell line ($2.5 \times 10^3$ cells/well) plated for 18 h, treated as described above and incubated with or without LPS for 24 h. Afterwards, cells were washed three times with incubation medium without AMP. The enzymatic reaction was then started by the addition of 200 μl of incubation medium as described above, without Tris–HCl, and with some differences: $MgCl_2$ (2 mM); glucose (10 mM) and HEPES (20 mM), pH 7.4. After 10 min of incubation, the reaction was stopped by collecting an aliquot of the incubation medium and transferring it into eppendorf tubes containing TCA (final concentration, 5% w/v), previously placed on ice. Controls to determine non enzymatic P*i* release were performed by incubating the cells in the absence of the substrate, or the substrate in the absence of the cells. To determine specificity, experiments were also performed in cells in the presence of the CD73 inhibitor, APCP. All samples were run in triplicate. The release of P*i* was measured by the malachite green method, using $KH_2PO_4$ as a P*i* standard, as described above. To have the net value of P*i* produced following enzymatic reaction, P*i* released by AMP into the assay medium without cells was subtracted from the total P*i* released by cells during incubation with AMP, and expressed as pmol/min.

### 2.8. Ultra-Performance Liquid Chromatography

In another series of experiments, CD73 activity on J774 macrophage cell line treated as described above was also assessed by quantifying the conversion of 1,$N^6$-etheno-adenosine-5′-*O*-monophosphate (ε-AMP, a bioactive, fluorescent analogue of AMP; BIOLOG, Bremen, Germany) to 1,$N^6$-etheno-adenosine (ε-adenosine) [25], using Ultra-Performance Liquid Chromatography (UPLC). Briefly, cells were washed three times with Hank's Balanced Salt Solution (HBSS; Gibco™, Thermo Fisher Scientific, Waltham, MA, USA) without ε-AMP. Afterwards the cells were incubated with ε-AMP (50 μM) in HBSS for 10 min at 37 °C. To determine specificity, a similar analysis was performed in cells untreated in the presence of the CD73 inhibitor, APCP (50 μM). To stop the reaction an aliquot of 60 μl of the incubation medium was transferred into an eppendorf tube on ice and centrifuged at 4 °C for 2.5 min at 550×*g*. Aliquots of 40 μl were applied to an ACQUITY UPLC® H-Class Bio (WATERS Corp. Milford, MA, USA) and ε-adenosine was separated by running a linear gradient of buffer A (150 mM KCl/150 mM $KH_2PO_4$ at pH 6) and buffer B (15% (v/v) solution of acetonitrile in buffer A) with a flow profile of 0,294 ml/min (0–0.54 min. 100% A; 0.54–1.24 min. 97% A and 3% B; 1.24–4.17 min. 91% A and 9% B; 4.17–7.45 min. 50% A and 50% B; 7.45–8.37 min. 50% A and 50% B; 8.37–8.45 min. 100% A) using a high pressure gradient mixing device. The performance was done on an Acquity UPLC™ BEH $C_{18}$ column (2.1 × 150 mm, 1.7 μm; WATERS Corp. Milford, MA, USA). The analyte ε-adenosine was detected at 254 nm and its concentration was calculated with reference to standards of known concentration (ε-adenosine; BIOLOG, Bremen, Germany) and expressed as pmol/min. The EMPOWER 3 software (WATERS Corp. Milford, MA, USA) was used for data analysis.

### 2.9. Transfection of J774 and real time PCR

For silencing of CD73 mRNA by small interfering RNA (siRNA), murine J774 cells were double shot transfected using HiPerfect (Qiagen, Germany) according to manufacturer's instructions, with a combination of two siRNAs (Sigma–Aldrich, Milan, Italy) specific for the CD73 or one siRNA (Sigma–Aldrich, Milan, Italy) specific for the green fluorescent protein (GFP). The sequences CD73 targeted by the siRNAs were: 5-CAUUGCAGCCUGAAGUAGA-3 and 5-GACAUUUGACCUCGUCCAA-3. The GFP sequence targeted by the siRNA was 5-CGGCAAGCUGACCCUGAAGUUCAU-3 and analysed for mRNA expression levels after 96 h. Total RNA was isolated from mouse J774 cells with TRI Reagent® (Sigma–Aldrich, Milan, Italy) and analysed spectroscopically. One microgram of RNA was retro-transcribed using Prime Script RT reagent Kit with gDNA eraser (Takara, Otsu, Japan) and amplified with specific primers described below. For CD73 mRNA fw: 5-GCGTGCATCGC TATGGCCAGTCC-3 and rv: 5-CCACCGTTGGCCAGATAGCTTGG-3 and fw: 5-AAAACCAACCCGGTGAGCTCCCTC-3 and rv: 5-CTCAGG CTCCCTCTCCGGAATCG-3 for 18 s, all primers were purchased from IDT (IDT, Germany). PCR amplification was carried out as by means of SYBR Premix Ex Taq (Takara, Otsu, Japan) according to manual instruction. PCR amplification of 18 s rRNA was used as the normalizer. Real-time PCR assays were performed using the Rotorgene RG-3000A cycle system (Qiagen, Germany). The programme was set to reveal the melting curve of each amplicon from 60 °C to 95 °C with a read every 0.5 °C.

### 2.10. Flow cytometry

The expression of the protein CD73 in mouse J774 silenced for 96 h and in untreated control was evaluated by flow cytometry platform FACS CANTO II (BD, Franklin Lakes, NJ, USA) after the

determination of nitrite release. Monoclonal antibody APC anti-mouse CD73 (clone: TY/11.8; Biolegend, San Diego, CA, USA) and the isotype-identical antibody as control were used for this purpose. Dead cells were excluded by 7-ADD (Sigma–Aldrich, St. Louis, MO, USA). Results were analysed with FCS Express 5.0 software (De Novo, Glendale, CA, USA).

### 2.11. Statistical analysis

All results were expressed as mean ± standard error of the mean (S.E.M.); each in vitro experiment was run in triplicate. Data were analysed by a one way ANOVA followed by Bonferroni's test for multiple comparisons or by Dunnett's test, as appropriate. Two way ANOVA was also utilised when appropriate. A value of $P < 0.05$ was taken as statically significant.

## 3. Results

### 3.1. Carrageenan-induced oedema

Treatment of rats with the selective $A_{2A}$ agonist, CGS21680 (2 mg/kg i.p.), significantly inhibited oedema formation monitored over a period of 6 h (Fig. 1A) and this effect was reversed by co-administration with the $A_{2A}$ antagonist, ZM241385 (3 mg/kg i.p.). In contrast treatment with ZM241385 alone did not modify oedema development (Fig. 1A). In animals receiving nimesulide (5 mg/kg i.p.), oedema formation was significantly reduced and this effect was partially reversed by treatment with ZM241385 (Fig. 1B). As shown in Fig. 1C, the anti-inflammatory effect of nimesulide was associated with a significant reduction of plasma $PGE_2$ levels, evaluated 4 h following oedema induction, consistent with COX-2 inhibition; however, $PGE_2$ plasma levels were only slightly, but not significantly, reduced by treatment with CGS21680.

### 3.2. AMPase in inflamed tissues and plasma

We also evaluated whether the anti-inflammatory effect of nimesulide would correspond to changes in AMPase activity in inflamed tissues and plasma. We found that in paws obtained from nimesulide-treated rats, AMP hydrolysis was significantly increased compared to the hydrolysis measured in paws from control animals (Fig. 2A). This increased AMPase activity was of functional significance since local injection of the CD73 inhibitor, APCP (400 μg/paw), did not affect oedema development (Fig. 2D) but reversed nimesulide effect (Fig. 2B). AMP hydrolysis was also significantly increased in plasma obtained from animals treated with nimesulide (Fig. 2C).

### 3.3. Nitrite production from J774

In initial experiments, we estabilshed that cell viability (>95%) was not affected by any of the treatments reported below (data not shown). Production of nitrite by un-stimulated J774 cells was undetectable (< 0.8 μM). Stimulation with LPS caused a substantial

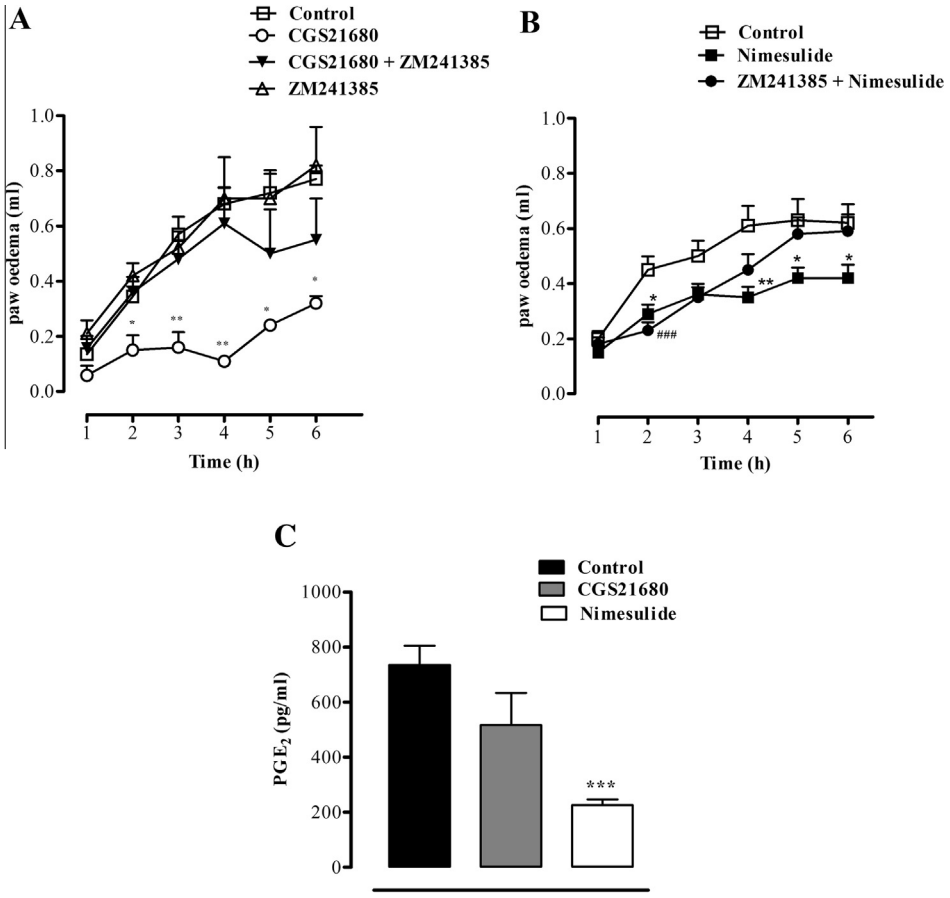


**Fig. 1.** The effect of drug treatments on carrageenan-induced rat paw oedema. (A) The $A_{2A}$ agonist, CGS21680 (2 mg/kg i.p.), inhibits carrageenan-induced rat paw oedema; the $A_{2A}$ antagonist ZM241385 (3 mg/kg i.p.) does not modify oedema development but reverses the effects of CGS21680. (B) Nimesulide (5 mg/kg i.p.) inhibits carrageenan-induced rat paw oedema and its effect is partially inhibited by co-administration of the $A_{2A}$ antagonist, ZM241385 (3 mg/kg i.p.). Results are the mean ± S.E.M ($n = 9–10$). (C) Plasma $PGE_2$ levels evaluated 4 h following oedema induction were reduced following treatment with nimesulide (5 mg/kg i.p.). Each bar represents the mean ± S.E.M. of $n = 3–5$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}$ and $^{\#\#\#}P < 0.001$ vs. control.

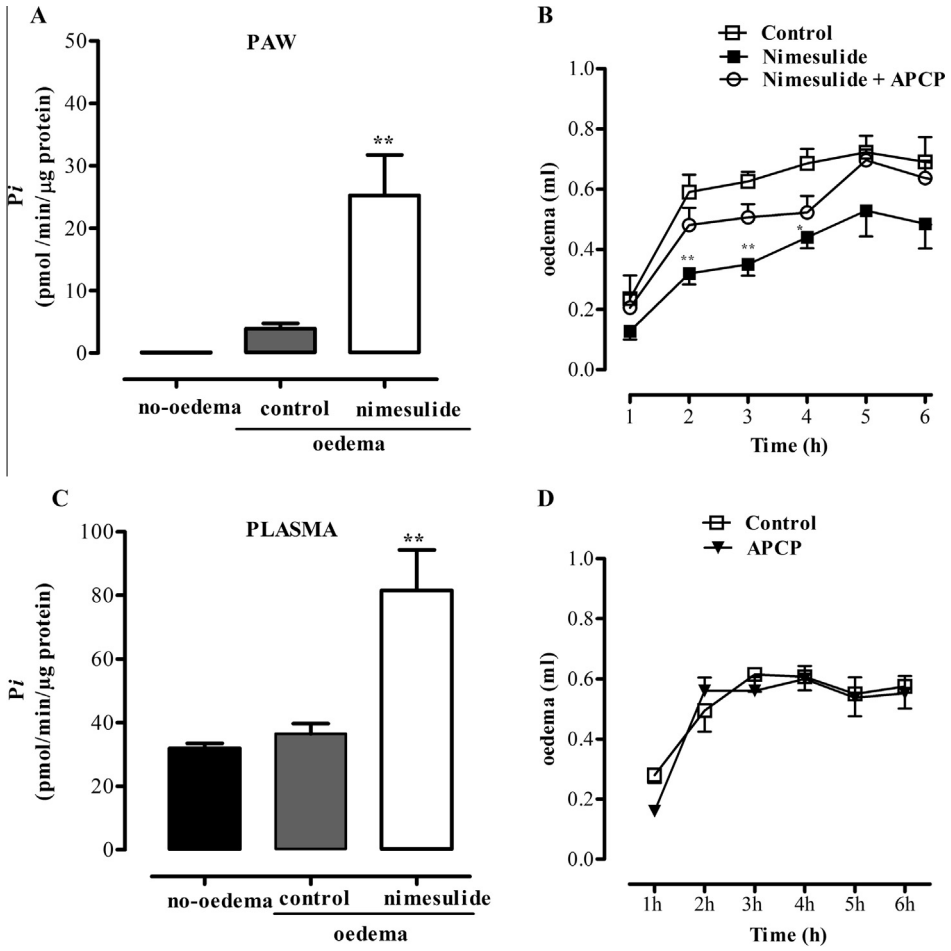


**Fig. 2.** Effect of systemic treatment with nimesulide on AMPase activity. AMP hydrolysis was evaluated as P*i* produced following sample (50 µg proteins) incubation with AMP (2 mM). Rat treatment with nimesulide (5 mg/kg i.p.) increases the extent of AMP hydrolysis in paws (A) and in plasma (C) obtained 4 h following injection of carrageenan. Each bar represents the mean ± S.E.M. of $n = 4–9$. $^{**}P < 0.01$ vs. control; one way ANOVA followed by Dunnett's test. (B) Administration of CD73 inhibitor, APCP (400 µg) into the rat paw, 1 h following carrageenan injection, inhibits the anti-inflammatory effect of nimesulide (5 mg/kg i.p.) treatment and (D) does not affect oedema in control rats. Each point represents the mean ± S.E.M. of $n = 5$. Data are analysed by two ways ANOVA followed by Bonferroni's test. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control.

release of nitrite (26.0 ± 0.9 µM, $n = 5$) that was significantly reduced by the adenosine $A_{2A}$ receptor agonist, CGS21680 (1 µM). The inhibitory effect of CGS21680 (1 µM) was reversed by cell co-treatment with the $A_{2A}$ antagonist, ZM241385 (10 µM; Fig. 3A). Similarly, nimesulide (100 µM) inhibited nitrite production from LPS-activated J774 and its effect was reversed by the $A_{2A}$ antagonist, ZM241385 (10 µM), 6 h following nimesulide treatment (Fig. 3B). ZM2141385 did not have any effect when J774 cells were treated together with nimesulide (data not shown). The inhibitory effect of nimesulide on nitrite production from LPS-activated J774 was also fully reversed by the CD73 inhibitor, APCP (5 µM, Fig. 3C). However, the inhibitory effect of celecoxib (10 µM) on nitrite accumulation from LPS-activated J774 was neither affected by ZM241385 nor by APCP (Fig. 3B and C).

### 3.4. Ecto-5′-nucleotidase in J774

Treatment with nimesulide (100 µM) increased AMPase activity in both unstimulated and LPS-activated J774 cells (Fig. 4A). The specificity of nimesulide effect was proved by evidence that it was blocked by APCP (5 µM). APCP also significantly reduced P*i* accumulation from naive cells (from 17.67 ± 1.4 µM, $n = 9$ to 3.7 ± 1.8 µM, $n = 3$; $P < 0.001$). AMP hydrolysis was not affected when J774 were incubated with celecoxib (Fig. 4B).

AMPase activity was also evaluated using ε-AMP as substrate. Similar to data described above, nimesulide (100 µM) increased the activity while it was unaffected by celecoxib (10 µM) (Fig. 5).

### 3.5. $PGE_2$ formation

There was a significant increase in $PGE_2$ production following J774 activation with LPS that was almost completely inhibited by nimesulide (100 µM). Either ZM241385 (10 µM) and APCP (5 µM) increased $PGE_2$ levels of nimesulide treated cells above control value (Fig. 6). Treatment of cells with CGS21680 (1 µM) did not significantly modify $PGE_2$ production from LPS-activated J774 (662.3 ± 53.28 pg/ml vs. 770.8 ± 42.56 pg/ml; $n = 6$).

### 3.6. SiRNA CD73 silencing

After siRNA treatment, CD73 transcripts were reduced by 69 ± 12% ($n = 3$) as revealed by real-time PCR (Fig. 7C). The reduced expression of CD73 protein (55.4 ± 20%, $n = 3$) was confirmed by means of flow cytometry, as shown in Fig. 7A and B. The effect of nimesulide (100 µM) on nitrite production was then assessed in siRNA CD73-treated cells. LPS significantly induced nitrite production in siRNA treated cells (10.0 ± 4.3 µM, $n = 3$), effect that was not significantly different from that of control cells (17.08 ± 2 µM, $n = 3$). Nimesulide (100 µM) significantly inhibited LPS effect in

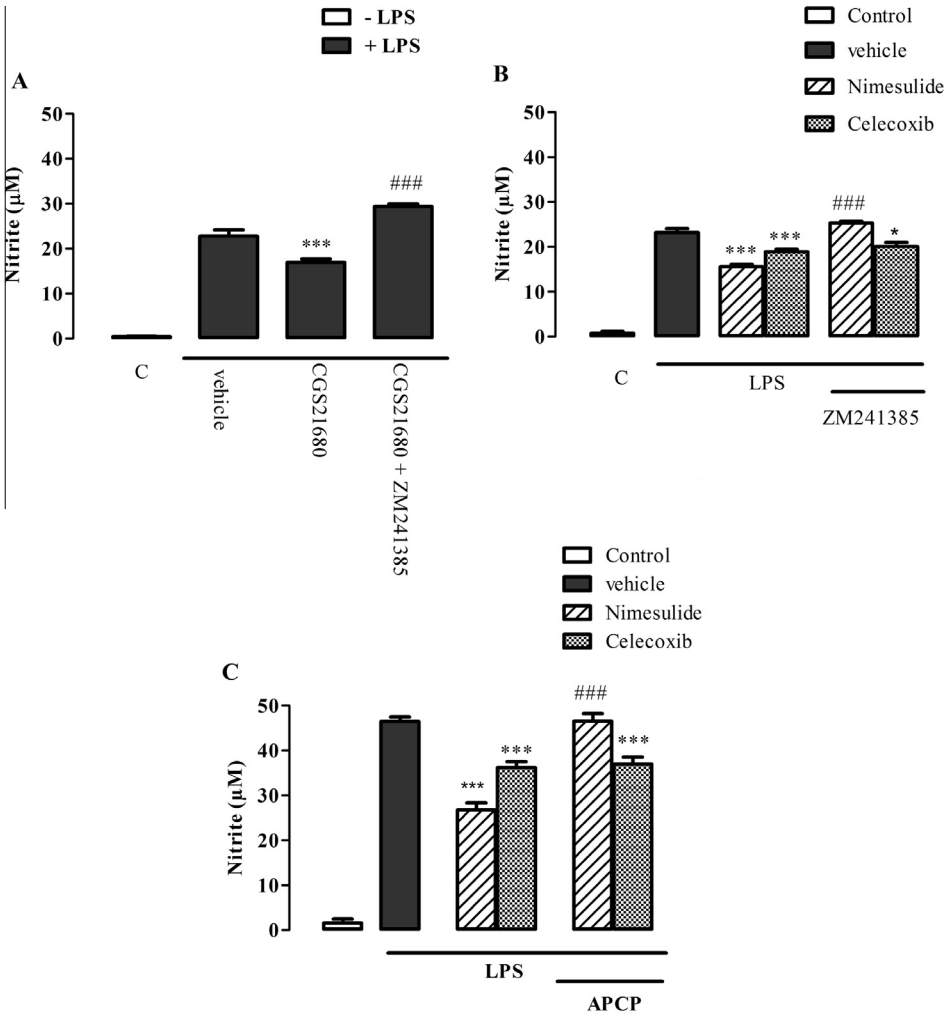


**Fig. 3.** Effect of treatments on nitrite production from LPS-activated J774 macrophages. (A) The $A_{2A}$ agonist, CGS21680 (1 μM), inhibited nitrite production from LPS-activated J774 and the effect was reversed by co-treatment with $A_{2A}$ antagonist, ZM241385 (10 μM). $^{***}P < 0.001$ vs. vehicle and $^{###}P < 0.001$ vs. CGS21680. (B) Nimesulide (100 μM) and celecoxib (10 μM) inhibited ($^{***}P < 0.001$ vs. vehicle) nitrite accumulation. The effect of nimesulide was reversed when ZM241385 (10 μM) was added after 6 h. $^{###}P < 0.001$ vs. nimesulide. (C) CD73 inhibitor, APCP (5 μM), reversed the inhibitory effect of nimesulide on nitrite accumulation. $^{###}P < 0.001$ vs. nimesulide and $^{***}P < 0.001$ vs. vehicle. Each bar represents the mean ± S.E.M. of $n = 5–8$.

control cells, while it did not impair nitrite production in siRNA treated cells (Fig. 7D).

## 4. Discussion

In this study we demonstrate that CD73/adenosine/$A_{2A}$ signalling pathway is involved in mediating the anti-inflammatory effect of nimesulide.

There is broad evidence that endogenous adenosine, generated through the action of two ectoenzymes, CD39 and CD73, exerts anti-inflammatory effects by activation of the $A_{2A}$ receptor subtype localised on several cell types [26]. Here, we have shown that the adenosine $A_{2A}$ receptor also modulates the inflammatory response in our in vivo model of acute inflammation. Similar results have been previously generated in other animal models of inflammation [27–32]. We found that the $A_{2A}$ agonist, CGS21680, strongly inhibited carrageenan-induced rat paw oedema and this effect was reversed by co-administration with the $A_{2A}$ antagonist, ZM241385. Similarly, we found that nimesulide inhibited oedema development and this effect was partially reversed by co-treatment with ZM241385. These results indicate that the adenosine $A_{2A}$ receptor is involved in the anti-inflammatory effect of nimesulide in vivo.

CD73 hydrolyses AMP to adenosine and forms extracellular adenosine which can contribute to the regulation of immune-inflammatory responses, either in vitro and vivo [3,8,33,34]. Interestingly, CD73 has also been shown to be required for the biological effect of several drugs that modulate the immune response [9,10,35–37]. In our model of carrageenan-induced paw oedema formation, AMP hydrolysis in nimesulide-treated rats was significantly increased. This reflects a functional role of the ecto-5′-nucleotidase (CD73) since local treatment with the CD73 inhibitor, APCP, significantly reversed the anti-inflammatory effect of nimesulide, without modifying the oedema development in control animals. These findings indicate that the increased CD73 activity, most likely via the formation of adenosine, contributes to the anti-inflammatory effect of nimesulide in the inflamed tissue.

Membrane bound 5′-nucleotidase can also be shedded into the plasma and can act in concert with a soluble form of NTPDase in the hydrolysis of extracellular ATP to adenosine [34,38]. Interestingly, we found that systemic administration of nimesulide also significantly increased plasma AMPase activity over basal controls.

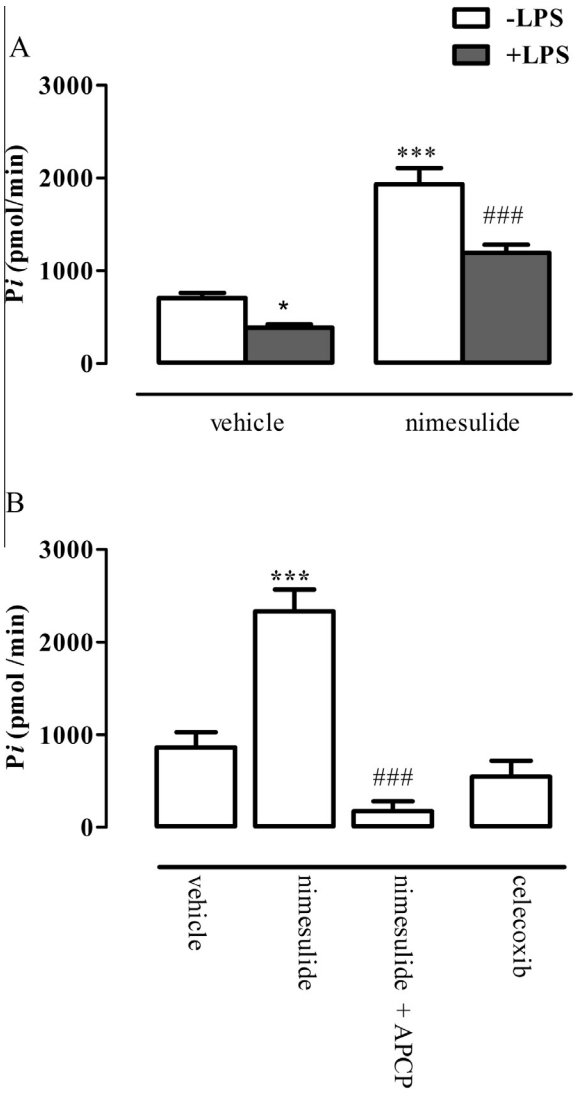


**Fig. 4.** Ecto-5′-nucleotidase activity in J774 evaluated as P*i* accumulation. (A) LPS-activated J774 ($250 \times 10^3$/well) show reduced AMPase activity evaluated as P*i* (pmol/min) accumulation following incubation with AMP (2 mM). Nimesulide (100 μM) increased P*i* accumulation in both non activated and LPS-activated cells. $^{**}P < 0.05$ and $^{***}P < 0.001$ vs. −LPS/vehicle; $^{\#\#\#}P < 0.001$ vs. +LPS/vehicle. (B) APCP (5 μM) inhibits the effect of nimesulide on P*i* accumulation. $^{***}P < 0.001$ vs. vehicle and $^{\#\#\#}P < 0.001$ vs. nimesulide. Each bar represents the mean ± S.E.M. of $n = 9–5$.

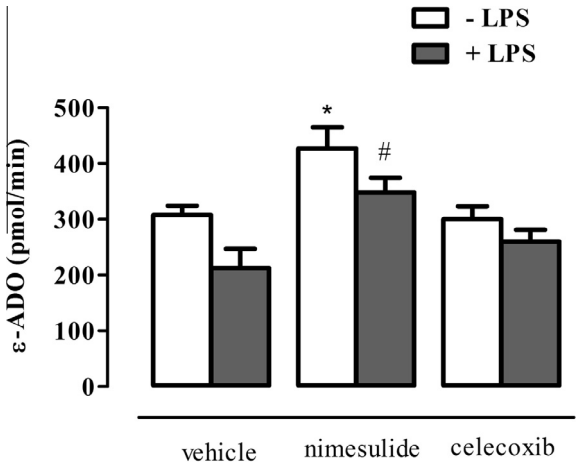


**Fig. 5.** Ecto-5′-nucleotidase activity in J774 evaluated as etheno adenosine-accumulation. Breakdown of etheno-AMP (ε-AMP) to etheno-adenosine (ε-ADO, pmol/min) by non activated and LPS-activated cells ($250 \times 10^3$/well) was increased by nimesulide. $^{*}P < 0.05$ vs. −LPS/vehicle; $^{\#}P < 0.05$ vs. +LPS/vehicle. Each bar represents the mean ± S.E.M. of $n = 6$.

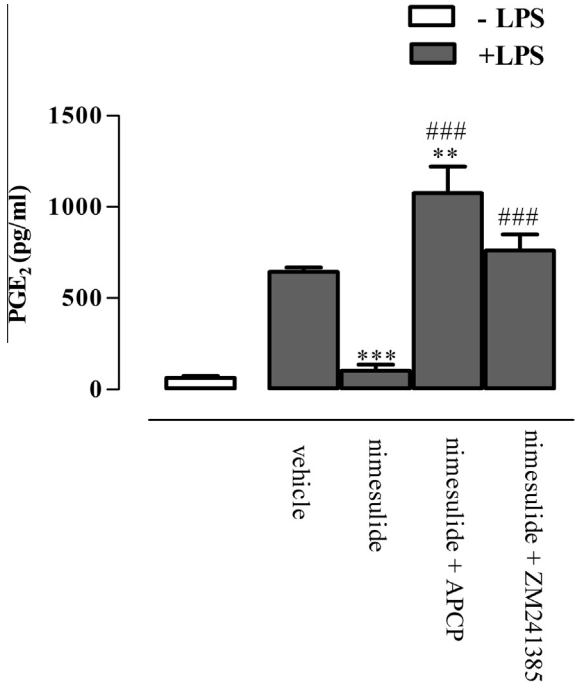


**Fig. 6.** $PGE_2$ accumulation in the culture medium. APCP (5 μM) and ZM241385 (10 μM) reversed the inhibitory effect of nimesulide (100 μM) on $PGE_2$ production from LPS-activated J774. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. vehicle; $^{\#\#\#}P < 0.001$ vs. nimesulide. Each bar represents the mean ± S.E.M. of $n = 6$.

The anti-inflammatory role of the adenosine $A_{2A}$ receptor was confirmed in the J774 macrophage cell line in that the $A_{2A}$ agonist, CGS21680, reduced nitrite production following cell activation with LPS and this effect was reversed by the $A_{2A}$ antagonist ZM241385. Nimesulide inhibited nitrite production from LPS-activated J774 cells and this effect again was reversed by ZM241385. Interestingly, the effect of ZM241385 was lost when added concomitantly to nimesulide (data not shown) but was only evident when it was added 6 h thereafter, suggesting that adenosine $A_{2A}$ receptor was likely involved but not directly activated by nimesulide. This result was also consistent with results obtained in vivo, where the reversal of nimesulide effect by ZM241385 was evident only after 4 h.

We found that CD73 activity was reduced following J774 activation with LPS, which is similar to findings in LPS-activated murine peritoneal macrophages [39]. Similarly, LPS was reported to down-regulate CD73 activity in cultured cortical astrocytes [40]. Here we found that pre-treatment with nimesulide strongly increased CD73 activity in naive J774 cells and this effect was still evident 24 h following LPS-induced cell activation. Enhanced CD73 activity in the presence of nimesulide was confirmed in independent experiments using ε-AMP as substrate.

Specific inhibition of CD73 with APCP reversed the nimesulide-induced anti-inflammatory effect as evidenced by the inhibition of nitrite accumulation. While the effect of CGS21680 on nitrite production in LPS-activated J774 was not accompanied by changes in $PGE_2$ release, nimesulide inhibited both nitrite and $PGE_2$ formation and both effects were reversed by ZM241385 or APCP. These results clearly suggest an important role of the CD73/adenosine/

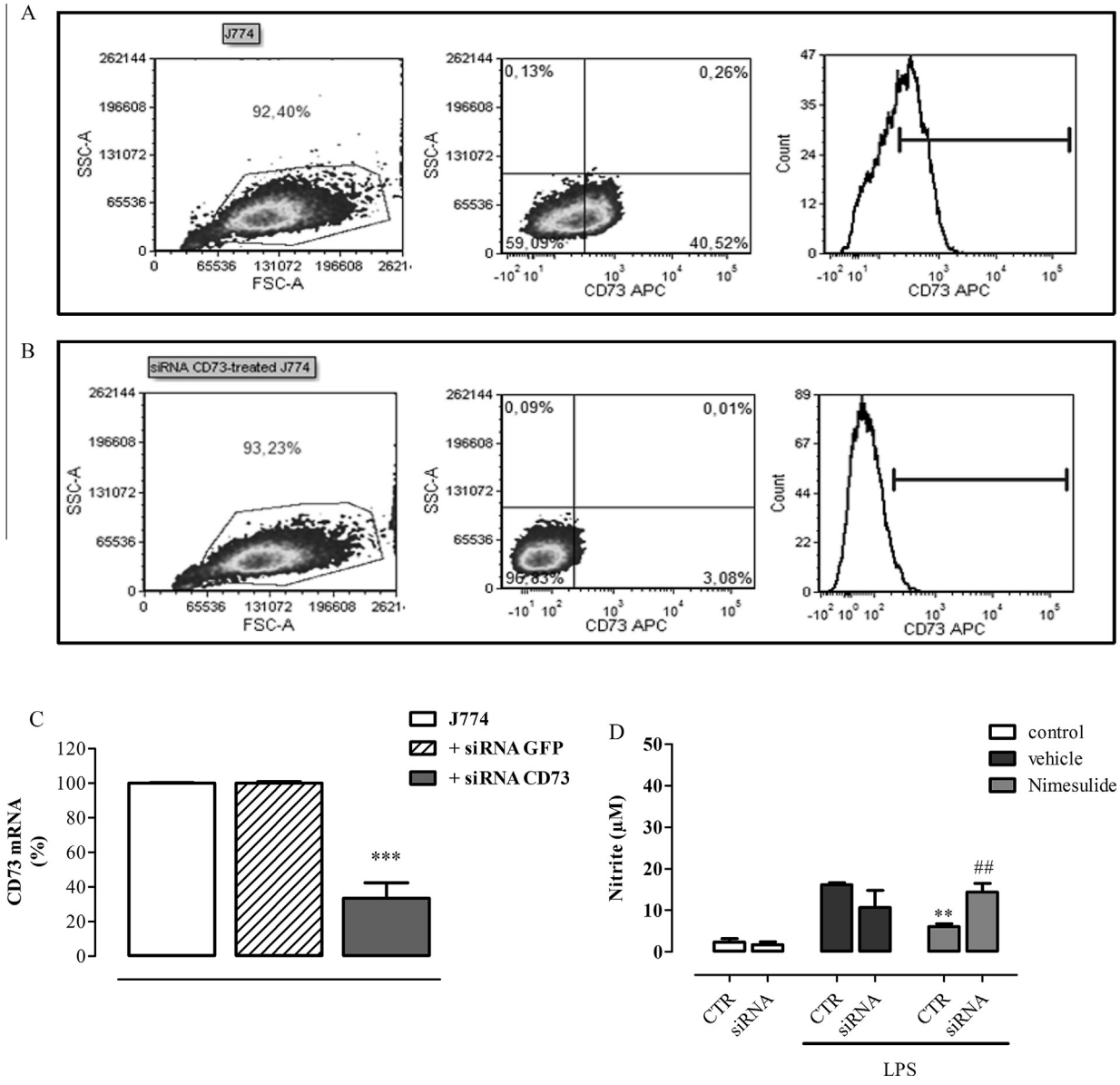


**Fig. 7.** Effect of nimesulide on siRNA CD73 J774. Representative flow cytometry (dot plots and histograms) outlining CD73 expression is shown. (A) control J774, (B) silenced J774 cells (siRNA-CD73-treated J774). (C) mRNA CD73 expression assessed by mean of real-time PCR. Results are reported as percent of untreated cells (J774). SiRNA-GFP, negative control; siRNA CD73, siRNA for CD73. $^{***}P < 0.001$ vs. J774 and vs. SiRNA-GFP; $n = 3$. (D) Effect of nimesulide (100 μM) on nitrite production in CTR and siRNA CD73-J774 (siRNA) stimulated with LPS (1 μg/ml). Each bar represents the mean ± S.E.M. of $n = 3$. $^{**}P < 0.01$ vs. CTR/vehicle, $^{\#\#}P < 0.01$ vs. CTR/nimesulide.

$A_{2A}$ axis to regulate macrophage function and, consequently, the innate immune response, as was already suggested [41,42]. It is therefore very likely that nimesulide by activating the CD73/adenosine/$A_{2A}$ pathway can inhibit macrophages shifting towards a pro-inflammatory phenotype following activation with LPS. This conclusion is in line with the observation that nimesulide was unable to impair nitrite production when CD73 in J774 cells was downregulated by siRNA.

The observed effect on CD73 is specific for nimesulide and is not shared by other COX-2 inhibitors such as celecoxib. The effect of celecoxib at a concentration that inhibited $PGE_2$ production and reduced nitrite accumulation from LPS-activated J774 was neither modified by ZM241385 nor by APCP. Furthermore, consistent with an independent effect of nimesulide on adenosine signalling, celecoxib did not alter CD73 activity in the J774 macrophage cell line. Thus, in addition to the COX-2/$PGE_2$ pathways, only nimesulide activates the CD73/adenosine/$A_{2A}$ axis. The molecular mechanisms by which nimesulide influences adenosine signalling is presently unclear but might involve a direct or indirect effect on CD73 activation. It is also possible that nimesulide may change CD73 activity by altering membrane fluidity [43,44].

Furthermore, by our data it appears that CD73/adenosine/$A_{2A}$ axis, once activated, is required by nimesulide to inhibit COX-2 with a mechanism that needs to be elucidated. There is evidence that $A_{2A}$ activation stimulates the expression and activity of ATP binding cassette transporter A1 (ABCA1) that regulates the transport of lipophilic molecules across membrane [45,46]; thus one could speculate that CD73/adenosine/$A_{2A}$ axis might regulate nimesulide intracellular concentration or, alternatively, might reduce phospholipid availability for the action of COX-2. This would explain the loss of COX-2 inhibition by nimesulide in the presence of APCP or ZM241385.

In summary, the in vivo experiments (carrageenan-induced paw oedema) and in vitro studies (J744 macrophage cell line) have shown that the anti-inflammatory effect of nimesulide involves the CD73/adenosine/$A_{2A}$ signalling pathway. These results are in support of a previous hypothesis suggesting that nimesulide may share a common mechanism with methotrexate also involving adenosine acting on immune cells [20]. Thus, the dual mode of anti-inflammatory activity of nimesulide demonstrated in the present study might be exploited in the future by synthesising COX-2 inhibitors which in addition display a more potent activity on adenosine signalling.

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