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In vivo activity of a PLC inhibitor, U73122, in acute and chronic inflammatory reactions

Cuifen Hou*, Thomas Kirchner, Monica Singer, Michele Matheis, Dennis Argentieri and Druie Cavender

Department of Drug Discovery, Johnson & Johnson Pharmaceutical Research and Development, L.L.C. 1000 Route 202, Raritan, New Jersey 08869, USA

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Running title: Characterization of U73122 *in vivo* in inflammation models

Correspondence to:
∗Cuifen Hou, Ph.D.
Johnson & Johnson Pharmaceutical Research and Development, L.L.C.
Drug Discovery,
1000 Route 202, Room B-110, PO Box 300,
Raritan, NJ 08869,
USA
Phone: (908) 704-5183
Fax: (908) 526-6469
E-mail: chou@prdus.jnj.com

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U73122:	1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione
U73343:	1-(6-((17β-3-methoxylestra-1,3,5(10)-trien-17yl)amino)hexyl)-2,5-pyrrolidine-dione

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ABSTRACT

To investigate the role of phospholipase C (PLC) in inflammatory processes, we tested U73122, a widely-employed PLC inhibitor, in several *in vitro* and *in vivo* assays. We first examined the effects of U73122 on human phospholipase C-β (PLC-β) isozymes and found that U73122 significantly inhibited recombinant human PLC-β_2, with an IC_{50} of ~6 μM. U73122 had little effect on PLC-β1, PLC-β3, or PLC-β4. Consistent with its ability to inhibit PLC-β2 enzymatic activity, U73122 reduced interleukin-8 (IL-8) and leukotriene B_4 ($LTB_{4)}$-induced Ca^{2+}-flux and chemotaxis in human neutrophils in a concentration-dependent manner. *In vivo*, U73122 blocked carrageenan-induced hind paw edema in rats, carrageenan-induced macrophage and lymphocyte accumulation into subcutaneous chambers in dogs, lipopolysaccharide (LPS)-induced macrophage, lymphocyte infiltration and PGE_2 production in a mouse peritonitis model, and 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced ear edema in mice. These results implicate PLC-dependent signaling pathways in the development of acute and chronic inflammatory responses *in vivo*.

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Inflammation is a series of well-coordinated dynamic events that depend on sequential arrival of inflammatory leukocytes to the site of inflammation, where neutrophils are the first cells to migrate into tissues in response to noxious stimuli. It is assumed that neutrophil infiltration or its related event might be crucial for subsequent macrophage infiltration. (Miyazaki, et al. 2000). Many chemoattractants have important roles in inflammatory reactions. Their receptors couple to the inhibitory heterotrimeric guanine nucleotide-binding proteins and elicit a wide range of responses in leukocytes (Baggiolini 1998; Premack and Schall 1996; Jung and Littman 1995; Zhong et al. 2000). There is evidence that the signaling pathways mediated by phospholipase C (PLC) and PI 3-kinase (PI-3K) are activated by chemoattractant receptors (Stoyanov et al. 1995; Stephens et al. 1997). The activation of PLC is one of the earliest key events in the regulation of various cell functions by a number of extracellular signaling molecules. PLC catalyzes the hydrolysis of a membrane phospholipid, phosphatidylinositol, 4,5-bisphosphate (PIP_2), to produce two intracellular messengers, diacylglycerol (DAG) and inositol-trisphosphate (IP_3), which, in turn, mediates the activation of protein kinase C (PKC) and intracellular Ca^{2+} release, respectively. Human neutrophils express abundant PLC. In leukocytes, cell-surface receptor activation leads to actin cytoskeleton reorganization that drives cell motility (Glogauer et al., 2000). The most well studied chemotactic receptors of leukocytes are the heterotrimeric G-protein-coupled, pertussis-toxin (PTX)-sensitive formyl peptide (*f*MLP) receptor and leukotriene B_4 (LTB_4) receptor and chemokine receptors. Chemokines are a large family of small (8-10 kDa) proinflammatory cytokines, which are produced by various cell types. Many chemokine receptors including interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1)

and macrophage inflammatory protein-1α (MIP-1α) receptors were reported to stimulate PLC by coupling to G protein (Charo et al. 1994; Franci et al. 1995; Jiang et al.1994). These receptors play important roles in the migration of neutrophils, monocytes and some T cells. It is thought that the G-protein βγ-linked pathway may account for the PTX-sensitive activation of PLC mediated by the IL-8 receptors in mature leukocytes (Kuang et al. 1996). However, despite the ample evidence, the role of PLC in the pathophysiology of inflammatory disorders is unclear.

To understand the role of PLC in the inflammatory responses in vivo, in the present study we investigated the effect of U73122, a membrane-permeable aminosteroid PLC inhibitor, on both acute and chronic inflammation models in rats, dogs or mice. U73122 was reported to selectively inhibit the PLC-dependent process in human platelets and neutrophils (Smith et al. 1990; Bleasdale et al., 1990; Jan, et al. 1998) and, therefore, has subsequently proven useful in the evaluation of the role that PLC plays in cell activation (Lockhart, et al. 1999). We have evaluated the effects of U73122 on carrageenan-induced hind paw edema in rats, carrageenan-induced macrophage accumulation in fluid exudates from subcutaneous chambers in dogs, LPS-induced macrophage infiltration into peritoneal lavage fluid of mice, TPA-induced ear edema in mice and LPS-induced macrophage infiltration in peritoneal fluid of mice. In addition, we have examined the effect of U73122 on PGE_2 production in LPS-induced peritonitis in mice. As part of our search for anti-inflammatory agents, we describe here for the first time the in vivo effect of U73122 on acute and chronic inflammation. The effects of U73122 on inflammatory parameters such as swelling and leukocyte infiltration, as well as specific PLC isozyme activities, are presented.

Materials and Methods

Animals

Male Sprague-Dawley rats, male Balb/C mice and Swiss Webster mice were purchased from ACE Animals, Boyertown, PA. Purpose bred dogs were purchased from Marshall Farms, North Rose, NY and housed in an American Association for Accreditation of Laboratory Animal Care (AAALAC) accredited facility. All procedures conformed to the requirements of the Animal Welfare Act and were conducted according to the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals. The studies were approved by the Institutional Animal Care and Use Committee. The animals were maintained on a 12-h light/dark cycle, the room temperature was set at 64-84°F and the humidity set at 30-70%. They were fed food and water ad libitum. The dogs were housed singly in 12 sq.ft. stainless steel cages and were fed Purina High Density Canine Diet once daily.

Chemicals and reagents

The PLC inhibitor U73122 and arachidonic acid (AA) were obtained from Biomol (Plymouth Meeting, PA). [^{3}H]PIP_2 and scintillation pre-coated 96-well plates were purchased from PerkinElmer Life Sciences (Boston, MA). Ficoll-pague and dextran T-500 were purchased from Pharmacia (Piscataway, NJ). Fluo-3 was purchase from Molecular Probes (Eugene, OR). Interleukin-8 (IL-8) was from R & D Systems (Minneapolis, MN). The cell culture media and serum were from CellGro (Kansas City, MO). The prostaglandin E_2 (PGE_2) EIA kit was from Assay Design (Ann Arbor, MI). The plethysmograph was from Buxco (Sharon, CT). Ionomycin was from CalBiochem (La

Jolla, CA). Leukotriene B_4 (LTB_4), chemotactic peptide *f*MLP, complement C5a, C3a, 12-O-tetradecanoylphorbol-13-acetate (TPA), carrageenan, indomethacin and the rest of the chemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

PLC assays

A scintillant pre-coated 96-well microplate (PerkinElmer Life Sciences, Boston, MA) was coated with 0.1 μCi of [^{3}H]PIP_2 (PerkinElmer Life Sciences, Boston, MA) in buffer and incubated at 4^{o}C for at least 72 hours, then aspirated and washed 3 times with PBS (Ca^{2+} and Mg^{2+} free) prior to use. The amount of [^{3}H]PIP_2 immobilized on the plates was determined by reading the plate in reaction buffer (50 mM Tris/HCl pH 7.2, 1 mM EDTA pH 7.3, 80 mM KCl, 10 mM LiCl, 0.04% DOC and 3 mM $CaCl_2$) on a microplate scintillation & luminescence counter (PerkinElmer Life Sciences, Boston, MA). The reaction was started with the addition of enzyme to wells in the presence or absence of U73122 with varying concentrations except the control wells. The wells were mixed and the plates were incubated at 37^{o}C for 1.5 hours. The plates were then read on a microplate scintillation & luminescence counter after termination of the reactions and these counts were used as the Postcounts for the assay. The percent inhibition of drugs was then calculated as a function of the amount of hydrolysis of the substrate (Hou, C et al. manuscript 2004).

Calcium mobilization assay in human neutrophils

Fresh human neutrophils were isolated from peripheral blood obtained from human volunteers, and loaded with 4 μM Fluo-3 (Molecular Probes, Eugene, OR), a

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calcium-sensitive fluorescence dye, in Hank's balanced salt solution (HBSS), pH 7.4, containing 20 mM HEPES, pH 7.5, 3.2 mM $CaCl_2$, 1% fetal bovine serum, 2.5 mM probenecid, and 0.04% pluronic acid, in the dark at room temperature for 45 min with gentle rocking. The excess dye was removed by washing cells with HBSS after centrifugation. The cells were then resuspended in HBSS buffer and added to clear bottom black 96-well plates. U73122 (Biomol, Plymouth Meeting, PA) with indicated concentrations was added to the neutrophils and incubated at 37°C for 30 min. Agonists in appropriate concentration were prepared. Changes in intracellular free Ca^{2+} concentration were measured with a FLIPR instrument immediately after the addition of agonist. The final concentration for IL-8 (R & D Systems, Minneapolis, MN) and LTB_4 (Sigma, St. Louis, MO) was 10 nM.

IL-8 and LTB_4-induced chemotaxis assay in human neutrophils

IL-8 or LTB_4 was prepared at a final concentration of 0.01 μg/ml in RPMI-1640 containing 0.5% BSA. Using Costar 24-well Transwell filter plates, medium containing IL-8 or LTB_4 was added to the bottom chambers of the plate. To the top chamber of the Transwell plate, 1 x 10^6 of freshly prepared human neutrophils was added. Next, testing compound or vehicle was added both to the cells and to the bottom chamber. The filters were loaded onto the bottom chambers of the plate and the plate was incubated for 3 hours at 37°C in a CO_2 incubator. The cells that have migrated into the bottom chambers of the plate were then counted using a hemocytometer.

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Carrageenan-induced macrophage accumulation in fluid exudate from subcutaneous chambers in dogs.

Carrageenan-induced inflammation assays in dogs were conducted as previously described (Kirchner et al. 1997). Groups of five beagle dogs of either sex, approximately one year of age, and weighing 8-13 kg were used in this study. After a post operative recovery period of at least 1 month, a 1 ml sample of exudate from each dog was aspirated from within the chamber using a 1 ml syringe and 20 gauge 1 inch needle inserted through one of the perforations in the ball. The area was cleansed with 70% ethanol before each sample was collected. Immediately after obtaining the sample, U73122 (administered to render 2 μM final concentration in the chamber, based on 25 ml average chamber exudate) was injected into the chamber. Then, an inflammatory response was generated by injecting 1.5 ml of 0.33% carrageenan (Sigma Chemical Co., St. Louis, MO) directly into the chamber. Fluid samples of 0.5 ml were obtained from within the chamber using a 21 gauge needle and 1 ml syringe. The skin area was treated with 70% ethanol prior to injections into and aspirations from the chamber. Exudate samples were obtained again at 1, 2, 3, and 4 days post carrageenan administration and were analyzed for macrophage accumulation as an indicator of inflammatory activity using a Technicon H*1E Hematology analyzer (Miles Technicon, Tarrytown, NY) to determine white blood cell (WBC) count. The H*1E is a laser light scattering flow system and colorimetric automated hematology analyzer that has four cytochemistry subsystem modules (RBC/PLT, Peroxidase, Baso, Hemoglobin). The instrument performs a complete blood cell count with differential leukocyte cell count and morphology observations on whole blood. Due to the nature of the exudate samples, only

the WBC value is used from the instrument. Differential cell counts were then performed manually. A cytospin centrifuge was used to spread the fluid out on a slide to make the cells easier to identify and the cells were then stained with Wright's stain. The percentage of cell types was determined after counting 100 cells under a microscope. Antibody and compound effects were compared to the chemotactic response of the control group. The exudate within the chambers was evacuated after the last sample was obtained and the dogs were allowed a recovery period of at least 4 weeks between carrageenan challenges. The dogs were used repeatedly and they served as their own controls when measuring treatment effects on the inflammatory responses elicited by various inflammatory provocations such as carrageenan, TNF-α, IL-8 and MCP-1 (Kirchner et al. 1997).

LPS-induced peritonitis and macrophage accumulation in mice

Swiss Webster mice (ACE Animals, Boyertown, PA) were dosed intraperitoneally with test compound in sterile saline. Thirty minutes later, they were injected intraperitoneally with 1 ml LPS (5 μg/ml) to induce peritonitis. The mice were dosed with U73122 30 mg/kg, *i.p.* on day 0, and once daily for a total of 9 doses. The animals were then sacrificed in a CO_2 chamber on Day 9 and the peritoneal cavities lavaged with 3 ml Dulbecco's PBS to collect cells. Lavage samples were analyzed by differential cell counts using the same procedure as described in the canine subcutaneous chamber model. Percent inhibition was evaluated by comparison to the increase in macrophage count between the baseline control group and the positive control group.

TPA-induced ear edema in mice

TPA-induced ear edema was performed by the method previously reported by Rao et. Al (1993). TPA (1.0 μg) dissolved in 20 μl acetone was applied to the dorsal surface of the right ear of mice. U73122 was administered intravenously before the TPA administration. Six hours later the animals were sacrificed and 7 mm biopsy punches were taken from each ear. The punches were weighed and the difference between treated and untreated ears determined. The percentage of inhibition was calculated by comparing the difference in ear weight of vehicle treated to compound treated mice.

Carrageenan-induced paw edema in rats

Male Sprague-Dawley rats (Ace Animals, Boyertown, PA) weighing ca. 200 grams were fasted overnight. 100 microliters of a 0.5 % carrageenan solution was injected into the subplantar tissue of one hind paw one hour after drug or vehicle pre-treatment. Paw volume displacement was measured using a mercury plethysmograph (Buxco Electronics) at 1, 3 and 5 h after induction of inflammation, and the edema was expressed as an increase in paw volume due to carrageenan injection. Indomethacin (10 mg/kg, *p.o.*), a cyclooxygenase (Cox) inhibitor, was used as a control.

In Vitro COX-2 Assay

Evaluation of specific COX-2 activity of the compound was performed using a whole cell assay with ECV-304 (human, endothelial, umbilical cord) cells (American Type Cell Culture, Rockville, MD) as previously described (Miralpeix et al., 1997 and Kirchner, 1997). These cells were cultured in Media 199/10% bovine serum albumin

(BioWhitaker, Walkersville, MD) at 37°C and 5% CO_2, then trypsinized and plated at a density of 9×10^4 cells per well of a 96-well plate before assay. Approximately 28 h later, 50 μg/ml PMA (Sigma, St. Louis, MO) and 2 μM ionomycin (Sigma, St. Louis, MO) (final concentration) were added to each well. Cells were incubated in the presence of vehicle or drug for 18 hr. PGE_2 production was determined via RIA (Assay Design, Ann Arbor, MI) after the addition of 30 μM arachidonic acid. The data were expressed as percent inhibition of products as compared to vehicle treatment.

Statistical analysis

The results of in vivo experiments were expressed as the mean ± S.E.M. Differences between means were tested for significance by Student's *t* test or (for multiple comparisons with the same control) by an analysis of variance (ANOVA) followed by the Bonferron's multiple comparison test (Prism, Graphpad, San Diego, CA). $^{*}P < 0.05$ or lower was considered to be statistically significant using Student's *t* test.

Results

Effects of U73122 on phospholipase C-β isozyme activities

We conducted our PLC assays by using the exogenously immobilized specific substrate, [^{3}H]PIP_2, and human PLC-β enzymes. U73343, a close analogue of U73122, was used as a negative control. U73122 did not significantly inhibit human recombinant PLC-β1 at concentrations $\leq$25 μM (Fig.1a). However, U73122 markedly inhibited the ability of human recombinant PLC-β2 to hydrolyze [^{3}H]PIP_2. The half-maximal and maximal inhibitory effect of U73122 on PLC-β2 was observed at 6 μM and 25 μM, respectively (Fig.1b). U73122 moderately inhibited the hydrolysis of the substrate by PLC-β3, isolated from human brain, with 35% inhibition at 25 μM (Fig. 1c). Similarly, up to 25 μM, U73122 did not significantly inhibit the hydrolysis of [^{3}H]PIP_2 by PLC-β4 (Fig.1d). The maximum inhibitory effect of U73122 on PLC-β4, isolated from a human retina cell line, was 30% inhibition. The rank order of potency among the isozymes for the inhibitory effect of U73122 was PLCβ2>PLCβ3≥PLCβ4 and PLCβ1. Therefore, our results demonstrate that U73122 preferentially inhibits PLC-β2 activity. U73343 had no effect on any of the four PLC-β isozymes.

Effect of U73122 on IL-8 and LTB_4 induced Ca^{2+} flux in human neutrophils

U73122 was then tested in cellular functional assays. As shown in the Fig. 2a and 2b, U73122 inhibited IL-8 and LTB_4-induced Ca^{2+}-fluxes in human neutrophils in a dose-dependent manner, with an IC_{50} of ~6 μM in each case, consistent with its potency in the PLCβ2 enzyme assay.

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Effect of U73122 on IL-8 and LTB_4-induced human neutrophil migration

It has been reported that cells from PLCβ2 KO mice show decreased chemoattractant-induced Ca^{2+} fluxes, consistent with our results above, but have normal or supranormal chemotactic responses. Therefore, we next tested U73122 in IL-8 and LTB_4 induced chemotaxis assays. As shown in Fig. 3a and Fig. 3b**,** IL-8 and LTB_4 were potent chemotactic factors for human neutrophils. Surprisingly, we found that U73122 markedly inhibited those chemotactic responses with IC_{50}s of ~5 μM, consistent with its effects in the enzymatic and Ca^{2+} mobilization assays described above.

Effect of U73122 on carrageenan-induced hind paw edema in rats

We examined the effect of U73122 on the development of inflammation in vivo in rats. Indomethacin was used as a positive control. At a dose of 30 mg/kg (ip), U73122 significantly inhibited carrageenan-induced inflammation and its effect was more complete than a 10 mg/kg (po) dose of indomethacin (Fig. 4). U73122 inhibited swelling by 65% and 80% at 1h and 3h post-carrageenan challenge, as shown in Fig. 4.

Effect of U73122 on COX-2 activity

To determine whether U73122 exhibits Cox-2 inhibitory activity, we evaluated U73122 in our in vitro Cox-2 assay using ECV-304 cells as previously described (Miralpeix et al., 1997; Kirchner et al., 1997). U73122 showed only 10% inhibition at 10 μM, indicating that the anti-inflammatory effect of U73122 was not due to the inhibition of the Cox-2 enzyme.

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Effect of U73122 on carrageenan-induced macrophage accumulation in dogs

We then evaluated U73122 for its anti-inflammatory effect on carrageenan-induced inflammation in s.c. chambers in dogs. We found that U73122 significantly inhibited the carrageenan-induced macrophage and lymphocyte infiltration into the exudates in s.c. chambers in dogs 24 h post challenge. The maximum effect of U73122 (0.1 mg/ml) on the macrophage and lymphocyte influx was 65% and 74% inhibition, respectively (Fig 5a and 5b). In contrast, U73122 did not have a significant effect on the influx of neutrophils into the chambers (Fig 5c).

Effect of U73122 on LPS-induced peritoneal macrophage accumulation in mice

In our LPS-induced mouse peritonitis model, the numbers of neutrophils in the peritoneal cavity rapidly decrease following LPS injection, whereas the macrophages and lymphocytes gradually increase and peak at day 9. As shown in Fig. 6a, U73122 (30 mg/kg, i.p.) totally inhibited the LPS-induced increase in macrophages. It also completely blocked the LPS-induced increase in lymphocytes (Fig 6b); in fact the lymphocyte count was below the background level in non-LPS-injected animals. However, U73122 did not inhibit the LPS-induced loss of neutrophils from the peritoneal cavity (Fig 6c).

Effect of U73122 on LPS induced PGE_2 production in peritoneal lavage fluid in mice

In addition, we studied the effect of U73122 on LPS-induced PGE_2 production in the same experimental model. As shown in Fig. 7, U73122 inhibited LPS-induced PGE_2 production in the peritoneal cavity by 80%.

Effect of U73122 on TPA-induced mouse ear edema

We also investigated the effect of U73122 on TPA-induced ear edema in mice. Administration of U73122 at 1, 3, and 10 mg/kg i.v. effectively suppressed TPA-induced ear edema in a dose-dependent manner (Fig. 8).

Effect of U73122 on glucocorticoid and other steroid receptor binding

Because U73122 is an aminosteroid, it is important to rule out the possibility that its anti-inflammatory effects were mediated by binding to one or more of the steroid receptors. We then tested the binding of U73122 to several steroid receptors, including glucocorticoid receptor (GR), estrogen receptor-α (ERα), estrogen receptor-β (ERβ) and androgen receptor (AR). As shown in Table 1, U73122 did not bind to the human glucocorticoid receptor at 10 μM. In constrast, the IC_{50} of dexamethasone was 26 nM. Similarily, U73122 had little or no binding affinity for ERα, ERβ and AR, respectively, at 10 μM while the appropriate control compounds had expected IC_{50}'s in the low nM range in each case.

Discussion

Activation of phospholipase C is a central component of the signal transduction in numerous cells, including leukocytes from human peripheral blood (Wu et al, 2000). However, the role of PLC in the inflammatory process in vivo is still largely unknown.

In this study, we demonstrated that a PLC inhibitor, U73122, inhibited recombinant human PLC-β2, with an IC_{50} of ~6 μM. U73122 had little effect on PLC-β1, PLC-β3 and PLC-β4, implying that U73122 preferentially inhibits PLC-β2 over PLC-β1, PLC-β3 and PLC-β4. Consistent with previous reports (Smith et al., 1990; Bleasdale et al., 1990), U73343 had no effect on any of the four PLC-β isozymes. In addition, we observed in our studies that U73122 blocked IL-8, LTB_4-induced Ca^{2+} mobilization and chemotaxis in human neutrophils.

Of particular interest, in vivo, we found, for the first time, that a PLC inhibitor, U73122, markedly inhibited LPS-induced leukocyte, macrophage and lymphocyte infiltration into lavage fluid of LPS-induced peritonitis in mice. In addition, U73122 inhibited LPS-induced PGE_2 production in lavage fluid of LPS-induced mouse peritonitis. The inhibition of peritoneal macrophage infiltration by U73122 correlated with the inhibition of the PGE_2 level in the lavage fluid of mouse peritonitis, indicating the anti-inflammatory effect of U71322 in vivo on the inflammatory models. In addition, it seems that U73122 could inhibit LPS-induced leukocyte and macrophage infiltration, but not the basal peritoneal leukocyte and macrophage in mice.

Furthermore, in our studies, U73122 inhibited carrageenan-induced macrophage and lymphocyte influx into subcutaneous chambers in dogs. In this model, carrageenan administration produces a significant influx of leukocytes into the exudates, which peaked at 24 h, and was maintained over a period of 3 or more days (Kirchner et al. 1997).

Next, we observed that U73122 significantly inhibited carrageenan-induced hind paw edema in rats and TPA-induced ear edema in mice, suggesting that U73122 was efficacious in vivo in inhibiting the inflammatory process.

Macrophages produce a variety of inflammatory mediators during inflammation (Guha, M. et al. 2001, Laskin, D.L. et al. 1995). One of the mediators is PGE_2 (Smith, W.L. et al 2000, Smith, W.L. 1996). Clinically, an increase in PGE_2 is associated with numerous pathophysiological conditions during inflammation (Clancy, R. et al 2000, Zhou, Y. Q. et al 2002). An acute inflammation is significantly suppressed by Cox-2 inhibitors and corticosteroids. With respect to this, we evaluated U73122 in our Cox-2 assay in vitro, using ECV-304 cells. Our data shows that U73122 showed only 10% inhibition of PGE_2 production at 10 μM, indicating that the anti-inflammatory effect of U73122 was not due to the inhibition of Cox-2. Thus, our hypothesis is that the anti-inflammatory effect of U73122 through the PLC pathway might contribute to the mechanisms of controlling PGE_2 synthesis in regulating inflammatory reactions.

We also demonstrated that U73122 did not bind to the human glucocortioid receptor at 10 μM. Similarly, no significant binding of U73122 to estrogen-α, estrogen-β and androgen receptor was observed at 10 μM. These results indicate that the anti-inflammatory activity of U73122 is not through glucocorticoid regulation of inflammatory signaling, but may be indeed through the PLC and PKC signaling pathways.

Cotransfection experiments in COS-7 and HEK293 cells suggest that PLC-β2 may function downstream of chemoattractant receptors. Transfection of receptors for complement C5a and fMet-Leu-Phe, interleukine-8 and monocyte chemoattractant protein-1 demonstrated that each of the receptors activates PLC-β2 through the pertussis toxin-sensitive G proteins. It was thought that PLC-β2 may be a primary signaling pathway in neutrophils, because the PLC activity elicited through chemoattractant receptors also appears to function through the Gi-mediated release of βγ subunits (Jiang et al. 1997). The role of PLC-β2 in chemoattractant-mediated responses was studied in mice lacking PLC-β2. These studies revealed that PLC-β2 deficiency blocked chemoattractant-induced Ca^{2+} release, superoxide production, Mac-1 up-regulation in neutrophils and regulation of protein kinases, but not chemotaxis (Jiang et al., 1997 and Li et al., 2000). To date there is no literature reports about the role of a PLC in the inflammatory process.

Our findings in the present study suggest that PLC-dependent signaling pathways are implicated in the development of acute and chronic inflammatory responses in vivo.

Alternatively spliced forms of PLC-β2 have been reported in hematopoietic cells (Mao et al., 2000). They differ in the carboxyl terminal sequence implicated in interaction of PLC-β enzymes with Gq, particulate association and nuclear localization. The PLC-β2 splice variants may be regulated differentially with distinct roles in signal transduction. The insight into the mechanism of the involvement of PLC pathways in the inflammatory reactions is currently under investigation.

In conclusion, our data provide direct evidence, for the first time, that PLC pathways may play an important role in leukocyte function at the cellular level and in animal model in vivo.

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References

Baggiolini M (1998) Chemokines and leukocyte traffic. *Nature (Lond)* **392:**565- 658.

Barrios-Rodiles M, Tiraloche G, and Chadee K (1999) Lipopolysaccharide Modulates Cyclooxygenase-2 Transcriptionally and Posttranscriptionally in Human Macrophages Independently from Endogenous IL-1ß and TNF-α. *J Immunol* **163:**963-969.

Bandeira-Melo, C, Serra MF, Diaz BL, Cordeiro R, Silva P, Lenzi HL, Bakhle YS, Serhan CN, and Martins MA (2000) Cyclooxygenase-2 derived prostaglandin E_2 and lipoxin A_4 accelerate resolution of allergic edema in *angiostrongylus costaricensis*-infected rats: relationship with concurrent eosinophilia. *J Immunol* **164:**1029-1036.

Bleasdale JE, Thakur NR, Gremban RS, Bundy GL, Fitzpatrick FA, Smith RJ, and Bunting S (1990) Selective inhibition of receptor-coupled phospholipase C-dependent processes in human platelets and polymorphonuclear neutrophils. *J Pharmacol Exp Ther* **255:**756-768.

Charo IF, Myers SJ, Herman A, Franci C, Connolly AJ, and Coughlin SR (1994) Molecular Cloning and Functional Expression of Two Monocyte Chemoattractant Protein 1 Receptors Reveals Alternative Splicing of the Carboxyl-Terminal Tails. *Proc Natl Acad Sci USA* **91:**2752-2756.

Franci C, Wong LM, Van Damme J, Proost P, and Charo IF (1995) Monocyte chemoattractant protein-3, but not monocyte chemoattractant protein-2, is a functional ligand of the human monocyte chemoattractant protein-1 receptor. *J Immunol* **154:**6511-6517.

Gilroy DW, Colville-Nash, PR, Willis D, Chivers J, Paul-Clark MJ and Willoughby DA (1999) Inducible cyclooxygenase may have anti-inflammatory properties. *Nat Med* **5:**698-701.

Glogauer M, Hartwig J, and Stossel T (2000) Two pathways through Cdc42 couple the N-formyl receptor to actin nucleation in permeablized human neutrophils. *J Cell Biol* **150:**785-796.

Guha M, and Mackman N (2001) LPS induction of gene expression in human monocytes, *Cell Signal* **13:**85-94.

Ianaro A, Ialenti A, Maffia P, Pisano B, and Di Rosa M (2001) Role of cyclopentenone prostaglandins in rat carrageenin pleurisy. *FEBS Lett* **508:**61-66.

Jan CR, Ho CM, Wu SN, and Tseng CJ (1998) The phospholipase C inhibitor U73122 increases cytosolic calcium in MDCK cells by activating calcium influx and releasing stored calcium. *Life Sci* **63:**895-908.

Jiang H, Wu D, and Simon MI (1994) Activation of phospholipase C beta 4 by heterotrimeric GTP-binding proteins. *J Biol Chem* **269:**7593-7596.

Jung S, and Littman DR (1999) Chemokine receptors in lymphoid organ homeostasis. *Curr Opin Immunol* **11:**319-325.

Kirchner T, Argentieri DC, Barbone AG, Singer M, Steber M, Ansell J, Beers SA, Wachter MP, Wu W, Malloy E, Stewart A, and Ritchie, DM (1997) Evaluation of the anti-inflammatory activity of a dual cyclooxygenase-2 selective/5-lipoxygenase inhibitor, RWJ 63556, in a canine model of inflammation. *J Pharmacol Exp Ther* **282:**1094-1101.

Kuang Y, Wu Y, Jiang H, and Wu D (1996) Selective G protein coupling by CC chemokine receptors. *J Biol Chem* **271:**3975-3978.

Laskin DL, and Pendino KJ (1995) Macrophages and Inflammatory Mediators in Tissue Injury. *Annu Rev Pharmacol Toxicol* **35:**655-677.

Lawrence T, Willoughby DA, and Gilroy DW (2002) Anti-inflammatory Lipid Mediators and Insights into the Resolution of Inflammation. *Nat Rev Immunol* **2:**787-795.

Li Z, Jiang H, Xie W, Zhang Z, Smrcka, AV, and Wu D (2000) Roles of PLC-β2 and -β3 and PI3K in chemoattractant-mediated signal transduction. Science (Wash DC) **287:**1046-1049.

Lockhart LK, and Mcnicol A (1996) The phospholipase C inhibitor U73122 inhibits phorbol ester-induced platelet activation. *J Pharmacol Exp Ther* **289:**721-728.

Mao GF, Kunapuli SP and Koneti RA (2000) Evidence for two alternatively spliced forms of phospholipase C-beta2 in haematopoietic cells. *Br J Haematol* **110:**402-408.

Mirapleix M, Camacho M, Lopez-Belmonte J, Canalias F, Beleta J, Palacios JM, and Vila, L (1997) Selective induction of cyclo-oxygenase-2 activity in the permanent human endothelial cell line HUV-EC-C: biochemical and pharmacological characterization. *Br J Pharmacol* **121:**171-180.

Miyazaki S, Matsukawa A, Ohkawara S, Takagi K, and Yoshinaga M (2000) Neutrophil infiltration as a crucial step for monocyte chemoattractant protein (MCP-1) to attract monocytes in lipopolysaccharide-induced arthritis in rabbits. *Inflamm. Res* **49:**673-678.

Muraoka RS, Sun WY, Colbert MC, Waltz SE, Witte DP, Degen JL, and Friezner Degen SJ (1999) The Ron/STK receptor tyrosine kinase is essential for peri-implantation development in the mouse. *J Clin Investig* **103:**1277-1285.

O'Neil GP, and Ford-Hutchinson AW (1993) Expression of mRNA for cyclooxygenase-1 and cyclooxygenase-2 in human tissues. *FEBS Lett* **330:**156-160.

Premack BA, and Schall, TJ (1996) Chemokine receptors: gateways to inflammation and infection. *Nat Med* **2:**1174- 1178.

Simon LS (1999) Role and regulation of cyclooxygenase-2 during inflammation. *Am J Med* **106:**37S-42S.

Smith RJ, Sam LM, Justen JM, Bundy GL, Bala GA, and Bleasdale**,** JE (1990) Receptor-coupled signal transduction in human polymorphonuclear neutrophils: effects of a novel inhibitor of phospholipase C-dependent processes on cell responsiveness. *J Pharmacol Exp Ther* **253:**688-697.

Smith WL, DeWitt DL, and Garavito RM (2000) CYCLOOXYGENASES: Structural, Cellular, and Molecular Biology. *Annu Rev Biochem* **69:**145-182.

Smith WL, Gavavito RM, and DeWitt DL (1996) Prostaglandin Endoperoxide H Synthases (Cyclooxygenases)-1 and –2. *J Biol Chem* **271:**33157-33160.

Stephens LR, Eguinoa A, Erdjument-Bromage H, Lui M, Cooke F, Coadwell J, Smrcka AS, Thelen M, Cadwallader K, Tempst P, and Hawkins PT (1997) The G beta gamma sensitivity of a PI3K is dependent upon a tightly associated adaptor, p101. *Cell* **89:**105-114.

Stoyanov B, Volinia S, Hanck T, Rubio I, Loubtchenkov M, Malek D, Stoyanova S, Vanhaesebroeck B, Dhand R, and Nurnberg B (1995) Cloning and characterization of

a G protein-activated human phosphoinositide-3 kinase. *Science* (Wash DC) **269:**690-693.

Thompson AK, Mostafapour SP, Denlinger LC, Bleasdale JE, and Fisher SK (1991) The aminosteroid U73122 inhibits muscarinic receptor sequestration and phosphoinositide hydrolysis in SK-N-SH neuroblastoma cells. *J Biol Chem* **266:**23856-23862.

Vickers, JD (1993) U73122 affects the equilibria between the phosphoinositides as well as phospholipase C activity in unstimulated and thrombin-stimulated human and rabbit platelets. *J Pharmacol Exp Ther* **266:**1156-1163.

Wang, JP (1996) U73122, an aminosteroid phospholipase C inhibitor, may also block Ca^{2+} influx through phospholipase C-independent mechanism in neutrophil activation. *NaunynSchmiedebergs Arch Pharmakol* **353:**599-605.

Wu D, Huang CK, and Jiang H (2000) Roles of phospholipid signaling in chemoattractant-induced responses. *J Cell Sci* **113:**2935-2940.

Zhou YQ, Chen YQ, Fisher JH, and Wang MH (2002) Activation of the RON receptor tyrosine kinase by macrophage-stimulating protein inhibits inducible cyclooxygenase-2 expression in murine macrophages. *J Biol Chem* **277:**38104-38110.

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Legends for Figures

Figure 1. Effects of U73122 and its analogue U73343 on the hydrolysis of [^{3}H]PIP$_2$ by PLC-β isozymes. The PLC-β isozyme protein was incubated with the vehicle or indicated concentrations of U73122 or U73343 at 37°C for 1.5h as described in Methods. The reduction in [^{3}H]PIP$_2$ level before and after the enzymatic reaction was quantitatively represented as percent inhibition. The effect of U73122 and U73343 on PLC-β1 (**Fig.1a**), PLC-β2 (**Fig.1b**), PLC-β3 (**Fig.1c**) and PLC-β4 activity (**Fig.1d**) was presented. The results are representative of three independent experiments.

Figure 2. Effect of U73122 on IL-8 and LTB$_4$ induced Ca^{2+} mobilization in human neutrophils. Fluo-3-loaded human neutrophils were pretreated with increasing concentrations of U73122 for 15 min followed by 100 nM of IL-8 or LTB$_4$. The changes in fluorescence representing the changes in Ca^{2+} concentration were measured by FLIPR at 37°C. Data shown were % inhibition of IL-8 or LTB$_4$ induced Ca^{2+} fluorescence counts by U73122. The inhibitory effect of U73122 on IL-8 (**Fig. 2a**) and LTB$_4$ (**Fig. 2b**) are presented. The results shown are representative of three separate studies.

Figure 3. Effect of U73122 on neutrophil migration in response to IL-8 and LTB$_4$. Using Costar 24-well filter plates, medium containing IL-8 or LTB$_4$ was added to the bottom chambers of the plate. 1 x 10^6 freshly prepared human neutrophils were added to the top chamber in the absence or presence of U73122. The transwell plate was incubated at 37°C for 3h. The cells that have migrated into the bottom chambers of the plate were

then counted using a hemacytometer. Data shown were percent inhibition of IL-8 (**Fig. 3a**) and LTB_4 (**Fig. 3b**) induced chemotaxis in human neutrophils by U73122. The results shown are representative of five separate studies.

Figure 4. Anti-inflammatory effects of U73122 and indomethacin on carrageenan-induced hind paw edema in rats. Vehicle or indomethacin (10 mg/kg, po) suspended in 0.5% methylcellulose solution or U73122 (30 mg/kg, ip) suspended in saline was administered as indicated in **Fig. 4**. Footpad edema was induced 1h later by subplantar injection of carrageenan (0.5% w/v in saline). Footpad volume was measured 3h after treatment as described in Methods. Values are the mean ± S.E.M. (n=6). The results are expressed the mean ± S.E.M. of 6 rats. $^{**}P < 0.01$ vs. control using Student's *t* test.

Figure 5. Effect of U73122 on carrageenan-induced inflammatory cell accumulation in fluid exudates from subcutaneous chambers in dogs. One ml of sample exudate from each dog was aspirated from within the chamber. Vehicle or U73122 suspended in 1.5 ml sterile saline was injected into the chamber. Then an inflammatory response was generated by injecting 1.5 ml 0.33% carrageenan directly into the chamber as described in the Methods session. Fluid samples of 0.5 ml were obtained from within the chamber 5h and 24h post carrageenan challenge, and analyzed for macrophage (**Fig. 5a**), lymphocyte (**Fig. 5b**) and neutrophil (**Fig. 5c**) accumulation via a Technicon H*1E hematology analyzer. The open bars represent the control levels 5h and 24h after treatment. The solid bars show the inhibitory effect of U73122 on the cell levels at 5h and

24h. Each bar represents the mean of levels from five dogs with S.E.M. $^{*}P < 0.05$ vs. control using Student's *t* test.

Figure 6. U73122 inhibits LPS-induced peritoneal inflammatory cell accumulation in mice. Vehicle or U73122 (30 mg/kg) suspended in sterile saline was administered i.p. 30 min prior to LPS (5 μg/ml, 1 ml, ip) challenge to induce peritonitis. The mice were dosed every day. On day 9 the peritoneal cavities were lavaged with 3 ml PBS to collect cells. Lavage samples were analyzed for macrophage (**Fig. 6a**), lymphocyte (**Fig. 6b**), and neutrophil (**Fig. 6c**) accumulation. The open bars show the control number of cells. The striped bar indicates the LPS-induced cell level. The solid bar represents the effect of U73122 on LPS-induced cell accumulation. The data are means ± S.E.M. of six samples in each group. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control using Student's *t* test.

Figure 7. U73122 inhibits LPS-induced PGE_2 production in peritoneal lavage fluid. The effect of U73122 on LPS-induced PGE_2 production was determined with the same lavage samples as in Fig. 6. The PGE_2 level was determined by using an EIA PGE_2 assay kit. The open bars represent the control level of PGE_2. The stripe bars show the LPS-induced PGE_2 production level. The solid bar indicates the inhibitory effect of U73122. The data are means ± S.E.M. of six samples in each group. $^{***}P < 0.001$ vs. control using Student's *t* test.

Figure 8. Effect of U73122 on TPA-induced ear edema in mice. Vehicle, control compound or indicated concentrations of U73122 was administered 1h prior to TPA

challenge. Twenty μl of TPA (1 μg) was topically applied onto the dorsal surface of the right mouse ear. Ear edema was measured 6h after challenge by using a 7 mm biopsy punch. The open bar represents the TPA-induced ear edema. The striped bars show the effect of tepoxalin on edema. The solid bars indicate the dose-dependent inhibitory effect of U73122 on TPA-induced mouse ear edema. The results are means ± S.E.M. of six samples. $^{*}P < 0.05$ vs. control using Student's *t* test.

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Table 1. Effect of U73122 on Binding of Steroid Receptors

Receptor	U73122 (μM)	% Inhibition	Control (Compound)	Control (IC_{50})
GR	10 μM	–	Dexamethasone	26 nM
ERα	10 μM	–	17β-Estradiol	3.9 nM
ERβ	10 μM	–	Ethinylestradio	4.6 nM
AR	10 μM	–	DHT	9 nM

The results represent the % inhibition of glucocorticoid receptor (GR), estrogen receptor-α (ERα), estrogen-receptor-β (ERβ) and androgen receptor (AR) binding by U73122 at 10 μM. Duplicate runs were performed against each receptor. The IC_{50s} of control compounds were as indicated.

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Fig. 1a

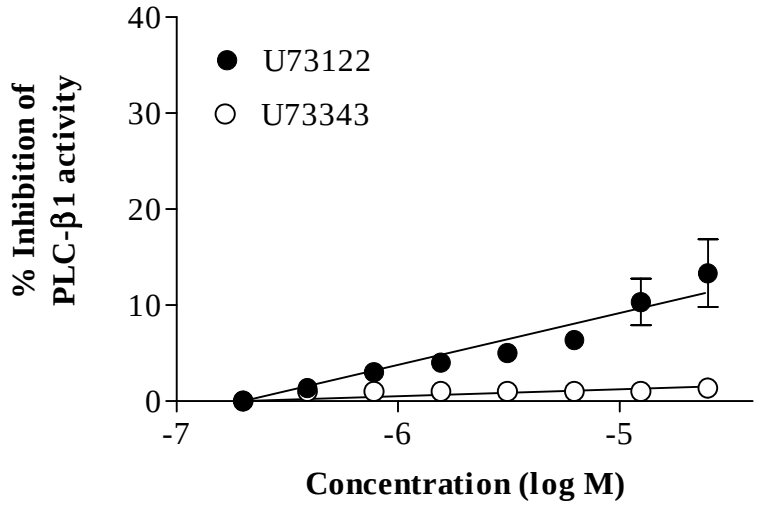

Fig. 1b

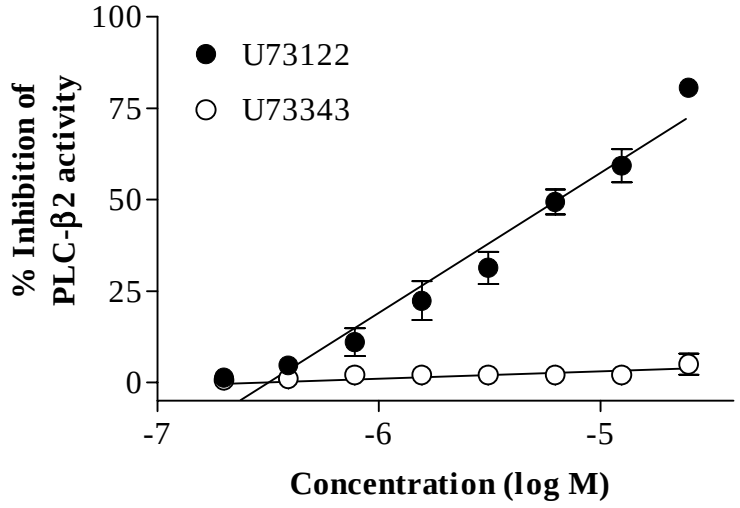

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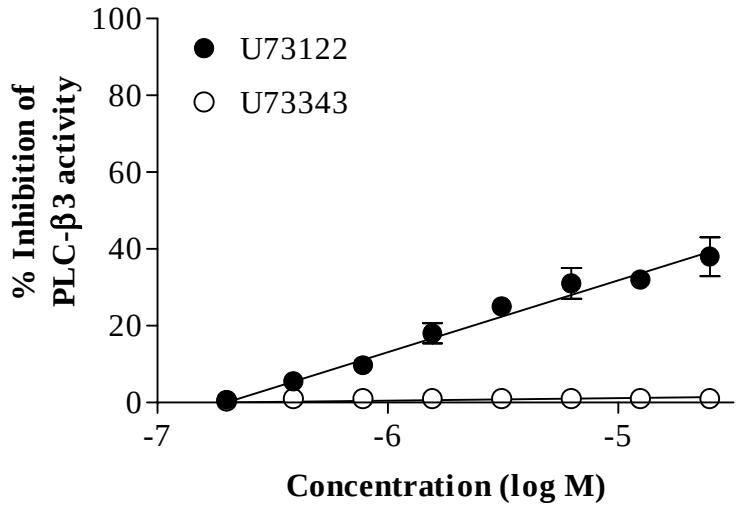
Fig .1c



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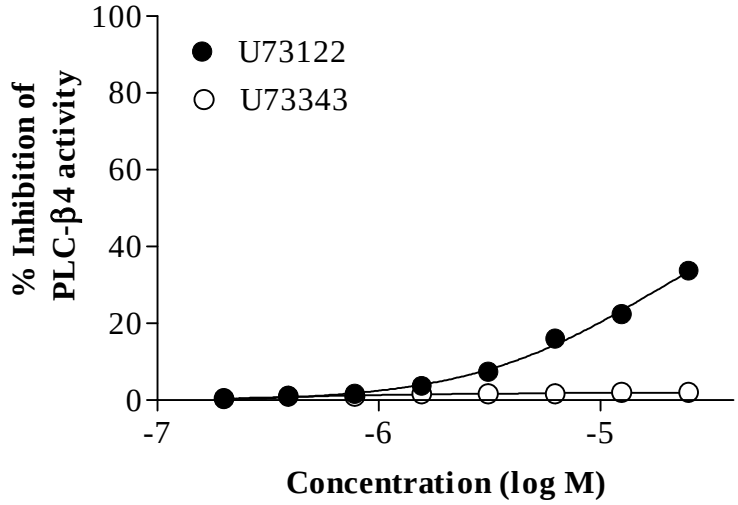

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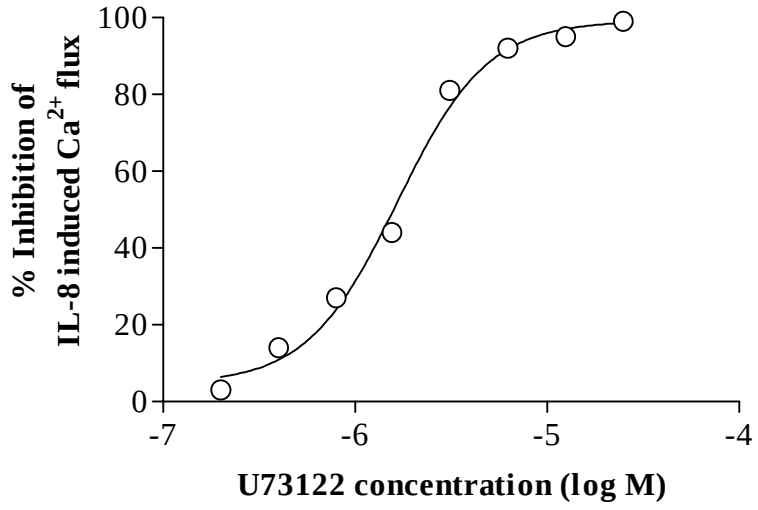
Fig. 2a



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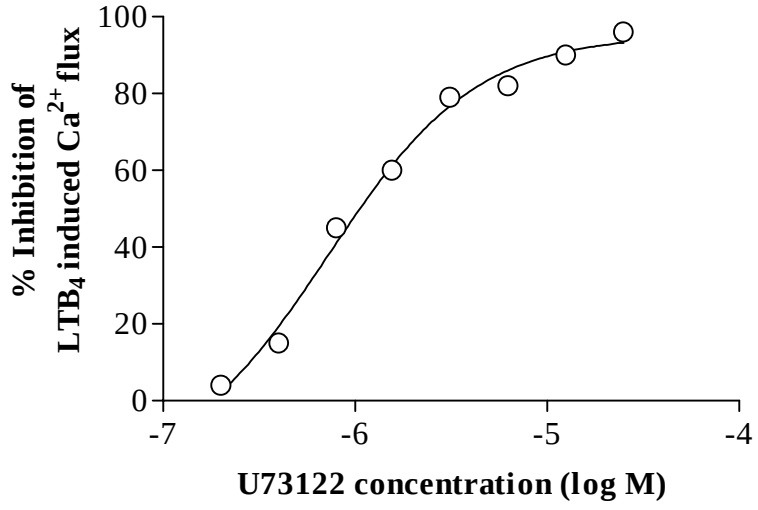

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Fig. 3a

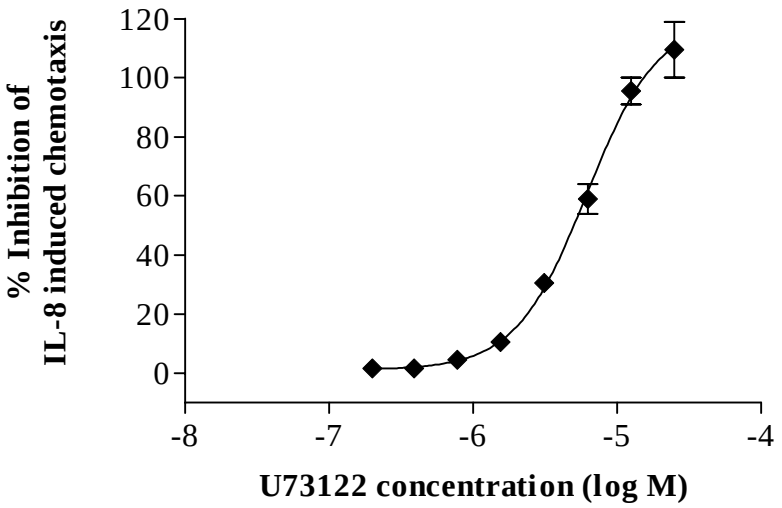

Fig. 3b

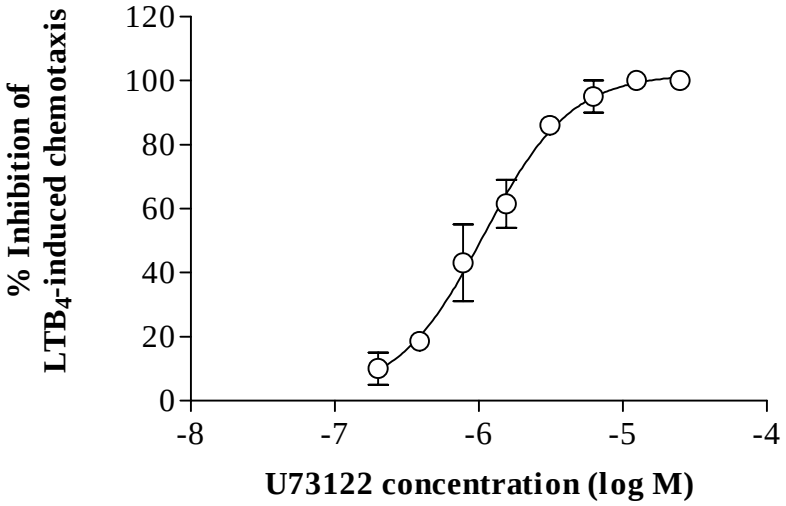

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Fig. 4

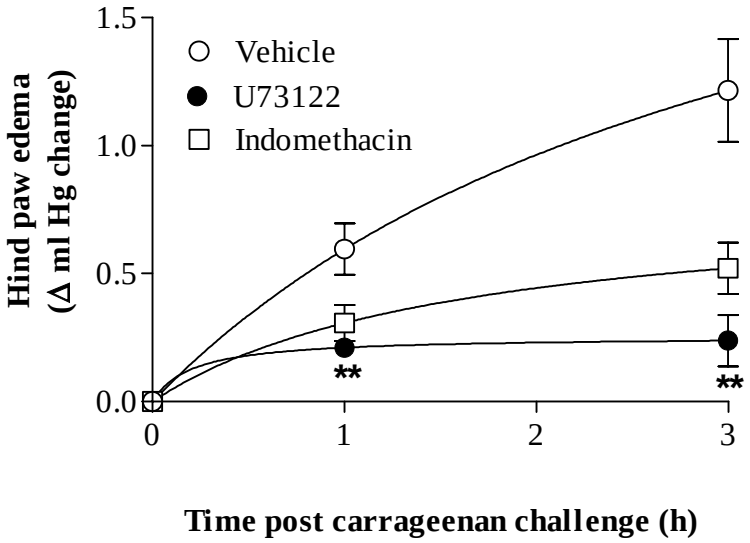

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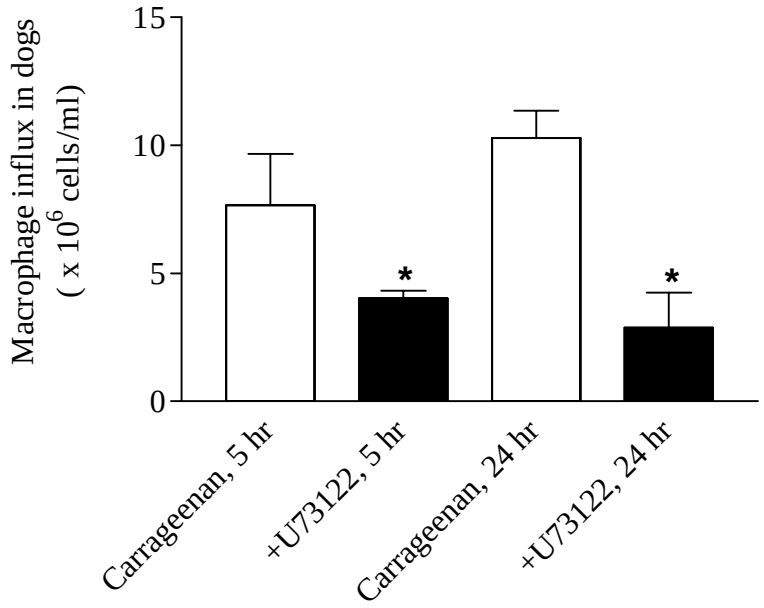
Fig. 5a



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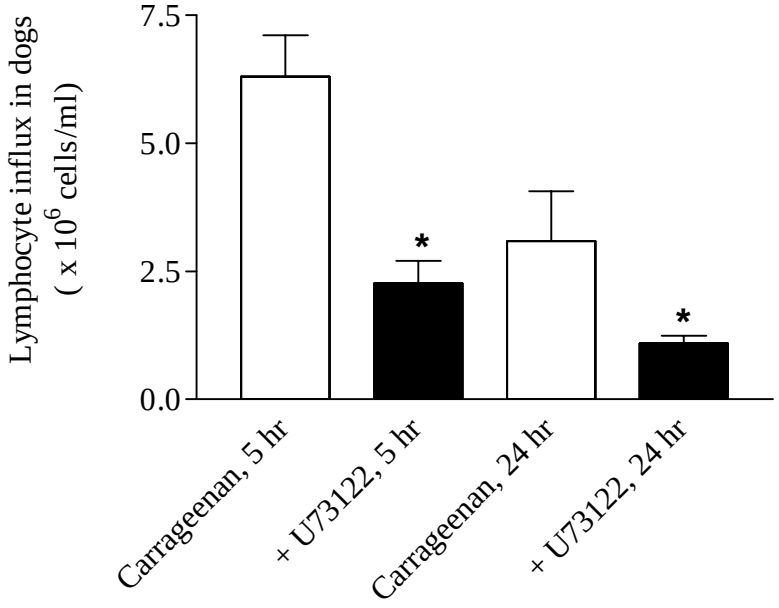

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Fig. 5c

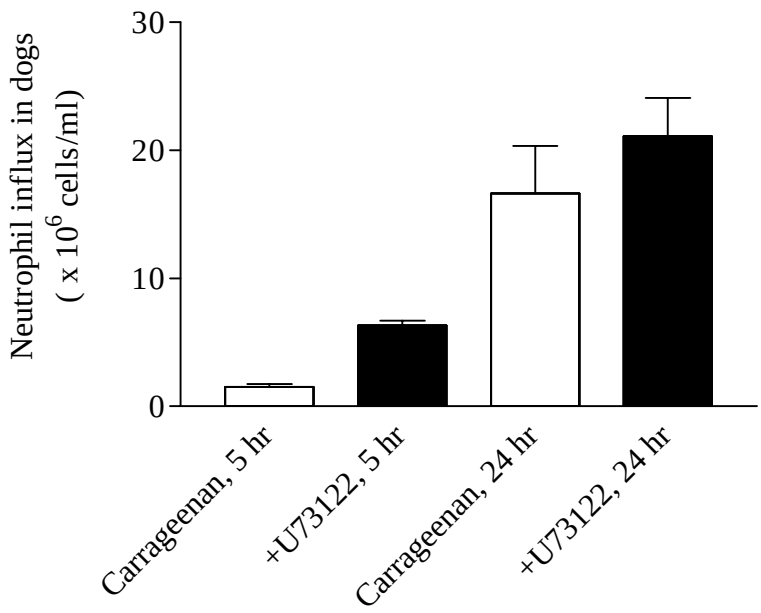

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Fig. 6a

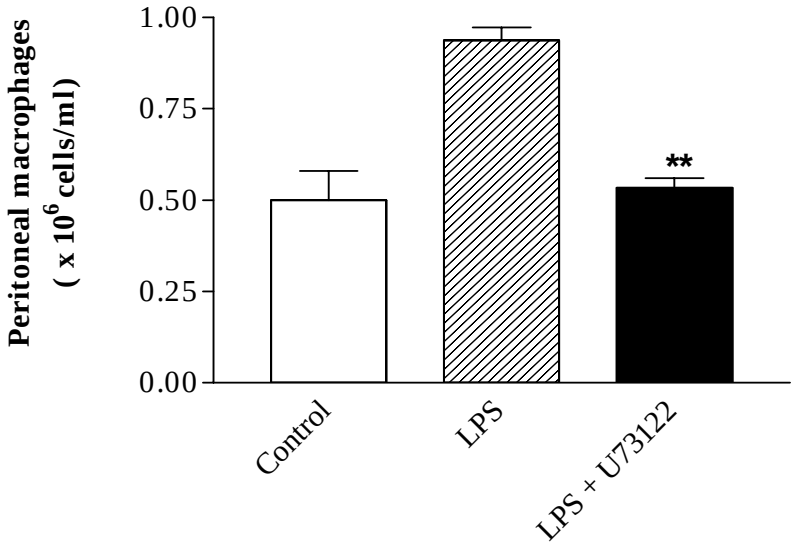
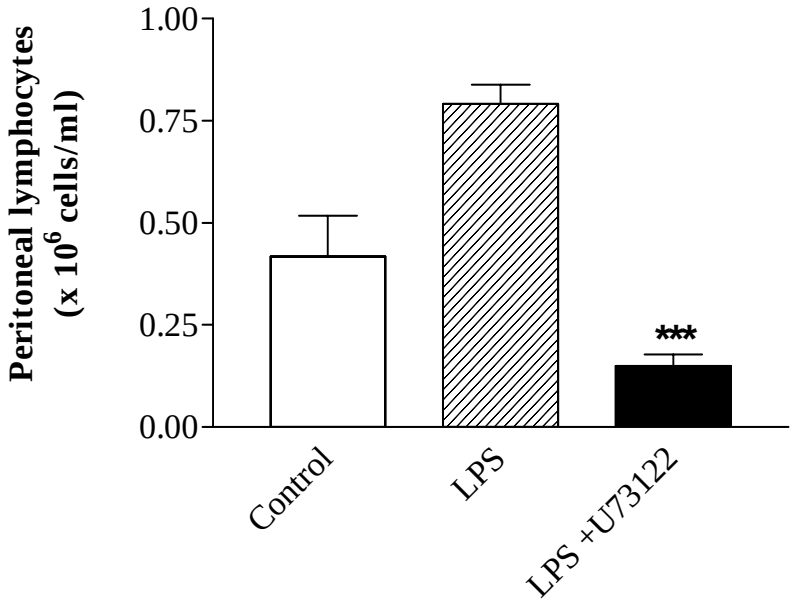


Fig. 6b



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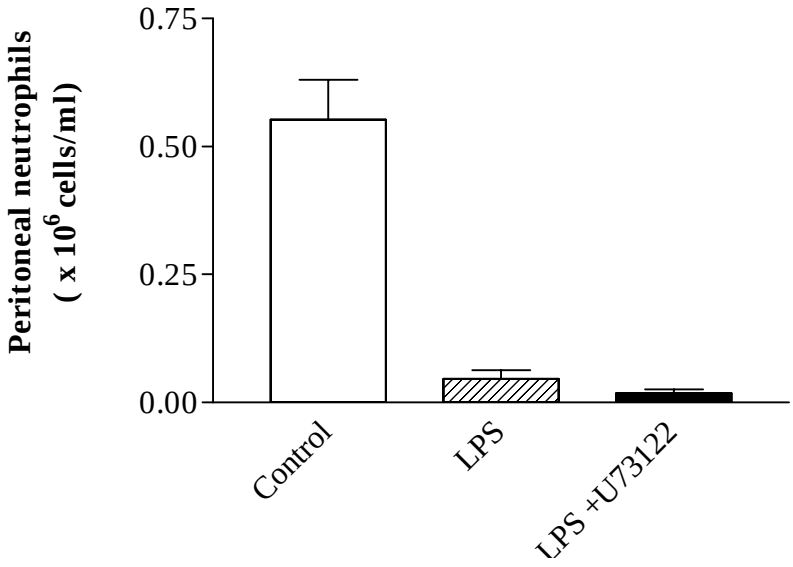

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Fig. 7

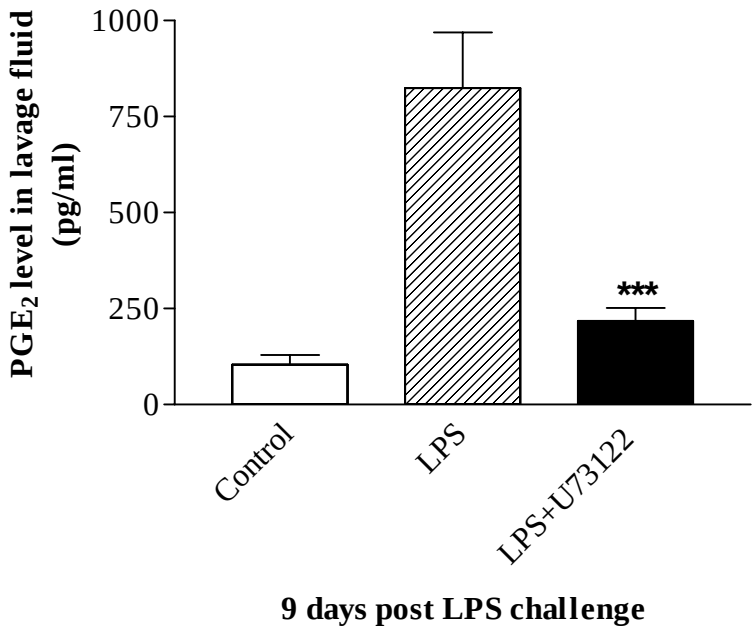

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Fig. 8