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Effects of dialkoxylphenyl compounds with oxime group on macrophage function and the proliferation of lymphocytes

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Abstract

Dialkoxyphenyl compounds have been reported to possess anti-inflammatory activity through inhibition of phosphodieseterase (PDE) type IV. In this study, a series of derivatives of dialkoxyphenyl compounds with an oxime group, which is generally known to be one of the biologically active functional groups, were prepared and evaluated for their ability to inhibit the production of inflammatory mediators in activated macrophages and the proliferation of lymphocytes. The structure–activity relationship (SAR) study with 12 compounds on tumour necrosis factor (TNF)-α inhibition, analysed by the oxime geometry and different size of spacers between the oxime and phenyl group, indicated that there might be at least three possible hydrogen bonding sites in the inhibitor binding pocket of PDE IV. Of them, compound **6** clearly displayed the highest inhibitory effect on in-vitro TNF-α production from lipopolysaccharide (LPS)-stimulated RAW264.7 cells. Compound **6** also suppressed in-vivo TNF-α release from LPS-primed mice, a level comparable with that of the standard PDE IV inhibitor, rolipram. In addition, oxime compounds also significantly inhibited both nitric oxide production from activated RAW264.7 cells and T lymphocyte proliferation elicited by concanavalin A but not IL-2. The data suggest that the oxime group may act as a functional group, capable of interacting with the inhibitor-binding pocket of target PDE IV. Therefore, it is conceivable that compound **6** may have the potential either to be developed as a new anti-inflammatory drug or to be used to develop more potent analogues.

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Introduction

Macrophages and lymphocytes are thought to play a central role in host acute and chronic inflammation (Panayi et al 1992; Correll et al 1997). These cells proliferate and are activated in response to inflammatory signals, such as some bacterial products, including lipopolysaccharide (LPS) (Weinstein et al 1992; Geng et al 1995). As a result, they secret a number of pro-inflammatory mediators, such as cytokines (tumour necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6) and eicosanoids (prostaglandin E_2 and leukotriene B_4) as well as reactive oxygen and nitrogen species, including nitric oxide (NO) (Ding et al 1988; Lee et al 1994). These pathological phenomena are being targeted as approaches for developing new anti-inflammatory drugs.

Phosphodiesterase (PDE) IV inhibitors are known to be promising anti-inflammatory drugs, which have been implicated in the inhibition of pro-inflammatory cytokines and inflammatory mediators (Palfreyman 1995; Barnette 1999; Essayan 1999). These compounds have demonstrated striking curative effects in in-vivo inflammatory animal models (Dinter et al 2000; Francischi et al 2000; Schmidt et al 2000). Nevertheless, it has been reported that this kind of compound exhibits adverse effects, including nausea and vomiting (Heaslip & Evans 1995; Duplantier et al 1996). To overcome the side effect, several approaches, introducing new chemical structures, are in trial (Dal Piaz & Gioyannoni 2000). In our laboratory, we have also designed numerous dialkoxylphenyl compounds, a representative class of PDE IV inhibitors, based on either cellular or enzymatic assays, to select more potent lead compounds (Park et al 2000a, b, 2001, 2002).

In this study, several compounds with a structural similarity were chosen to evaluate their immunomodulatory property towards inflammatory mediators. Since it is of particular importance to exactly evaluate the anti-inflammatory property of the tested drugs, pharmacological tests at the cellular level, rather than the enzymatic level, should be carried out in screening potent therapeutic drugs. This may help in considering several real factors, including membrane permeability of the drugs in evaluating the drug's pharmacology. To do this, several cellular-based models, such as TNF-α production and NO release from activated macrophages and the proliferation of T lymphocytes, were used to address whether these novel compounds are capable of modulating inflammatory responses. The data generated with these assays demonstrated that compound **6**, selected on the basis of a structure–activity relationship (SAR) study, strongly inhibits in-vitro and in-vivo TNF-α production, and significantly suppresses NO release and T cell proliferation.

Materials and Methods

Materials

Compounds (**1**: 3-(3,4,5-trimethoxyphen yl)-propenal oxime *(trans)*; **2**: 3-(3,4,5-trimethoxyph enyl)-propenal oxime *(cis)*; **3**: 3-(3,4-dimethoxypheny l)-propenal oxime *(trans)*; **4**: 3-(3,4-dimethoxyphenyl)-propenal oxime *(cis)*; **5**: 3-(3-cyclopentyloxy-4-methox yphenyl)-propenal oxime *(trans)*; **6**: 3-(3-cyclopentyloxy-4 -methoxyphenyl)-propenal oxime *(cis)*; **7**: 6-[2-(3-cyclopentyloxy -4-methoxyphenyl)-vinyl]-pyridine-2-carbaldehy de oxime *(trans)*; **8**: 6-[2-(3-cyclopentyloxy-4- methoxyphenyl) -vinyl]-pyrid ine-2-carbald ehyde oxime *(cis)*; **9**: 2-(3-cyclopentyloxy-4-me thoxyphenyl)-3-oxo-2,3-dihydro-1 *H*-isoindole-5-carbaldeh yde oxime *(trans)*; **10**: 2-(3-cyclopentyloxy-4-meth oxyphenyl)-3-oxo-2,3-dihydro-1 *H*-isoindole-5-carbaldeh yde oxime *(cis)*; **11**: 2-(3-cyclopentyloxy-4 -methoxyphenyl)-6-(2-[1 ,3] dioxolan-2-yl-vinyl)-2,3-di hydro-isoindol-1-one *(trans)*; and **12**: 2-(3-cyclopentyloxy-4 -methoxyphenyl)-6-(2-[1 ,3] dioxolan-2-yl-vinyl)-2,3-di hydro-isoindol-1-one *(cis)*) (Table 1) and A77,1726 (an active metabolite of leflunomide) were synthesized in the Chemistry Department of Daewoong R & D center (Sungnam, Korea) (Baik et al 1998; Cho et al 2000a; Park et al 2002 (and unpublished data)). Sulfanilamide, pentoxifylline, staurosporin, herbimycin A, dibutyryl cyclicAMP (dbcAMP), *N*-(1-naphthyl)-ethylenedi amine dihydrochloride, nitro-L-arginine methyl ester (L-NAME), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), concanavalin A (Con A), lipopolysaccharide (LPS, *Escherichia coli* 0111:B4) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Chemical Co. (St Louis, MO). Recombinant human interferon-γ (IFN-γ) and interleukin (IL)-2 were obtained from Boehringer Mannheim (Ottweiler, Germany). Rolipram was purchased from Calbiochem (La Jolla, CA). Fetal bovine serum (FBS), penicillin, streptomycin and RPMI1640 were obtained from GIBCO (Grand Island, NY). RAW264.7, Sup-T1 and CTLL-2 cells were purchased from American Tissue Culture Center (Rockville, MD). All other chemicals were of Sigma grade.

Animals

Eight-week-old BALB/c male mice were purchased from B & K Universal (Fremont, CA). The BALB/c mice were maintained in plastic cages under conventional conditions and were allowed free access to water and pelleted diets (Samyang, Daejeon, Korea). Animal care in this study conformed to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

Cell culture

RAW264.7 and Sup-T1 cells were maintained in RPMI1640 supplemented with 100 U mL^{-1} of penicillin, 100 μg mL^{-1} of streptomycin and 10% FBS. CTLL-2 cells were cultured with the same medium containing 25 U mL^{-1} of IL-2. Cells were grown at 37 °C and 5% CO_2 in humidified air.

TNF-α production in-vitro

Twelve dialkoxyphenyl compounds, solubilized with dimethyl sulfoxide, were diluted with RPMI1640 before use. The inhibitory effect of the 12 dialkoxyphenyl compounds and standard compounds (rolipram, prednisolone, dbcAMP, staurosporin and herbimycin A) on TNF-α production from LPS-treated RAW264.7 cells was determined as described previously (Cho et al 2000a). Differentiation and stimulation of U937 cells for the human TNF-α assay were performed as reported previously (Sajjadi et al 1996; Cho et al 2000a). The PMA-differentiated U937 cells (2×10^6 cells/mL) were treated with LPS (1 μg mL^{-1}) for 6 h in the presence or absence of compound **6** or positive control drugs (rolipram, dbcAMP and pentoxifylline). The supernatants were harvested and assayed for TNF-α content using mouse or human TNF-α ELISA kit (Amersham, Little Chalfont, UK). The ELISA displayed good reproducibility and accuracy with inter- or intra-assay error in the range 2–10%.

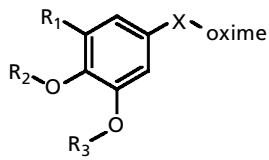
TNF-α production in-vivo

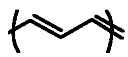
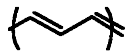
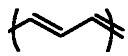
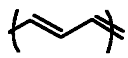
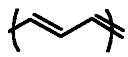
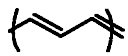
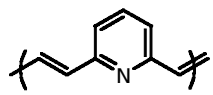
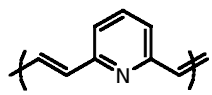
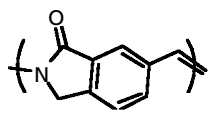
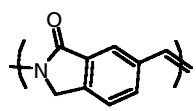
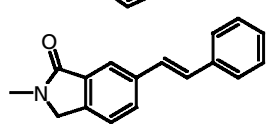
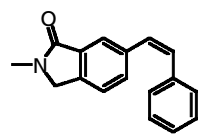
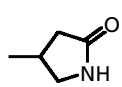
The method of Novogrodsky et al (1994) was used for LPS induction of TNF-α in-vivo. Fasted mice were orally administered with the test compounds (compounds **5** and **6**, rolipram and prednisolone) suspended in 0.5% sodium carboxylmethylcellulose 2 h before challenge with intraperitoneal injection of LPS. After 90 min, blood was collected and serum samples were used to measure TNF-α levels by means of an ELISA kit.

Determination of NO production

After pre-incubation of RAW264.7 cells (1×10^6 cells/mL) for 18 h, the various concentrations of test compounds (compounds **3**–**6**, rolipram and L-NAME) with LPS

Table 1 Inhibitory effect of oxime-containing dialkoxylphenyl compounds on TNF-α production in LPS-stimulated RAW264.7 cells.



Compound	Activity (%)[a]			R_1	R_2	R_3	X	Geometry of oxime
	0.1 μM	0.5 μM	1 μM					
1	3.1	5.4	8.9	OCH_3	CH_3	CH_3		*trans*
2	2.6	5.1	11.3	OCH_3	CH_3	CH_3		*cis*
3	1.9	6.9	12.0	H	CH_3	CH_3		*trans*
4	13.8	26.7	40.3	H	CH_3	CH_3		*cis*
5	62.2	73.5	81.8	H	CH_3	cyclopentyl		*trans*
6	76.3	89.7	92.4	H	CH_3	cyclopentyl		*cis*
7	16.7	32.4	43.9	H	CH_3	cyclopentyl		*trans*
8	5.4	17.2	37.3	H	CH_3	cyclopentyl		*cis*
9	21.4	56.4	87.4	H	CH_3	cyclopentyl		*trans*
10	19.8	45.3	82.4	H	CH_3	cyclopentyl		*cis*
11	9.7	8.9	12.9	H	CH_3	cyclopentyl		
12	−11.4	5.8	−13.5	H	CH_3	cyclopentyl		
Rolipram	53.5	76.1	94.4	H	CH_3	cyclopentyl		

[a]Mean value of three separate observations.

($1\,\mu g\,mL^{-1}$) or IFN-γ ($50\,U\,mL^{-1}$) were incubated for 24 h as reported previously (Ding et al 1988; Cho et al 2000a). Nitrite in culture supernatants was measured by adding 100 μL of Griess reagent (1% sulfanilamide and 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride in 5% phosphoric acid) to 100-μL samples of medium. The nitrite assay showed good reproducibility and accuracy with inter- or intra-assay error in the range 3–12%.

Splenocyte proliferation assay

Splenocytes were prepared from the spleens of mice killed by cervical dislocation under sterile conditions, as described previously (Cho et al 2000a). Splenocytes (5×10^6 cells/mL) were cultured in flat-bottom 96-well microtitre plates (Corning Glass, Corning, NY) in the presence and absence of T lymphocyte mitogen ($1\,\mu g\,mL^{-1}$ of Con A) with test compounds (compounds **3**–**6**, rolipram, dbcAMP, A77,1726 and pentoxifylline) in a total volume of 200 μL/well at the same conditions for 48 h. The proliferation assay was performed by MTT assay (colorimetric assay).

IL-2-dependent cell proliferation assay

CTLL-2 cells were harvested from IL-2-containing growth medium and washed twice with RPMI 1640 without FBS and IL-2. They were re-suspended in growth medium without IL-2 to 5×10^5 cells/mL. Fifty microlitres of cell suspension was placed into each well of a 96-well tissue culture plate and incubated in the presence of test compounds and $50\,U\,mL^{-1}$ of IL-2 for 48 h. The proliferation of the cells was measure by MTT assay.

MTT assay (colorimetric assay)

The cytotoxicity of test compounds (compounds **1**–**12**) and lymphocyte proliferation were evaluated by MTT assay with minor modification as reported previously (Cho et al 2000a). Four hours before culture termination, 10 μL of MTT solution ($10\,mg\,mL^{-1}$ in phosphate-buffered saline, pH 7.4) was added to the culture in each well and cells were continuously cultured until termination. The culture was stopped by addition of 15% sodium dodecyl sulfate (SDS) into each well for solubilization of formazan. The optical density (OD) at 570 nm ($OD_{570-630}$) was measured by a microplate Spectramax 250 microplate reader (Molecular Devices, Sunnyvale, CA).

Statistical analysis

Unless otherwise stated, all measurements were made from four or ten observations and at least three separate experiments. Data were expressed as mean $\pm$ s.e.m. For statistical comparison, results were analysed using analysis of variance/Scheffe's post-hoc test and Kruskal–Wallis/Mann–Whitney test. A P value < 0.05 was considered a statistically significant difference. All statistical tests were carried out using the computer program STATISTICA, version 4.5 (StatSoft Inc, Microsoft corporation, OK).

Results

Cytotoxicity of test compounds

As a first step, the cytotoxicity of test compounds in the presence or absence of LPS was evaluated using MTT assay. Pre-treatment of both stimulated and unstimulated RAW264.7 cells up to testing concentrations did not significantly affect the cell viability (data not shown).

SAR study

To better characterize the pharmacophore of dialkoxy phenyl compounds with an oxime group, which acts as a hydrogen bonding donor, 12 representative oxime-containing compounds were tested by TNF-α production assay. Basically, oxime has a geometrical isomer, such as *cis*- and *trans*-isomer, as seen in many pharmaceutical compounds. A series of derivatives of 3,4-dialkoxy phenyl *cis*- or *trans*-oxime compounds were prepared by different combination of functional groups at R_1, R_2 and R_3 positions (Table 1), and evaluated for their TNF-α inhibitory effects.

Certainly, most of the compounds containing the oxime group showed significant inhibitory effects (Table 1). Especially, the geometry of oxime was necessary to give these compounds a strong suppressive effect, depending on the length of spacer. Thus, for compounds **2**–**6**, which have a pronenal spacer on X, the *cis*-isomers (**4, 6**) were more active than the *trans*-isomers (**3, 5**). In contrast, compounds **7**–**9** and **10**, having a long spacer of 6 carbons, displayed an opposite effect. In addition, the 3-cyclopentyl group of R_3 potentiated the inhibitory activity of compounds **5** and **6**, compared with the methyl group (compounds **3** and **4**), as reported previously in terms of hydrophobicity. Furthermore, the isoindolinone spacer (compounds **9** and **10**), possessing a carbonyl group as a hydrogen bonding source, showed better effect than the pyridine group of compounds **7** and **8**. The importance of the oxime group as a hydrogen-bond donor was confirmed by two compounds (**11** and **12**), *cis*- or *trans*-phenyl isomer without the hydrogen-bonding source. Both compounds displayed a lower inhibitory effect than compounds **9** and **10**, indicating that oxime is a biologically active group involved in the formation of hydrogen bonds. On the other hand, compounds **6** and **9** also strongly suppressed the PDE IV activity with IC50 values (concentration producing 50% inhibition) of 100 and 160 nM, respectively, suggesting that the inhibitory mode of action is apparently due to inhibition of PDE IV activity.

Of them, compounds **6** could thus be regarded as a potential therapeutic agent for those inflammatory conditions in which TNF-α is known to be an important contributor. Accordingly, the candidate compound was further carefully tested for the ability to attenuate other inflammatory mediators or regulators, such as NO production and CD4+ and CD8+ lymphocyte proliferation. Furthermore, other compounds (**3, 4** and **5**) displaying structural similarity were also evaluated under the same conditions, to confirm the in-vitro SAR obtained by TNF-α assay.

Dose–response on in-vitro TNF-α production

To evaluate the efficacy of oxime-containing compounds in TNF-α inhibition, the dose dependency was evaluated under the same conditions. Compounds **3, 4, 5** and **6** strongly inhibited LPS-induced TNF-α production in murine macrophages in a dose-dependent manner (Figure 1A) with IC50 values of 8.8, 1.1, 0.14 and 0.024 μM, respectively. The inhibitory pattern of these compounds depended on structural feather decided by oxime geometry. Positive control drugs, dbcAMP, pentoxifylline and rolipram, used in this experiment also significantly suppressed TNF-α production in a dose-dependent manner with IC50 values of 28.9, 246 and 0.082 μM, respectively.

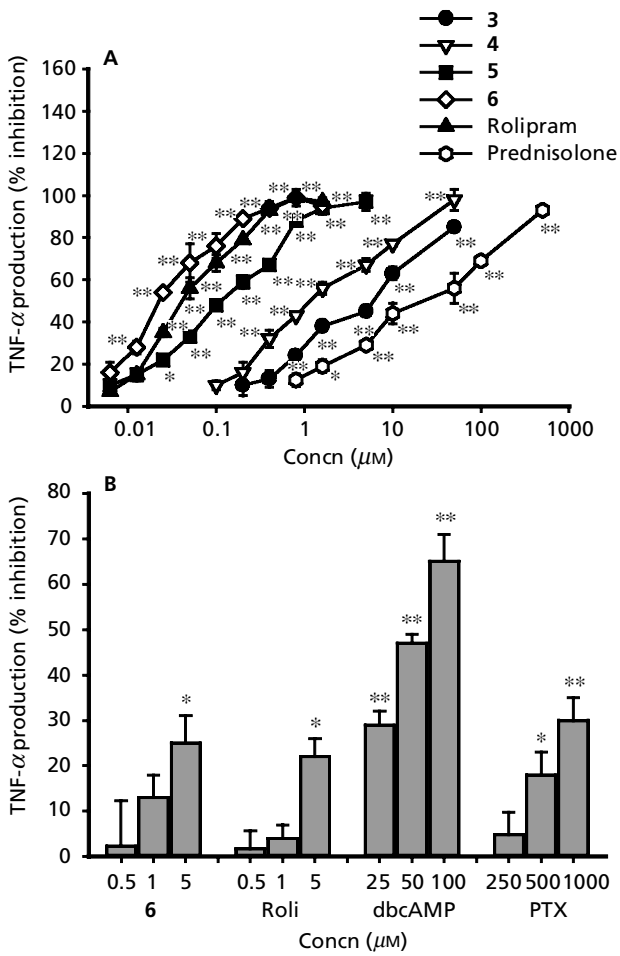


Figure 1 Effect of compounds **3, 4, 5** and **6** on in-vitro TNF-α production in RAW264.7 (A) and PMA-differentiated U937 cells (B) stimulated by 1 $\mu g\,mL^{-1}$ of LPS for 6 h. RAW264.7 (1×10^6 cells/mL; A) or PMA-differentiated U937 (1×10^6 cells/mL; B) cells were stimulated by 1 $\mu g\,mL^{-1}$ of LPS with various concentrations of test compounds (compounds **3–6**, dbcAMP, pentoxifylline (PTX), rolipram (Roli) and prednisolone). Supernatants were collected after 6 h and assayed by ELISA to determine TNF-α concentration. Data represent mean ± s.e.m. of 3 separate observations (n = 4 at each experiment). $^*P < 0.05$, $^{**}P < 0.01$ compared with control group (LPS alone).

To confirm the inhibitory effect of compound **6** on TNF-α production in human macrophages, human monocyte cell line U937 cells were used after inducing differentiation with PMA. Indeed, differentiated U937 cells produced a comparable level of TNF-α upon LPS exposure (Cho et al 2000a). Compound **6** significantly suppressed human TNF-α production (Figure 1B), although the inhibitory potency was not comparable with the murine model. However, the lower inhibitory effect was also shown with rolipram (also pentoxifylline), suggesting that the PMA/LPS-stimulated U937 model is not a sensitive method for evaluating the action of PDE IV inhibitors.

Effect of compounds 5 and 6 on in-vivo TNF-α release

To expand the in-vitro inhibitory effect of compound **6** on TNF-α production to in-vivo efficacy, the in-vivo TNF-α inhibitory effect was examined in LPS-primed mice orally administered with compounds **5** and **6**. First, we tested more time points, displaying higher serum TNF-α release. LPS injection induced maximum serum TNF-α release at 1.5 h (Figure 2A). Hence, in the next experiment we chose 1.5 h for evaluating in-vivo suppressive activity of compound **6**. Compound **6** potently, and dose-dependently, inhibited serum TNF-α release in LPS-primed mice (dose producing 50% of maximum effect (ED50) = 6.8 $mg\,kg^{-1}$) (Figure 2B). Additionally, compound **5** also showed an inhibitory effect, but it was less than that of compound **6**. Rolipram also attenuated the release of serum TNF-α (ED50 = 7.8 $mg\,kg^{-1}$). Another standard compound, prednisolone (10 $mg\,kg^{-1}$), strongly blocked in-vivo TNF-α release.

Combination treatment of compound 6 with known TNF-α inhibitors

To assess the additive or synergic effect of compound **6**, the compound (at a concentration producing 40% inhibition) and several well-known TNF-α inhibitors were used to co-treat LPS-activated RAW264.7 cells. All test inhibitors, including protein kinase C inhibitor (staurosporin), protein tyrosine kinase inhibitor (herbimycin A) and protein kinase A activator (dbcAMP) showed additive inhibitory effects (more than 60 %) (Figure 3). In contrast, treatment of compound **6** with the same kind of PDE IV inhibitor, rolipram, did not increase the inhibitory potency of compound **6** alone (Figure 3), suggesting that these compounds are competitive in recognising the same target enzyme (PDE IV) (Cho et al 1999b).

Effects of compounds 3, 4, 5 and 6 on NO production and NO-mediated cytotoxicity

To evaluate the effect of test compounds on NO production, the stable NO oxidation product, nitrite, was measured from the cell supernatant containing 0.5–1 μM of NO as a basal level. When RAW264.7 cells were stimulated with 1 $\mu g\,mL^{-1}$ of LPS for 24 h, about 30–55 μM of

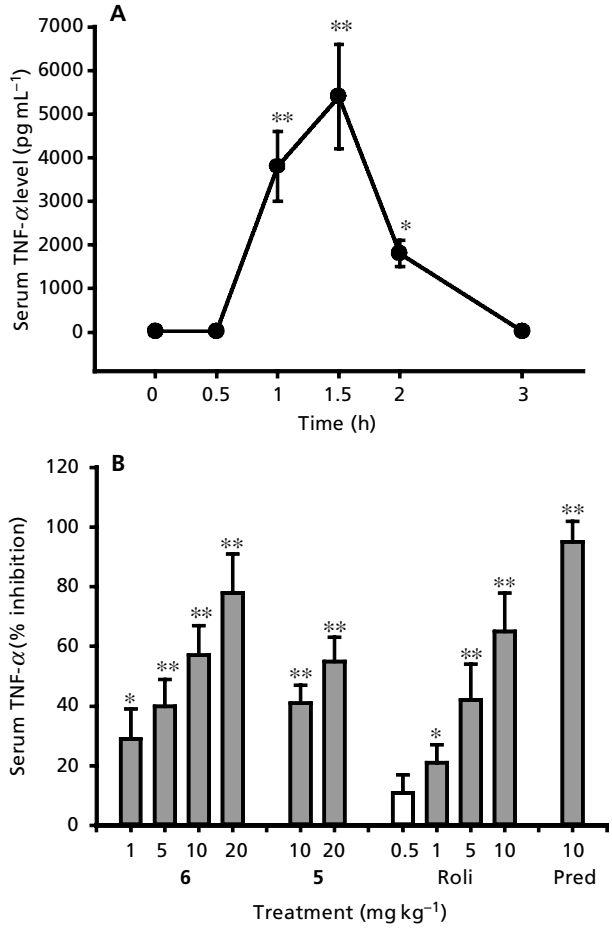


Figure 2 Effect of compounds **5** and **6** on in-vivo TNF-α release from LPS-primed mice. Serum TNF-α level was tested from LPS-primed mice at different time points (A). Test compounds (compounds **5** and **6**, rolipram (Roli) and prednisolone (Pred)) were orally administered 30 min before LPS injection and then LPS was injected peritoneally and blood was collected after 1.5 h (B). Serum TNF-α released was assayed by ELISA. Data represent mean ± s.e.m. of 3 separate observations (n = 10). $*P < 0.05$, $**P < 0.01$ compared with control group (LPS alone).

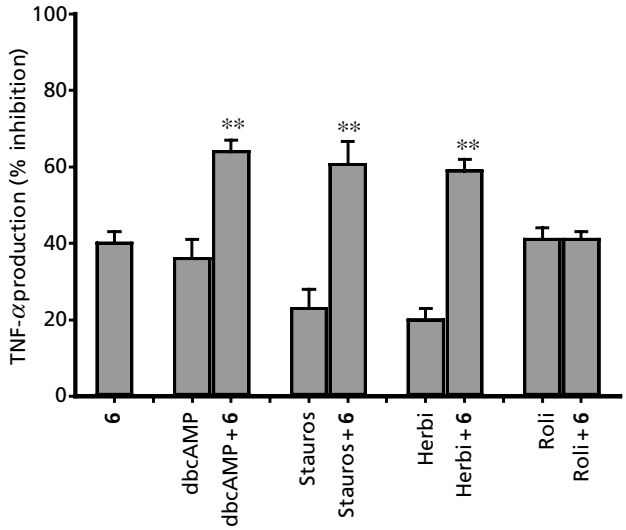


Figure 3 Effect of known TNF-α antagonists on compound-**6**-mediated TNF-α inhibition. Protein kinase C inhibitor (staurosporin, Stauros, 25 nM), protein tyrosine kinase inhibitor (herbimycin A, Herbi, 500 nM) and cyclic AMP elevating agent (dbcAMP, 25 μM and rolipram, Roli, 1 μM) were added alone, or in combination with compound **6**, to LPS-activated RAW264.7 cells. Culture supernatants were then collected after 6 h and assayed by ELISA to determine TNF-α concentration. Data represent mean ± s.e.m. of 3 separate observations (n = 4). $**P < 0.01$ compared with control group (compound **6** alone).

NO was produced in the culture medium, more than 30 ~ 100-fold greater than the basal level. Compounds **6** and **5** and rolipram significantly inhibited NO production by LPS signals in a dose-dependent manner (Figure 4). Contrary to the anti-TNF-α effect, however, the potency was relatively less. In the absence of stimuli, test compounds did not stimulate or inhibit NO production (data not shown). L-NAME also inhibited NO production in LPS-stimulated RAW264.7 cells (IC50 = 180 μM).

Furthermore, NO produced upon LPS exposure is known to induce cell cytotoxicity via inducing apoptosis. Therefore, inhibition of NO release leads to suppression of NO-mediated cytotoxicity. As Figure 4B indicates, LPS exposure for 24 h blocked viability of RAW264.7 cells and L-NAME strongly abolished NO cytotoxicity. The protective effect of oxime-containing compounds was revealed in compounds **5, 6** and rolipram.

Effect of compounds 3, 4, 5 and 6 on lymphocyte proliferation

We examined the inhibitory effect of these compounds on lymphocyte proliferation from splenocytes in the presence of common T cell mitogen Con A by MTT assay. The proliferation of T lymphocytes treated by Con A was significantly increased by 3–4 fold compared with untreated cells. Compounds **6** and **5** strongly inhibited Con A-induced T cell proliferation (Figure 5A), with IC50 value of 1.1 and 12.4 μM, respectively. Standard drugs, rolipram, A77,1726 and dbcAMP in Con A-stimulated splenocytes had IC50 values of 1.9, 6.7 and 603.5 μM, respectively. Because Con A is a non-specific T cell mitogen by which CD4+ and CD8+ T cells are proliferated and a higher concentration of these compounds only showed around 65% inhibition, we further checked whether compound **6** and rolipram were able to modulate CD8+ T cell proliferation, using an IL-2 dependent cytotoxic (CD8+) T cell line, CTLL-2. However, these two compounds did not strongly block IL-2 dependent cell proliferation (Figure 5B), suggesting that PDE IV inhibitors may not block IL-2 dependent CD8+ T cell proliferation. Finally, to confirm whether the inhibitory effect of Con A-induced lymphocyte proliferation by compound **6** and rolipram was due to non-specific cytotoxicity, human CD4+ T cell line Sup-T1 cells were used. Figure 5C indicates that the inhibitory effect of compound **6** and rolipram was not mediated by non-specific

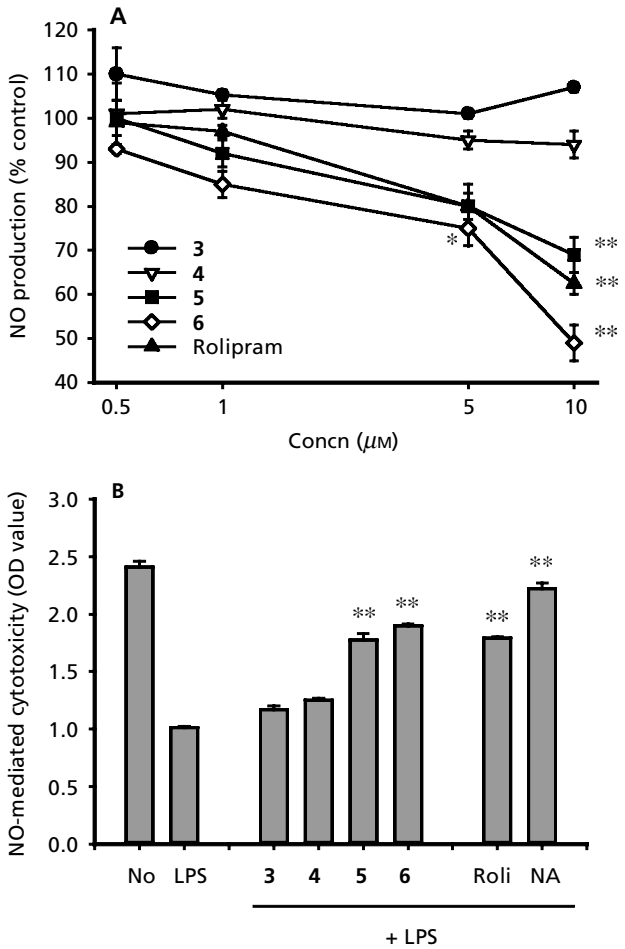


Figure 4 Effects of compounds **3, 4, 5** and **6** on LPS-induced NO production (A) and NO-mediated cytotoxicity of RAW264.7 cells (B). RAW264.7 cells (1×10^6 cells/mL) were incubated with various concentrations of compounds **3, 4, 5** and **6**, and rolipram in the presence or absence of $1\,\mu g\,mL^{-1}$ LPS for 24 h. For NO determination (A), supernatants were collected and assayed for nitrite. For NO-mediated cytotoxicity assay (B), viability of the cells treated with compounds **3, 4, 5** and **6**, rolipram (Roli) and L-NAME (NA) in the presence of $1\,\mu g\,mL^{-1}$ LPS was assayed by conventional MTT method. Data represent mean ± s.e.m. of 3 separate observations (n = 4). $^*P < 0.05$, $^{**}P < 0.01$ compared with control group (LPS alone).

cytotoxicity, because the proliferation of Sup-T1 cells was not blocked by these two PDE IV inhibitors.

Discussion

TNF-α is an important cytokine secreted from activated monocytes or macrophages for host immune defence. However, over-production of TNF-α is known to cause many different inflammatory diseases, such as septic shock, rheumatoid arthritis and asthma (Beutler 1995; Renzetti et al 1996; Sekut & Connolly 1996; Newton & Decicco 1999). Indeed, anti-TNF-α antagonists, including

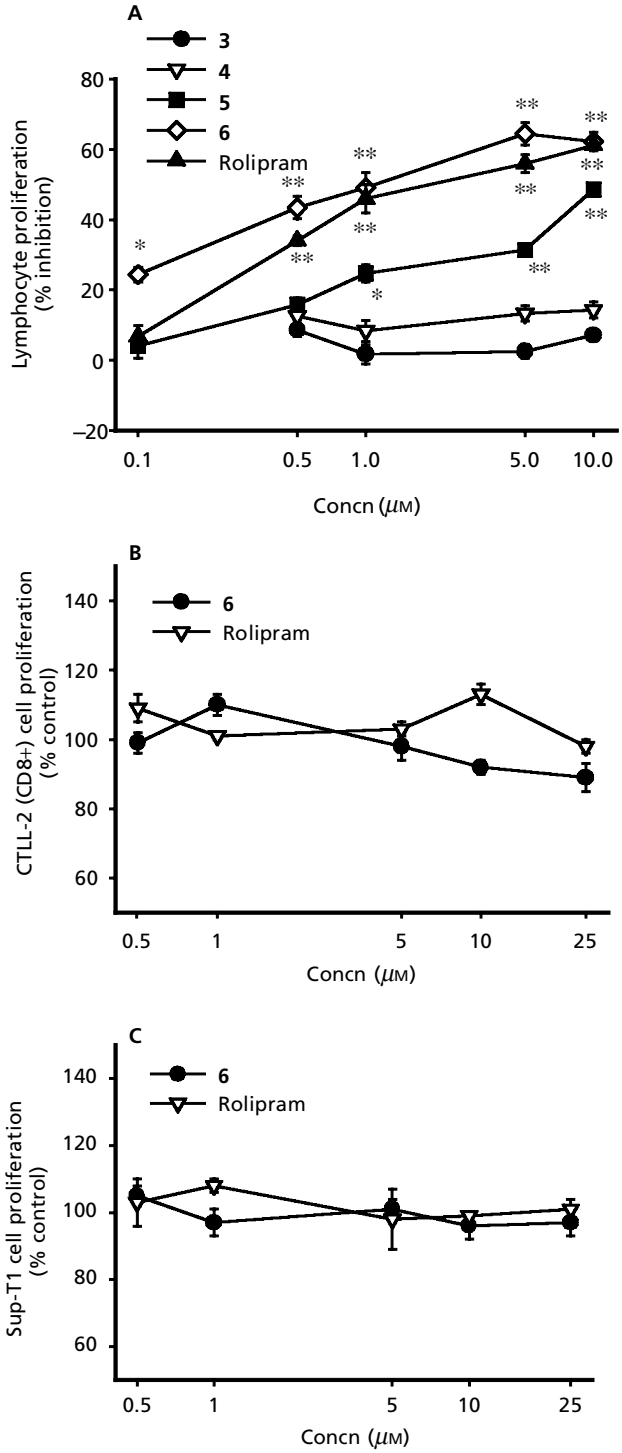


Figure 5 Effect of compounds **3, 4, 5** and **6** on lymphocyte proliferation. Splenocytes (5×10^6 cells/mL; A) or CTLL-2 cells (5×10^5 cells/mL; B) were incubated with compounds **3, 4, 5** and **6**, and rolipram in the presence or absence of $1\,\mu g\,mL^{-1}$ of Con A or IL-2 ($50\,U\,mL^{-1}$) for 48 h. Sup-T1 cells (5×10^5 cells/mL; C) were incubated with compound **6** and rolipram for 48 h. Cell proliferation was assayed by conventional MTT method. Data represent mean ± s.e.m. of 3 separate observations (n = 4). $^*P < 0.05$, $^{**}P < 0.01$ compared with control group (Con A, IL-2 or medium alone).

a soluble receptor (etanercept) for TNF-α and an anti-TNF-α antibody (infliximab), have been demonstrated to be effective in regulating TNF-α-mediated diseases (Garrison & McDonnell 1999; Harriman et al 1999). Because of adverse immune responses by these kinds of proteinaceous inhibitors, development of small antagonists against TNF-α production has been regarded as an important work for the next generation of TNF-α therapy.

cAMP is a well-known second messenger for intracellular signaling from interaction of receptor and stimuli. Since many investigators have reported down-regulation of TNF-α production by intracellular cAMP, the inhibitory mechanism by which TNF-α gene expression is suppressed through inhibiting nuclear factor (NF)-κB activation has been more or less demonstrated (Soderling & Beavo 2000; Jimenez et al 2001). By this reasoning, cAMP-enhancing agents, such as cAMP PDE inhibitors and adenylate cyclase activators, are now widely used for, or are being developed for the treatment of TNF-α-mediated diseases (D'Hellencourt et al 1996; Barnette 1999; Essayan 1999; Park et al 2001, 2002). Of PDEs, in particular, PDE IV is known to be one of the most striking pharmacological targets, since not only is it highly expressed in inflammatory cells, but also the specific inhibitors showed a strong anti-inflammatory activity (Beavo et al 1994; Schudt et al 1995; Soderling & Beavo 2000). With this regard, a large number of dialkoxylphenyl compounds (e.g. rolipram), which are known to selectively inhibit PDE IV, was synthesized and selected on the basis of SAR studies from our group and others (Baures et al 1993; Brackeen et al 1995; Park et al 2000a, b, 2001, 2002). Of them, the pharmacological potency of 4 kinds of structurally similar compounds with an oxime group was evaluated concerning their suppressive effect on inflammatory mediator production at the cellular level.

Using oxime functionality, our study suggested novel information for the target pharmacophore site. The common pharmacophore for dialkoxyphenyl compounds is still less understood, but several points have already been explored by SAR studies using rolipram and its derivatives. For examples, the dialkoxyphenyl form was more effective than the trialkoxy in terms of proper hydrophobicity (Baures et al 1993; Brackeen et al 1995). The carbonyl group from rolipram acted as a powerful hydrogen bonding source (Baures et al 1993; Brackeen et al 1995). Our data (Table 1) indicate that there may possibly be two new hydrogen bonding sites in the inhibitor binding pocket. Thus, compounds **3**–**6** with a 3-carbon spacer, which can be distinguished from the pyrrolidinone ring of rolipram, showed better effect (*cis*-form of oxime geometry is higher than *trans*-form), suggesting that there may be a new hydrogen bonding site at the *cis*-type oxime group. Moreover, the inhibitory activity of compounds **7**–**10**, having a long spacer and displaying strikingly different hydrogen-bond donors from rolipram and compounds **3**–**6**, may also suggest that another possible hydrogen bonding site participates in suppressing TNF-α production. In these compounds, the carbonyl group of the isoindolinone ring may act as a hydrogen-bond acceptor, similar to the carbonyl group of rolipram (pyrrolol isomerism, which acts as a hydrogen-bond donor). The effect is also clearly confirmed by compounds **11** and **12**, without a hydrogen-bond donor. Moreover, the inhibitory effects of dialkoxyphenyl compounds seem to be distinguished by different spacers, suggesting that there may be several approaches to improving inhibitory potency by introducing another spacer either to interact with functional groups from target enzymes or to support direct interaction between oxime and its counter part from PDE IV. Dialkoxyphenyl compounds with a different structure type to rolipram have been recently studied because of rolipram's side effects, such as nausea and emetic potency (Heaslip & Evans 1995; Duplantier et al 1996). Therefore, combination of oxime's functionality with non-rolipram type PDE IV inhibitors will be an interesting approach as a new PDE antagonist. We are now undertaking these new approaches and are also currently examining the other in-vivo biological activity and side effects of compound **6**.

According to the immunopharmacological data presented in this paper, compound **6** can exert a significant immunomodulatory effect on mammalian immune responses in-vitro and in-vivo. Compound **6** significantly blocked the ability of macrophages and lymphocytes in response to mitogens such as Con A. The effects seem not to be due to the non-specific cytotoxicity, since compound **6** did not affect cell viability at the pharmacologically effective doses (data not shown). In particular the inhibitory potency of compound **6** on TNF-α was higher than those of the standard compound rolipram and other naturally occurring compounds, such as arctigenin, eudesmin, ginsenosides, woorenosides and cynaropicrin, reported previously (Cho et al 1999a, b, 2000a, b, 2001). Of the mitogenic responses of activated macrophages and lymphocytes evaluated, TNF-α production seems to be the most significant target of the dialkoxyphenyl compounds (Figure 1). Compound **6** and rolipram exerted a strong inhibitory activity, with IC50 values in the range 25–100 nM against TNF-α production (Figure 1), but not NO and T cell proliferation (Figures 4 and 5), although there is a discrepancy between the in-vitro and in-vivo effects of compound **6** and rolipram, which may be due to weak solubility. These results suggest that compound **6** selectively possesses therapeutic activity against TNF-α-mediated diseases, such as rheumatoid arthritis and septic shock (Beutler 1995; Sekut & Conolly 1996; Jackson et al 1998). As these types of compounds have been known to show severe side effects, however, their use may be restricted. Interestingly, numerous reports have recently pointed out the involvement of TNF-α in the late phase of cancerous states such as tumour metastasis. Indeed, some agents, such as thalidomide and linolemide, which inhibit TNF-α production, have also been demonstrated to suppress tumour metastasis and neovascular formation in the tumour-occupied tissue (Qin & Blankenstein 1996; Ferrara 2000). Thus, such facts may suggest the possibility that diphenylalkoxyl compound **6** with an oxime group (also other PDE IV inhibitors) is a new class of anti-cancer drug, which targets TNF-α production. The possibility may be also expanded to treatment with combination of

compound **6** and chemotherapeutic agents. We postulate that the combination treatment may lead to both decreased side effects of PDE IV inhibitors and, simultaneously, improvement of total therapeutic potential against cancer. The enhancement of pharmacological potency of PDE IV as an anti-TNF-α inhibitor was demonstrated by combination of compound **6** with well-known TNF-α inhibitors (Figure 3). In addition, oxime (ketone) functionality may be more enforced in tumour cells, which have low pH, because, under this condition, functional ketones can be selectively released in tumour cells (Dimmock et al 1992). The potential significance of compound **6** in anti-cancer therapy should now be carefully evaluated.

Conclusion

In conclusion, we have shown that a series of derivatives of dialkoxylphenyl compounds with an oxime group significantly modulated several inflammatory phenomena (TNF-α production, NO release and lymphocyte proliferation) in a structure-related manner at cellular level. In particular, the SAR study with 12 oxime-containing compounds on TNF-α inhibition indicated that there might be at least three possible hydrogen bonding sites in the inhibitor binding pocket of PDE IV. Of them, compound **6** displayed the highest inhibitory effects on the inflammatory phenomena, at a level comparable with that of the standard PDE IV inhibitor, rolipram. Therefore, these data suggest that the oxime structure may act as a function-regulating group of target PDE IV and that compound **6** may be suitable to be developed as a new anti-inflammatory drug or to be used to develop more potent analogues.

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