JPP Journal of Pharmacy and Pharmacology

JPP 2006, 58: 219–226
© 2006 The Authors
Received July 28, 2005
Accepted October 31, 2005
DOI 10.1211/jpp.58.2.0009
ISSN 0022-3573

Evaluation of in-vitro anti-inflammatory activity of some 2-alkyl-4,6-dimethoxy-1,3,5-triazines

Chiara Dianzani, Massimo Collino, Margherita Gallicchio, Simona Samaritani, Giovanni Signore, Rita Menicagli and Roberto Fantozzi

Abstract

The ability of some 2-alkyl(aryl)-4,6-dimethoxy-1,3,5-triazine derivatives to interfere with production of reactive oxygen species (ROS) by human phagocytes was evaluated in an in-vitro cell model. Superoxide anion ($O_2^{-\cdot}$) production by human polymorphonuclear cells (PMNs), challenged by the chemotactic agent *N*-formylmethionyl-leucyl-phenylalanine (FMLP), was inhibited in a dose-dependent manner by all the compounds tested, compounds **3**, **4** and **5** being statistically the most active. Adhesion of PMNs to vascular endothelial cells (ECs) is a critical step in recruitment and infiltration of leucocytes into tissues during inflammation, and the effects of 1,3,5-triazine derivatives on PMN adhesion to ECs from the human umbilical vein (HUVEC) were also investigated. Triazines were incubated with PMNs and HUVEC; adhesion was quantitated by computerized micro-imaging fluorescence analysis. The 1,3,5-triazines tested inhibited the adhesion evoked by pro-inflammatory stimuli, such as platelet activating factor (PAF), FMLP, phorbol myristate acetate (PMA), tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) in a dose–response manner over the concentration range 10^{-9} to 10^{-4} M, compounds **5** and **6** being the most active. Both of these compounds inhibited PMN adhesion to HUVEC, even when endothelial or PMN stimuli were used. Indeed, when both cell populations were activated contemporarily, the anti-adhesive effect was enhanced. The study suggests that 2-aryl-4,6-dimethoxy-1,3,5-triazines deserve further evaluation as anti-inflammatory agents.

Department of Anatomy, Pharmacology and Forensic Medicine, University of Turin, V. P. Giuria 9, 10125 Torino, Italy

Chiara Dianzani, Massimo Collino, Margherita Gallicchio, Roberto Fantozzi

Department of Chemistry and Industrial Chemistry, University of Pisa, Via Risorgimento 35, 56126 Pisa, Italy

Simona Samaritani, Giovanni Signore, Rita Menicagli

Correspondence: C. Dianzani, Department of Anatomy Pharmacology and Forensic Medicine, Via Pietro Giuria, 9, 10125 Torino, Italy. E-mail: chiara.dianzani@unito.it

Acknowledgments and funding: This research was supported by the Turin University funding (ex-60%). We thank the Anatomo-Pathology Unit and the Obstetric and Gynecology Unit at Martini Hospital, Turin, for providing human umbilical cords and the Blood Bank at the Molinette Hospital, Turin, for providing human blood.

Introduction.

2,4,6-Tris-(heteroalkyl)-1,3,5-triazine derivatives have long been the subject of investigation in many fields of medicinal chemistry, with regard to their possible use as hypolipaemic agents (D'Atri et al 1984), modulators of multidrug resistance (Dhainaut et al 1992), anti-trypanosomal drugs (Klenke et al 2001), VLA-4 integrin antagonists (Porter et al 2002) and antiviral drugs (Wang et al 2003). Recently, 2-alkyl (aryl, alk-1′-ynyl)-4,6-diheteroalkyl-1,3,5-triazines have shown interesting anti-HIV and anti-cancer properties (Kukla et al 1998; Menicagli et al 2004). In the context of our studies on the cytotoxicity of these heteroaromatic compounds towards some tumour cell lines (Menicagli et al 2004), we synthesized 2-alkyl(aryl)-4,6-dimethoxy-1,3,5-triazines **1**–**7** and investigated them for potential anti-inflammatory activity (Samaritani et al 2005). The 1,3,5-triazine compounds were selected on the basis of the nature of the alkyl and aryl C-C-bonded residues and of their electronic effects on the heteroaromatic ring. In particular, 2-alkyl-4,6-dimethoxy-1,3,5-triazines characterized by a linear primary (**1**), an α-branched primary (**4**) and a secondary alkyl moiety (**2**) were tested. As far as the 2-aryl derivatives are concerned, in addition to 2-phenyl-4,6-dimethoxy-1,3,5-triazine (**3**), compounds containing an electron-donating heteroaromatic (**5**) or aromatic (**6**) substituent, as well as an electron-withdrawing aromatic substituent (**7**), were investigated.

There is currently considerable therapeutic interest in novel anti-inflammatory drugs with a mode of action different from that of the classical nonsteroidal anti-inflammatory drugs (NSAIDs), mainly for use in patients with arthritis of varying degrees of severity. The classical NSAIDs do not prevent the progression of this disease and cause irritant side-effects on the gastric mucosa.

Inflammation is a fundamental response to tissue injury or invasion by microbes, but is noxious in some clinical inflammatory disorders. Leucocytes are very important players in

the inflammatory response because of their antimicrobial, secretory and phagocytic activity. They are recruited at the inflamed site by sequential adhesive interactions between leucocytes and the endothelium, which are mediated by cell-adhesion molecules (CAMs) on the surface of the interacting cells. Leucocyte extravasion is due to the cooperative activity of several molecules acting on at least four steps (Cines et al 1998; Muller 2002): firstly, leucocyte rolling on the endothelium, which is mediated by constitutively functional adhesion receptors, such as selectins and mucin-like molecules; secondly, activation of inactive adhesion receptors by chemoattractants present on the vascular endothelial cells (ECs); thirdly, leucocyte arrest mediated by molecules such as the integrins, activated in the previous step; and finally, extravasation mediated by molecules such as CD31 and integrins, which direct leucocyte motility towards the tissue. At the same time, the ECs control leucocyte migration by expressing distinct patterns of adhesion molecules and producing or presenting chemoattractants to the leucocytes.

The effects of many inflammatory drugs may at least in part be ascribed to their inhibition of CAMs expression (Ulbrich et al 2003) or alternatively they might influence leucocyte adhesion and migration.

This study evaluates the potential anti-inflammatory properties of 2-alkyl(aryl)-4,6-dimethoxy-1,3,5-triazines **1**–**7**. The test used aimed to mime in-vitro what occurs in-vivo when polymorphonuclear cells (PMNs) adhere to the ECs before migration to the inflammatory site; adhesion of PMNs to fetal calf serum (FCS)-coated plastic wells was also studied. PMNs recruited at the inflammation site produce free radicals that contribute to the epithelial injury. Therefore activity of triazines **1**–**7** on superoxide anion ($O_2^{-\cdot}$) production by the PMNs was also determined.

Materials and Methods

Materials

Dextran T500 was from Pharmacia Biotech (Uppsala, Sweden). FCS (endotoxin tested) was from Hyclone Laboratories Inc. (Logan, UT, USA). Trypsin was from Difco Laboratories Inc. (Detroit, MI, USA). Histopaque 1077, fluorescein diacetate, M199 (endotoxin tested), platelet-activating factor (PAF), *N*-formylmethionyl-leucyl-phenylalanine (n-FMLP), interleukin (IL)-1β, phorbol myristate acetate (PMA), tumour necrosis factor (TNF)-α, cytochrome C, superoxide dismutase, cytochalasin B, human serum albumin (HSA) and methylthiazolyldiphenyl-tetrazolium bromide (MTT) were from Sigma-Aldrich (St Louis, MO, USA). Monoclonal antibody (mAb) LFA-1 was a gift of Prof. U. Dianzani (University of Piemonte Orientale, Novara, Italy). mAb OKM-1 was obtained from American Type Culture Collection (Rockville, MD, USA). Each mAb was used at the concentration that demonstrated maximal inhibitory effects in adhesion assay, (20 μg mL^{-1}) (Zimmerman et al 1992). All other reagents and solvents were from Merck (Darmstadt, Germany).

The 1,3,5-triazine derivatives were synthesized as reported by Samaritani et al 2005. Each was dissolved in dimethyl sulfoxide (DMSO). Stock solutions were prepared daily and diluted in M199 to the appropriate concentrations before each experiment. The final concentration of DMSO was not above 0.1%. The same amount of DMSO was added to the control samples and did not affect either absolute control adhesion or superoxide anion production ($O_2^{-\cdot}$).

Cell cultures

PMNs were prepared from citrated venous blood obtained from healthy subjects at a local hospital bank, using the standard technique of dextran sedimentation followed by Histopaque 1077 gradient centrifugation. Residual erythrocytes were removed by hypotonic lysis and PMNs were resuspended in buffered salt solution (BSS) (composition in mM: 138 NaCl, 2.7 KCl, 8.1 Na_2HPO_4, 1.5 KH_2PO_4, 1 $MgCl_2$, 1 $CaCl_2$, pH 7.4) supplemented with 1 mg mL^{-1} glucose and 1 mg mL^{-1} HSA. Purity of the final cell suspension and cell viability, assessed by the trypan blue exclusion test, was > 95% in all cases. Cell viability was not affected by compound treatment, measured with the MTT test for ECs from the human umbilical vein (HUVEC) and with the trypan blue exclusion test for PMNs (test duration: 0–60 min).

HUVEC were isolated from human umbilical veins by trypsin treatment (1%) and cultured in M199 medium with the addition of 20% FCS and 10 ng mL^{-1} human fibroblast growth factor (FGF). Purity of the HUVEC preparation, evaluated by morphology and immunostaining for factor VIII, was > 95%. Contaminant leucocytes were detected by immunostaining for CD45.

HUVEC were grown to confluence in flasks and used at the 3rd to 5th passage.

Superoxide anion ($O_2^{-\cdot}$) production

The PMNs were suspended in the BSS. PMNs (1×10^6 cells/mL) were pretreated with cytochalasin B (5 μg mL^{-1}) for 5 min at 37°C, to maximize the measured response, and then challenged with the substances for 15 min at 37 °C before exposure to 10^{-7} M FMLP for a further 5 min. $O_2^{-\cdot}$ production was determined spectrophotometrically by measuring the superoxide dismutase-inhibitable reduction of cytochrome C reduced/10^6 PMNs/min. Compounds **1**–**7** were checked for interference in the assay by measuring their effects on cytochrome C reduction with a xanthine oxidase superoxide generating system. None of the substances tested interfered with the spectrophotometric assay. Assays were carried out in the same buffer, with 100 μM cytochrome C, 150 μM hypoxanthine, 0.01 U xanthine oxidase per mL, and appropriate concentrations of each compound.

Adhesion assay

HUVEC were grown to confluence in 24-well plates. PMNs (10^7 cells/mL) were labelled with fluorescein diacetate (5 μg mL^{-1}) for 30 min at 37°C, washed with BSS, and plated at 10^6 cells/well in a final volume of 0.25 mL BSS on HUVEC pretreated with the compounds (10^{-9} to 10^{-4} M) for 10 min and challenged with different stimuli: FMLP and PAF (both at 10^{-7} M) or PMA (10^{-8} M) for 10 min or IL-1β and TNF-α (both at 10 ng mL^{-1}) for 1 h. After incubation,

non-adherent PMNs were removed by washing three times with 1 mL BSS. The centre of each well was analysed by fluorescence image analysis (Dianzani et al 2003). Adherent cells were counted employing Image Pro Plus Software for micro-imaging (Media Cybernetics, version 5.0). Single experimental points were assayed in quadruplicate, and standard error of the four replicates was below 10% in all cases. Data are presented as percentage adhesion versus the control value, control adhesion being measured on HUVEC that underwent no treatment. Control adhesion was 55 ± 8 cells/microscope field ($n = 30$).

The direct effect on PMNs was assessed by seeding the cells on 24-well EC-free plates for 10–20 min at 37°C, in the presence of the compounds and 10^{-7} M PAF. The plates had previously been coated with heat-inactivated calf serum for three hours to reduce spontaneous adhesion to the plastic wells. Percentage inhibition of adhesion was calculated as follows: $[100-(a)/(b)]\times 100$, where a is adhesion measured in the presence of the compound plus stimulus minus basal adhesion and b is adhesion elicited by stimulus minus basal adhesion.

Statistical analysis

Results are expressed as means ± s.e.m.; n indicates the number of experiments. Data in Table 1 were analysed by one-way analysis of variance to ascertain whether differences among the means were significant. Tukey's multiple comparison post-test was then used to determine significant differences between specific mean pairs. Data in Figures 1–5 were analysed by two-way analysis of variance; Bonferroni multiple comparison post-test was then applied to determine significant differences between specific mean pairs. The molar concentration of each compound that reduced response to the stimulus by 50% (IC50) was calculated with a non-linear regression model using Origin version 6.0 software (Microcal Software, MA, USA). The IC50 data in Figures 1, 4 and 5 were analysed using Student's *t*-test; IC50 data in Figures 2 and 3 were analysed using one-way analysis of variance, followed by the Tukey multiple comparison post-test. Differences were considered to be statistically significant at $P < 0.05$. All statistical analyses were done using GradPadPrism 3.0 software (GraphPad Software, San Diego, CA, USA).

Results and Discussion

Effect on $O_2^{-\cdot}$ production induced by FMLP on PMNs

The effects of compounds **1–7** on $O_2^{-\cdot}$ production by FMLP-challenged human PMNs are reported in Table 1. None of the tested derivatives alone induced $O_2^{-\cdot}$ production by PMNs in the concentration range 10^{-8} to 10^{-4} M (data not shown). On the contrary, PMNs challenged with 10^{-7} M FMLP released $O_2^{-\cdot}$ (1.8 ± 0.3 nmol cytochrome C reduced/10^6 cells/min; $n = 5$). This concentration was selected as suitable to produce optimal $O_2^{-\cdot}$ generation by human PMNs (Dianzani et al 1996). When PMNs were incubated with increasing concentrations of compounds **1–7** (10^{-6} to 10^{-4} M) for 10 min and then challenged with 10^{-7} M FMLP, an inhibitory effect on $O_2^{-\cdot}$ production was detected. All compounds dose-dependently inhibited $O_2^{-\cdot}$ production evoked by 10^{-7} M FMLP. Maximal inhibition was obtained at 10^{-4} M, with inhibition values of 75–99%, compounds **3**, **4** and **5** being statistically more potent than the others ($IC50 = 3.1 \pm 0.3 \times 10^{-6}$ M, $4.5 \pm 0.6 \times 10^{-6}$ M and $1.6 \pm 0.4 \times 10^{-6}$ M, respectively). 2-Chloroadenosine, a well-known modulator of PMN function, used as positive control (Dianzani et al 1994), caused greater inhibition than the tested compounds, with a maximum effect of 70 ± 4% inhibition, and $IC50 = 87 \pm 6 \times 10^{-9}$ M. Even when a higher concentration of FMLP was used (3×10^{-6} M FMLP: 4.0 nmol/cytochrome C reduced/10^6 cells/min; data not shown) all compounds tested still inhibited the higher FMLP-induced $O_2^{-\cdot}$ production, with a dose–response curve quantitatively and qualitatively equal to those reported in Table 1.

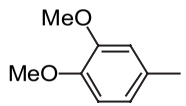
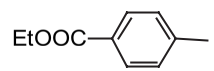
Reactive oxygen species (ROS), such as $O_2^{-\cdot}$, are produced in all aerobic organisms during respiration and exist in the cell in balance with endogenous antioxidants (e.g. glutathione, vitamins A, C and E). Excess ROS production alters the cellular redox balance. ROS react with many macromolecules causing structural and functional modifications, cytotoxicity and mutagenic damage (Jabs 1999). ROS exert genomic effects and modulate cell proliferation, by activating transcription factors, such as AP-1, AP-2 and NF-κB (Dalton et al 1999). Leucocytes, particularly PMNs and monocytes, and ECs provide a rich source of ROS that can contribute to the development of degenerative diseases (e.g. atherosclerosis, diabetes, Alzheimer's disease, arthritis, multiple sclerosis). ROS also play an important role in evolving organ injury (e.g. cerebral, cardiac, intestinal damage), which characterizes the pathophysiology of ischaemia–reperfusion (I/R) (Lucchesi 1990). The ability of triazine derivatives to inhibit $O_2^{-\cdot}$ production thus demonstrates their role in modulating oxidative stress and may suggest their potential use in different degenerative diseases.

Effect of 1,3,5-triazine derivatives on PMN adhesion to HUVEC induced by FMLP

Adhesion and transendothelial migration of leucocytes into the surrounding tissues are crucial steps in inflammation, immunity and atherogenesis (Li et al 1993; Jang et al 1994; Springer 1994). These experiments were designed to ascertain whether compounds **1–7** modulate PMN adhesion to HUVEC. Considering that the bacterial peptide FMLP only activates PMN adhesive machinery, this stimulus was selected to evaluate the effect of these compounds on PMN adhesion to HUVEC. It has been shown that the concentration of FMLP tested (10^{-7} M) gives near-maximal activation of PMNs (Tonnesen et al 1984; Zimmerman et al 1985; Avanzi et al 1998). HUVEC were pretreated for 10 min with the compounds under study (10^{-6} to 10^{-4} M) and then co-incubated with 10^{-7} M FMLP and PMNs for a further 10 min.

The control value of adhesion was 55 ± 8 cells/microscope field (mean ± s.e.m., $n = 30$) and FMLP-induced adhesion was 283 ± 28% of the control value. None of the compounds (at 10^{-6} to 10^{-4} M) affected PMN adhesion of resting cells (data not shown).

Table 1 Effect of 1,3,5-triazine derivatives **1–7** on FMLP-evoked $O_2^{-\cdot}$ production by PMNs

Concentration	% Inhibition of $O_2^{-\cdot}$ production induced by derivatives 1–7						
	1	**2**	**3**	**4**	**5**	**6**	**7**
	Et	i.Pr	Ph	(S)-2(Me)Bu			
10^{-4} M	85 ± 4	91 ± 7	82 ± 5	83 ± 5	83 ± 4	99 ± 1	76 ± 7
3×10^{-5} M	60 ± 3	60 ± 3	75 ± 4	75 ± 5	78 ± 2	75 ± 4	31 ± 2
10^{-5} M	30 ± 2	31 ± 8	58 ± 3	49 ± 5	58 ± 4	37 ± 3	12 ± 3
10^{-6} M	11 ± 5	10 ± 2	22 ± 3	12 ± 6	32 ± 1	4 ± 2	3 ± 2
10^{-7} M	2 ± 1	2 ± 1	3 ± 1	3 ± 1	15 ± 1	—	—
10^{-8} M	—	—	—	—	2 ± 1	—	—
IC50	$1.3 \pm 0.3 \times 10^{-5}$ M	$1.6 \pm 0.3 \times 10^{-5}$ M	$3.1 \pm 0.3 \times 10^{-6}$ M*	$4.5 \pm 0.6 \times 10^{-6}$ M*	$1.6 \pm 0.4 \times 10^{-6}$ M*	$1.3 \pm 0.1 \times 10^{-5}$ M*	$5.9 \pm 0.3 \times 10^{-5}$ M

The results are expressed as percentages of inhibition of $O_2^{-\cdot}$ production evoked by 10^{-7} M FMLP in the absence of triazine derivatives: this production amounted to 1.8 ± 0.3 nmol cytochrome C reduced/10^6 cells/min and was taken as 100%. Data are expressed as mean ± s.e.m., n = 5. IC50 are expressed as mean ± s.e.m. Compounds **3**,**4** and **5** were found to be more potent than all the other compounds in a statistically significant fashion (*$P < 0.05$), compound **5** being the most potent.

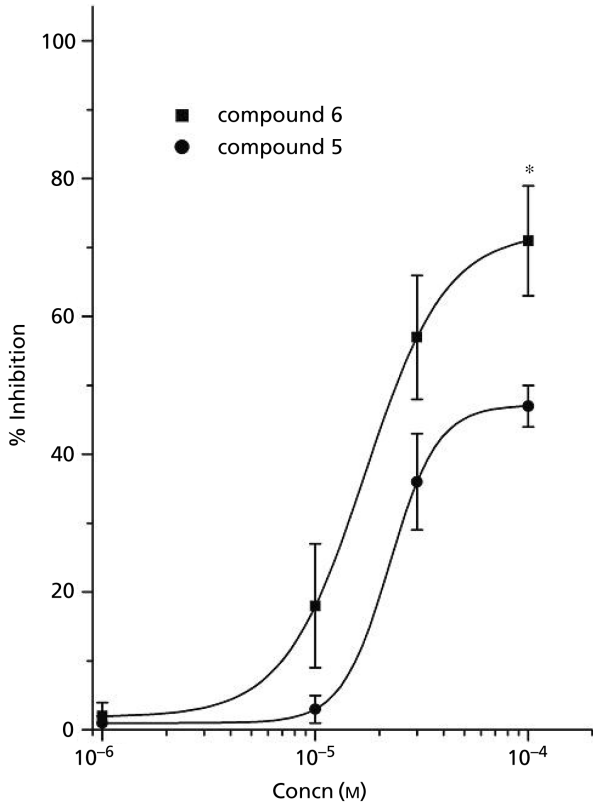


Figure 1 Effect of 1,3,5-triazine derivatives **5** and **6** on PMN adhesion to HUVEC evoked by FMLP. HUVEC were pretreated with increasing concentrations (10^{-6} to 10^{-4} M) of the tested compounds for 10 min at 37°C and then challenged with 10^{-7} M FMLP and PMNs for a further 10 min. Data are expressed as percentages of inhibition versus control adhesion. Control adhesion was 51 ± 10 cells/microscope field (mean ± s.e.m., n = 26). Data are expressed as means ± s.e.m., n = 5. *$P < 0.05$, inhibition of compound **6** versus compound **5**. IC50 values were: $1.4 \pm 0.3 \times 10^{-5}$ M for compound **6** and $2.6 \pm 0.3 \times 10^{-5}$ M for compound **5.**

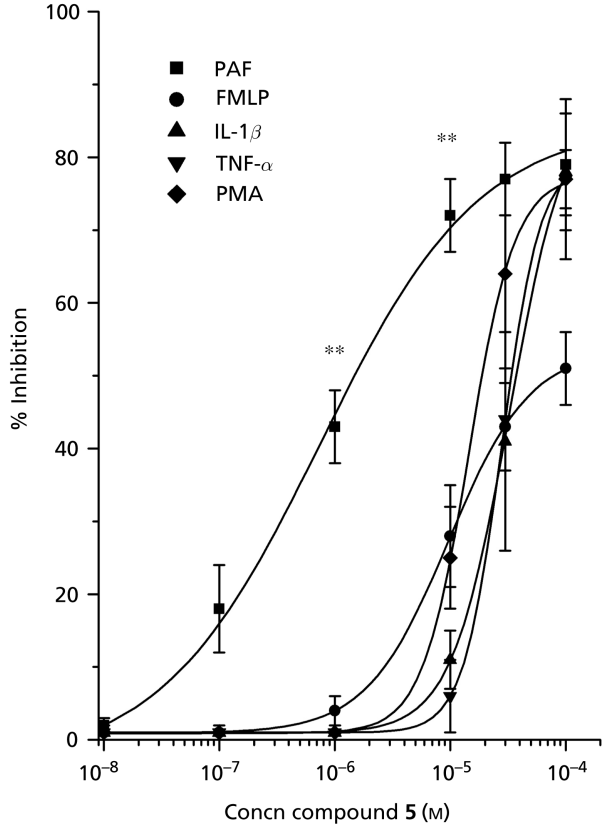


Figure 2 Effect of compound **5** on PMN adhesion to HUVEC evoked by PAF, FMLP, IL-1β, TNF-α and PMA. HUVEC were pretreated with increasing concentrations (10^{-8} to 10^{-4} M) of the tested compound for 10 min at 37°C and then challenged with PAF, FMLP (both at 10^{-7} M) or PMA (10^{-8} M) and PMNs for a further 10 min; IL-1β and TNF-α (both at 10 ng mL^{-1}) were incubated for 1 h with pretreated HUVEC and then challenged with PMNs. Data are expressed as percentages of inhibition versus control adhesion, as mean ± s.e.m., n = 5. **$P < 0.01$, inhibition using PAF 10^{-6} to 10^{-5} M as stimulus versus the same concentration of FMLP, IL-1β, TNF-α and PMA. IC50 values were: $7.0 \pm 0.5 \times 10^{-7}$ M, $7.9 \pm 0.4 \times 10^{-6}$ M, $1.2 \pm 0.2 \times 10^{-5}$ M; $3.3 \pm 0.3 \times 10^{-5}$ M and $3.7 \pm 0.3 \times 10^{-5}$ M, when the stimulus used was PAF, FMLP, PMA, IL-1 β and TNF-α, respectively. IC50 values obtained with PAF were statistically different from all others ($P < 0.001$).

Only compounds **5** and **6** showed dose-dependent inhibition in the concentration range 10^{-6} to 10^{-4}M (Figure 1). Compound **4** displayed an inhibitory effect that was not dose-dependent, showing a non-specific effect (data not shown). Almost complete inhibition (71±8%) was produced by compound **6**, while the maximum inhibition for compound **5** was only 48±3%, with statistically significant difference only at the highest concentration tested (10^{-4}M, $P<0.05$). None of the other compounds displayed any effect on stimulated PMN adhesion to HUVEC and we thus focused our investigation only on compounds **5** and **6**. In the same experimental condition, mAbs against the integrin adhesion molecules, LFA-1 and OKM-1 (Altieri et al 1990), used as positive control, exert 92±3% and 94±5% of inhibition, respectively.

The role of CAMs in modulating PMN adhesion to ECs is well known. The CAMs involved in leucocyte trafficking constitute excellent targets for pharmacological modulation of the cellular response in inflammation. Several mechanisms can modulate the function of inflammatory CAMs, including competitive blockade, altered expression on the cell surface and interference with receptor activation. Several groups of pharmaceutical agents in use clinically interfere with the function of CAMs either directly or indirectly. Many clinical trials of anti-adhesion therapies have used humanized antibodies, but low-molecular-weight compounds have several advantages over antibodies and are less likely to trigger adverse immune reactions (Ulbrich et al 2003). Therefore, further direct evidence linking compounds **5** and **6** to their potential ability to interact with CAMs would be very useful to clarify their mechanism of action.

Effects of compounds 5 and 6 on PMN adhesion to HUVEC induced by different stimuli: PAF, IL-1β, TNF-α and PMA

To investigate the effects of compounds **5** and **6** on other pro-inflammatory stimuli, we challenged PMNs and HUVEC

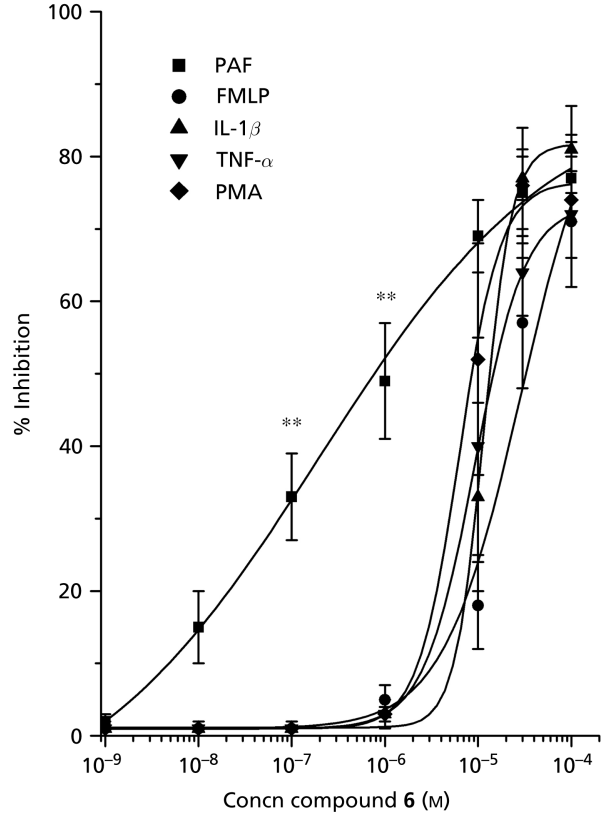


Figure 3 Effect of compound **6** on PMN adhesion to HUVEC evoked by PAF, FMLP, IL-1β, TNF-α and PMA. HUVEC were pretreated with increasing concentrations (10^{-9} to 10^{-4} M) of the tested compound for 10 min at 37°C and then challenged with PAF, FMLP (both at 10^{-7} M) or PMA (10^{-8} M) and PMNs for a further 10 min; IL-1β and TNF-α (both at 10 ng mL^{-1}) were incubated for 1 h with pretreated HUVEC and then challenged with PMNs. Data are expressed as percentages of inhibition versus control adhesion, as mean ± s.e.m., n = 5. $^{**}P < 0.01$, inhibition using PAF as stimulus versus FMLP, IL-1β, TNF-α and PMA. IC50 values were: $2.3 \pm 0.7 \times 10^{-7}$ M, $5.5 \pm 0.3 \times 10^{-5}$ M, $8.5 \pm 0.4 \times 10^{-6}$ M; $8.1 \pm 0.5 \times 10^{-6}$ M and $9.5 \pm 0.3 \times 10^{-6}$ M, when the stimulus used was PAF, FMLP, PMA, IL-1 β and TNF-α, respectively. IC50 values obtained with PAF were statistically different from all others ($P < 0.001$).

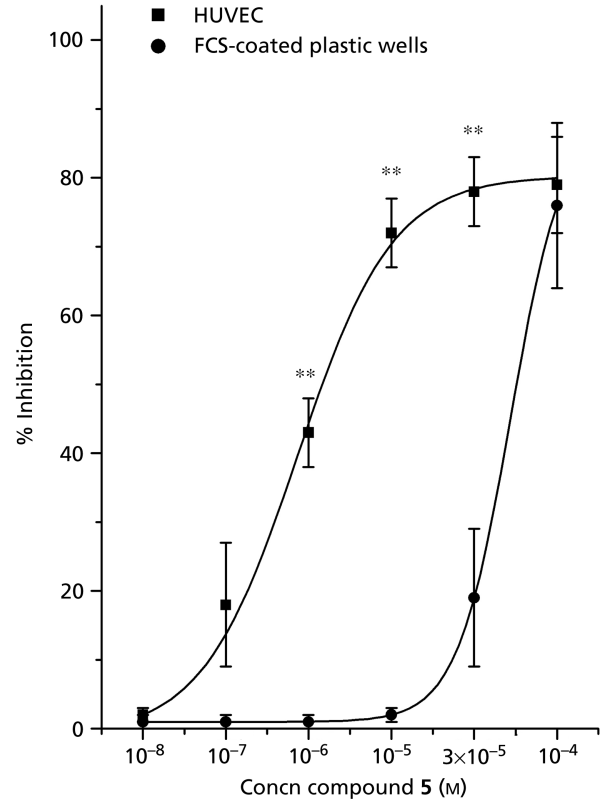


Figure 4 Effect of compound **5** on PMNs adhesion to FCS-coated plastic wells and compared with PMN adhesion to HUVEC. PMNs were incubated with increasing concentrations (10^{-8} to 10^{-4} M) of the tested compound for 10 min at 37°C and then challenged with PAF 10^{-7} M for 10 min. Data are expressed as percentages of inhibition versus control adhesion, as mean ± s.e.m., n = 5. $^{**}P < 0.01$, inhibition of adhesion to HUVEC versus FCS-coated plastic wells. IC50 values obtained with and without HUVEC, were, respectively, $7.0 \pm 0.5 \times 10^{-7}$ M and $4.4 \pm 0.4 \times 10^{-5}$ M ($P < 0.001$).

with PAF 10^{-7} M, IL-1β, TNF-α (both at 10 ng mL^{-1}) and PMA (10^{-8} M), basing our selection on other reports (Avanzi et al 1998; Choi et al 2004; Yang et al 2004). The concentrations of these stimuli were selected on the basis of experimental results so as to obtain adhesion close to that achieved with FMLP. The inhibitory effects exerted by compound **5** when the cells were stimulated with IL-1β, TNF-α and PMA were similar to those obtained when PMNs were challenged with FMLP, depicting a dose-dependent curve, in the range of concentration 10^{-6} to 10^{-4} M (Figure 2), but with higher inhibition at the higher concentration (10^{-4} M). PAF induced a stronger inhibition of the PMN adhesion than did the other stimuli: inhibition occurred over the range of concentration 10^{-8} to 10^{-4} M and the maximum value was at 10^{-4} M (80%); the inhibition curve was statistically different to that obtained with FMLP as stimulus ($P < 0.01$), while only inhibition values at concentrations between 10^{-6} and 10^{-5} M were statistically different from those obtained with the other stimuli ($P < 0.01$; Figure 2). Similar results were obtained with compound **6**: using PAF (10^{-7} M) as stimulus, the inhibitory effects obtained were markedly higher; the compound was effective in the range of concentration 10^{-9} to 10^{-4} M and there were statistically significant differences at 10^{-7} to 10^{-6} M in comparison with all other stimuli tested ($P < 0.01$; Figure 3).

Effects of compounds 5 and 6 on PMN adhesion to FCS-coated plastic wells

Since PAF was found to be active on both PMNs and HUVEC, we hypothesized that the 1,3,5-triazine derivatives tested may act on both cell populations. To evaluate whether compounds **5** and **6** acted preferentially on PMNs, or also acted on HUVEC, the effects of the two substances on PMN adhesion to HUVEC were compared with their effect on adhesion to FCS-coated plastic wells, using PAF as stimulus. In this experimental system, compound **5** induced a very slight dose–response curve (10^{-5} to 10^{-4} M, Figure 4; 10 or 20 min incubation gave the same dose–response curve) when the PMN population alone was used in the test, whereas it was very marked when both

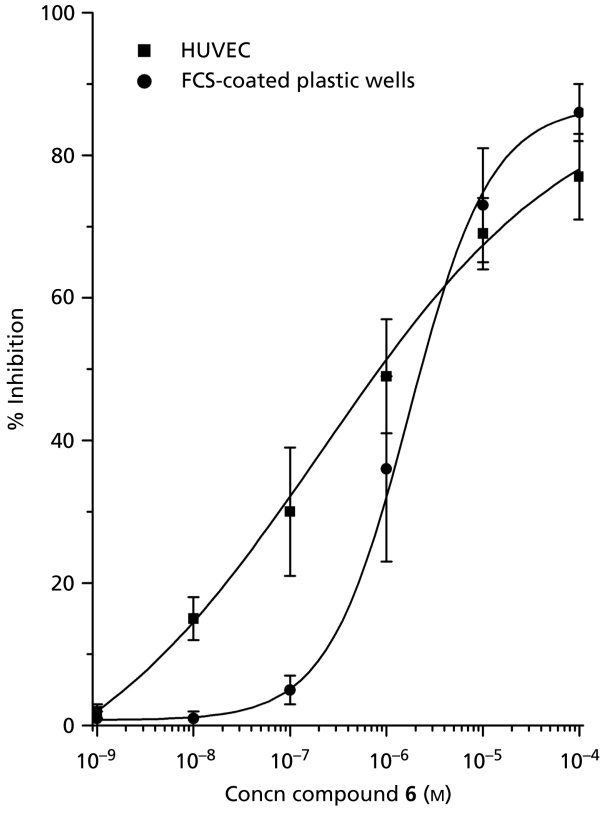


Figure 5 Effect of compound **6** on PMNs adhesion to FCS-coated plastic wells and compared with PMN adhesion to HUVEC. PMNs were incubated with increasing concentrations (10^{-9} to 10^{-4} M) of the tested compound for 10 min at 37°C and then challenged with PAF 10^{-7} M for 10 min. Data are expressed as percentages of inhibition versus control adhesion, as mean ± s.e.m., n = 5. *$P < 0.05$, inhibition of adhesion to HUVEC versus FCS-coated plastic wells. IC50 values obtained with and without HUVEC, were, respectively, $2.3 \pm 0.7 \times 10^{-7}$ M and $2.1 \pm 0.5 \times 10^{-6}$ M.

populations were present and stimulated (IC50 = $7.0 \pm 0.5 \times 10^{-7}$ M and $4.4 \pm 0.4 \times 10^{-5}$ M, respectively, with and without HUVEC), with statistically significant difference at 3×10^{-5} to 10^{-6} M ($P < 0.01$). On the contrary, for compound **6** the dose–response curves with and without HUVEC were similar (Figure 5; IC50 = $2.3 \pm 0.4 \times 10^{-7}$ M and $2.1 \pm 0.5 \times 10^{-6}$ M, respectively). We may thus hypothesize that compound **6** acts equally on PMNs or HUVEC: in fact the same results were observed when PMNs alone or HUVEC alone (with adhesion stimuli PMA, IL-1β or TNF-α) were stimulated. Moreover, the effect was significantly enhanced when both populations were activated by PAF at the same time.

The ability of compound **5** to inhibit PMN adhesion may be regarded as a direct effect, particularly on HUVEC. This is supported by the finding that PAF-mediated inhibition of PMN adhesion to HUVEC was significantly stronger than that to FCS-coated plastic wells. Moreover, with regard to PMN adhesion to HUVEC, inhibition of PAF-induced adhesion was significantly stronger than that of FMLP-induced inhibition, since this compound exerts its effect by activating only the PMN adhesive mechanism. The finding that this inhibition was also stronger than was achieved using endothelial stimuli (PMA, TNF-α or IL-1β) suggests that compound **5** may act synergistically when both cell types are stimulated.

The different effects on HUVEC activated with different stimuli induced by the compounds tested were not surprising, as the mechanisms used by PAF, PMA, IL-1β and TNF-α to potentiate PMN adhesion to HUVEC are, to some extent, different to that used by FMLP (Nick et al 1997). Resting ECs contain small amounts of PAF, which is not released. PAF synthesis and release by ECs is induced by PMA, and by PAF itself, within 2–5 min (Bussolino et al 1995). PMA activity may thus be mediated by endogenous PAF, which is both an autocrine and a paracrine modulator of EC adhesion. By acting on PMNs, PAF up-regulates integrin adhesion and cell polarization; by contrast, in HUVEC, PAF induces expression of P-selectin, rapid loss of sulfated proteoglycan, and change of cell shape through rearrangement of cytoskeletal structures, with a rapid decrease in F-actin content and redistribution of vinculin (Tonnesen et al 1984; Zimmerman et al 1985). IL-1β and TNF-α display their action especially on ECs, while, as has been said, the chemotactic peptide FMLP displays a selective effect on PMNs (Tonnesen et al 1984; Zimmerman et al 1985).

Leucocyte extravasion is due to the cooperative activity of several molecules acting on both PMNs and HUVEC. The short time required by the tested compounds to exert their inhibition of adhesion might indicate the existence of a steric block of adhesion molecules activated by proadhesive stimuli on PMNs or on HUVEC. It is noteworthy that synthetic compounds directed against CAMs might be therapeutically effective, partly because they block intracellular signaling events that are crucial for numerous immune cell activities, such as motile responses, exocytosis, cytokine production and the respiratory burst (Berton & Lowell 1999).

Summary and conclusion

In summary, our results indicate that all the 1,3,5-triazine derivatives tested inhibit the $O_2^{-\cdot}$ production evoked by FMLP in PMNs. Compounds **5** and **6** appear to be the most interesting of the compounds tested, combining an anti-adhesive effect in an in-vitro model of PMN adhesion to HUVEC and inhibition of $O_2^{-\cdot}$ production. It is interesting that in both compounds **5** and **6** the effect of the C-C-bonded aromatic substituent is to enhance the electronic charge density of the heteroaromatic ring through a mesomeric electron-donating effect, and that this seems to be necessary for anti-adhesive activity. Similar activity was not observed for 2-alkyl-4,6-dimethoxy-1,3,5-triazine derivatives in which the electron-donating effect of the alkyl residue is inductive and weaker than in compounds **5** and **6**, nor in compound **7**, whose 2-substituent exerts an electron-withdrawing mesomeric effect on the heteroaromatic ring. Although specific investigation is required to clarify a possible mechanism of interaction between active compounds and suitable receptors, the collected data appears to suggest that the presence of aromatic electron-donating substituents could be the basis of the structure–activity relationship. Compounds **5** and **6** exert their anti-adhesive effects very quickly, inhibiting PMNs adhesion to HUVEC within 10 min. Moreover, a marked increase in

the anti-adhesive effect occurred when both populations were activated simultaneously. The inhibitory effect detected in the presence of IL-1β and TNF-α is also particularly interesting, since these experimental conditions may be regarded as mimetic of pathophysiological mechanisms of the inflammatory diseases. These features, together with the absence of toxicity, may be relevant for the design and development of innovative anti-inflammatory molecules among the 2-aryl-4,6-dimethoxy-1,3,5-triazines.

References

Altieri, D. C., Agbanyo, F. R., Plescia, J., Ginsberg, M. H., Edgington, T. S., Plow, E. F. J. (1990) A unique recognition site mediates the interaction of fibrinogen with the leukocyte integrin Mac-1(CD11b/CD18). *J. Biol. Chem*. **265**: 12119–12122

Avanzi, G. C., Gallicchio, M., Bottarel, F., Gammaitoni, L., Cavalloni, G., Buonfiglio, D., Bragardo, M., Bellomo, G., Albano, E., Fantozzi, R., Garbarino, G., Varnum, B., Aglietta, M., Saglio, G., Dianzani, U., Dianzani, C. (1998) GAS6 inhibits granulocyte adhesion to endothelial cells. *Blood* **91**: 2334–2340

Berton, G., Lowell, C. A. (1999) Integrin signaling in neutrophils and macrophages. *Cell Signal*. **11**: 621–635

Bussolino, F., Camussi, G. (1995) Platelet-activating factor produced by endothelial cells. A molecule with autocrine and paracrine properties. *Eur. J. Biochem*. **229**: 327–337

Choi, J. S., Choi, Y. J., Park, S. H., Kang, J. S., Kang, Y. H. (2004) Flavones mitigate tumor necrosis factor-alpha-induced adhesion molecule upregulation in cultured human endothelial cells: role of nuclear factor-kappa B. *J. Nutr*. **134**: 1013–1019

Cines, D. B., Pollak, E. S., Buck, C. A., Loscalzo, J., Zimmerman, G. A., McEver, R. P., Pober, J. S., Wick, T. M., Konkle, B. A., Schwartz, B. S., Barnathan, E. S., McCrae, K. R., Hug, B. A., Schmidt, A. M., Stern, D. M. (1998) Endothelial cells in physiology and in the pathophysiology of vascular disorders. *Blood* **91**: 3527–3561

Dalton, T. P., Shertzer, H. G., Puga, A. (1999) Regulation of gene expression by reactive oxygen. *Annu. Rev. Pharmacol. Toxicol*. **39**: 67–101

D'Atri, G., Gomarasca, P., Resnati, G., Tronconi, G., Scolastico, C., Sirtori, C. R. (1984) Novel pyrimidine and 1,3,5-triazine hypolipemic agents. *J. Med. Chem*. **27**: 1621–1629

Dhainaut, A., Regnier, G., Atassi, G., Pierre, A., Leonce, S., Kraus-Berthier, L., Prost, J. F. (1992) New triazine derivatives as potent modulators of multidrug resistance. *J. Med. Chem*. **35**: 2481–2496

Dianzani, C., Brunelleschi, S. M., Viano, I., Fantozzi, R. (1994) Adenosine modulation of primed human neutrophils. *Eur. J. Pharmacol*. **263**: 223–226

Dianzani, C., Parrini, M., Ferrara, C., Fantozzi, R. (1996) Effect of 4-hydroxynonenal on superoxide anion production from primed human neutrophils. *Cell Biochem. Funct*. **14**: 193–200

Dianzani, C., Collino, M., Lombardi, G., Garbarino, G., Fantozzi, R. (2003) Substance P increases neutrophil adhesion to human umbilical vein endothelial cells. *Br. J. Pharmacol*. **139**: 1103–1110

Jabs, T. (1999) Reactive oxygen intermediates as mediators of programmed cell death in plants and animals. *Biochem. Pharmacol*. **57**: 231–245

Jang Y., Lincoff, A. M., Plow, E. F., Topol, E. J. (1994) Cell adhesion molecules in coronary artery disease. *J. Am. Coll. Cardiol*. **24**: 1591–1561

Klenke, B., Stewart, M., Barrett, M. P., Brun, R., Gilbert, I. H. (2001) Synthesis and biological evaluation of *s*-triazine substituted polyamines as potential new anti-trypanosomal drugs. *J. Med. Chem*. **4**: 3440–3452

Kukla, M. J., Ludovici, D. W., Janssen, P. A. J., Heeres, J., Moereels, H. E. L. (1998) Substituted diamino-1,3,5-triazine derivatives. *European Patent Application* EP 0 834 507 A1 *Chem. Abstr*. **128**: 257449f

Li, H., Cybulski, M. I., Gimbrone, M. A., Libby, P. (1993) An atherogenic diet rapidly induce VCAM-1, a cytokine-regulatable mononuclear leukocyte adhesion molecule, in rabbit aortic endothelium. *Atherioscler. Thromb*. **13**: 197–204

Lucchesi, B. R. (1990) Modulation of leukocyte-mediated myocardial reperfusion injury. *Annu. Rev. Physiol*. **52**: 561–576

Menicagli, R., Samaritani, S., Signore, G., Vaglini, F., Dalla Via, L. (2004) In vitro cytotoxic activities of 2-alkyl-4,6-diheteroalkyl-1,3,5-triazines: new molecules in anticancer research. *J. Med. Chem*. **47**: 4649–4652

Muller, W. A. (2002) Leukocyte-endothelial cell interactions in the inflammatory response. *Lab Invest*. **82**: 521–533

Nick, J. A., Avdi, N. J., Young, S. K., Knall, C., Gerwins, P., Johnson, G. L., Worthen, G. S. (1997) Common and distinct intracellular signaling pathways in human neutrophils utilized by platelet activating factor and FMLP. *J. Clin. Invest*. **99**: 975–986

Porter, J. R., Archibald, S. C., Brown, J. A., Childs, K., Critchley, D., Head, J. C., Hutchinson, B., Parton, T. A. H., Robinson, M. K., Shock, A., Warrellow, J., Zomaya, A. (2002) Discovery and evaluation of N-(triazin-1,3,5-yl) phenylalanine derivatives as VLA-4 integrin antagonists. *Bioorg. Med. Chem. Lett*. **12**: 1591–1594

Samaritani, S., Signore, G., Malanga, C., Menicagli R. (2005) Organometallic alkylation of 2-chloro-4,6-dimethoxy-1,3,5-triazine: a study. *Tetrahedron* **61**: 4475–4483

Springer, T. A. (1994) Traffic signals for lymphocyte recirculation and leucocyte emigration: the multistep paradigm. *Cell* **76**: 301–304

Tonnesen, M. G., Smedly, L. A., Henson, P. M. (1984) Neutrophil-endothelial cell interactions. Modulation of neutrophils adhesiveness induced by complement fragments C5a and C5a des arg and formyl-methionyl-leucyl-phenylalanine in vitro. *J. Clin. Invest*. **74**: 1581–1592

Ulbrich, H., Eriksson, E. E., Lindbom, L. (2003) Leucocyte and endothelial cell adhesion molecules as targets for therapeutic interventions in inflammatory disease. *Trends Pharmacol. Sci*. **24**: 640–647

Wang, Q., Liu, G., Shao, R., Huang, R. (2003) Synthesis and antivirus activity of 1,3,5-triazine derivatives. *Heteroatom. Chem*. **14**: 542–545

Yang, Y. Y., Hu, C. J., Chang, S. M., Tai, T. Y., Leu, S. J. (2004) Aspirin inhibits monocyte chemoattractant protein-1 and interleukin-8 expression in TNF-alpha stimulated human umbilical vein endothelial cells. *Atherosclerosis* **174**: 207–213

Zimmerman, G. A., McIntire, T. M., Prescott, S. M. (1985) Thrombin stimulates the adherence of neutrophils to human endothelial cells in vitro. *J. Clin. Invest*. **76**: 2235–2246

Zimmerman, B. J., Anderson, D. C., Granger, D. N. (1992) Neuropeptides promote neutrophil adherence to endothelial cell monolayers. *Am. J. Physiol*. **263**: G678–G682