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Title Page

Discovery of BRL 50481, a Selective Inhibitor of Phosphodiesterase 7: *In vitro* Studies in Human Monocytes, Lung Macrophages and $CD8^+$ T-Lymphocytes

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Running title page

Running title: BRL 50481, PDE7 and human pro-inflammatory cells

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Abstract

The biochemical and pharmacological characteristics in human pro-inflammatory cells of BRL 50481, a novel and selective inhibitor of phosphodiesterase (PDE) 7, is described. BRL 50481 inhibited the activity of hrPDE7A1 expressed in baculovirus-infected Sf9 cells in a competitive manner (*K*i = 180nM) and was 416- and 1884-times less potent against PDE3 and 38- and 238-times less potent against PDE4 at a substrate concentration of 1μM and 50nM cAMP respectively. Western blotting identified HSPDE7A1 but not HSPDE7A2 in three human cell types that are implicated in the pathogenesis of chronic obstructive lung disease, namely $CD8^+$-T-lymphocytes, monocytes and lung macrophages. BRL 50481 had no effect on the proliferation of $CD8^+$ T-lymphocytes, and only marginally (~2-11%) reduced the generation of TNFα from blood monocytes and lung macrophages. However, in the presence of BRL 50481 the inhibitory effect of rolipram was enhanced on all three cell types. The expression of HSPDE7A1 was increased in a time-dependent manner in monocytes that were "aged" in culture medium. Under this condition BRL 50481 now inhibited TNFα generation in a concentration-dependent manner. In "aged" monocytes, rolipram, Org 9935 (a PDE3 inhibitor) and PGE_2 inhibited TNFα generation in a concentration-dependent manner and interacted additively with BRL 50481. These data demonstrate that BRL 50481 is the first fully documented PDE7 inhibitor that has acceptable selectivity for *in vitro* studies. Furthermore, although BRL 50481 had only a modest inhibitory effect *per se* on the pro-inflammatory cells studied, it acted at least additively with other cAMP-elevating drugs especially when HSPDE7A1 was up-regulated.

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Introduction

Phosphodiesterase (PDE) 4 is the most extensively studied PDE (Conti and Jin, 1999, Houslay et al., 1998, Houslay and Adams, 2003). Enzymes within this family are found in most pro-inflammatory and immune cells where they are important regulators of cAMP metabolism (Giembycz, 1992, Torphy, 1998). Moreover, PDE4 inhibitors abrogate inflammation in animal models of respiratory diseases, which has led to the view that PDE4 may represent a target amenable to therapeutic intervention with small molecule inhibitors (Torphy, 1998). While the use of PDE4 inhibitors for the treatment of airways inflammatory diseases is based on a conceptually robust hypothesis, the clinical efficacy of the current generation of compounds is compromised by dose-limiting side effects (Giembycz, 2000) of which nausea and vomiting are the most common and worrisome (Giembycz, 2001, Giembycz, 2002, Torphy et al., 1999). These adverse effects represent an extension of the pharmacology of PDE4 inhibitors (Duplantier et al., 1996) and improving their therapeutic ratio has proved a major challenge that is still on-going.

Several strategies have been considered to dissociate the beneficial from detrimental effects of PDE4 inhibitors (Giembycz, 2000, Torphy, 1998) with some degree of success (Giembycz, 2001, Torphy et al., 1999). However, compounds with an optimal pharmacophore still have not been described. An alternative approach is to target other cAMP PDE families that are expressed in pro-inflammatory and immune cells in the hope that therapeutic activity can be retained with a reduced side-effect profile. One such candidate is PDE7, which was first isolated at the gene level in 1993 from a human glioblastoma cDNA library and expressed in a cAMP-deficient strain of the yeast *Saccharomyces cerevisiae* (Michaeli et al., 1993). *PDE7A* encodes a cAMP-specific PDE that is insensitive to cGMP and inhibitors of PDE3 and PDE4, and has an amino acid sequence distinct from other cAMP PDEs (Michaeli et al., 1993). Two PDE7 genes (*PDE7A* and *PDE7B*) have been identified in humans (Gardner et al., 2000, Hetman et al., 2000, Michaeli et al., 1993, Sasaki et al., 2000). Transcription of *PDE7A* can give rise to three isoenzymes (PDE7A1, PDE7A2, PDE7A3) as a consequence of alternative mRNA splicing (Glavas et al., 2001, Han et al., 1997). In contrast, PDE7B exists as a single

isoenzyme in humans with approximately 70% sequence similarity to, and distinct kinetic properties from, PDE7A (Gardner et al., 2000, Hetman et al., 2000, Sasaki et al., 2000)**.**

We have reported recently that PDE7A1 mRNA and protein are distributed ubiquitously across human pro-inflammatory and immune cells (Smith et al., 2003). Conversely, PDE7A2, which is generated by 5'-splicing and differs from PDE7A1 at its N-terminus (Bloom and Beavo, 1996, Han et al., 1997), was never detected at the protein level despite unequivocal identification of its mRNA (Smith et al., 2003). The distribution of PDE7A3 is largely unknown but it has been found in human T-lymphocytes (Glavas et al., 2001) and may also be present in many PDE7A1-expressing cells as both transcripts are probably regulated by the same promoter (Torras-Llort and Azorin, 2003). In contrast, PDE7B is abundant in the brain, liver, heart, thyroid glands and skeletal muscles, but is not found in leukocytes (Gardner et al., 2000). Taken together, the distribution of PDE7A1 across human pro-inflammatory and immune cells mirrors the expression pattern of PDE4 and raises the possibility that it could be exploited to therapeutic advantage to suppress chronic inflammation that characterises several airway diseases including asthma and COPD. This hypothesis is supported by the finding that anti-CD3/anti-CD28-driven IL-2 production by, and proliferation of, human naïve peripheral blood-derived T-lymphocytes are associated with induction of PDE7A1, and that delivery to these cells of antisense oligonucleotides directed against PDE7A prevented these responses (Glavas et al., 2001, Li et al., 1999). Despite these data, the functional role of PDE7A is still largely unexplored. Here we report the discovery of a sulphonamide PDE7 inhibitor, BRL 50481 (Figure 1), and describe its pharmacological activity on pro-inflammatory responses in human blood monocytes, lung macrophages and $CD8^+$ T-lymphocytes that are believed to contribute to the inflammation that is a characteristic feature of COPD.

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Materials and Methods

Patients providing blood samples gave written informed consent. The Ethics Committee of the Royal Brompton and Harefield National Health Service Trust and National Heart and Lung Institute approved this study.

Purification of leukocytes: Blood was collected from normal healthy individuals by antecubital venepuncture into acid citrate dextrose (disodium citrate 160 mM, glucose 11 mM - pH 7.4) and leukocytes were purified as described below.

T-Lymphocytes **-** $CD8^+$ T-Lymphocytes were purified from the peripheral blood mononuclear cell (PBMC) fraction, which was obtained from density gradient centrifugation of anti-coagulated blood on Ficoll-Hypaque (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). Blood was diluted 1:1 with Hanks'-balanced salt solution (HBSS), layered onto Ficoll-Hypaque (1.077 g/ml) and centrifuged (400 x *g*, 20 min) at room temperature. PBMCs were harvested from the plasma/Ficoll-Hypaque interface, washed twice in HBSS and further purified by negative immunoselection using the MACS system (Miltenyi Biotech, Bisley, Surrey, UK) according to the manufacturer's instructions to obtain highly purified leukocytes. The cocktail used to isolate the $CD8^+$ T-lymphocytes included antibodies against CD4, CD11b, CD16, CD19, CD36 and CD56.

Monocytes **-** Monocytes were isolated from peripheral blood, centrifugation on discontinous Percoll gradients and purified either by adherence to tissue culture plastic (Seldon et al., 1995) or by negative immunoselection using the Miltenyi MACS system (Bisley, UK). Cells were then cultured RPMI-1640 medium for the times indicated in the text for TNFα and western blotting experiments.

Lung macrophages **-** Macroscopically normal lung tissue, obtained from patients undergoing surgical resection for carcinoma, was lavaged with RPMI-1640 medium containing 5 mM EDTA, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin. The cells were washed, resuspended in 2 ml of PBS and layered on top of a discontinuous Percoll density gradient [65%:25% (v/v)]. After centrifugation (20 min; 20^{o}C; 500 x *g*), the macrophage-enriched fraction was collected at the 65% (v/v) and 25% (v/v) Percoll interface and the cells were washed twice in PBS and

resuspended in RPMI-1640 medium supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin. After 2 h of incubation in 24 well cell culture plates, the non-adherent cells were removed and fresh medium added. The adherent purified macrophages were incubated overnight and the medium changed the next day prior to the beginning of the experiment.

Assessment of $CD8^+$ T-Lymphocyte Proliferation*:* Isolated $CD8^+$ T-lymphocytes were re-suspended at 5 x 10^5 cells/ml in supplemented RPMI-1640. Unless stated otherwise, 100 μl of the T cell suspension were added to each well of a 96-well plate containing IL-15 as indicated. Cells were incubated in a final volume of 200 μl at 37°C under an atmosphere of 5% CO_2 in air for 60 h. At this time 1 μCi of [^{3}H]-thymidine in 20 μl of RPMI-1640 was added to each well for a further 18 h. Typically, inhibitors were diluted in supplemented RPMI-1640 and added to the cells at the start of the incubation 30 min prior the addition of mitogen and were present throughout the experiment. At 72 h cells were harvested (Packard Filtermate 196 harvester) onto Packard GF/C 96 well filter plates pre-wetted with distilled water, washed with excess water, rinsed with 70% (v/v) ethanol and allowed to air dry. Packard Microscint-20 (50 μl) was then added to each well, the plates shaken for approximately 5 min and radioactivity incorporated into cellular DNA was counted using a Packard TopCount scintillation counter. Proliferation determined using this method was confirmed by manual counting.

Measurement of TNFα: Monocytes and macrophages were pre-treated for 30 min with PDE inhibitor(s) or 5 min with PGE_2 before being exposed to LPS (3 ng/ml) for 24 h and were present throughout the experiment. The medium was removed and assayed for TNFα by ELISA (human matched pair antibodies, R & D Systems Europe, Abingdon, UK) according to the manufacturer's instructions. The detection limit of this assay is 16 pg/ml.

Measurement of cAMP: MOLT-4 cells (ATCC) in 96-well plates were treated for 30 min with PDE inhibitor as indicated in the text. The cAMP content was then determined by an immunospecific ELISA. Results are expressed as a percentage of the response effected by 100 μM IBMX.

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Measurement of PDE Activity: PDE assays were conducted at room temperature and initiated by the addition of partially purified enzyme to a reaction buffer (100 µl total volume) containing (final concentration): 50 mM Tris-HCl - pH 7.5, 8.3 mM $MgCl_2$ and 1 µM or 50 nM of either cAMP or cGMP (supplemented with 50,000 dpm/pmole of [^{3}H]cAMP or [^{3}H]cGMP respectively) with or without added inhibitors. The assay was allowed to proceed for 1 h after which the reaction was terminated by the addition of 100 µl of 0.1M TES - pH 8.0, containing 25 mM EDTA and 100 µM [^{14}C] 5'-AMP/5'-GMP (s.a. ~ 300 dpm nmol/100 µl TES "stop" solution) to estimate recovery. The sample was applied to a column containing dry (unwashed) neutral alumina and unhydrolyzed substrate was eluted with 2 ml plus 10 ml of 0.1 M TES, pH 8.0 and discarded. The 5'-nucleotide products were eluted with 2 ml 2 M NaOH into 15 ml of Gold Ultima XR liquid scintillation cocktail and the radioactivity was counted by liquid scintillation spectrometry. The protein concentration in the cytosolic and particulate fractions was adjusted such that less than 20% of the substrate was hydrolysed and cyclic nucleotide hydrolysis was linear for at least 60 min.

Determination of kinetics constants: K_m and V_{max} were determined by varying the amount of unlabelled cAMP in the reaction cocktail in the presence of a fixed concentration of cyclic nucleotide tracer. Appropriate corrections were made for the changes in specific activity of the substrate. K_I values were derived by the method of Dixon using substrate and inhibitor concentrations that spanned the K_m and estimated K_I.

Western immunoblot analysis: Cells (3 x 10^6) were lysed in ice-cold buffer (10 mM Tris HCl - pH 7.4, 150 mM NaCl, 1mM EDTA, 1% (v/v) Nonidet P-40, 0.25% (w/v) sodium deoxycholate, 0.1% (w/v) SDS and 0.25% (v/v) Triton X-100) supplemented with PMSF (0.1 mg/ml), leupeptin (10 µg/ml), aprotinin (25 µg/ml), pepstatin (10 µg/ml), sodium orthovanadate (10 µg/ml), sodium fluoride (100 µg/ml) and sodium pyrophosphate (200 µg/ml) and centrifuged (12000 x *g*, 10 min) to remove insoluble material. The lysates were diluted (5:1) in sample buffer (62.5 mM Tris, 10% (v/v) glycerol, 1% (w/v) SDS, 1% (v/v) β-mercaptoethanol and 0.01% (w/v) bromophenol blue, pH 6.8) and boiled for 5 min. Denatured proteins were subsequently separated by electrophoresis upon 4-12% (w/v)

gradient SDS polyacrylamide vertical gels and transferred to Hybond ECL membranes (Amersham) in 50 mM Tris base - pH 8.3, 192 mM glycine, 20% (v/v) methanol. Non-specific binding sites were blocked by immersing the membranes in nonfat milk (5% w/v in TBS/Tween 20) for 1 h at room temperature. PDE7A expression was detected using a rabbit anti-PDE7A antibody, which is specific for a 15 amino acid sequence at the C-terminal end of HSPDE7A1 and HSPDE7A2. Primary labelling was performed at room temperature using 1 μg/ml of the rabbit anti-PDE7A in nonfat milk (5% w/v in TBS/Tween 20) for 1 h at room temperature. Following washing in TBS/Tween 20, the membranes were incubated for 60 min with a peroxidase-conjugated goat anti-rabbit antibody (Dako), washed again and the antibody-labelled proteins were visualised by enhanced chemiluminescence (Amersham).

Construction of a NFκB plasmid: An NFκB-dependent reporter, 6NFκBtk.luc, was constructed containing three tandem repeats of the sequence 5'-AGC TTA CAA GGG ACT TTC CGC TGG GGA CTT TCC AGG GA-3', which harbors two copies of the NFκB binding site (underlined) upstream of a minimal thymidine kinase promoter (-105 to +51) driving a luciferase gene. Neomycin resistance was conferred by ligating a *Hin*cII (blunted)/*Pvu*I fragment from pMC1neoPoly(A) (Stratagene, Cambridge, UK) into the *Pvu*I site of 6NFκBtk.luc downstream of the luciferase gene producing 6NFκBtk.luc.neo.

Stable transfection and luciferase assay: Subconfluent (60%) A549 cells (ATCC) in T-75 flasks were washed and incubated in medium containing 8 μg of plasmid and Tfx50 (Promega, UK) for 2 h. Cells were washed again, grown in fresh medium for a further 16 h and then seeded in T-175 flasks containing 500 μg/ml G-418 (Life Technologies, UK). In the continued presence of G-418, foci (~1000) of stable transfected cells developed after approximately 14 days of culture. To create a heterogeneous cell population with regard to integration site, multiple clones were harvested and used in experiments over eight additional passages while being maintained in medium containing 500 μg/ml G-418. After being incubated overnight in serum-free medium in the absence of G-418 selection, cells in 23 well plates were stimulated with IL-1β as indicated in the text and figure legends and the effect of PDE inhibitors on NFκB-dependent transcription was determined.

Luciferase activity was measured using a commercially available Luciferase Assay System according to the manufacturer's instructions (Promega, UK).

Cell viability: At the end of each experiment, cell viability was determined colorimetrically by measuring the reduction of the tetrazolium salt, MTT, to formazan by mitochrondial dehydrogenases.

Drugs and analytical reagents*:* PGE_2, rolipram and human recombinant interleukin (IL)-15 were obtained from Sigma-Aldrich (Poole, Dorset, UK), Calbiochem (Merck Biosciences, Nottingham, UK) and Peprotech (Rocky Hill, New Jersey, USA) respectively. [^{3}H]Thymidine, [^{3}H]cAMP, [^{3}H]cGMP, [^{14}C]5'-AMP and [^{14}C]5'-GMP were from Amersham-Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). Human recombinant PDE7A1, hrPDE4A4, siguazodan and BRL 50481 (3-(*N*,*N*-dimethylsulphonamido)-4-methyl-nitrobenzene) were made "in-house" at GlaxoSmithKline. PDE1B, PDE1C, PDE2, PDE3 and PDE5 were purified from canine trachea as described previously (Torphy and Cieslinski, 1990). Org 9935 (4,5-dihydro-6-(5,6-dimethoxybenzo[b]thien-2-yl-5-methyl-3(2H)-pyridazinone) was donated by Organon Laboratories (Newhouse, Lanarkshire, UK). The PDE7A antibody was provided by Celltech Chiroscience (Cambridge, UK). All other reagents were of the highest commercial grade available.

Data and statistical analyses: Data points, and values in the text and figure legends, represent the mean ± s.e. mean of '*n*' independent determinations using cells from different donors. Concentration-response curves were analysed by least-squares, non-linear iterative regression with the 'PRISM' curve fitting program (GraphPad software, San Diego, USA) and EC_X and IC_X values were subsequently interpolated from curves of best-fit. Where appropriate log-transformed data were analysed statistically using Student's paired *t*-test or by one-way ANOVA/Newman-Keuls multiple comparison test. The null hypothesis was rejected when $P < 0.05$.

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Results

Kinetic characteristics of hrPDE7A1 and sensitivity to BRL 50481: The hydrolysis of cAMP by hrPDE7A1 expressed in baculovirus-infected *Spodoptera fugiperda* (Sf9) cells followed simple Michaelis-Menten kinetics from which a K_m of 9.4 ± 0.7 nM and a V_{max} of 74.5 ± 1.8 μmol/min/mg protein were derived (Figure 2). At substrate concentrations of 50 nM and 1 μM, BRL 50481 inhibited the hydrolysis of cAMP by hrPDE7A1 with IC_{50} values of 0.26 μM and 2.4 μM respectively, whereas siguazodan (PDE3 inhibitor) and rolipram (PDE4 inhibitor) were > 400-fold less active (Table 1). BRL 50481 was a selective inhibitor of hrPDE7A1 being 416- and 1884-times less potent against PDE3 and 38- and 238-times less potent against hrPDE4A4 at 1 μM and 50 nM cAMP respectively (Table 1). Similarly, BRL 50481 failed to significantly inhibit PDE1B, PDE1C, PDE2 and PDE5 at concentrations below 100 μM (Table 1). IBMX also inhibited hrPDE7A1 but was non-selective yielding similar IC_{50} values for the inhibition of PDE3 and PDE4 (Table 1). Kinetic analysis demonstrated that BRL 50481 was a competitive inhibitor (with respect to substrate) of hrPDE7A1 (Figure 3a) with a K_I, derived from secondary (Dixon) plots, of 180 ± 10 nM (Figure 3b).

Identification of PDE7A1: Western blotting was used to establish PDE7A isoform expression in human monocytes, tissue macrophages and $CD8^+$ T-lymphocytes. For this purpose a rabbit polyclonal antibody raised against the 15 amino acid sequence (FELNSQLLPQENRLS) at the extreme C-terminus of PDE7A, which is common to both PDE7A1 and PDE7A2, was employed. In these experiments the T-cell line, HUT-78, or U-937 monocytic cells were used as positive controls (Bloom and Beavo, 1996, Smith et al., 2003). As shown in figure 4, the anti-PDE7A antibody labelled a peptide in $CD8^+$ T-lymphocytes, monocytes and macrophages that migrated as a 57 kDa band on SDS polyacrylamide gels. On a protein basis, $CD8^+$ T-cells and macrophages expressed similar amounts of PDE7A relative to monocytes where expression was very weak. In each cell type, these immunoreactive proteins were identical in size to PDE7A1 expressed by HUT-78 cells and of a molecular mass consistent with hrPDE7A1 expressed in Sf9 insect cells (Bloom and Beavo, 1996). PDE7A2 (50 kDa protein) was never detected in any of the three cell types studied.

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Effect of BRL 50481 on the cAMP content in the MOLT-4 T-cell line: Rolipram (100 nM to 100 μM) increased in a concentration-dependent manner the cAMP content of MOLT-4 T-cells with a pEC_{50} of 5.31 ± 0.05 (Figure 5). At the maximally effective concentration (30 μM) the increase in cAMP mass amounted to 18.1 ± 1.9% of the response elicited by IBMX (100 μM). BRL 50481 also increased the cAMP content (19.1 ± 6.2% of IBMX response at 300 μM) but was considerably less potent (EC_{50} >> 100 μM). When the effect of BRL 50481 (10 to 300 μM) on cAMP mass was examined in the presence of a submaximal concentration of rolipram (10 μM; ~EC_{80}), synergy was observed such that the mean BRL 50481 concentration-response curve was displaced upwards of a magnitude significantly greater than the sum of the cAMP response effected by rolipram and BRL 50481 alone (Figure 5). Thus, at the highest concentration of BRL 50481 used, the cAMP content amounted to 57.6 ± 3.8% of the IBMX maximum (Figure 5).

Effect of BRL 50481 on human $CD8^+$-T-lymphocyte proliferation: Exposure of $CD8^+$-T-lymphocytes to the T-cell mitogen, IL-15 (Grabstein et al., 1994), increased the amount of [^{3}H]thymidine incorporated in to DNA in a concentration-dependent manner with a pEC_{50} (g/ml) of 7.99 ± 0.02 (Figure 6a). Pre-treatment (30 min) of $CD8^+$-T-lymphocytes with rolipram (10 μM) suppressed proliferation by 39.0 ± 4.8%, which was associated with a small (two-fold) but significant ($P < 0.05$) decrease in the potency of the mitogen (pEC_{50} (g/ml): 7.74 ± 0.3). In contrast, BRL 50481 (30 μM) failed to suppress proliferation by itself (data not shown) but significantly potentiated the effect of rolipram. Thus, [^{3}H]thymidine incorporation was reduced by 67.0 ± 3.6% without a further reduction in the potency of IL-15 (pEC_{50} (g/ml): 7.63 ± 0.02: Figure 6a).

Pre-treatment of $CD8^+$ T-lymphocytes with rolipram (30 nM to 10 μM) prevented IL-15 (100 ng/ml; ~EC_{95})-induced proliferation in a concentration-dependent manner with a pIC_{50} (M) of 5.56 ± 0.11 (Figure 6b). At the highest concentration (10 μM) of rolipram tested the cellular incorporation of [^{3}H]thymidine was reduced by 60.0 ± 4.3% (Figure 5b). BRL 50481 (30 μM) had no effect on IL-15-induced proliferation but augmented the inhibitory effect of rolipram. Thus, the potency of rolipram was increased four-fold (pIC_{50} (M): 6.17 ± 0.06; $P < 0.05$) and the maximum inhibition of proliferation was enhanced to 77.1 ± 2.5% (Figure 6b).

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Effect of BRL 50481 on TNFα release from human monocytes: The amount of TNFα released spontaneously from human monocytes after they had been cultured for 24 h in RPMI-1640 was low or below the detection limit of the ELISA. However, treatment of these cells with LPS (3 ng/ml; ~EC_{90} in freshly isolated cells) resulted in appreciable TNFα production that amounted to 233 ± 32 pg/ml.

Pre-treatment (30 min) of monocytes with rolipram inhibited LPS-induced TNFα release in a concentration-dependent manner with a pEC_{50} (M) of 7.06 ± 0.29 and maximum inhibition of 94.2 ± 2.0% (Figure 7a). Pre-treatment (30 min) of human monocytes with BRL 50481 had, by itself, a negligible (~ 2-10%) inhibitory effect on TNFα output at all concentrations tested. However, BRL 50481 (30 μM) displaced five-fold to the left and in a parallel manner the rolipram concentration-response curve that described the inhibition of TNFα release, which was statistically significant (pEC_{50} (M): 7.76 ± 0.17; $P < 0.05$; Figure 7a).

BRL 50481 also potentiated the inhibitory effect of PGE_2 on LPS-induced TNFα release (Figure 7b). Indeed, the mean PGE_2 concentration-response curve was displaced to the left in an apparently parallel fashion by BRL 50481 (30 μM) increasing the prostanoid's potency approximately four-fold (pEC_{50} values (M): PGE_2 = 8.37 ± 0.14; PGE_2 + BRL 50481 = 9.02 ± 0.16, $P < 0.05$). In contrast, neither Org 9935 *per se* nor Org 9935 in the presence of BRL 50481 (30 μM) modified TNFα release accept at high concentrations where isoenzyme selectivity may be compromised (Shahid et al., 1991) (Figure 7c).

Effect of BRL 50481 on TNFα release from human tissue macrophages: Figure 8a and b shows the inhibitory effect of rolipram and PGE_2 on TNFα release from LPS-stimulated human tissue macrophages. Both drugs suppressed TNFα release in a concentration-dependent manner but were less effective (maximum inhibition < 50%) and potent when compared to their activity on human blood monocytes (*cf*. Figure 7a & b). The PDE3 inhibitor, Org 9935, which was essentially inactive on human monocytes, also incompletely suppressed TNFα release from tissue macrophages at concentrations (≤ 10 μM) of the compound where isoenzyme selectivity is preserved (Figure 8c).

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In contrast, BRL 50481 (30 μM) was a very weak inhibitor of TNFα output (4 to 11% in the experiments shown in Figure 8). However, when BRL 50481 (30 μM) was combined with rolipram, Org 9935 or PGE_2 an additive (or possibly synergistic) effect was seen where the maximum inhibition of TNFα output and potency of rolipram and Org 9935 were significantly increased (Figure 8).

Effect of PDE inhibitors on κB-dependent transcription: The effect of BRL 50481, rolipram and Org 9935 on κB-dependent transcription was assessed in A549 cells stably expressing a reporter plasmid, 6NFκBtk.luc.neo, that features two copies of the NFκB binding site upstream of a minimal thymidine kinase promoter driving a luciferase gene. IL-1β (1 ng/ml) increased luciferase expression 19.0 ± 3.5-fold (n = 4) over baseline. Pre-treatment of cells with rolipram and Org 9935 inhibited the induction of the luciferase gene in a concentration-dependent manner with pIC_{30} (M) values of 5.34 ± 0.13 and 5.97 ± 0.18 respectively (Figure 9). At the highest concentration where selectivity for PDE3 and PDE4 is preserved, Org 9935 and rolipram repressed the luciferase gene by 52.1 ± 7.2% and 51.9 ± 1.9% respectively (Figure 9). In contrast, BRL 50481 had no significant effect by itself on κB-dependent transcription (5.6 ± 1.9% inhibition at 30 μM) and failed to enhance the effect of rolipram (maximum inhibition: 52.9 ± 2.7%; pIC_{30}: 5.33 ± 0.12).

Effect of "ageing" monocytes on the expression of PDE7A1 and sensitivity to BRL 50481: Culture of monocytes in RPMI 1640 for 36 h was associated, after an approximate 6 h lag, with a time-dependent increase in the expression of PDE7A1 when compared to freshly isolated cells (Figure 10). Maximal up-regulation (5.7 ± 0.6-fold) of PDE7A1 was seen after 24 h of culture and this remained stable for the duration of the experiment. Functionally, BRL 50481 suppressed, in a concentration-dependent manner, LPS-induced TNFα release in monocytes in which PDE7A1 was induced (21.7 ± 1.6% inhibition at 30 μM at the 12 h time-point; Figure 11a). Kinetic studies showed that the maximal inhibition of TNFα output effected by BRL 50481 was reached by 12 h and was steady at all further time-points examined (Figure 11b). In contrast, while the ability of rolipram to reduce LPS-induced TNFα release was preserved in "aged" monocytes, the characteristics of the inhibition now resembled those seen in human tissue macrophages (*cf.* Figure 7d & 8a). Thus, rolipram was 11-fold more potent in the aged (12 h) population of cells (pEC_{50} (M): 7.86 ± 0.53) than in macrophages (pEC_{50} (M): 6.82 ±

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0.36), whereas the maximum inhibition achieved was similar (~52% *vs*. 41%) and considerably less than that effected in freshly purified monocytes (> 95%; Figure 7a). As shown in figure 11b, culture of monocytes beyond 12 h was not associated with any further reduction in the maximum inhibitory effect of rolipram and, accordingly, all further experiments were performed at this time.

In the presence of BRL 50481 (30 μM), which in this set of experiments suppressed the elaboration of TNFα by 26.2 ± 5.9%, the rolipram concentration-response curve in "aged" monocytes was displaced downwards by an amount that was purely additive (Figure 7d). Thus, the 86% inhibition of TNFα output produced by BRL 50481(30 μM) and rolipram (10 μM) in combination amounted to the sum of the individual effect of BRL 50481 (26.2%) and rolipram (60.1% at 10 μM). Accordingly, this was reflected as equivalence in the potency of rolipram (pEC_{50} (M): 7.97 ± 0.62).

On "aged" human monocytes the PDE3 inhibitor, Org 9935, evoked a very modest inhibitory effect on the elaboration of TNFα (mean pEC_{25} (M): 5.13), which was in-keeping with its weak of activity on freshly isolated cells (*cf*. Figure 7c & 7f). In contrast, BRL 50481 (30 μM) suppressed TNFα output by 25.5 ± 4.7% and displaced downwards the Org 9935 concentration-response curve in an additive manner (Figure 7f). In this respect the data were reminiscent of those obtained with rolipram (Figure 7d). Thus, the 52% inhibition of TNFα release produced by BRL 50481 (30 μM) and Org 9935 (10 μM) in combination amounted to the sum of the individual effect of BRL 50481 (25.5%) and Org 9935 (27.7% at 10 μM). Accordingly, this was reflected as equivalence in the potency of Org 9935 (mean pIC_{25} (M): 5.26).

Consistent with the rolipram and Org 9935 results described above, BRL 50481 interacted with PGE_2 in, at most, an additive manner (Figure 7e). However, because PGE_2 abolished TNFα output, additivity was seen only at low agonist concentrations as the asymptotes of the PGE_2 and PGE_2/BRL 50481 concentration-response curves converged at 100% inhibition (Figure 7e). Nevertheless, the pEC_{50} (M) of PGE_2 in the presence of the PDE7A inhibitor was 8.71 ± 0.17, which was not significantly different from the potency of PGE_2 alone (pEC_{50} (M): 8.98 ± 0.27; $P > 0.05$).

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Discussion

Phosphodiesterase 4 inhibitors provide a promising new approach for alleviating the chronic inflammation that characterises COPD (Giembycz, 2001, Giembycz, 2002, Torphy et al., 1999). However, despite some evidence of clinical efficacy (Compton et al., 2001, Gamble et al., 2003), this class of compound is compromised by dose-limiting side effects that are proving difficult to overcome (Giembycz, 2000). Theoretically, an alternative way of evoking anti-inflammatory activity in the lung and airways is to target, with small molecule inhibitors, other cAMP PDE families that share a similar pulmonary cellular distribution to PDE4 in the hope that an improved therapeutic ratio will be realised. Of the novel cAMP PDEs thus far identified, PDE7A is a prime candidate as it is expressed in essentially all pro-inflammatory and immune cells (Smith et al., 2003) and may modulate human T-cell function (Li et al., 1999). As an initial step to testing this hypothesis, we describe here the discovery and pharmacology in human $CD8^+$-T-lymphocytes, monocytes and macrophages of BRL 50481, a selective PDE7 inhibitor.

At the protein level, human pro-inflammatory and immune cells express PDE7A1 but not PDE7A2 (Smith et al., 2003). Accordingly, the inhibition by BRL 50481 of hrPDE7A1 (engineered in Sf9 cells) was characterised and its selectivity assessed against hrPDE4A4 and PDEs 1 to 5 purified from canine trachealis. Enzyme kinetic analyses established that BRL 50481 was a competitive inhibitor (with respect to substrate) of hrPDE7A1 with a *K*i in the high nanomolar range. At a substrate concentration of 50 nM, where the IC_{50} of cAMP PDE inhibitors closely approximates to their *K*i (Lee et al., 2002), BRL 50481 was at least 200-fold selective for hrPDE7A1 over all other PDEs examined (Table 1). BRL 50481 was not tested against PDE7A2, PDE7A3 or PDE7B in the present study. However, as the three PDE7A splice variants differ only at their extreme amino and carboxy termini it is likely that they will all be inhibited with similar potency (see Giembycz and Smith, 2004). Similarly, BRL 50481 is a catalytic site inhibitor and comparable activity would be expected against PDE7B in the same way that rolipram does not readily discriminate between PDE4 isoenzymes.

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Few compounds have been reported with inhibitory activity against PDE7. Martinez *et al*., (2000) have described a series of benzothieno and benzothiadiazine dioxides that inhibit hrPDE7A expressed in yeast. However, none of the compounds tested showed statistically significant selectivity for PDE7A over other cAMP PDE isoenzymes. Nevertheless, a 3-dimensional quantitative structure-activity relationship study using comparative molecular field analysis of 19 compounds within these two series has allowed the design, *in silico*, of inhibitors with theoretically enhanced activity towards PDE7A (Castro et al., 2001). More recently, data on ICOS corporation's IC242 was disclosed. This compound has an IC_{50} against PDE7A of 370 nM (at 32 nM cAMP) and has a similar isoenzyme selectivity profile to BRL 50481 (Lee et al., 2002). Several series of compounds from Pfizer and Bristol-Myers Squibb with PDE7 inhibitory activity are also available (Pitts et al., 2004; Vergne et al., 2004; Lorthios et al., 2004). One of these, BMS-586353, is a potent (IC_{50} = 8 nM), bioavailable inhibitor of PDE7 with excellent selectivity relative to other PDE isoenzyme families (3722-, 6277-, 1250-, 1231- and 553-fold less potent against PDE1, PDE3, PDE4, PDE5 and PDE6 respectively (Yang et al., 2003)). Finally, chemists from Celltech have found that 8-bromo-9-substituted derivatives of guanine are selective inhibitors of PDE7A (Barnes et al., 2001). In particular, incorporation of a bromo-substituted tetralin ring at position 9 of the guanine template results in a compound with an IC_{50} for HUT-78 PDE7A of 1.3 μM with weak activity against PDE3 and PDE4 (14% and 10% inhibition at 10 μM respectively). Thus, several selective PDE7 inhibitors have now been described including BRL 50481, which are suitable for *in vitro* pharmacological testing (see below).

Despite the unequivocal identification of PDE7A1 in $CD8^+$ T-cells, BRL 50481 had no effect on IL-15-driven proliferation even though the cAMP content in MOLT-4 T-cells was significantly elevated over the basal level. This was an unexpected finding given that Beavo and colleagues have reported that IL-2 production by, and proliferation of, human blood T-lymphocytes evoked by anti-CD3 and anti-CD28 antibodies is associated with induction of PDE7A1 and prevented by antisense oligonucleotides directed against PDE7A (Glavas et al., 2001, Li et al., 1999). Nevertheless, our data are consistent with results obtained in a more recent investigation where proliferation, and Th1 and Th2 cytokine production evoked by ligation of CD3/CD28 was preserved in T-lymphocytes taken from *PDE7A*

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knockout mice and from wild type animals treated with BMS-586353 (Yang et al., 2003). It is not clear why these results fail to corroborate data reported by Li *et al*., (1999) but based on current evidence we suggest that it is unlikely to be species related or to a redundant mechanism in mice that compensates for the deficiency in *PDE7A.* However, two additional possibilities may account for the discrepancy. In the present study $CD8^+$ T-lymphocytes were isolated from other leukocytes by negative immunoselection using a mixture of antibodies against CD4, CD11b, CD16, CD19, CD36 and CD56. In the experiments described by Li *et al*., (1999), antibodies against CD25 and HLA-DR were also used, which will remove all activated and proliferating T-cells. Thus, it is possible that naïve T-lymphocytes are regulated differently by PDE7A than their activated and proliferating counterparts. Alternatively, the use of naked antisense oligonucleotides, as used by Li *et al.*, (1999), may not have targeted specifically the mRNA of interest or, alternatively, evoked toxic effects that were sequence non-specific (Stein, 2001).

Consistent with other reports, rolipram suppressed the cellular uptake of [^{3}H]thymidine into $CD8^+$-T-lymphocytes providing further support that PDE4 is a major regulator of T-cell growth and division (Averill et al., 1988, Giembycz et al., 1996, Robicsek et al., 1991, Staples et al., 2001). In contrast, BRL 50481 was inert at all concentrations tested. To determine if inhibition of PDE7A could enhance the anti-mitogenic activity of a PDE4 inhibitor, rolipram concentration-response curves were constructed in the absence and presence of a fixed concentration (30 μM) BRL 50481 where selectivity for PDE7 in intact cells is preserved. As shown in figure 6, BRL 50481 significantly enhanced the anti-mitogenic activity of rolipram in a manner that was reminiscent of the behaviour of PDE3 inhibitors in human $CD8^+$ T-lymphocytes (Giembycz et al., 1996). Moreover, in MOLT-4 T-cells BRL 50481 (30 μM) produced only a very modest increment in cAMP mass when compared to rolipram. However, when used in combination BRL 50481 and rolipram acted synergistically increasing cAMP to a level that was significantly greater than that elicited by a maximally effective concentration of rolipram. Thus, PDE7A regulates proliferation and the cAMP content of human $CD8^+$ T-cells provided PDE4 is inhibited concomitantly.

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On human monocytes and lung macrophages BRL 50481 was also inactive or, at most, poorly active (2-11% inhibition) in suppressing LPS-induced TNFα release, but interacted with rolipram, PGE_2 and Org 9935 in at least an additive manner. This profile of activity closely resembles the behaviour of BRL 50481 on human T-lymphocytes and demonstrates that inhibition of PDE7A can negatively regulate the pro-inflammatory activity of cells from the monocytes/macrophage lineage when PDE4 is inhibited.

Many cAMP-elevating agents are known to inhibit the induction of a distinct set of NFκB-regulated genes including *Tnf*. To determine if BRL 50481 also shares this mechanism, a NFκB reporter plasmid, 6NFκBtk.luc.neo, was stably transfected in to A549 cells and the effect of PDE inhibitors on IL-1β-induced κB-dependent transcription assessed. Consistent with previous reports (Jimenez et al., 2001; Ollivier et al., 1996; Takahashi et al., 2002), rolipram and the PDE3 inhibitor, Org 9935, effectively repressed luciferase gene expression. In contrast, BRL 50481 was inactive and also failed to enhance the inhibitory effect of rolipram, which was contrary to the behaviour of these two PDE inhibitors on TNFα output. How cAMP blocks κB-dependent transcription has not been fully elucidated and controversy still prevails. In human monocytes, we have found previously that rolipram has no effect on IL-1β-induced IκB degradation or on NFκB/DNA binding (K.K. Meja, M.A. Giembycz – unpublished observations). These data are consistent with the results of comparable studies with human umbilical vein endothelial cells and Jurkat T-lymphocytes and may be explained by the recent demonstration that cAMP blocks κB-dependent transcription in susceptible cells by modifying, directly or indirectly, the transactivation domain of the p65 subunit of NFκB independently of the PKA recognition site at S^{276} (Ollivier et al., 1996; Takahashi et al., 2002). However, blockade of NFκB may not be the primary mechanism of action through which cAMP-elevating drugs suppress TNFα release from monocytes. Indeed, IL-1β-driven, κB-dependent luciferase expression in A549 cells was relatively insensitive (~20% inhibition) to rolipram at a concentration (3 μM) that reduced IL-1β-induced TNFα output from monocytes by >90%. Furthermore, Org 9935 effectively inhibited the reporter construct at concentrations where TNFα release was unaffected. Although these comparisons

are made across two different cells type, these results may, nevertheless, account for the lack of effect of BRL 50481 on IL-1β-stimulated luciferase expression.

Culture of human monocytes in RPMI-1640 for 36 h resulted in ~six-fold up-regulation of PDE7A1 and conferred functional sensitivity to BRL 50481 such that LPS-induced TNFα release was now significantly inhibited. Moreover, in monocytes where PDE7A1 was up-regulated the ability of rolipram, PGE_2 and Org 9935 to prevent TNFα release was enhanced by BRL 50481 in a purely additive manner. Previous studies have found that ageing monocytes is also associated with a down-regulation of PDE4 activity that is primarily attributable to PDE4D family members (e.g. Gantner et al., 1997; Shepherd et al., 2004) and that this enzyme remodelling accounts for the reduction in the maximum inhibition of TNFα output produced by rolipram (Gantner et al., 1997). Therefore, collectively, these data demonstrate that with respect to the regulation of TNFα release, the increased expression of PDE7A1 compensates, at least in part, for the down-regulation of PDE4. Theoretically, this could be exploited to therapeutic advantage in inflammatory diseases where cells of the monocyte/macrophages lineage play a pathogenic role.

It is important to emphasise that the ability of BRL 50481 to suppress TNFα output at the concentration (i.e. 30 μM) used in the combination experiments was not due to the inhibition of PDE4 as the rolipram and rolipram plus BRL 50481 concentration-response curves did not converge at high concentrations. Taken together, these data imply that PDE7A can regulate the responsiveness of monocytes and possibility other pro-inflammatory and immune cells under circumstances when PDE7A is highly expressed, such as conditions of chronic inflammation. In this respect many cytokines relevant to the pathogenesis of airway inflammatory diseases signal, in part, through a protein kinase C-dependent mechanism (Kontny et al., 2000) and Torras-Llort and Azorin (2003) have reported that the human PDE7A1 promoter is activated by the phorbol ester, PMA.

In conclusion, the results of the present investigation demonstrate that BRL 50481 is a PDE7 inhibitor with nanomolar potency and is sufficiently selective against other PDE isoenzyme families for *in vitro* pharmacological studies. Functionally, BRL 50481 was poorly active in suppressing human T-cell

proliferation and TNFα release from monocytes and macrophages but, nevertheless, acted in at least an additive manner with the PDE4 inhibitor, rolipram. These findings suggest that hybrid PDE4/PDE7 inhibitors may be more efficacious and display a superior therapeutic index than a PDE4 inhibitor alone. This is an important consideration as the two most clinically advanced PDE4 inhibitors in trials of COPD are compromised by dose-limiting side-effects (see Introduction). In this respect it noteworthy that several pharmaceutical companies have filed patents claiming for dual PDE4/PDE7 inhibitors with potential anti-inflammatory activity (Hatzelmann et al., 2004, Pitts et al., 2002). Finally, up-regulation of PDE7A1 in monocytes conferred increased sensitivity to BRL 50481 indicating that inhibitors of this isoenzyme family *per se* could be effective in inflammatory indications where *HSPDE7A* is induced.

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Legends for figures

Figure 1. Structure of BRL 50481 (3-(*N*,*N*-dimethylsulphonamido)-4-methyl-nitrobenzene)

Figure 2 Kinetics of cAMP hydrolysis catalysed by hrPDE7A1. Lineweaver-Burke plots of the same data are shown in the inset and are typical of five independent determinations.

Figure 3 Kinetic analysis of the inhibition by BRL 50481 of hrPDE7A1. Panel (*a*) shows Lineweaver-Burk plots of cAMP hydrolysis catalysed by hrPDE7A1 in the absence and presence of BRL 50481 (50 – 600 nM). In panel (*b*), a Dixon plot is illustrated from which a *K*i of 185 nM was obtained in this experiment. These data are typical of three independent determinations.

Figure 4 Identification of PDE7A1 in human pro-inflammatory cells by western analysis. Highly purified cells were lysed, insoluble proteins were removed and 20 μg of soluble extract denatured and subjected to electrophoresis on 4-12% (w/v) SDS polyacrylamide gels. Proteins were transferred onto nitrocellulose and incubated with a rabbit anti-PDE7A antibody. The nitrocellulose was subsequently incubated with a peroxidase-conjugated goat anti-rabbit Ig antibody and labelled proteins were detected by enhanced chemiluminescence. The gels are representative of five independent experiments. See Methods for further details. Key: **1** Macrophage; **2** Monocyte; **3** HUT-78; **4** $CD8^+$ T-lymphocyte

Figure 5 Effect of BRL 50481 on the cAMP content of MOLT-4 T-cells. Cells were treated for 30 min with BRL 50481 (10 to 300 μM), rolipram (100 nM to 100 μM) and rolipram (10 μM) in the presence of BRL 50481 (10 to 300 μM). The cAMP content was then determined by immunospecific ELISA and expressed as a percentage of the response produced by IBMX (100 μM). Each data point represents the mean ± s.e. mean of four independent determinations.

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Figure 6 Effect of BRL 50481 on IL-15-induced proliferation of human $CD8^+$ T-lymphocytes. Cells in 96-well plates were pre-treated for 30 min PDE inhibitors or vehicle. IL-15 was added and the cells were incubated at 37°C for 60 h under a 5% CO_2 atmosphere and then for a further 18 h in the presence of [^{3}H]thymidine (1 µCi). At 72 h cells were harvested on to GF/C filters, which were washed and counted. In panel (*a*) the effect of rolipram (10 µM) and rolipram and BRL 50481 (30 µM) in combination is shown on IL-15 (1 to 400 ng/ml)-induced [^{3}H]thymidine uptake. Panel (*b*) shows the anti-mitogenic action of rolipram (300 nM to 10 µM) in the absence and presence of BRL 50481 (30 µM) on IL-15 (100 ng/ml)-induced [^{3}H]thymidine uptake. Each data point represents the mean ± s.e. mean of four independent determinations using cells from different donors. Note that BRL 50481 (30 µM) did not suppress IL-15-induced proliferation (data not shown). See Methods for further details.

* $P < 0.05$, significant inhibition of proliferation

** $P < 0.05$, significant inhibition of proliferation over the effect of rolipram alone.

Figure 7 Effect of BRL 50481 on the inhibition by rolipram, PGE_2 and Org 9935 of TNFα release from human monocytes. Freshly isolated cells (panels *a, b* & *c*) or cells aged in culture for 24 h (panel *d, e & f*) were pre-treated (30 min) rolipram (1 nM to 10 µM), PGE_2 (0.1 nM to 10 µM), Org 9935 (1 nM to 10 µM) in the absence and presence of BRL 50481 (30 µM). LPS (3 ng/ml) was then added and the TNFα elaborated in to the culture medium determined 24 h later by ELISA. Each data point is the mean ± s.e mean of five and four independent determinations from different donors for fresh and aged cells respectively. See Methods for further details.

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Figure 8 Effect of BRL 50481 on the inhibition by rolipram, Org 9935 and PGE_2 of TNFα release from human lung macrophages. Freshly isolated cells were pre-treated (30 min) with BRL 50481 (30 μM) by itself, or BRL 50481 in the absence and presence of rolipram (10 nM to 10 μM; panel *a*), PGE_2 (1 nM to 10 μM; panel *b*) or Org 9935 (10 nM to 10 μM; panel *c*). LPS (3 ng/ml) was then added and the amount of TNFα elaborated in to the culture medium was then determined 24 h later by immunospecific ELISA. Each data point is the mean ± s.e mean of six, four and five independent determinations from different donors for rolipram, Org 9935 and PGE_2 respectively. See Methods for further details.

Figure 9 Effect of PDE inhibitors on IL-1β-induced activation of a κB-dependent reporter construct. A549 cells were stably transfected with a reporter plasmid, 6NFκBtk.luc.neo, featuring two copies of the NFκB binding site upstream of a minimal thymidine kinase promoter driving a luciferase gene. Cells were pre-treated (30 min) with Org 9935, rolipram, BRL 50841 or a combination of rolipram and and BRL 50481, exposed to IL-1β (1 ng/ml) and luciferase activity measured 8 h later. Each data point is the mean ± s.e mean four independent determinations. See Methods for further details.

Figure 10 Effect of ageing monocytes on the expression of HSPDE7A1. Cells (3×10^6) were cultured for 1 to 36 h in RPMI-1640, lysed and the soluble extract denatured and subjected to electrophoresis on 4-12% (w/v) SDS polyacrylamide gels. Proteins were transferred onto nitrocellulose and incubated with a rabbit anti-PDE7A antibody. The nitrocellulose was subsequently incubated with a peroxidase-conjugated goat anti-rabbit Ig antibody and labelled proteins were detected by enhanced chemiluminescence and shown as fold-increase over expression at the "0" time-point. Panels *a* and *b* show a representative gel and a bar chart of the mean s.e. ± mean of four determination using cells from different donors. HUT-78 and U-937 cells were used as positive controls. See Methods for further details.

* $P < 0.05$, significant induction of PDE7A1 in aged vs. freshly harvested cells.

Figure 11 Effect of BRL 50481 and rolipram on LPS-induced TNFα generation from human aged monocytes. Cells were aged in culture for up to 48 h, treated (30 min) with either BRL 50481 or rolipram as indicated, exposed to LPS (3 ng/ml) and the TNFα elaborated in to the culture medium determined 24 h later by immunospecific ELISA. Panel *a* show the inhibitory effect of BRL 50481 in monocytes aged for 24 h relative to freshly isolated cells. Panel *b* shows the inhibitory effect of a fixed concentration of rolipram (10 μM) and BRL 50481 (30 μM) on LPS-induced TNFα release in fresh and aged monocytes. Each data point is the mean ± s.e mean five independent determinations using cells from different donors. See Methods for further details.

* $P < 0.05$, significantly greater inhibition of TNFα release effected by BRL 50481 in aged *vs*. freshly isolated monocytes.

** $P < 0.05$, significant change in maximal TNFα output compared to release at the "0" time-point.

Table

Table 1 PDE isoenzyme selectivity of BRL 50481

		Inhibition of Cyclic Nucleotide Hydrolysis (IC_{50} - µM)						
		hrPDE7A1	**PDE1B*	**PDE1C*	**PDE2*	**PDE3*	*hrPDE4A4*	**PDE5*
	cAMP/cGMP Concentration (µM)							
BRL 50481	0.05	0.26	>100	>100	>100	490	62	>100
	1	2.4	>100	>100	>100	>1000	92	>100
IBMX	0.05	5.7	ND	ND	ND	0.87	11	ND
	1	53	ND	ND	ND	5.1	14	ND
Siguazodan	0.05	>1000	ND	ND	ND	0.25	>1000	ND
	1	>1000	ND	ND	ND	1.1	>1000	ND
Rolipram	0.05	280	ND	ND	ND	300	0.07	ND
	1	>1000	ND	ND	ND	>1000	0.17	ND

* Isoenzymes purified from canine trachealis.

ND: not determined

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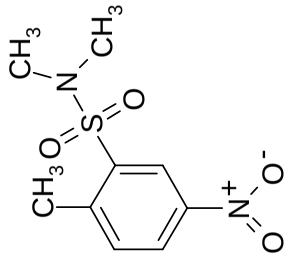
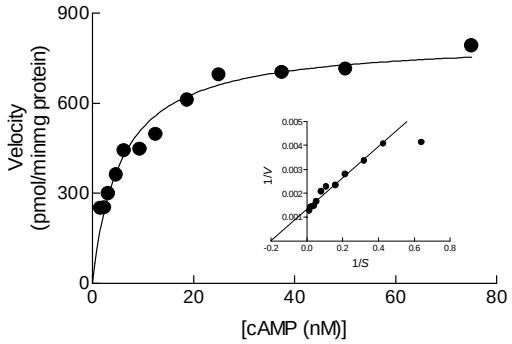


Figure 1

Figure 2



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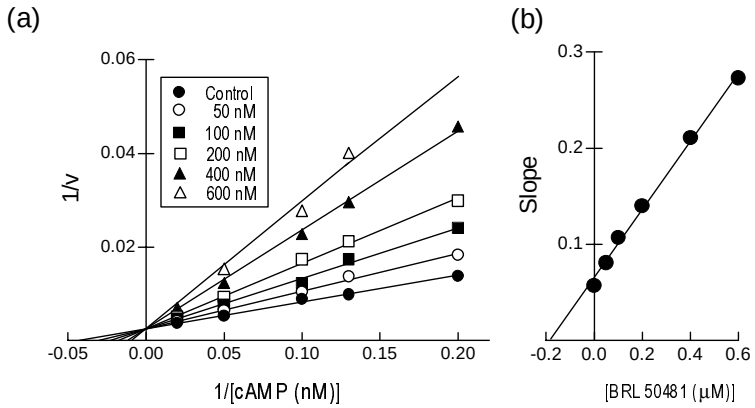

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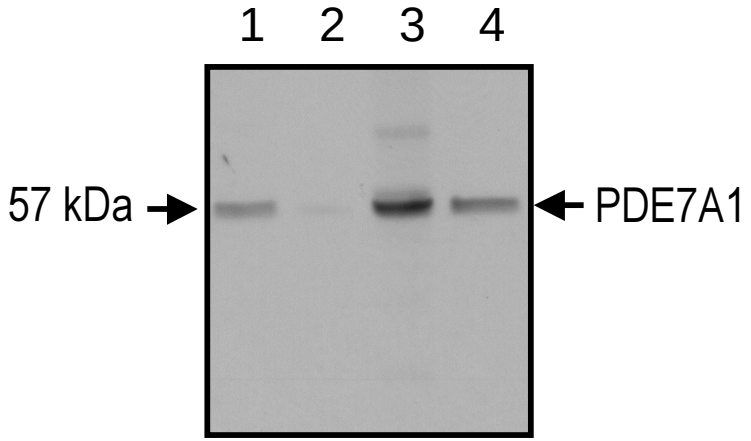

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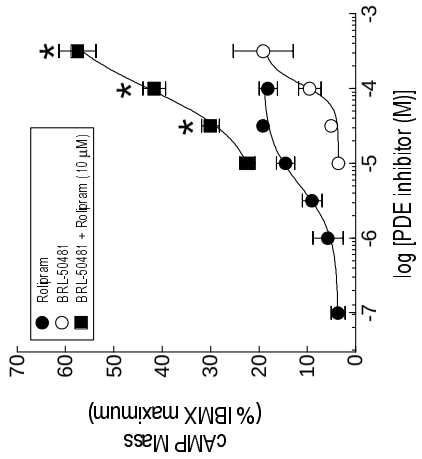
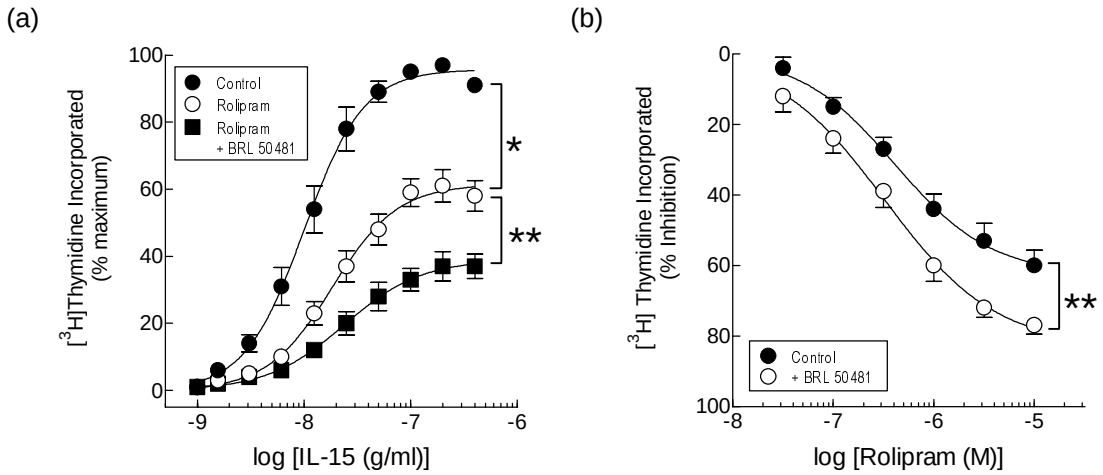


Figure 5

Figure 6



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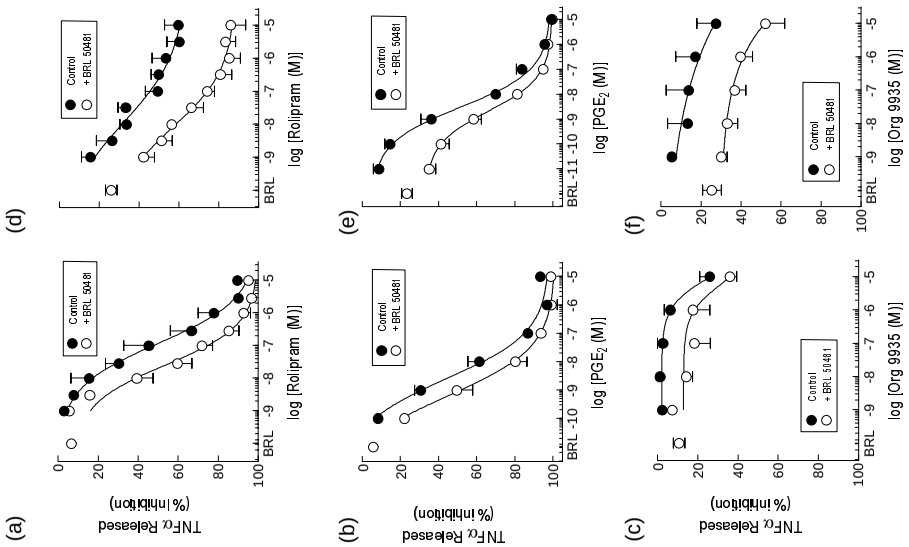
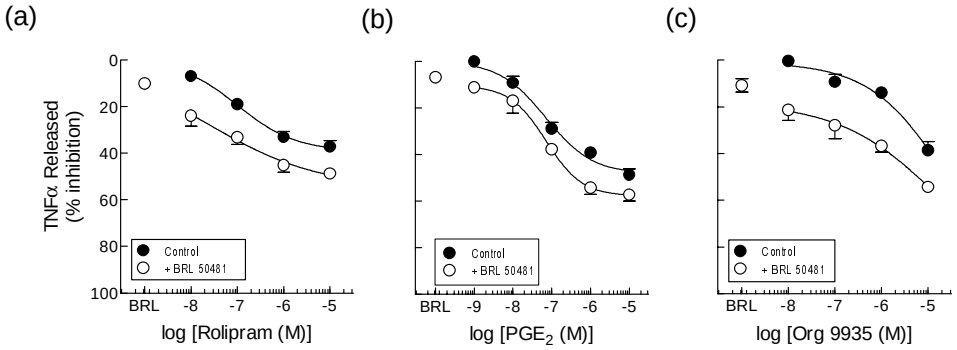


Figure 7

Figure 8



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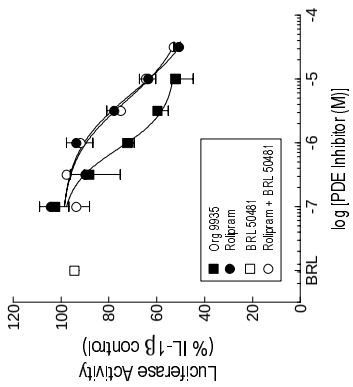

Figure 9

Figure 10

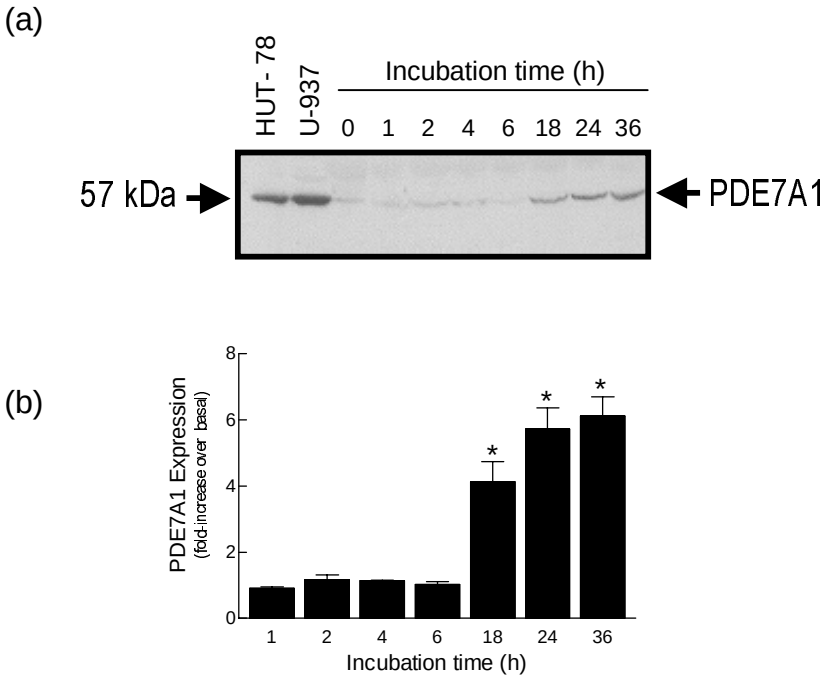

Figure 11