Biochemical Pharmacology, Vol. 59, pp. 1305–1314, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00252-5

Inhibitory Action of 1-(2-Chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide (PK 11195) on Some Mononuclear Phagocyte Functions

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ABSTRACT. Peripheral benzodiazepine receptors (PBRs) are widely distributed throughout the body, but their functions are unknown. They are found on mononuclear phagocytes, and they are up-regulated in a number of neurological and other disease states. We explored the functional consequences of PBR ligand binding to mononuclear-derived cells using the high-affinity ligands 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide (PK 11195) and 4′-chlorodiazepam (7-chloro-5-(4′-chlorophenyl)-1,3-dihydro-1-methyl-2*H*-1,4-benzodiazepin-2-one; Ro 5–4864). The functions were the following: respiratory burst; secretion of glutamate, interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α); toxicity of culture supernatants towards SH-SY5Y human neuroblastoma cells; and expression of the inflammatory surface markers HLA-DR and Fcγ RII (CDw32). PK 11195 inhibited the respiratory burst response, reduced release of glutamate and IL-1β, and suppressed secretion of products cytotoxic to neuronal cells. Selectivity was suggested by the failure of PK 11195 to influence TNF-α secretion or expression of HLA-DR and CDw32. Powerful ligands of PBRs, such as PK 11195, may be useful inhibitors of selective macrophage functions, retarding both local and systemic inflammation. Since PK 11195 readily enters the brain, it may be beneficial in treating central as well as peripheral inflammatory diseases. BIOCHEM PHARMACOL **59**;10:1305–1314, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. cytokines; glutamate; inflammation; macrophages; peripheral benzodiazepine receptors; reactive oxygen intermediates

There are two classes of benzodiazepine receptors in mammalian tissues, central and peripheral. Ligands for CBRs† are in wide clinical use due to their anti-convulsant, muscle relaxant, sedative, and anxiolytic properties (for reviews, see Refs. 1 and 2). They are believed to act by enhancing activity of the inhibitory neurotransmitter GABA through binding to a subtype of GABA receptors. In contrast, ligands of PBRs have no established pharmacological properties. PBRs are widely distributed throughout the body, including the CNS [3]. Several molecules have been identified as possible endogenous ligands of PBRs, but the physiological functions of the endogenous ligands and their receptors are still largely a mystery (for a review, see Ref. 4).

The two most widely studied synthetic ligands of PBRs are the isoquinolinecarboxamide derivative 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide (PK 11195) and the benzodiazepine derivative 4′-chlorodiazepam (7-chloro-5-(4′-chlorophenyl)-1,3-dihydro-1-methyl-2*H*-1,4-benzodiazepin-2-one; Ro 5–4864). Affinities of these substances for PBRs are in the low nanomolar range, with PK 11195 having the highest affinity (e.g. [5, 6]).

PK 11195 binding is up-regulated in various human disease states. This up-regulation has permitted labeled PK 11195 to be used as a positron emission tomography imaging agent to detect CNS pathology. It can demonstrate the lesions in multiple sclerosis, gliomas, Huntington's disease, and Alzheimer's disease [7–12].

PK 11195 binding is also up-regulated in several animal models such as experimental autoimmune encephalitis [12], motor neuron axotomy [13, 14], kainic acid administration, ischemic lesions [11, 15], and sciatic nerve degeneration and regeneration [16]. Microglial cells appear to express the highest density of PBRs among brain cells [12, 13, 15]. PBRs have been demonstrated to exist on peritoneal macrophages [6] and peripheral blood mononuclear cells [5, 17, 18], as well as mouse microglial [19] and human monocytic THP-1 and U937 cells [5, 20].

The up-regulation of PK 11195 binding in these various inflammatory states led us to explore whether PK 11195

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† *Abbreviations*: CBR, central benzodiazepine receptor; DMEM-F12, Dulbecco's modified Eagle's medium-nutrient mixture F12 Ham; FBS, fetal bovine serum; Fcγ RII, Fc gamma receptor II; GABA, γ-aminobutyric acid; HBSS, Hanks' balanced salt solution; IFN-γ, interferon-γ; IL, interleukin; INT, *p*-iodonitrotetrazolium violet; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OZ, serum-opsonized zymosan; PBR, peripheral benzodiazepine receptor; PMA, phorbol myristate acetate; and TNF, tumor necrosis factor.

Received 21 July 1999; accepted 14 October 1999.

possessed anti-inflammatory properties. We found that it had both preventative and therapeutic effects in the MRL-lpr mouse rheumatoid arthritis model [21]. To explore those properties further, we undertook an *in vitro* study of the pharmacological effects of PK 11195 on several parameters associated with macrophage functions, using the human THP-1 cell line and rat peritoneal macrophages as representative cells. The effects were compared with those observed with several known macrophage inhibitors. The functions included respiratory burst; secretion of glutamate, IL-1β, and TNF-α; toxicity of mononuclear phagocyte culture supernatants to SH-SY5Y neuroblastoma cells; and expression of the inflammatory surface markers HLA-DR and the Fcγ RII (CDw32).

MATERIALS AND METHODS

Reagents

PK 11195 was obtained from the Sigma Chemical Co. and from Tocris Cookson. The following substances, applied to cell preparations, were obtained from Sigma: carbamazepine, Ro 5–4864, dexamethasone, nordihydroguaiaretic acid, bacterial LPS (from *Escherichia coli* 055:B5), PMA, and zymosan particles from *Saccharomyces cerevisiae*. Human recombinant IFN-γ was purchased from Bachem California. OZ was prepared by incubation of zymosan particles (10 mg mL^{-1}) with 20% human or rat serum in HBSS at 37° for 30 min.

The following substances used in the enzymatic and immunological assays were supplied by Sigma: diaphorase (EC 1.8.1.4, from *Clostridium kluyveri,* 5.8 U mg^{-1} solid), INT, L-glutamic dehydrogenase (EC 1.4.1.3 from bovine liver, 30 U mg^{-1} solid), NAD^+, MTT, avidin–alkaline phosphatase conjugate, and phosphatase substrate Sigma 104. Mouse anti-human TNF-α monoclonal antibodies (clones MAb1 and biotinylated MAb11) were purchased from PharMingen; human recombinant TNF-α was obtained from Biosource International. Polyclonal anti-human IL-1β antibodies, raised in rabbits, were a gift from Dr. H. Ziltener, The Biomedical Research Centre. Culture supernatants containing monoclonal anti-human IL-1β antibodies were obtained from Dr. A. E. Berger, The Upjohn Co. Alkaline phosphatase-labeled goat anti-mouse antibodies were purchased from GIBCO BRL, Life Technologies, and human recombinant IL-1β was from Bachem California. Anti-human HLA-DR (CR3/43) mouse monoclonal antibody was purchased from DAKO. Anti-human Fcγ RII (CDw32) mouse monoclonal antibody was from AMAC.

Cell and Tissue Culture

Peritoneal macrophages were obtained from Wistar male rats injected with 1% sterile casein 4 days before harvesting. Cells were washed three times with HBSS by centrifugation at 250 *g* for 10 min. They were resuspended at a concentration of 2×10^7 cells mL^{-1} and kept on ice until used. The human monocytic THP-1 cell line was obtained from the American Type Culture Collection, and cells were grown in RPMI-1640 medium containing 10% FBS. The human neuroblastoma SH-SY5Y cell line [22] was a gift from Dr. R. Ross, Fordham University. These cells were grown in DMEM-F12 supplemented with 10% FBS. Both cell lines were used without initial differentiation. The viability of monocytic cells in the presence of various drugs was monitored visually with a phase-contrast microscope and also by staining with 0.1% trypan blue. Cell viability was also evaluated by the release of LDH following lysis (see below).

Measurement of Oxygen Consumption

The oxygen consumption of stimulated and unstimulated cells was measured by a Clark-type oxygen electrode (Rank Brothers). For each measurement, HBSS containing 5 mM HEPES buffer (pH 7.4) was added to the electrode cell and equilibrated to 37°. Peritoneal macrophages or THP-1 cells were added, followed 10 min later by OZ or PMA, to give as final concentrations in a volume of 1 mL: cells, 2×10^6 mL^{-1}; OZ, 0.5 mg mL^{-1}; and PMA, 0.5 μg mL^{-1}. The effect of various drugs on the oxygen consumption rate was studied by adding these drugs 5 min before the stimulation of cells. In some cases, drugs were also added following OZ stimulation at the point where the oxygen consumption rate had reached its maximum (see Ref. 23). In all cases, the linear oxygen consumption rate was measured and expressed as nanomoles per 10^6 cells per minute.

Measurement of Glutamate Production

The concentration of L-glutamic acid (glutamate) in cell-free supernatants was measured by an enzymatic assay [24, 25] as described previously [26, 27]. Briefly, cells were seeded into 96-well plates at 2×10^5 cells per well in 100 μL of DMEM, containing 4 mM L-glutamine and 0.2 mM L-cystine, but no L-glutamate. Cells were allowed to adhere for 90 min before various concentrations of drugs were added to triplicate wells, followed 30 min later by activation with OZ (200 μg mL^{-1}). Experiments with THP-1 cells were performed in the same manner by using the initial concentration of 2×10^5 cells per well and using RPMI-1640 medium containing 10% FBS. After incubation for 24 hr in a humidified 5% CO_2, 95% air atmosphere at 37°, 10 μL of cell supernatant was transferred to a new plate and mixed with 180 μL of triethanolamine buffer (80 mM triethanolamine, 20 mM potassium phosphate, pH 8.6) containing INT, NAD^+, and diaphorase. The enzymatic reaction was started by the addition of 10 μL of glutamic dehydrogenase solution, and, after a 60-min incubation at room temperature, optical densities were measured by a model 450 microplate reader (Bio-Rad Laboratories) with a 490 nm filter. The final concentrations of reagents were as follows: 71.4 μM INT; 0.4 mM NAD^+; 0.26 U mL^{-1} of diaphorase; 7.5 U mL^{-1} of glutamic

dehydrogenase. Concentrations of glutamate in the experimental samples were calculated according to the optical densities obtained from wells containing standards of glutamate solution in corresponding media. The amount of glutamate produced by cells was calculated after subtracting the values obtained from samples containing medium alone. It was confirmed that the media used did not interfere with the assay, as assessed by comparing values obtained in standard glutamate dilutions in distilled water and media [25], and that cell lysis was not a contributing factor to the increase in glutamate concentration in cell supernatants (see Refs. 26 and 27).

Measurement of IL-1β and TNF-α

Cells were seeded into 24- or 12-well culture plates (5×10^5 cells mL^{-1}). They were exposed for 64 hr to 1 μg mL^{-1} of LPS for measurement of TNF-α, and to a combination of 0.5 μg mL^{-1} of LPS and 150 U mL^{-1} of IFN-γ for measurement of IL-1β. The concentration of cytokines in cell-free supernatants was measured by an ELISA. In the case of TNF-α, the procedure recommended by the antibody supplier (PharMingen) was used [28]. This procedure was modified for the detection of IL-1β as follows: rabbit polyclonal IL-1β capture antibodies were diluted 1:1000 in 0.1 M bicarbonate coating buffer, pH 8.2. Aliquots (50 μL) were added to each well of 96-well plates (Easy Wash, Corning) and incubated overnight at 4°. Nonspecific binding sites were blocked by incubation of the wells with 200 μL of 3% BSA in PBS for 2 hr at room temperature. Samples and recombinant IL-1β standards diluted in PBS/3% BSA were added at 100 μL per well, and plates were incubated overnight at 4°. Mouse monoclonal antibodies were used for the detection of IL-1β. A culture supernatant was diluted 1:50 in PBS/3% BSA and added at 100 μL to each well. Plates were incubated for 1 hr at room temperature. The alkaline phosphatase-labeled anti-mouse antibody was added (1:3000 dilution) in PBS/3% BSA at 100 μL per well, followed by 45 min of incubation at room temperature. After each of the above experimental steps, plates were washed 2–8 times with 0.5% Tween in PBS, pH 7.0. Optical density at 405 nm was read after a 120-min incubation of wells with substrate buffer containing 1 mg mL^{-1} of Sigma 104 phosphate substrate in 0.1 M diethanolamine buffer, pH 9.8. A microplate reader with a 405 nm filter was used to measure optical density of each sample. Concentrations of IL-1β in the experimental samples were calculated according to the optical densities obtained from wells containing standards of recombinant cytokine.

Measurement of Cell Surface Receptor Expression

THP-1 cells were seeded into 96-well culture plates (2×10^5 cells per well) in 160 μL of RPMI-1640 medium containing 10% FBS. Various substances were added to the quadruplicate wells in 20-μL aliquots, and, after 30 min of incubation, another 20-μL aliquot containing IFN-γ (final concentration 333 U mL^{-1}) was added to each well. Plates were incubated in a humidified 5% O_2, 95% air atmosphere at 37° for 48 hr. The measurement of the expression of THP-1 cell surface receptors was performed as follows. First, incubation medium was removed, and the cells were fixed by air-drying. Subsequently, the plates were blocked at room temperature with 3% BSA in PBS for 2 hr, incubated with one of two monoclonal antibodies diluted 1:500 in blocking solution for 1 hr, washed briefly four times in PBS, and then incubated with goat anti-mouse IgG alkaline phosphatase conjugate diluted 1:4000 in blocking solution for 1 hr. After washing six more times with PBS, the presence of surface receptors in each of the wells was estimated using a microplate reader. Optical densities were measured after incubating the wells for 1 hr with substrate buffer containing 1 mg mL^{-1} of Sigma 104 phosphate substrate in 0.1 M diethanolamine buffer, pH 9.8, using a 405-nm filter.

Cytotoxicity of THP-1 Cells towards SH-SY5Y Neuroblastoma

Human monocytic THP-1 cells were seeded in 24-well plates at a concentration of 5×10^5 per well in 1 mL of DMEM-F12 medium containing 5% FBS. Cells were incubated in the presence or absence of various drugs for 30 min prior to the addition of a stimulus (1 μg mL^{-1} of LPS with 333 U mL^{-1} of IFN-γ). After 24 hr of incubation in a humidified 5% CO_2, 95% air atmosphere at 37°, 0.5-mL samples of cell-free supernatants were transferred to the wells containing SH-SY5Y cells that had been plated 24 hr earlier at a concentration of 1×10^5 per well in 1 mL of DMEM-F12 medium containing 5% FBS. The cell culture medium was sampled for LDH content, and an MTT assay was performed after a 72-hr incubation period.

Cell Viability Assays: LDH Release

Cell death was evaluated by LDH release. LDH activity in cell culture supernatants was measured by an enzymatic test as described by Decker and Lohmann-Matthes [29], in which formation of the formazan product of INT was followed colorimetrically. Briefly, 100-μL samples of cell culture supernatants were pipetted into the wells of 96-well plates, followed by the addition of 15 μL of lactate solution (36 mg mL^{-1} in PBS) and 15 μL of INT solution (2 mg mL^{-1} in PBS). The enzymatic reaction was started by the addition of 15 μL of NAD^+/diaphorase solution (3 mg mL^{-1} of NAD^+; 2.3 mg solid diaphorase mL^{-1}). After 15 min of incubation, the reaction was terminated by the addition of 15 μL of oxamate (16.6 mg mL^{-1}). Optical densities were measured by a microplate reader with a 490-nm filter, and the amount of LDH that had been released was expressed as a percentage of the value obtained in comparative wells where the remaining cells were 100% lysed by 1% Triton X-100.

Cell Viability Assays: Reduction of Formazan Dye (MTT)

The MTT assay was performed as described by Mosmann [30] and by Hansen *et al.* [31]. This method is based on the ability of viable, but not dead cells to convert the tetrazolium salt (MTT) to colored formazan. The viability of SH-SY5Y cells was determined by adding MTT to the SH-SY5Y cell cultures to reach a final concentration of 1 mg mL^{-1}. Following a 2-hr incubation at 37°, the dark crystals formed were dissolved by adding to the wells an equal volume of SDS/DMF extraction buffer (20% SDS and 50% *N*,*N*-dimethylformamide, pH 4.7). Subsequently, plates were placed overnight at 37°, and optical densities at 570 nm were measured by transferring 100-μL aliquots to 96-well plates and using the plate reader with a corresponding filter to record values. The viable cell value was calculated as a percentage of the value obtained from cells incubated with fresh medium only.

Statistical Analysis

Data are presented as means ± SEM. The data were evaluated statistically by ANOVA, followed by the Bonferroni test for multiple comparisons, or by the randomized blocks design ANOVA. In those cases where data are presented as a percentage of control values, statistical analyses were performed before transformation of data.

TABLE 1. Inhibition of respiratory burst in cultured rat peritoneal macrophages by benzodiazepine receptor ligands and anti-inflammatory agents

Substance	Concentration (M)	Oxygen consumption rate (% of control)
PK 11195	10^{-4}	48.9 ± 5.4* (3)
	10^{-5}	87.9 ± 2.5† (4)
	10^{-6}	99.0 ± 3.3 (5)
	10^{-7}	104.2 ± 0.9 (4)
	0	100.0 ± 6.6 (8)
Ro 5-4864	10^{-4}	76.1 ± 1.6* (6)
	10^{-5}	91.3 ± 5.9 (3)
	0	100.0 ± 2.2 (8)
Carbamazepine	10^{-4}	75.1 ± 3.4‡ (5)
	10^{-5}	82.0 ± 2.4 (4)
	0	100.0 ± 6.4 (5)
Nordihydroguaiaretic acid	10^{-4}	18.0 ± 7.9* (3)
	10^{-5}	52.5 ± 3.8* (4)
	10^{-6}	86.8 ± 4.9 (5)
	0	100.0 ± 5.8 (16)

Rat peritoneal macrophages (2×10^6) were preincubated with substances at the indicated concentrations for 5 min, followed by injection of OZ (0.5 mg mL^{-1}). The linear oxygen consumption rate was measured and expressed as a precentage of the oxygen consumption rate in control samples incubated with an appropriate vehicle solution only. The absolute control values varied between experiments from 0.47 ± 0.03 to 0.79 ± 0.02 nmol 10^6 $cells^{-1}$ min^{-1}. The overall oxygen consumption rate of unstimulated cells was 0.17 ± 0.06 nmol 10^6 $cells^{-1}$ min^{-1} (N = 9). Data are presented as means ± SEM, and the number of samples from at least three independent experiments is shown in parentheses.

*–‡Significantly different from the control samples containing no drugs (Bonferroni test for multiple comparisons): *$P < 0.001$, †$P < 0.05$, and ‡$P < 0.01$.

RESULTS

Table 1 shows that PK 11195, at a concentration equal to or higher than 10 μM, inhibited the respiratory burst of rat peritoneal macrophages. The effects of Ro 5–4864 and the tricyclic-like anti-epileptic carbamazepine on this parameter were weaker. Both these substances failed to exceed a maximum of 25% inhibition, and then only at 100 μM concentrations. In this assay, PK 11195 was less effective than the lipoxygenase inhibitor and antioxidant nordihydroguaiaretic acid.

Table 2 compares the inhibition of respiratory burst by PK 11195 under various activation conditions. The magnitude of its inhibitory action remained at approximately the same level whether it was added to rat peritoneal macrophages before or after stimulation by the complement receptor ligand OZ, or before cells were stimulated by the protein kinase C activator PMA. The inhibition was also comparable with OZ-stimulated human monocytic THP-1 cells. This is in contrast to nordihydroguaiaretic acid, which inhibits reactions generated by arachidonic acid at the cell surface. This agent was much less effective when added after OZ stimulation than before. This surface-directed agent was also less effective against the intracellular activator PMA (data not shown).

The effects of PK 11195, compared with dexamethasone, on glutamate production by rat peritoneal macrophages and human monocytic THP-1 cells are summarized in Table 3. Incubation of both cell types with OZ resulted in a significant increase of glutamate concentration in cell supernatants. The inhibitory effect of PK 11195 on this parameter of cell activation was much smaller than on the respiratory burst of these cells. In addition, it appeared that the effect was somewhat stronger on peritoneal macrophages than on THP-1 cells. Dexamethasone also had a weak inhibitory effect on glutamate secretion in both rat peritoneal cells and THP-1 cells, although the level did not meet $P < 0.05$ significance criteria in the case of peritoneal cells. The effectiveness of Ro 5–4864 was not tested in this assay.

The effects of PK 11195 on secretion by THP-1 cells of the two cytokines IL-1β and TNF-α were different. Table 4 shows that PK 11195 significantly inhibited IL-1β secretion, although it was a much weaker inhibitor than the steroid dexamethasone. Both Ro 5–4864 and carbamazepine were ineffective in this assay. Meanwhile, PK 11195 at the concentrations tested (1–50 μM) had no significant effect on TNF-α secretion by THP-1 cells stimulated for 64 hr with either IFN-γ or LPS, whereas dexamethasone was an effective inhibitor of this parameter (data not shown).

PK 11195 (1–10 μM) on its own did not affect cell surface expression of HLA-DR or Fcγ RII on THP-1 cells (see Table 5). IFN-γ treatment significantly up-regulated both these proteins, and this effect was not inhibited or

TABLE 2. Inhibition of respiratory burst by PK 11195 in cultured rat peritoneal macrophages and human monocytic THP-1 cells

	Oxygen consumption rate (% of control)			
	Peritoneal macrophages			THP-1 cells
Concentration (M)	Before OZ (A)	After OZ (B)	Before PMA (C)	Before OZ (D)
10^{-4}	48.9 ± 5.4* (3)	47.1 ± 6.8* (4)	65.7 ± 4.3* (3)	37.8 ± 3.2* (5)
10^{-5}	87.9 ± 2.5† (4)	83.5 ± 4.3† (4)	83.5 ± 5.5† (3)	66.7 ± 10.7† (3)
10^{-6}	99.0 ± 3.3 (5)	97.9 ± 0.2 (3)	100.3 ± 2.7 (3)	88.7 ± 7.0 (4)

Rat peritoneal macrophages or THP-1 cells (2×10^6 cells in 1 mL medium) were preincubated with PK 11195 for 5 min before OZ (0.5 mg mL^{-1}, A and D) or PMA (0.5 μg mL^{-1}, C) was added. In a different experiment (B), OZ (0.5 mg mL^{-1}) was first added to cells and, after the linear oxygen consumption rate was established, the various concentrations of PK 11195 were injected. The linear oxygen consumption rate was measured and expressed as a percentage of the oxygen consumption rate in control samples injected with an appropriate vehicle solution only. The absolute values of control (N = 6–8) were as follows: A and B, 0.79 ± 0.02; C, 0.52 ± 0.02; and D, 0.18 ± 0.05 nmol 10^6 $cells^{-1}$ min^{-1}. Data are presented as means ± SEM, and the number of samples from at least three independent experiments is shown in parentheses.

*,†Significantly different from the control samples containing no PK 11195 (Bonferroni test for multiple comparisons): *$P < 0.001$ and †$P < 0.05$.

enhanced by PK 11195. Dexamethasone also failed to modify significantly the levels of Fcγ RII, while it effectively inhibited the HLA-DR up-regulation.

Figure 1 shows the effects of PK 11195 and Ro 5–4864 on THP-1 cell secretions that cause the death of SH-SY5Y cells. The drugs were administered to THP-1 cells 30 min before stimulating them with LPS + IFN-γ. These stimulating agents at the concentrations tested did not have any direct effects on SH-SY5Y cells. According to two independent cytotoxicity parameters, MTT reduction by viable cells (Fig. 1A), and LDH release by damaged cells (Fig. 1B), both PK 11195 and Ro 5–4864 partially inhibited the cytotoxicity of the harvested THP-1 cell supernatants in a concentration-dependent manner (2–50 μM). Carbamazepine had a very small effect only at a 200 μM concentration, whereas dexamethasone was ineffective (data not shown). In each of the above experimental systems, the toxicity of the drugs used was monitored by measuring the LDH levels in cell supernatants. None of the substances, at the concentrations used in these experiments, caused any significant changes in the viability of rat peritoneal macrophages or THP-1 cells.

DISCUSSION

We have shown previously that the powerful PBR ligand PK 11195 is an inhibitor of joint inflammation in a well-established mouse model of rheumatoid arthritis [21]. The model involves priming mice of the MRL-lpr strain with Freund's adjuvant, after which they develop a severe autoimmune arthritic condition. PK 11195 has a preventative action against this disorder at a dose of 1 mg kg^{-1} day^{-1} and a therapeutic effect against established disease at a dose of 3 mg kg^{-1} day^{-1}. Thus, PK 11195 suppresses inflammation *in vivo*.

We have used *in vitro* parameters to try to establish which phagocytic functions may be influenced by PK 11195. The secretion of toxic materials was explored, as well as the expression of two widely used activation markers highly sensitive to stimulation of phagocytic cells, namely the

TABLE 3. Inhibition of glutamate production by PK 11195 and dexamethasone in cultured rat peritoneal macrophages and human monocytic THP-1 cells

		Glutamate production (% of control)	
Conditions	Concentration (M)	Peritoneal macrophages	THP-1 cells
OZ only		100.0 ± 1.1 (22)	100.0 ± 1.2 (21)
Without OZ		79.8 ± 2.7* (13)	53.3 ± 5.5* (8)
OZ + PK 11195	10^{-4}	56.7 ± 11.0* (7)	83.9 ± 3.2† (6)
OZ + PK 11195	10^{-5}	94.1 ± 2.8 (7)	98.6 ± 2.4 (6)
OZ + PK 11195	10^{-6}	101.0 ± 3.2 (7)	95.3 ± 4.1 (6)
OZ + Dexamethasone	10^{-4}	91.8 ± 4.9 (7)	89.6 ± 5.4‡ (7)
OZ + Dexamethasone	10^{-5}	102.8 ± 4.1 (7)	93.7 ± 2.1 (7)
OZ + Dexamethasone	10^{-6}	101.6 ± 6.0 (5)	96.3 ± 1.8 (5)

Rat peritoneal macrophages or THP-1 cells (2×10^5 per well) were seeded in 96-well plates in the presence or absence of PK 11195 or dexamethasone for 30 min before OZ (0.2 mg mL^{-1}) was added. After 24 hr of incubation, the glutamate levels in cell-free supernatants were measured and expressed as a percentage of the glutamate concentration in control samples incubated with OZ only. The concentration of glutamate in the supernatants of cells stimulated by OZ reached the following values: 204.9 ± 10.5 μM for rat peritoneal macrophages and 434.5 ± 23.5 μM for THP-1 cells. Data are presented as means ± SEM, and the number of triplicate samples from 5–8 independent experiments is shown in parentheses.

*–‡Significantly different from the control samples containing OZ only (Bonferroni test for multiple comparisons): *$P < 0.001$, †$P < 0.01$, and ‡$P < 0.05$.

TABLE 4. Inhibition of IL-1β production by THP-1 cells

Substance	Concentration (M)	IL-1β production (% of control)
LPS + IFN-γ only		100.0 ± 8.1 (21)
Without LPS + IFN-γ		7.7 ± 5.2* (10)
PK 11195	5×10^{-5}	32.7 ± 8.2† (6)
	5×10^{-6}	110.4 ± 13.5 (6)
	5×10^{-7}	91.3 ± 12.0 (6)
Ro 5-4864	10^{-4}	85.5 ± 17.6 (6)
	10^{-5}	99.0 ± 23.5 (6)
Carbamazepine	10^{-4}	110.9 ± 43.4 (5)
	10^{-5}	116.8 ± 36.2 (5)
Dexamethasone	10^{-6}	26.5 ± 12.9† (4)
	10^{-7}	35.5 ± 14.4‡ (4)
	10^{-8}	52.8 ± 16.9‡ (4)

Human monocytic THP-1 cells (2.5×10^5 per well) were seeded in 24-well plates in the presence or absence of various drugs for 30 min before a combination of LPS (0.5 μg mL^{-1}) and IFN-γ (150 U mL^{-1}) was added. After 64 hr of incubation, the IL-1β levels in cell-free supernatants were measured and expressed as a percentage of the IL-1β concentration in control samples incubated in the absence of drugs. The concentration of IL-1β in the supernatants of stimulated cells was 2.22 ± 0.18 U mL^{-1}. Data are presented as means ± SEM, and the number of samples from 3 independent experiments is shown in parentheses.

*–‡Significantly different from the control samples containing LPS and IFN-γ only (Bonferroni test for multiple comparisons): *$P < 0.001$, †$P < 0.01$, and ‡$P < 0.05$.

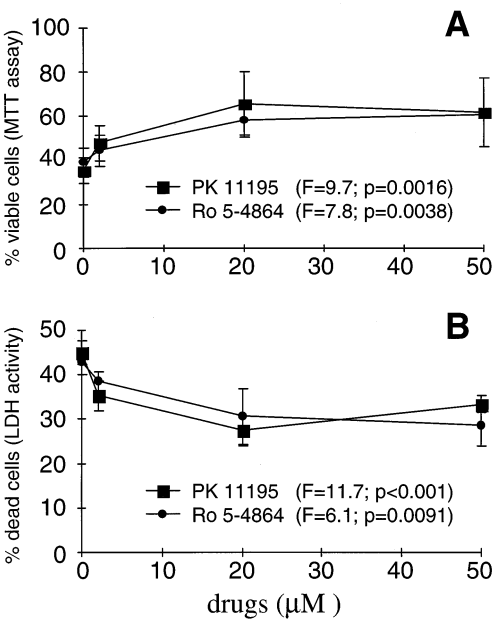

FIG. 1. Inhibition of THP-1 cell toxicity towards human SH-SY5Y neuroblastoma cells by benzodiazepine receptor ligands. THP-1 cells were pretreated with various concentrations of PK 11195 or Ro 5–4864 (shown on the abscissa) for 30 min before stimulation with LPS (1 μg mL^{-1}) and IFN-γ (333 U mL^{-1}). After a 24-hr incubation, the cell-free supernatants of THP-1 cells were transferred to the wells containing SH-SY5Y cells. Viability of SH-SY5Y cells was assessed after 72 hr by MTT assay (A), and also by measuring the LDH activity in the supernatants (B). Supernatants of unstimulated THP-1 cells were not cytotoxic to SH-SY5Y cells, and the percent values of viable and dead cells were 107.8 ± 9.2 (N = 6) and 3.2 ± 1.2 (N = 6), respectively. Data are presented as means ± SEM from 4–5 independent experiments. Significance levels for the concentration-dependent effect of the drug were calculated by randomized blocks design ANOVA.

major histocompatibility complex antigen HLA-DR and the immunoglobulin receptor Fcγ RII [32, 33].

The results presented in Table 2 show that PK 11195 was an effective inhibitor of both PMA- and OZ-induced respiratory bursts, reducing activity by about 50%. The respiratory burst is an important phagocytic function promoting host defense. This system is characterized by NADPH-dependent conversion of molecular oxygen into reactive oxygen intermediates such as superoxide anion and hydrogen peroxide. At high concentrations, reactive oxygen species can be toxic to host cells (see Refs. 34 and 35). Unlike some inhibitors of the respiratory burst, PK 11195 was equally effective in slowing down oxygen consumption whether added before or after stimulation by OZ. This suggests that receptors for activated fragments of C3, which mediate the effect of OZ, are an unlikely target for PK 11195, and supports the concept that PK 11195 is acting at

TABLE 5. Modification of cell surface receptors of THP-1 cells by IFN-γ, PK 11195, and dexamethasone

Conditions	Concentration (M)	HLA-DR (% of control)	Fcγ RII (% of control)
IFN-γ only		100.0 ± 4.0 (18)	100.0 ± 8.1 (12)
Without IFN-γ		7.3 ± 0.6* (18)	75.3 ± 5.0† (12)
IFN-γ + PK 11195	10^{-5}	96.4 ± 8.6 (10)	92.0 ± 12.5 (9)
IFN-γ + PK 11195	10^{-6}	91.8 ± 8.4 (10)	93.1 ± 13.6 (9)
IFN-γ + Dexamethasone	10^{-4}	73.9 ± 5.8‡ (9)	84.1 ± 9.2 (8)
IFN-γ + Dexamethasone	10^{-5}	80.0 ± 7.8† (10)	95.1 ± 12.3 (8)
IFN-γ + Dexamethasone	10^{-6}	85.5 ± 8.0 (10)	96.9 ± 9.0 (8)

THP-1 cells (2×10^5 per well) were seeded in 96-well plates in the presence or absence of PK 11195 or dexamethasone for 30 min before human recombinant IFN-γ (333 U mL^{-1}) was added. After 48 hr of incubation, a density of cell surface receptors was determined by using monoclonal antibodies against HLA-DR antigens and Fcγ receptors II. The absolute values of optical densities obtained in control samples stimulated by IFN-γ only were as follows: 0.31 ± 0.04 U for HLA-DR and 0.25 ± 0.03 U for Fcγ RII. Data (means ± SEM) are presented as a percentage of values obtained in control samples, and the number of quadruplicate samples from eight independent experiments is shown in parentheses.

*–‡Significantly different from the control samples containing IFN-γ only (Bonferroni test for multiple comparisons): *$P < 0.001$, †$P < 0.05$, and ‡$P < 0.01$.

some downstream, intracellular target. Several earlier studies, using different types of phagocytes, have also shown modulation of respiratory burst by the ligands of PBRs [36–39].

PK 11195 also inhibited glutamate secretion, although the effect was weaker than that on respiratory burst. Glutamate is another neurotoxin secreted by mononuclear phagocytes (e.g. [25]). In peritoneal macrophages, the effective concentration for inhibiting glutamate secretion was 100 μM (43% inhibition). Inhibition of glutamate secretion by THP-1 cells was 16%. This small inhibitory effect of PK 11195 is similar to that observed previously for dexamethasone in both THP-1 cells and mouse microglia (Table 3; [25, 27]).

In addition to THP-1 cells, rat peritoneal macrophages were also used to study the effect of PK 11195 on glutamate secretion and the respiratory burst response. The effects were very similar on these monocyte-derived cells from two different species. Such a correlation reinforces *in vivo* data from the murine model and human disease states.

PK 11195 at 50 μM inhibited IL-1β secretion by human THP-1 monocytic cells, while Ro 5–4864 had no significant effect (Table 4). Both these substances were without effect on THP-1 cell secretion of TNF-α in the concentration range of 0.1 to 50 μM. It had been reported previously that Ro 5–4864 (0.001 to 100 μM) inhibits TNF-α secretion by mouse macrophages, while PK 11195 has no effect at 10 μM [40]. The data presented here, and by others (see Refs. 40–42), show that PK 11195 and Ro 5–4864 can act similarly on some macrophage functional parameters, while in other cases the effects of these two PBR ligands differ. These data could point to the existence of several PBR subtypes that have different functions and affinities towards various ligands, as well as different cellular expression.

Unlike other parameters studied, THP-1 cell supernatant cytotoxicity towards the neuronal cell line SH-SY5Y does not measure a defined secretory product. It is most likely that a mixture of compounds is responsible for this phenomenon, which, nevertheless, may be the assay most relevant to neuroinflammatory processes. The inhibitory effect of PK 11195 in this assay was partial, and in the 2–50 μM concentration range, it reached a maximum of approximately 40% protection. The effect of Ro 5–4864 in this assay system was almost identical to that of PK 11195. The exact nature of the cytotoxic substances responsible for the neuronal death induced by phagocytes has not yet been established in spite of the fact that this phenomenon has been demonstrated in a number of *in vitro* preparations (e.g. [43–48]).

The described assay system uses human monocytic THP-1 cells as representatives of mononuclear phagocytes, and human SH-SY5Y cells as neuronal representatives. While they are clearly different from primary human microglia and mature human neurons, they have the advantage of being species-specific. THP-1 cells have been accepted as a good model for human monocytes/macrophages because of their extensive metabolic and morphological similarities. Moreover, similarities between the properties of THP-1 cells and postmortem human microglia have been established directly, and the ability of these cells to secrete neurotoxic products has already been characterized after stimulation by several agents, including the β-amyloid protein of Alzheimer's disease, prion protein peptides, LPS, IFN-γ, and infection with human immunodeficiency virus-1 (see Refs. 43, 46, and 47). The usefulness of SH-SY5Y cells, along with another human neuronal cell line, LA-N-2, as surrogates for mature human neuronal cells has also been established previously. They have been demonstrated previously to be suitable as targets for THP-1 cell toxicity [47].

The inhibitory effects of PK 11195 were observed after stimulating mononuclear phagocytes either by OZ (respiratory burst and glutamate secretion), or by a combination of LPS and IFN-γ (secretion of IL-1β and cytotoxic products). Different stimulating agents were required to achieve optimal phagocyte activation in all the assay systems used. It is likely that OZ and LPS + IFN-γ activate different intracellular pathways, since, for example, LPS + IFN-γ are unable to induce a respiratory burst response. It is also possible that, when cells were stimulated by different stimulating agents, PK 11195 could be influencing different pathways. Although OZ, which acts through complement receptors, and IFN-γ are relevant as stimuli to a number of peripheral inflammatory conditions, as well as such CNS disease as multiple sclerosis, it is possible that the effects of PK 11195 on macrophages activated by other stimuli (e.g. Alzheimer β-amyloid protein and prion protein) would differ from the effects observed here.

In our experiments PK 11195 and Ro 5–4864 were effective in the micromolar range. This exceeds the affinity for PBRs, which is reported to be in the low nanomolar range for isolated membranes [5, 6, 49, 50]. It is possible that with intact cells higher concentrations of the drugs are needed before the ligands can reach their intracellular receptors. The effective concentration range of PK 11195 in these *in vitro* experiments is comparable to the *in vivo* concentration that would be achieved from the dosage schedules utilized in the mouse arthritic model experiments [21]. It is also important to note that the plasma concentration of clinically used benzodiazepines, including the mixed PBR/CBR ligand diazepam, reaches the micromolar range [51, 52].

The cellular effects of PBR ligands most probably are mediated by PBRs and their downstream signaling pathways. PBRs are believed to consist of three proteins of 18, 30, and 32 kDa. The 18-kDa protein preferentially binds isoquinolinecarboxamide compounds such as PK 11195. Both the 18- and the 32-kDa protein are required for binding of a subset of benzodiazepines including Ro 5–4864. Both the 18- and the 32-kDa protein, also known as a voltage-dependent anion channel or porin, are located on the mitochondrial outer membrane. While these two proteins seem to be associated functionally, the third

30-kDa protein, also known as the adenine nucleotide carrier, which co-purifies with these proteins, does not appear to play a role in ligand binding (see Refs. 53 and 54). PBRs have been reported to be linked to DNA synthesis and lipid metabolism [55], and to such intracellular signaling pathways as those involving cAMP [56], the apoptosis inhibitory oncoprotein Bcl-2 [57], and cyclooxygenase [58]. One that appears particularly relevant to these studies is the calcium–calmodulin pathway (see Refs. 42 and 59). Calmodulin inhibitors reduce IL-1β secretion by murine macrophages, but have no effect on TNF-α secretion [60, 61]. In our studies, PK 11195 reduced IL-1β secretion but had no effect on TNF-α secretion in THP-1 cells. Calmodulin has been implicated in the respiratory burst response [62] and cytotoxicity [43, 63], which are two other functions inhibited by PK 11195. Finally, calmodulin is not involved in the induction of HLA-DR following IFN-γ stimulation in several cell types, including THP-1 and U937 cells [64, 65]. Data from this study show that PK 11195 was unable to inhibit IFN-γ induction of HLA-DR expression. Thus, the data reported in this study are all consistent with PK 11195 inhibiting directly, or through PBRs, inflammatory phagocytic functions associated with calcium and calmodulin. Further studies are required to test this hypothesis directly.

In summary, the data presented show that the peripheral benzodiazepine ligand PK 11195 partially inhibited several mononuclear phagocyte functions including the respiratory burst response and secretion of glutamate and IL-1β. It also had a modest inhibitory effect on cytotoxicity towards neuronal cells, whereas secretion of TNF-α and the expression of two surface membrane markers were unaffected. It is suggested that the ligands of PBRs, such as PK 11195, could be useful inhibitors of selective macrophage functions, and therefore, may be beneficial in treating inflammatory diseases in the CNS as well as peripheral tissues.

This work was supported by a grant from the Arthritis Society of Canada. The authors wish to thank Dr. M. Schulzer for advice on statistical analysis

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