4-Hydroxy-oxyphenbutazone is a potent inhibitor of cytokine production

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ABSTRACT. 4-hydroxy-oxyphenbutazone (4OH-OPB), is currently in phase II trials for its immunosuppressive effect in patients with rheumatoid arthritis. 4OH-OPB and other compounds related to phenylbutazone were tested for their effect on *in vitro* cytokine production by monocytes and lymphocytes present in peripheral mononuclear cells (PBMC) or whole blood (WB) cultures, and compared against phenylbutazone and oxyphenbutazone, two known anti-inflammatory drugs. In PBMC cultures, 4OH-OPB was by far the most potent inhibitor, and both monokines and Th1 and Th2 lymphokines were efficiently inhibited at low concentrations. In WB cultures, 4OH-OPB was less effective than in PBMC cultures, but was still the best inhibitor of lymphokine production and, furthermore, was the only inhibitor of monokine production. The increase in 4OH-OPB concentration needed to induce the same inhibition of cytokine production in WB as in PBMC culture could be mimicked by the addition of erythrocytes to the PBMC cultures. Experiments with radioactively-labeled 4OH-OPB suggest that 4OH-OPB is taken up very rapidly into erythrocytes and is secreted by the erythrocytes with much slower kinetics *via* a multidrug-resistance-associated protein. The secreted compound is most likely structurally different from 4OH-OPB, as in PBMC and WB cultures, the inhibition of cytokine production seems to be caused by a different mechanism. In PBMC cultures, the inhibition of cytokine production is accompanied by a loss of cell viability, while this is not the case when 4OH-OPB inhibits cytokine production in WB. Our data suggest that 4OH-OPB may be useful as an immunosuppressive drug for patients with inflammatory diseases.

Keywords: immunosuppressive drug, 4-hydroxy-oxyphenbutazone, phenylbutazone, inflammatory cytokines, PBMC, whole blood

Immunosuppressive and anti-inflammatory drugs are widely used to treat a variety of human diseases. In every situation where over-stimulation of cells of the immune system causes disease, immunosuppressive and anti-inflammatory drugs are used. The cause of most chronic inflammatory diseases, such as rheumatoid arthritis, psoriasis and inflammatory bowel disease, is not completely understood. Established chronic inflammation often seems to be an interplay between several immune system components, therefore a drug which affects both the activity of monocytes and T lymphocytes would be helpful for suppressing such diseases.

Most immunosuppressive drugs used nowadays, such as Cyclosporin A, FK506, rapamycin, methotrexate and mycophenolic acid exert their main effect on T lymphocytes [1-3] and not on monocytes. Corticosteroids are often used because of their potent anti-inflammatory effects; they inhibit the production of cytokines by both T lymphocytes and monocytes [4], however, the toxicity of corticosteroids is a major limitation of their use.

Non-steroidal anti-inflammatory drugs (NSAIDs), and, more recently, specific inhibitors of cyclo-oxygenase, are also frequently used as anti-inflammatory drugs. They reduce formation of prostaglandins and leukotriens, which are important regulators of inflammation [5]. Phenylbutazone (PB) was one of the first NSAIDs to be introduced in the 1950s. PB and oxyphenbutazone (OPB), a metabolite

Abbreviations:

Ab	antibody
BSA	bovine serum albumin
ELISA	enzyme linked immunosorbent assay
FCS	fetal calf serum
GM-CSF	granulocyte macrophage colony stimulating factor
IL	interleukin
IMDM	Iscove's modified Dulbecco's medium
IFN	interferon
LOS	lipo-oligosaccharide
MRP	multidrug-resistance-associated protein
NSAID	non-steroidal anti-inflammatory drug
OPB	oxyphenbutazone
PB	phenylbutazone
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
RA	rheumatoid arthritis
SEM	standard error of the mean
TNF	tumor necrosis factor
WB	whole blood
4OH-OPB	4-hydroxy-oxyphenbutazone

of PB [6], which differs from PB in having an extra hydroxyl group attached to the benzene ring, are effective in treating the symptoms of rheumatoid arthritis (RA) [7]. However the use of these drugs is restricted clinically because of side effects such as damage to the gastric mucosal membrane, hepatoxicity and bone marrow depression [8-10].

As side effects are, in most cases, the limitation of the use of immunosuppressive or anti-inflammatory drugs, ongoing research for new and less harmful drugs is important. New and highly specific drugs, the so-called biologicals, that modulate the immune system are being developed. Although some of these biologicals have yielded promising results in clinical studies *e.g.* anti-TNF antibodies in RA and Crohn's disease [11, 12], there is still a huge need for specific, non-toxic, financially attractive immunosuppressive and anti-inflammatory drugs.

4-Hydroxy-oxyphenbutazone (4OH-OPB) is, at the moment, in phase II clinical trials as an immunosuppressive drug in patients with RA. In this study, we investigated the effect of 4OH-OPB and a panel of other compounds related to PB, for their effect on monokine and lymphokine production in peripheral blood mononuclear cell (PBMC) *in vitro*. 4OH-OPB was shown to be the most effective compound as regards the inhibition of cytokine production. We therefore also studied this compound in whole blood (WB) cultures and compared it with the known drugs PB and OPB.

MATERIALS AND METHODS

Materials

The compounds tested are listed in *table 1*. The 8 compounds are produced by Syntagon (Södertälje, Sweden). PB and OPB were obtained from ICN biomedicals (Aurora, Ohio, USA). Twenty mM stocks of the compounds were made in DMSO, which were kept at 4°C. [Phenol ring –U-^{14}C] 4OH-OPB was synthesized by Nycomed Amersham plc. (Little Chalfont, England) and had a specific activity of 63 mCi/mmol. Indomethacin stock solution was prepared in DMSO.

Culture medium

The medium used throughout this study was Iscove's modified Dulbecco's medium (IMDM, Bio Whittaker, Verviers, Belgium), containing 100 U/mL penicillin, 100 mg/mL streptomycin (Gibco, Merelbeke, Belgium) and 50 µM 2-mercaptoethanol (Sigma-Aldrich, Steinheim, Germany). For WB cultures, 0.1% heat-inactivated, fetal bovine serum (FCS, Bodinco, Alkmaar, the Netherlands) and 15 U/mL heparin (Leo Pharmaceutical products, Weesp, The Netherlands) were added to the culture medium. PBMC were cultured in IMDM containing 5% FCS and 20 µg/mL of human transferrin (Sigma-Aldrich). All compounds used were endotoxin-free.

Table 1
Chemical structures and names of compounds tested
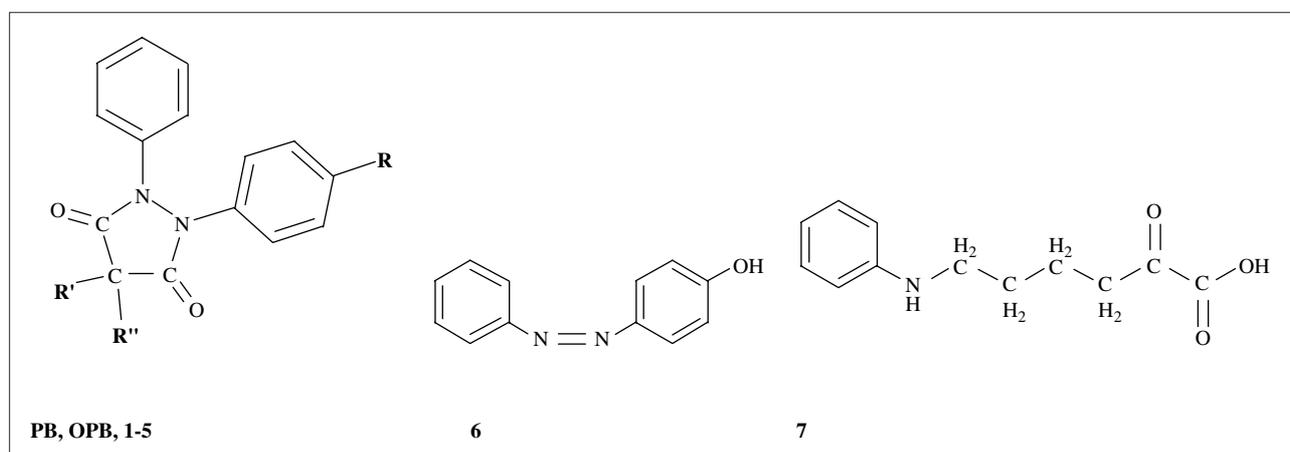
Compounds	R	R'	R"	Name
PB	-H	-H	-C$_4$H$_9$	4-(1-butyl)-1,2-diphenyl-3,5-pyrazol-idinedione *(phenylbutazone)*
OPB	-OH	-H	-C$_4$H$_9$	4-(1-butyl)- 1-phenyl-2-hydroxyphenyl-3,5-pyrazol-idinedione *(oxyphenbutazone)*
4OH-OPB	-OH	-OH	-C$_4$H$_9$	4-(1-butyl)-4-hydroxy-1-phenyl-2-hydroxyphenyl-3,5-pyrazol-idinedione *(4-hydroxy oxyphenbutazone)*
1	-H	-OH	-C$_4$H$_9$	4-(1-butyl)-4-hydroxy-1,2-diphenyl-3,5-pyrazol-idinedione
2	-H	-OH	-phenyl	4-phenyl-4-hydroxy-1,2-diphenyl-3,5-pyrazol-idinedione
3	-H	-OH	-phenyl-p-CH$_3$	4-(p-methylphenyl)-4-hydroxy-1,2-diphenyl-3,5-pyrazol-idinedione
4	-OH	-OH	-C$_2$H$_4$SO- phenyl	4-ethylsulfonphenyl-4-hydroxy-1-phenyl-2-hydroxyphenyl-3,5-pyrazol-idinedione
5	-OCH3	-H	-C$_4$H$_9$	4-(1-butyl)-4-hydroxy-1-(4-methoxyphenyl-2-phenyl-3,5-pyrazol-idinedione
6				p-hydroxyazobenzene
7				2-oxo-hexanoic acid phenylamide

Purification of PBMC and erythrocytes

PBMC were isolated from freshly drawn blood from a healthy volunteer and separated over a Percoll gradient (d = 1.078, Pharmacia Fine Chemicals AB, Uppsala, Sweden). The fraction on top of the Percoll contained the PBMC. Erythrocytes were obtained by washing the red cell fraction present underneath the Percoll layer with culture medium.

Stimulation of PBMC and WB cultures

PBMC (2x 10^5 cells/mL) were cultured in 200 μL IMDM at 37 °C in the presence of 5% CO_2 in 96-well, flat-bottom plates (Nunc, Roskilde, Denmark). In the case of WB cultures, heparinized blood was diluted 1:10 in culture medium. A combination of 1 μg/mL anti-CD3 and anti-CD28 (CLB.T3/4.E res. CLB.CD28/1, CLB, Amsterdam, The Netherlands) was used for T lymphocyte stimulation. For monocyte stimulation, 100 pg/mL lipo-oligosaccharide (LOS, a kind gift from Dr J. Poolman, RIVM, Bilthoven, The Netherlands) was used.

Cytokine production

Cytokine production of T lymphocytes was measured 3 days after anti-CD3/anti-CD28 stimulation. Twenty-four hours after LOS stimulation of the monocytes, cytokine production was measured. At the indicated times, supernatant was harvested and stored at -20 °C until used. Interleukin (IL)-1β, IL-6, IL-8, IL-10, IL-12, IL-13, tumor necrosis factor (TNF) α and interferon (IFN)γ were detected with enzyme linked immunosorbent assay (ELISA) kits (PeliKine-compact, CLB, Amsterdam, The Netherlands) according to the protocol. Summarized briefly, mAbs were coated onto flat-bottomed microtitre plates (Nunc, Maxisorb), overnight in 100 μL 0.1 m sodium bicarbonate at pH 9.6. All incubations were performed in 100 μL, at room temperature. Plates were washed 5 times with PBS + 0.02% Tween (Mallinckrodt Baker, Deventer, The Netherlands). Samples were incubated together with a biotinylated mAb, for 2 hours in High Performance ELISA buffer (CLB). Plates were washed 5 times with phosphate-buffered saline (PBS) + 0.02% Tween, and incubated with streptavidin-labeled poly-horseradish peroxidase (CLB), 1:1000 diluted in PBS + 2% skimmed milk for 30 minutes. After 5 wash steps, the plates were developed with TMB-substrate containing 0.003% H_2O_2, 100 μg/mL 3,5,3',5'-tetramethylbenzidine (Merck, Darmstadt, Germany) in 0.11 m sodium acetate, pH 5.5. The reaction was stopped with an equal volume of 2 m H_2SO_4. The plates were measured in an ELISA-reader at 450 nm, using 540 nm for background measurements. Granulocyte macrophage colony stimulating factor (GM-CSF) was measured using a protocol similar to that used for the other cytokines. The GM-CSF Abs were a kind gift from Dr G. Trinchieri (the Wistar Institute, Philadelphia, PA, USA). In this assay, the coating Ab was anti-GM-CSF 9.1 (used at 2 μg/mL), the biotinylated Ab was anti-GM-CSF 16.3 (0.1 μg/mL). rGM-CSF (Sandoz, Basel, Switzerland) and HGF 22.10 (CLB) were used for preparation of a standard curve.

Incubation of erythrocytes with ^{14}C-4OH-OPB

Erythrocytes (2x 10^8/mL) were incubated with 1 μM ^{14}C-4OH-OPB at 37 °C in culture medium. After the incubation, the erythrocytes were spun down and the amount of radioactivity present in the supernatant was determined using Ultima Gold scintillation fluid (Perkin Elmer) and a liquid scintillation analyzer 1900 TR (Packard).

FACS analysis of cell viability

PBMC or WB cultures were washed in PBA (PBS with 0.5% BSA) and stained with fluorescent conjugated antibodies against CD4 and CD8 for 30 minutes (Becton Dickinson, San Jose, CA, USA) in order to gate for T lymphocytes. Cell viability was detected in PBMC culture by use of propidium iodide (0.5 μg/mL final concentration, Sigma), which was added just before measuring the samples on the FACScalibur (Becton Dickinson, San Jose, CA, USA). Since in the FACS analysis of WB, the erythrocytes are lysed by treatment with FACS lysing solution, and, as a result, some of the lymphocytes become permeable, we could not use propidium iodide for the detection of cell death. Therefore, for the WB cultures, cell viability was detected by staining the cells with a biotinylated antibody directed against nucleosomes (DNA#2) [13], which is only able to stain the nuclei of cells which are permeable. Previous experiments on PBMC have shown that the nucleosome-positive population is the same as the propidium iodide-positive population (Zwart *et al.*, manuscript in preparation). The stained cells were washed and incubated with streptavidin-APC (1:750, Pharmingen). Subsequently, the cells were washed, and erythrocytes were lysed with FACS lysing and permeabilization buffer (Becton Dickinson, San Jose, CA, USA) after which the samples were analyzed on the FACScalibur. Appropriate isotype control mAbs were used in each experiment.

RESULTS

4-hydroxy-oxyphenbutazone is the best inhibitor of IL-6 and GM-CSF production by PBMC *in vitro*

4OH-OPB, which has an extra hydroxyl group attached to the pyrazolidinedione ring as compared to OPB, is in a phase II clinical trial for its immunosuppressive effect in the treatment of RA. In order to see if 4OH-OPB shows an immunosuppressive effect *in vitro*, we studied 4 OH-OPB and a panel of 7 other compounds related to PB *(table 1)*, together with PB and OPB, for their effect on cytokine production by LOS and anti-CD3/anti-CD28-stimulated PBMC. A three-day incubation of anti-CD3/anti-CD28-stimulated PBMC in the presence of a series of concentrations (up to 40 μM) of the different compounds showed that the GM-CSF production by the T lymphocytes present in the PBMC cultures was impressively inhibited when cells were incubated with 4OH-OPB. At a concentration of 1.25 μM, total inhibition was seen *(table 2, figure 1A)*. Compound number 4, PB and OPB also inhibited GM-CSF production, but at a higher concentration, with an IC_{50} value around 30 μM *(table 2, figure 1A)*. Compound number 6 also inhibited GM-CSF production but with a different dose-response (more gradual) than seen for compound

Table 2
IC$_{50}$ (in μM) of tested compounds of LOS-induced, IL-6 production
and the anti-CD3/anti-CD28-induced GM-CSF production

Compound name/number (n)	aCD3/aCD28 stimulation	LOS stimulation
PB (8)	32 ± 12	> 40
OPB (8)	31 ± 11	36 ± 27
4OH-OPB (8)	0.38 ± 0.27	0.16 ± 0.13
1 (3)	> 40	> 40
2 (3)	> 40	> 40
3 (3)	> 40	> 40
4 (3)	30 ± 10	13 ± 4
5 (3)	> 40	> 40
6 (3)	8 ± 4	> 40
7 (3)	> 40	> 40

IC$_{50}$ values (the concentration of the compound needed to inhibit 50% of the maximum response) were determined by using 2-fold titration curves of the compounds. The minimum concentration tested was 0.15 μM and the maximum concentration was 40 μM. Results represent the mean ± standard deviation (see first column for number of donors, n). IL-6 in the culture supernatant was determined after 24h of LOS stimulation of the PBMC by ELISA. GM-CSF in the culture supernatant was determined after 3 days of anti-CD3/anti-CD28 stimulation of the PBMC by ELISA.

4OH-OPB *(table 2)*. A slight inhibition by this compound is already seen around 2.5 μM, but at 40 μM there is still no total inhibition (data not shown).

Also, the LOS-induced IL-6 production by the monocytes present in the PBMC culture could be inhibited at a very low concentration of 4OH-OPB. The inhibiting concentration was lower than for the anti-CD3/anti-CD28-induced GM-CSF production *(table 2, figure 1B)* by a factor of 2. Again, a much higher concentration of compound number

4 and OPB was needed compared to 4OH-OPB *(table 2)*. In contrast to their effect on T cell-induced GM-CSF production, PB and compound number 6 did not show an effect on LOS-induced IL-6 production at 40 μM *(table 2, figure 1B)*. None of the other compounds showed an inhibitory effect in the concentration range tested for both the anti-CD3/anti-CD28 and LOS stimulation *(table 2)*.

As 4OH-OPB was by far the most effective compound in inhibiting cytokine production and is in a phase II clinical trial for its immunosuppressive effect, we investigated this compound in more detail. The effect of 4OH-OPB on LOS-induced IL-6 production was compared with the production of other monokines produced by the PBMC. All monokines (IL-1β, IL-6, IL-8, IL-10, IL-12 and TNFα) were similarly inhibited by 4OH-OPB (data not shown). The same holds true for the effect of 4OH-OPB on the production of lymphocyte-specific cytokines (IFNγ, TNFα, IL-13) by PBMC, both Th1 and Th2 lymphokines were inhibited (data not shown).

Effect of 4OH-OPB in WB cultures

We investigated whether 4OH-OPB was also able to inhibit cytokine production in a WB culture system. A diluted WB culture may be the most appropriate conditions in which to study the influence of drugs on cytokine production *in vitro*, as this is a better mimic of the drug environment. In the WB cultures, much more 4OH-OPB was needed to induce the same inhibition of cytokine production as in PBMC cultures. This was the case for both the monokines (IL-6) and lymphokines (GM-CSF) pro-
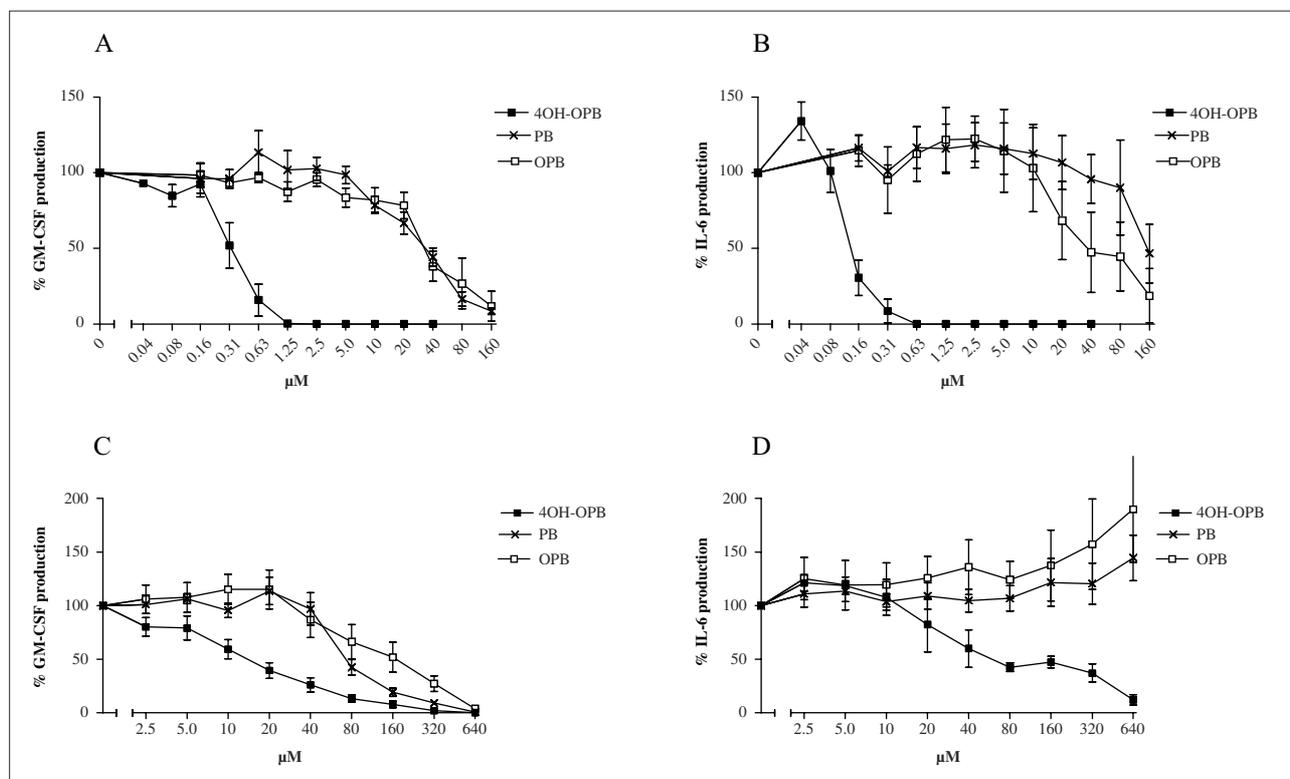
Figure 1
The effect of PB, OPB and 4OH-OPB on cytokine production in PBMC and WB cultures.
PBMC (**A, B**) or WB (**C, D**) of healthy volunteers were stimulated with anti-CD3/anti-CD28 (**A, C**) or LOS (**B, D**) and incubated with a concentration range of the tested compounds for 3 days or 24 hours respectively. The GM-CSF (**A, C**) or IL-6 (**B, D**) production of these cells was determined by ELISA, the value without drug was set at 100%. (Mean of 7 donors ± standard error of the mean (SEM)).
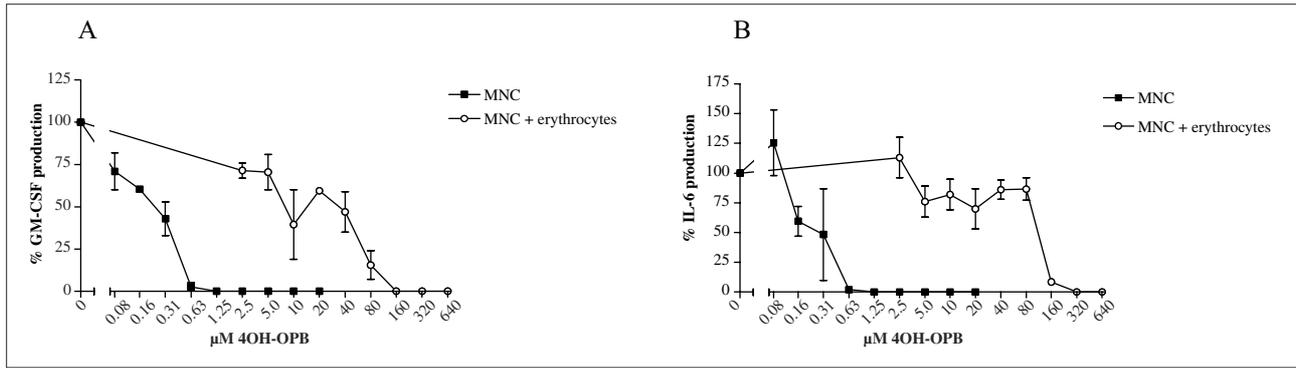
Figure 2
The effect of the presence of erythrocytes during PBMC culture on the inhibitory capacity of 4OH-OPB.
PBMC of healthy volunteers in the absence or presence of 5x 10^7 erythrocytes were stimulated with anti-CD3/anti-CD28 (**A**) or LOS (**B**) and incubated with a concentration range of 4OH-OPB for 3 days or 24 hours respectively. The GM-CSF (**A**) or IL-6 (**B**) production of these cells was determined by ELISA, the value without drug was set at 100% (mean of 2 donors ± SEM).

duced; about 400- and 40-fold more 4OH-OPB was needed to induce 50% inhibition, respectively *(figure 1C and D)*. OPB did not inhibit the monokine production in WB in the concentration range tested, and about 5 times more OPB was necessary to cause 50% inhibition of lymphokine production when compared to PBMC cultures. The effect of PB on the inhibition of cytokine production was almost the same in WB as in PBMC cultures: no inhibition of the monokine IL-6 was detected and just a 2-fold higher concentration of PB was necessary for inhibition of the lymphokine GM-CSF *(figure 1C and D)*. In conclusion, these data show that 4OH-OPB can inhibit monokine and lymphokine production in WB cultures, although at higher concentrations than found in PBMC cultures. Furthermore, 4OH-OPB was the only compound which could inhibit monokine production in WB cultures. In WB cultures, 4OH-OPB was about 4 and 8 times more effective in inhibiting lymphokine production compared to PB and OPB, respectively.

In a 1 to 10 dilution of the WB culture, around the same number of PBMC are present as in the PBMC culture. Therefore, the number of PBMC present in WB cannot be the cause of this large difference in the concentration of drug required to induce similar inhibition of cytokine production. One clear difference between the two *in vitro* culture conditions is the presence of erythrocytes. To see if the presence of erythrocytes could cause the difference in the 4OH-OPB concentration needed between the PBMC and WB cultures, we cultured isolated PBMC in the presence of erythrocytes *(figure 2)*. In the presence of erythrocytes, indeed a higher concentration of 4OH-OPB was needed for inhibition of cytokine production, comparable to the situation seen in the WB cultures.

Interaction of 4OH-OPB with erythrocytes

To explain the effect of erythrocytes on the inhibition of cytokine production by 4OH-OPB, we studied the interaction of 4OH-OPB with erythrocytes in more detail. Erythrocytes were incubated with ^{14}C-labeled 4OH-OPB. We observed that within 10 to 15 minutes of the incubation, around 60% of the radioactivity was associated with the erythrocytes, but that the amount of radioactivity associated with the erythrocytes decreased upon longer incubation times *(figure 3)*. This dissociation of radioactivity

from the erythrocytes had much slower kinetics than the initial association *(figure 3)*.

In order to make a distinction between binding or uptake of 4OH-OPB by the erythrocytes, we added indomethacin to the incubation of 4OH-OPB with the erythrocytes. Indomethacin is an inhibitor of multidrug-resistance-associated proteins (MRP), which are able to pump drugs out of cells [14, 15]. The addition of indomethacin inhibited the dissociation of radioactivity *(figure 3)*, indicating that 4OH-OPB is taken up by the erythrocytes and can not leave the cell because of blockage of MRP. We obtained the same results by using other MRP antagonists, benzbromarone and MK571 (data not shown).

In order to see if the erythrocyte-secreted 4OH-OPB, and/or its metabolites are still able to inhibit cytokine production in PBMC, we loaded the erythrocytes by incubating them for 10 minutes with 4OH-OPB. The free 4OH-OPB was washed away, and after 4 hours the supernatant, containing 4OH-OPB-derived products, was harvested and added to freshly isolated PBMC. Unfortunately we could not use indomethacin-treated erythrocytes as a control, as at the concentration used, indomethacin by itself had already inhibited the cytokine production (data
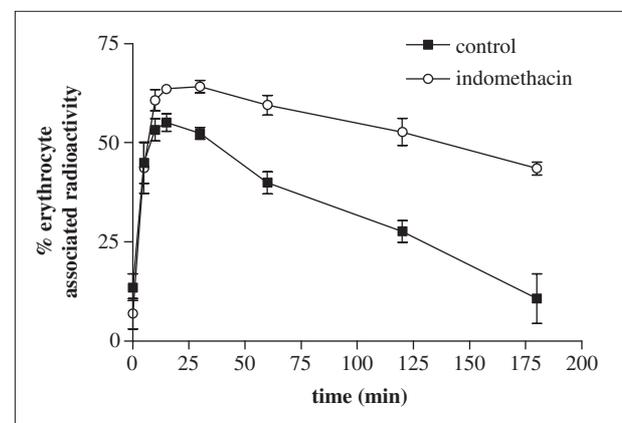

Figure 3
Interaction of ^{14}C labeled 4OH-OPB with erythrocytes.
4×10^7 erythrocytes were incubated with 1 μM ^{14}C-4OH-OPB at 37^0C, in the presence or absence of 250 μM indomethacin. After the incubation, the erythrocytes were spun down and the amount of radioactivity present in the supernatant was determined by scintillation counting (mean of 5 donors ± SEM).
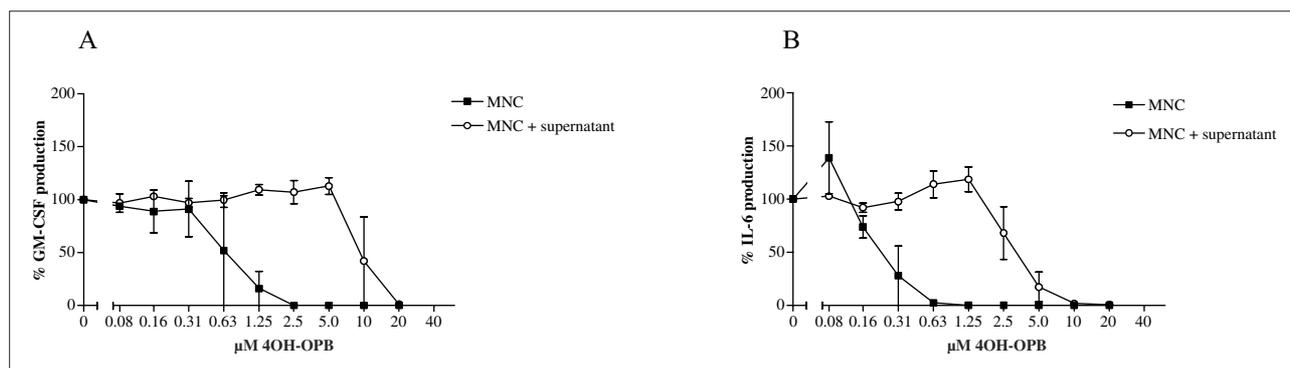
Figure 4

4OH-OPB secreted by erythrocytes is still able to inhibit cytokine production by stimulated PBMC.

1×10^9 erythrocytes were loaded with 20 µM 4OH-OPB for 10 minutes, after which the cells were washed three times and incubated for another 4 hours. Supernatants of the erythrocytes or fresh 4OH-OPB were added to PBMC cultures. PBMC were stimulated with anti-CD3/anti-CD28 (**A**) or LOS (**B**) and incubated for 3 days or 24 hours respectively. The GM-CSF (**A**) or IL-6 (**B**) production of these cells was determined by ELISA, the value without drug was set at 100%. (Mean of 2 donors ± SEM).

not shown). As depicted in *figure 4*, the secreted 4OH-OPB-derived product in the supernatant of the erythrocytes was still able to inhibit cytokine production, although at a higher concentration than 4OH-OPB, which was not incubated with erythrocytes.

Effect of 4OH-OPB on cell viability of lymphocytes in PBMC and WB cultures

To have some idea about the way 4OH-OPB induces the inhibition of cytokine production, we studied whether or not this was due to a lack of viability of the lymphocytes after 3 days of anti-CD3/anti-CD28 stimulation by FACS analysis. The inhibition of GM-CSF production by 4OH-OPB in PBMC occurred almost simultaneously with the loss of viability of the lymphocytes *(figure 5A)*. In contrast, in the WB cultures, at the 4OH-OPB concentration at which the GM-CSF production was inhibited by 50%, no induction of cell death could be detected *(figure 5B)*. At higher concentrations of 4OH-OPB some loss of cell viability could be detected.

DISCUSSION

The screening of a panel of compounds related to PB for their cytokine-inhibiting properties showed that 4OH-

OPB was, by far, the best compound for inhibiting both monokine and lymphokine production in PBMC culture, even better than PB and OPB, which were marketed many years ago as anti-inflammatory drugs [7]. At 160 µM, PB showed almost no inhibition of monokine production and the IC_{50} value for the inhibition of lymphokine production was about 30 µM, while the IC_{50} of 4OH-OPB for the inhibition of cytokine production was between 0.2 and 0.4 µM. The IC_{50} of OPB for the inhibition of cytokine production was around 35 µM. However, when tested in a 1 to 10 dilution of WB culture, which is probably a better *in vitro* system with which to study the effect of immunosuppressive drugs as it better mimics the situation in the body, it was remarkable that a more than 40-fold higher concentration of 4OH-OPB was needed to inhibit cytokine production to the same extent as in PBMC cultures. The difference in the drug concentration needed between PBMC and WB culture was not that dramatic for PB or OPB and similar observations have been made with corticosteroids and Cyclosporin A (unpublished results). Although higher concentrations of 4OH-OPB needed in WB to induce inhibition of cytokine production, 4OH-OPB was still 4 and 8 times more effective in inhibiting lymphokine production in WB cultures than PB and OPB, respectively. Furthermore, 4OH-OPB is the only compound of the three which can inhibit monokine production in WB cultures.
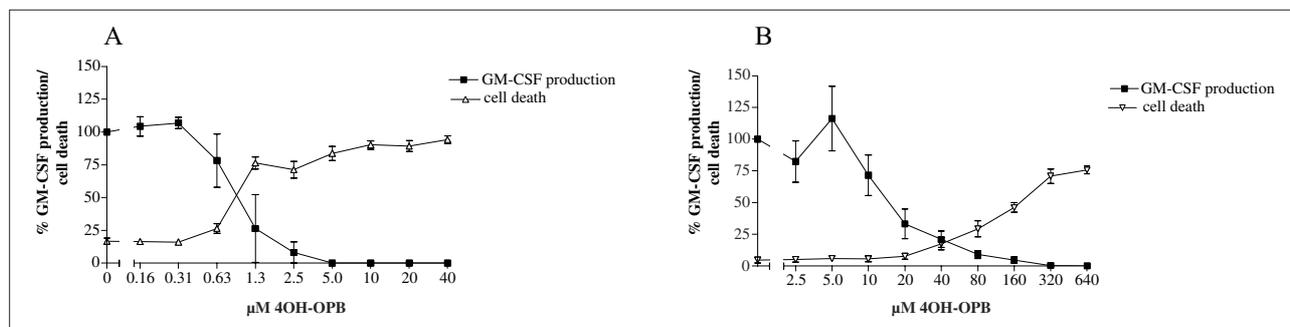

Figure 5

The effect of 4OH-OPB treatment on lymphocyte viability during PBMC or WB culture.

PBMC (**A**) or WB (**B**) of healthy volunteers were stimulated with anti-CD3/anti-CD28 and incubated with a concentration range of 4OH-OPB for 3 days. After 3 days, the GM-CSF production was determined by ELISA, the value without drug was set at 100%, and the cell viability of the lymphocytes was determined as described in materials and methods. The percentage of death cell is depicted (mean of 3 donors ± SEM).

Thus, 4OH-OPB is a better inhibitor of cytokine production than OPB, which in turn, is a better inhibitor of cytokine production than PB. OPB is a metabolite of PB [6]; they differ from each other only in one hydroxyl group at the para position of the phenyl ring. Furthermore, *in vitro* oxidation of OPB leads to the formation of 4OH-OPB; these compounds differ also by only one hydroxyl group, which is attached to the pyrazolidinedione ring [16, 17]. Thus the addition of both hydroxyl groups to PB leads to a drug that is more capable of inhibiting cytokine production *in vitro*. Both hydroxyl groups seem to be required for this activity, as compound numbers 1 and 4, which have only a single hydroxyl group at the pyrazolidinedione ring, have no or less effect on the cytokine production in PBMC compared to 4OH-OPB.

The interaction of 4OH-OPB with erythrocytes seems to be the explanation for the higher concentration of 4OH-OPB needed to induce cytokine inhibition in WB cultures, as this could be mimicked by the addition of erythrocytes to the PMBC culture *(figure 2)*. Furthermore, indicative for the interaction of 4OH-OPB with erythrocytes in WB is the fact that radioactively-labeled 4OH-OPB associated with erythrocytes within 10 minutes of incubation. The fact that blockade of MRP by indomethacin prevents dissociation of the radioactivity from the erythrocytes supports the notion that erythrocytes take up 4OH-OPB.

The compound which is secreted after loading the erythrocytes with 4OH-OPB could still inhibit cytokine production. Although a 16 times higher concentration was needed to establish the same inhibition of cytokine production. This difference in concentration can partly be explained by the fact that not all of the 4OH-OPB was taken up by the erythrocytes before they were washed, and furthermore that not all 4OH-OPB-derived product is transported out of the erythrocytes within 4 hours *(figure 4)*. Thus, the concentration which is actually present in the supernatant is less than expected in theory, but this is most likely in the range of a 2- to 3-fold difference. Therefore, it seems that a higher concentration of 4OH-OPB secreted by the erythrocytes is necessary to induce the same inhibition of cytokine production by PBMC than for 4OH-OPB itself, indicating that the 4OH-OPB-derived product, which is secreted by the erythrocytes, is unlikely to be 4OH-OPB. This is supported by the fact that in WB cultures, cytokine production seems to be inhibited *via* mechanism different from that in PBMC cultures. In PBMC culture, the inhibition by 4OH-OPB of GM-CSF production by the lymphocytes is accompanied by the induction of cell death, while in the WB cultures, no cell death was detected at the IC_{50} value of GM-CSF inhibition after 3 days of incubation. We were not able to study the induction of cell death in the monocyte fraction in the PBMC and WB cultures, as monocytes adhere to the plastic culture wells during culturing and therefore we were not able to analyze them during FACS analysis. However, considering the fact that the LOS-induced IL-6 production by monocytes and the anti-CD3/anti-CD28-induced GM-CSF production by lymphocytes showed the same inhibition profile in PBMC and WB, we believe that the loss of cell viability would also be comparable for lymphocytes and monocytes. Since 4OH-OPB is taken up by erythrocytes, it is possible that the compound has an effect on erythrocyte function. 4OH-OPB did not induce lysis of the erythrocytes at the highest concentration tested (data not shown), but other effects on erythrocyte function can not be ruled out and should be further explored.

In conclusion, 4OH-OPB seems to be a very promising immunosuppressive drug, which is more sensitive than PB, which itself has been used successfully as an anti-inflammatory drug [7]. 4OH-OPB is a better inhibitor of cytokine production than PB; 4OH-OPB is able to inhibit monokine production in WB cultures, while PB was not able to do so in the concentration range tested, and a more than 4-fold less 4OH-OPB than PB is needed for inducing a 50% inhibition of lymphokine production. In addition, taking into consideration the difference in behavior of 4OH-OPB in PBMC and WB cultures and the fact that 4OH-OPB is capable of inhibiting monokine production, while PB does not, it is very likely that 4OH-OPB is a totally different drug from PB. This suggests that 4OH-OPB might lack the side effects seen for PB [8-10, 18]. This is further supported by the fact that Portoghese *et al.* found that 4OH-OPB is not an inhibitor of prostaglandin synthesis as has been ascribed to PB [16].

In view of this, 4OH-OPB could be useful for treating patients with chronic inflammation such as rheumatoid arthritis, patients with acute inflammation such as sepsis, and other diseases in which activation of the immune system contributes to the pathology. Therefore, the mechanism by which 4OH-OPB inhibits cytokine production in both PBMC and WB cultures, which metabolite of 4OH-OPB is involved in the inhibition of cytokine production in WB, and the effect of 4OH-OPB on immune suppression *in vivo* should be further explored.

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