Chemical Synthesis and Biological Activity of Bromohydrin Pyrophosphate, a Potent Stimulator of Human γδ T Cells*

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Small phosphorylated metabolites from mycobacteria stimulate human γδ T lymphocytes. Although such phosphoantigens could prove useful in the composition of vaccines involving γδ T cell-mediated immunity, their very low abundance in natural sources limits such applications. Here, we describe the chemical production, purification, and bioactivity of a phosphorylated bromohydrin (BrHPP) analogue that mimics the biological properties of natural phosphoantigens. This compound can be obtained in gram amounts, is easy to detect, and is of high stability in aqueous solutions. Whereas unspecific binding of BrHPP to a wide panel of cell surface receptors is not detected even at micromolar concentrations, nanomolar concentrations specifically trigger effector responses of human γδ T lymphocytes. Thus, BrHPP is a novel molecule enabling potent immunostimulation of human γδ T lymphocytes.

Stimulating ligands for αβ T lymphocytes are usually composed of single peptides complexed at the surface of major histocompatibility complex molecules. Some small non-peptidic structures, however, may also constitute specific agonist ligands for T cells, particularly γδ T lymphocytes. In human blood, about 3% of T cells initiate their physiological function upon recognition of small phosphorylated non-peptide antigens (phosphoantigens). This cognate interaction involves on the one hand phosphoantigens in the absence of major histocompatibility complex-presenting molecules, and on the other hand, highly selective receptors (TCR) of γδ subtype. In nature, phosphoantigens that can activate human γδ T cells at nanomolar concentrations are produced by Gram-positive and Gram-negative bacteria and also by some eukaryotic parasites and plants. Synthetic analogues of natural phosphoantigens are also known, but their stimulating concentrations for the reactive cells never go below the micromolar range. Mycobacterium tuberculosis, the agent of human tuberculosis, produces four distinct phosphoantigens. These molecules share a moeity that is responsible for the potent stimulation of γδ cells seen in tuberculosis patients (1). The structure of this common core is 3-formyl-1-butyl-pyrophosphate, a recently described phosphoester (2). Its metabolic production might be related to the non-mevalonate (or so-called Rohmer’s) pathway for isoprenoid precursor biosynthesis (3). 3-formyl-1-butyl-pyrophosphate is produced in very small amounts in slow-growing mycobacteria such as Mycobacterium tuberculosis and only accumulates to submicromolar concentrations in culture media from fast-growing mycobacterial species (4). Getting large amounts of highly bioactive phosphoantigens by purification routes from such natural sources is therefore hard to conceive.

Such molecules could prove therapeutically useful for immunotherapeutic approaches involving γδ T cell-mediated immunity, such as elicitation of anti-infectious protection or antitumor immunity (5, 6). To address the need for readily available highly bioactive phosphoantigens, we have developed a synthetic reagent called bromohydrin pyrophosphate (BrHPP), whose biological properties on human T cells are optimized compared with those of 3-formyl-1-butyl-pyrophosphate.

MATERIALS AND METHODS

Chemical Synthesis—All glassware and equipment were dried for several hours prior to use. Unless otherwise stated, the reagents and starting material were from Fluka. Trisodium (R)-S-(bromomethyl)-3-butan-1-yl-diphosphate (BrHPP) was produced as white amorphous powder by the following procedure. Tosyl chloride (4.8 g, 25 mmol) and 4-(N,N-dimethylamino)-pyridine (3.4 g, 27.5 mmol; Aldrich) were mixed under magnetic stirring with 90 ml of anhydrous dichloromethane in 250-ml three-necked flask cooled in an ice bath. A solution of 3-methyl-3-butene-1-ol (2.2 g, 25 mmol) in about 10 ml of anhydrous dichloromethane was then slowly introduced with a syringe through a septum in the flask, and the ice bath was then removed. The reaction was monitored by silica gel TLC (pentane/ethyl acetate, 85:15 (v/v)). After 2 h with constant stirring, the mixture was precipitated by dilution into 1 liter of hexane and filtered, and the filtrate was concentrated under reduced pressure. This filtration/suspension step was repeated using diethyl ether, and the resulting oil was purified by liquid chromatography on silica gel (pentane/ethyl acetate, 85:15 (v/v)), yielding a yellowish oil of 3-methyl-3-butene-1-yl-tosylate (5.6 g, 23.5 mmol, 94% yield) kept under dry N2 at 4 °C (positive mode ESI-MS: m/z 241 [M + H]+; m/z 258 [M + Na]+; MS2 of m/z 258: m/z 190 (C5H12O, loss)).

To a suspension of dibydrogen pyrophosphate (51.5 mmol, 11.1 g) dissolved in 100 ml of deionized water (adjusted to pH 9 with NH4OH) was passed over a cation exchange DOWEX 50WX8 (42 g, 200 meq of form H+)-column and eluted with 150 ml of deionized water (pH 9). The collected solution was neutralized to pH 7.3 using tetra-n-butyl ammonium hydroxide and lyophilized. The resulting hygroscopic powder was solubilized in anhydrous acetone and further dried by repeated evaporation under reduced pressure. The resulting Tris (tetra-n-butyl ammonium) hydrogenopyrophosphate (97.5% purity by HPAEC, see below) was stored (concentration, ~0.5 M) at ~20 °C in anhydrous conditions under molecular sieves. 100 ml of a solution containing 50 mmol of Tris (tetra-n-butyl ammonium) hydrogenopyrophosphate (0.5 M, 2.5 eq) in anhydrous acetone was lyophilized in a 250-ml
three-necked flask cooled in an ice bath were slowly mixed with 20 mmol (4.8 g) of 3-methyl-3-buten-1-yloxytosylate introduced via a septum with a syringe. After 20 min, the ice bath was withdrawn, and the reaction was left under agitation at room temperature for 24 h. The reaction was analyzed by HPAEC (see below), evaporated, and diluted into an acidic solution (pH 2.1) of ammonium hydrogen carbonate (25 mM) and 2-propanol (2 volume %). The resulting mixture was passed over a cation exchange DOWEX 50WX8 (NH4+, 750 meq) column formerly equilibrated with 200 ml of the solution (98 % volume) of ammonium hydrogen carbonate (25 mM) and 2-propanol (2 volume %). The column was eluted with 250 ml of the same solution at a slow flow and collected in a flask kept in an ice bath. The collected liquid was lyophilized. The resulting white powder was solubilized in 130 ml of ammonium hydrogen carbonate (0.1 M) and completed by 320 ml of acetonitrile/2-propanol (v/v). After agitation, the white precipitate of inorganic pyro- and mono-phosphates was eliminated by centrifugation (2100 × g, 10 °C, 8 min). This procedure was repeated three times, the supernatant was collected and dried, and the resulting oil was eluted in 120 ml of water. Remains of unreacted tosylate were obtained by this procedure (75% yield) and were then dissolved in 120 ml of water for oxidation. For 6 mmol of 3-methyl-3-buten-1-ylpyrophosphate triammonium salt were obtained by this procedure (75% yield) and were then dissolved in 120 ml of water and separated from bromides by passing through Dionex OnGuard-Ag (2 meq/unit) cartridges and an on-line column (100 meq, 21 g) DOWEX 50WX8–200 (10 nM) eluted by milli-Q water. Colorless stock solutions of BrHPP (NaBrHPP) were filtered over Acrodisc 25 membranes of 0.2 µm and kept as aliquots at −20 °C.

**HPLC**—Final purification of BrHPP was achieved by HPLC (Spectra system P1000 XR device) on an analytic Symmetry 5 µ C18 column (Waters) eluted at 1 ml/min and 20 °C with the ternary gradient indicated below. Upstream of detectors, a split of eluent distributions (µm/min) was measured with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) was added dropwise until appearance of a persistent yellowish color, yielding after evaporation 5.8 mmol (2.3 g) of an acidic solution (pH 2.1) of BrHPP, which was immediately neutralized by passing over DOWEX 50WX8–200 (NH4+, 48 meq). The ammonium salt of BrHPP obtained after lyophilization was dissolved in water and separated from bromides by passing through Dionex OnGuard-Ag (2 meq/unit) cartridges and an on-line column (100 meq, 21 g) DOWEX 50WX8–200 (10 nM) eluted by milli-Q water. Colorless stock solutions of BrHPP (NaBrHPP) were filtered over Acrodisc 25 membranes of 0.2 µm and kept as aliquots at −20 °C.

Chemical Synthesis of BrHPP—The procedure for BrHPP was conveniently derived from oxidation of isopentenyl pyrophosphate (8–10) by ethyl pyrophosphate. In addition to these parameters, the bromohydrin phosphate BrHPP was selected for its good superimposition to 3fbPP (arbitrarily referred as to the acidification rate). In each experiment, 32 × 10^5 γδ T cells were resuspended in 30 µl of low buffered medium plus 10 µl of melted low temperature-melting agarose at 37 °C. 10 µl of the mixture were rapidly spotted on a cytosensor cell capsule. After 10 min the cell capsule was assembled and loaded in the sensor chamber of a microphysiometer. The experiments were run at 37 °C, and the low buffered medium (pH 7.4) was perfused at 100 µl/min.

**RESULTS**

**Molecular Overlay of 3-Formyl-1-butyl-pyrophosphate and BrHPP—**Former structure-activity relationship studies of natural and synthetic phosphoantigens have shown that among monoesters of pyrophosphate (8, 9), several organic esters with chemical reactivity (e.g. of Sn-2 type) (10) presented γδ cell-stimulating bioactivities higher than that of single chain alkyl, such as ethyl pyrophosphate. In addition to these parameters, a topological fit of the alkyl chain clearly contributes to optimize recognition by the γδ TCR. To select a synthetic phosphoantigen matching 3-formyl-1-butyl-pyrophosphate (3fBP) as much as possible, we over laid this latter compound and several synthetic compounds. The bromohydrin phosphate BrHPP was selected for its good superimposition to 3fBP (arbitrarily referred as to the acidification rate). In each experiment, 32 × 10^5 γδ T cells were washed in phosphate-buffered saline containing 5% fetal calf serum and incubated for 30 min at 4 °C with anti-CD3-PE and anti-82-fluorescein isothio cyanate monoclonal antibodies (Biosource, Camarillo, CA).

**Microphysiometry—**The cell acidification rate was monitored using a cytosensor microphysiometer (Molecular Devices, Crawley, UK), which measures pH of extracellular fluid using a silicon-based method (7). The raw data from sensor output give mV = f(t), which may be converted to pH = f(t). The system allows cells (8 × 10^5) disposed in sensor chambers to be irrigated (flow on period, 90 s) by low buffered RPMI medium (Molecular Devices) containing phosphoantigen or not; then, during a flow off period (30 s), the sensor data are used to calculate a slope, giving ΔpH and referred as to the acidification rate. In each experiment, 32 × 10^5 γδ T cells were resuspended in 30 µl of low buffered medium plus 10 µl of melted low temperature-melting agarose at 37 °C. 10 µl of the mixture were rapidly spotted on a cytosensor cell capsule. After 10 min the cell capsule was assembled and loaded in the sensor chamber of a microphysiometer. The experiments were run at 37 °C, and the low buffered medium (pH 7.4) was perfused at 100 µl/min.

**Phenotype Analysis by Flow Cytometry—**5 × 10^5 cells were washed in phosphate-buffered saline, 5% fetal calf serum and immediately acquired by an EPICS XL flow cytometer (Beckman Coulter).

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**Molecular Overlay of 3-Formyl-1-butyl-pyrophosphate and BrHPP—**Former structure-activity relationship studies of natural and synthetic phosphoantigens have shown that among monoesters of pyrophosphate (8, 9), several organic esters with chemical reactivity (e.g. of Sn-2 type) (10) presented γδ cell-stimulating bioactivities higher than that of single chain alkyl, such as ethyl pyrophosphate. In addition to these parameters, a topological fit of the alkyl chain clearly contributes to optimize recognition by the γδ TCR. To select a synthetic phosphoantigen matching 3-formyl-1-butyl-pyrophosphate (3fBP) as much as possible, we overlaid this latter compound and several synthetic compounds. The bromohydrin phosphate BrHPP was selected for its good superimposition to 3fBP (arbitrarily referred as to the acidification rate). In each experiment, 32 × 10^5 γδ T cells were washed in phosphate-buffered saline containing 5% fetal calf serum and incubated for 30 min at 4 °C with anti-CD3-PE and anti-82-fluorescein isothiocyanate monoclonal antibodies (Beckman Coulter) or isotypic controls. Samples were then washed in phosphate-buffered saline, 5% fetal calf serum and immediately acquired by an EPICS XL flow cytometer (Beckman Coulter).
synthesis was modified from Ref. 11 and is summarized in Fig. 2. A fully detailed description of this synthesis, which yields the racemate of BrHPP, due to asymmetry of the C3 position, is given under “Materials and Methods.” Thus, in the absence of further enantiomer resolution of the synthetic mixture, the produced BrHPP compound corresponds to the racemic structure represented in Fig. 2. The produced BrHPP was essentially devoid of unreacted reagents when using the separation scheme based on differential solvent reprecipitation, as described under “Materials and Methods.” 4–20% of the recovered material still corresponded to other products (e.g., phosphate), warranting a final step of HPLC separation (see below). At this step, BrHPP was obtained as a triammonium salt, which was found to interfere with several cell culture assays (data not shown). It was then converted to BrHPP (Na+ form) by cation exchange. This latter form is stable in aqueous solutions and can be stored at −20 °C for 4 months without detectable structural degradation.

Chromatographic and Structural Assignment of BrHPP—Because of the halohydrin structure of BrHPP, its chromatographic analysis could not be undertaken by HPAEC as described for natural phosphoantigens (12), because hydroxide eluents of HPAEC rapidly catalyzed an epoxide rearrangement by HBr elimination (data not shown). Therefore, a chromatographic procedure for BrHPP analysis was based upon use of near-neutral pH eluents. The stock solutions of BrHPP were analyzed by ion pair reverse-phase C18 HPLC and monitored by UV-visible diode array and MS detection following a procedure described previously, with minor modifications (see “Materials and Methods” and Ref. 1). As shown in Fig. 3A, several UV-absorbing and phosphorylated contaminants eluted close to the major peak of BrHPP (sample injected: 0.75 mM, Rt = 5.3 min.). Ion-trap MS in negative mode of the collected fractions gave a unique set of signals at m/z 341 and 343, as expected for the pseudomolecular anion of BrHPP. Its composition, C9H12P2O3Br, was supported by the relative abundance of natural bromine isotopes evidenced by zoom scan (Fig. 3B). Negative mode MS2 of m/z 341 showed a unique fragment of m/z 261 corresponding to an epoxide rearrangement after HBr loss. Subsequent MS3 of m/z 341, corresponding to MS2 of m/z 261, showed its [(M−H)−H2O] fragment (m/z 243) as well as diagnostic ions for pyrophosphate and phosphate (m/z 159, P2O5H2; m/z 97, PO4H2−) moieties, respectively. 31P NMR (D2O) confirmed the BrHPP structure: δ 1.41 (s, 31P), Me; 2.06 (t, J = 6 Hz, 2H), CH2; 3.56 and 3.60 (AB, J = 11 Hz, 2H), CH2Br; 4.10 (m, 2H), CH2O. 31P NMR (D2O) was as follows: δ −10.38 (d, J = 19.5 Hz); −11.04 (d, J = 19.5 Hz).

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**Fig. 1. Molecular envelopes of synthetic BrHPP and of some natural phosphoantigens: 3-formyl-1-butyl-pyrophosphate and isopentenyl pyrophosphate.** Molecular envelopes of mycobacterial 3-formyl-1-butyl-PP, isopentenyl-PP, and BrHPP were computed for molecules in triacidic form under stretched conformation. Molecular volumes were as follows: 3-formyl-1-butyl-PP, 198 Å3; isopentenyl-PP, 191 Å3; BrHPP, 199 Å3. Molecular surfaces were as follows: 3-formyl-1-butyl-PP, 170 Å2; isopentenyl-PP, 163 Å2; BrHPP, 176 Å2. Computing and images were generated with Alchemy 2000 (Tripos) and Swiss PDBViewer 6.3.

**Fig. 2. Scheme of chemical synthesis of BrHPP.**

**Fig. 3. Chromatographic isolation and mass spectrometry of BrHPP.** A, BrHPP was analyzed using reverse-phase HPLC and diode array detection in UV over the λ range of 200–400 nm. The major fraction absorbing at low wavelength (226 nm) and eluting around 5 min corresponds to BrHPP, as identified by MS analysis. The dotted box shows the BrHPP fraction collected for MS and biological analysis. B, ion trap MS analysis of the BrHPP fraction in negative ionization mode. Full scan analysis of the HPLC eluate was obtained using the standard LC-MS coupling and ESI source, whereas MS2 and zoom scan were done by nanospray of the purified BrHPP. The main BrHPP spectrum shows two (M−H)+ pseudomolecular ions with equal relative abundance (zoom scan) due to natural bromine isotopes. Their respective MS2s show only HBr loss, both leading to the same epoxide rearrangement (m/z 261). The latter ion fragments further in MS3 by water loss and generation of both P and PP daughter ions.

**Biological Properties of BrHPP: Receptor Profiling of BrHPP—**To search for further putative receptors for BrHPP, a high concentration (10 μM) of this molecule was assayed for inhibition of the selective binding of various reference radioligands on their nominal receptors (13). BrHPP was not found to interfere significantly with any of the 70 binding assays tested (see “Appendix”). This suggested that mammalian receptors for BrHPP might be restricted to the formerly characterized Vγδ/ 
Vδ2-encoded antigen receptors borne by phosphoantigen-reactive human γδ T lymphocytes (1, 8, 9, 14–16).

BrHPP Activates Proliferation and Cytokine Release by TCR γδ T Lymphocytes—T lymphocytes respond to antigenic activation by proliferating, secreting cytokines, and/or mediating toxicity for target cells. Accordingly, when they are stimulated by the mycobacterial phosphoantigen 3fbPP, γδ T cells expressing the Vγ9/Vδ2-encoded TCR expand specifically in culture and secrete TNF-α (2). Thus we tested whether increasing concentrations of BrHPP would lead to the selective outgrowth of the Vγ9/Vδ2 T lymphocytes in in vitro cultures of PBL. The result of a representative experiment (of 10 independent ones) is shown in Fig. 4A. BrHPP proved to be as potent a stimulus as whole mycobacterial extract for the in vitro expansion of the Vγ9/Vδ2 T cells. This property was abrogated by dephosphorylation of BrHPP using alkaline phosphatase in the culture, demonstrating that BrHPP owes its bioactivity on γδ T cells to its pyrophosphate moiety, a characteristic hallmark of all natural and synthetic phosphoantigens (1, 17). In addition to proliferative responses, human γδ T cells reactive to phosphoantigens frequently secrete cytokines in response to antigen stimulation. When γδ T cells were exposed to BrHPP, the production of high levels of interferon-γ (Fig. 4C) and TNF-α (Fig. 4D) by cultured γδ T cells in response to BrHPP.
Perfusion of γδ T cells with BrHPP stimulates a rapid metabolic activation witnessed by early extracellular acidification.

A, pulsing of γδ T cells in cytosensor chambers with increasing concentrations of BrHPP induce a sustained extracellular acidification. B, microphysiometric titration of the BrHPP dose effect on γδ cells. The area below each acidification curve over 1 h in the presence of BrHPP was calculated, normalized to 100% using the largest response, and plotted as a function of BrHPP concentration. C, a physiological delay of around 10 s separates exposure to BrHPP and the metabolic response of γδ cells, evidenced by a relative drop in redox potential (mV) of the stimulated versus unstimulated trace (arrows).
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BrHPP presents the same T cell-stimulating property as natural phosphoantigens.

**Bromohydrin Pyrophosphate Causes Early Activation of Specific γδ T Cells**—Intracellular transduction of activating signals delivered by the encounter of the natural phosphoantigen 3βPP involves extracellular acid release by reactive γδ T cells (2). A similar pathway of signal transduction could be expected for recognition of a phosphoantigen mimicking 3βPP, such as BrHPP. We tested this using Cytosensor® microphysiometry, to detect whether γδ T cell activation with BrHPP led to early acid release, as already reported for αβ T cells (18). Fig. 5A shows that a transient (10 min) pulse of γδ cells with BrHPP does result in acid release (7). As illustrated in Fig. 5B, the dose-response relationship in this experiment matched the dose responses for cytokine release (Fig. 4, C and D). Whereas this early metabolic response was not sustained at low BrHPP concentrations (5 and 25 nM), the initial burst was followed by sustained signaling for at least 1 h when cells were triggered by high BrHPP concentrations (50–100 nM, Fig. 5A). In this latter case, the metabolic burst presented a particularly rapid onset, as evidenced by comparing the raw data from an unstimulated chamber (Fig. 5C, lower trace) and from a chamber perfused with BrHPP (Fig. 5C, upper trace). When taking into account the delay for BrHPP diffusion onto the cells (7 s in these assays), the mV collapse recorded in the perfused chamber indicated that extracellular acidification by γδ T cells had occurred about 10 s after exposure to BrHPP. Thus, under saturating concentrations, phosphoantigen recognition by the γδ TCR leads to very rapid activating events.

**DISCUSSION**

This paper details the structure, chemical synthesis, and biological property of bromohydrin pyrophosphate, a novel molecule activating human γδ T lymphocytes. Based on the molecular overlay of this compound with the natural γδ T cell ligand found in mycobacteria, it was hoped that this synthetic compound could mimic the biological properties of the naturally occurring 3βPP, i.e., activation of a specific γδ T cell subset in human blood (see Ref. 19 for a review). We describe a convenient and straightforward mode of synthesis for producing BrHPP. This simple method is based on pyrophosphorylation of the tosylated C5 precursor, followed by stoichiometric oxidation of the pyrophosphoester product in aqueous bromine. Because the compound carries a chiral C3, the resulting product is a racemic mixture used without further resolution of enantiomers. This straightforward and inexpensive synthesis is followed by a purification scheme involving solvent precipitation, LC, and HPLC to eliminate residual inorganic phosphate and bromide. The final stock solutions of BrHPP (Na+) salts are very stable and can be stored for several months without degradation. Little information about structural changes of the organic moiety of phosphorylated metabolites is usually drawn from HPLC-MS in negative mode, thereby limiting its use as an analytical tool. The mass spectral data of BrHPP presented here demonstrate a highly sensitive detection of BrHPP in aqueous phases, and its bromine content enables unambiguous detection for pharmacological follow-up studies. For the reasons listed above, BrHPP appears to be a promising lead candidate for therapeutic explorations among synthetic phosphoantigens.

**In vitro** cultures of bulk human lymphocytes carried out in the presence of 100 nM BrHPP and IL-2 lead to the systematic expansion of T lymphocytes that express the phosphoantigen-reactive γδ82 TCR, and no other cell subset. This has been described previously for total lymphocyte populations stimulated by crude extracts from *M. tuberculosis* and is known to rely upon the presence of several stimulating phosphoantigens. Here, BrHPP also acts as a phosphoantigen agonist, because BrHPP dephosphorylation abolishes this bioactivity. Exposure to BrHPP also elicits TNF-α and interferon-γ release, indicating that the full range of γδ T cell effector responses is activated by this ligand. Microphysiometric analysis showed that this activation results from exposure to a typical agonist (20). Its early signal transduction involves a BrHPP dose-dependent extracellular acid release, as was shown for αβ T cells stimulated with peptide-major histocompatibility complex tetramers (21). Whereas high bioactive doses of BrHPP triggered a strong acidification burst followed by a sustained intracellular signaling, suboptimal BrHPP concentrations (5 nM) led to barely detectable signaling. When rapidly exposed to saturating BrHPP concentrations, γδ cells respond by extracellular acidification within about 10 s, indicating that little (if any) intermediate processing of the stimulating BrHPP occurs prior to triggering the T cell reaction.

In summary, although synthetic BrHPP presents the same biological properties as natural phosphoantigens, the possibility of synthesizing gram amounts from simple procedures bypasses the production drawbacks of the natural counterparts. This makes BrHPP an attractive candidate for investigations of selective γδ T cell-based immunomodulation approaches. Future studies will evaluate the potential of this novel immunostimulating molecule in subunit vaccines where γδ T cell contribution *in vivo* is expected to be beneficial, either as antituberculous immunity and protection against acute leukemia.

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**Table I**

Receptor-antigens unaffected by BrHPP

| Receptor | Origin | Ligand | Conc. | Non-specific | Incubation |
|---------|--------|--------|-------|-------------|------------|
| AγδT (3) | human recombinant (CD3 ωδε) | 3βPPCPA | 1 nM | DCPA (1 μM) | 60 min/20°C |
| Aαβ (4) | human recombinant (HBE 715 cells) | 3βPPCPA | 6 nM | NCA (10 μM) | 10 min/22°C |
| AγδT (5) | human recombinant (HBE 715 cells) | 3βPPCPA | 1 nM | NCA (1 μM) | 60 min/22°C |
| δ2 (non-selective) | rat, cerebral cortex | 3βPPCPA | 0.25 nM | (styrene-β-cyclodextrin) | 30 min/22°C |
| δ2 (selective) | rat, cerebral cortex | 3βPPCPA | 0.3 nM | (styrene-β-cyclodextrin) | 30 min/22°C |
| β (6) | human recombinant (HBE cells) | 3βPPCPA | 0.13 μM | allopurinol (0 μM) | 60 min/22°C |
| δ (7) | human recombinant (HBE cells) | 3βPPCPA | 0.13 μM | allopurinol (0 μM) | 60 min/22°C |
| δ (8) | mouse recombinant (HDC 5 cells) | 3βPPCPA | 0.9 μM | allopurinol (0 μM) | 60 min/22°C |
| δ (9) | human recombinant (HDC cells) | 3βPPCPA | 0.05 μM | allopurinol (0 μM) | 60 min/22°C |
| δ (10) | mouse recombinant (HDC cells) | 3βPPCPA | 0.05 μM | allopurinol (0 μM) | 60 min/22°C |
| δ (11) | mouse recombinant (HDC cells) | 3βPPCPA | 0.05 μM | allopurinol (0 μM) | 60 min/22°C |
| δ (12) | mouse recombinant (HDC cells) | 3βPPCPA | 0.05 μM | allopurinol (0 μM) | 60 min/22°C |
| δ (13) | mouse recombinant (HDC cells) | 3βPPCPA | 0.05 μM | allopurinol (0 μM) | 60 min/22°C |
| δ (14) | mouse recombinant (HDC cells) | 3βPPCPA | 0.05 μM | allopurinol (0 μM) | 60 min/22°C |
| δ (15) | mouse recombinant (HDC cells) | 3βPPCPA | 0.05 μM | allopurinol (0 μM) | 60 min/22°C |
| δ (16) | mouse recombinant (HDC cells) | 3βPPCPA | 0.05 μM | allopurinol (0 μM) | 60 min/22°C |
| δ (17) | mouse recombinant (HDC cells) | 3βPPCPA | 0.05 μM | allopurinol (0 μM) | 60 min/22°C |
| δ (18) | mouse recombinant (HDC cells) | 3βPPCPA | 0.05 μM | allopurinol (0 μM) | 60 min/22°C |
Receptor/ligand bindings that were tested and found to be unaffected by 10 μM BrHPP are listed in Table I. The binding assays were performed as follows. For each receptor binding assay, the respective reference ligand was tested in duplicate at a minimum of eight concentrations to obtain a competition curve to validate the titration. In parallel, 10 μM BrHPP was tested in duplicate in each assay. Following incubation, the membranes or cells in suspension were rapidly harvested onto glass fiber filters with an ice-cold buffer using a cell harvester. Bound radioactivity was then measured with a scintillation counter (Betaplate, Wallac). The specific radio-ligand binding to the receptors is defined as the difference between total binding and nonspecific binding determined in the presence of an excess of unlabelled ligand. Results are expressed as percent inhibition of control specific binding obtained in the presence of BrHPP. Conc. represents the concentration causing a half-maximal inhibition of control specific binding.

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