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Enhancement of Th1 type cytokine production and primary T cell activation by PBI-1393

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Received 19 January 2007; accepted with revision 27 July 2007

Available online 12 September 2007

KEYWORDS

PBI-1393;
IL-2;
IFN- γ ;
Th1 cytokine;
Immunostimulant;
Concanavalin A

Abstract In previous reports, we have shown that PBI-1393 (formerly BCH-1393), *N,N*-Dimethylaminopurine pentoxycarbonyl *D*-arginine, stimulates cytotoxic T-lymphocyte (CTL) responses both *in vitro* and *in vivo* in normal immune status and immunosuppressed mice. Additionally, PBI-1393 was tested for anticancer activity in syngeneic mouse experimental tumor models and it displayed significant inhibition of tumor outgrowths when given in combination with sub-therapeutic doses of cytotoxic drugs (cyclophosphamide, 5-fluorouracil, doxorubicin and *cis*-platinum). However, the mechanism of action of PBI-1393 was still unknown. Here, we report that PBI-1393 enhances IL-2 and IFN- γ production in human activated T cells by 51% and 46% respectively. PBI-1393 increases also IL-2 and IFN- γ mRNA expression as shown by RT-PCR. The physiological relevance of IL-2 and IFN- γ gene modulation by PBI-1393 is illustrated by the advantageous increase of T cell proliferation ($39 \pm 0.3\%$ above control) and human CTL response against prostate (PC-3) cancer cells ($42 \pm 0.03\%$). The enhancement of human T cell proliferation and CTL activation by PBI-1393 demonstrates that this compound potentiates the immune response and in this regard, it could be used as an alternative approach to IL-2 and/or IFN- γ therapy against cancer.

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Introduction

Therapeutic agents or immunostimulants that are able to restore or enhance the performance of the immune system are of interest in clinical practice. The most appropriate immunostimulants are those used by the organism in physiological conditions, in particular cytokines. A wide array of cytokines are known to be involved in immune

response. However, two of them, interleukin-2 (IL-2) and interferon- γ (IFN- γ), present a particular interest. Indeed, they play a predominant role in adaptive immune response and host defense against infectious agents and malignant cells.

IL-2 was originally described as a T cell growth factor. It promotes the proliferation, activation and differentiation of T and B cells [1,2]. IL-2 can also stimulate natural killer (NK) and inflammatory cells including monocyte/macrophages and neutrophils [3–5]. The ability of IL-2 to activate NK cells and to enhance the response of CTL against tumor cells has attracted the interest of clinicians. IL-2 based therapy was

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approved since two decades by the FDA for the treatment of advanced renal carcinoma [6,7]. Despite the relative success of this therapy, the administration of IL-2 was limited to 1 out of 10 patients in the USA due to the resulting toxicities [8].

IFN- γ is a potent immunomodulatory cytokine predominantly produced by immune and inflammatory cells including CD4⁺, CD8⁺ and NK cells [9]. IFN- γ is involved in a variety of immune responses including T cell proliferation and differentiation [10,11], monocyte/macrophage activation [4], pro-inflammatory cytokine production (TNF- α , IL-1 β) [12,13], and IFN- γ induced up-regulation of major histocompatibility complex class I and II expression [14,15].

Used as a therapeutic agent, IFN- γ showed significant efficacy for the treatment of chronic granulomatous disease [16]. However, similar to IL-2, administration of IFN- γ to patients induces several side effects including cardiotoxicity and depression [17,18].

As an alternative to cytokine therapy, synthetic immunostimulants such as levamisole and tucaresol have been proposed [19]. Both compounds have shown a significant activity on T cell lineage similar to thymic hormone. However, they showed a slow efficacy and serious side effects when used in clinical trials [20,21]. Other compounds, particularly hypoxanthine derivatives that are nontoxic, were also used as immunostimulants [22]. However, they are usually not very active.

Based on hypoxanthine derivatives, PBI-1393 was designed to be a more active and less toxic immunostimulant [23]. *In vitro* and *in vivo* assessment of PBI-1393 activity in mice demonstrated a significant increase in CTL responses [23]. In addition, PBI-1393 induced a significant increase of the relative percentage of CD4⁺, CD8⁺, NK and monocyte subsets without any evidence of toxicity. More importantly, PBI-1393 exhibited a synergistic anti-tumor activity when used in combination with cyclophosphamide, doxorubicin and 5-fluorouracil (5-FU) in breast (DA-3) and colon (MC38) tumor models in mice [24].

Based on the ability of PBI-1393 to stimulate CTL activity, we hypothesized that this compound may increase Th1 cytokine production. In this work, it is shown that PBI-1393 enhances both IL-2 and IFN- γ production in activated T cells. The physiological relevance of this effect is illustrated by an increase of human T cell proliferation and CTL activation. The stimulatory activity of PBI-1393 on Th1 cytokine production suggests that this compound may be used as an adjunct in viral or cancer therapy.

Materials and methods

RPMI 1640, fetal bovine serum (FBS), concanavalin A (con A) and protease inhibitor cocktail were purchased from Sigma Chemical Co. (St. Louis, MO). Alamar Blue was from Biosource International (Camarillo, CA). IL-2 and IFN- γ ELISA were from BD Biosciences Canada. ERK1/2 was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Jurkat E6-1 and PC-3 cells were from American Type Culture Collection (Rockville, MD). Ficoll-hypaque and ⁵¹Cr were from GE-Amersham (Canada). PBI-1393 was synthesized as previously described [23]. Trizol reagent, Moloney murine leukemia virus reverse transcriptase (M-MLV), dNTPs, oligo-dT, dithiothreitol RNAs inhibitor (RNAsOUT) and Taq DNA polymerase were from Invitrogen Life technology

(Canada). Anti-CD3 and anti-CD28 antibodies were from R&D systems (Minneapolis, MN).

Cell culture

The human leukemic T cell line Jurkat, clone E6.1 and PC-3 cell lines were grown in RPMI-1640 medium supplemented with 10% heat inactivated FBS, 10 mM HEPES and 1 mM sodium pyruvate.

Determination of secreted cytokines by ELISA

Human peripheral blood mononuclear leucocytes (PBML) and Jurkat cells were incubated (2×10^6 cells/ml) in RPMI-1640 medium supplemented with 10% FBS. Cells were then treated with con A and/or PBI-1393 and incubated for appropriate periods of time. Cell suspensions were then centrifuged briefly and cell-free supernatants were analyzed for their content in cytokines using commercially available specific ELISA kits, according to manufacturers' instructions.

Isolation of total RNA and RT-PCR

Total RNA was isolated from freshly harvested cells using Trizol reagent. RNA samples (2 μ g) were heated at 65 °C for 10 min and reverse transcribed for 1 h at 37 °C using M-MLV reverse transcriptase in 20 μ l of reaction buffer containing 0.5 mM dNTPs, 1 μ M oligo-dT, 10 mM dithiothreitol, 40 U RNAsOUT, 45 mM Tris-HCl pH 8.3, 70 mM KCl and 3 mM MgCl₂. Samples were denatured at 94 °C for 2 min and stored at -20 °C. Resulting cDNA (2 μ l) were used as a template for PCR amplification with Taq DNA polymerase. The primer sequences are: for human IL-2: forward primer, 5' CAC TAC TCA CAG TAA CCT CAA CTC CTG 3'; and reverse primer, 5' GTG GGA AGC ACT TAA TTA TCA AGT CAG TG 3' (Product size, 521 base pairs). Primers for human IFN- γ : forward primer, 5' GTT TTG GGT TCT CTT GGC TGT 3'; and reverse primer, 5' ATG TAT TGC TTT GCG TTG GAC 3' (Product size, 350 base pairs). Primer for human *c-fos*: forward primer, 5' AAG GAG AAT CCG AAG GGA AAG GAA TAA GAT GGC T3', reverse primer, 5' AGA CGA AGG AAG ACG TGT AAG CAG TGC AGC T 3' (Product size, 612 base pairs). Primer for human *c-jun*: forward primer, 5' TCC CCG CGC GAA CGG AAC GTT GGA CT 3', reverse primer, 5' CT CCC CCC GCT TTG TGT TCT TAA GGA 3' (Product size, 170 base pairs). Primer for β -actin: forward primer, 5'-CGT GGA CAT CCG TAA AGA CC-3', reverse primer, 5'-ACA TCT GCT GGA AGG TGG AC-3' (Product size, 209 base pairs). Samples were amplified at 94 °C for 1 min, 60 °C for 1 min and 72 °C for 2 min. The number of cycles ensuring that the reaction remained in the linear range was previously determined. The final PCR products were analyzed by electrophoresis on 2% agarose gel containing ethidium bromide along with DNA markers. Band density was analyzed using Instant Imager System 2000.

Quantification of PCR amplicons by ELISA-PCR

Quantification of PCR products was performed as described by Puntschart et al. [25]. Briefly, PCR amplicons were labeled during the amplification by the incorporation of digoxigenin-dUTP (PCR DIG labeling mix, Boehringer Mannheim), PCR

products (10 μ l) were then denatured by adding 40 μ l of 0.1 μ M of biotinylated probe (5'GCC CAA GAA GGC CAC AGA ACT G for IL-2; 5' GGC TGT TAC TGC CAG GAC CCA for IFN- γ ; 5' CCC GGA GTC TGA GGA GGC CTT for *c-fos* and 5' TAC CCT GGC ATT GCC GAC AGG A for β -actin) diluted in 0.1 M of NaOH. Then 50 μ l of hybridization buffer were added [6 \times saline–sodium citrate (SSC), 10 μ M Tris HCl (pH 7.4), 10 mM EDTA, and 0.2 M HCl], and samples were incubated at room temperature for 1 h. 50 μ l from each well were then transferred to a streptavidin-coated microplate to allow binding of hybrids to streptavidin during 30 min of incubation. Plates were washed with TBST, 50 μ l of alkaline phosphatase-conjugated anti-digoxigenin antibody (Fab fragments, Roche) was added to each well and the incubation was performed for 30 min at room temperature. Then 50 μ l of freshly prepared substrate were added: 10% (vol/vol) diethanolamine, 0.5 mM MgCl₂, 0.08 M HCl, and 4 mg/ml of *p*-nitrophenyl phosphate (Boehringer). After incubation for 30 min, absorbance at 405 nm was determined in an ELISA reader (Bio-Rad). The control without template served as background.

T cell proliferation assay

T cell proliferation assay was performed in 96-well plates in triplicate, in a final volume of 200 μ l of RPMI-1640 supplemented with 10% FBS. Human purified PBML (2.5×10^4 cells/well) were cultured with PBS (negative control), PBI-1393 (10^{-7} – 10^{-5} M), con A (5 μ g/ml) or with a combination of con A and PBI-1393. Cultures were incubated at 37 °C in 5% CO₂ atmosphere, for 48 h. Alamar Blue was then added and fluorescence was measured after 4 h according to the manufacture's instructions. Relative Fluorescence Unit that is proportional to cell proliferation was measured using Tecan Genios Plus instrument. Results were expressed as relative proliferation (fold increase) to basal level.

Generation of human CTL

PBML were separated from heparinized venous blood by Ficoll-Hypaque density gradient centrifugation. Cells were then resuspended in fresh RPMI-1640 supplemented with 10% FBS and 120 μ g/ml of PC-3 cell lysate was added to the cell suspension. Cells were then seeded at 2×10^6 cells/ml/well in 12 well plates and incubated for 6 days in the presence of IL-2 (10 ng/ml) or PBI-1393 (10^{-7} – 10^{-5} M). PBML were harvested and used as effector cells in a ⁵¹Cr cytotoxicity assay.

⁵¹Cr cytotoxicity assay

Adherent target cells (PC-3) were labeled with 100 μ Ci of Na₂⁵¹CrO₄ per 10⁶ cells for 1 h at 37 °C. Labeled PC-3 cells were mixed with effector cells (1:100) and incubated for 4 h at 37 °C. The radioactivity present in the supernatant was then determined in a gamma counter. ⁵¹Cr total release was determined by treatment of target cells with triton X-100. CTL activity was calculated as the specific release of ⁵¹Cr from target cells using the following equation: specific release (%) = (ER – SR) / (TR – SR) \times 100% where ER = experimental release, TR = total release and SR = spontaneous release.

Preparation of nuclear extracts

Cells were treated with con A (5 μ g/ml), PBI-1393 and/or a combination of con A and PBI-1393 (10^{-5} M) for 1 h and then harvested by centrifugation. Nuclear extracts (NE) were prepared as previously described [26]. Briefly, cells were harvested, washed twice in cold PBS, and suspended in 200 μ l of buffer A (10 mM KCl, 10 mM HEPES (pH 7.9), 0.1 mM EGTA (pH 7.9), 0.1 mM EDTA (pH 7.9), 1 \times protease inhibitor cocktail, 1 mM DTT, 1 mM sodium orthovanadate and 0.5% Nonidet P-40) for 3 min on ice. After immediate centrifugation at 16,000 \times g, 10 min, the supernatant was collected as cytosolic extract (CE). The pellet was washed twice in buffer A and incubated with 100 μ l of buffer C (420 mM NaCl, 20 mM HEPES (pH 7.9), 1 mM EGTA (pH 7.9), 1 mM EDTA (pH 7.9), protease inhibitor cocktail, 1 mM DTT, and 1 mM sodium orthovanadate) for 2 h with constant shaking at 4 C. After incubation, NE was collected by centrifugation at 16,000 \times g for 20 min. The protein concentrations of NE were determined using Bradford's reagent (Bio-Rad, Munich, Germany). NE supernatant fractions were stored in small aliquots at –20 °C.

Analysis of *c-fos* DNA binding activity in nuclear extract

To analyze *c-fos* DNA binding activity associated with PBI-1393 treatment, the *in vitro* TransFactor Kit was used (BD Biosciences, Palo Alto, CA). The provided positive-control NE were used along with the prepared NE according to the manufacturer's standard protocol. Briefly, the provided 96-well TransFactor plate was blocked with a blocking buffer supplied by the manufacturer for 15 min, then removed and replaced with 50 μ l of NE (10 μ g of protein) and incubated at room temperature for 1 h. Plates were then washed three times with blocking buffer, incubated with provided primary antibodies and washed three times. Bound primary antibodies were detected through incubation with HRP-conjugated secondary antibody and the addition of TMB substrate. Absorbance measurements were detected at 655 nm on a Tecan Genios Plus instrument. Results were expressed as relative DNA binding activity to control (fold increase).

Analysis of AP-1 signaling pathway activation by PBI-1393

To analyze the activation of AP-1 signaling pathway by PBI-1393, the *in vitro* inducible reporter plasmids containing the luciferase reporter gene driven by a basic promoter element (TATA box) plus an AP-1 inducible *cis*-enhancer element (cis-PathDetect kit from Stratagene) was used. Transient transfection of Jurkat cells and luciferase assay were performed following manufacturer's instructions. Briefly, Jurkat cells (2×10^5 cells/well in 96 well plates) were transiently transfected with pAP-1-Luc plasmid or pCIS-CK negative control plasmid (containing luciferase reporter gene without any *cis*-acting DNA element) using the Fugene reagent (Roche D). Each sample (total 2 μ g DNA/well) was performed in triplicate. After 24 h of incubation (37 °C, 5% CO₂), cells were treated with con A, PBI-1393 and/or a combination of con A and PBI-1393 for 6 h. Luciferase assay was then

performed using bright-Glo Luciferase Assay System provided by the manufacturer. Luciferase activity (relative units) was measured using Tecan Genios Plus instrument. Luciferase activity present in cells transfected with pAP-1-Luc plasmid and treated with vehicle, con A, PBI-1393 and/or a combination of con A and PBI-1393 was normalized with Luciferase activity present in cells transfected with negative control plasmid. Results were expressed as relative AP-1 activation to control (fold increase).

Statistical analysis

Data are presented as means \pm S.E.M. Student's *t*-test was used to determine statistical significance of the data. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$.

Results

Effect of PBI-1393 on IL-2 secretion in Jurkat cells

In order to determine the ability of PBI-1393 to induce IL-2 production in resting or activated T cells, IL-2 was measured in culture medium of cells treated with this compound. Jurkat cells were used as the T cell line with con A as an activator. Several concentrations of con A (0.5–10 $\mu\text{g/ml}$) were tested and it was determined that con A at 5 $\mu\text{g/ml}$ induced optimal production of IL-2 (data not shown). As illustrated in Table 1, while con A (5 $\mu\text{g/ml}$) alone induced a significant increase of IL-2 by Jurkat cells, PBI-1393 alone had no effect. PBI-1393 was then tested at increasing concentration (10^{-7} – 10^{-5} M) in combination with con A for a potential co-stimulatory effect on Jurkat cells. The results showed that PBI-1393 increased in a dose-dependent manner IL-2 production. Maximum release of IL-2 (51.4 \pm 0.10%) was observed with 10^{-5} M of PBI-1393 in the presence of con A (Fig. 1).

Effect of PBI-1393 on IL-2 gene expression in Jurkat cells

Inducible expression of IL-2 is tightly regulated by multiple transcription factors that bind at distinct sites on the IL-2 promoter including activator protein-1 (AP-1), nuclear factor- κB (NF- κB) and the nuclear factor of activated T cells (NF-AT) [27]. PBI-1393 alone did not stimulate IL-2 production but its co-stimulatory effect with con A suggests a

Table 1 Effect of PBI-1393 and con A on IL-2 production by Jurkat cells

| | IL-2 (pg/ml) |
|-----------------------------|---------------------|
| Control | 0.03 \pm 0.01 |
| PBI-1393 (10^{-5} M) | 0.06 \pm 0.07 |
| Con A (5 $\mu\text{g/ml}$) | 1925.31 \pm 43.64 |

IL-2 production by Jurkat cells treated with con A or PBI-1393. Cells were treated with PBS, PBI-1393 or con A for 24 h. The supernatant were then harvested by centrifugation and IL-2 production was measured by ELISA. Results represent mean \pm S.E.M. of three separate experiments.

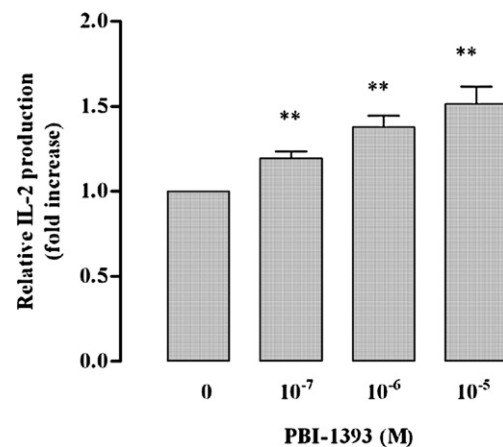


Figure 1 Effect of PBI-1393 on IL-2 production by con A-activated Jurkat cells. Cells were treated with PBI-1393 at the indicated concentration and stimulated with con A (5 $\mu\text{g/ml}$). IL-2 present in the supernatant was measured by ELISA after 24 h of incubation. Results are expressed as increase of IL-2 production (fold increase) relative to basal level obtained with con A alone. Results represent the mean \pm S.E.M. of 3 separate experiments (**: $P < 0.01$).

potential role in the activation of IL-2 gene expression. The effect of PBI-1393 on IL-2 mRNA expression was analyzed by quantitative RT-PCR. As shown in Fig. 2A, no IL-2 mRNA expression was seen in cells treated with PBI-1393 alone. However, PBI-1393 (10^{-5} M), in combination with con A, significantly increased the level of IL-2 mRNA expression by approximately 59% comparatively to cells treated with con A alone.

It has been shown that T cell stimulation with con A leads to induction of the *c-fos* proto-oncogene [28]. *c-Fos* is a member of the AP-1 complex that is known to be an activator of IL-2 gene expression [29]. The enhancement of IL-2 gene expression seen in the presence of PBI-1393 may result from an increase of *c-fos* expression. Subsequently, *c-fos* mRNA expression was analyzed by quantitative RT-PCR. As shown in Fig. 2B, PBI-1393 in the presence of con A induced a significant increase of *c-fos* mRNA expression (by two fold). However, there was no effect of PBI-1393 on *c-jun* mRNA expression (Fig. 2C).

We have also tested the ability of PBI-1393 to modulate IL-2 mRNA stability. We have measured by quantitative RT-PCR the rate of decay of IL-2 mRNA in Jurkat cells treated with con A (5 $\mu\text{g/ml}$) alone or con A combined with PBI-1393 (10^{-5} M) at 60, 90 and 120 min after treatment with Actinomycin D (5 $\mu\text{g/ml}$). As shown in Fig. 2D, IL-2 mRNA degradation rate in cells treated with con A and in cells treated with con A and PBI-1393 are comparable.

These results demonstrate that PBI-1393 did not affect IL-2 mRNA stability.

Effect of PBI-1393 on transcriptional activation of *c-fos*

The increase of *c-fos* expression by PBI-1393 in con A activated Jurkat cells lead to an assessment of its interaction with the corresponding DNA binding element and the

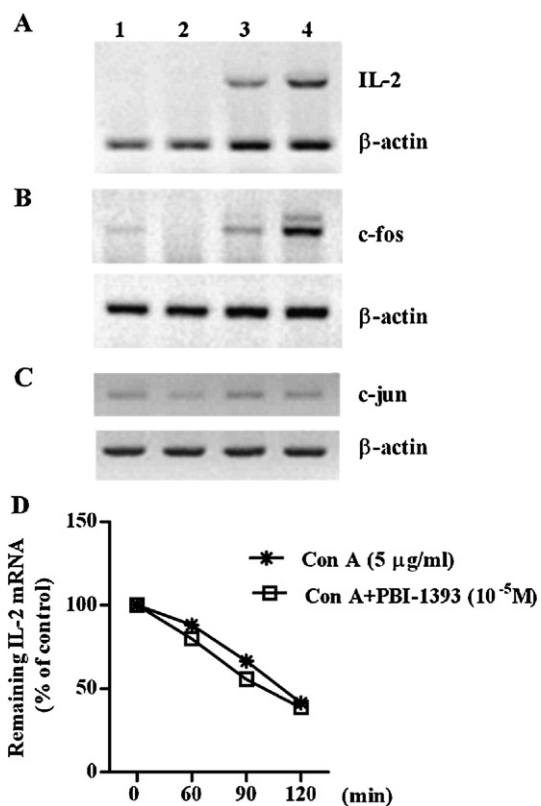


Figure 2 Effect of PBI-1393 on IL-2 gene expression. Jurkat cells were treated with PBS (lane 1), PBI-1393 (10^{-5} M, lane 2), con A ($5 \mu\text{g/ml}$, lane 3) or con A + PBI-1393 (lane 4). (A) IL-2 mRNA expression analyzed by RT-PCR after 16 h of incubation. (B, C) *c-fos* and *c-jun* mRNA expression analyzed by RT-PCR after 45 min of incubation. (D) IL-2 mRNA stability: cells were treated with con A ($5 \mu\text{g/ml}$) alone or in combination with PBI-1393 (10^{-5} M). After 4 h of incubation, Actinomycin D ($5 \mu\text{g/ml}$) was added to the medium and the incubation was prolonged for 60, 90 and 120 min. IL-2 mRNA was then analyzed by quantitative RT-PCR. β -actin mRNA expression was used to normalize IL-2, *c-fos* and *c-jun* mRNA expression. Results are representative of three separate experiments.

resulting activation. Using a transfactor kit, it was shown that PBI-1393, in combination with con A, induced a significant increase of *c-fos* binding to its DNA consensus sequence comparatively to cells treated with con A alone (Fig. 3A). To confirm the increase of *c-fos* activation, an AP-1-driven luciferase reporter plasmid was used as described in Materials and methods. The increase of *c-fos* activation may lead to an enhancement of AP-1 activity and subsequently luciferase gene expression in transfected cells will be increased. As shown in Fig. 3B, while PBI-1393 alone did not stimulate AP-1 activation, its combination with con A induced a significant increase of luciferase gene expression when compared to con A alone.

Effect of PBI-1393 on MAPK activation

As shown above, PBI-1393 in the presence of con A increased IL-2 mRNA and protein expression. The enhancement of IL-2 gene expression was associated with an increase in *c-fos*

activation. It was then of interest to analyze the effect of PBI-1393 on up-stream gene expression. It is known that *c-fos* activation is regulated by phosphorylation of mitogen-activated protein (MAP) kinase, in particular the phosphorylation of extracellular-regulated kinases (ERKs) 1 and 2. An immunoblotting experiment was performed with protein extract from cells treated with con A, PBI-1393 and/or a combination of con A and PBI-1393. As shown in Fig. 4, while PBI-1393 alone did not affect ERK1/2 phosphorylation, its combination with con A induced a significant increase in the activation of these MAP kinases. p38 and JNK activation was also analyzed but PBI-1393 did not affect these two MAPKs (data not shown).

Effect of PBI-1393 on IL-2 and IFN- γ expression in human con A-activated PBML

As shown above, PBI-1393 is able to increase IL-2 production in con A-activated Jurkat cells and so it was reasonable to expect that this compound may stimulate both IL-2 and IFN- γ expression in human con A-activated PBML. Subsequently, ELISA and RT-PCR were used to assess the expression of IL-2 and IFN- γ .

As shown in Fig. 5A, PBI-1393 in the presence of con A significantly increased IL-2 mRNA and protein expression. Maximal increase of IL-2 production in the presence of PBI-1393 and con A occurred after 16 h of incubation (2556 ± 894 pg/ml in cells treated with the combination of con A and PBI-1393 versus 1914 ± 713 pg/ml in cells treated with con A alone). In parallel, IL-2 mRNA expression was also significantly increased in cells treated with a combination of con A and PBI-1393 versus con A alone (Fig. 5B).

With regard to IFN- γ , PBI-1393 increased production of this cytokine and its mRNA expression as shown in Fig. 6. In cells treated with PBI-1393 in combination with con A, IFN- γ production and mRNA expression were increased by $46 \pm 0.27\%$ and $42 \pm 0.12\%$ respectively, compared to cells treated with con A alone.

Human PBML contain several subsets of cells able to produce IFN- γ and since PBI-1393 was shown to increase *in vitro* CTL activity in mice [23], it was then of interest to examine if the effect of PBI-1393 on IFN- γ production affected only CD8⁺ cells or all PBML cell subsets. IFN- γ production was then assessed in CD8⁺-enriched and CD8⁺-depleted PBML after treatment with con A and/or PBI-1393. As shown in Fig. 7, PBI-1393 in the presence of con A, increased significantly IFN- γ production in CD8⁺ and CD8⁻ cells by 30% and 39% respectively ($P < 0.01$) compared to cells treated with con A alone.

Effect of PBI-1393 on T cell proliferation and human CTL

The increase of IL-2 and IFN- γ by PBI-1393 in human PBML suggested an effect on T cell proliferation and CTL activity, since it is known that both IL-2 and IFN- γ play a key role in these two physiological activities. As shown in Fig. 8, PBI-1393 in the presence of con A induced in a dose dependent manner an increase of T cell proliferation. Maximal response (38%, $P < 0.05$) was reached with 10^{-5} M of PBI-1393.

With regard to CTL activity, human PBML primed with PC-3 antigenic lysate in the presence of PBI-1393 showed a

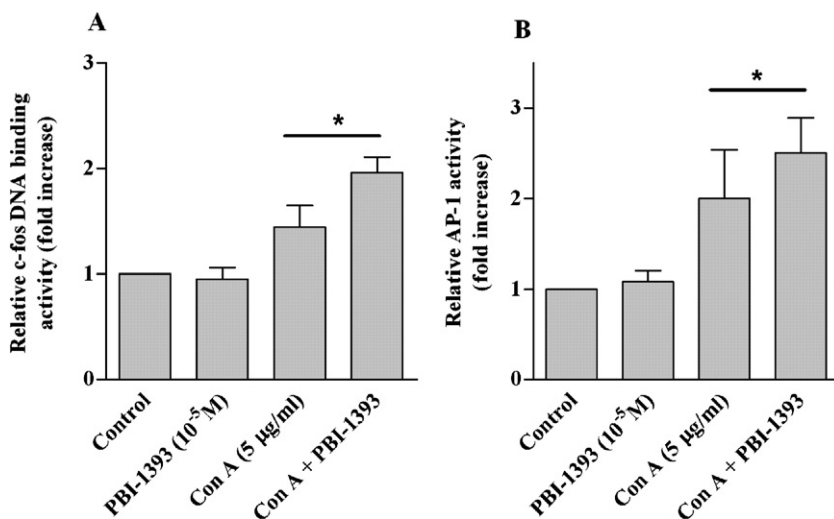


Figure 3 Effect of PBI-1393 on *c-fos* DNA binding activity and on AP-1 activation. (A) Jurkat cells were treated with PBS (control), con A (5 μg/ml), PBI-1393 (10⁻⁵ M) or con A+PBI-1393. Nuclear extract was prepared as described in Materials and methods and were assayed for the presence of phosphorylated *c-fos* using Transfactor profiling kit from BD. (B) Jurkat cells transiently transfected with AP-1 path detect system or control vector were treated with PBS, con A (5 μg/ml), PBI-1393 (10⁻⁵ M) or con A+PBI-1393 for 6 h. Luciferase gene activity was then assessed. Results were expressed as mean ± S.E.M. of relative *c-fos* binding activity or relative AP-1 activity to basal level (fold increase) from three separate experiments (*: *P*<0.05).

significant increase of their cytotoxic activity toward living PC-3 cells. As shown in Fig. 9, CTL activity of human PBML was increased by 42±0.03% (*P*<0.01) in the presence of 10⁻⁵ M of PBI-1393. Compared to IL-2 stimulation, PBI-1393 activity represents 63% of the CTL activity obtained by 10 ng/ml of IL-2.

Effect of PBI-1393 on IL-2 production by anti-CD3/anti-CD28 antibodies activated T cells

We have demonstrated that PBI-1393 plays a co-stimulatory effect in con A-activated T cells. It is of interest to see if this

compound induces the same effect in more physiological conditions. We tested PBI-1393 in both Jurkat cells and human PBML stimulated with anti-CD3 antibody (αCD3) alone or in combination with anti-CD28 (αCD28). As shown in Fig. 10A, PBI-1393 was able to increase in a dose-dependent manner, IL-2 production induced by αCD3 (5 μg/ml) in Jurkat cells. Maximal increase of IL-2 production (43.5%) was reached with 10⁻⁵ M of PBI-1393.

In human PBML we used the same concentration of PBI-1393 (10⁻⁵ M) to test its co-stimulatory effect when combined with αCD3 at the concentration of 1.5, 2.5 and 5 μg/ml. PBI-1393 showed maximal costimulatory effect at

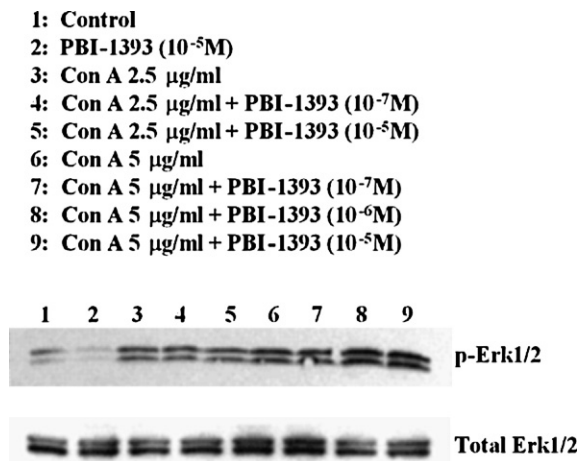


Figure 4 Effect of PBI-1393 on ERK1/2 activation: Jurkat cells were stimulated with con A and incubated in the presence or absence of PBI-1393 for 10 min. After protein extraction, ERK1/2 phosphorylation was analyzed by immunoblotting as described in Materials and methods using specific antibodies recognizing phosphorylated ERK1 and ERK2 and antibody against total ERK. Results are representative of three separate experiments.

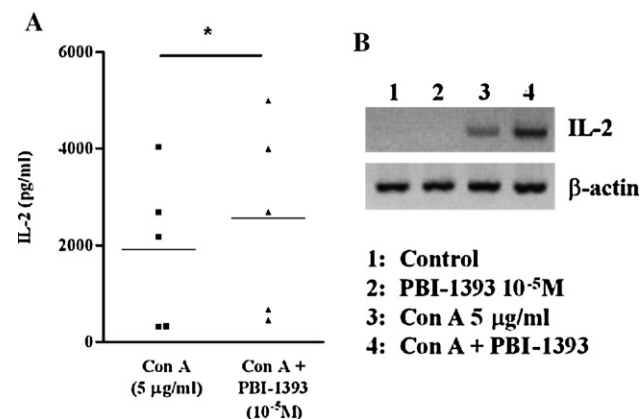


Figure 5 Effect of PBI-1393 on IL-2 protein and mRNA expression in human PBML. Human purified PBML were stimulated with PBS, PBI-1393 (10⁻⁵ M), con A (5 μg/ml), or con A+PBI-1393 for 16 h. (A) IL-2 production measured by ELISA and (B) IL-2 mRNA expression assessed by RT-PCR (one representative result). β-actin mRNA expression was used to normalize IL-2 mRNA expression (*: *P*<0.05).

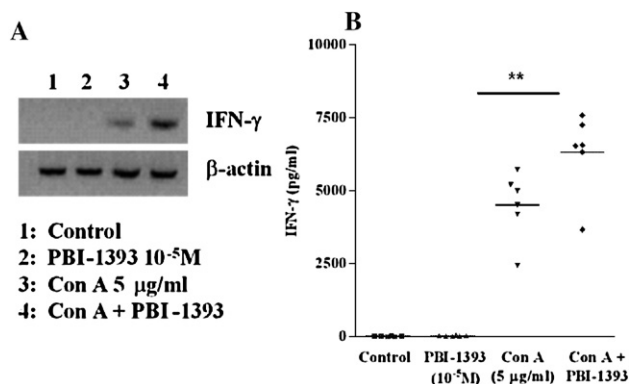


Figure 6 Effect of PBI-1393 on IFN- γ mRNA and protein expression. (A) Human PBML were treated with PBS (control), PBI-1393 (10^{-5} M), con A ($5 \mu\text{g/ml}$), or con A+PBI-1393 for 48 h. After total RNA extraction, RT-PCR was performed as described in Materials and methods to analyze IFN- γ mRNA expression. β -actin mRNA expression was used to normalize IFN- γ mRNA expression. (B). IFN- γ production was measured in the supernatant by ELISA (**: $P < 0.01$).

the concentration of $2.5 \mu\text{g/ml}$ of αCD3 antibody (66.7%; Fig. 10B). We have also tested the co-stimulatory effect of PBI-1393 on human PBML activated by αCD3 combined with αCD28 . A low concentration of αCD28 ($1 \mu\text{g/ml}$) was combined with three concentrations of αCD3 (1.5 , 2.5 or $5 \mu\text{g/ml}$). PBI-1393, tested in these three conditions, did not show any effect on IL-2 production induced by $\alpha\text{CD3}/\alpha\text{CD28}$ activation (Fig. 10C). The addition of $1 \mu\text{g/ml}$ of αCD28 to αCD3 led to maximal response of human PBML with regard to IL-2 production.

Discussion

In this study, it was demonstrated that PBI-1393 increases IL-2 and IFN- γ production in human activated lymphocytic cells.

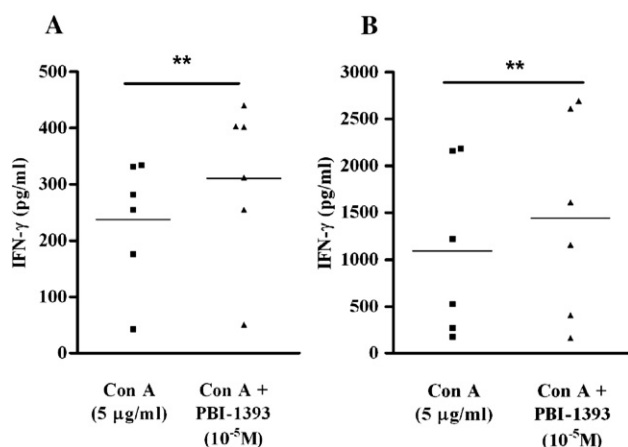


Figure 7 Effect of PBI-1393 on IFN- γ production in human CD8^+ cells (panel A) and CD8^- cells (panel B). CD8^+ cells were separated from whole human PBML using an EasySep kit. CD8^+ and CD8^- cells were treated with con A ($5 \mu\text{g/ml}$) alone or in the presence of PBI-1393 (10^{-5} M). After 48 h of incubation, IFN- γ production was measured in the supernatant by ELISA (**: $P < 0.01$).

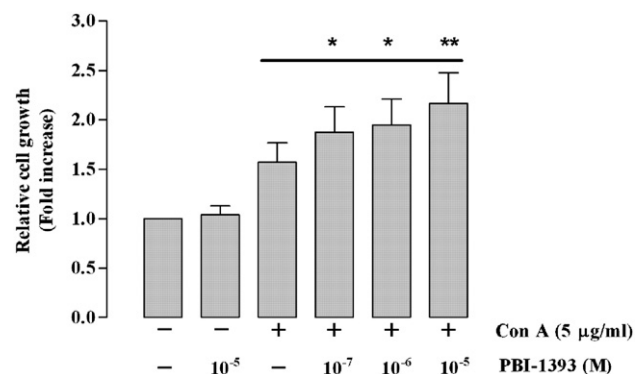


Figure 8 Effect of PBI-1393 on T cell proliferation. Human purified PBML were treated with PBS, PBI-1393 (10^{-5} M), con A ($5 \mu\text{g/ml}$) or con A+PBI-1393. After 72 h of incubation cell proliferation was measured using Alamar Blue. Cell proliferation was expressed as relative growth (fold increase) compared to non-treated cells. Results represent the mean \pm S.E.M. of four experiments performed with PBML from four different donors (*: $P < 0.05$; **: $P < 0.01$ and ***: $P < 0.005$).

The physiological relevance of this effect was illustrated by an increase of T cell proliferation and CTL activation against tumor cells. PBI-1393 alone did not stimulate Th1 cytokine production but it displays a co-stimulatory activity when combined with con A or αCD3 antibody.

Previously, PBI-1393 was tested for immunostimulatory and anti-tumor activities. We have shown that PBI-1393 exhibits potent activation of murine CTL and inhibits growth of syngenic mouse breast and colon carcinomas when co-administered with cyclophosphamide (Cy) [23,24]. Furthermore, when injected in mice, PBI-1393 induces an increase of immune cell subsets including CD4^+ , CD8^+ , NK and monocytes [23]. The mechanism of action of PBI-1393 is not determined but all these data suggest a relationship with Th1 cytokine activities in particular IL-2 and IFN- γ .

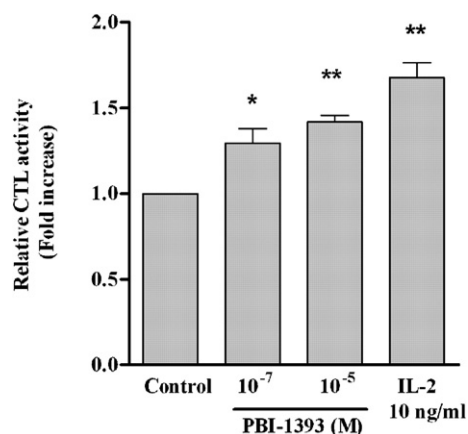


Figure 9 Effect of PBI-1393 on human CTL activity. Human T cells were primed as described in Materials and methods and used as effector cell against PC-3 cells. PC-3 cell lysis was measured using the ^{51}Cr method. Results are expressed as relative CTL activity as compared to control (fold increase). Results represent the mean of CTL activity obtained with PBML from four individuals (*: $P < 0.05$; **: $P < 0.01$).

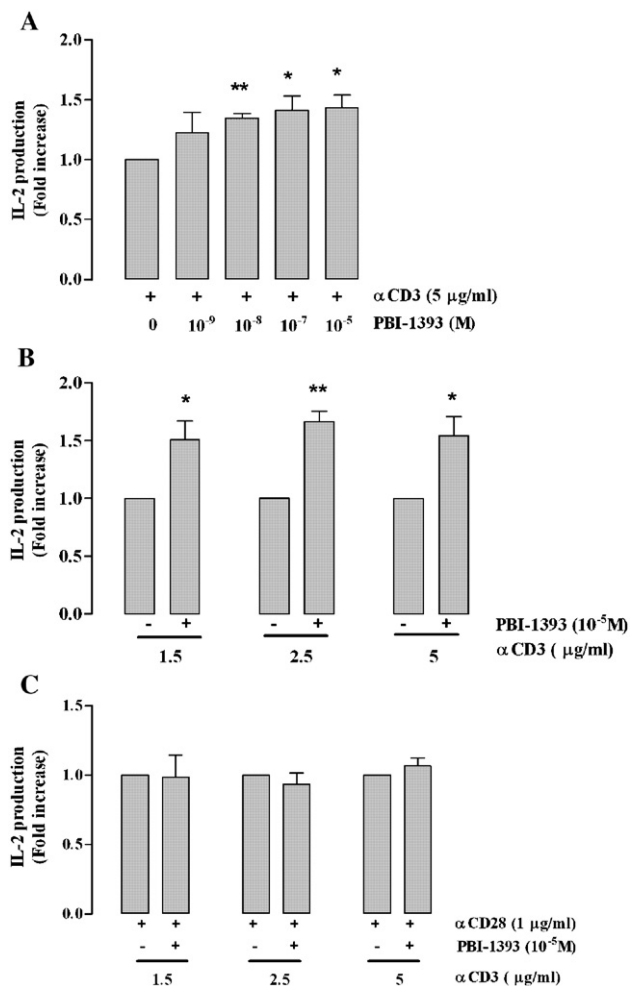


Figure 10 Co-stimulatory effect of PBI-1393 on Jurkat and human PBML cells activated by α CD3. (A) Dose–response curve of the costimulatory effect of PBI-1393 (10^{-9} – 10^{-5} M) on α CD3-activated Jurkat cells. (B) Co-stimulatory effect of PBI-1393 (10^{-5} M) on human α CD3 activated PBML. PBI-1393 was tested at 3 concentrations of α CD3 (1.5, 2.5 and 5 μ g/ml). (C) Relative IL-2 production by human α CD3-activated PBML in the presence of PBI-1393 and α CD28. IL-2 production was measured by ELISA after 48 h of incubation (*: $P < 0.05$, **: $P < 0.01$).

While IL-2 is known to be a growth factor for lymphocytes, IFN- γ is a key mediator in CTL activity [30,31]. The previously demonstrated immunostimulatory and anti-tumor activity of PBI-1393 could then be explained by an increase of IL-2 and IFN- γ availability *in vivo*. Based on this hypothesis, we undertook our study and confirmed that PBI-1393 increases in a co-stimulatory manner IL-2 and IFN- γ production both in Jurkat cells and human PBML. However, while maximal increase of IL-2 production is obtained 24 h after treatment with PBI-1393, IFN- γ production reaches its maximum 48 h after treatment. IL-2 and IFN- γ are known to be coordinately expressed [32,33] and their genes share regulatory regions [34,35]. Furthermore, IL-2 has been shown to promote IFN- γ production in NK cells [36]. In this respect, it appears that PBI-1393 enhances first IL-2 gene and protein expression and subsequently increases IFN- γ production.

IL-2 gene expression is modulated by at least three major transcription factors including NF-AT, NF- κ B and AP-1 (a complex of *c-jun* and *c-fos* protein) [27]. In our study, only *c-fos* mRNA was up-regulated by con A and this result is in agreement with the one reported by Gu et al. [28]. Up-regulation of *c-fos* only in T cells is sufficient to enhance their activation, proliferation and IL-2 expression [37,38]. PBI-1393 may act through the same signaling pathway of con A. Indeed, *c-fos* gene regulation is tightly regulated by MAP kinases, in particular ERK1/2 and our results show that PBI-1393 enhances also the phosphorylation of MAP kinases induced by con A. Taken together, it is likely that PBI-1393 may act up-stream on a Ras–Raf–Erk pathway or potentiates its signaling leading to an increase of AP-1 complex formation by newly synthesized *c-fos* and the preformed *c-jun* already present in the cell. The enhancement of *c-fos* gene expression by PBI-1393 in the presence of con A leads to the enhancement of AP-1 activation as demonstrated with the use of pAP-1-luc system in our study and subsequently the enhancement of IL-2 production.

The enhancement of T cell activation, proliferation and Th1 cytokine production by PBI-1393 in con A-treated cells mimics a co-stimulatory effect that was confirmed when T cells were activated with α CD3 antibody. However, PBI-1393 did not show synergistic effect when used in combination with α CD28. We were probably in saturation conditions with regard to cell activation and no effect of PBI-1393 in the presence of α CD28 could be observed.

The fact that PBI-1393 alone did not activate T cells but it enhances their activation by con A or α CD3, suggests that PBI-1393 is involved in a parallel pathway. It is unlikely that this compound interacts with known receptors for co-stimulatory molecules such as CD28. Indeed, PBI-1393 contains a purine moiety and, in this regard, it may interact with adenosine receptors (ADRs) that modulate cAMP through adenylyl cyclase. It has been shown that ADRs are involved in the inhibition by adenosine of T cell activation, cell proliferation and IL-2 production [39,40,41]. ADRs that regulate these events are those that mediate the increases of cAMP levels, i.e. A2A and/or A2B [42,43]. Conversely, decrease of cAMP level leads to an increase of cell proliferation and IL-2 expression [44,45]. In addition, recent reports have further elucidated the role of cAMP in T cell activation and co-stimulation via CD28 receptor. Indeed, cAMP generated upon T cell activation leads to the activation of protein kinase A which in turn mediates negative feed back and inhibits TCR-induced IL-2 secretion [46,47]. Conversely, upon TCR/CD28 co-ligation, phosphodiesterase 4 (PDE4) is recruited to decrease cAMP levels and enhance T cell activation [46]. Based on these reports and on preliminary results obtained in our laboratory showing that PBI-1393 activity is modulated *in vitro* by adenosine deaminase, it is hypothesized that this compound enhances T cell activation through ADRs.

In a previous study, PBI-1393 and cyclophosphamide (Cy) were compared as treatments for anti-tumor therapy [24]. It was demonstrated that the combination of PBI-1393 and Cy enhances the anti-tumor effect of established MC38 colon carcinoma tumors in mice compared to Cy treatment alone [24]. Cy is known to be a chemotherapeutic agent that kills tumor cells and unspecifically immune cells [48]. Based on

the results reported herein, PBI-1393 used in combination with Cy may increase the immune response against tumors via IL-2 and IFN- γ production and in this regard, PBI-1393 may be used as potent adjunct drug to chemotherapy.

Acknowledgment

We would like to thank Lyne Marcil for the preparation of the manuscript.

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