

**The thromboxane receptor antagonist PBT-3, a hepoxilin stable analog,
selectively antagonizes the TP α isoform in transfected COS-7 cells**

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Running Title: PBT-3 antagonizes the TP α isoform of the thromboxane receptor

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Abbreviations: TP, thromboxane A₂ ; PBT, hepoxilin cyclopropane analogs; PBT-3, 10(S)-hydroxy-11,12-cyclopropyl-eicosa-5Z,8Z,14Z-trienoic acid methyl ester.

Number of text pages 14 (excluding references)

Number of Tables 1

Number of Figures 5

Number of References 40

Number of Words: Abstract 240

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ABSTRACT: The hepoxilin analog, PBT-3, was previously shown to inhibit the aggregation of human platelets and to antagonize the binding of the thromboxane receptor agonist, I-BOP, in human platelets (Pace-Asciak et al., 2002). We show herein that PBT-3 inhibits, to different degrees, binding of the TP receptor antagonist, [³H] SQ 29,548, to the TP receptor isoforms in TP α and TP β transfected COS-7 cells. These isoforms possess a different tail length, the α being shorter than the β isoform. In contrast, SQ 29,548 shows no selection for the two TP isoforms. IC₅₀ for PBT-3 = $2.0 \pm 0.3 \times 10^{-7}$ M was observed for TP α , while this was one-sixth less active on the TP β isoform (IC₅₀ = $1.2 \pm 0.2 \times 10^{-6}$ M), suggesting selectivity for the TP α isoform. To investigate whether the tail contributes to the difference in competition binding by PBT-3, we investigated the tailless TP isoform expressed in transfected COS-7 cells. Its IC₅₀ was similar to that of the TP α isoforms. In additional studies we investigated the effect of PBT-3 on the collagen and I-BOP evoked intracellular calcium release, and on the collagen and I-BOP evoked phosphorylation of pleckstrin. PBT-3 blocked both pathways further demonstrating its TP receptor antagonist activity. These results demonstrate that the action of PBT-3 in inhibiting platelet aggregation is mediated via inhibition of the TP α isoform of the thromboxane receptor and that the tail may play an important role in recognition of this TP receptor antagonist.

Thromboxane A₂ (TXA₂) has various pharmacological effects which modulate the physiological responses of several cells and tissues (Halushka et al., 1995). It is a product of sequential metabolism of arachidonic acid by the cyclooxygenases and TXA₂ synthase (Hamberg and Samuelsson, 1974; Smith, 1992). TXA₂ formation can result from activation of various cell types, including platelets, macrophages and vascular smooth muscle cells.

Activation of TXA₂ (TP) receptors induces platelet aggregation, constriction of vascular and bronchiolar smooth muscle cells, as well as mitogenesis and hypertrophy of vascular smooth muscle cells (Moncada and Vane, 1979; Packham, 1993). TXA₂ formation is increased in thrombotic disorders (Fitzgerald et al., 1987) and has been implicated in a wide variety of cardiovascular diseases (Smith, 1992).

TXA₂ exerts intracellular effects by interaction with selective members of the G protein-coupled receptor family (Coleman et al., 1994; Watson and Arkininstall, 1994). The receptor appears to be encoded by a single gene that can be alternatively spliced in the carboxyl-terminal tail (C-tail) leading to two variants, termed TP α and TP β , which share the first 328 amino acids. Complementary DNAs for the 343-amino acid TP α were cloned from placental and megakaryocytic sources (Hirata et al., 1991), whereas a

cDNA for the 407-amino acid TP β was isolated from a vascular endothelial library (Raychowdhury et al., 1994). The receptors have been shown to have different C-tails which are likely to play a role in G-protein-coupling specificity and different desensitization characteristics (Hirata et al., 1996).

The TP receptor is abundantly expressed at both the mRNA and protein level in tissues of relevance to TXA₂ biology, such as platelets, vascular and uterine smooth muscle, uterus and placental tissue, endothelium, epithelium, trophoblasts, thymus, liver and small intestine with TP α expression predominating in most tissues examined (Miggin and Kinsella, 1998). There is evidence that mRNA for both isoforms is co-expressed in platelets, endothelial cells and a number of other cell/tissue types with significantly greater levels of TP α than TP β expressed. Isoform selective antibodies permitted detection of TP α , but not TP β , in human platelets, leading to the suggestion that TP α may be the predominant isoform in platelets, despite the presence of mRNA for both isoforms in these cells (Miggin and Kinsella, 1998).

TP receptors have also been cloned from K562 cells, HEL cells, endothelial cells, mouse lung, rat kidney and rat astrocytes (Namba et al., 1992; D'Angelo et al., 1994; Abe et al., 1995; Kitanaka et al., 1995; Allan et al., 1996). The cloned receptor has been expressed in several cell lines, including HEK293, CHO cells, COS-1 and COS-7 cells (Ushikubi et al., 1989; Funk et al., 1993). These cell lines have been used in the past to express and characterize cloned receptors. COS-7 cells are a SV40-transformed African

Green monkey kidney cell line that has been used extensively for the transient expression of cloned receptors (Becker et al., 1998) and can produce desirable expression efficiency.

Hepoxilins are hydroxy epoxide metabolites of arachidonic acid formed via the 12-lipoxygenase pathway (Pace-Asciak et al., 1995). We have previously shown that the hepoxilins possess a variety of biological actions *in vitro* as well as *in vivo*; namely, they cause the release of intracellular calcium from calcium stores in human neutrophils (Dho et al., 1990; Laneuville et al., 1993), and the release of insulin from pancreatic beta cells *in vitro* (Pace-Asciak and Martin, 1984) and into the circulation of rats *in vivo* (Pace-Asciak et al., 1999). Since the native hepoxilins are rather unstable, we prepared a series of chemically stable analogs for *in vivo* use (Demin and Pace-Asciak, 1993). During more comprehensive screening for *in vitro* biological actions, we discovered that one of these analogs, PBT-3, potently inhibited collagen-evoked aggregation of human platelets (Pace-Asciak et al., 2002).

The present study was designed to further investigate PBT-3 binding to TP receptor isoforms transiently expressed in COS-7 cells. Our results demonstrate significant selectivity of PBT-3 to the TP α isoform which is the isoform most abundant in human platelets.

Methods

Materials. PBT-3 was prepared in our laboratory as previously described (Demin and Pace-Asciak, 1993). I-BOP ([1S-[1 α ,2 α (Z),3 β (1E,3S*),4 α]]-7-[3-[3-hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid), I-SAP (Iodophenyl sulfonyl amino pinane thromboxane A₂), pinane thromboxane A₂ (9 α , 11 α -(dimethyl)methylene-15S-hydroxy-11a-deoxy-11a-methylene-thromba-5Z,13E-dien-1-oic acid) and SQ 29,548 ([1S-[1 α ,2 α (Z),3 α ,4 α]]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid) were purchased from Cayman Chemical (Ann Arbor, MI), and Indo-1-AM and ionomycin from Calbiochem (La Jolla, CA). All reagent-grade chemicals for buffers were purchased from Sigma-Aldrich (Oakville, Ontario). [³²P]orthophosphate (400-800 mCi/ml) was obtained from ICN Biomedical Research Products. Anti-rabbit IgG, horseradish peroxidase linked whole antibody, ECL western blotting detection reagents were purchased from Amersham Life Sciences (Baie d'Urfe, Quebec). Prestained SDS-PAGE marker broad range was from New England Biolabs (Mississauga, ON). Rabbit serum containing a polyclonal anti-human pleckstrin antibody was prepared and kindly supplied by Dr. Richard J. Haslam, McMaster University (Sloan et al., 2002). [³H] SQ 29,548 and SQ 29,548 were from Cayman Chemical (Ann Arbor, MI). DEAE-dextran, chloroquine, indomethacin and HEPES were obtained from Sigma. dRhodamine terminatorcycle sequencing kit was from PE Applied Biosystems (Foster City, USA). TP alpha, beta and tailless cDNA were as previously described (Allan et al., 1996; Becker et al., 1998). The tailless cDNA is truncated at amino acid 328.

Cell culture and transient transfection. COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum and 1% of solution containing 10,000 units/ml penicillin G, 10,000 $\mu\text{g/ml}$ streptomycin, and 25 $\mu\text{g/ml}$ amphotericin (Cellegro). Cells were grown at 37 °C in a humidified atmosphere of 95% O₂, and 5% CO₂. cDNAs for the TP α and TP β were subcloned into pcDNA3, the resultant plasmid, pcDNA3:TP α and pcDNA3:TP β were introduced into COS-7 cells (5 $\mu\text{g}/100$ mm plate) by the DEAE-dextran/chloroquine method. After 48 hours post-transfection, cells were harvested by centrifugation at 500 x g for 5 min and washed 3 times in phosphate-buffered saline (PBS). Protein concentrations were determined using the Bradford assay (Bradford, 1976). Cells were resuspended in buffer containing 25 mM HEPES/ 125 mM NaCl/ 10 μM indomethacin, pH 7.4, and kept on ice for the binding study. TP expression was determined according to Miggin and Kinsella (1998) using [³H] SQ 29,548 (10 nM) and a single saturating amount of SQ 29,548 (10 μM).

Radioligand binding assay. Competition binding curves were carried out on COS-7 cells expressing TP receptor isoforms. Binding reactions were carried out on 5×10^5 cells in a total volume of 0.2 ml in the above buffer with 10 nM [³H] SQ 29,548 added to all tubes in triplicate, containing various concentration of PBT-3 (10^{-8} - 10^{-5} M) in 1 μl ethanol. Additional tubes containing excess unlabeled SQ 29,548 (10 μM) were included to assess the extent of nonspecific binding. Binding was allowed to take place for 30 min at 37 °C; free radioligand was removed by rapid vacuum filtration through

Whatman (Maidstone, UK) GF/B glass fiber filters pre-washed with the cell suspension buffer. The tubes and the filters were rapidly washed with ice-cold buffer (three times with 3 ml). The radioactivity on the filters containing the ligand-receptor complexes was counted in 10 ml of Ecolite scintillation fluid (ICN, St.Laurent, Quebec) in a Beckman (Model LS 3800) liquid scintillation counter.

Sequencing Analysis. TP α , TP β and TPtailless sequences were confirmed on both strands using the ABI Big Dye Terminator Cycle Sequencing Ready Reaction kit, and the products were resolved on an ABI Prism 310 Genetic Analyzer (Perkin-Elmer/Applied Biosystems). The sequences were assembled and analyzed using the ClastalW Sequence analysis.

Preparation of human platelet suspension. Blood samples were collected from healthy volunteers who had not taken NSAIDs for at least two weeks. Blood was drawn in 60 mL plastic syringes containing ACD (1.36% citric acid, 2% glucose, 2.5% trisodium citrate dihydrate) in the absence or presence of aspirin (ASA, 1 mM) (ratio: 9 volume blood for 1 volume ACD), and centrifuged at 200 x g for 15 minutes at room temperature. The platelet-rich-plasma (PRP) supernatant was used for dye loading cells (next paragraph) or further centrifuged at 400 x g for 5 minutes to prepare platelet suspension. The resulting platelet pellet was resuspended in fresh medium containing 137 mM NaCl, 1 mM KCl, 0.4 mM NaH₂PO₄, 5.5 mM glucose and 20 mM HEPES, pH 7.4.

Measurement of intracellular calcium ($[Ca^{2+}]_i$) mobilization in platelets. The method previously described with human neutrophils was modified to utilize human platelets (Dho et al., 1990). Briefly, PRP was incubated with 3 mM (final concentration 3 μ M) of the acetoxymethyl ester precursor of the calcium indicator Indo-1 during 45 minutes at 37°C. The platelets were centrifuged (400 x g for 5 minutes). The supernatant containing excess dye was removed and the dye-loaded platelet pellet was gently resuspended in the same fresh medium (containing no $CaCl_2$). Aliquots of 350×10^6 cells were placed in a plastic cuvette (Diamed Lab, Toronto, Canada) and equilibrated, when necessary with 1 mM $CaCl_2$ or EGTA. The cell suspension was continuously stirred magnetically and the temperature was controlled at 37°C. Intracellular calcium concentrations were monitored with a Perkin-Elmer fluorescence spectrophotometer (model 650-40) and recorded on a chart recorder (LKB model 2210) set at 1 cm/min. The excitation wavelength was set at 331 nm, the emission wavelength at 410 nm, with slits of excitation and emission set at 3 and 15 nm respectively. Each sample was stirred for 1 minute in the spectrofluorometer before any addition. Typical measurement was initiated by addition of 1 μ l glass distilled ethanol or test compound in ethanol followed 2 minutes later by collagen (2 μ g) or I-BOP (2 ng). The resulting effect was recorded for the next 6 minutes. At the end of the test, a calibration was carried out to determine the maximal fluorescence by adding ionomycin at 1 mM (final concentration 1 μ M) and minimal fluorescence by adding $MnCl_2$ (final concentration 3mM).

Platelet labeling and stimulation. Platelets were prepared as described above. They were resuspended at 7×10^8 cells/ml in a phosphate-free Hepes tyrode's buffer without calcium (20 mM Hepes, 1mM KCl, 5.5 mM Glucose, 125 mM NaCl, pH 7.4), and labeled with 250 μ Ci/ml [32 P]orthophosphate for one hour at room temperature (Markus et al., 1999). For stimulation, unless otherwise indicated, the [32 P] labeled platelet suspension was incubated (final 1 mM calcium concentration) at 37°C with vigorous shaking. Where appropriate, SQ 29,548, PBT-3 or vehicle was added prior to stimulation for 5 minutes. Thereafter, selective agonists or vehicle were added for 2-5 minutes. At the time indicated, the reaction was terminated by transferring an aliquot to a denaturing solution (6% SDS, 2% 2-mercaptoethanol, 30% glycerol, 3 mM EDTA, 12 mM EGTA, 0.03% bromophenol blue, 450 mM Tris; pH 6.8). The samples were denatured by boiling for 3 min and separated on a 12% SDS-PAGE gel for autoradiography and western blot analysis of pleckstrin (Habib et al., 1999).

SDS-PAGE, western immunoblotting. Platelet proteins were analyzed on 12% SDS-PAGE for the separating gel in the presence of prestained protein markers. The gels were transferred to a Trans-Blot Nitrocellulose membrane (Bio-Rad). Visual protein bands on the nitrocellulose membranes were checked with Ponceau S-staining to ensure equivalent protein loading/transfer comparing different samples. Membranes were blocked with nonfat dry milk (5%, w/v) in PBS containing 0.5% (v/v) Tween-20 for one hour at room temperature and then incubated with 1:20,000 dilution of anti-pleckstrin antibody overnight at 4°C. The secondary antibody of horseradish peroxidase anti-rabbit

antibody was used at 1:2,000 dilution. Bound antibodies were detected using enhanced chemiluminescence (ECL) kit and exposed to Hyperfilm.

Autoradiography and densitometric analysis. The above membrane exposed to ECL was kept in strong light overnight to eliminate the chemiluminescence, then it was exposed to Hyperfilm at -70 °C for autoradiography. The intensity of the autoradiograph bands was analyzed using FluorChem software. The areas were integrated using the same program, and the results were expressed in arbitrary units as % of control (untreated) bands.

Statistical Analysis. Values stated are the mean \pm S.D. of the number of observations (n) indicated. Analysis of statistical significance was performed using Student's t test employing a Macintosh StatView software program. Competition binding were fitted to a line of best fit through a Michaelis-Menten-like hyperbolic treatment with a Kaleidagraph statistical software package. The TP expression rates are expressed as picomoles of [³H] SQ 29,548 incorporated per milligram of cell protein \pm S.D., where n = 3-5.

Results

1. PBT-3 Dose-Dependently Inhibits Intracellular Calcium Release Evoked by collagen and I-BOP in Human Platelets *In Vitro*. Free cytoplasmic Ca^{2+} concentrations in human platelets were measured by the Indo-1-AM fluorescent dye method. The experiments were carried out in the absence of extracellular calcium ions to demonstrate that the observed $[\text{Ca}^{2+}]_i$ transients were exclusively derived from intracellular stores.

1A. Effects on collagen: Dose response curves comparing the actions of PBT-3 and two TP receptor antagonists, SQ 29,548 and pinane thromboxane A_2 on $[\text{Ca}^{2+}]_i$ release evoked by collagen are shown in Fig. 1A. IC_{50} values for inhibition by these three compounds are: 6.8×10^{-8} M, 1.2×10^{-9} M and 6.5×10^{-8} M, respectively for the three agents. Hence PBT-3 appears to have similar potency to pinane thromboxane A_2 , but SQ 29,548 is about 50-fold more active in inhibiting $[\text{Ca}^{2+}]_i$ release evoked by collagen (see Table 1).

1B. Effects on I-BOP; I-BOP (8×10^{-9} M) evoked a fast rise in $[\text{Ca}^{2+}]_i$ from baseline (152 ± 5 nM) reaching a maximum (564 ± 10 nM above baseline) within seconds before decreasing back to the baseline level. This I-BOP-evoked increase in $[\text{Ca}^{2+}]_i$ was markedly inhibited by PBT-3 (2.85×10^{-7} M), added 2 minutes before I-BOP and this inhibition was dose related as shown in Fig. 1B. The estimated IC_{50} value for the inhibition of $[\text{Ca}^{2+}]_i$ release evoked by I-BOP was 7.0×10^{-9} M. The TP receptor

antagonists, SQ 29,548 and I-SAP dose-dependently inhibited $[Ca^{2+}]_i$ release evoked by I-BOP (Fig. 1B) with IC_{50} values estimated at 0.5×10^{-9} M and 0.6×10^{-9} M for SQ 29,548 and I-SAP respectively (see Table 1). These results show that PBT-3 is about one-tenth less active as an antagonist of I-BOP evoked $[Ca^{2+}]_i$ release than SQ 29,548 or I-SAP.

2. PBT-3 Inhibits Phosphorylation of Pleckstrin.

2A. Effects of Collagen: Collagen caused the incorporation of $[^{32}P]$ into a P47 protein (pleckstrin) in human platelets. A reduction in $[^{32}P]$ incorporation took place in the presence of ASA indicating that pleckstrin phosphorylation followed TP receptor activation by the endogenous thromboxane formed during collagen challenge (Fig. 2). PBT-3 blocked the collagen-evoked phosphorylation, both in the presence and absence of ASA. Fig. 2A shows an autoradiogram of newly formed phosphorylated proteins separated on 12% SDS-PAGE gel, with total pleckstrin protein (a measure of loading) being detected by a rabbit anti-human pleckstrin-selective antibody (bottom panels). Since the collagen effect is a thromboxane mediated event, the presence of ASA reduced the incorporation of $[^{32}P]$ into phosphorylated pleckstrin (P47) as shown by comparing the left panels with the right panels. PBT-3 blocks this event quite well. Note that pleckstrin expression did not change by the various treatments, only the extent of newly phosphorylated pleckstrin did (x-ray film). The inhibitory actions of PBT-3 were dose dependent (Fig. 3) within the concentration range of 0.06 to 6 μ M. The IC_{50} value was estimated to be 3×10^{-7} M (see Table 1).

2B. Effects of I-BOP: I-BOP at 4 ng/ml strongly induced the incorporation of [³²P] into pleckstrin (Fig. 4, autoradiogram, top panel, A). PBT-3 effectively blocked this phosphorylation within the tested concentration range of 0.0006 to 6 μM. The expression levels of the P47 phosphorylated pleckstrin protein were unchanged by the various treatments (Fig. 4, lower panel, B) as determined by western blot analysis and densitometry. The IC₅₀ value is estimated to be 1 x 10⁻⁸ M (see Table 1).

3. Inhibition of binding by PBT-3 of [³H]SQ 29,548 to TPα, TPβ and TPtailless transfected COS-7 cells. Since the message for the TPβ isoform has been found in platelets, there may be a low expression of the receptor. Hence, we decided to determine the potency of PBT-3 on the two receptors. Additionally, we decided to investigate the effect of the TPtail on PBT-3 antagonism of binding of SQ 29,548 by expressing TPtailless in COS-7 cells. To establish the efficiency of transfection and to confirm sustained protein expression at 48 hours post-transfection, for each independent experiment COS-7 cells were transfected with the vector only. Routinely, TPα expression was 1.6 ± 0.1, TPβ was 1.2 ± 0.1 and TPtailless was 1.8 ± 0.2 pmol of [³H] SQ 29,548/mg cell protein, respectively. PBT-3 effectively competed with [³H] SQ 29,548 for binding to the TP receptor isoforms. PBT-3 displaced SQ 29,548 with different potencies depending on which isoform was studied. Competition curves are shown for TPα and TPβ, from which the IC₅₀ values were derived (Fig. 5). PBT-3 was about 6 times more potent in competing with [³H] SQ 29,548 binding for the TPα than TPβ. The IC₅₀ value for TPα was 2.0 ± 0.3 x 10⁻⁷M, for TPβ was about 1.2 ± 0.2 x 10⁻⁶ M. Due to the different potency for the two isoforms, we decided to investigate whether

the tail was responsible for the difference, by removing it. The IC_{50} for the TPtailless was $9.1 \pm 0.2 \times 10^{-8}$ M, similar to the TP α isoform which possesses a short tail.

Discussion

The results demonstrate that PBT-3 is an effective antagonist to the effects of collagen and I-BOP in stimulating intracellular calcium release and phosphorylation of pleckstrin. Stimulation of the phosphorylation of pleckstrin via the TP receptor represents a new intracellular event coupled to this receptor. The TP receptor is abundantly expressed at both the mRNA and protein level in tissues of relevance to TXA₂ biology, such as platelets, vascular and uterine smooth muscle, uterus and placental tissue, endothelium, epithelium, trophoblasts, thymus, liver and small intestine with TP α expression predominating in most tissues examined (Miggin and Kinsella, 1998). There is evidence that mRNA for both isoforms is co-expressed in platelets, endothelial cells and a number of other cell/tissue types with significantly greater levels of TP α than TP β expressed. Isoform selective antibodies permitted detection of TP α , but not TP β , in human platelets, leading to the suggestion that TP α may be the predominant isoform in platelets, despite the presence of mRNA for both isoforms in these cells (Miggin and Kinsella, 1998). From our previous work, we demonstrated that PBT-3 is an antagonist of the human platelet TP receptor resulting in the inhibition of aggregation (Reynaud et al., 2001). We further show herein that the effect of PBT-3 in inhibiting platelet aggregation may involve inhibition of pleckstrin phosphorylation and, in addition, inhibition of intracellular calcium release.

TP α and TP β display similar binding affinities for the selective TP receptor antagonist SQ 29,548, with respect to K_d values of 11.2 ± 1.4 and 12.4 ± 1.8 nM, indicating that the C-tails do not affect the binding properties of these receptors to this TP receptor antagonist. (Parent et al., 1999). In contrast, the inhibition of binding of both isoforms show significant difference for PBT-3 with $2.0 \pm 0.3 \times 10^{-7}$ M for TP α , and $1.2 \pm 0.2 \times 10^{-6}$ M for TP β . Even at the high concentration of PBT-3 (200 μ M), the inhibition of binding of TP β only reached 76% in comparison with 100% inhibition of TP α at 10 μ M. These results raise the interesting possibility that the tail of the TP receptor participates in the selective binding of this receptor antagonist. Previous studies of the TP receptor have raised the possibility that the binding of agonists and antagonists may be at different sites, but not in the tail. The different tails of the TP α and TP β receptors have been thought to confer differences in desensitization and internalization but not binding (Spurney and Coffman, 1997; Spurney, 1998; Parent et al., 1999). Indeed, previous studies have focused on either intramembraneous domains or extracellular domains as potential ligand binding sites (Dorn et al., 1997; Turek et al., 2002). As PBT-3 may have different inhibition mechanisms on the two isoforms, studies on the signaling pathways affected by PBT-3 warrants further investigation. Since the distribution of the TP α and TP β receptor isoforms differ, selective inhibition of the TP α isoform in the platelets may provide an important selectivity for a potential therapeutic benefit that has not been seen with other TP receptor antagonists.

New drugs that act both as TXA₂ synthase inhibitors and as TP receptor antagonists have been developed, such as picotamide or ridogrel. These compounds

were noted to have good clinical efficacy in patients with thrombotic disorders (Keith et al., 1994; Neirotti et al., 1994). Terbogrel exhibits an equipotent (IC_{50} of about 10 nM) activity as TXA_2 synthase inhibitor and TP receptor antagonist (Muck et al., 1998). In our study, we demonstrated that PBT-3, a novel combined TXA_2 synthase inhibitor/TP receptor antagonist (Pace-Asciak et al., 2002), selectively inhibits TXA_2 -dependent platelet activation but exhibits a more potent activity as TP receptor antagonist (IC_{50} for inhibition of [125 I]-BOP binding, intracellular calcium release, platelet aggregation are 8, 7 and 56 nM respectively) compared to its activity as TXA_2 synthase inhibitor (IC_{50} for inhibition of TXA_2 formation and platelet aggregation evoked by collagen are 0.4 and 0.64 μ M). In this study we have found that PBT-3 is selective towards the $TP\alpha$ isoform present in platelets. PBT-3 may therefore be an excellent candidate for further *in vivo* testing as a novel therapeutic in the treatment of thrombotic disorders or thromboxane-mediated diseases.

Acknowledgements

We wish to thank Prof. Richard Haslam for reading our manuscript and for his constructive suggestions.

References

- Abe T, Takeuchi K, Takahashi N, Tsutsumi E, Taniyama Y and Abe K (1995) Rat kidney thromboxane receptor: molecular cloning, signal transduction, and intrarenal expression localization. *J. Clin. Invest.* **96**:657-664.
- Allan CJ, Higashiura K, Martin M, Morinelli TA, Kurtz DT, Geoffroy O, Meier GP, Gettys TW and Halushka PV (1996) Characterization of the cloned HEL cell thromboxane A₂ receptor: evidence that the affinity state can be altered by G_α13 and G_αq. *J. Pharmacol. Exp. Ther.* **27**:1132-1139.
- Becker KP, Ullian M and Halushka PV (1998) Cloning and characterization of an endogenous COS-7 cell thromboxane A₂ receptor. *Biochim. Biophys. Acta Mol. Cell. Res.* **1403**:109-114.
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248-254.
- Coleman RA, Smith WL and Narumiya S (1994) International union of pharmacology classification of prostanoid receptors: Properties, distribution, and structure of the receptors and their subtypes. *Pharmacol. Reviews* **46**:205-229.
- D'Angelo DD, Davis MG, Ali S and Dorn II GW (1994) Cloning and pharmacologic characterization of a thromboxane A₂ receptor from K562 (human chronic myelogenous leukemia) cells. *JPET* **271**:1034-1041.

Demin PM and Pace-Asciak CR (1993) Synthesis of racemic 11,12-cyclopropyl analogs of hepoxilins A₃ and B₃. *Tetrahedron Lett.* **34**:4305-4308.

Dho S, Grinstein S, Corey EJ, Su WG and Pace-Asciak CR (1990) Hepoxilin A₃ induces changes in cytosolic calcium, intracellular pH and membrane potential in human neutrophils. *Biochem. J.* **266**:63-68.

Dorn GI 2nd, Davis M and D'Angelo D (1997) Structural determinants for agonist binding affinity to thromboxane/prostaglandin endoperoxide (TP) receptors. Analysis of chimeric rat/human TP receptors. *J. Biol. Chem.* **272**:12399-12405.

Fitzgerald GA, Healy C and Daugherty J (1987) Thromboxane A₂ biosynthesis in human disease. *Fed. Proc.* **46**:154-158.

Funk CD, Furci L, Moran N and Fitzgerald GA (1993) Point mutation in the seventh hydrophobic domain of the human thromboxane A₂ receptor allows discrimination between agonist and antagonist binding sites. *Mol. Pharmacol.* **44**:934-939.

Habib A, FitzGerald GA and Maclouf J (1999) Phosphorylation of the thromboxane receptor α , the predominant isoform expressed in human platelets. *J. Biol. Chem.* **274**:2645-2651.

Halushka PV, Allan CJ and Davis-Bruno KL (1995) Thromboxane A₂ receptors. *J. Lipid Mediat. Cell. Signal.* **12**:361-378.

Hamberg M and Samuelsson B (1974) Prostaglandin endoperoxides: novel transformations of arachidonic acid in human platelets. *Proc. Natl. Acad. Sci. USA* **71**:3400-3404.

- Hirata T, Ushikubi F, Kakizaka A, Okuma M and Narumiya S (1996) Two thromboxane A₂ receptor isoforms in human platelets. Opposite coupling to adenylyl cyclase with different sensitivity to Arg60 to Leu mutation. *J. Clin. Invest.* **97**:949-956.
- Hirata M, Hayashi Y, Ushikubi F, Yokota Y, Kageyama R, Nakanishi S and Narumiya S (1991) Cloning and expression of cDNA for a human thromboxane A₂ receptor. *Nature* **349**:617-620.
- Keith JJ, Endo Y, Warwick K, Keith K, Brugh S and Rowles T (1994) Ridogrel improves maternal/fetal homeostasis in an ovine model of pregnancy-induced hypertension. *Prostaglandins* **47**:247-263.
- Kitanaka J, Hashimoto H, Sugimoto Y, Sawada M, Negishi M, Suzumura A, Marunouchi T, Ichikawa A and Baba A (1995) cDNA cloning of a thromboxane A₂ receptor from rat astrocytes. *Biochim. Biophys. Acta Mol. Cell. Res.* **1265**:220-223.
- Laneuville O, Reynaud D, Grinstein S, Nigam S and Pace-Asciak CR (1993) Hepoxilin A₃ inhibits the rise in free intracellular calcium evoked by formyl-methionyl-leucyl-phenylalanine, platelet-activating factor and leukotriene B₄. *Biochem. J.* **295**:393-397.
- Markus B, Michaela R, jonathan I, Petra M, Markus E, Martin A, Steve P and Wolfgang S (1999) Dichotomous regulation of myosin phosphorylation and shape change by Rho-kinase and calcium in intact human platelets. *Blood* **94**:1665-1672.
- Miggin SM and Kinsella BT (1998) Expression and tissue distribution of the mRNAs encoding the human thromboxane A₂ receptor (TP) α and β isoforms. *Biochim. Biophys. Acta* **1425**:543-559.

- Moncada S and Vane JR (1979) Pharmacology and endogenous roles of prostaglandin endoperoxides, thromboxane A₂ and prostacyclin. *Am. Soc. Pharmacol. Exptl. Therap.* **30**:293-331.
- Muck S, Weber A-A and Schror K (1998) Effects of turbogrel on platelet function and prostaglandin endoperoxide transfer. *Eur. J. Pharmacol.* **344**:45-48.
- Namba T, Sugimoto Y, Hirata M, Hayashi Y, Honda A, Watabe A, Negishi M, Ichikawa A and Narumiya S (1992) Mouse thromboxane A₂ receptor: cDNA cloning, expression and northern blot analysis. *Biochim. Biophys. Res. Commun.* **184**:1197-1203.
- Neirotti M, Molashi M and Ponzetto M (1994) Hemodynamic, hemorheologic and hemocoagulative changes after treatment with picotamide in patients affected by peripheral arterial disease (PAD) of the lower limbs. *Angiology* **45**:137-141.
- Pace-Asciak CR, Demin PM, Estrada M and Liu G-Y (1999) Hepoxilins raise circulating insulin levels *in vivo*. *FEBS Lett.* **461**:165-168.
- Pace-Asciak CR and Martin JM (1984) Hepoxilin, a new family of insulin secretagogues formed by intact rat pancreatic islets. *Prostagl. Leukotriene and Med.* **16**:173-180.
- Pace-Asciak CR, Reynaud D, Demin P, Aslam R and Sun A (2002) A new family of thromboxane receptor antagonists with secondary thromboxane synthase inhibition. *J. Pharmacol. Exper. Ther.* **301**:618-624.
- Pace-Asciak CR, Reynaud D and Demin PM (1995) Hepoxilins: A review on their enzymatic formation, metabolism and chemical synthesis. *Lipids* **30**:1-8.
- Packham MA (1993) Role of platelets in thrombosis and hemostasis. *Can. J. Physiol. Pharmacol.* **72**:278-284.

- Parent JL, Labrecrue P, Orsini MJ and Benovic JL (1999) Internalization of the TXA₂ receptor alpha and beta isoforms. Role of the differentially spliced COOH terminus in agonist-promoted receptor internalization. *J. Biol. Chem.* **274**:8941-8948.
- Raychowdhury MK, Yukawa M, Collin LJ, McGrail SH, Kent KC and Ware JA (1994) Alternative splicing produces a divergent cytoplasmic tail in the human endothelial thromboxane A₂ receptor. *J. Biol. Chem.* **269**:19256-19261, *Correction - J. Biol. Chem.*, 270 (1995), 7011.
- Reynaud D, Sun A, Demin PM and Pace-Asciak CR (2001) Novel platelet antiaggregating substances. *Biochem. Biophys. Res. Commun.* **284**:580-582.
- Sloan DC, Wang P, Bao X and Haslam RJ (2002) Translocation of pleckstrin requires its phosphorylation and newly formed ligands. *Biochem. Biophys. Res. Commun.* **293**:640-646.
- Smith WL (1992) Prostanoid biosynthesis and mechanisms of action. *Am. J. Physiol.* **263**:F181-F191.
- Spurney RF (1998) Role of C-terminal serines in desensitization and phosphorylation of the mouse thromboxane receptor. *J. Biol. Chem.* **273**:28496-28503.
- Spurney RF and Coffman TM (1997) The C-terminus of the thromboxane receptor contributes to coupling and desensitization in a mouse mesangial cell line. *J. Pharmacol. Exp. Therap.* **283**:207-215.
- Turek JW, Halmos, T, Sullivan NL, Antonakis K, and Le Breton GC (2002) Mapping of a ligand-binding site for the human thromboxane A₂ receptor protein. *J. Biol. Chem.* **277**:16791-16797.

Ushikubi F, Nakajima M, Hirata M, Okuma M, Fujiwara M and Narumiya S (1989)

Purification of the thromboxane A₂/prostaglandin H₂ receptor from human blood platelets. *J. Biol. Chem.* **264**:16496-16501.

Watson S and Arkinstall S (1994) (Eds) In the G-protein linked receptor factsbook.

Academic Press, London.

Footnotes

This study was supported by funds to CRP-A from the CIHR (MT-4181) and to PVH from NIH (NIH HL36838)

Legends for Figures

Figure 1: Dose-responses showing inhibition by PBT-3 of $[Ca^{2+}]_i$ release induced by collagen (A) and I-BOP (B). Also shown is a comparison of the effects of PBT-3 with those of the TP receptor antagonists SQ 29,548 and pinane thromboxane A_2 (panel A), and with SQ 29,548 and I-SAP (panel B). Inhibition data were fitted to a line of best fit through a Michaelis-Menten-like hyperbolic treatment using Kaleidagraph statistical software package for Macintosh. IC_{50} values represent data from 3 separate experiments \pm SD.

Figure 2: PBT-3 inhibition of collagen-induced phosphorylation of pleckstrin in the presence or absence of ASA treatment. Platelets were labeled with $[^{32}P]$, pretreated with or without ASA and were incubated with PBT-3 (2 μ g/ml) for 5 min at 37 °C. They were stimulated with collagen (4 μ g/ml) for 3-5 minutes. Protein in the samples was resolved on 12% SDS-PAGE and then it was transferred to a nitrocellulose membrane. A: the membrane was autoradiographed, the arrowhead indicating phosphorylated pleckstrin (47 kDa, P47). Densitometry analysis of the $[^{32}P]$ incorporated into pleckstrin is reported by the bar diagram as mean \pm SD (n=3), and B: Pleckstrin immunoblotting analysis showing similarity in the amount of pleckstrin protein loaded in each well.

Figure 3: Dose-responses showing inhibition by PBT-3 of the collagen (4 μ g/ml)-evoked phosphorylation of pleckstrin (P47) in human platelets *in vitro*. Experimental conditions were described in Fig. 2.

Figure 4: Dose-responses showing inhibition by PBT-3 of the I-BOP (8 x 10⁻⁹ M) evoked phosphorylation of pleckstrin (P47) in human platelets *in vitro*. Experimental conditions were described in Fig. 2.

Figure 5. Competition dose-response curves comparing the inhibition by PBT-3 of the binding of [³H] SQ 29,548 to TP α , TP β and TPtailless transfected COS-7 cells. Note the greater selection by PBT-3 for TP α  TP β . The similarity in competition between the TP α isoform and the TPtailless is suggestive that the tail length may play an important role in PBT-3 inhibition of binding. n=3 separate experiments.

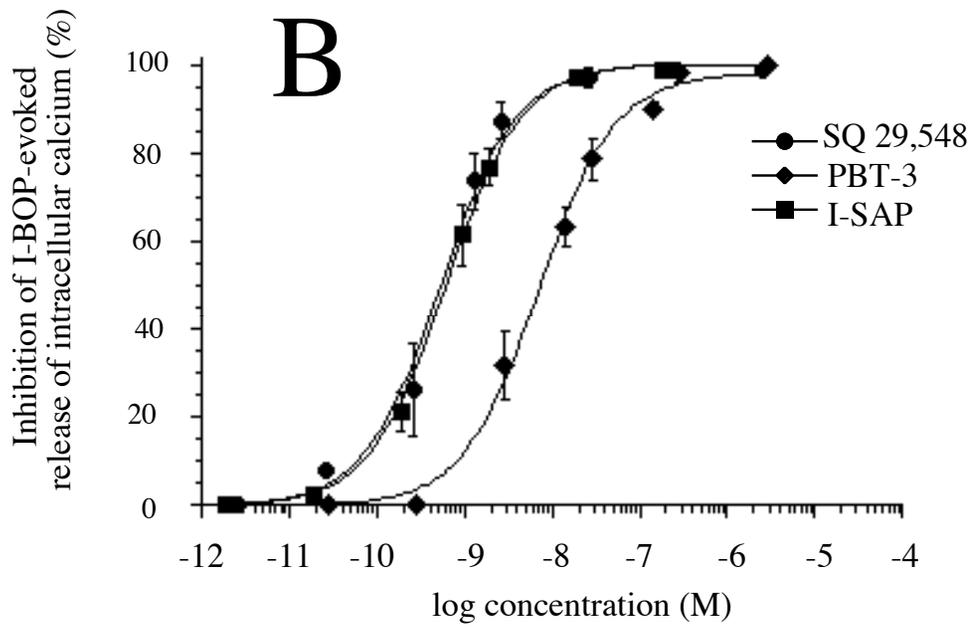
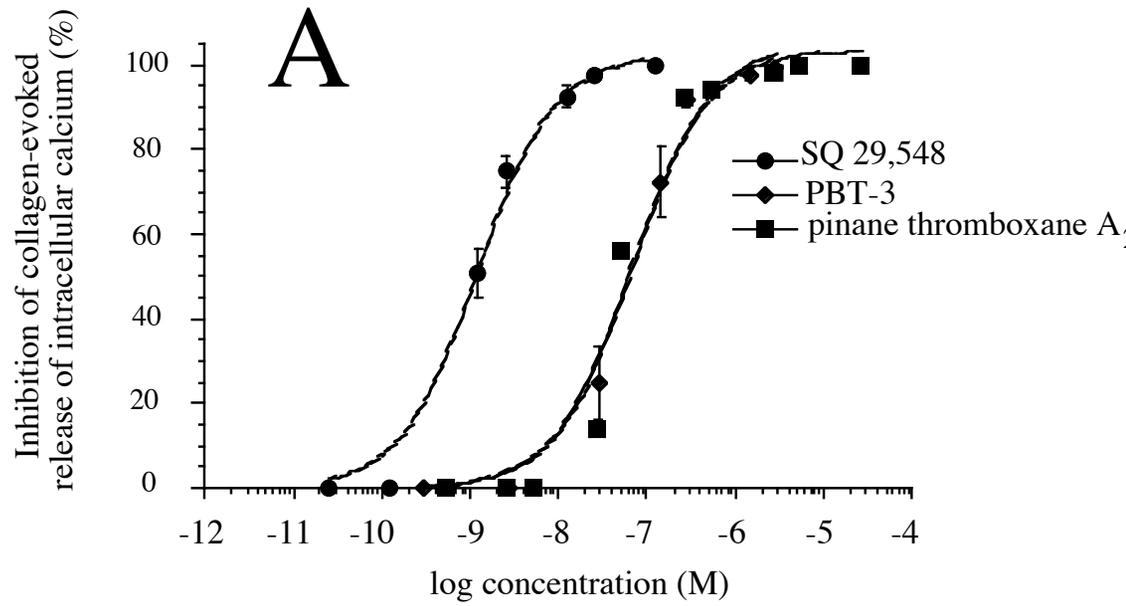


FIG. 1

A

aspirin	+	+	+	+	-	-	-	-
collagen (4 μ g/ml)	-	+	+	-	-	+	+	-
PBT-3 (6 μ M)	-	-	+	+	-	-	+	+

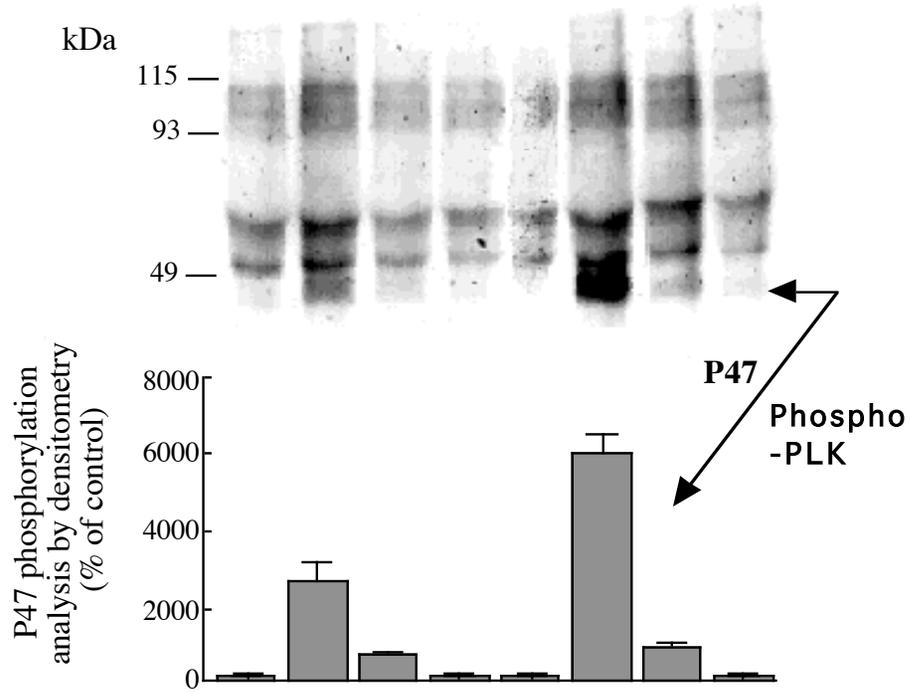
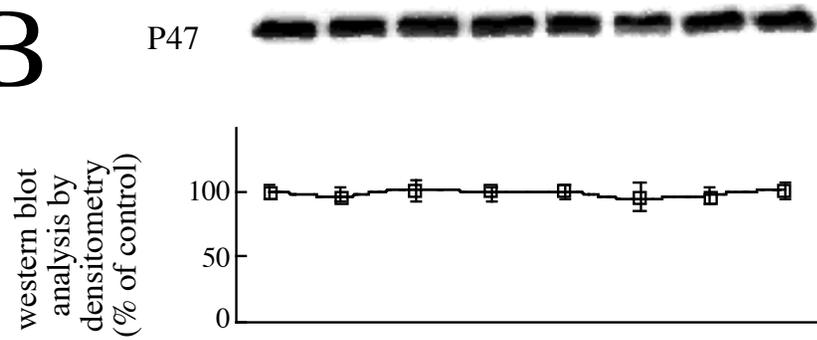
**B**

FIG. 2

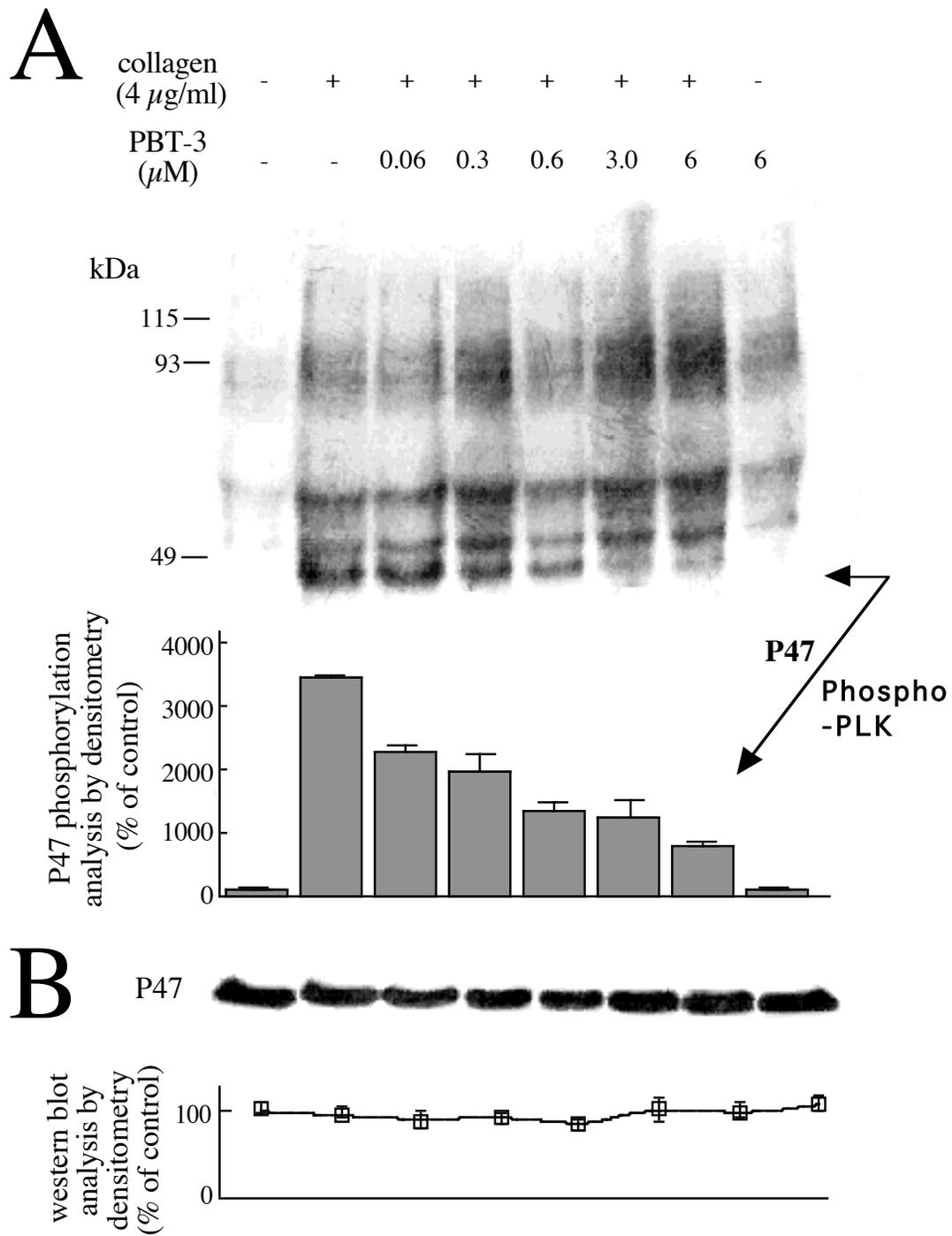
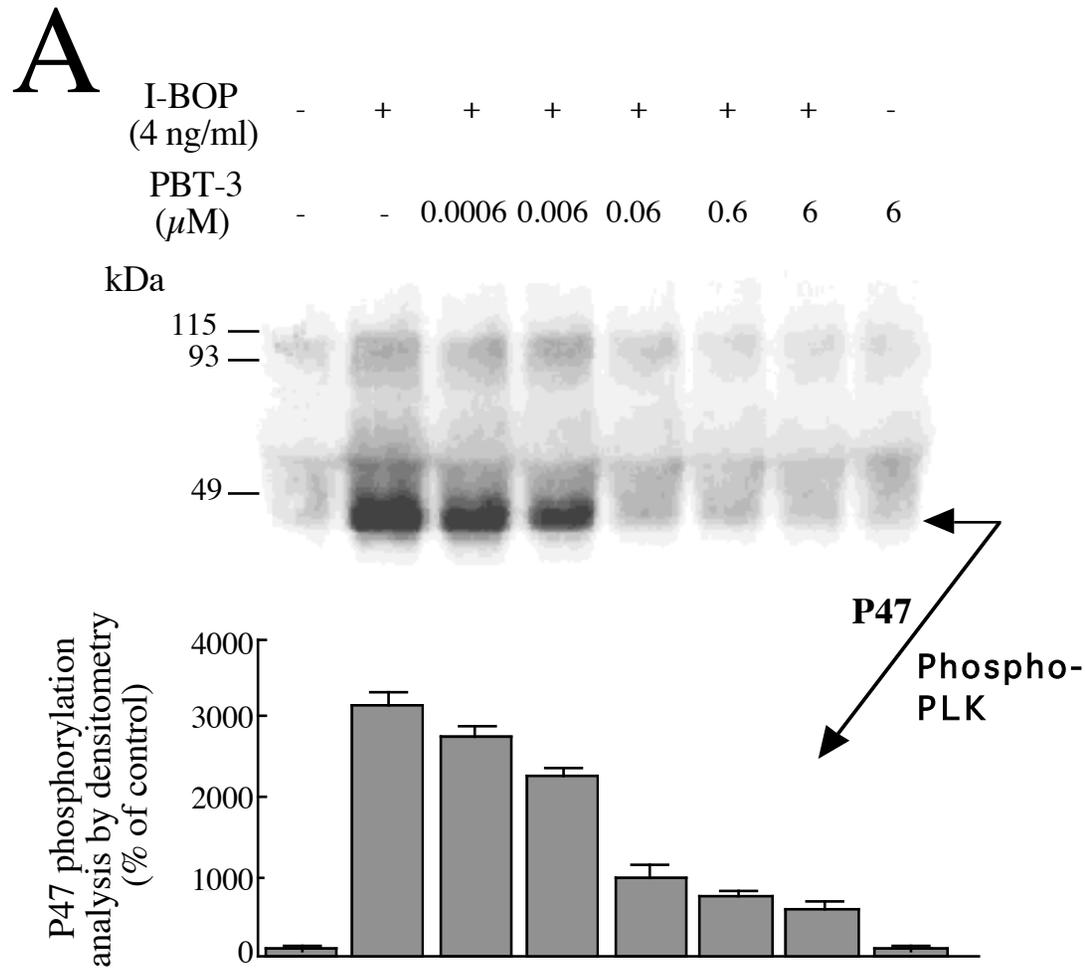


FIG. 3



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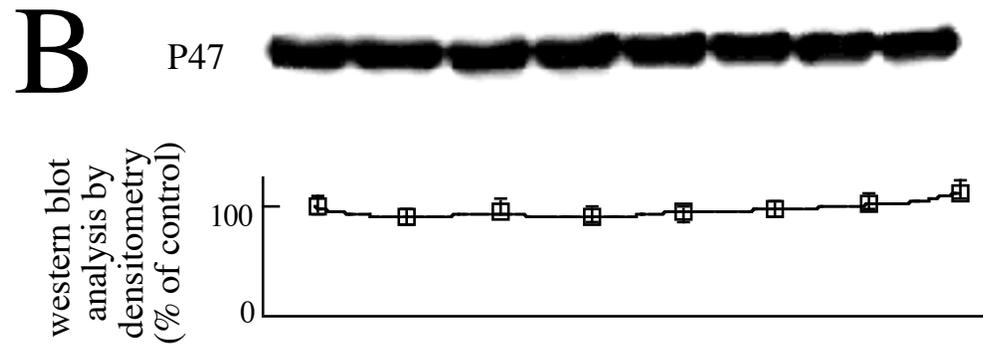


FIG. 4

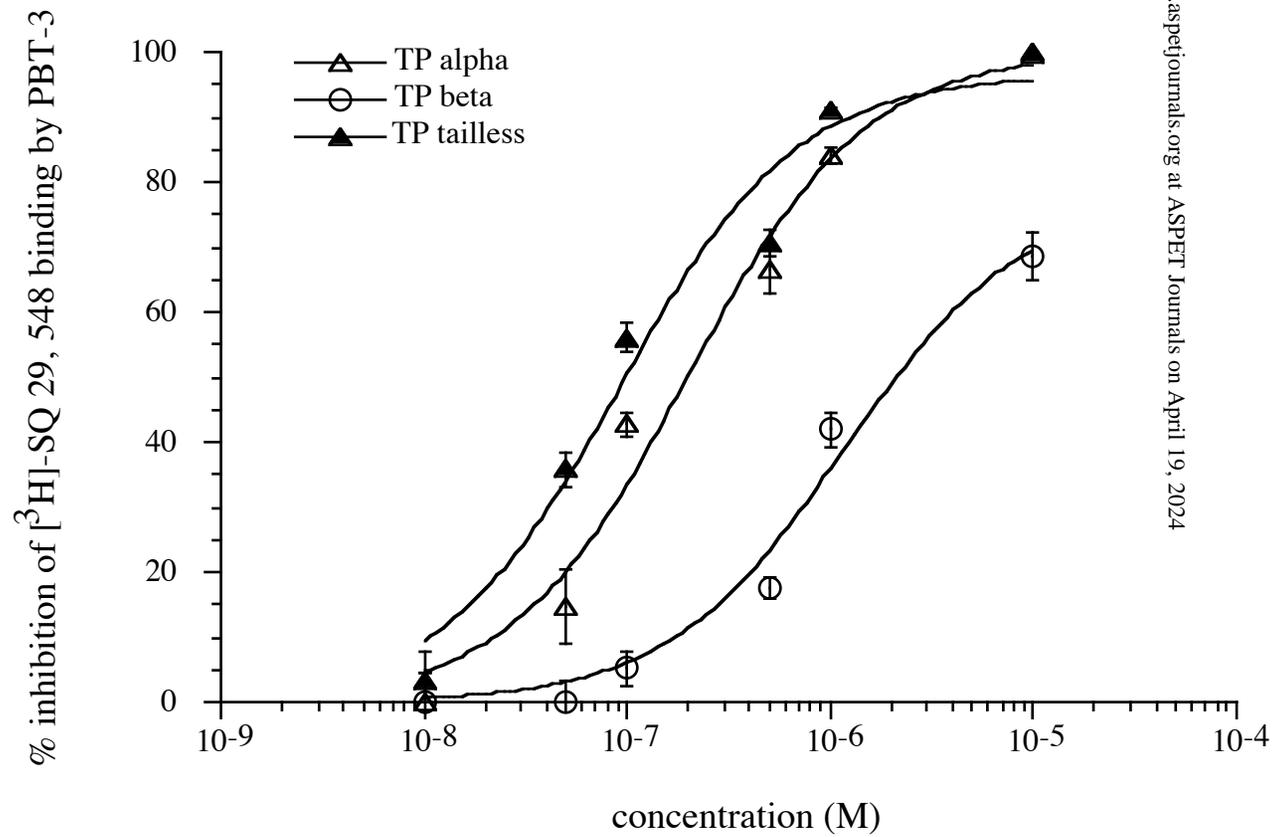


FIG. 5

Table 1. IC₅₀ values comparing the inhibitory effects of PBT-3 and other thromboxane receptor antagonists on collagen- and I-BOP- evoked intracellular calcium release and pleckstrin phosphorylation in human platelets.

Drug	[Ca ²⁺] _i release evoked by		Pleckstrin phosphorylation evoked by	
	collagen (M)	I-BOP (M)	collagen (M)	I-BOP (M)
PBT-3	6.8 x 10 ⁻⁸	7.0 x 10 ⁻⁹	3.0 x 10 ⁻⁷	1.0 x 10 ⁻⁸
SQ 29,548	1.2 x 10 ⁻⁹	0.5 x 10 ⁻⁹		
Pinane TxA ₂	6.5 x 10 ⁻⁸			
I-SAP		0.6 x 10 ⁻⁹		
	Figure 1	Figure 1	Figure 3	Figure 4

JPET #56705