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

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Abbreviations: TP, thromboxane A_2 ; PBT, hepoxilin cyclopropane analogs; PBT-3, 10(S)-hydroxy-11,12-cyclopropyl-eicosa-5Z,8Z,14Z-trienoic acid methyl ester.

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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, [^{3}H] SQ 29,548, to the TP receptor isoforms in TPα and TPβ 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. IC_{50} for PBT-3 = 2.0 ± 0.3 x 10^{-7}M was observed for TPα, while this was one-sixth less active on the TPβ isoform (IC_{50} = 1.2 ± 0.2 x 10^{-6} M), suggesting selectivity for the TPα 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 IC_{50} was similar to that of the TPα 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 TPα isoform of the thromboxane receptor and that the tail may play an important role in recognition of this TP receptor antagonist.**

Thromboxane A_2 (TXA_2) 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 TXA_2 synthase (Hamberg and Samuelsson, 1974; Smith, 1992). TXA_2 formation can result from activation of various cell types, including platelets, macrophages and vascular smooth muscle cells.

Activation of TXA_2 (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). TXA_2 formation is increased in thrombotic disorders (Fitzgerald et al., 1987) and has been implicated in a wide variety of cardiovascular diseases (Smith, 1992).

TXA_2 exerts intracellular effects by interaction with selective members of the G protein-coupled receptor family (Coleman et al., 1994; Watson and Arkinstall, 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 TPα and TPβ, which share the first 328 amino acids. Complementary DNAs for the 343-amino acid TPα were cloned from placental and megakaryocytic sources (Hirata et al., 1991), whereas a

cDNA for the 407-amino acid TPβ 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 TXA_2 biology, such as platelets, vascular and uterine smooth muscle, uterus and placental tissue, endothelium, epithelium, trophoblasts, thymus, liver and small intestine with TPα 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 TPα than TPβ expressed. Isoform selective antibodies permitted detection of TPα, but not TPβ, in human platelets, leading to the suggestion that TPα 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 TPα 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-[1α,2α (Z),3ß(1E,3S*),4α]]-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 A_2), pinane thromboxane A_2 (9α, 11α-(dimethyl)methylene-15S-hydroxy-11a-deoxy-11a-methylene-thromba-5Z,13E-dien-1-oic acid) and SQ 29,548 ([1S-[1α,2α (Z),3α,4α]]-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). [^{32}P]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). [^{3}H] 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 µg/ml

streptomycin, and 25 µg/ml amphotericin (Cellegro). Cells were grown at 37 °C in a

humidified atmosphere of 95% O_2, and 5% CO_2. cDNAs for the TPα and TPβ were

subcloned into pcDNA3, the resultant plasmid, pcDNA3:TPα and pcDNA3:TPβ were

introduced into COS-7 cells (5 µg/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 [^{3}H] 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 x 10^5

cells in a total volume of 0.2 ml in the above buffer with 10 nM [^{3}H] 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. TPα, TPβ 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 NaH$_2$PO$_4$, 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 µM) 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 x

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 µl glass distilled ethanol or test compound in

ethanol followed 2 minutes later by collagen (2 µg) 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 µM) 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 µCi/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 ± 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 [^{3}H] SQ 29,548 incorporated per milligram of cell protein ±

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 $[Ca^{2+}]_i$ transients were exclusively derived from intracellular stores.

<u>1A. Effects on collagen</u>: Dose response curves comparing the actions of PBT-3 and two TP receptor antagonists, SQ 29,548 and pinane thromboxane A_2 on $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ release evoked by collagen (see Table 1).

<u>1B. Effects on I-BOP</u>; I-BOP (8×10^{-9} M) evoked a fast rise in $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 µM. 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 [^{32}P] 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 IC$_{50}$ value is estimated to be 1 x 10^{-8} M (see Table 1).

3. Inhibition of binding by PBT-3 of [^{3}H]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 [^{3}H] SQ

29,548/mg cell protein, respectively. PBT-3 effectively competed with [^{3}H] 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 IC$_{50}$ values were derived (Fig. 5). PBT-3 was about 6

times more potent in competing with [^{3}H] SQ 29,548 binding for the TPα than

TPβ. The IC$_{50}$ value for TPα was 2.0 ± 0.3 x 10^{-7}M, 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 ± 0.2 x 10^{-8} M, similar to the TPα 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 TXA_2 biology, such as platelets, vascular and uterine smooth muscle, uterus and placental tissue, endothelium, epithelium, trophoblasts, thymus, liver and small intestine with TPα 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 TPα than TPβ expressed. Isoform selective antibodies permitted detection of TPα, but not TPβ, in human platelets, leading to the suggestion that TPα 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.

TPα and TPβ 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 TPα, and $1.2 \pm 0.2 \times 10^{-6}$ M for TPβ. Even at the high concentration of PBT-3 (200 µM), the inhibition of binding of TPβ only reached 76% in comparison with 100% inhibition of TPα at 10 µM. 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 TPα and TPβ 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 TPα and TPβ receptor isoforms differ, selective inhibition of the TPα 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 TXA_2 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 µM). In this study we have found that PBT-3 is selective towards the TPα 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.

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Footnotes

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 µg/ml) for 5 min at 37 °C. They were stimulated with collagen (4 µg/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 (4μg/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 [^{3}H] SQ 29,548 to TPα, TPβ and TPtailless transfected COS-7 cells. Note the greater selection by PBT-3 for TPα□□□□□□□□□ TPβ□□□□□□□. The similarity in competition between the TPαisoform and the TPtailless is suggestive that the tail length may play an important role in PBT-3 inhibition of binding. n=3 separate experiments.
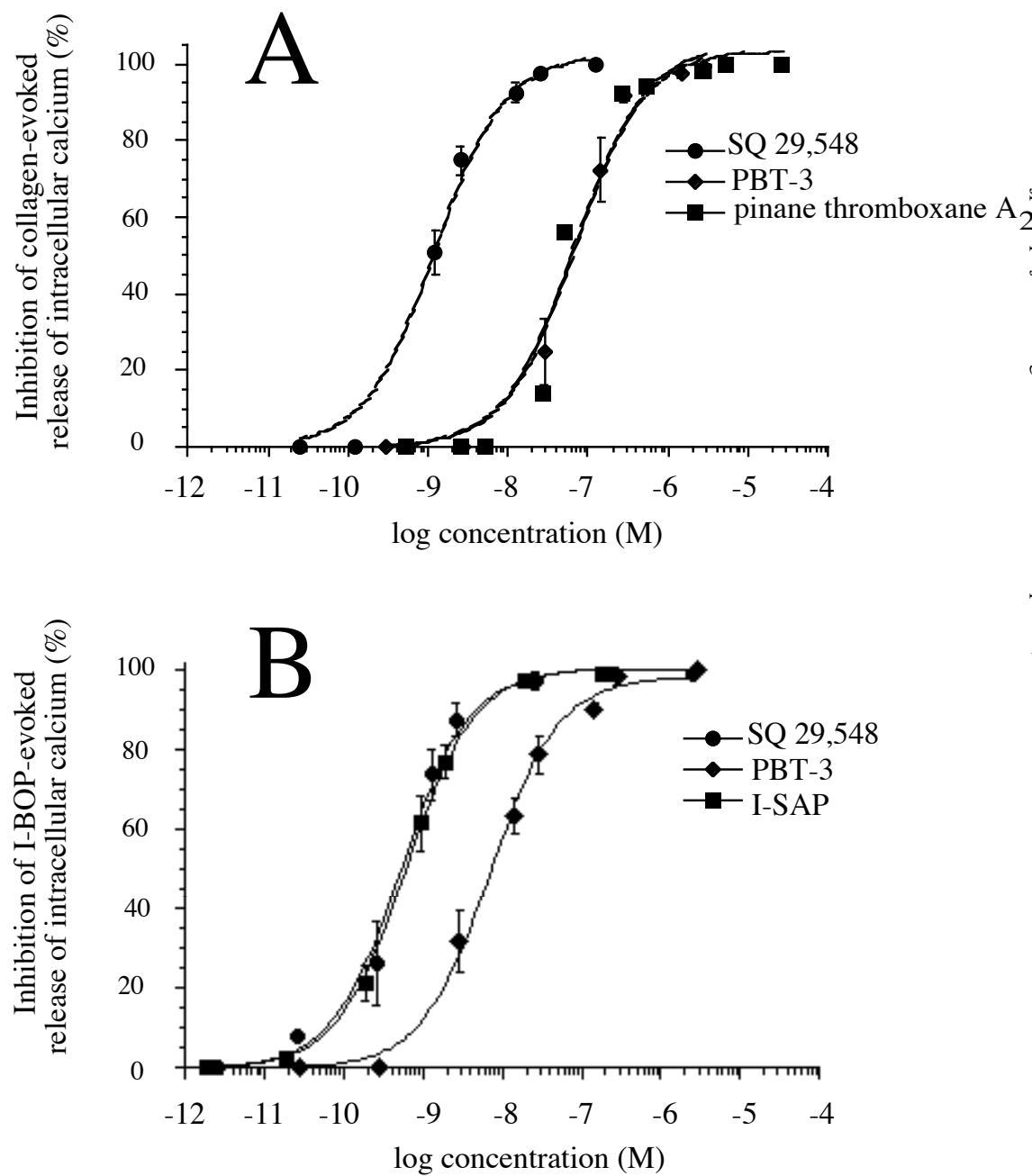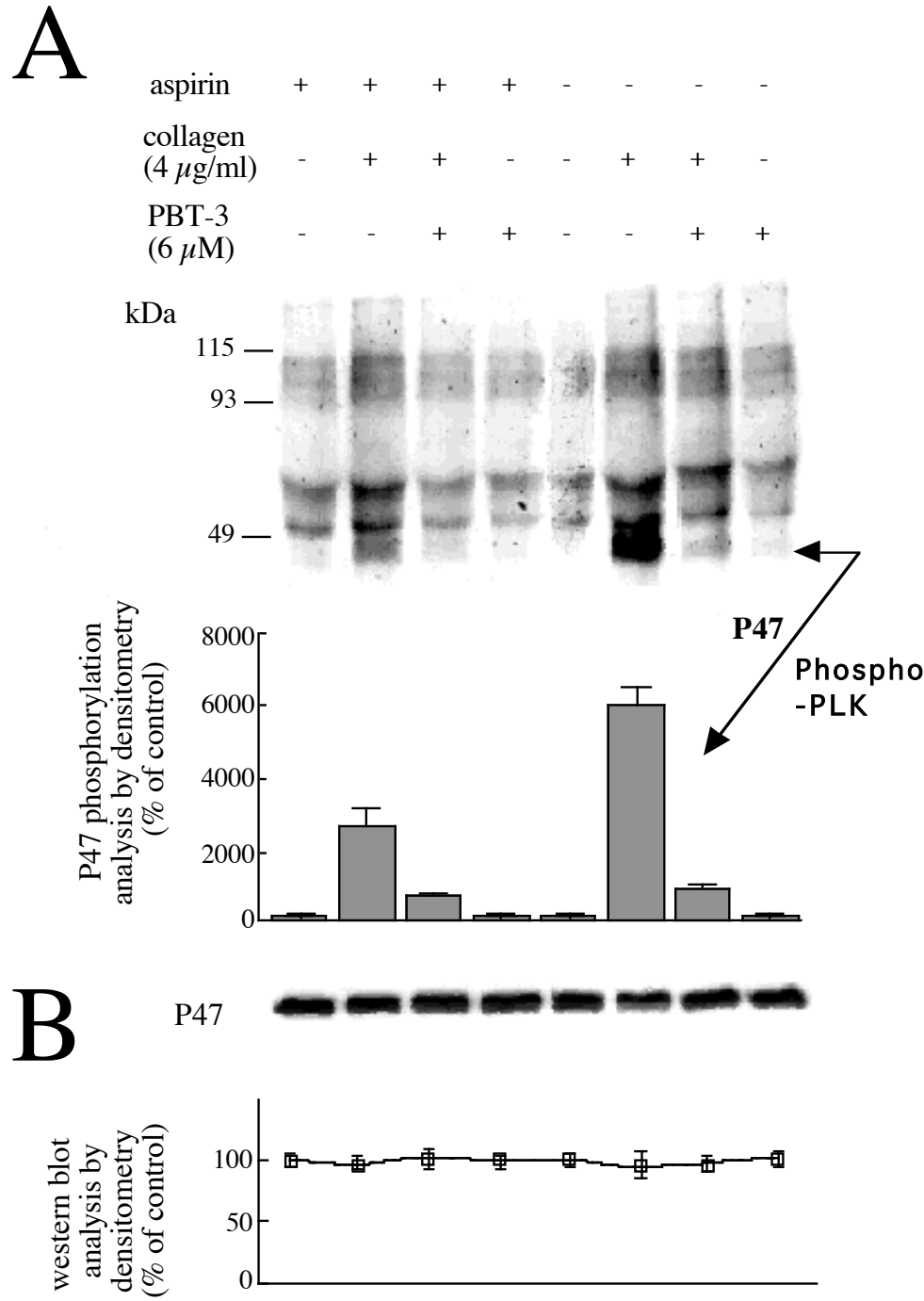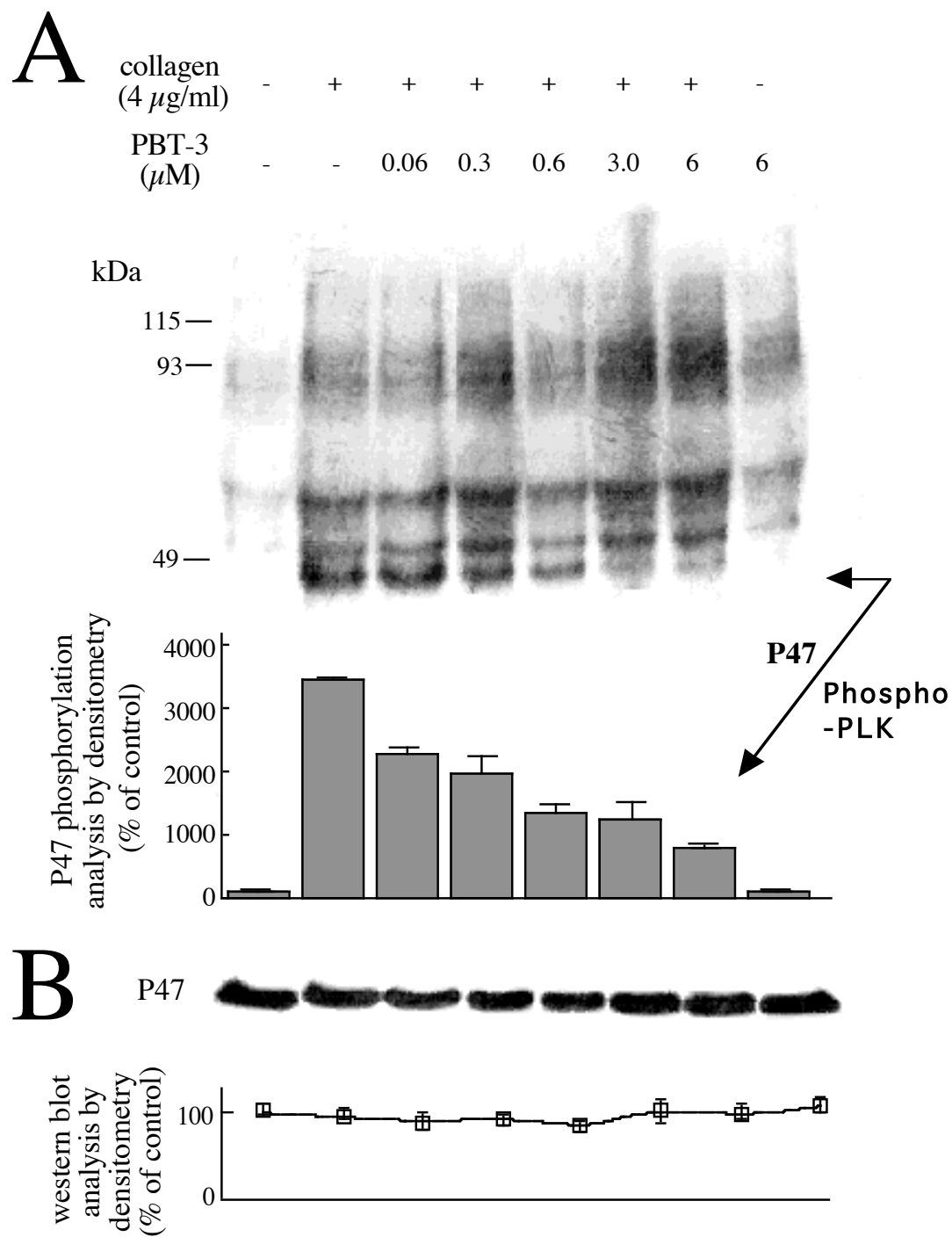
FIG. 1

A

aspirin	+	+	+	+	-	-	-	-
collagen (4 μg/ml)	-	+	+	-	-	+	+	-
PBT-3 (6 μM)	-	-	+	+	-	-	+	+

kDa

115 —

93 —

49 —

P47

Phospho-PLK

P47 phosphorylation analysis by densitometry (% of control)

B

P47

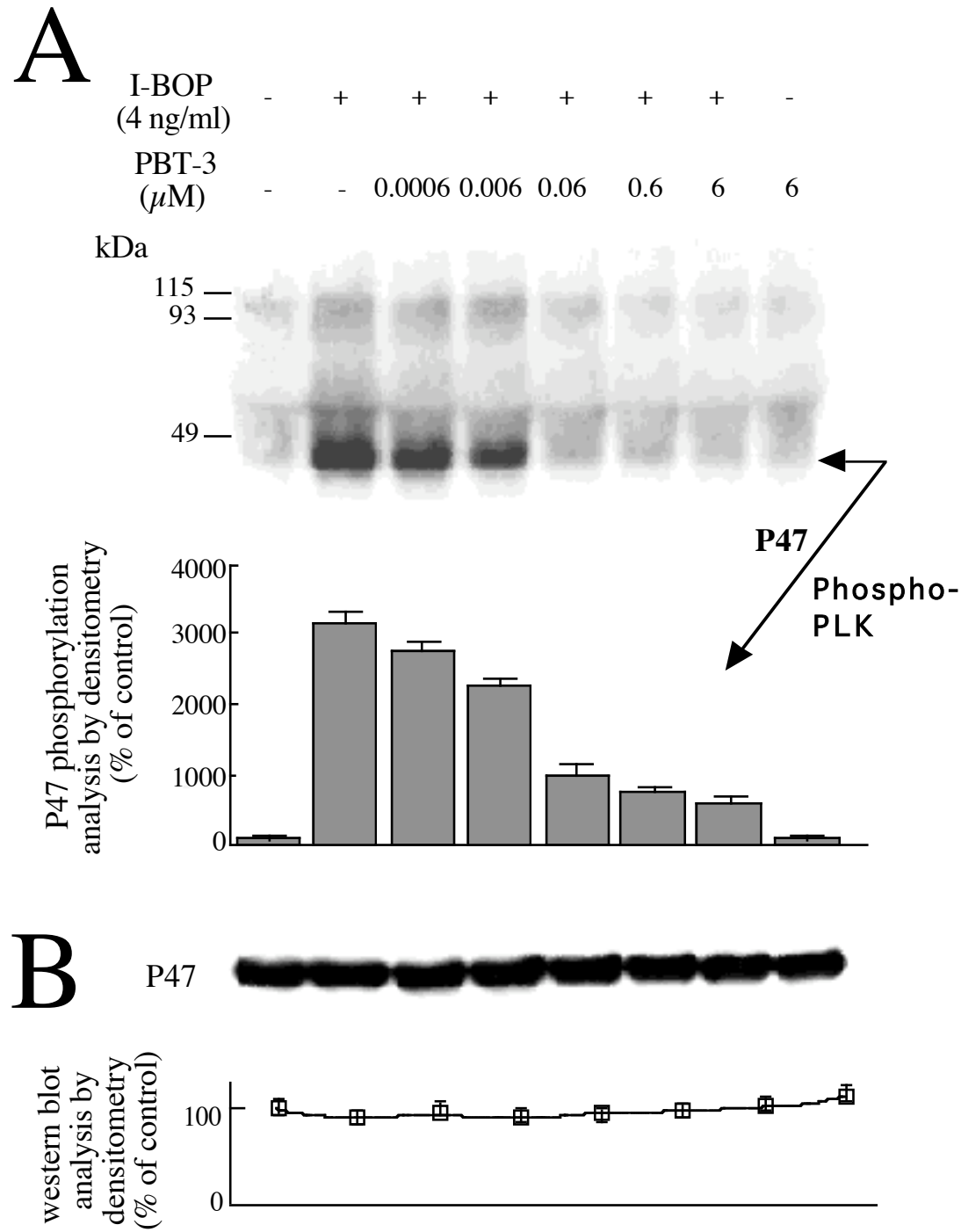western blot analysis by densitometry (% of control)

FIG. 2

FIG. 3

FIG. 4
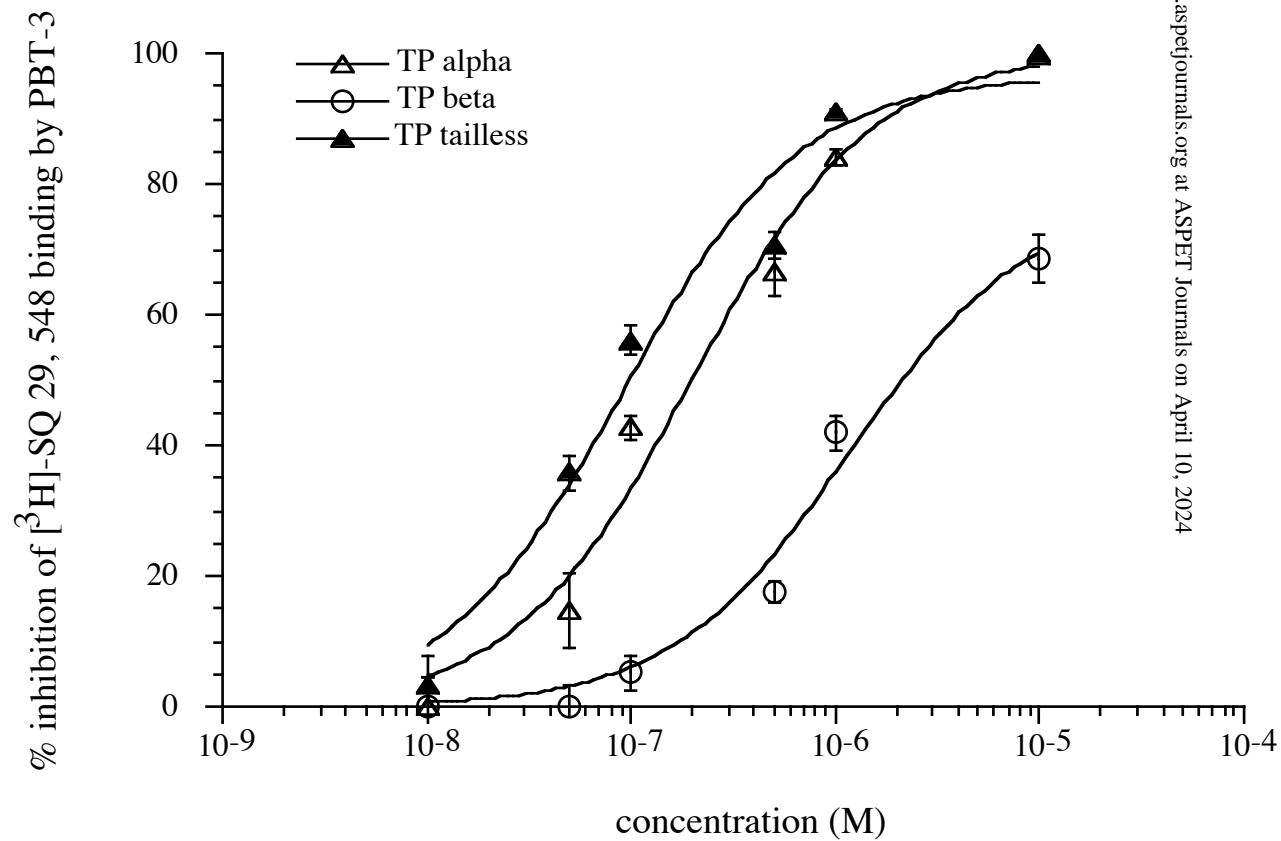
FIG. 5

Table 1. IC$_{50}$ 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	collagen (M)	I-BOP
PBT-3	6.8×10^{-8}	7.0×10^{-9}	3.0×10^{-7}	1.0×10^{-8}
SQ 29,548	1.2×10^{-9}	0.5×10^{-9}		
Pinane TxA$_2$	6.5×10^{-8}			
I-SAP		0.6×10^{-9}		
	Figure 1	Figure 1	Figure 3	Figure 4

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