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RESEARCH PAPER

Inhibition of vascular ectonucleotidase activities by the pro-drugs ticlopidine and clopidogrel favours platelet aggregation

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BACKGROUND AND PURPOSE

After conversion to their active forms by the liver, ticlopidine and clopidogrel exert antiplatelet effects through irreversible inhibition of the $P2Y_{12}$ receptor. Concentrations of nucleotides such as ADP, the physiological agonist at platelet $P2Y_1$ and $P2Y_{12}$ receptors, are regulated by vascular ectonucleotidases, mainly nucleoside triphosphate diphosphohydrolase (NTPDase)1 and ecto-5′-nucleotidase. Here we evaluate the effect of these pro-drugs on vascular ectonucleotidase activity and on the natural function of these enzymes in regulating platelet aggregation.

EXPERIMENTAL APPROACH

Nucleotidase assays were performed by HPLC and by P_i determination, using human umbilical vein endothelial cells (HUVEC) and protein extracts from transfected COS-7 cells as sources of enzymes. Platelet aggregation was assayed using human platelet-rich plasma.

KEY RESULTS

Each pro-drug inhibited endothelial ectonucleotidase activities and decreased their ability to block platelet aggregation *in vitro*. At their therapeutic concentrations, ticlopidine (60 μM) and clopidogrel (20 μM) inhibited ADP hydrolysis by HUVEC by about 80%, and AMP hydrolysis by one-third. Accordingly, these compounds showed a mixed-type inhibition of recombinant human NTPDase1 with an apparent K_i ($K_{i,app}$) of 10 μM (clopidogrel) and 14 μM (ticlopidine). Recombinant rat ecto-5′-nucleotidase, but not its human orthologue, was inhibited by ticlopidine with a $K_{i,app}$ of 4.5 mM.

CONCLUSIONS AND IMPLICATIONS

These pro-drugs facilitated platelet aggregation via the inhibition of vascular NTPDase1 *in vitro*. Further studies should be performed to assess whether this effect also occurs *in vivo*, especially at the beginning of treatment, before sufficient levels of active metabolites are produced by the liver.

Abbreviations

NTPDase, nucleoside triphosphate diphosphohydrolase; HUVEC, human umbilical vein endothelial cells

Introduction

The imbalance of extracellular purine levels in blood observed in various cardiovascular diseases is associated with complications characterized by uncontrolled blood clot formation (Ajjan and Grant, 2006; Vorchheimer and Becker, 2006), blood pressure fluctuations (Duprez and Cohn, 2006) and changes in the physiology of endothelial, smooth muscle and blood cells (Nadar *et al.*, 2004; Corradi *et al.*, 2005).

Extracellular nucleotides such as ATP and ADP exert their effects via the activation of purinoceptors collectively referred to as P2 receptors: the

ligand-gated ion channels P2X1-7 and the G protein-coupled receptors $P2Y_{1,2,4,6,11-14}$ (Ralevic and Burnstock, 1991; receptor nomenclature follows Alexander *et al.*, 2009). Similarly, adenosine, the dephosphorylated product of ATP and ADP, is biologically active via other G protein-coupled P1 receptors ($A_{1,2A,2B,3}$) that modulate adenylate cyclase activity (Yaar *et al.*, 2005). All these molecules, namely ATP, ADP and adenosine, modulate cardiovascular functions. ADP is a major platelet-recruiting factor that activates haemostasis via $P2Y_1$ and $P2Y_{12}$ receptors (Hechler *et al.*, 2005). ATP stimulates endothelial cells to produce nitric oxide and prostacyclin, which have the ability to act as both vasodilators and inhibitors of platelet aggregation (Motte *et al.*, 1995). Finally, adenosine inhibits platelet aggregation by activating the A_{2A} receptor on platelets and induces vasodilation via A_{2B} on smooth muscle cells (Hourani, 1996; Ansari *et al.*, 2007).

The purine/nucleotide pool in the blood originates from different sources including exocytosis (platelets), shear stress (red blood cells), cell activation (platelets, endothelial cells) and cell lysis (Goldsmith *et al.*, 1995; Morrell *et al.*, 2005). In the blood, hydrolysis of these nucleotides is regulated by membrane-bound nucleoside triphosphate diphosphohydrolase (NTPDase)1[responsible for the hydrolysis of ATP &ADP] and ecto-5′-nucleotidase [responsible for the hydrolysis of AMP] at the surface of the vascular endothelium (Pearson *et al.*, 1980; Kaczmarek *et al.*, 1996; Marcus *et al.*, 1997; Sévigny *et al.*, 1997; Enjyoji *et al.*, 1999; Kawashima *et al.*, 2000). The pertinent hydrolytic products of the nucleotides selectively activate P1 and P2 pathways. AMP, the product of NTPDase1-driven hydrolysis of ATP and ADP, is the substrate for ecto-5′-nucleotidase that produces adenosine (Colgan *et al.*, 2006). Hence, ectonucleotidases turn off P2 pathways, and simultaneously switch on the activation of P1 pathways (Koszalka *et al.*, 2004; Robson *et al.*, 2006), underlining the importance of both NTPDase1 and ecto-5′-nucleotidase as regulators of haemostasis (Kawashima *et al.*, 2000; Sévigny *et al.*, 2002). Therefore, the nucleotide pathways involved in the regulation of platelet aggregation may be targeted in order to prevent thrombus formation.

Thienopyridine drugs, such as ticlopidine (Tyklid) and clopidogrel (Plavix), exert their anti-aggregatory effects by irreversibly inhibiting the $P2Y_{12}$ receptor (Foster *et al.*, 2001; Hollopeter *et al.*, 2001; Cattaneo, 2006; Braun *et al.*, 2007). Clinical studies have shown that these anti-platelet agents become effective 24 to 48 h after their administration, with a maximal effect achievable between 3 and 5 days, that is, only after a sufficient fraction of each pro-drug has been converted into an active drug by microsomal oxygenases in the liver (Kam and Nethery, 2003). Long-term treatment with thienopyridines is required to reach a 40–60% reduction in platelet aggregation (Kam and Nethery, 2003). Unfortunately, these drugs also demonstrate some undesirable side effects (Quinn and Fitzgerald, 1999). Ticlopidine therapy may be accompanied by the development of thrombotic thrombocytopaenic purpura-haemolytic uraemic syndrome, cutaneous exanthema, diarrhoea and reversible leucopaenia/agranulocytosis in patients with cerebrovascular diseases (Oster *et al.*, 1994; Chinnakotla *et al.*, 2000; Sempere *et al.*, 2000). Clopidogrel has been reported to cause gastrointestinal haemorrhage (Hallas *et al.*, 2006) and/or red and purple skin rashes (Makkar *et al.*, 2006).

In this study, we report that ticlopidine and clopidogrel, in their pro-drug forms, directly affect the hydrolysis of nucleotides by vascular ectonucleotidases, which might lead to an undesired effect on platelet aggregation.

Methods

Plasmids

The plasmids encoding human NTPDase1 (GenBank accession no. U87967) and rat ecto-5′-nucleotidase (NM_021576) have been described in published reports (Misumi *et al.*, 1990; Kaczmarek *et al.*, 1996).

The cDNA encoding human ecto-5′-nucleotidase was cloned as follows. The cDNA was synthesized with SuperScript®II (Invitrogen) from 500 ng of total human liver RNA (AMBIOV; a gift from Dr C. Guillemette, CRCHUL) with oligo$(dT)_{18}$ as the primer, in accordance with manufacturer's instructions (Invitrogen). For amplification, 10% of the reverse transcription reaction was used as template in a final volume of 50 µL of a reaction mixture containing 0.6 µM primer, 400 µM dNTP and 3.5 U Expand High Fidelity PCR System (Roche, Laval, Canada). The following primers were designed based on the human ecto-5′-nucleotidase sequence from the NCBI database (NM_002526): forward sequence 5′-AGTTCACGCGCCACAGCTAT-3′ and reverse sequence 5′-AAGATGGTTTTTGCTGTCA-3′. Amplification was started by incubating the reaction mixture for 2 min at 94°C followed by 30 cycles of denaturation for 30 s at 94°C, annealing for 1 min at 60°C and primer extension for 1 min at 72°C, with a final incubation of 7 min at 72°C. The PCR products of approximately 1.7 kb were purified on a 1% agarose gel using the QIAEX II gel extraction kit (Qiagen, Missisauga, Ontario, Canada) and ligated into the expression vector pcDNA3.1/V5-His

(Invitrogen). Plasmids were purified with the QIAprep Spin Miniprep kit (Qiagen) and orientation of the inserts was determined by restriction enzyme mapping. One clone was amplified, fully sequenced in one direction and deposited in Gene Bank: DQ186653. These plasmids were used for transfection and activity assays.

Cell transfection and protein preparation

COS-7 cells were transfected in 10 cm plates using Lipofectamine (Invitrogen) as described (Kaczmarek *et al.*, 1996). Protein extracts from these transfected cells were also prepared as before (Kukulski *et al.*, 2005). Briefly, Tris-saline buffer-washed cells were collected by scraping in harvesting buffer (95 mM NaCl, 0.1 mM PMSF, 10 $\mu g \cdot mL^{-1}$ aprotinin and 45 mM Tris at pH 7.5) and sonicated. Nuclei and cellular debris were discarded by centrifugation and the supernatant, herein called the cell lysate or protein extract, was aliquoted and stored at –80°C until used for activity or platelet assays. Protein concentration was estimated by the Bradford microplate assay using bovine serum albumin as standard (Bradford, 1976).

Ectonucleotidase activities and kinetics

Enzyme activity of protein extracts was determined as previously described (Kukulski *et al.*, 2005). Briefly, assays were carried out at 37°C in buffer A, for NTPDases, which consisted of 5 mM $CaCl_2$ and 80 mM Tris/HCl, pH 7.4, with or without the indicated concentration of ticlopidine or clopidogrel (stock solutions in 80 mM Tris/HCl, pH 6.8). Assays for ecto-5′-nucleotidase were carried out in buffer B that consisted of the buffer A as above but $CaCl_2$ was replaced by 5 mM $MgCl_2$. Protein extracts were pre-incubated with the incubation mixture at 37°C for 3 min. The reaction was initiated by the addition of 0.5 mM ATP or ADP as substrate (down to 0.05 mM for kinetic assays), and stopped with malachite green reagent. For controls (consisting of the appropriate substrate and pro-drug), protein extracts were added after mixing the other components with the malachite green reagent. The activity at the surface of intact human umbilical vein endothelial cells (HUVECs, passage 2) or ectonucleotidase-transfected cells was measured in 24-well plates with the buffers indicated above supplemented with 125 mM NaCl. The reactions were initiated as above and stopped by transferring an aliquot of the reaction mixture to a tube containing the malachite green reagent. Net cell surface enzyme activity was calculated after subtracting the value measured in the control cell reaction mixture where the substrate was added after the malachite green reagent. Release of inorganic phosphate (P_i) was measured according to Baykov *et al.* (1988). All experiments were performed in triplicate. One unit of enzymatic activity corresponds to the release of 1 µmol $P_i \cdot min^{-1} \cdot mg^{-1}$ of protein or 1 µmol $P_i \cdot min^{-1}$ per well at 37°C for protein extracts or intact cells respectively. Kinetic parameters ($K_{m,app}$, $K_{i,app}$ and V_{max}) were determined by Eadie-Hofstee/Hanes-Woolf transformations and the Dixon method and the values were calculated using the GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). For some experiments, nucleotides were analysed in reaction samples by HPLC as described earlier (Kukulski *et al.*, 2005).

Platelet aggregation assays

The ethical investigation standards conformed to principles outlined in the Helsinki declaration (Puri *et al.*, 2009). Platelet-rich plasma (PRP) was prepared from the blood of healthy volunteers according to standard procedures (Sévigny *et al.*, 2002). Briefly, whole blood was collected in heparinized tubes, centrifuged at room temperature at 300× *g* for 12 min, and the upper layer comprising the PRP was collected. The interval between blood collection and the beginning of platelet aggregation assays never exceeded 2 h. Platelet aggregation was either measured using an AggRAM aggregometer, or by spectrophotometry, using a microplate reader (Molecular Devices). The extent of platelet aggregation corresponded to the increase in light transmission or decrease in optical density at 600 nm in 0.6 mL (aggregometer) or 0.2 mL (spectrophotometer) of PRP samples maintained at 37°C. Platelet-poor plasma (PPP) was used as the reference standard. Platelet aggregation was initiated with 5 to 20 µM ADP. Where indicated, lysates from NTPDase1-transfected COS-7 cells (4–6 µg diluted in incubation buffer A with 145 mM NaCl) or HUVECs treated or not with a CD39-specific shRNA or a scrambled shRNA (2×10^6 HUVECs scraped into a 135 mM KCl/15 mM sodium citrate solution, washed twice and resuspended in incubation buffer A in the presence of 145 mM NaCl with or without test drugs, were added to PRP. Note that appropriate control experiments contained either incubation buffer with intact COS-7 cells, protein extracts from non-transfected COS-7 cells, or an equivalent amount of the water used to dilute the pro-drugs.

For the parallel assays using light microscopy, 100 µL of the above reaction mixture were put on microscope slides 10 min after the initiation of platelet aggregation by ADP addition. These slides were then air dried at 37°C and stained using the Diff-Quick kit (Dade Behring Inc., Deerfield, IL, USA). The remainder of the reaction mixture was spun at 300× *g* for 3 min, and free platelets in the supernatant were counted.

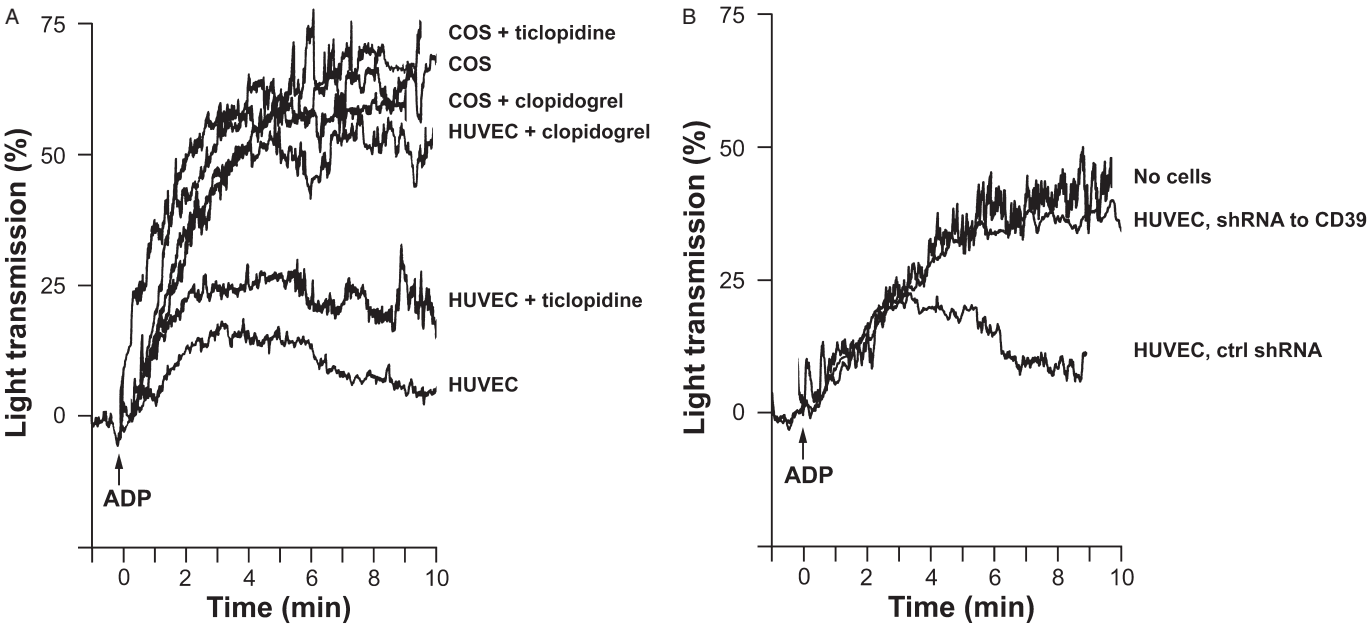


Figure 1

Ticlopidine and clopidogrel decrease the ability of HUVEC (2×10^6 cells) to block ADP-induced platelet aggregation. Where indicated, cells with or without 60 μM ticlopidine or 20 μM clopidogrel were pre-incubated in 0.6 mL platelet-rich plasma (PRP) 3 min before stimulation with 8 μM ADP. The ability of HUVECs to block platelet aggregation was diminished by ticlopidine, clopidogrel (A), and by a specific shRNA that partly inhibits NTPDase1 expression, but was not affected by a control (ctrl) shRNA (B). In control assays, COS-7 cells, which are devoid of ADPase and AMPase activity, did not modify ADP-induced platelet aggregation. This number of intact HUVECs released 6 or 4.8 μmol $P_i \cdot min^{-1}$ with ADP or AMP as substrate respectively. A representative experiment is shown. All 12 experiments performed showed similar trends with these thienopyridines. NTPDase, nucleoside triphosphate diphosphohydrolase; HUVEC, human umbilical vein endothelial cell.

Statistics

Comparisons between the shapes of the different platelet aggregation curves and of the ectonucleotidase assays were drawn, using the two-tailed *t*-test for unpaired data. A value of $P < 0.01$ was considered as statistically significant.

Materials

ATP, ADP, AMP, ticlopidine, clopidogrel, bovine serum albumin, KCl, malachite green and phenylmethylsulfonyl fluoride were purchased from Sigma-Aldrich (Oakville, ON, Canada). Ammonium molybdate, NaCl, Tris and Tween 20 were provided by EMD Chemicals (Gibbstown, NJ). $CaCl_2$ and $MgCl_2$ were acquired from Fisher Scientific (Ottawa, ON, Canada), DMEM/F12 from Invitrogen (Burlington, ON, Canada) and fetal bovine serum from Wisent Bioproducts (St-Bruno, QC, Canada).

Results

The pro-drugs ticlopidine and clopidogrel do not inhibit $P2Y_{12}$ receptors directly, that is, without metabolic activation (Foster *et al.*, 2001; Kam and Nethery, 2003), so we assessed whether or not they might influence the process of haemostasis. The concentration of the pro-drug in the blood of patients treated by oral or i.v. administration has been reported to be in the order of 60 μM for ticlopidine and 20 μM for clopidogrel (Kam and Nethery, 2003). At these concentrations, pro-drugs decreased the ability of HUVECs to block ADP-stimulated platelet aggregation (Figure 1A), which correlated with changes in the catabolism of purines by NTPDase1 (CD39) at the cell surface. Ticlopidine and clopidogrel each inhibited the hydrolysis of ADP and ATP by about 80% and 30% respectively (Figure 2A). Notably, this inhibition did not increase after a longer pre-incubation of HUVECs with pro-drugs prior to the assay, as demonstrated by the 3 min, and 3, 7 and 32 h results (Figure 2B and data not shown). Both ticlopidine and clopidogrel also inhibited HUVEC-driven AMP hydrolysis by about 37% and 17% respectively (Figure 2A). HPLC analyses of nucleotides and nucleosides at the end of the incubations confirmed the inhibition of HUVEC ATPase, ADPase and AMPase activities by thienopyridines (data not shown). As a control, knock-down of the expression of NTPDase1, that is, the enzyme responsible for the hydrolysis of ADP at the surface of HUVECs, using a specific shRNA, similarly

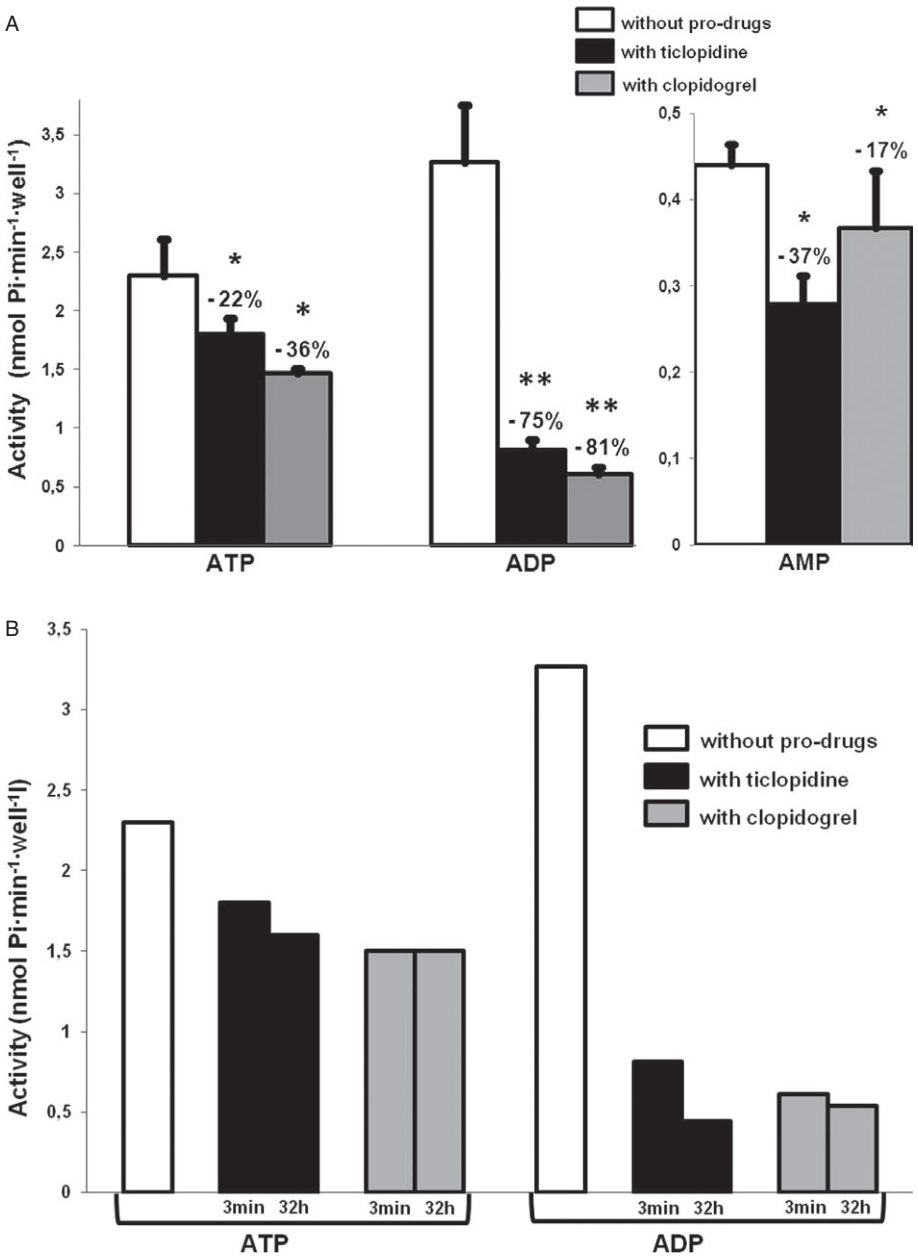


Figure 2

Therapeutic concentrations of ticlopidine (60 μM) and clopidogrel (20 μM) inhibit directly the ectonucleotidase activity of intact human umbilical vein endothelial cells (HUVECs). Cells at the second passage from three donors were tested, each in duplicate. Hydrolysis of ATP, ADP or AMP was examined with or without ticlopidine or clopidogrel. Enzymic units are expressed as the mean ± SD in nmol P_i·min^{-1} per well. The percentage of inhibition is indicated above each bar. (A) ATP, ADP and AMP hydrolysis. (B) Influence of the pre-incubation time of thienopyridines with HUVEC on ATP and ADP hydrolysis at their surface. Statistical differences between assays with or without pro-drugs *$P < 1 \times 10^{-3}$, **$P < 1 \times 10^{-4}$.

reduced the ability of these cells to block platelet aggregation (Figure 1B). The efficacy of the shRNA was evaluated by FACS (13% decrease of CD39 cell surface expression) and by an activity assay using ADP as substrate (30% decrease). A scrambled shRNA did not affect these parameters when compared with untreated HUVEC (Figure 1B and data not shown).

We next examined the effect of ticlopidine and clopidogrel on the activity of two recombinant vascular ectonucleotidases, namely NTPDase1 and ecto-5′-nucleotidase, using protein extracts from transfected COS-7 cells. As expected, these pro-drugs inhibited ADP hydrolysis more efficiently than that of ATP (Figure 3A). Significantly, ticlopidine, at the concentration reported in the blood of treated

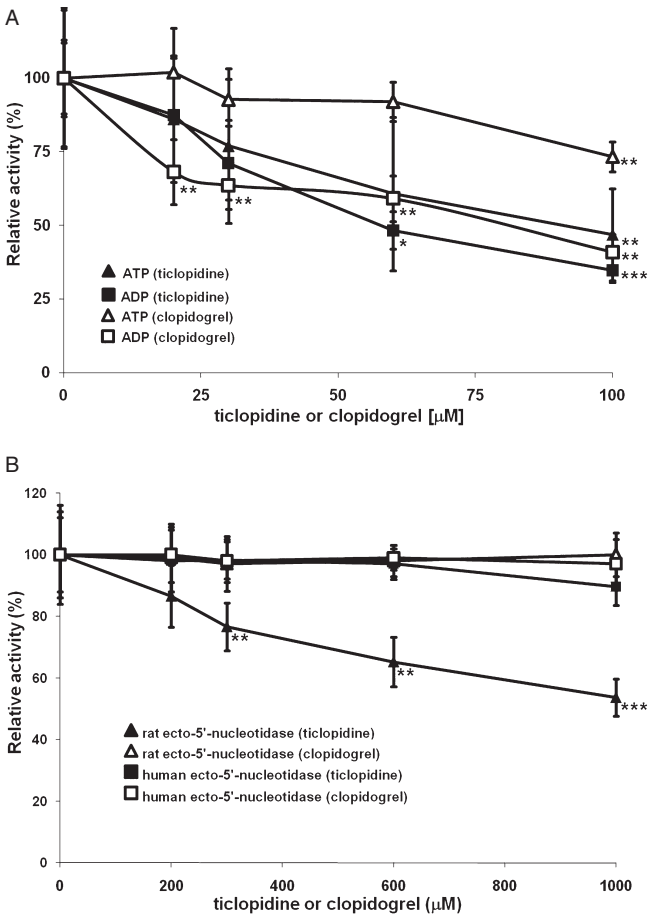


Figure 3

Influence of ticlopidine and clopidogrel on recombinant ectonucleotidase activities. Enzymic assays were carried out using protein extracts from COS-7 cells transfected with an expression vector encoding each enzyme separately. The activity of human NTPDase1 with either ATP or ADP as substrate (A), as well as of human and rat ecto-5′-nucleotidases with AMP as substrate (B), were evaluated in the presence of ticlopidine or clopidogrel. (A) Activity of 100% for human NTPDase1 corresponded to 150 and 120 nmol P_i·min^{-1}·(mg protein)$^{-1}$ with ATP and ADP respectively. (B) Activity of 100% corresponded to 6800 and 1000 nmol P_i·min^{-1}·(mg protein)$^{-1}$ with AMP as substrate for the rat and the human ecto-5′-nucleotidase respectively. Relative activities are expressed as the mean ± SD of four independent experiments, each performed in triplicates. Statistical differences between assays with and without pro-drugs are $^*P < 1 \times 10^{-2}$, $^{**}P < 1 \times 10^{-3}$, $^{***}P < 1 \times 10^{-4}$. NTPDase, nucleoside triphosphate diphosphohydrolase; HUVEC, human umbilical vein endothelial cell.

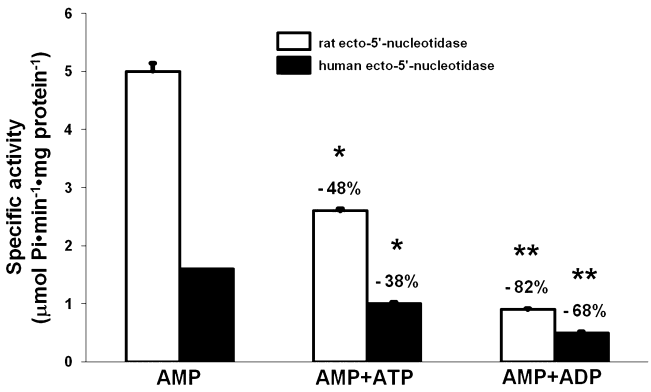


Figure 4

Influence of ATP and ADP on recombinant ecto-5′-nucleotidase activity from rat and human sources. The concentration of each nucleotide was set at 0.5 mM. Enzymic activities are expressed as the mean ± SD ($n = 4$) in μmol P_i·min^{-1}·(mg protein)$^{-1}$. Statistical differences between assays with and without inhibitors (ATP or ADP) are $^*P < 1 \times 10^{-4}$, $^{**}P < 1 \times 10^{-5}$.

patients (60 μM), inhibited the hydrolysis of ADP by recombinant human NTPDase1 by about 50%, an effect that was observed with concentrations of clopidogrel ranging from 10 to 60 μM. Interestingly, ticlopidine did not affect human recombinant NTPDase2, 3 and 8 at concentrations up to 0.1 mM (data not shown), thus excluding an effect of ticlopidine on these enzymes in HUVECs. Because the AMPase activity in HUVECs was slightly inhibited by both thienopyridines (Figure 2A), we tested the effect of these pro-drugs on recombinant ecto-5′-nucleotidases. While clopidogrel did not affect rat ecto-5′-nucleotidase activity, a concentration of ticlopidine of 300 μM was required to inhibit ~25% of the enzyme activity, that is, a level estimated to be fivefold higher than the clinical concentration found in the plasma of ticlopidine-treated patients (Figure 3B). We next assessed whether the activity of human ecto-5′-nucleotidase was also affected by thienopyridines. For this purpose, we first cloned an ecto-5′-nucleotidase cDNA from liver RNA via RT-PCR using primers derived from the database sequence (NM_002526). The cDNA sequence thereby obtained was deposited to GenBank (DQ186653) and was found to be nearly identical to the original sequence NM_002526 except for an A > G change at nucleotide 1145, resulting in a Thr > Ala exchange at residue 386. Clopidogrel and ticlopidine had no effect on human ecto-5′-nucleotidase activity (Figure 3B). The basis for the difference in the effect of ticlopidine on human versus rat recombinant ecto-5′-nucleotidase is not clear as these isoforms are highly similar, with >90% amino acid homology and have similar substrate kinetics and show similar inhibition patterns by their natural inhibitors, ATP and ADP (Table 1 and Figure 4).

We further characterized the kinetic parameters for the inhibition of NTPDase1 and ecto-5′-nucleotidase by thienopyridines. Both enzymes showed mixed-type inhibition, indicating that the inhibition was mainly competitive and therefore partly reversible. The estimated apparent K_i ($K_{i,app}$) values for human NTPDase1 (14 μM for ticlopidine and 10 μM for clopidogrel) were about 300-fold

Table 1

Kinetic parameters of vascular ectonucleotidases in presence or absence of thienopyridines

	Ecto-5′-nucleotidase		Human NTPDase1 (ADP as substrate)
	Rat	Human	
K_m (μM)	20	18	22*
V_{max} (U·mg protein^{-1})	5.9	1.1	0.75*
$K_{i,app}$ for ticlopidine (mM)	4.5	Not inhibited	0.014
$K_{i,app}$ for clopidogrel (mM)	Not inhibited	Not inhibited	0.010

The kinetic parameters were evaluated using protein extract from the indicated recombinant enzymes transiently expressed in COS-7 cells.
*From Kukulski *et al.* (2005).
NTPDase, nucleoside triphosphate diphosphohydrolase.

Figure 5

Therapeutic concentration of ticlopidine and clopidogrel reduce the ability of recombinant human NTPDase1 to block platelet aggregation induced by ADP. Human NTPDase1 (hNTPDase1) was obtained from a protein extract of transfected COS-7 cells (6 μg of protein). Proteins from non-transfected COS-7 cells (ctrl COS; 6 μg) were used as controls. Platelet aggregation in PRP samples in the presence or absence of the indicated protein extract and drug was initiated with 8 μM ADP. (A) Platelet aggregation curves of a representative experiment, with ticlopidine (60 μM), clopidogrel (20 μM) and ARL 67156 (100 μM), out of 10 (all showing a similar trend) are shown. The control curves with clopidogrel and ARL 67156 (without hNTPDase1) were similar to ctrl COS plus ticlopidine. They were omitted to simplify the figure. (B) Therapeutic concentration of ticlopidine (60 μM) decreases the ability of recombinant hNTPDase1 (6 μg of protein) to block platelet aggregation induced by ADP (8 μM). Proteins from non-transfected COS-7 cells (COS prot.; 6 μg) were used as controls. The difference between the curves ($n = 10$), in presence of hNTPDase1 with and without ticlopidine, was statistically different with a $P < 1 \times 10^{-2}$ (*) from 100 s to the end of the 10 min recorded assay. (C) Qualitative assessment of ticlopidine (60 μM) and clopidogel (20 μM) on platelet aggregation, with and without hNTPDase1, by light microscopy (40×). The reaction was performed for 10 min. The pictures of a representative experiment out of four are shown. The control without platelets is represented by platelet-poor plasma (PPP) and with non-aggregated platelets by platelet-rich plasma (PRP). (D) Non-aggregated platelet number after 10 min of reaction with or without pro-drugs and hNTPDase1. 100% was set at 3.15×10^6 cells in 1 mL. *$P < 1 \times 10^{-2}$ and **$P < 1 \times 10^{-3}$, significantly different. HUVEC, human umbilical vein endothelial cell.

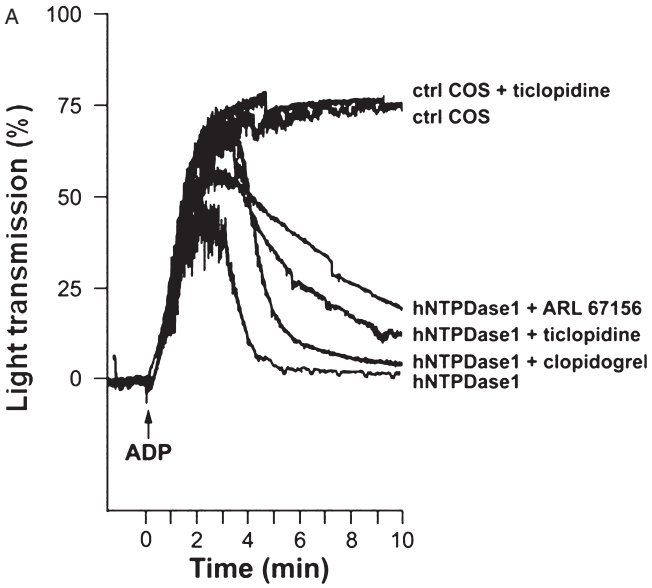
lower than for rat ecto-5′-nucleotidase (4.5 mM for ticlopidine; Table 1) and fourfold lower than the expected concentration of ticlopidine in the blood of treated patients. These calculations show that ticlopidine inhibits NTPDase1 more potently than ecto-5′-nucleotidase, and further support the view that the inhibition of NTPDase1 by these pro-drugs may have therapeutic relevance (cf. 'Discussion').

We next estimated the consequence of NTPDase1 inhibition on platelet aggregation using the recombinant enzyme. As expected, the inhibition of platelet aggregation by human NTPDase1 was attenuated in the presence of ticlopidine (Figure 5A). The difference between the curves obtained in the presence of NTPDase1 with or without ticlopidine was statistically different ($P < 0.01$) from 100 s to the end of the 10 min recorded assay, as determined in separate experiments with a plate reader (Figure 5B). This effect was immediate and similar to that obtained with the NTPDase1 inhibitor ARL 67156, used as an additional control (Figure 5A). Note that this inhibitor only partially inhibits NTPDase1 activity (Lévesque *et al.*, 2007). Pre-incubation of NTPDase1 with ticlopidine for 3 or 10 min showed similar results (data not shown). In agreement with its weaker inhibition of recombinant NTPDase1, clopidogrel was less efficient to reduce the ability of NTPDase1 to block platelet aggregation (Figure 5A).

Light microscopy further confirmed the above observation. Additionally, it allowed a more systematic quantification of these effects. With this method we have shown that, in the presence of thienopyridines, human NTPDase1 did not prevent the formation of platelet aggregates (Figure 5C) and the number of non-aggregated platelets in the mixture with human NTPDase1 was reduced to the levels observed in absence of the enzyme (Figure 5D).

Discussion

Although thrombogenesis is an essential function of platelets, under certain conditions complications may lead to vascular disorders (Cutlip *et al.*, 2001; Evens *et al.*, 2002). Ticlopidine and clopidogrel are routinely administered to patients as part of an antithrombotic therapy (Lee *et al.*, 2005). Various studies have documented a delay of 3 and 5 days for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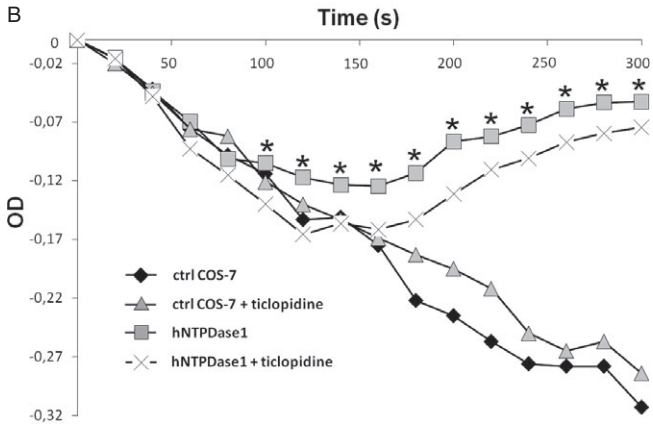
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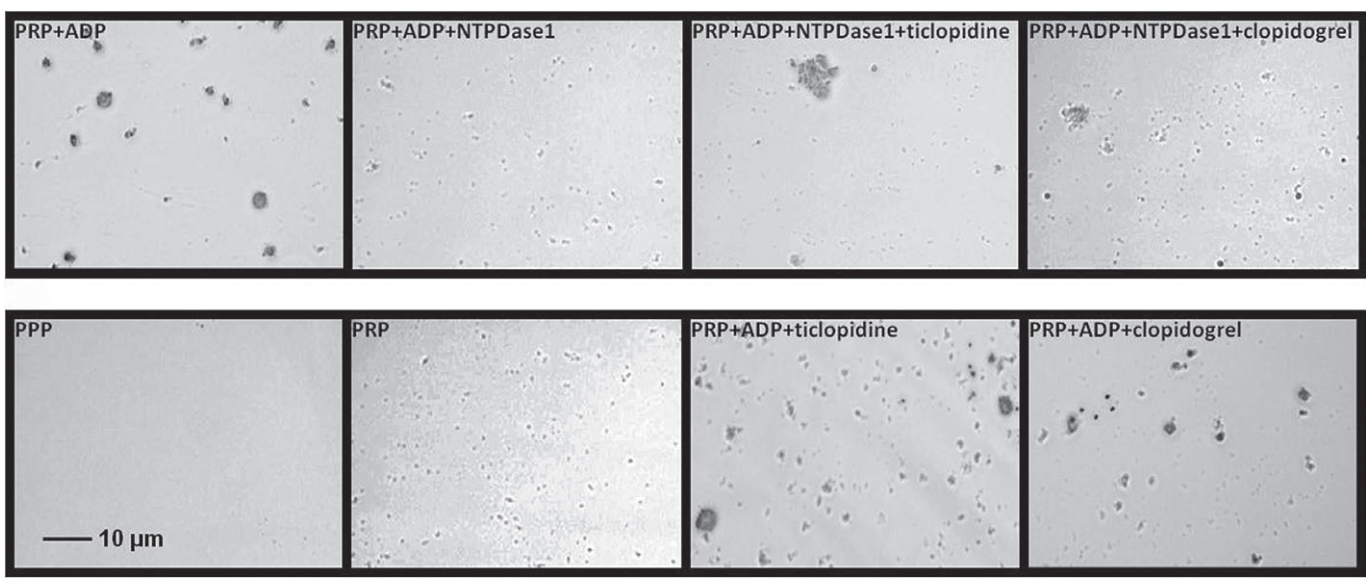
PRP+ADP
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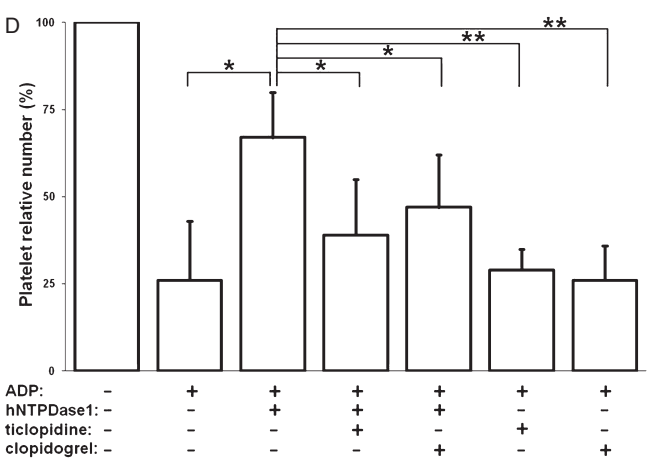
PRP+ADP+ticlopidine
PRP+ADP+clopidogrel
10 µm


D
Platelet relative number (%)
100
75
50
25
0
*
*
*
**
**
ADP: - + + + + + +
hNTPDase1: - - + + + - -
ticlopidine: - - - + - + -
clopidogrel: - - - - + - +

clopidogrel and ticlopidine, respectively, to become fully effective (Di Minno *et al.*, 1985; Boneu and Destelle, 1996; Kam and Nethery, 2003). Upon intravenous or oral administration, these pro-drugs are converted into their reactive thiol derivatives via a cytochrome P450 pathway in the liver (Savi *et al.*, 1994). Furthermore, only a small percentage of the daily dose of these pro-drugs (250 mg for ticlopidine or 75 mg for clopidogrel) is transformed to the respective active drug, explaining both the delay in achieving their steady state effectiveness *in vivo*, and their lack of activity *in vitro* (Kam and Nethery, 2003).

Nucleoside- and nucleotide-regulated haemostasis in blood may be an important modulator of the development of cardiovascular and immunological complications (Hourani, 1996; Hechler *et al.*, 2005; Waehre *et al.*, 2006; Ansari *et al.*, 2007; Armstrong *et al.*, 2009). Hence, vascular endothelium-derived ectonucleotidases NTPDase1 and ecto-5′-nucleotidase that regulate the concentration of nucleosides and nucleotides in the blood may also be considered as critical regulators of these complications (Kaczmarek *et al.*, 1996; Marcus *et al.*, 1997; Enjyoji *et al.*, 1999; Kawashima *et al.*, 2000; Sévigny *et al.*, 2002; Guckelberger *et al.*, 2004; Koszalka *et al.*, 2004; Colgan *et al.*, 2006). In this work we observed that thienopyridines, in their respective pro-drug form, facilitate platelet aggregation by inhibiting NTPDase1 activity (Figures 1 and 5). At the concentration expected in the plasma of treated patients (60 μM ticlopidine and 20 μM clopidogrel), these pro-drugs reduced the efficiency of vascular endothelial cells to prevent platelet aggregation by inhibiting (by 80%) ADP hydrolysis by HUVECs as a result of NTPDase1 inhibition (Figures 2A and 3A). We previously reported that NTPDase1 prevents not only ADP-induced platelet aggregation but also the aggregation induced by collagen and low concentrations of thrombin, emphasizing the importance of ADP (mostly derived from platelet granules) in promoting efficient platelet aggregation (Enjyoji *et al.*, 1999).

Kinetic analysis of the recombinant NTPDase1 showed a mixed-type inhibition by ticlopidine and clopidogrel with $K_{i,app}$ values of 14 and 10 μM (Table 1) that is, ~25% and 50% of their effective therapeutic concentrations respectively. Such inhibition of NTPDase1 activity might facilitate thrombus formation due to the accumulation of ADP, the natural ligand of $P2Y_1$ and $P2Y_{12}$ (Hechler *et al.*, 2005). Of note, ticlopidine and clopidogrel also blocked AMP hydrolysis to adenosine by 37% and 17%, respectively (Figure 2A), which might also be expected to promote platelet aggregation. HPLC analysis of HUVEC supernatants incubated with equimolar concentrations of ATP and ADP confirmed that ADP hydrolysis was inhibited in the presence of thienopyridines, and that adenosine formation was reduced (data not shown). The decrease in adenosine concentration was due to the inhibition of AMPase by the pro-drugs, as well as to the presence of higher concentrations of ATP and ADP, which are endogenous inhibitors of ecto-5′-nucleotidase (Figure 4 and data not shown). However, this partial inhibition of ecto-5′-nucleotidase activity in HUVECs by thienopyridines could not be reproduced with a protein extract from the recombinant form of the enzyme (Figures 2A and 3B). The reason for this difference is unclear. It is noteworthy that the inhibition of the ectonucleotidase activities for all purines (ATP, ADP and AMP) was more pronounced with intact HUVECs than with cell lysates, containing the individual recombinant enzymes. The observed effect was direct and did not require any pre-incubation (Figure 2B). This observation suggests that the microenvironment in the vascular endothelial cells could make these enzymes more sensitive to inhibition by the pro-drugs, thereby facilitating platelet aggregation.

Of interest, the data presented here suggest that ticlopidine might be used as a selective inhibitor of human NTPDase1. Indeed, at 100 μM, ticlopidine did not affect the activity of other plasma membrane-bound NTPDases (data not shown), whereas it inhibited recombinant NTPDase1 by >50% (Figure 3A). This finding is of great potential interest as there are very few specific inhibitors so far that are selective for a limited subset of the different ectonucleotidases.

In summary, therapeutic concentrations of ticlopidine and clopidogrel inhibit vascular NTPDase1 activity and the enzymes' ability to block platelet aggregation *in vitro*. These data suggest that these effects also occur *in vivo*, especially at the beginning of ticlopidine and clopidogrel therapy, before sufficient levels of the active metabolites are produced by the liver to inhibit platelet aggregation. This intriguing hypothesis warrants future studies.

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Conflict of interest

None to declare.

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