A small-molecule inhibitor of integrin α2β1 introduces a new strategy for antithrombotic therapy

Liisa Nissinen[1*]; Olli T. Pentikäinen[2*]; Annukka Jouppila[3*]; Jarmo Käpylä[4]; Marika Ojala[1]; Jonna Nieminen[1]; Anu Lipsanen[6]; Heli Lappalainen[6]; Beate Eckes[7]; Mark S. Johnson[5]; Riitta Lassila[3,8]; Anne Marjamäki[1]; Jyrki Heino[4]

[1]Biotie Therapies Corp., Turku, Finland; [2]Nanoscience Center, Department of Biological and Environmental Science, University of Jyväskylä, Finland; [3]Clinical Research Institute, Helsinki University Central Hospital, Finland; [4]Department of Biochemistry, University of Turku, Finland; [5]Department of Biochemistry and Pharmacy, Åbo Akademi University, Turku, Finland; [6]CNServices, Kuopio, Finland; [7]Department of Dermatology, University of Cologne, Germany;, [8]Department of Hematology, Coagulation Disorders, and Laboratory Division (HUSLAB), Helsinki University Central Hospital, Finland

Summary

Interaction of blood platelets with vascular collagen is an initiating event in haemostasis and thrombus formation. Based on molecular modelling of human integrin α2I domain and cell-based screening assays we have developed sulfonamide derivatives, a mechanistically novel class of molecules. These molecules show antiplatelet efficacy by selectively inhibiting α2β1 integrin-mediated collagen binding. One sulfonamide derivative, named BTT-3016, showed inhibitory capacity in several assessments of human platelet interaction with collagen. It inhibited about 90% of the aggregation of gel-filtered magnesium-supplemented platelets and 70% of aggregation in PPACK-anticoagulated platelet-rich plasma when stimulated with collagen but not with ADP. The antiplatelet activity of BTT-3016 was dependent on α2β1 integrin, since in collagen binding test BTT-3016 had no effect on the platelets derived from α2 integrin null mice. When tested in an *in vivo* model in mice, BTT-3016 clearly reduced thrombus formation on the vessel wall after vascular injury. Furthermore, BTT-3016 prolonged tail-bleeding time in a manner comparable to aspirin. We show that new α2β1 inhibitors exert collagen-specific antiplatelet activity and regulate thrombus growth *in vivo* without compromising primary haemostasis more than aspirin. We suggest that the α2β1 inhibiting strategy could be further developed for the prevention and treatment of arterial thrombosis.

Keywords

Integrin, collagen, platelet, antithrombotic therapy

Correspondence to:
Dr. Jyrki Heino, MD, PhD
Professor, Biochemistry
University of Turku
FI-20014 Turku, Finland
Tel.: +358 2 333 6879
E-mail: jyrki.heino@utu.fi

* These authors contributed equally.

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Introduction

Integrin α2β1, also called as glycoprotein (GP) Ia/IIa, and GPVI are primary collagen receptors on platelets but their respective roles are still contradictory. As such, spontaneous bleeding has not been detected in α2-deficient animals (1, 2), which show only mild bleeding tendency upon vascular injury (3). However, α2 deficiency has been reported to attenuate the growth of thrombus after endothelial injury (4, 5). Moreover, one human individual lacking α2β1 integrin activity expressed a mild bleeding disorder (6, 7). In all, α2β1 has been suggested to be a relatively minor player in collagen-mediated platelet activation, and many experimental thrombosis models have stressed the importance of GPVI (8, 9). In addition specific plasma proteins, such as von Willebrand factor, thrombospondin and fibronectin, may recognise collagenous proteins and thus interfere with platelet interaction (10).

Epidemiological studies have suggested the role of α2β1 integrin in vascular diseases in man (11). High levels of α2β1 have been associated with acute coronary syndrome, including myocardial infarction (12, 13), stroke in young individuals (14), diabetes-associated retinopathy (15) and retinal vein occlusion (16). However, it should be noted that many studies have also failed to show such connections (17, 18). Monoclonal antibodies against human α2β1 can block platelet-collagen interaction in *in vitro* assays (19), but their species specificity limits their utility in animal models. Therefore, the idea that an antithrombotic strategy could be based on the inhibition of α2β1 should be tested by selective small molecule inhibitors. Recently, the first small molecules blocking recombinant α2I domain function *in vitro* have been presented (20–22).

Here we describe novel sulphonamide derivatives that recognise the collagen binding metal ion-dependent adhesion site (MIDAS) in human α2I domain and are potent inhibitors of α2β1-mediated cell adhesion to collagen. One of these derivatives, BTT-3016 (sodium salt of 4-({[3-(4-fluorophenyl)phenyl]sulfonyl}amino)phenyl phenyl ketone), was shown to be an effective in-

hibitor of collagen-induced aggregation of human platelets. In animal models its *in vivo* antiplatelet action was confirmed with assessments of tail bleeding time and thrombus formation following carotid artery injury. Our results indicate that platelet function can be regulated *in vivo* by α2β1 inhibitors without compromising primary haemostasis more than aspirin. We suggest that this strategy could be further developed for the prevention and treatment for arterial thrombosis.

Methods

Protein structures, ligand discovery and ligand docking

The three-dimensional structure of the integrin α2I domain in the "closed" conformation (PDB access code:1aox; 23) was obtained from the Protein Data Bank (24). Partial *de novo* ligand discovery was made by using visualisation with the program Bodil (25), in combination with energy minimization of ligands and protein-ligand complexes in Sybyl (Tripos Inc, St. Louis, MO, USA). Ligands were docked flexibly into the "closed" conformation of the integrin α2I domain with the program FlexX in Sybyl (Tripos Inc).

Cell assays

CHO (Chinese hamster ovary), PC-3 (human prostate cancer), Jurkat (T lymphocytes) and MG-63 and Saos-2 (human osteosarcoma) cells were obtained from the American type Culture Collection (ATCC, Manassas, VA, USA). Saos cells were transfected to express human α2 integrin (26). CHO cells were transfected to express either wild-type (wt) or variant human α2 integrin (CHO-α2Y285F) (21). The expression of integrins on the surface of cells was studied by flow cytometry (27) with anti-α2 (1:50; 12F1, BD Biosciences, Pharmingen, San Diego, CA, USA) and anti-α1 (1:50; SR-84, BD Biosciences, San Jose, CA, USA) antibodies. In adhesion assays cells (150,000/well) were allowed to attach on rat tail collagen I (Becton Dickinson Labware), fibronectin (5 μg/cm^2; Sigma-Aldrich, St. Louis, MO, USA), ICAM-1 (12.5 μg/ml, R&D Systems Inc, Minneapolis, MN, USA), laminin-5, vitronectin, and fibronectin α-chymotryptic fragment of 120 kDa (10 μg/ml, Chemicon, Temecula, CA, USA) for 2 hours (h) at 37°C. Number of adherent cells was measured with WST-1 (Roche Applied Science, Madison, WI, USA) according to the manufacturer's protocol. Jurkat cells were treated with TPA (12-*O*-tetradecanoylphorbol-13-acetate, 100 ng/ml, Sigma) for 5 minutes (min) before the adhesion assay. The EC50 (concentration required for half maximal effect) and Emax (maximal inhibitory effect) values were determined using Graph Pad Prism (Graph Pad Software Inc, La Jolla, CA, USA).

Cytotoxicity assay performed with CytoTox-ONE™ (Promega, Madison, WI, USA) followed the manufacturer's protocol. Cell spreading (28) and invasion of PC-3 cells through matrigel (29) were assayed as described.

Determination of binding site

The binding site of α2β1 blockers was studied using a [^{14}C]-labelled hydrochloride salt of [4-(dimethylamino)phenyl]{[3-(4-fluorophenyl)phenyl]-sulfonyl}methylamino ([^{14}C]-335) shown to inhibit α2β1 integrin in the similar manner. [^{14}C]-labelling was performed by Amersham Biosciences (Fairfield, CT, USA). Integrin α2I and α1I domains (26, 30) and soluble α2 integrin (Biomarket, Turku, Finland) were incubated with 10 μM [^{14}C]-335 in the presence or absence of 50 μM L3008 (anhydromaggiemycin, an aromatic polyketide, which binds equally to α1I and α2I domains) (21) or with BTT-3016 (at EC50 concentration) for 90 min in 50 mM Tris-HCl buffer pH 7.4 containing 2 mM $MgCl_2$ at RT. The samples were analyzed by SDS-PAGE followed by autoradiography.

Collection of human blood samples

Blood samples from healthy donors were collected after an overnight fast, as reported (31). As anticoagulant for blood perfusion studies 40 μM D-phenylalanyl-L-prolyl-L-arginine chloromethylketone (PPACK, Calbiochem, EMD Biosciences, San Diego, CA, USA), for platelet rich-plasma (PRP) 3.2% sodium citrate or 40 μM PPACK, for preparing gel-filtered platelets (GFP) 85 mM acidic (pH 4.5) citrate dextrose were used.

Platelet preparations

PRP was obtained from human blood as reported (32). GFP was prepared using the method described (33) and supplemented with 2 mM $MgCl_2$ in HEPES buffer (pH 7.35) to foster α2β1-mediated interactions (19, 34). The platelet number in PRP and GFP was adjusted to 300 x 10^6/ml.

Human platelet aggregation

Aggregation of PRP and GFP was studied with turbidometric method (35) using the PAP-4D-aggregometer (Bio-Data Corporation, Horsham, PA, USA). Platelet aggregation (at 37°C, 1,000 rpm) was induced with either fibrillar collagen (Horm collagen, mainly type I collagen fibrils, Nycomed, Zurich, Switzerland), CRP (collagen related peptide; kindly provided by Prof. Richard Farndale, University of Cambridge, UK) or ADP (Sigma-Aldrich). During aggregation the final concentrations of inhibitors used were

20 μM for BTT-3016 and ARMX (AR-C69931MX, AstraZeneca, Wilmington, DE, USA) and 0.8–2.0 μg/ml for human α2 mAb (P1H5, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA). For vehicle controls, DMSO or 70% polyethylene glycol 400 (PEG400) 1:1,000 was used. Agonist-induced aggregation of platelets was targeted between 40–65% (~ED50). The lag time (s) and maximal aggregation (% change of light transmission) were measured at 5 min.

Human whole blood perfusion

For perfusion studies, a Turitto´s chamber with defined rheological characteristics was used (36). Human platelets were labeled with 7 nM 5-hydroxy tryptamine (21 Ci/mmol, Amersham Biosciences) (34). Collagen-coated coverslips (Permanox, NUNC, Rochester, NY, USA) were used as an adhesive surface (34, 37). Prior to perfusion, blood aliquots were incubated for 10 min at 37°C with either BTT-3016 (20 μM), 70% PEG400 (1:1,000) or α2 mAb, (P1H5 0.8–2.0 μg/ml). Blood was perfused over collagen once for 2 min at a shear rate of 3,200 s^{-1} after which the surface was rinsed for 30 seconds with PBS (pH 7.35). Platelet deposition on collagen per area was calculated based on the [^{3}H]-activity on the coverslips (32, 35).

Mouse platelet adhesion in flow conditions

The Cellix microfluidic platform (Cellix Ltd, Dublin, Ireland), a dynamic set-up mimicking physiological flow conditions was used to measure mouse platelet adhesion on collagen chips under flow. Whole blood samples were collected from wt and α2-deficient mice (Strain C57Bl/6, α2-deficient mice strain was kindly provided by Dr. Beate Eckes, University of Cologne, Germany) (1) using 40 μM PPACK (Calbiochem, Gibstown, NJ, USA) and 7.5 U/ml Heparin (Leo Pharma, Ballerup, Denmark) as anticoagulant. Mouse whole blood was stained with 1 μM 3,3´-dihexyloxacarbocyanine iodide (DiOC6(3), Invitrogen, San Diego, CA, USA) and the adhesion of platelets to fibrillar collagen (60 μg/ml) coated capillary (Cellix Ltd) were detected with fluorescent microscope, 20x magnification (Carl Zeiss Inc, Jena, Germany). The mouse whole blood was incubated with or without the inhibitor BTT-3016 (20 μM) for 5 min. The blood was run through the capillary with the constant shear rate (120 dynes/cm2, Mirus 1.0 Nanopump, Cellix Ltd) for 4 min and capillary was washed with JNL buffer (6 mM Dextrose, 0.13 M NaCl, 9 mM $NaHCO_3$, 10 mM Na Citrate, 10 mM Tris base, 3 mM KCl, 0.81 mM KH_2PO_4, 0.9 mM $MgCl_2$; pH was adjusted to 7.35 with 19 mM Citrate acid anhydrous, 37 mM Sodium citrate, 67 mM Dextrose) with the constant shear rate for 2 min. The adhesion of platelets on the capillary wall was analysed with DucoCell analysis program (Cellix Ltd).

Activated partial thromboplastin time (APTT) and prothrombin time (PT)

APTT was measured using the IL Test™ APTT Lyophilized silica kit (ILS Laboratories, Scandinavia Ltd, Jonkoping, Sweden) and the PT was determined using Owren's coagulation reagent (Medi-Rox, Nykoping, Sweden) in the ACL 7000 automated coagulation analyser (Instrumentation Lab. Company, Lexington, MA, USA). For APTT, heparin (0.5 U/ml) was used as a control for anticoagulation activity.

In vivo experiments in rodents

The antiplatelet activity of BTT-3016 in mouse tail-bleeding model was investigated as described previously (3). Mice (Balb/c) at 8–10 weeks of age were administered i.v. with 30% hydroxypropyl-β-cyclodextrin (HB-β-CD, Roquette) vehicle, aspirin (20 mg/kg, Weylchem GmbH, Elgin, SC, USA) or BTT-3016 (20 mg/kg). Wilcoxon two-sample test (SAS/STAT, SAS Institute Inc, Cary, NC, USA) was used for statistical analysis. The study was approved by the Animal Care and Use Committee of the University of Turku, approval number 1619/06.

Carotid artery thrombosis was induced as described previously (4). Briefly male mice (C57Bl/6) at 9–13 weeks of age (wt or α2 integrin-deficient) were anesthetised with Equithesin (5 ml/kg) (Kuopio University Hospital, Kuopio, Finland). The right common carotid artery (CCA) was carefully exposed via midline incision and Laser Doppler probe (probe 403, Perimed, Jarfalla, Sweden) was placed above for measuring blood flow. Laser beam (RLDD 532–5–3, 532 nm, 5 mW) was focused on the common carotid artery. Rose Bengal (50 mg/kg, Sigma-Aldrich) was injected i.v. after a steady baseline blood perfusion was achieved during illumination. Flow in the vessel was monitored up to 60 min after induction of thrombosis. Animals were administered with vehicle (30 % Cremophor EL, BASF, Ludwigshafen, Germany), BTT-3016 in 30 % Cremophor EL p. o. (dose corresponds to 90 μg/ml in plasma) and α2 mAb (15 mg/kg, clone Ha 1/29, BD Bioscience) i.p. 24 and 2 h before the experiment. Kruskall-Wallis test followed by Mann-Whitney test was used for statistical analysis. The study was approved by the Animal Ethics Committee (Hämeenlinna, Finland), approval number ESLH-2008–05541/Ym-23.

Results

Computer-aided protein structure-based molecular discovery of BTT-3016

Integrin α2β1 binds to collagen using its metal ion-dependent adhesion site (MIDAS) in the αI domain. Integrin αI domains can be either in a low-affinity "closed" conformation or a high-affinity/ligand-bound "open" conformation (38). In order to block col-

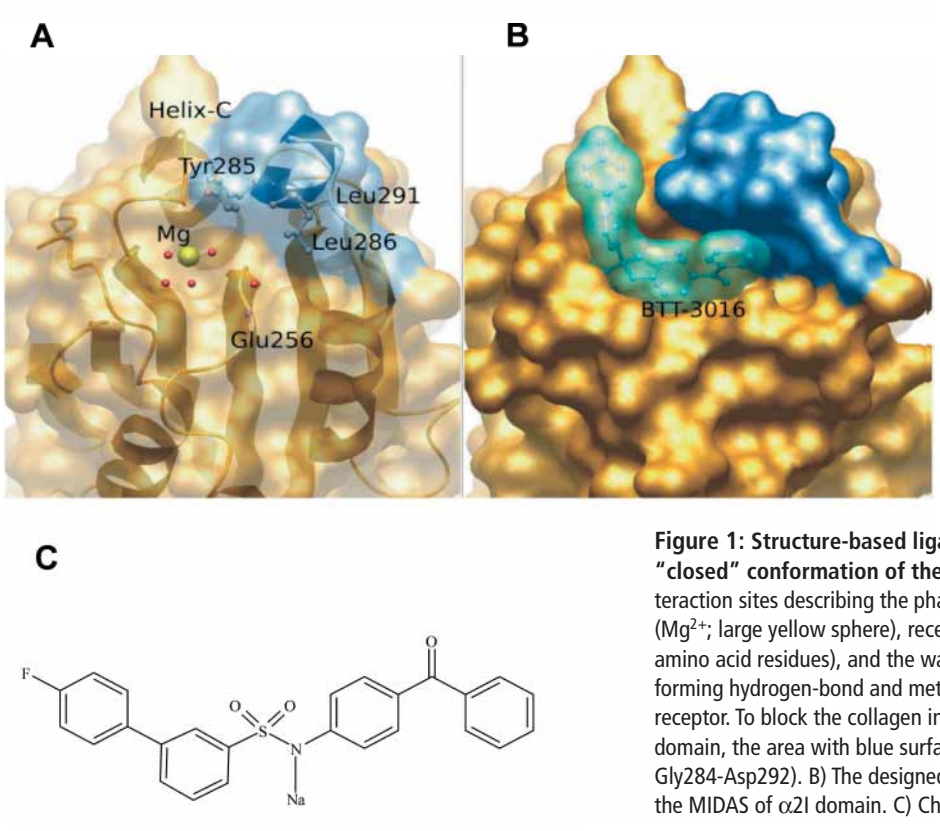

Figure 1: Structure-based ligand discovery used to target the "closed" conformation of the integrin α2I domain. A) The key interaction sites describing the pharmacophore model: the metal ion (Mg^{2+}; large yellow sphere), receptor atoms (small spheres within the amino acid residues), and the water molecules (red spheres) that are forming hydrogen-bond and metal-coordination interactions with the receptor. To block the collagen induced conformational change of α2I domain, the area with blue surface should be stabilised (amino acids Gly284-Asp292). B) The designed ligand, BTT-3016, docked flexibly into the MIDAS of α2I domain. C) Chemical structure of BTT-3016.

lagen binding a small molecule should restrain the flexibility of the α2I domain by stabilising the "closed" conformation (▶Fig. 1; [21]). We have set criteria that a putative α2I domain binder should fulfil (23). Here, this pharmacophore model was used in partial *de novo* ligand discovery, where ligands were built atom-by-atom, by using computer graphics. The resulting sulfonamides, e.g. BTT-3016 (Fig. 1), were suggested for synthesis. The chemical characteristics of the synthesised compound BTT-3016 are summarised in ▶Table 1.

Table 1: Chemical characteristics of BTT-3016.

IUPAC name	Sodium salt of 4-({[3-(4-fluorophenyl) phenyl]sulfonyl} amino) phenyl phenyl ketone
Molecular weight (Na-salt)	453.47 g/mol (Free acid 431.49)
pKa	~ 10
LogP at neutral pH	> 5
Chemical stability	Stable against heat, acid, base, oxidation and daylight.
Protein binding (HSA)	> 70%

IUPAC, International Union of Pure and Applied Chemistry. LogP, octanol-water partition constant; pKa, -log10Ka ; Ka, Dissociation constant. Log P, -log10P ; P, partition coefficient (Octanol/water). HSA, human serum albumin.

BTT-3016 inhibits specifically human α2β1 integrin-mediated cell adhesion to collagen I

Cell-based assays utilising PC-3 cells expressing endogenous human α2β1 integrin and CHO cells transfected to overexpress human α2β1, were used for identification of potential α2β1 integrin inhibitors from the synthesis program. In cell-invasion assays BTT-3016 inhibited the interaction between PC-3 cells and basement membrane with EC50 of 160 nM and the Emax of ~100 %

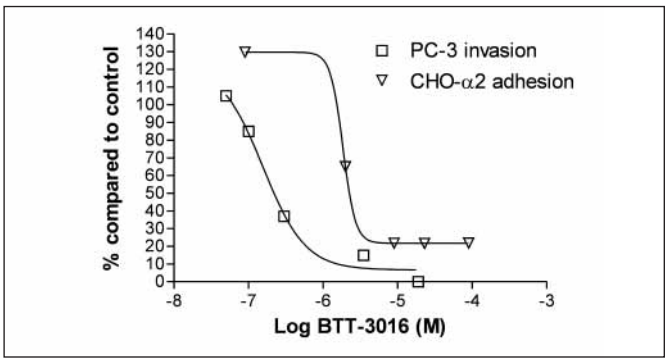

Figure 2: BTT-3016 is a potent inhibitor of α2β1 integrin. CHO-α2β1 adhesion to rat tail collagen I and PC-3 cell invasion through Matrigel in the presence of BTT-3016.

(▶Fig. 2A). In addition BTT-3016 inhibited the adhesion of α2 integrin overexpressing CHO cells on rat tail collagen I (Fig. 2A) with EC50 of 2 μM and the Emax of 90%. BTT-3016 was not cytotoxic for cells at the tested concentrations up to 200 μM.

The effect of BTT-3016 was shown to be specific for collagen receptors since it was not able to inhibit the adhesion of CHO wt cells on fibronectin (▶Table 2). Interaction of CHO wt cells with fibronectin is known to be mediated by other β1 integrins. The selectivity of BTT-3016 was also determined in MG-63 cell adhesion assays to different matrices and in Jurkat cell adhesion assay to ICAM-1. BTT-3016 (at EC50 concentration, 2 μM) did not inhibit the adhesion of MG-63 cells to vitronectin or 120 kDa fibronectin indicating specificity over αV and α5β1 integrins, respectively (Table 2). BTT-3016 neither did inhibit adhesion of Jurkat to ICAM-1 indicating specificity over leukocyte αLβ2 integrin (Table 2).

The selectivity of BTT-3016 was also studied in the Saos cell spreading assay on rat tail collagen I. Human Saos wt cells do not

Table 2: The effect of BTT-3016 (2 mM) on cell adhesion (%) to different matrices and recombinant human ICAM-1.

Cell line	MG-63		CHO wt		Jurkat
Matrix/recombinant protein	120 kDa FN	VN	FN	Col I	ICAM-1
Adhesion (%) in the presence of BTT-3016	102 ± 3.6%	96.7 ± 2.9%	108.0 ± 1.7%	121.8 ± 3.9%	99.2 ± 18.2%
Mean ± SD, n=3, FN, fibronectin; VN, vitronectin; Col I, rat tail collagen type I.					

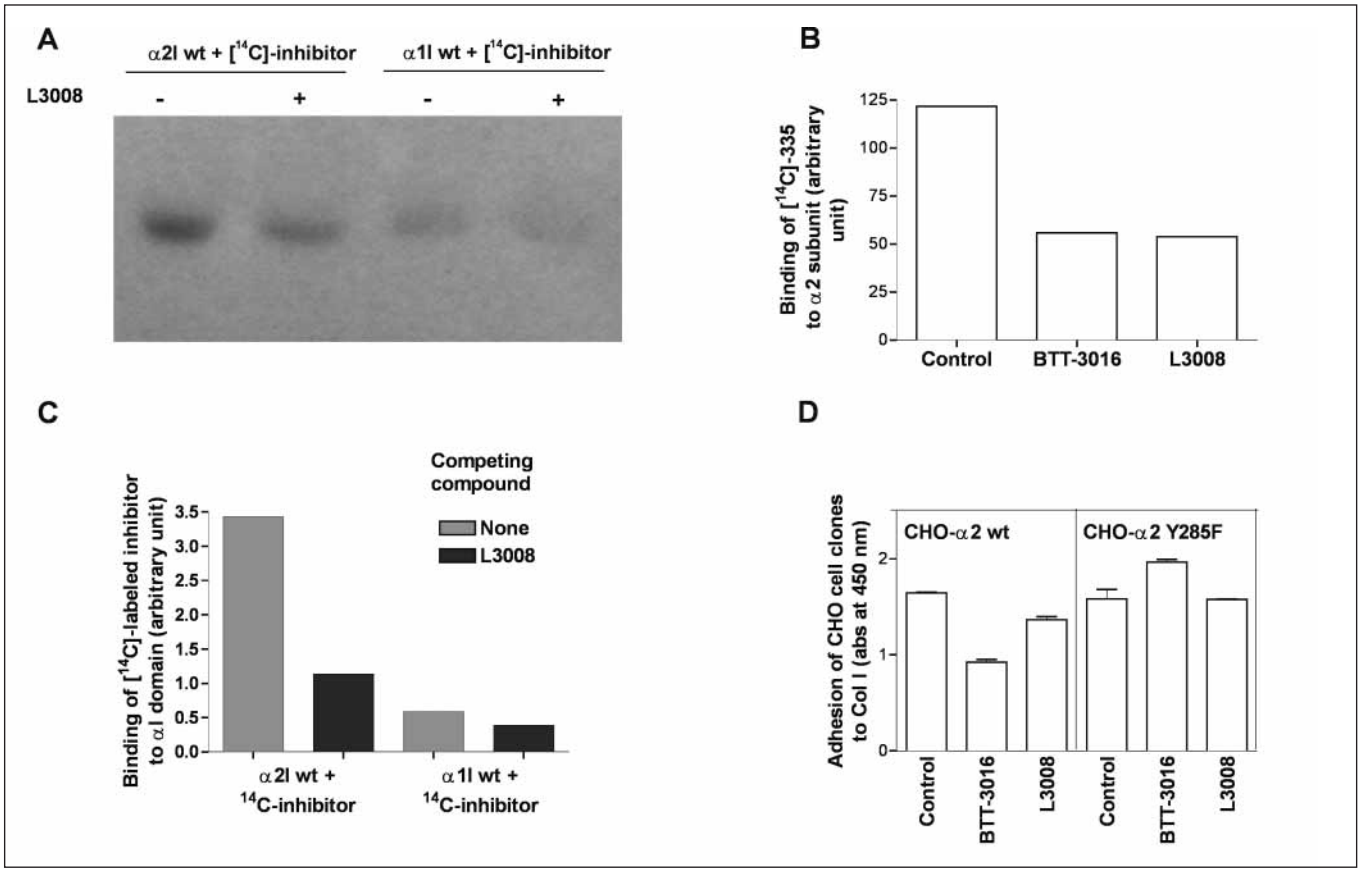

Figure 3: Sulfonamide derivatives bind to α2I domain. A) The binding site of sulphonamides in α2β1 integrin was studied with a BTT-3016 analogue [14C]-335. Compound 335 was used instead of BTT-3016 due to a lack of suitable chemistry allowing labelling site. A) Integrin α2I and α1I domains were incubated with 10 μM [14C]-335 in the presence or absence of 50 μM L3008, known to equally bind to both α2I and α1I domains. The samples were analysed by electrophoresis on SDS-PAGE followed by autoradiography. In lower panel the quantitated levels of αI domains are shown. B) Integrin α2 subunit was incubated with 10 μM [14C]-335 in the presence or absence of 50 μM L3008 or 2 μM BTT-3016. The samples were analysed by electrophoresis on SDS-PAGE followed by autoradiography. The quantitated levels of α2 subunits are shown. C) CHO-α2 wt and -α2Y285F adhesion to rat tail collagen I in the presence of BTT-3016 (2 μM) or L3008 (10 μM) (n=3).

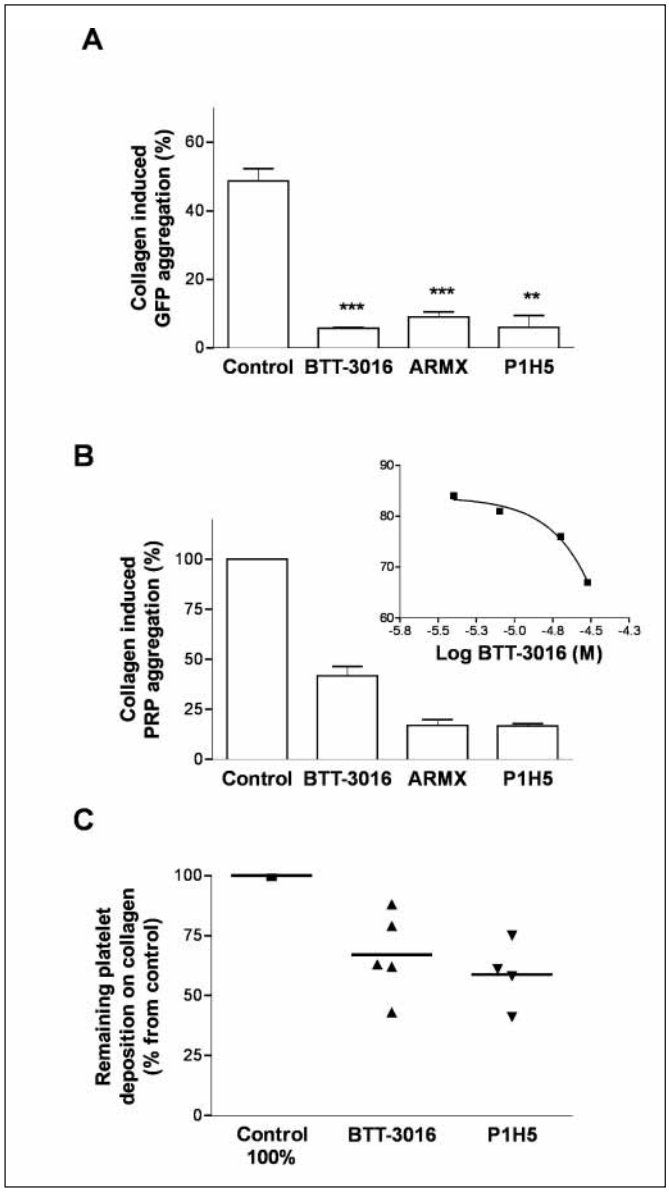

Figure 4: BTT-3016 inhibits human platelet interaction with collagen *in vitro*. A) Collagen (2 μg/ml) induced aggregation of gel-filtrated platelets (GFP) supplemented with magnesium ions (2 mM) and in the presence or absence of BTT-3016 (20 μM), AR-C69931MX (ARMX; 20 μM), or α2 mAb (P1H5, 0.8 μg/ml). Maximal aggregation was measured. Unpaired t-test, ***p<0.0007, **p<0.002. B) Collagen induced (0.3–0.6 μg/ml) aggregation of platelet-rich plasma (PRP) in the presence or absence of BTT-3016 (20 μM; Insert: dose response curve), AR-C69931MX (ARMX, 20 μM) and α2 mAb (P1H5, 0.8 μg/ml). Maximal aggregation was measured. C) Platelet deposition after perfusion over collagen. The blood was incubated with or without BTT-3016 (20 μM) or α2 mAb (P1H5, 2 μg/ml) for 10 min before perfusion. The representative data of responding donors are presented.

express the α2 integrin subunit, whereas the other known collagen binding integrin α subunits, namely α1, α10 and α11, are present (39). In Saos cells transfected with human α2 integrin cDNA (Saos-α2β1) α2β1 is the main collagen receptor. BTT-3016 (2 μM) was about six-fold better inhibitor of Saos-α2β1 spreading than Saos-wt spreading indicating specificity over other collagen binding β1 integrins.

Integrin α2I domain contains a binding site for the sulfonamide derivatives

The ability of integin α2I wt domain to bind the sulfonamide derivatives was determined by utilising [^{14}C]-335, which is structurally analogous and functionally similar to BTT-3016 (EC50 and Emax for compounds 335 and BTT-3016 were EC50 5 μM, Emax 80% and EC50 2 μM, Emax 90%, respectively). Compound 335 was used instead of BTT-3016 due to lack of suitable chemistry allowing labelling of BTT-3016. [^{14}C]-335 bound 5.8-fold more efficiently to human recombinant integrin α2I domain than to α1I domain (▶Fig. 3A), indicating its selectivity. We have previously shown that L3008, an aromatic polyketide, can bind to MIDAS in both α1I and α2I domains with equal potency (21). The binding of the [^{14}C]-335 to the α2I domain was inhibited by L3008 (Fig. 3A), proposing that the sulfonamides may bind to α2I MIDAS. Both L3008 and BTT-3016 could also inhibit [^{14}C]-335 binding to soluble α2 integrin (Fig. 3B). However, unlike L3008 (21), sulfonamides were only week inhibitors of recombinant α2I domain binding to collagen.

Based on molecular modeling both BTT-3016 (Fig. 1) and L3008 (21) are supposed to interact with tyrosine residue 285 (Y285), which is located close to MIDAS in α2I domain. CHO cells transfected to express variant α2 integrin, CHO-α2Y285F, have previously been shown to bind to rat tail collagen I (21). However, the binding of CHO-α2Y285F cells to collagen I cannot be inhibited by L3008 (21) (Fig. 3C). Here, BTT-3016 could not prevent CHO-α2Y285F cell adhesion to collagen when tested at EC50 concentration (Fig. 3C), confirming that the effect of BTT-3016 is also dependent on Y285 in α2I domain.

BTT-3016 inhibits collagen-induced human platelet aggregation

In the absence of plasma proteins BTT-3016 strongly inhibited platelet aggregation by 90 ± 1% (mean inhibition % ± SD, n=7; ▶Fig. 4A) in Mg^{2+}-supplemented (2 mM)-GFP stimulated with fibrillar collagen. Furthermore, BTT-3016 blocked collagen-induced aggregation in PPACK-anticoagulated PRP by 71 ± 20% (n=6; Fig. 4B) and in citrated PRP by 58 ± 13% (n=7). The platelet inhibitory effect of BTT-3016 was dose-dependent (Fig. 4B). Similarly α2 mAb (P1H5) strongly inhibited collagen-induced aggregation by 84 ± 7% in GFP (n=3; Fig. 4A) and by 83 ± 3% (n=6) in citrated PRP and by 88 ± 19% (n=4) in PPACK-anticoagulated PRP (Fig. 4B). Interestingly, both BTT-3016 and α2 mAb inhibited CRP (40–150 ng/ml) induced aggregation in citrated PRP in 6/9 and 6/7 blood donors, respectively. Using submaximal CRP concentration

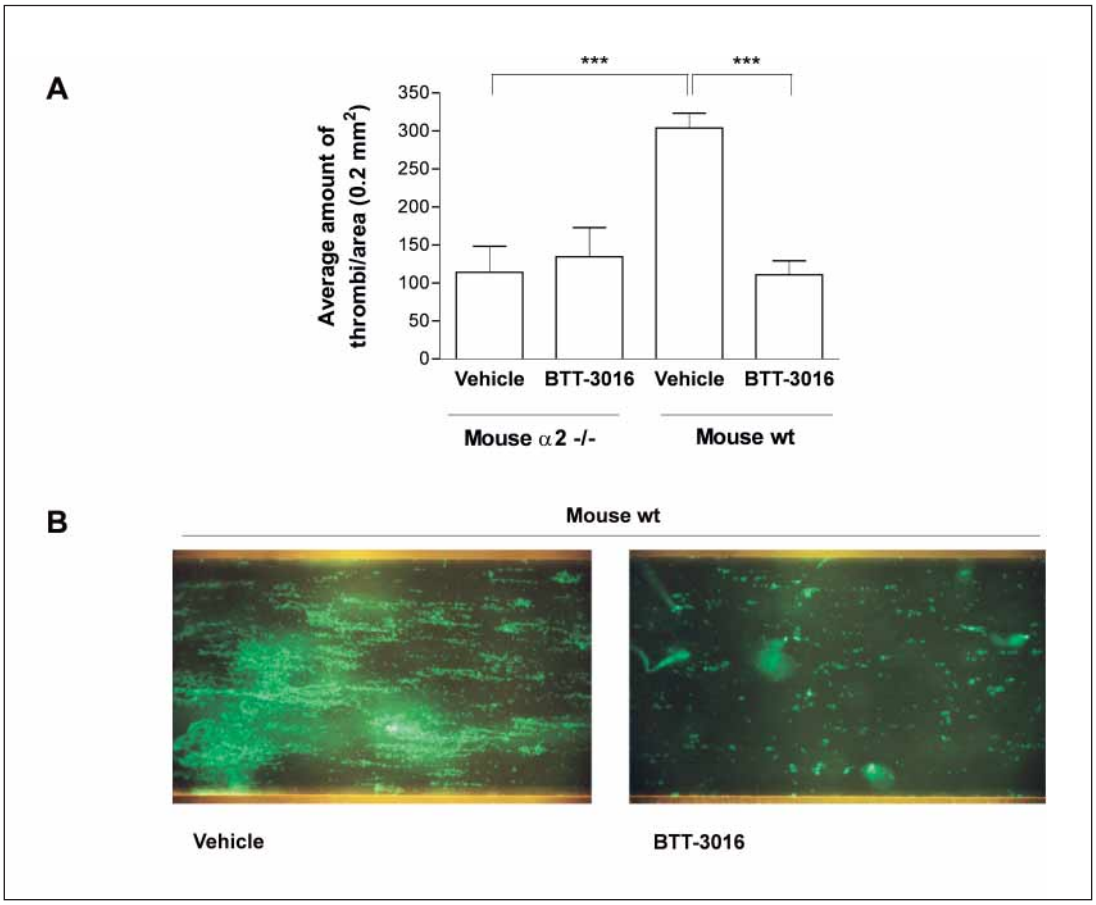

Figure 5: BTT-3016 has no inhibitory effect on thrombus formation of α2-/- mice. The adhesion of platelets to fibrillar collagen (60 μg/ml) coated capillary under the flow. The mouse whole blood stained with 1 μM DiOC6(3) was incubated with or without the inhibitor BTT-3016 (20 μM) and run through the capillary with the constant shear rate (4 min, 120 dynes/cm²). Capillary were washed (2 min, 120 dynes/cm²). A) The amount of thrombi on the capillary wall was analyzed with DucoCell analysis program. Unpaired t-test, ***p<0.0001 n=7; ***p<0.0001 n=12. B) Representative staining of collagen-coated capillary walls from the vehicle versus BTT-3016 treated whole blood from wild-type (wt) mice are shown (20x magnification).

the inhibition observed in responding individuals was 44 ± 10% and 75 ± 11% for BTT-3016 and α2 mAb, respectively. BTT-3016 did not significantly inhibit ADP-induced aggregation (5 ± 9%, n=3, in citrated PRP and 16 ± 9%, n=4, in PPACK-anticoagulated PRP), whereas the same concentration of ARMX (20 μM) inhibited ADP-induced platelet aggregation 97–100%, in both citrated (n=3) and PPACK (n=3) anticoagulated PRP. High dose ARMX also blocked collagen-induced aggregation by 83 ± 4% (n=3) in GFP (Fig. 4A) and by 96–100% in PRP (n=5; Fig. 4B).

Blood from 11 donors was perfused over collagen to investigate the effect of BTT-3016 on platelet-collagen interactions under flow conditions. BTT-3016 inhibited platelet deposition on the collagen surface in seven out of 11 donor blood. In those seven samples the inhibition was 37 ± 17% (Fig. 4C). The inhibitory effect (49 ± 20%) of α2 mAb (P1H5) was exclusively observed in the same seven samples that responded to BTT-3016 (Fig. 4C). Thus, BTT-3016 inhibits platelet-collagen interaction under arterial shear forces. Importantly, by using the Cellix microfluidic platform, the platelets derived from donors that showed no response in other assay systems, could still be inhibited with α2 mAb and BTT-3016 (data not shown).

PT and APTT were measured from the untreated control, vehicle- and BTT-3016– treated whole blood. BTT-3016 had no effect on PT and APTT and thus did not influence global plasma coagulation.

BTT-3016 does not inhibit collagen-induced platelet activation in α2-deficient mice

The adhesion of platelets derived from wt and α2-deficient mice was determined on collagen-coated capillary under constant flow (120 dynes/cm²) in the presence and absence of BTT-3016 (20 μM). BTT-3016 inhibited the wt platelet adhesion on collagen by 65%, but did not affect deposition of α2-/- mouse-derived platelets (▶Fig. 5A and B). Platelets from α2-/- mice showed weaker attachment on collagen-coated capillaries in comparison with the platelets from wt mice (Fig. 5A)

Antithrombotic efficacy of BTT-3016 *in vivo*

To test whether BTT-3016 could also inhibit the α2β1 integrin function *in vivo*, its effect on tail-bleeding time in wt mice (Balb/c, n=20) was studied. BTT-3016 significantly prolonged the bleeding time when compared to the vehicle control (HB-β-CD; Wilcoxon two-sample test p<0.05; ▶Fig. 6). The effect of BTT-3016 was comparable to one seen with aspirin (Fig. 6). In addition the tail-bleeding time was determined with another mouse strain (C57BL/6, n=22). BTT-3016 was shown to significantly

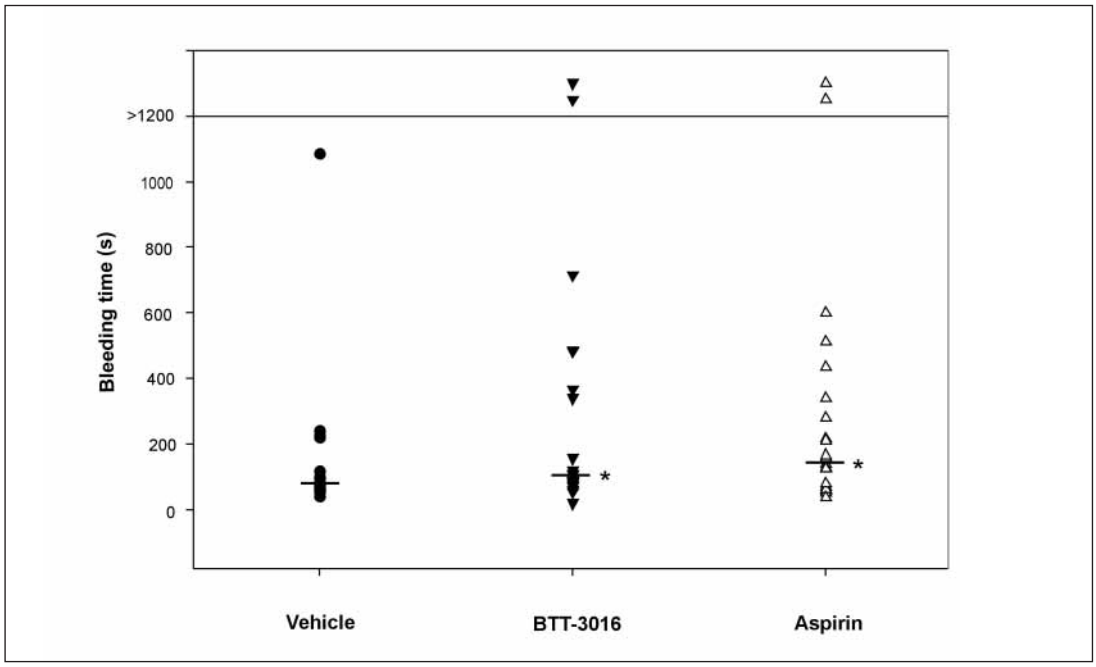

Figure 6: Integrin α2β1 inhibitor BTT-3016 prolongs the mouse tail-bleeding. Tail-bleeding times of Balb/c mice were determined. Animals were administered (i.v.) with hydroxypropyl-β-cyclodextrin (HB-β-CD) vehicle, aspirin (20 mg/kg) or BTT-3016 (20 mg/kg). The bleeding was monitored (up to 20 min) and determined when bleeding ceased. Each point represents an individual animal (n=20) and horizontal bars indicate the mean tail-bleeding time. Wilcoxon two-sample test, *p<0.05

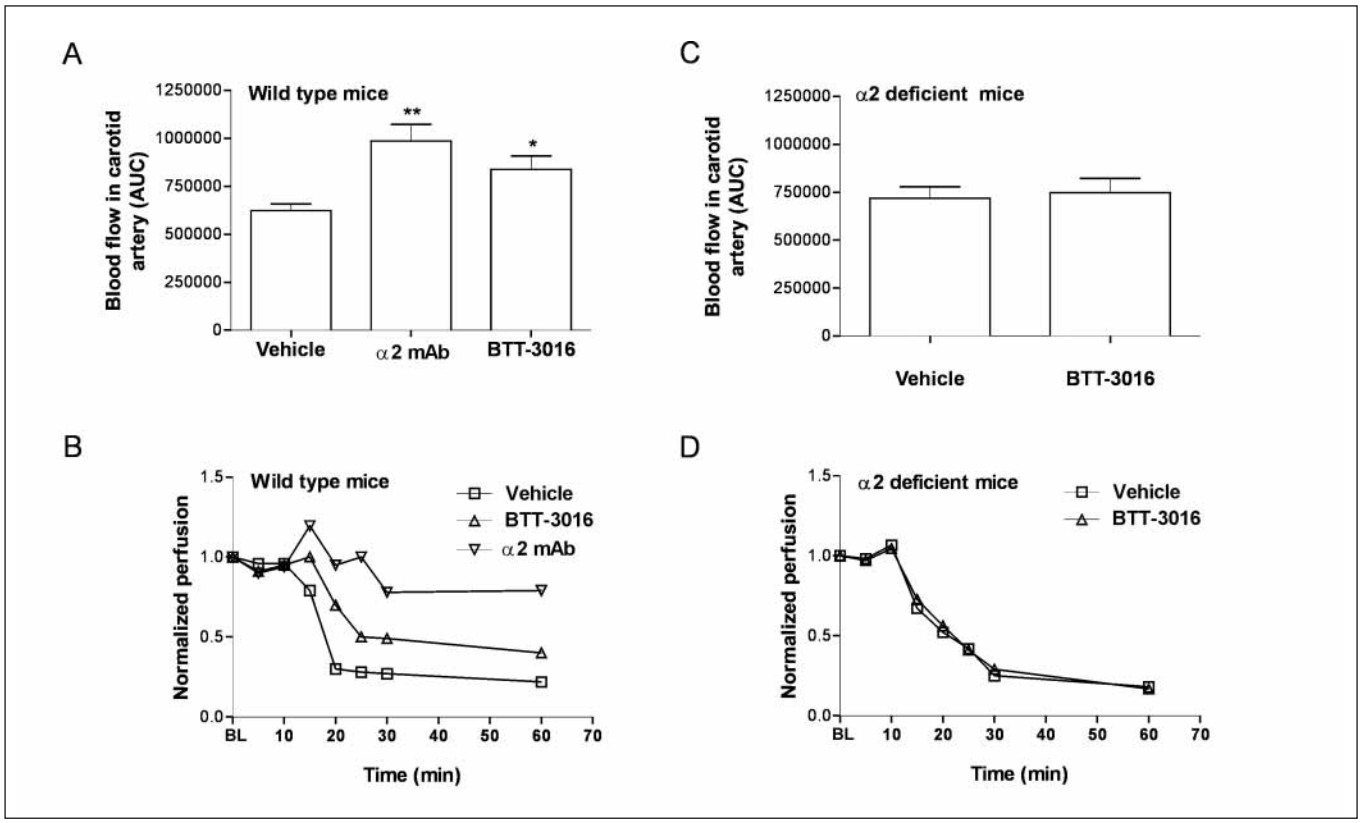

Figure 7: BTT-3016 shows integrin α2-specific antithrombotic effect *in vivo*. The mice (n=10–11) were subjected to photochemical injury of the right common carotid artery (CCA). Carotid artery blood flow was monitored throughout the experiment and the area under curve was quantified. A) The area under curve (AUC) in vehicle (30% Cremophor EL), BTT-3016 (serum level 90 μg/ml), and α2 mAb (Ha 1/29, 15 mg/kg) treatment groups in wild-type (wt) mice is presented. Kruskall-Wallis test followed by Mann-Whitney test, *p<0.05, **p<0.01. B) The normalised perfusion from segmented data in vehicle, BTT-3016, and α2 mAb (Ha 1/29) treatment groups in wt mice is presented. C) In integrin α2-deficient mice the area under curve (AUC) in vehicle (30% Cremophor EL) and BTT-3016 (serum level 90 μg/ml) treatment groups is presented. D) In integrin α2-deficient mice the normalised perfusion from segmented data in vehicle and BTT-3016 treatment groups is presented. BL, baseline.

prolong the bleeding time also in Balb/c mice ($p<0.05$) (data not shown).

To confirm the antithrombotic efficacy of BTT-3016, another *in vivo* model of thrombus formation was used. The wt mice (vehicle n=10, BTT-3016 n=10 and α2 mAb, Ha 1/29 n=11) or integrin α2-deficient mice (vehicle n=10, BTT-3016 n=13) were subjected to photochemical injury of the right CCA. Carotid artery blood flow was monitored throughout the experiment with a Laser Doppler probe. The area under curve (AUC) describing the perfusion time (▶Fig. 7A, C) and normalised perfusion from segmented data (Fig. 7B, D) in the vehicle (Cremophor EL), BTT-3016 and α2 mAb treatment groups are presented. BTT-3016 (90 μg/ml in plasma) and α2 mAb (15 mg/kg) could significantly increased the perfusion time ($p<0.05$ and $p<0.01$, respectively) in wt mice, Kruskall-Wallis test followed by Mann-Whitney test; Fig. 7). Importantly, BTT-3016 could not increase the perfusion time in α2-deficient mice (Fig. 7).

Discussion

The BTT-3016 was found to be a unique tool to test the putative effects of α2β1-blocking on platelet function. Some previous epidemiological studies have proposed that individuals, especially those with high α2β1 expression levels on platelets, are vulnerable to the development of myocardial infarction, stroke and diabetes-associated retinopathy (12–15) and could benefit from inhibition of this receptor. BTT-3016 was assessed in collagen-dependent activation and aggregation of human platelets. The inhibitory effect of BTT-3016 was most obvious in GFP, favoring α2β1-dependent conditions (Mg^{2+}) (35), but it was also present in PRP both under citrate- and PPACK-anticoagulation. Interestingly, also CRP-induced aggregation was somewhat inhibited by BTT-3016, suggesting an interplay between α2β1 and GPVI receptors. These findings were compatible with the α2β1-blocking antibody P1H5, which seemed even stronger and more uniform inhibitor of collagen-induced platelet aggregation than another α2 mAb 6F1 (19, 40). These two Abs interact with distinct sites at the α2I domain and they may have distinct effects on the activation signals mediated by α2β1. The extent of antiplatelet action of ARMX, an ADP antagonist, and BTT-3016 *in vitro* seemed comparable, but the inhibitory mechanism of BTT-3016 was collagen-specific. The pharmacological properties of BTT-3016 were suitable for *in vivo* models. Furthermore, our results indicate that BTT-3016 can even be orally administrated (Marjamäki et al., unpublished data).

The sulfonamide BTT-3016 represents a novel group of integrin inhibitors. Previously, the lack of suitable small molecule inhibitors for α2β1 integrin has made it difficult to test whether antithrombotic therapy could be tailored to inhibition of this target in platelets. We and others have recently described other α2β1 inhibitors (20–22, 41). These compounds have been useful in characterising the structural requirements of α2β1 inhibition. The published arylamide derivatives (20) were shown to recognise a hydrophobic cleft on the side of the α2I domain. The α2β1 integrin inhibitors belonging to the group of prolyl-2,3-diaminopropionic acid derivatives were proposed to bind to the MIDAS in β1 I-like domain (22). Both of these inhibitor scaffolds were shown to inhibit α2β1 integrin binding to collagen I and prevent the human platelet adhesion *in vitro*. No information on their antithrombotic efficacy *in vivo* has been shown. However, these studies suggest that these alternative binding sites could serve as a target for drug development. The previously published nucleoside derivative (41) isolated from unidentified fungus was shown to inhibit α2β1 integrin binding to collagen. The publication did not disclose any information on the binding site of the compound or its possible antiplatelet activity. The group of aromatic polyketides previously described by us (21) were the first α2β1 integrin inhibitors that were shown to directly bind to the MIDAS in α2I domain. However, the poor solubility and possible toxicity with high concentrations related to the compounds limited the use of these inhibitors in animal models predicting anti-thrombotic efficacy (Marjamäki et al., unpublished data).

The existing small-molecule inhibitors for leukocyte αI domain integrins (αLβ2 and αMβ2) act in an allosteric manner (42). One group of molecules binds into a "lovastatin" site in the αI domain, but an identical pocket does not exist in the collagen receptor αI domains. Another group of β2 integrin inhibitors recognises the MIDAS-like site at the interface of the β2I domain and the β propeller domain in the α subunit. Our results indicate that Y285 in the α2I domain is essential for the full effect of BTT-3016. However, we cannot exclude the possibility that BTT-3016 has, in addition to the α2I domain, other binding sites on the α2β1 integrin, too. The EC50 of BTT-3016 is relatively high, especially when compared with the allosteric inhibitors of β2 integrins. Importantly, at the concentrations used BTT-3016 seems to be selective for α2β1 integrin, with only a slight effect on other collagen receptor integrins, α1β1, α10β1 and α11β1, and without an effect on, e.g. fibronectin and vitronectin receptors, such as α5β1 and αV integrins, and ADP receptor P2Y12. Furthermore, BTT-3016 had no effect on the binding of leukocyte αI domain integrin αLβ2 to ICAM-1.

The efficacy of BTT-3016 on inhibiting platelet collagen-interaction under high shear forces was confirmed with an *in vitro* perfusion assay of human blood. Interestingly, variation in the response of platelets from different blood donors was detected. However, we were able to demonstrate that inhibitory effect of BTT-3016 was observed exclusively in the same donors that responded to function blocking α2 mAb (P1H5). Interestingly when the measurements were performed in Cellix microfluidic platform, the platelets derived from donors that showed no response with other methods, could still be inhibited with α2 mAb and BTT-3016. In platelets the effect of BTT-3016 was dependent on the presence of α2β1 integrin, since platelets derived from α2 null mice failed to show response. This also confirms the specificity of BTT-3016 as an α2β1 integrin inhibitor that cannot block the other collagen receptors on platelets.

The pharmacological properties of BTT-3016 allowed the testing of its *in vivo* effects and, whether the attenuation of α2β1 binding to collagen could be developed as a novel strategy in antithrombotic therapy. BTT-3016 was shown to prolong the bleeding time in accordance with previous observations in the complete absence

of α2β1 integrin in α2 null mice (3). *In vivo* thrombus formation on the vessel wall following carotid artery injury of mice was inhibited by BTT-3016. This observation is in agreement with the experiments in integrin α2-deficient mice indicating the critical role of α2β1 in thrombus formation following vascular injury (4).

During the preparation of this manuscript Miller et al. (43) reported small-molecules that are allosteric inhibitors of α2β1 integrin. These molecules are unrelated to BTT-3016. Similarly to BTT-3016 they could inhibit arterial thrombosis *in vivo* (43).

To conclude, we have described a novel group of sulfonamides as selective inhibitors of α2β1 integrin. These sulfonamides could be used to rationally test whether the α2β1 integrin is a potential target for development of antithrombotic drugs. In support of this hypothesis, one of the sulfonamides, BTT-3016, showed individual antithrombotic potential both *in vitro* and *in vivo* models confirming that even in the presence of other platelet collagen receptors, the inhibitor of α2β1 may suppress collagen-dependent platelet activation. BTT-3016 could be further developed as a specific platelet inhibitor with the potential to control arterial thrombosis, while other collagen receptors may sustain the activation capacity of platelets and assist in preserving adhesive capacity of platelets and primary haemostasis.

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What is known about this topic?

- Based on previous animal models the significance of α2β1 integrin as a platelet collagen receptor is not clear. Some published reports have suggested that the role of α2β1 integrin is marginal when compared to another collagen receptor, GPVI. However, other papers have reported that the receptors act together.
- Epidemiological data suggest that high expression levels of α2β1 integrins on platelets may increase the risk of myocardial infarctions and stroke.
- Recently, allosteric small-molecule inhibitors of α2β1 integrin were shown to inhibit arterial thrombosis *in vivo*.

What does this paper add?

- We describe sulfonamide derivatives as a structurally and functionally novel group of α2β1 integrin inhibitors.
- We report the effect of BTT-3016, a new sulfonamide compound, on platelets. The effect of BTT-3016 is dependent on α2β1 integrin, since the compound has no effect on platelet function in α2 integrin-deficient animals. BTT-3016 shows inhibitory capacity in several assessments of human platelet interaction with collagen and in an *in vivo* model in mice it reduces thrombus formation on the vessel wall after vascular injury.
- Based on the experiments with BTT-3016 we suggest that the α2β1 inhibiting strategy could be further developed for the prevention and treatment of arterial thrombosis.

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