

ORIGINAL ARTICLE

Sulfonamide inhibitors of $\alpha 2\beta 1$ integrin reveal the essential role of collagen receptors in in vivo models of inflammation

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Introduction

The members of the integrin family mediate adhesion to extracellular matrix, cell–cell adhesion and the binding of platelets and inflammatory cells to plasma proteins. Platelet fibrinogen receptor, $\alpha\text{IIb}\beta 3$ integrin, and leukocyte integrins $\alpha 4\beta 1/\beta 7$ and $\alpha\text{L}\beta 2$ have been targets for intensive and successful drug development (Goodman and Picard 2012). The collagen receptors form a special subgroup among the integrin family (Heino 2007). These heterodimeric receptors share a common $\beta 1$ subunit in complex with $\alpha 1$, $\alpha 2$, $\alpha 10$, or $\alpha 11$ subunit. The α subunits bind to collagens and other ligands via their αI (also called αA) domains (Emsley et al. 2000).

Integrin $\alpha 2\beta 1$ is expressed on platelets, epithelial cells and large variety of mesenchymal cells, including endothelial cells (Zutter and Santoro 1990). Genetic mouse models have indicated that the deficiency of $\alpha 2\beta 1$ leads to impaired

Abstract

Small molecule inhibitors of $\alpha 2\beta 1$ integrin, a major cellular collagen receptor, have been reported to inhibit platelet function, kidney injury, and angiogenesis. Since $\alpha 2\beta 1$ integrin is abundantly expressed on various inflammation-associated cells, we tested whether recently developed $\alpha 2\beta 1$ blocking sulfonamides have anti-inflammatory properties. Integrin $\alpha 2\beta 1$ inhibitors were shown to reduce the signs of inflammation in arachidonic acid-induced ear edema, PAF stimulated air pouch, ovalbumin-induced skin hypersensitivity, adjuvant arthritis, and collagen-induced arthritis. Thus, these sulfonamides are potential drugs for acute and allergic inflammation, hypersensitivity, and arthritis. One sulfonamide with potent anti-inflammatory activity has previously been reported to be selective for activated integrins, but not to inhibit platelet function. Thus, the experiments also revealed fundamental differences in the action of nonactivated and activated $\alpha 2\beta 1$ integrins in inflammation when compared to thrombosis.

Abbreviations

ANOVA, analysis of variance; HP- β -CDX, hydroxypropyl- β -cyclodextrin; MC, methylcellulose; PAF, platelet-activating factor.

platelet binding to collagen (Chen et al. 2002; Holtkötter et al. 2002), accelerated neoangiogenesis (Grenache et al. 2007; Zweers et al. 2007; Zhang et al. 2008) and defects in innate immunity (McCall-Cullbreath et al. 2008). Integrin $\alpha 2\beta 1$ has also been shown to regulate collagen synthesis in vitro (Ivaska et al. 1999) and in vivo (Borza et al. 2012). This collagen receptor was originally named as very late activation antigen 2 (VLA-2) because its expression started on T lymphocytes only several days after activation (Hemler et al. 1985). The fact that $\alpha 2\beta 1$ is a dominating collagen receptor on neutrophils (Werr et al. 2000), mast cells (Edelson et al. 2004) and Th17 lymphocytes (Boisvert et al. 2010) may explain its role during infection and inflammation. New observations have also indicated that $\alpha 2\beta 1$ is a marker for type 1 T regulatory cells, that have an essential role in promoting and maintaining tolerance (Gagliani et al. 2013). Furthermore, in transmigration from blood to bone marrow $\alpha 2\beta 1$ is essential for CD4⁺ memory T-cell

precursors (Hanazawa *et al.* 2013). In addition to various collagen subtypes, $\alpha 2\beta 1$ can mediate cell adhesion to numerous other extracellular matrix proteins, such as tenascin, laminins, and proteoglycans (Heino, 2007). Moreover, integrin $\alpha 2\beta 1$ can also recognize collectin family members (Zutter and Edelson 2007).

Integrin $\alpha 2\beta 1$ has successfully been used as a target molecule in development of direct and allosteric inhibitors (Choi *et al.* 2007; Käpylä *et al.* 2007; Nissinen *et al.* 2010; Momic *et al.* 2015) as well as inhibitors of $\alpha 2\beta 1$ synthesis (Mita *et al.* 2011). Inhibitors of $\alpha 2\beta 1$ function and expression are generally considered as potential drugs for many human diseases. Animal models support this opinion, since specific monoclonal antibodies can inhibit cancer-related angiogenesis (Senger *et al.* 1997), contact hypersensitivity reaction (de Fougères *et al.* 2000), experimental colitis (Gillberg *et al.* 2013) and collagen-induced arthritis (El Azreq *et al.* 2013). A monoclonal antibody against $\alpha 2\beta 1$ integrin (Vatelizumab; GBR 500) is in Phase II clinical trials for acute relapse in multiple sclerosis and inflammatory bowel disease (www.glenmarkpharma.com).

There are still several open questions related to the role of $\alpha 2\beta 1$ integrin in inflammatory diseases and the usefulness of small molecule inhibitors as drug molecules. In animal models $\alpha 2\beta 1$ antibodies have shown anti-inflammatory effects (Senger *et al.* 1997; de Fougères *et al.* 2000; El Azreq *et al.* 2013; Gillberg *et al.* 2013), but often the influence of the blocking antibodies has been moderate and only simultaneous blocking of both $\alpha 1\beta 1$ and $\alpha 2\beta 1$ has given more remarkable effects (Senger *et al.* 1997; de Fougères *et al.* 2000). An important unanswered question is, which $\alpha 2\beta 1$ conformation should be targeted in drug development? The three major activation states of integrins are considered to represent (i) bent, αI domain closed conformation, (ii) extended, αI domain closed conformation, and (iii) extended, αI domain open conformation (Luo *et al.* 2007). Our results with blood platelets indicate that most effective inhibitors are the ones that actually recognize the nonactivated conformation (Nissinen *et al.* 2012).

We have previously described two sulfonamide-type molecules that both inhibit $\alpha 2\beta 1$ function *in vitro* by about similar EC₅₀ value (Nissinen *et al.* 2012). However, the two molecules have different binding mechanism to $\alpha 2\beta 1$ integrin and they also have shown selectivity in the recognition of different $\alpha 2\beta 1$ conformations (Nissinen *et al.* 2012). Here, we have tested three sulfonamides in different *in vivo* models of inflammation. Our results indicate that blocking of $\alpha 2\beta 1$ function is an effective strategy to suppress inflammation. Furthermore, the ability to block the nonactivated $\alpha 2\beta 1$ integrin is not critical in inflammation-associated cellular mechanism as it is on platelets. Thus, $\alpha 2\beta 1$ integrin may function in a fundamentally different manner in inflammation when compared to thrombosis.

Materials and Methods

Ethics statement

The animal experiments were approved by the Committee on Animal Care and Use of the Free State of Saxony and carried out following the German Law on the Protection of Animals. Local ethical guidelines for experimental investigations in animals used in the rat adjuvant arthritis model were followed and the regional ethical committee of Szeged University, Hungary, approved the experimental protocol.

Drugs

BTT-3016, BTT-3033, and BTT-3034 were obtained from Pharmatory Oy (Oulu, Finland), dexamethasone and Diclofenac-Na were purchased from Fluka Chemie (Buchs, Switzerland) and Sigma (Budapest, Hungary), respectively. The $\alpha 2$ -antibody (Hal/29) was obtained from BD Biosciences Pharmingen (San Diego, CA). No significant adverse effects for thromboxane A₂ or cyclooxygenase COX-1 were detected for BTT-3016, BTT-3033, and BTT-3034 (MDS Pharma Services, Taipei, Taiwan).

Animals

Depending on the experimental model, different mice strains were used: NMRI mice, BALB/cAnNCrl (both from Charles River, Sulzfeld, Germany), and DBA/1JNCrlj (Janvier Labs, Le Genest St. Isle, France). They were housed in groups of five to six under standard conditions on a 12 h light/dark cycle (light on at 06:00 h) with *ad libitum* access to water and food (ssniff M/R 15, Spezialdiäten GmbH, Soest, Germany). Guinea pigs obtained from Charles River were housed in groups of four under standard conditions with *ad libitum* access to food and water (ssniff MS-H 4 mm, hey cobs 15 mm, Spezialdiäten GmbH, Germany). The Lewis rats used in the adjuvant arthritis were obtained from Charles River (Budapest, Hungary). They were housed in groups of five animals in plastic cages (Macrolon 2) in the same room under temperature and light-controlled conditions at the rodent facility. Rats were maintained on a standard pellet rodent diet (Bioplan Ltd, Isaszeg, Hungary) with tap water available *ad libitum*. The number of animals that were used in each experiment is given in the method description.

Drug administration

For oral administration BTT-3016 was suspended in Tween 80/0.5% hydroxyethylcellulose (2/98 v/v). BTT-3033 and BTT-3034 were dissolved in PEG 400/30% hydroxypro-

pyl- β -cyclodextrin (HP- β -CDX) or 30% captisol (80/20 v/v). Dexamethasone was suspended in PEG 300/0.5% hydroxyethylcellulose (10/90 v/v). Diclofenac-Na was prepared in 0.25% of methylcellulose (MC) each day. All compounds were administered orally with an administration volume of 10 mL/kg, 5 mL/kg and 2 mL/kg in mice, rats and guinea pigs, respectively. Except, BTT-3016, which was administered using dosing volume of 10 mL/kg in rats. The monoclonal antibody Hal/29 was directly administered into air pouch. Pretreatment time was different depending on the different experimental settings.

PAF-stimulated air pouch model

To induce an air pouch, a volume of 5 mL of sterile filtered air was injected subcutaneously under the back skin of anesthetized female NMRI mice (22.5–31.3 g; Charles River) on day 1. The procedure was repeated on day 3 and day 6 (2.5 mL of air into the existing pouch). On day 7 inflammation was induced by application of 0.7 mL of a 10^{-6} mol/L/mL platelet-activating factor (PAF, Sigma Aldrich, Deisenhofen, Germany) in Phosphate buffered saline, pH 7.4 (PBS) into the pouch. About 6 h after PAF stimulation the animals were euthanized and the air pouches were lavaged with 1 mL ice-cold PBS. The number of leukocytes in the lavage was counted under microscope. Test or reference compounds or the corresponding vehicle were administered orally 24 h and 2 h prior to application of the inflammatory stimulants (group size: $n = 8$).

Arachidonic acid-induced ear edema model

Before the experiment the basal thickness of both ears of each female NMRI mouse (23.2–29.3 g; Charles River) was measured (group size: $n = 6$). The inflammatory response was induced by administration of 20 μ L arachidonic acid (Sigma Aldrich) solution (164 mmol/L in acetone) on the left ears. The right ears were treated with the same volume of acetone and served as a negative control. 60 min after the arachidonic acid challenge ear thickness was measured again. Test or reference compound and the corresponding vehicle were given once daily 48 h, 24 h and 3 h before the challenge with arachidonic acid, respectively. Formulation used has no effect when compared the sham control (data not shown).

Mouse skin hypersensitivity model

Female BALB/cAnNCrI mice (16.5–22.2 g; Charles River) were sensitized subcutaneously twice with 0.4 mL of saline containing 100 μ g ovalbumin (grade VI; Sigma

Aldrich) and 3.3 mg Al(OH)₃ (Sigma Aldrich) on day 1 and day 8. Starting on day 13, mice were treated with test or reference compound and the corresponding vehicle 48 h, 24 h, and 2 h before ovalbumin challenge (group size: $n = 10$). On day 15 basic thickness of both ears of each mouse was measured. After that the right ear was treated with 10 μ L PBS, while the left one was treated with 10 μ L of 1% ovalbumin solution, both intradermally. About 24 h and 48 h later ear thickness was measured again.

Guinea pig skin allergic inflammation model

At two consecutive days male HA guinea pigs (387.9–514.4 g; Charles River) were sensitized by intraperitoneal injections of 20 μ g ovalbumin (grade VI; Sigma Aldrich) and 20 mg Al(OH)₃ (Sigma Aldrich). Fourteen days later the guinea pigs were challenged with 50 μ L of a 0.1% ovalbumin solution in four different locations under the back skin. The response was measured by determining the diameter of the allergic wheal that developed 60 min after the challenge. Based on the data nonresponders were excluded from further testing. The responders were allowed to rest for 7 days. Prior to second antigen challenge the guinea pigs were treated orally with the test or reference compound or the corresponding vehicle once daily for 3 consecutive days (group size: $n = 6$). Last treatment was given 2 h prior to second antigen challenge. Each location was then challenged by intradermal injection of 50 μ L of 0.05% OVA solution. Sixty minutes after antigen challenge, the area of the allergic wheals was measured as described above.

Arthritis models

In adjuvant arthritis model male Lewis inbred rats (157–186 g, age 6–7 weeks; Charles River) were injected intradermally, 2 cm from the base of the tail with 0.5 mg of killed *Mycobacterium butyricum* (Difco, BD Biosciences, San Jose, CA) in 0.1 mL of liquid paraffin. The severity of the disease was determined prior to adjuvant injection and then on days 4, 7, 11, 14, 18, 21, 25, and 28 by scoring by two independent examiners. On days 0, 10, 21, and 28 the volume of both left and right hind paws was measured.

In collagen-induced arthritis model 150 μ L of emulsion containing in 2:1 ratio bovine type II collagen solution (MD Biosciences, Zürich, Switzerland) and complete Freund's adjuvant (Sigma Aldrich) was injected intradermally into the base of the tail of male DBA/1JNCrI mice (20.6–27.3 g; Janvier Lab). The same injection was repeated at day 21. Test compounds, the reference compound or vehicle were administered orally once or twice daily from

day 21 until day 44 (group size: $n = 10$). The severity of arthritis was evaluated every day for 26 days. Scoring from 0 to 4 was done using a published scale (Holmdahl et al., 2002). Additionally, the thickness of hind paw was measured three times a week.

Plasma concentration

To investigate the pharmacokinetic profile of BTT-3033 and BTT-3034, male DBA/1 mice were orally administered with 10 mg/kg formulated in PEG400/captisol (80/20 v/v). Plasma concentrations of three mice per sampling point were determined by LC/MS/MS. Based on that, pharmacokinetic parameters were calculated from the composite profile using a noncompartmental pharmacokinetic model.

Statistical analysis

The data were analyzed by Student's *t*-test (two groups) or by one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc comparisons tests (several groups). Two-way ANOVA with the post hoc Tukey's test for individual comparison was used in the collagen-induced arthritis model. ANOVA followed by Newman-Keuls multiple comparison test was used in the adjuvant-induced arthritis model.

Results

Integrin $\alpha 2\beta 1$ function blocking sulfonamides BTT-3016, BTT-3033 and BTT-3034 have anti-inflammatory effects in PAF-stimulated air pouch model

Sulfonamide BTT-3016 is an inhibitor of $\alpha 2\beta 1$ integrin function (Nissinen et al. 2010). It is selective for $\alpha 2\beta 1$ over other collagen receptor integrins, when $\alpha 1^+ \alpha 2^- \alpha 11^+$ Saos-wt are compared to their $\alpha 2$ overexpressing counterparts. BTT-3016 is an effective inhibitor of platelet binding to collagen in ex vivo assays and in vivo thrombosis models (Nissinen et al. 2010).

To analyze the effects of BTT-3016 on acute inflammation, we used a mouse skin air pouch model. This model measures, for example, the function of polymorphonuclear leukocytes, known to use $\alpha 2\beta 1$ as their collagen receptor (Werr et al. 2000). BTT-3016 dose dependently suppressed the recruitment of leukocytes into the air pouch. With 100 mg/kg (oral administration) the effect size was in range of that of 0.1 mg/kg dexamethasone indicating a moderate anti-inflammatory effect (not shown). To further study the dose dependency of BTT-3016 effect we repeated the experiment. Low doses of BTT-3016 (1 and 3 mg/kg, p.o.) were not able to affect

the number of leukocytes in inflamed air pouch of mice compared to untreated control group (Fig. 1A). However, higher doses (10 and 30 mg/kg) dose dependently and in a statistically significant manner reduced the number of leukocytes after PAF treatment (Fig. 1A). Importantly, specific monoclonal antibody against $\alpha 2$ integrin (Ha1/29) was only moderately more effective than BTT-3016. To conclude, our results indicate that orally administrated $\alpha 2\beta 1$ blocking sulfonamides are anti-inflammatory agents in vivo.

BTT-3033 and BTT-3034 represent more potent $\alpha 2\beta 1$ inhibiting sulfonamides (Nissinen et al. 2012). The two molecules have different binding mechanisms to $\alpha 2$ I domain and we have reported that they have interesting differences in their action. BTT-3033 seems to be a better inhibitor of non-activated $\alpha 2\beta 1$ conformation than BTT-3034, whereas BTT-3034 seems to be more potent inhibitor of the activated integrin conformation, and only BTT-3033 can inhibit platelet binding to collagen under shear stress (Nissinen et al. 2012). The selectivity of BTT-3033 (8-fold) for $\alpha 2\beta 1$ integrin when compared to $\alpha 1\beta 1$ integrin is greater than that of BTT-3034 (twofold) (Nissinen et al. 2012).

To test the effects of BTT-3033 and BTT-3034 (1 or 10 mg/kg, per oral) on acute inflammation, we used mouse air pouch model (Fig. 1B). BTT-3033 reduced the infiltration of leukocytes by about 50% at 10 mg/kg dose, while it had no significant effect at the dose of 1 mg/kg. BTT-3034 was considered to be more effective than BTT-3033, since it had similar effect at the smaller dose (1 mg/kg). Dexamethasone (0.1 mg/kg) was used as a reference substance and it reduced leukocyte infiltration about 70% (Fig. 1B).

Importantly, the results indicate that BTT-3034 can inhibit inflammation despite the fact that in previously published experiments it had no inhibitory effect on platelet function under flow (Nissinen et al. 2012). Thus, $\alpha 2\beta 1$ integrin seems to function by a different mechanism in inflammation than in thrombosis. The fact that BTT-3034 can also block $\alpha 1\beta 1$ integrin function may partially explain the difference between BTT-3033 and BTT-3034, since $\alpha 1$ integrin antibodies can also potentiate the effects of $\alpha 2$ integrin antibodies (de Fougerolles et al. 2000). Furthermore, the pharmacokinetic profiles of two sulfonamides indicate that BTT-3034 plasma levels are higher during the first 1 h after oral administration (Fig. 1C; $P < 0.05$; Student's *t*-test, $n = 3$).

BTT-3033 and BTT-3034 have moderate anti-inflammatory effects in arachidonic acid-induced ear edema model

In the further experiments, the anti-inflammatory effects of BTT-3033 and BTT-3034 were tested using arachidonic

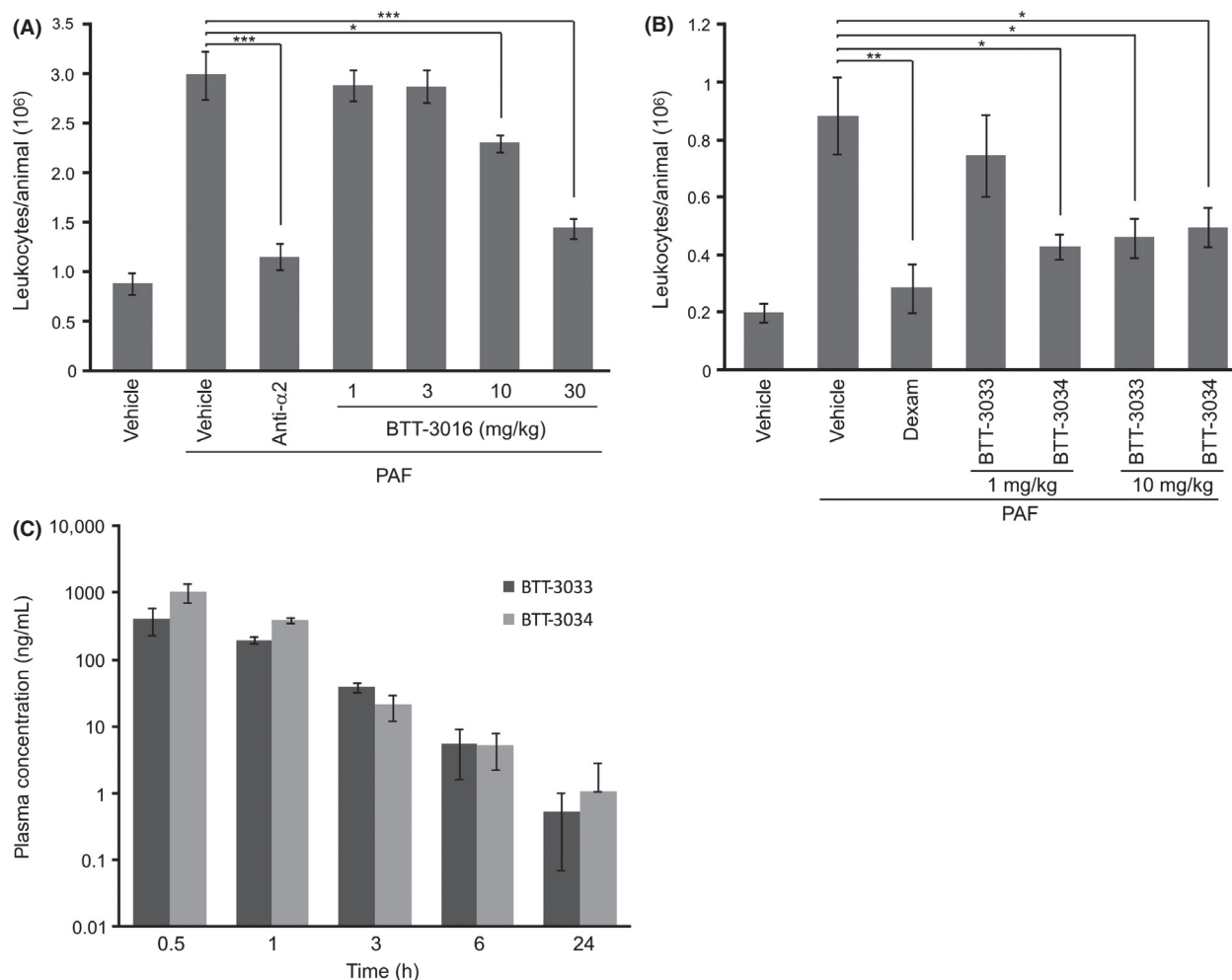


Figure 1. BTT-3016, BTT-3033, and BTT-3034 small molecular inhibitors of $\alpha 2\beta 1$ integrin present anti-inflammatory effect in PAF-induced air pouch model. An air pouch was induced by injecting sterile air to the back of female NMRI mice. Platelet-activating factor (PAF, 0.7 mL of 10^{-6} mol/L solution/air pouch) was used to induce inflammation. (A) Indicated concentrations of BTT-3016 were administered p.o. and anti- $\alpha 2$ antibody (anti- $\alpha 2$, Ha1/29, 25 μ g) directly into air pouch 24 h and 2 h before PAF induction. Number of mice in each group was 10. $*P < 0.05$; $***P < 0.001$ (One-way ANOVA followed by Bonferroni's post hoc comparisons tests). (B) BTT-3033 and BTT-3034 (both 1 mg/kg, $n = 8$; 10 mg/kg, $n = 8$), dexamethasone (Dexam, 0.1 mg/kg, $n = 8$), nonstimulated vehicle ($n = 7$) and PAF-stimulated vehicle ($n = 8$) were administered p.o. 24 h and 2 h before PAF induction. $*P < 0.05$; $**P < 0.01$ (One-way ANOVA followed by Bonferroni's post hoc comparisons tests). (A and B) The number of leukocytes was counted under microscope. The data are shown as mean \pm SEM. (C) The pharmacokinetic profiles of BTT-3033 and BTT-3034 were studied in male DBA/1 mice by administering single oral dose (10 mg/kg) of the compounds. Plasma concentrations of three animals per sampling time points are presented as mean \pm SD. In 1 h time point the difference is statistically significant ($P < 0.05$; Student's *t*-test).

acid-induced ear edema (Fig. 2), a mouse model of acute inflammation (Young et al., 1984; Chang et al., 1986; Van der Mey et al., 2003). Sulfonamides were administered orally 48 h, 24 h, and 3 h before ear swelling was induced by application of arachidonic acid. When ear swelling was measured 60 min later both BTT-3033 (10 mg/kg, $P < 0.01$) and BTT-3034 (2.5 mg/kg, $P < 0.001$; 10 mg/kg, $P < 0.001$) were clearly effective. However, they did not reach the potency of a reference compound (dexamethasone, 0.1 mg/kg). Thus, again inhibition of $\alpha 2\beta 1$ integrin function was shown to have moderate

anti-inflammatory effects and BTT-3034 was more effective than BTT-3033.

BTT-3034 inhibits inflammation in a mouse skin hypersensitivity model

To investigate the effects of the integrin inhibitor BTT-3034 on allergic inflammation, mice were sensitized with ovalbumin. This model measures delayed type hypersensitivity reaction. Ear thickness was determined 3 h, 24 h, and 48 h after the challenge. Dexamethasone significantly

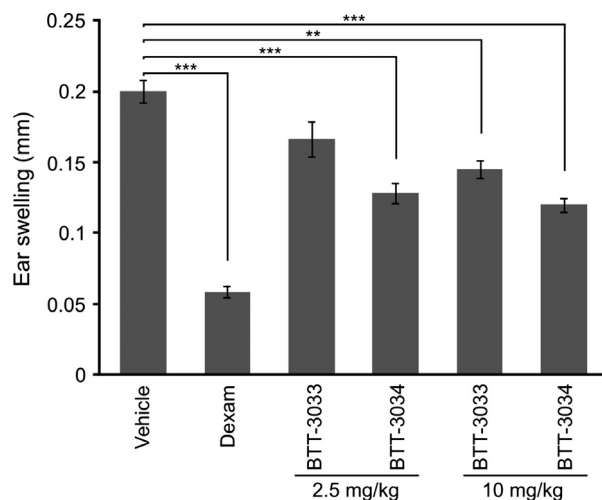


Figure 2. Both BTT-3033 and BTT-3034 demonstrate moderate anti-inflammatory effect in an acute inflammation model of mouse. The inflammatory response was induced with arachidonic acid (20 μ L of 164 mmol/L solution in acetone) on left ears. The indicated concentrations of BTT-3033 and BTT-3034, dexamethasone (Dexam, 0.1 mg/kg, $n = 6$), and vehicle were administrated p.o. once daily 48 h, 24 h, and 3 h before arachidonic acid challenge. The number of mice in each group was 6. Ear thickness was measured 60 min after arachidonic acid challenge. The data are presented as mean \pm SEM. ** $P < 0.01$; *** $P < 0.001$ (One-way ANOVA followed by Bonferroni's post hoc comparisons tests).

reduced inflammation (about 53% at 3 h, about 40% at 24 h, and about 47% at 48 h). BTT-3034 had also significant but slightly weaker inhibitory effect on inflammation at 48 h time point (about 35%) (Fig. 3). BTT-3033 was not effective at 10 mg/kg but reduced ear thickness at 40 mg/kg at 24 h and 48 h time point (data not shown).

BTT-3034 inhibits inflammation in guinea pig skin allergic inflammation model

BTT-3034 was further tested using a guinea pig allergic skin inflammation model. For this experiment, guinea pigs were sensitized to ovalbumin in a way (i.p.) to promote allergic immune responses. BTT-3034 inhibited the ovalbumin-induced increase in the area of allergic wheals when administrated in doses 0.75 mg/kg, 2.5 mg/kg or 10 mg/kg (Fig. 4). Thus, BTT-3034 inhibited both hypersensitivity and allergic inflammation.

Inhibitors of $\alpha 2\beta 1$ integrin have anti-inflammatory effects in arthritis models

To test, whether $\alpha 2\beta 1$ integrin inhibitors can inhibit the signs of chronic inflammation we tested the potency of intraperitoneally administered BTT-3016 (5 mg/kg, once

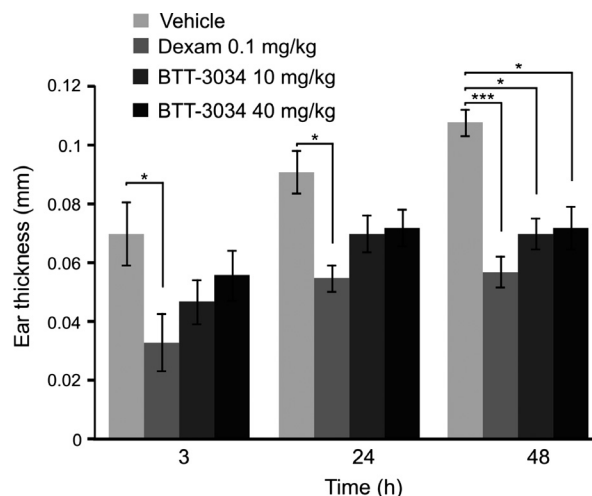


Figure 3. BTT-3034 inhibits inflammatory response in mouse skin hypersensitivity model. Subcutaneous sensitization with ovalbumin and Al(OH)₃ (100 μ g and 3.3 mg/400 μ L, respectively) was done on day 1 and 8 for female BALB/cAnNCrl mice. On day 13 vehicle, dexamethasone (0.1 mg/kg) and the indicated concentrations of BTT-3034 were administrated p.o. 48 h, 24 h, and 2 h before ovalbumin challenge. There were 10 mice in each group. Ear thickness was measured 3 h, 24 h, and 48 h after ovalbumin challenge. * $P < 0.05$; *** $P < 0.001$ (One-way ANOVA followed by Bonferroni's post hoc comparisons tests). The data are shown as mean \pm SEM.

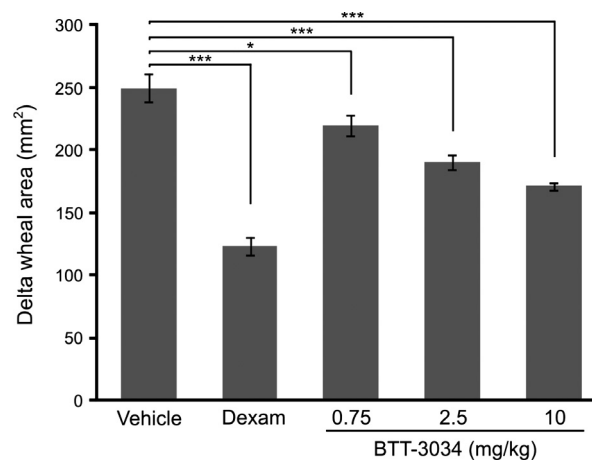


Figure 4. BTT-3034 inhibits inflammatory response in guinea pig skin allergic inflammation model. Male HA guinea pigs were sensitized intraperitoneally with ovalbumin and Al(OH)₃ (20 μ g and 20 mg, respectively). In the final experiment after 3 weeks the guinea pigs were challenged with 0.1% ovalbumin solution on the back skin. Vehicle ($n = 6$), dexamethasone (Dexam, 0.1 mg/kg, $n = 5$), and indicated concentrations of BTT-3034 ($n = 6$ in both groups) were administrated orally once a day for 3 days before the ovalbumin treatment. The response was measured 60 min after the challenge by measuring the diameter of allergic wheals. The data are presented as change in the area of allergic wheals. * $P < 0.05$; *** $P < 0.001$ (One-way ANOVA followed by Bonferroni's post hoc comparisons tests). The data are shown as mean \pm SEM.

a day) in a rat model of adjuvant-induced arthritis. Orally administered diclofenac (2 mg/kg, twice a day) was used as a positive control. BTT-3016 treatment reduced substantially and statistically significantly the score reflecting the clinical severity of the arthritis (Fig. 5A). The effect was comparable with that caused by the reference compound diclofenac (Fig. 5A). BTT-3016 also significantly decreased the volume of angle edema, but the effect was smaller than that of diclofenac (Fig. 5B). Thus, in principle small molecule inhibitors of $\alpha 2\beta 1$ integrin are effective anti-inflammatory compounds in chronic inflammation.

In another series of experiments arthritis in DBA/1J mice was induced by two immunizations with bovine collagen type II in the interval of 3 weeks. This disease model is influenced by the activity of $\alpha 2\beta 1$ -positive Th17 cell. Furthermore, the antibodies against $\alpha 2\beta 1$ have been reported to decrease the severity of arthritis (El Azreq *et al.* 2013). Dexamethasone significantly reduced paw edema, thickness, and redness. BTT-3033 (10 mg/kg) had no statistically significant effects. BTT-3034 (10 mg/kg) significantly suppressed paw thickness (Fig. 6A) but not paw redness (Fig. 6B) or paw edema (Fig. 6C). The

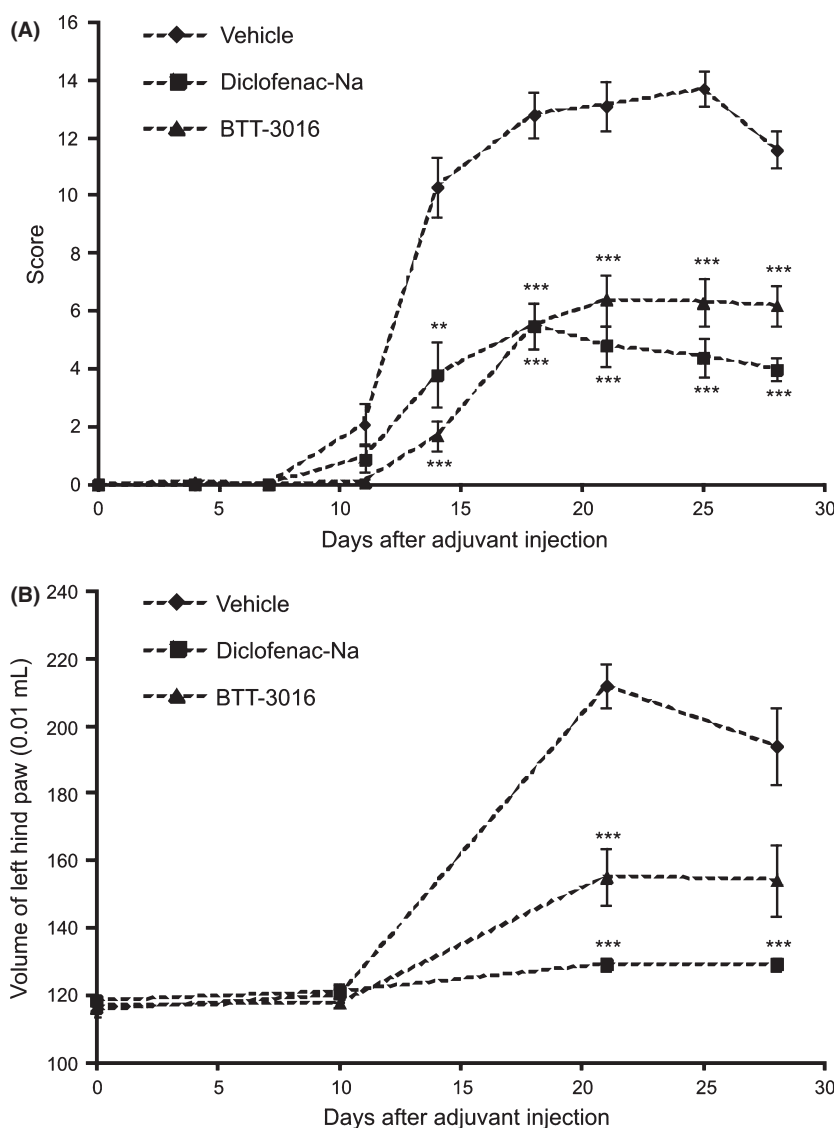


Figure 5. Inhibition of $\alpha 2\beta 1$ integrin has anti-inflammatory effect in adjuvant-induced arthritis model. The male Lewis inbred rats were injected with 0.5 mg of killed *Mycobacterium butyrium*. Vehicle ($n = 10$), Diclofenac-Na (2 mg/kg, $n = 10$) and BTT-3016 (5 mg/kg, $n = 10$) were administered i.p. once a day from day 1 to 27 after the challenge. The severity of the disease was determined by (A) scoring and by (B) measuring the volume of left hind paw at time points indicated. (A, B) The data are represented as mean \pm SEM. ** $P < 0.01$; *** $P < 0.001$ (ANOVA followed by Newman-Keuls multiple comparison test).

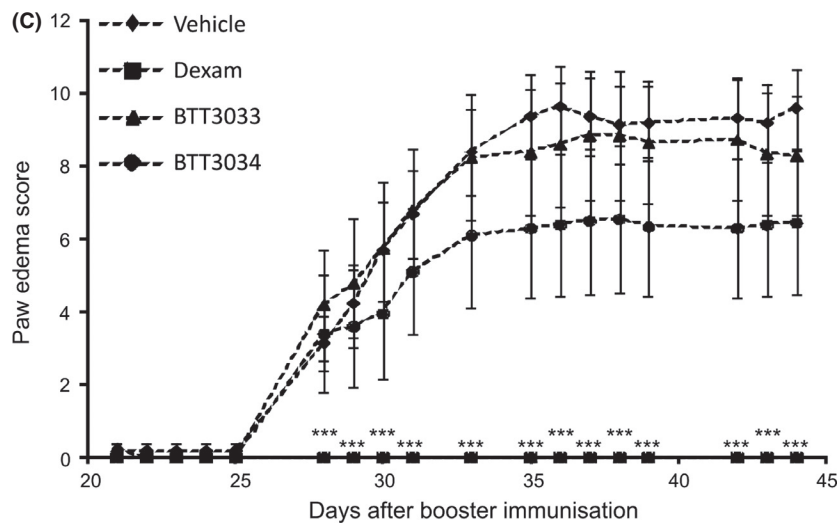
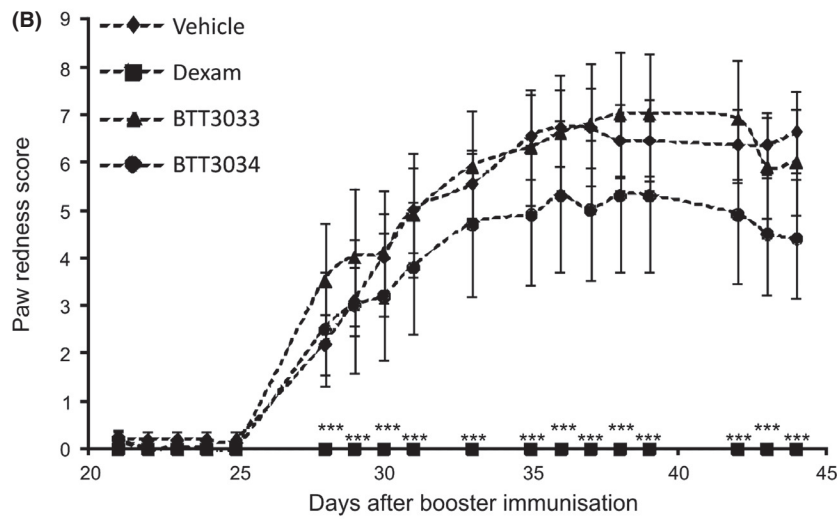
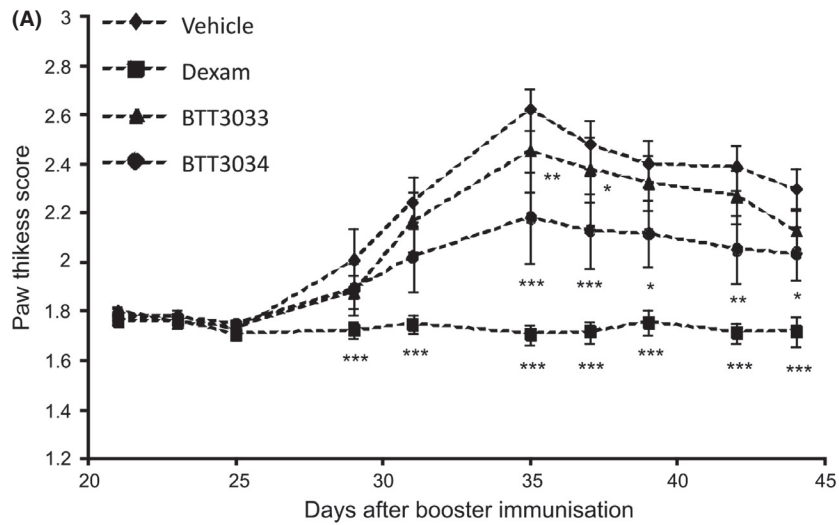


Figure 6. $\alpha 2\beta 1$ integrin inhibitor BTT-3034 presents anti-inflammatory effect in collagen-induced arthritis model. Male DBA/1JNCrj mice were challenged with bovine type II collagen and Freud's adjuvant. Vehicle ($n = 11$), BTT-3033 (10 mg/kg, $n = 10$) and BTT-3034 (10 mg/kg, $n = 10$) were administered orally twice a day and dexamethasone (Dexam, 0.1 mg/kg, $n = 10$) once a day from day 1 to 26 after the challenge. The severity of arthritis was evaluated with (A) paw thickness score, (B) paw redness score, and (C) paw edema score at time points indicated. (A–C) The data are shown as mean \pm SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (Two-Way ANOVA with the post hoc Tukey's test).

results with BTT-3034 confirm the data from BTT-3016 experiments. Weaker potency of BTT-3034 is most probably due to its relative short half-life in plasma.

Discussion

Small molecule inhibitors of $\alpha 2\beta 1$ integrin have been used in in vivo animal models of human diseases and shown to inhibit thrombosis (Miller et al. 2009; Nissinen et al. 2010), cancer-related angiogenesis (San Antonio et al. 2009), and kidney injury (Borza et al. 2012). Here, we report that sulfonamide-type inhibitors are also potent anti-inflammatory agents.

Small molecule inhibitors are also valuable tools in studies on integrin biology. Firstly, the use of small size, nonprotein ligands gives new insight when compared to function blocking antibodies. Even more so, because in many cases the in vivo results obtained with specific antibodies are in conflict with integrin knockout phenotypes. For example $\alpha 2\beta 1$ -binding proteins and antibodies against $\alpha 2\beta 1$ prevent angiogenesis (Senger et al. 1997; Woodall et al. 2008), but $\alpha 2$ deficiency leads to accelerated angiogenesis (Grenache et al. 2007; Zweers et al. 2007; Zhang et al. 2008). Secondly, small molecules can be selective to different integrin conformations and therefore be used to study the biology of different activation stages of integrins (Nissinen et al. 2012). New insight into the integrin function is also essential for drug development.

Skin air pouch model has been reported to give conflicting information about the role of $\alpha 2\beta 1$ integrin depending, whether the experiments are based on the use of specific antibodies against $\alpha 2\beta 1$ integrin (de Fougerolles et al. 2000) or genetic deficiency of this collagen receptor (Zweers et al. 2006). Here, we have shown that different sulfonamide-type inhibitors of $\alpha 2\beta 1$ are effective anti-inflammatory agents. Thus, our data indicate that the previous observations received with specific antibodies are based on the inhibition of integrin function and not any antibody related secondary effect, for example, induction of integrin clustering. We also noticed that, in the air pouch model BTT-3034 was more potent inhibitor than BTT-3033. This result is opposite to what we have previously detected in experiments measuring platelet function (Nissinen et al. 2012). We have proposed that under shear stress nonactivated platelet $\alpha 2\beta 1$ integrin, presumably in bent conformation, can mediate binding to collagen and that BTT-3033 inhibits this interaction.

Here, our observations based on several models of acute and chronic inflammation, indicate that in inflammation $\alpha 2\beta 1$ behaves in a fundamentally different manner. Thus, we propose that in the inflammation regulating cells $\alpha 2\beta 1$ must be preactivated to be functional.

In adjuvant arthritis the potency of sulfonamide BTT-3016 was comparable to that of diclofenac, stressing the important role of $\alpha 2\beta 1$ in joint inflammation. This is in accordance with previous observations based on the use of function blocking antibodies (de Fougerolles et al. 2000; El Azreq et al. 2013). Similarly, genetic deficiency of $\alpha 2$ integrin suppresses inflammation and cartilage destruction (Peters et al. 2012). The abundant expression of $\alpha 2\beta 1$ on various leukocytes, for example, neutrophils, T lymphocytes and mast cells (Werr et al. 2000; Edelson et al. 2004; Boisvert et al. 2010; Gagliani et al. 2013), and the large number of its putative ligands (Heino et al., 2007) makes it difficult to speculate, what is the most critical step that requires the presence of $\alpha 2\beta 1$ in distinct animal models. BTT-3034 also inhibited hypersensitivity and allergic inflammation. Integrin $\alpha 2\beta 1$ has been reported to augment T-cell receptor-dependent production of interferon- γ in T cells and therefore suggested to control Th1 response (Boisvert et al. 2007). Furthermore, $\alpha 2\beta 1$ is required for IL-6 secretion by mast cells (McCall-Culbreath et al. 2011).

As a summary, we have shown using sulfonamide-type $\alpha 2\beta 1$ integrin inhibitors, that the collagen receptor plays an essential role in in vivo animal models of acute inflammation, allergy, hypersensitivity, and arthritis. Furthermore, our data propose a fundamental difference in $\alpha 2\beta 1$ function when inflammation is compared to thrombosis (Nissinen et al. 2012). We suggest that in inflammation, unlike on platelets, $\alpha 2\beta 1$ integrins are preactivated before ligand binding.

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Author Contributions

Liisa Nissinen, Anne Marjamäki, Jarmo Käpylä, and Jyrki Heino defined the experimental design of this study and wrote the final version of this manuscript.

Marika Ojala, Barbara Langen, and Rita Dost designed, managed, and analyzed the experimental in vivo animal models and development of formulations used in drug administration under supervision of Anne Marjamäki. They also contributed in description of the experimental procedures of this study and writing of this manuscript.

Marjo Pihlavisto and Anne Marjamäki were responsible for design, synthesis, and chemical characterization of small molecule inhibitors.

Liisa Nissinen and Jarmo Käpylä were responsible for pharmacological in vitro characterization of compounds selected for in vivo experiments.

Conflicts of Interest

Marjo Pihlavisto is a current employee of Biotie Therapies Corp. Liisa Nissinen, Marika Ojala, and Anne Marjamäki are former employees of Biotie Therapies Corp. Jyrki Heino is a former consultant of Biotie Therapies Corp. Barbara Langen and Rita Dost are former employees of Biotie Therapies GmbH and current employees of Bio-Crea GmbH (Radebeul, Germany).

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