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Topical administration of the pan-Src kinase inhibitors, dasatinib and LCB 03-0110, prevents allergic contact dermatitis in mice

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Conflicts of interest
None declared.

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Summary

Background Allergic contact dermatitis (ACD) is a delayed type of T cell-mediated cutaneous inflammatory response, in which multiple cell types are involved. Dasatinib and LCB 03-0110 are small molecule multityrosine kinase inhibitors, and they share remarkably similar target kinases such as the c-Src family, Btk and Syk, which play key roles in the cell signalling of T cells and other inflammatory cells.

Objectives To test the anti-ACD activity of dasatinib and LCB 03-0110 and compare it with that of tacrolimus (FK506) and triamcinolone acetonide (a glucocorticoid), which are widely used for topical treatment of ACD, and to examine the two compounds for their capacity to induce skin atrophy, a side-effect.

Methods ACD was induced on the ears of mice by repeated topical application of oxazolone. Each test compound was then topically applied on the ear. Ear swelling, epidermal thickness and levels of inflammatory cytokines were measured. The skin atrophy induced by the compounds was tested during prolonged application on the dorsal skin of hairless mice, followed by haematoxylin and eosin staining.

Results Dasatinib and LCB 03-0110 suppressed the symptoms of ACD such as ear swelling, increase in epidermal thickness and synthesis of inflammatory cytokines (i.e. interleukin-1β, tumour necrosis factor-α and interferon-γ) in a dose-dependent manner. The two compounds showed near-equal potency to tacrolimus; however, their potency was lower than that of triamcinolone acetonide. Prolonged treatment with the two compounds did not induce any skin atrophy, whereas use of steroidal agents induced severe atrophy.

Conclusions Dasatinib and LCB 03-0110 could be used as effective agents for the treatment of ACD without the adverse side-effect of skin atrophy.

Allergic contact dermatitis (ACD) is one of the most common occupation-associated inflammatory skin diseases in industrialized countries, and its incidence is steadily increasing.[1,2] It is associated with allergic signs and symptoms of the skin, such as redness, oedema, warmth and pruritus, followed by scaling and dryness. Optimal ACD reactions in mouse models were observed through two phases, the sensitization phase and the elicitation phase. These events involve multiple cell types, inflammatory mediators and cytokines.[3] Haptens stimulate keratinocytes and Langerhans cells to secret tumour necrosis factor (TNF)-α and interleukin (IL)-1β, which stimulate migration and maturation of Langerhans cells to become professional antigen-presenting cells[4–6] and enhance T-cell trafficking.[7] Studies in experimental murine models have revealed that CD8+ T cells are the main antigen-specific effector cells in ACD.[8,9] They stimulate the recruitment of neutrophils and T cells, which in turn induce the morphological and clinical symptoms of ACD.[10,11] Moreover, they secrete interferon (IFN)-γ and IL-17, which are important for promoting ACD.[12–14]

Current topical therapies for most inflammatory skin diseases utilize various types of glucocorticoids and calcineurin inhibitors containing FK506 (i.e. tacrolimus and pimecrolimus).[15–18] Although steroidal drugs have been widely used

for many decades, their use is sometimes limited by their adverse side-effects, of which skin atrophy is the most prominent.[19,20] Moreover, the use of tacrolimus and pimecrolimus is still controversial as they have been implicated as causes of skin cancer.[21,22] Therefore, safer and more effective agents for the treatment of inflammatory skin diseases are required.

Dasatinib (BMS-354825, Sprycel®; Bristol Myers Squibb, Uxbridge, U.K.) is a small-molecule inhibitor targeting multityrosine kinases including the c-Src family.[23] Dasatinib is used as an anticancer drug to treat chronic myeloid leukaemia by virtue of its potent inhibition of Abl tyrosine kinase and its mutated variants.[24] LCB 03-0110 is a pan-discoidin domain receptor (DDR)/Src family tyrosine kinase inhibitor, and is characterized by its ability to inhibit fibroinflammation by suppressing activation of macrophages and myofibroblasts.[25] Interestingly, dasatinib and LCB 03-0110 share similar *in vitro* kinase inhibition profiles: both potently inhibit kinases of the c-Src family, the DDR family, Btk, Syk, c-Abl and others. However, their chemical structures, including the pharmacophore moiety, are quite distinct.[25–27] Of these kinases, the c-Src family, Btk and Syk are key targets for anti-inflammatory drugs as they are involved in cell signalling for various immune cell activation pathways in inflammatory processes.[28] In fact, dasatinib was demonstrated to suppress the synthesis of inflammatory cytokines in mast cells and other immune cells by its potent inhibition of Btk kinase.[29] Moreover, it inhibits T-cell activation via inhibition of Lck kinase.[30] Further, dasatinib suppressed TNF-α production following stimulation of Toll-like receptor signalling with lipopolysaccharide (LPS).[31]

Dasatinib and LCB 03-0110 could have merit for suppressing inflammatory pathologies, as inflammatory reactions include diverse responses of multiple inflammatory cells and the two compounds target multityrosine kinases that are involved in various inflammatory processes. In this study, we evaluated the anti-ACD activity of dasatinib and LCB 03-0110 in a murine model and compared their activity with that of tacrolimus and the glucocorticoid, triamcinolone acetonide.

Materials and methods

Chemicals

The synthesis of LCB 03-0110 has been described previously.[25] Dasatinib and tacrolimus were purchased from LC Laboratories (Woburn, MA, U.S.A.) and oxazolone, triamcinolone acetonide and clobetasol-17-propionate (clobetasol) from Sigma-Aldrich (St Louis, MO, U.S.A.).

Experimental murine allergic contact dermatitis model

Eight-week-old female BALB/c mice, weighing 16–18 g, were purchased from Taconic (Germantown, NY, U.S.A.). ACD was induced using oxazolone as described previously.[32] Briefly, mice were sensitized by applying 50 μL of 30 mg mL^{-1} oxazolone in acetone : olive oil (4 : 1 v/v) on a shaved dorsal area on day 0. The sensitized mice were challenged on both sides of the ear with 20 μL of 10 mg mL^{-1} oxazolone in acetone : olive oil (4 : 1) on days 7, 10 and 13. The test reagents dissolved in ethanol (20 μL of each) were topically applied to both sides of the ear 30 min before and 3 h after each challenge of oxazolone on days 7, 10 and 13. The thickness of the central portion of each ear lobe was measured using a digital micrometer (Mitutoyo Corp, Tokyo, Japan) before challenge with oxazolone. On day 16, the mice were sacrificed and 6-mm punch biopsies including a central strip of the ear were obtained. The animal experiments were approved by the Institutional Ethics Committee for Animal Care of the Korea Institute of Science and Technology and followed the *Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by the National Institutes of Health (http://www.nap.edu/openbook.php?record_id=5140).

Experimental murine skin atrophy model

Seven-week-old hairless mice were purchased from Charles River Laboratories (Wilmington, MA, U.S.A.). One-hundred microlitres of each reagent dissolved in ethanol was applied daily for 5 weeks on 6 cm^2 dorsal skin of the mice. They were subsequently sacrificed to obtain 6-mm punch biopsies of the treated skin area.

Histological analysis

Biopsied tissue was fixed in 4% formalin and embedded in paraffin. The deparaffinized 5-μm thick slices were used for haematoxylin and eosin (H&E) staining. Using photographs taken under light microscopy, epidermal thickness was estimated as the distance from the bottom of the stratum corneum to the basement membrane in the interfollicular epidermis. The stained slides were examined by certified pathologists for histological changes in epidermis and dermis.

Isolation of total protein and enzyme-linked immunosorbent assay for cytokines

Biopsied tissue samples frozen in liquid nitrogen were homogenized in 1 mL of lysis buffer containing 20 mmol L^{-1} Tris (pH 7·5), 150 mmol L^{-1} NaCl, 1 mmol L^{-1} ethylenediamine tetra-acetic acid, 1% Triton-X-100 and protease inhibitor cocktail (Roche, Indianapolis, IN, U.S.A.). The samples were then sonicated for 5 × 10 s and centrifuged at 10 000 **g** for 10 min at 4 °C. The supernatant was used for enzyme-linked immunosorbent assay (ELISA) analysis of TNF-α, IL-1β and IFN-γ. The ELISA kits were purchased from eBioscience (San Diego, CA, U.S.A.), and each assay was performed in triplicate according to the manufacturer's instructions.

Statistics

The statistical analysis was performed using an unpaired Student's *t*-test. Data were expressed as mean ± SD. $P < 0{\cdot}05$ was considered statistically significant.

Results

Dasatinib and LCB 03-0110 suppress allergic contact dermatitis with a potency that is similar to that of tacrolimus in the experimental murine model

Dasatinib and LCB 03-0110 both strongly inhibit tyrosine kinases of all eight members of the Src family, and Syk and Btk, as summarized in Table S1 (see Supporting information). We aimed to test whether dasatinib and LCB 03-0110 showed anti-ACD activity in the experimental mouse model, compared with that of tacrolimus and triamcinolone acetonide. Repeated challenge with oxazolone on the ears of sensitized mice on days 7, 10 and 13 caused significant time-dependent ear swelling, leading to a 3·5-fold increase in the average ear thickness by day 16, compared with the untreated control group (Fig. 1). However, co-treatment with dasatinib, LCB 03-0110 and tacrolimus at concentrations of 0·02%, 0·05% or 0·1% reduced the ear swelling significantly in a dose-dependent manner and the three compounds showed very similar dose-dependent potencies for the reduction of ear swelling (Figs 1a–c and S1; see Supporting information). On the other hand, triamcinolone acetonide exhibited the strongest inhibition against ear swelling among the tested compounds (Fig. 1d). Taken together, these results suggest that dasatinib and LCB 03-0110 can suppress ACD in the experimental mouse model, and their inhibition potency is almost equal to tacrolimus but less than that of triamcinolone acetonide.

The anti-ACD activity of the compounds was further evaluated by H&E staining of skin tissue treated with concentrations of 0·1% of each compound and biopsied at day 16. The epidermis of the ear tissue from the ACD mice treated with the vehicle only showed a 6·8-fold increase in thickness compared with that of non-ACD control mice (Fig. 2). However, treatment with 0·1% dasatinib, LCB 03-0110 and tacrolimus reduced the increase in thickness significantly, by 50·3%, 57·5% and 57·1%, respectively; there was no statistically significant difference in the reduction observed with these three treatments. Triamcinolone acetonide reduced the thickness increase by 89·8%, giving them a thickness that was only slightly increased compared with that of the tissue from non-ACD control mice. In addition, when we estimated the ear thickness from the stained tissues, the result was virtually the same with that obtained by measuring it in the animal (Fig. S2). Therefore, this histochemical analysis confirmed again that ACD is suppressed by dasatinib and LCB 03-0110 with a potency similar to that of tacrolimus; however, this suppression was less effective than that of triamcinolone acetonide.

On the other hand, we observed a large increase in stained inflammatory cells in epidermal and dermal areas in the ACD-induced mice (Fig. 2a). However, the number of inflammatory cells was reduced notably and similarly in the tissues treated with dasatinib, LCB 03-0110 or tacrolimus. The reduction was greatest in the tissues treated with triamcinolone

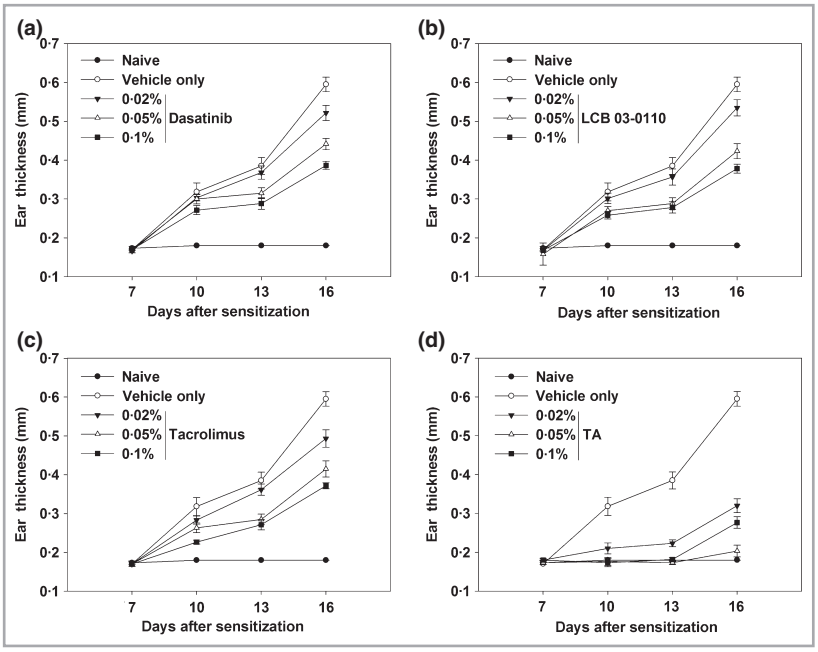

Fig 1. Dose-dependent inhibition of the induction of ear swelling by (a) dasatinib, (b) LCB 03-0110, (c) tacrolimus and (d) triamcinolone acetonide (TA) in the allergic contact dermatitis (ACD) model mice. Mice were sensitized with oxazolone on day 0, then elicited with oxazolone three times, on days 7, 10 and 13. Twenty microlitres of ethanol (vehicle only) or each compound at three different concentrations dissolved in ethanol was topically applied to the ear 30 min before and 3 h after each elicitation with oxazolone. Ear thickness was measured before each treatment and at day 16 (day of sacrifice). Each group contained 6 mice (n = 12 per group). Values are mean ± SD. Naive, normal mice without ACD.

acetonide. This result suggests that the reduction of epidermal and dermal expansion by dasatinib and LCB 03-0110 is associated with their anti-inflammatory activity, at least in part, as with tacrolimus and triamcinolone acetonide.

The antiallergic contact dermatitis activity of dasatinib and LCB 03-0110 is associated with the inhibition of the synthesis of inflammatory cytokines

In order to test whether the anti-ACD activity of dasatinib and LCB 03-0110 seen in the murine model is due to their anti-inflammatory activity, we measured the alteration of inflammatory cytokines in the ear tissue. We estimated the amount of TNF-α, IL-1β and IFN-γ in the lysate of ear tissue biopsied at day 16 after treatment with 0·1% concentrations of the four compounds being tested. IL-1β was significantly increased by an average 5·1-fold in ACD mice treated with vehicle only, compared with that in non-ACD mice (Fig. 3a). However, treatment with dasatinib and LCB 03-0110 significantly reduced the induction by an average of 63·4% and 66·1%, respectively. Tacrolimus treatment resulted in almost the same degree of reduction (70·9%); there were no statistically significant differences between the three compounds. Triamcinolone acetonide resulted in the largest suppression (90·7%). Similar to IL-1β, TNF-α was increased by 6·1-fold in ACD mice treated with vehicle only, but it was suppressed by dasatinib, LCB 03-

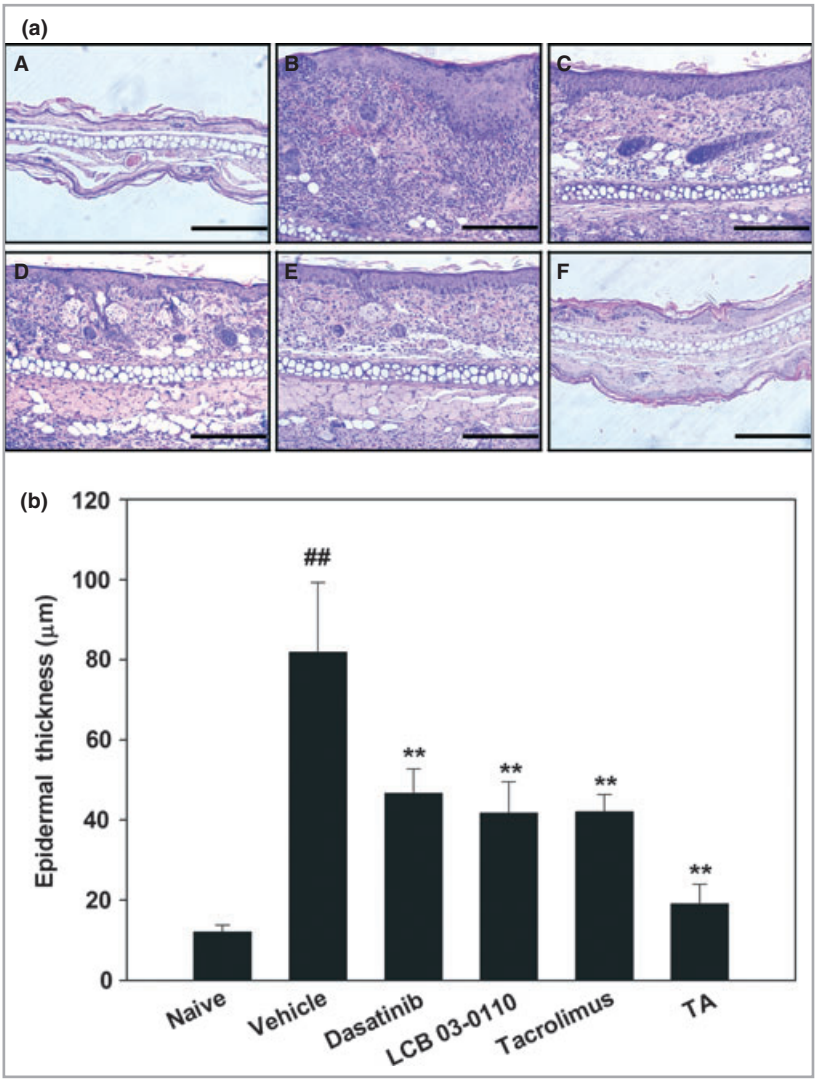

Fig 2. Haematoxylin and eosin (H&E)-stained histological sections and estimation of epidermal thickness. Ear tissue of allergic contact dermatitis (ACD) model mice treated with ethanol or 0·1% of each compound was biopsied on day 16, fixed and paraffin-imbedded. Slices (5 μm) were stained with H&E. (a) Representative images: (A) naive (normal); (B) treated with vehicle (ethanol) on ACD skin; (C) 0·1% dasatinib; (D) 0·1% LCB 03-0110; (E) 0·1% tacrolimus; (f) 0·1% triamcinolone acetonide (TA). Photographs were taken under light microscopy at 200× magnification. Scale bar: 200 μm. (b) Epidermal thicknesses were estimated and averaged using five photographs taken from each H&E-stained tissue section. Each bar represents mean ± SD ($n = 6$ per group); ##significantly different from the naive group ($P < 0·01$); **significantly different from the vehicle group ($P < 0·01$).

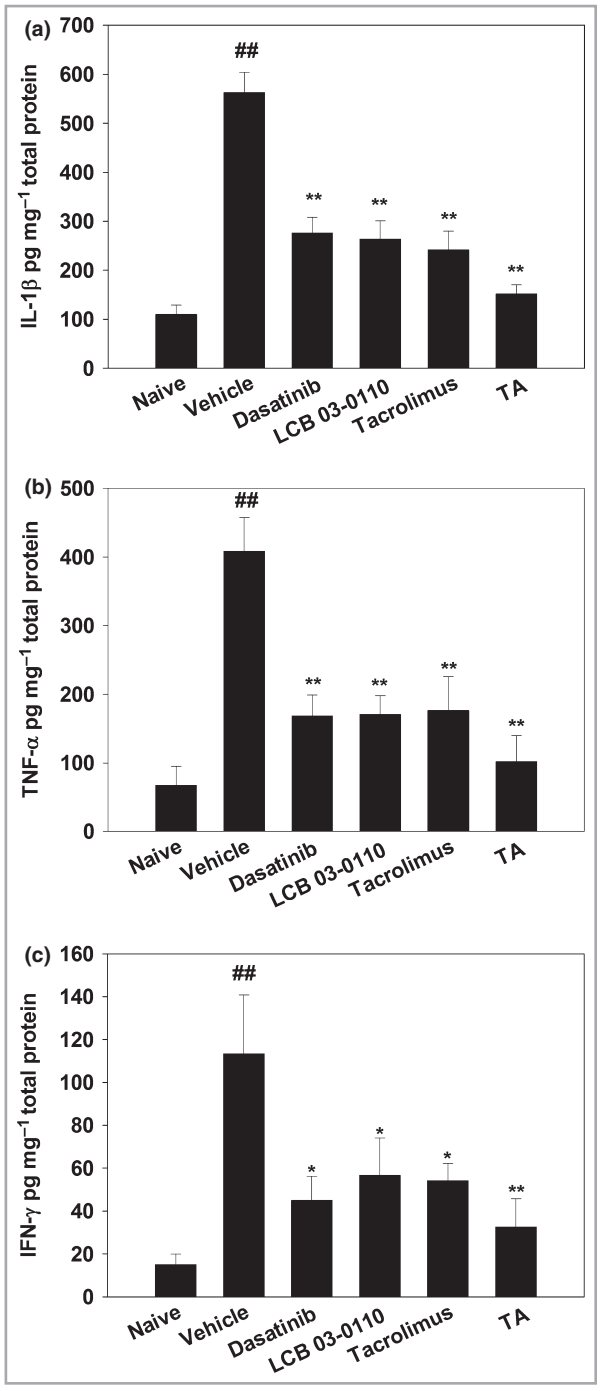

Fig 3. Suppressive effect of the test compounds on the inflammatory cytokine levels in the lysed ear tissue of allergic contact dermatitis (ACD) model mice. Ear tissue of ACD model mice treated with ethanol (vehicle) or 0·1% of each compound was biopsied on day 16, and whole protein lysate was prepared for estimation of (a) interleukin (IL)-1β, (b) tumour necrosis factor (TNF)-α and (c) interferon (INF)-γ using enzyme-linked immunosorbent assay. Each bar represents mean ± SD ($n = 6$); ##significantly different from the naive group ($P < 0·01$); ***significantly different from the vehicle group (*$P < 0·05$; **$P < 0·01$).

0110 and tacrolimus with similar potencies (reductions of 70·4%, 69·7%, and 68·1%, respectively). However, it was reduced by 89·9% by triamcinolone acetonide treatment (Fig. 3b). For IFN-γ, a 7·6-fold induction was observed in ACD mice treated with vehicle only, but the induction was significantly reduced by treatment with dasatinib, LCB 03-0110 and tacrolimus (by 69·5%, 57·6% and 60·2%, respectively); there were no statistically significant differences between the three compounds. On the other hand, triamcinolone acetonide suppressed IFN-γ by 82·2% (Fig. 3c). These results indicate that dasatinib and LCB 03-0110 inhibited inflammatory reactions, as did tacrolimus and triamcinolone acetonide, and therefore the results support the histological observations (as shown Fig. 2a). In addition, these results are consistent with the inhibition pattern of the ear thickness seen after treatment with the four compounds (as shown in Fig. 1). Therefore, it can be concluded that the inhibition of inflammatory reactions by dasatinib and LCB 03-0110 contributes to their suppression of ACD, and their inhibitory activities are similar to those of tacrolimus, but less than those of triamcinolone acetonide.

LCB 03-0110 and dasatinib do not induce the adverse side-effect of skin atrophy, even after prolonged topical treatment

We investigated whether prolonged treatment with dasatinib and LCB 03-0110 causes skin atrophy. We applied either dasatinib or LCB 03-0110 topically at the elevated concentration of 0·3% on the dorsal skin of hairless mice for 6 days per week for 5 weeks. We treated control mice with 0·1% of the glucocorticoids, triamcinolone acetonide or clobetasol. Skin wrinkles started to appear in the mice treated with glucocorticoids after 2 weeks, and the skin became severely winkled and looked transparent and fragile after 3 weeks. However, we did not observe any morphological changes in the skin of mice treated with 0·3% dasatinib or LCB 03-0110 during 5 weeks of administration. Reductions of both epidermal and dermal thickness were clearly observed in the H&E-stained dorsal skin tissue after treatment with the glucocorticoids, while the histological morphology of the tissues treated with either 0·3% dasatinib or LCB 03-0110 appeared similar to those of untreated controls (Fig. 4a). When we estimated the epidermal and dermal thickness in the H&E-stained tissues, both were significantly decreased by treatment with each glucocorticoid; however, they were not significantly altered in the tissues treated with either dasatinib or LCB 03-0110 compared with that in the controls (Fig. 4b,c). Therefore, this result suggests that dasatinib and LCB 03-0110 do not provoke skin atrophy after prolonged treatment, even at higher-than-normal concentrations.

Discussion

We have demonstrated that dasatinib and LCB 03-0110, both pan-Src inhibitors targeting multityrosine kinases, can suppress

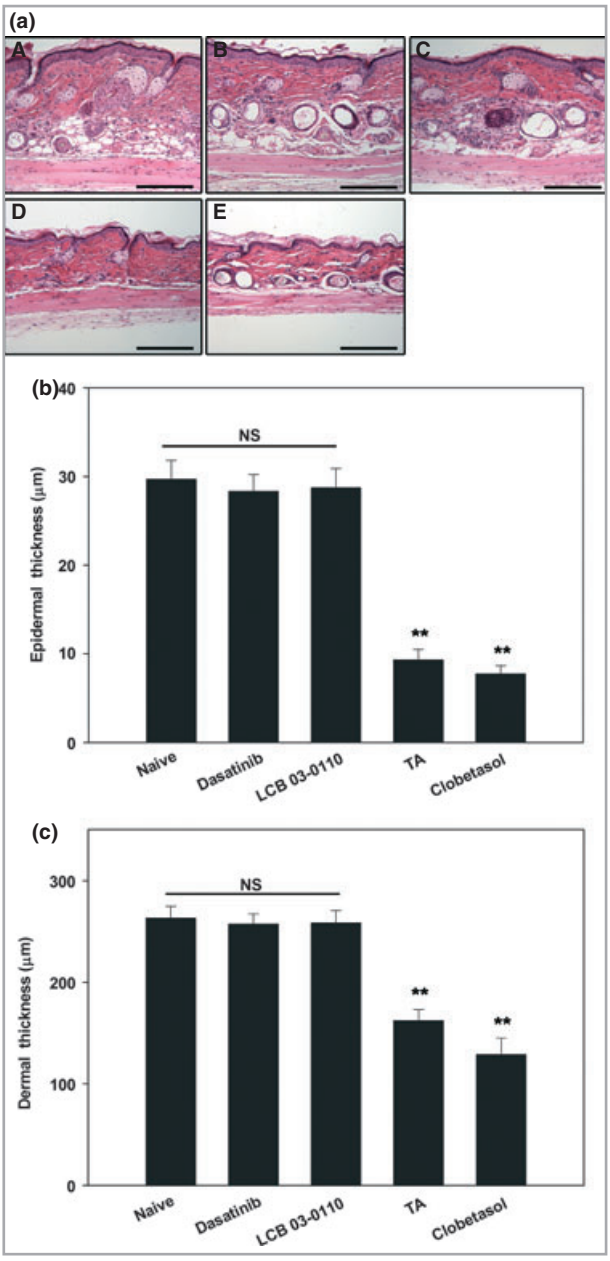

Fig 4. Lack of skin atrophy after prolonged topical treatment with the pan-Src kinase inhibitors. Dorsal skin of hairless mice was treated with each compound for 6 days per week for 5 weeks, and the biopsied tissues were haematoxylin and eosin (H&E) stained. (a) Representative images at 200× magnification (scale bars = 200 μm): (A) normal skin control (naive); (B) 0·3% dasatinib; (C) 0·3% LCB 03-0110; (D) 0·1% triamcinolone acetonide (TA); (E) 0·1% clobetasol. The epidermal (b) and dermal (c) thicknesses were estimated and averaged from five photographic areas of each stained skin tissue specimen. Each bar represents mean ± SD ($n = 6$ per group); *$P < 0·05$; **$P < 0·01$; NS, no statistically significant difference between groups of normal skin and that treated with dasatinib and LCB 03-0110.

ACD with potent efficacy in a murine model when they were applied topically on the skin in the elicitation phase of ACD. These two compounds showed almost identical potency against ACD; moreover, their activity was very similar to that of tacrolimus. Although their efficacy in suppressing ACD was less than that of triamcinolone acetonide, we think that dasatinib and LCB 03-0110 might offer advantages over steroidal drugs in treating inflammatory skin disease because they do not induce skin atrophy, the major adverse side-effect of steroidal drugs. Additionally, we propose that this type of compound might be superior to tacrolimus when considering the concerns over skin cancer, as we expect that they would have potent anticancer activity.[33] In fact, dasatinib and LCB 03-0110 have been shown to suppress skin cancer development[23,34] (S.H. Jung, unpublished observation).

Although dasatinib and LCB 03-0110 showed an anti-ACD activity in our study using a rodent model, there is a limitation for predicting their activity in humans, as chronic skin inflammatory responses are different between animal models and humans at the cellular and molecular level. For example, the repertoire of leucocyte subsets involved in skin dermatitis might not be equal between them and the difference in the expression pattern of adhesion molecules could also affect lymphocyte homing.[35,36] In fact, there have been many cases of compounds showing a minimal activity in humans although they have a potent antiskin inflammatory activity in an animal model study.[37] Although we observed no atrophogenic activity of dasatinib and LCB 03-0110 in our test using hairless mice, this could also vary in humans. The hairless rodent model is known to be highly sensitive to atrophy assay compared with humans.[38] However, there are clear anatomical differences between rodent and human skin.[39] In addition, the difference of atrophogenic potential among the compounds we observed in the hairless rodent model could simply be due to their different anti-inflammatory activities, as it was reported previously that the two activities correlated well when glucocorticoids were evaluated in a hairless rodent model.[40]

We showed that anti-inflammatory activity is associated with the suppression of ACD by dasatinib and LCB 03-0110. As CD8+ T cells function as the main antigen-specific effector cells, blocking the cell signalling that activates T cells is one of the best strategies to suppress ACD. In fact, the pharmacological mode of action of glucocorticoids and calcineurin inhibitors includes potent inhibition of T-cell signalling and function.[41,42] The c-Src family of tyrosine kinases, such as Lck and Fyn, are critically involved in the downstream signalling cascade of the T-cell receptor (TCR).[43–45] Both dasatinib and LCB 03-0110 inhibit Lck and Fyn potently. Therefore, the potent inhibition of Lck and Fyn, and thus the inhibition of the TCR signalling cascade, might be one of the important reasons why dasatinib and LCB 03-0110 can suppress ACD. Along with CD8+ T cells, other inflammatory cells such as macrophages and neutrophils are suggested as targets in the treatment of ACD.[46] Hck, Fgr and Lyn, which belong to the c-Src family, are important when macrophage cells are activated by LPS to produce immune cytokines such as TNF-α, IL-1β and IL-6.[47] Moreover, Hck and Fgr are necessary to promote migration and attachment of macrophage cells to the sites of

inflammation by mediating the cell signalling from integrin.[48] Integrin-dependent activation and adhesion of neutrophils requires Fgr and Lyn,[49,50] while both Fgr and Hck are associated with the adhesion-dependent degranulation of neutrophils.[51] Therefore, the inhibition of various inflammatory cells such as T cells, macrophages and neutrophils through the potent inhibition of the Src family tyrosine kinases, and Syk and Btk kinases (as shown in Table S1), may be the critical mode of action by which dasatinib and LCB 03-0110 can suppress ACD with similar potency.

What's already known about this topic?

- Dasatinib and LCB 03-0110 are small-molecule inhibitors targeting multiple tyrosine kinases including the c-Src family, Btk and Syk, which play key roles in inflammatory cell signalling.
- c-Src kinases are important for the downstream signalling of T-cell receptors, and CD8+ T cells are the main effector cells in allergic contact dermatitis (ACD).
- Topical steroidal drugs can induce the adverse side-effect of skin atrophy.

What does this study add?

- Topical treatment with dasatinib and LCB 03-0110 inhibits ACD in a murine model with equal potency to tacrolimus; however, the potency is less than that of triamcinolone acetonide, a glucocorticoid.
- Dasatinib and LCB 03-0110 do not induce any skin atrophy, even after prolonged treatment.
- Dasatinib and LCB 03-0110 could be used as anti-ACD agents as they are safer than the steroidal agents.

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Supporting Information

Additional supporting information may be found with the online version of this article.

Fig S1. Comparison of efficacy in reducing ear thickness between the four test compounds at their three different concentrations in allergic contact dermatitis mice model on day 16.

Fig S2. Estimation of ear thickness from haematoxylin and eosin-stained histological sections.

Table S1 Comparison of IC_{50} values against target kinases between dasatinib and LCB.