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Bortezomib, a proteasome inhibitor, alleviates atopic dermatitis by increasing claudin 1 protein expression

Yong-Eun Kim ^a, Namjoon Cho ^a, Seonghye Cheon ^a, Kee K. Kim ^{a,*}^a Department of Biochemistry, Chungnam National University, Daejeon, 34134, Republic of Korea

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ABSTRACT

Atopic dermatitis (AD) is a chronic inflammatory skin disease. Many studies investigating AD pathogenesis and its therapy have been conducted but none have been successful. One of the causes of AD is dysfunction of tight junctions through reduction of claudin 1 expression in the epidermal barrier of the skin. In the present study, we investigated the role of bortezomib (BTZ) in the restoration of the reduced expression of claudin 1. Immunoblot and immunofluorescence analyses revealed that BTZ increased the protein expression level of claudin 1 in the human keratinocyte cell line HaCaT, thereby forming paracellular barriers. Furthermore, repeated application of BTZ alleviated atopic symptoms on the backs and ears of 2, 4-dinitrochlorobenzene (DNCB)-induced AD mice, and led to the formation of normal tight junctions in the epidermal barrier of DNCB-induced mice skin. Taken together, these results demonstrate that BTZ-induced claudin 1 expression may be a valuable therapeutic approach for AD.

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1. Introduction

Atopic dermatitis (AD), also known as atopic eczema, is a chronic inflammatory skin disease prevalent in children. AD is characterized by dry eczematous skin lesions, chronic and relapsing dermatitis, and pruritus. The cause of AD is still poorly understood; it seems to be correlated with interactions among susceptibility genes, environment factors, pharmacological abnormalities, and immunological factors [1]. In general, AD develops through Th2-driven inflammatory processes, and the compromised skin barrier helps T cells penetrate the AD lesions. Thus, defects of the skin barrier are key to disease development. The most well-known protein in the development of the skin barrier is filaggrin, which forms the stratum corneum, the outermost layer of the epidermis. One-third of European patients with AD have a defective filaggrin gene [2]. Additionally, tight junctions are considered responsible for the epidermal barrier defects seen in AD [3].

Tight junctions are highly complex structures that form paracellular barriers to regulate the passage of water, ions, and solutes. They are composed of a number of transmembrane proteins, including occludin, claudin family, tricellulin, and the peripheral

membrane proteins—ZO-1, ZO-2, and ZO-3. Claudins are divided into two groups according to their functions: those having paracellular barrier functions (claudins 1, 3, 4, 5, 8, 11, 14, 18, and 19), and those having paracellular channel functions (claudins 2, 7, 10, 15, 16, and 17) [4]. Claudin 1 is expressed in various organs, such as the liver, brain, lungs, heart, kidneys, testis, and skin. Especially in the skin, claudin 1 is expressed predominantly in the tight junctions of the epidermis. The fundamental role of claudin 1 in the skin has been demonstrated by the death of claudin 1 knock-out mice within one day after birth, due to severe dehydration by excessive transdermal water loss through the skin [5]. Claudin 1 expression is decreased in human and mouse AD skin lesions [6–8] due to pro-allergic cytokines, including IL-4, IL-13, and IL-31, suggesting that it is triggered by inflammation [9]. Therefore, claudin 1 is a valuable target for developing therapeutic agents for AD.

The ubiquitin-proteasome pathway is responsible for maintaining protein homeostasis. Accordingly, the proteasomes that regulate protein degradation have essential roles in a variety of cellular process, including cell survival, apoptosis, cell signaling, DNA repair, and cell-cycle progression [10]. Proteasome inhibitors, such as bortezomib (BTZ), carfilzomib, and ixazomib, are approved for the treatment of multiple myeloma [11]. These proteasome inhibitors are also effective in the treatment of mantle-cell and non-Hodgkin lymphomas [12,13]. Among them, BTZ inhibits chymotrypsin- and caspase-like active sites of the 26 S proteasome. BTZ directly induces the apoptosis of tumor cells, inhibits the activation

* Corresponding author. Department of Biochemistry, College of Natural Sciences, Chungnam National University, Daejeon 305-764, Republic of Korea.

E-mail address: kimkk@cnu.ac.kr (K.K. Kim).

of NF- κ B, induces cell cycle arrest, and blocks the production and intracellular signaling of IL-6 in myeloma cells by stabilizing proteins, including Bid, Bax, p53, I κ B α , p21, and p27 [14].

In this study, we investigated both *in vitro* and *in vivo* effects of BTZ on AD. BTZ increased claudin 1 expression in both human keratinocytes and DNCB-induced AD skin lesions. Consequently, claudin 1 led to the formation of well-organized epidermal tight junctions in the skin of DNCB-induced AD mice. Furthermore, the symptoms of AD were diminished by the topical application of BTZ in DNCB-induced AD mice.

2. Materials and methods

2.1. Cell culture

The immortalized human keratinocyte cell line, HaCaT (ThermoFisher Scientific, Waltham, MA) was maintained using Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 1% penicillin-streptomycin, at 37 °C in a humid 5% CO₂ atmosphere.

2.2. Animals

Male BALB/c mice, aged 8 weeks, purchased from Daehan Biolink (Eumseong, Korea) were housed under controlled conditions (22 $\pm$ 2 °C, relative humidity 50 $\pm$ 5%, and 12 h light/dark cycle), and were given *ad libitum* access to standard rodent chow and water. All mice were acclimated for 7 days before experiment initiation. All animal experiments were approved by the Animal Experimental Ethics Committee of Chungnam National University (Daejeon, Korea), and were performed in accordance with the institutional guidelines.

2.3. Induction of AD-like skin lesions and treatment

For aiding topical application of DNCB (Sigma-Aldrich, St. Louis, MO), the backs of mice were shaved with a clipper and a depilatory cream, and were washed with sterilized PBS. The mice were divided into 4 groups (n = 5): (1) control (Vehicle), (2) DNCB, (3) DNCB + 50 nM BTZ, and (4) DNCB + 500 nM BTZ. To induce AD-like symptoms and skin lesions, 250 μ L of 1% DNCB dissolved in an acetone:jojoba oil mixture (3:1 v/v), was applied on the shaved backs of mice on the first day, followed by application of 250 μ L of 0.2% DNCB every 2 days. BTZ (LC Laboratories, Woburn, MA) was dissolved in an acetone:jojoba oil mixture (3:1 v/v), and 250 μ L of either 50 nM or 500 nM BTZ was applied daily on the backs of mice, according to the schematic representation of the experimental procedure described in Fig. 2A.

2.4. Induction of AD on mice ears and treatment

To induce AD on mice ears, DNCB was applied on the outer and inner surfaces of the ears. Mice were randomly divided into 4 groups (n = 5): (1) control (Vehicle), (2) DNCB, (3) DNCB + 50 nM BTZ, and (4) 50 nM BTZ. To induce AD-like symptoms and skin lesions in the mice ears, 40 μ L of 1% DNCB was applied on the first day, and then 40 μ L of 0.2% DNCB was applied every 3 days. BTZ was applied daily on the outer and inner surfaces of the ears, according to the schematic representation of the experimental procedure described in Fig. 3A. Ear thickness was measured weekly using micro-calipers.

2.5. Immunoblot analysis

HaCaT cells were isolated using a lysis buffer [10 mM Tris-HCl

(pH 7.5), 100 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100] containing a complete protease inhibitor cocktail (Roche, Basel, Switzerland). Mouse skin tissues were homogenized using 3 vol of lysis buffer. Protein concentration was determined using the Qubit[®] protein assay kit (ThermoFisher Scientific). For each sample, 30 μ g of lysate was loaded and separated using 15% SDS-PAGE. Immunoblotting was performed as described previously [15]. Anti-claudin 1 (Cell Signaling Technologies, Danvers, MA) and anti-GAPDH (Meridian Life Science, Memphis, TN) antibodies were used in the immunoblot analysis.

2.6. H and E staining

Mice of each group were sacrificed 24 h after the final application of BTZ, and the dorsal skin and ear tissues were isolated, fixed in 4% paraformaldehyde for at least 24 h, followed by cryoprotection with 30% sucrose in PBS. Frozen samples embedded in Tissue-Tek[®] O.C.T. Compound (Sakura[®] Finetek, Torrance, CA) were sectioned 12 μ m thick and mounted on slides that were gently rinsed with PBS to remove the O.C.T. compound. For the measurement of epidermal thickness, the skin and ear sections were conventionally stained with Hematoxylin and eosin Y (Sigma-Aldrich). Slides were observed under a light microscope (Nikon, Tokyo, Japan) at a fixed magnification of 100 $\times$. Randomly, 16 slides were selected and the epidermal thickness was reported as a mean of 3 measurements in a 3-part visual field.

2.7. Immunofluorescence microscopy

HaCaT cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.5% Triton X-100 in PBS for 15 min. The fixed cells were blocked with 5% goat serum and 0.1% bovine serum albumin, followed by overnight incubation with anti-claudin 1 (Cell Signaling Technologies) and anti-claudin 6 antibodies (Santa Cruz Biotechnology, Dallas, TX). Alexa Fluor 488- and 532-conjugated goat antibodies against rabbit and mouse IgG (ThermoFisher Scientific), respectively, were used as secondary antibodies. For staining of the mouse skin tissues, anti-claudin 1 (Santa Cruz Biotechnology) and anti-NMHC II-A (Covance, Princeton, NJ) were used. Alexa Fluor 488- and 532-conjugated goat antibodies against mouse and rabbit IgG were used as secondary antibodies, respectively. Nuclei were stained with DAPI (ThermoFisher Scientific) and the images were captured by an LSM 510 Live Configuration Vario Two VRGB confocal laser-scanning microscope (Carl Zeiss, Oberkochen, Germany).

2.8. Statistical analysis

Data are presented as mean $\pm$ SD. The statistical significance of differences between values of BTZ-treated and BTZ-nontreated groups was determined using the Student's *t*-test. *P* < 0.05 was considered to be significant, and has been indicated in the figures with asterisks.

3. Results

3.1. BTZ increases claudin 1 expression

The expression of claudin 1 is regulated by many factors to maintain homeostasis of the epithelial barrier. Claudin 1 expression is up-regulated by tumor necrosis factor alpha (TNF α) or via histone deacetylase-mediated increase in its mRNA stability, whereas its expression is down-regulated by transforming-growth factor β involved in epithelial-mesenchymal transition [16–18]. In addition, claudin 1 protein undergoes proteasomal degradation after

ubiquitination by the E3 ubiquitin ligase, LNXp80, in the tight junctions of MDCK epithelial cells [19]. Two recent studies revealed that the expression of claudin 1 decreased markedly in the epidermal layer of AD skin lesions [6,7]. Therefore, we investigated whether or not the expression of claudin 1 is increased by BTZ treatment in the human keratinocyte cell line HaCaT. Immunoblot analysis using an anti-claudin 1 antibody showed that the expression of claudin 1 increased upon BTZ treatment in a time- and concentration-dependent manner (Fig. 1A and B). To examine whether other proteasome inhibitors also influence on claudin 1 expression, immunoblot analysis was performed after the cells were treated with MG132 and VR23. The increase in claudin 1 expression after MG132 or VR23 treatment was lesser than that after BTZ treatment (Supplementary Figs. S1A and B). Further, to investigate whether BTZ-induced claudin 1 proteins are located in the cellular membranes that constitute tight junctions, we performed immunofluorescence microscopy using the anti-claudin 1 antibody. We observed that in the presence of BTZ, claudin 1 proteins organized themselves in the cellular membranes to form paracellular barriers, whereas in the absence of BTZ, they were found diffused in both the cytoplasm and cell membranes (Fig. 1C). In addition, claudin 6 level slightly increased after BTZ treatment in HaCaT cells. These results showed that the inhibition of proteasomal degradation by BTZ increased claudin 1 protein stability, resulting in increased paracellular barrier formation.

3.2. BTZ ameliorates the symptoms of DNCB-induced AD in mice

To investigate the effect of BTZ on AD-like lesions *in vivo*, AD was induced by repeated topical application of 2,

4-dinitrochlorobenzene (DNCB) in mice. The DNCB-induced AD mouse model that exhibits significantly increased levels of serum immunoglobulin E (IgE) and cytokines, such as IL-4 and IL-13, in the lesions, is a well-established AD model [20]. We topically applied two different doses of BTZ daily on the DNCB-induced AD lesions and scored the severity of AD lesions weekly by observation (Fig. 2A). Repeated application of DNCB in the control group induced erythema and scarring on the dorsal skin of mice. However, erythema and scarring in the skin of BTZ-treated mice were markedly reduced (Fig. 2B). Furthermore, BTZ treatment significantly reduced the total lesion-severity score in a dose-dependent manner after 35 days in comparison to that without any treatment (Fig. 2C). Further, hematoxylin and eosin (H and E) staining was performed to investigate the effect of BTZ on the histopathological features of the dorsal skin in the DNCB-induced AD lesions. Histopathological analysis revealed that BTZ treatment significantly reduced the epidermal thickness in comparison to that without any treatment (Fig. 2D and E). Ear swelling and hyperplasia are commonly seen when DNCB is applied on the ear. Thus, DNCB-induced ear swelling test was used to confirm the effect of BTZ on AD lesions. As expected, DNCB caused redness and swelling in the ear, which was ameliorated by BTZ treatment (Fig. 3B). Moreover, BTZ treatment significantly reduced the thickness of the DNCB-induced ear (Fig. 3C), which was also seen after H and E staining (Fig. 3D). To identify adverse effects of BTZ treatment after topical application, we applied BTZ alone on the dorsal skin of normal mice; BTZ treatment did not cause any histopathological change (data not shown). Taken together, these results suggest that BTZ has a therapeutic effect on the skin lesions in the DNCB-induced AD mouse model.

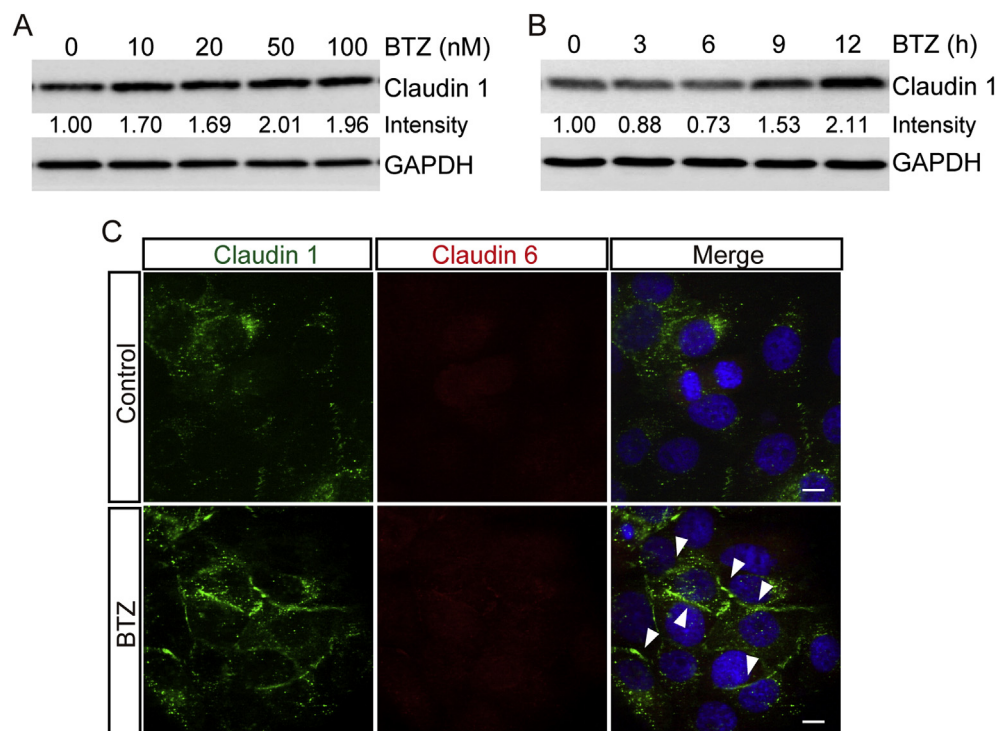


Fig. 1. Increase in claudin 1 expression by BTZ (A) Human keratinocyte HaCaT cells were treated with the indicated concentrations of BTZ for 12 h (B) or treated with 50 nM BTZ for the indicated time. The total cell extracts were prepared for the immunoblot analysis of claudin 1. GAPDH served as a loading control. Relative intensities of blots are indicated for claudin 1 expression. (C) HaCaT cells were treated with 50 nM BTZ for 12 h and subjected to immunofluorescence analysis using anti-claudin 1 (green) and anti-claudin 6 (red) antibodies. Overlay images include nuclear staining (blue). Arrowheads indicate claudin 1-containing tight junctions. Bars: 10 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

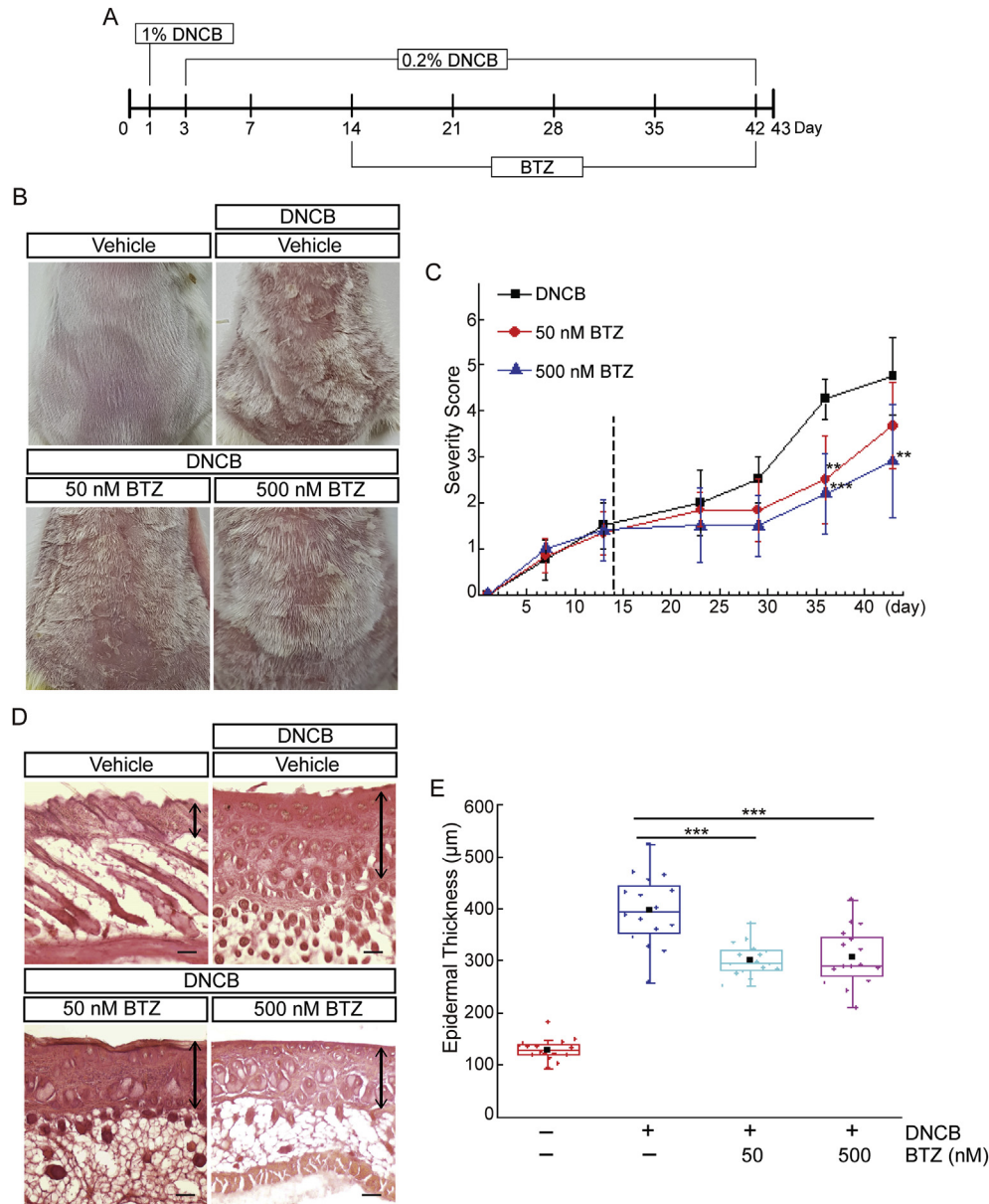


Fig. 2. Effect of BTZ on DNCB-induced AD in mice (A) Schematic diagram for the whole procedure of the *in vivo* experiment. (B) Images of skin lesions in mice from the different groups taken on the last day of the experiment. (C) Total lesion-severity score was defined as the sum of the individual scores for each of the following four signs: i) erythema and hemorrhage, ii) edema, iii) erosion (excoriation), and iv) scaling (dryness); score 0 (no symptoms), 1 (mild symptoms), 2 (moderate symptoms), 3 (severe symptoms). Each value represents the mean $\pm$ SD with 5 mice in each group; ** $P < 0.05$ and *** $P < 0.001$ vs DNCB group. The dashed line indicates the day BTZ treatment was started. (D) H and E staining. The pictures are representative of each group of mice ($n = 5$). Arrows indicate the measured epidermal thickness. Bars: 100 μ m. (E) The epidermal thickness in 16 randomly chosen visual fields of images of the H and E stained slides. *** $P < 0.001$ vs DNCB group.

3.3. BTZ increases claudin 1 in the tight junctions of skin in DNCB-induced AD mice

To investigate the changes in claudin 1 expression by BTZ treatment in the dorsal skin tissues of DNCB-induced AD mice, immunoblot analysis was performed using an anti-claudin 1 antibody. We observed that claudin 1 level was decreased in the skin of DNCB-induced AD mice, as seen in human patients with AD [8]; however, it was restored by BTZ treatment in the DNCB-induced skin tissues (Fig. 4A). Interestingly, even though BTZ as a protease inhibitor was known to prevent proteasomal degradation, topical application of BTZ also restored the mRNA expression level of claudin 1 in the DNCB-induced AD skin lesions (Supplementary

Fig. S2). However, we did not observe significant changes in the mRNA expression of tight junction-related genes, including ZO-1, claudin 7, and occludin (Supplementary Fig. S2). Further, we investigated claudin 1 expression in the epidermal barrier of the skin tissues of DNCB-induced AD mice using immunofluorescence microscopy. The tight junctions formed by claudin 1 in the skin tissue of normal mice were in the form of a thin layer, whereas those formed by claudin 1 in the skin tissues of DNCB-induced AD mice appeared to have severe defects and formed an abnormally thick stratum corneum (Fig. 4B). However, BTZ treatment restored claudin 1 expression resulting in a normal epidermal barrier of the skin tissues in DNCB-induced AD mice (Fig. 4B).

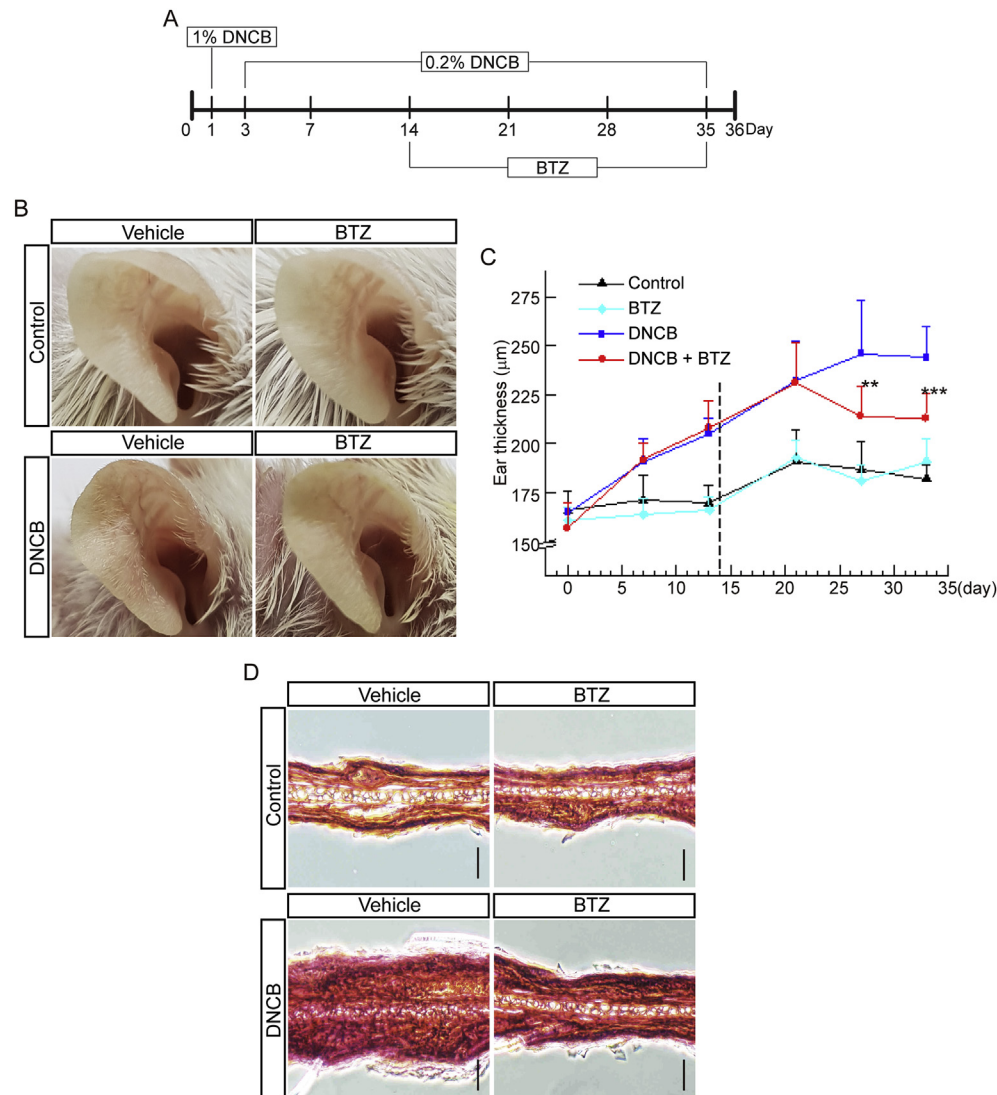


Fig. 3. Effect of BTZ on DNCB-induced ear swelling in mice (A) Schematic diagram for the whole procedure of the *in vivo* experiment. (B) Images of skin lesions in mice from the different groups taken on the last day of the experiment. (C) The results of H and E staining. The pictures are representative of each group of mice ($n = 5$). Bars: 50 μm . (D) The ear thickness was measured weekly. Dashed line indicates the day BTZ treatment was started. Each value represents the mean $\pm$ SD with 5 mice in each group. ** $P < 0.05$ and *** $P < 0.001$ vs DNCB group.

4. Discussion

Although there has been a significant amount of research on AD, no effective drug has been developed. Anti-inflammatory agents, such as topical corticosteroids and topical calcineurin inhibitors, have been used for treating AD, an inflammatory and allergic disease. However, these drugs may cause various adverse effects, including skin atrophy, rebound dermatitis, telangiectasia, striae, dyspigmentation, and skin burning. Particularly, prolonged topical corticosteroid use in children causes serious adverse effects [21,22]. Therefore, we need to identify novel approaches for the development of therapeutic agents for AD.

Recent studies demonstrated that the expression of claudin 1, a major component of tight junctions in the skin barrier, is decreased in AD lesions [6–8]. Thus, we focused on identifying drugs that restore the expression of claudin 1. Since claudin 1 is degraded through the ubiquitin-proteasome pathway [19], we investigated whether agents inhibiting the activity of proteasomes may increase the expression of claudin 1. Proteasome inhibitors, especially BTZ,

increased the expression of claudin 1 in human keratinocytes and skin of DNCB-induced AD mice, thereby alleviating atopic symptoms. Increased claudin 1 enhanced the formation of tight junctions, preventing water loss from the skin and the penetration of allergens, such as bacteria, viruses, pollens, dust-mites, and microbes. Thus, restoring the expression of claudin 1 that is reduced in AD, is indispensable to cure this skin disease.

We examined whether proteasome inhibitors increase the expression of claudin 1 in human keratinocytes, and observed that treatment with BTZ increased claudin 1 expression to an extent greater than that seen with other proteasome inhibitors, including MG132 and VR23. This might have been due to their differences in the permeability, stability, or proteolytic activities in the cells [23–25]. However, the activity of proteasome inhibitors on claudin 1 expression *in vivo* needs to be investigated. In addition, proteasome inhibitors may be associated with the reduction in the IgE-mediated allergic reaction that targets antibody-producing plasma cells, which is one of the causes of AD. However, the effects of proteasome inhibitors on the allergic immune responses in

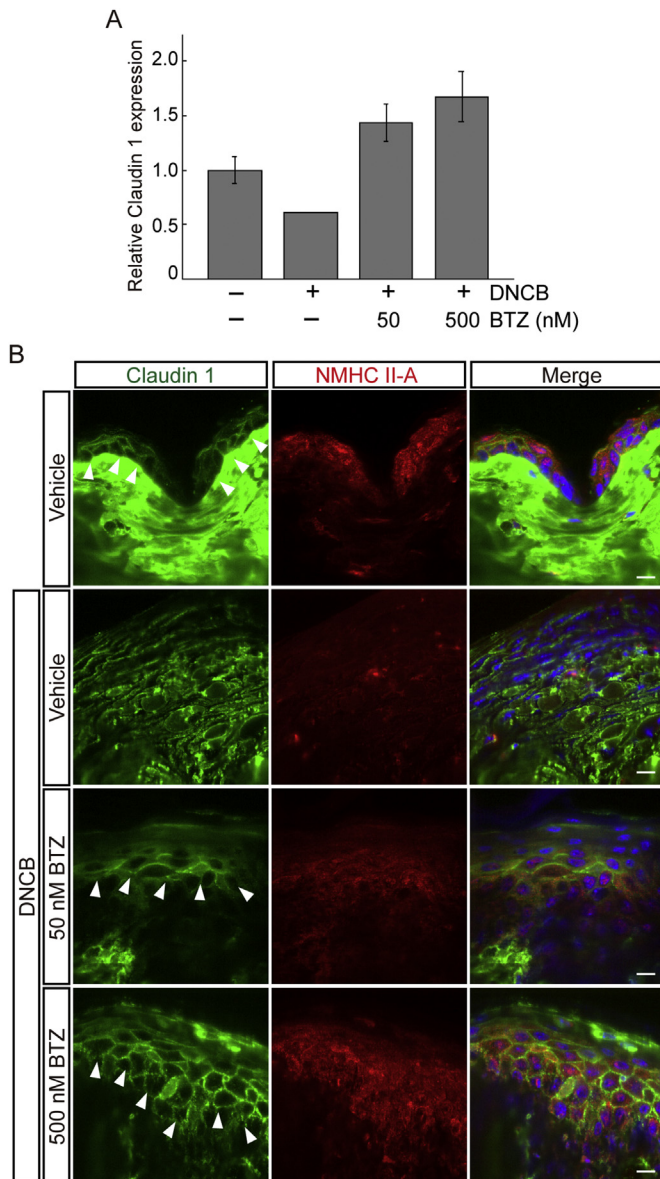


Fig. 4. Reduced claudin 1 expression in DNCB-induced AD was restored by BTZ treatment (A) Total cell lysates were prepared from the mouse skin tissues after the indicated treatments. The lysates were then subjected to immunoblot analysis of claudin 1 protein. The data shown are representative of 5 independent experiments from 5 different mice. (B) Immunofluorescence analysis of claudin 1 (green), NMHC II-A (red), and DAPI for nuclei (blue). Arrowheads indicate claudin 1-containing tight junctions. Bars: 10 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

skin diseases like AD, have not been explored. Consistent with the effects of BTZ on the immune function and skin barrier regulation in an ovalbumin driven dermatitis mouse model, serum IgE antibody titers decreased in the blood and the CD4⁺ and CD8⁺ cells, related to the Th1 and Th2 responses, decreased in the skin lesions. However, the expression of skin barrier genes, including transglutaminase-1 and filaggrin, decreased unexpectedly [26]. This decrease in the expression of skin barrier genes by BTZ treatment is inconsistent with our results that revealed an increase in claudin 1 expression. We assume that the expression of claudin 1 increases through direct inhibition of proteasomes by topical application of BTZ, whereas expression of transglutaminase-1 and filaggrin decreases through other gene regulation pathways,

regulated only by intravenous administration of BTZ. Therefore, we speculate that topical application of BTZ could be clinically more effective and less toxic.

Therapeutic agents targeting tight junctions for AD therapy have been underexplored. In particular, no attempt has been made to ameliorate the atopic symptoms by up-regulating the reduced claudin 1 protein levels in the epidermal barrier of the skin. Therefore, BTZ that increases the expression of claudin 1 may be effective in the treatment of skin diseases, such as AD.

Conflict of interest

The authors have no financial conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.bbrc.2017.08.120>.

Transparency document

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SUPPLEMENTARY MATERIALS AND METHODS

RNA preparation and quantitative reverse transcription-PCR (qRT-PCR)

Total RNA from the skin lesions of mice were isolated using the eCube Tissue RNA Mini Kit (PhileKorea, Seoul, Korea) according to manufacturer's instructions. Reverse transcription-PCR was conducted with the M-MLV reverse transcriptase (Promega, WI, USA) using random hexamers. The PCR reaction was performed using the GoldHot Start Taq PCR master mix (Bioneer, Daejeon, Korea) on the Eppendorf MasterCycler EP S Thermal Cycler (Eppendorf, Hamburg, Germany) equipment. The following PCR primers were used: claudin 1, 5'-GCA GAT CCA GTG CAA AGT CT-3' and 5'-CAT ACA CTT CAT GCC AAC GG-3'; claudin 7, 5'-ATG TAC AAG GGG CTG TGG AT-3' and 5'-CAC CAG GGA GAC CAC CAT TA-3'; ZO-1, 5'-AGA GCA CAG CAA TGG AGG AA-3' and 5'-GAC GTT TCC CCA CTC TGA AA-3'; Occludin, 5'-TTT GTG GGA CAA GGA ACA CA-3' and 5'-ATG CCA TGG GAC TGT CAA CT-3'; HNRNPL, 5'-ACA AAC CCC AAT CTC AGT GG-3' and 5'-CCC TCA TCA TGG TAA TGG CT-3'. The PCR products were separated by 1.5% agarose gel electrophoresis. Intensity of each band was analyzed using the NIH Image J program.

Reagents

MG132 (Z-Leu-Leu-Leu-al) and VR23 (7-Chloro-4-(4-(2,4-dinitrophenylsulfonyl)piperazin-1-yl)quinolone) was purchased from Sigma Aldrich(MO, USA). These compounds were dissolved in DMSO.

Supplementary Figure 1

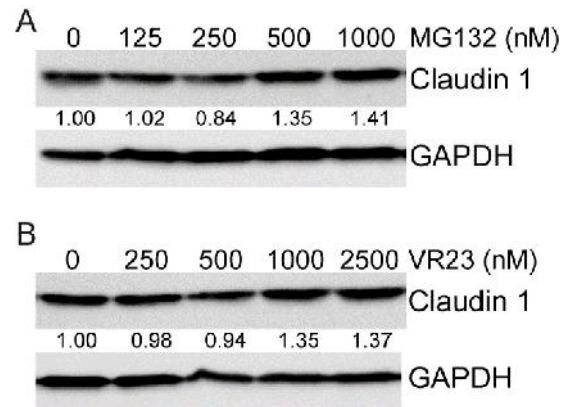


Fig. S1. Proteasome inhibitors increased claudin 1 expression in keratinocytes.

Human keratinocyte HaCaT cells were treated with different concentrations of (A) MG132 and (B) VR23 for 12 h. The total cell extracts were then prepared for immunoblot analysis of claudin 1. GAPDH served as a loading control. Relative intensities of the blots are presented for claudin 1.

Supplementary Figure 2

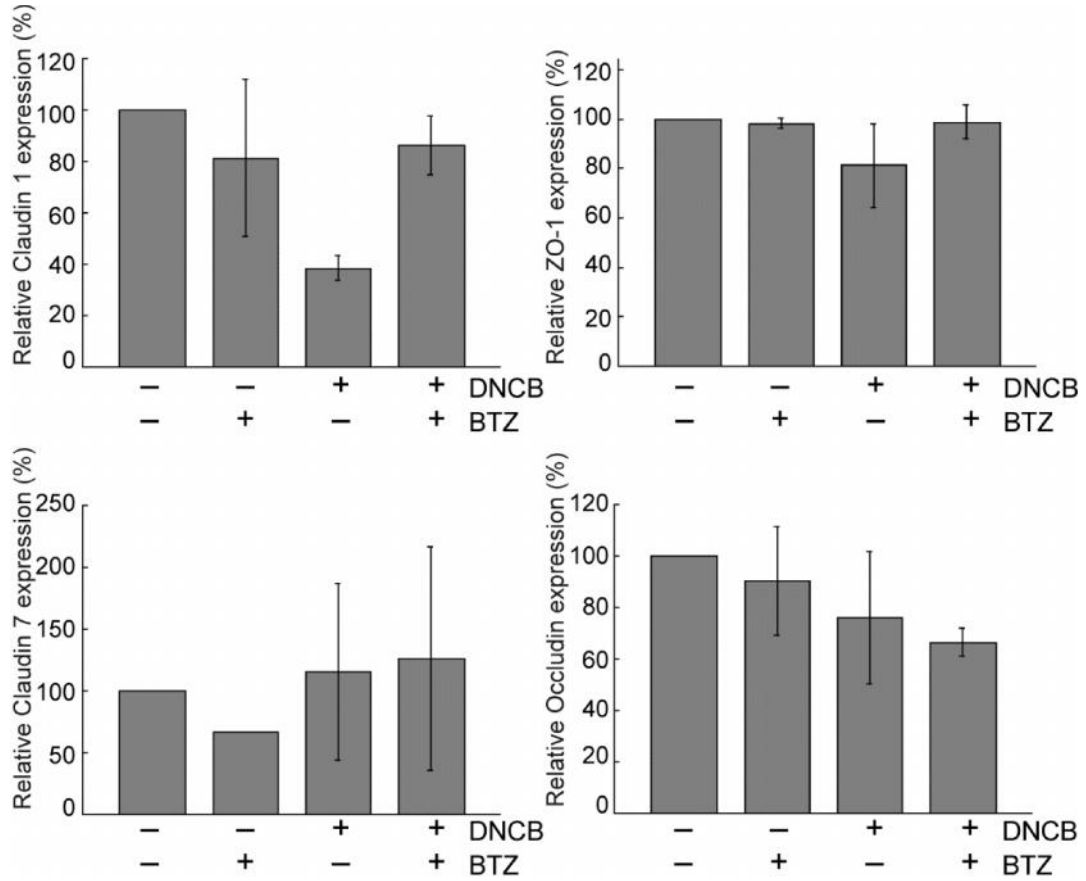


Fig. S2. BTZ regulates tight junction-related genes at the transcriptional level in DNCB-induced AD. Total RNA from the skin lesions of mice were isolated after the indicated treatments and analyzed by PCR for expression of the tight junction-related genes. The intensities of each mRNA expression normalized by HNRNPL are presented.