Effect of lonidamine derivatives on the inhibition of transformed cell area expansion

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ABSTRACT

Expansion of transformed cell area is regulated by the surrounding nontransformed cells. Lonidamine (LND) was recently found to regulate transformed cell area expansion through suppressing the cell motility of nontransformed cells; however, the structure–activity relationship between LND and this inhibitory activity has yet to be elucidated. We synthesized several LND derivatives and evaluated their inhibitory activity against the expansion of transformed cell area and found that the halogenation pattern on the benzene ring moiety, the carboxylic acid moiety, and the overall hydrophobicity of the molecule were correlated with inhibition activity. We also found that the localization of the tight junction protein, zonula occludens-1 (ZO-1), in nontransformed cells was significantly altered after treatment with the LND derivatives that displayed inhibitory activity. Further studies with LND derivatives and monitoring the localization of ZO-1 may help to develop more active compounds for suppressing transformed cell area expansion and lead to new anticancer treatments.

1. Introduction

The fate of cancer cells is controlled by the surrounding "normal" cells. In fibroblasts and keratinocytes, the proliferation of transformed cells is inhibited by neighboring nontransformed cells [1,2]. In epithelial tissues, mutation-acquired cells are physically eliminated by the surrounding nontransformed cells, a phenomenon known as epithelial defense against cancer [3]. Despite the existence of such cancer-suppressive mechanisms, these defense mechanisms can be subverted by environmental factors, such as inflammation. When the surrounding nontransformed cells are prevented from functioning properly, mutated cancer cells can remain in the nontransformed cell layer [4]. Indeed, recent developments in sequencing technology have revealed that tissues can be remodeled by the expansion of multiple cell clones containing mutations in different oncogenes (mutant clones) [5, 6]. Functionally and histologically "normal" mutant clones that exist

near the cancer cells will regulate cancer cell proliferation. The frequency of some acquired mutations can be higher in normal tissues than in cancerous tissues, and such positively selected mutant clones can promote tumorigenesis [7] or conversely, suppress tumorigenesis in a noncell-autonomous manner [8]. These findings suggest that the cancer prevention mechanisms employed by "normal" cells surrounding cancerous cells may play an important role in tumorigenesis starting from as early as the acquisition of a single genetic mutation.

To study the phenomenon in which the proliferation of transformed cells is regulated by surrounding nontransformed cells, we established a mixed culture focus formation assay by mixing nontransformed NMuMG cells with NMuMG cells expressing an active mutant KRAS (transformed cells). Chemical screening and further analysis using this assay revealed that 1 μM or less of lonidamine (**1**, LND, 1-(2,4-dichlorobenzyl)-1H-indazole-3-carboxylic acid) inhibited expansion of the transformed area (focus) through suppressing the cell motility of nontransformed cells

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[9].

LND, initially identified as an antispermatogenic agent [10], is a derivative of 1H-indazole-3-carboxylic acid. Although LND exhibits limited anticancer activity when used as a monotherapy, LND can be used in combination with conventional therapeutic strategies (chemotherapy or radiation therapy) to enhance the therapeutic efficacy [11]. Unlike other anticancer drugs, LND does not inhibit the synthesis of nucleic acids or proteins in cancerous cells. Interestingly, LND has been reported to impair various cellular energy metabolism pathways, cause mitochondrial membrane permeability transitions, and induce apoptosis [12,13]. Considerably more than 1 μM of LND was needed for effective inhibition of the identified targets in the reported studies; therefore, a yet undiscovered LND target protein is likely responsible for the mechanism of focus expansion inhibition.

Furthermore, how the LND structure itself is important for focus expansion inhibition remains unclear. Therefore, we synthesized 19 LND derivatives and evaluated their inhibitory activity for focus expansion to perform a structure–activity relationship analysis. Studies performed using the LND derivatives revealed that focus area expansion inhibition was correlated to both enhanced actin stress fiber formation and changes in the localization of the tight junction protein zonula occludens-1 (ZO-1).

2. Materials and methods

2.1. Cell culture

NMuMG cells were cultured according to a previously reported procedure without modification [9].

2.2. Chemical reagents

Indazole (HC-2811), indazole-3-carboxylic acid (OR-0885), and 1-methlyindazole-3-carboxylic acid (OR-4312) were purchased from Combi-blocks (San Diego, CA, USA). Adjudin (22,939) was purchased from Cayman Chemical (Ann Arbor, MI, USA). 1H-Indazole-3-carboxylic acid, 1-(phenylmethyl)- (AG00I836) was purchased from Angene Chemical (China). All reagents used in the chemical synthesis of LND derivatives were purchased from Sigma Aldrich (St. Louis, MO, USA). All compounds were dissolved in dimethyl sulfoxide (DMSO, 049–07213, Fujifilm Wako Pure Chemicals) and stored at −20 °C until use.

2.3. Mixed culture focus formation assay

For the mixed culture focus formation assay, 2.0×10^5 NMuMG cells and 200 NMuMG cells that express KRASG12D through the Tet-On system were seeded in 12-well culture plates. Two days after seeding, after the cells had formed a confluent cell layer, the growth medium was replaced with a fresh medium containing 50 ng/mL doxycycline and the test compound at the indicated concentration or just the vehicle (DMSO). Thereafter, the culture medium was replaced every other day. After 7 days of culture, cells were fixed in methanol and stained with 0.01% crystal violet. Quantification of the area in focus was performed using Open CV [14].

2.4. Immunofluorescent staining

Immunofluorescent staining was performed according to the procedure described in the reference without modification [9]. Briefly, Anti-ZO-1 (21773-1-AP, Proteintech, Rosemont, IL, USA) diluted at 1:2000 for primary antibody and goat anti-rabbit IgG [H + L] cross-adsorbed secondary antibody and Alexa Fluor™ 568 (A-11011, Invitrogen, Waltham, MA, USA) diluted at 1:1000 for secondary antibody were used. For phalloidin staining, Alexa Fluor™ 568 phalloidin (A12380, Invitrogen) diluted at 1:1000 was used. For nuclear staining, DAPI (DAPI solution, EZ150, Dojindo) was used.

2.5. Statistical analysis

All statistical analyses were performed using the GraphPad Prism 9 program (GraphPad Software, San Diego, CA, USA). Statistical results are presented as the mean ± standard deviation (SD). Welch's *t*-test was employed for comparisons between two groups.

2.6. Synthetic methods for lonidamine derivatives

2.6.1. 1-Benzyl 1H-indazole-3-carboxylic acids 1–10

2.6.1.1. General procedure. 1H-Indazole-3-carboxylic acid (1 eq.) was dissolved in THF and cooled to 0 °C under an Ar atmosphere. Thereafter 60% NaH in oil (2.2 eq.) was added and the reaction mixture was stirred for 1 h at 0 °C. To the reaction mixture, various benzyl chloride derivatives (1.2 eq. to 1H-Indazole-3-carboxylic acid) was added dropwise at 0 °C. The resulting reaction mixture was allowed to warm to ambient temperature with stirring for 12 h and then acidified with diluted HCl. The resulting solution was poured into brine and extracted with ethyl acetate 3 times. The combined organic layer was dried over MgSO₄, filtered, and concentrated *in vacuo*. Silica gel column chromatography of the residue (CH₂Cl₂: MeOH = 10:1) afford the desired compound.

2.6.2. 1-(2,4-dichlorobenzyl)-1H-indazole-3-carbaldehyde (11)
To a solution of compound **12** (100 mg, 326 μmol) in CH₂Cl₂ (50 mL) was pyridinium dichromate (PDC, 245 mg, 652 μmol). The reaction mixture was stirred for 12 h at ambient temperature and then quenched with silica gel. The resulting slurry was concentrated *in vacuo* and loaded directly for silica gel column chromatography (*n*-hexane: ethyl acetate = 5: 1) to afford aldehyde **11** (45 mg) in 45% yield.

The syntheses of alcohol **12**, tetrazole **13**, carboxamides **14** and **15**, amine **16**, and nitrile **18** were conducted according to previously reported methods without modification [10].

2.6.3. (E)-3-(1-(2,4-dichlorobenzyl)-1H-indazol-3-yl)acrylic acid (17)
Trimethyl phosphonoacetate (36 mg, 0.2 mmol) was dissolved in anhydrous THF (2 mL) and cooled to −78 °C under an Ar atmosphere. To the cooled solution was added *n*-BuLi (1.6 M, 160 μL, 0.25 mmol) dropwise at −78 °C under an Ar atmosphere and the reaction mixture was stirred for 1 h at −78 °C. When the color of the solution changed to yellow, compound **11** (33 mg, 0.10 mmol) in THF (0.5 mL) was added to the solution and the reaction mixture was stirred at 0 °C for 3 h. The resulting mixture was quenched with saturated aqueous NH₄Cl and extracted with ethyl acetate three times. The combined organic layer was washed with brine, dried over MgSO₄, filtered, and concentrated *in vacuo*. The resulting residue was purified by silica gel column chromatography (*n*-hexane: ethyl acetate = 6: 1) to afford the desired methyl carboxylate (29 mg) in 88% yield. The methyl carboxylate was immediately dissolved in THF (2 mL) and treated with aqueous NaOH (1 M, 5 mL) for 30 min at ambient temperature with stirring. The reaction mixture was neutralized with aqueous HCl (1 M) concentrated *in vacuo*. The obtained residue was purified by silica gel column chromatography (*n*-hexane: ethyl acetate = 3:1) to afford the compound **17** (27 mg) in 88% yield over 2 steps.

2.6.4. 1-(2,4-dichlorobenzyl)-1,4,5,6,7,8-hexahydrocyclohepta [c] pyrazole-3-carboxylic acid (19)
1,4,5,6,7,8-hexahydrocyclohepta [c]pyrazole-3-carboxylic acid (100 mg, 0.66 mmol) was dissolved in DMF (1 mL) and cooled to 0 °C under an Ar atmosphere. 60% NaH in oil (58 mg, 1.4 mmol) was slowly added the resulting mixture was stirred at ambient temperature for 1 h. Thereafter, the solution was again cooled to 0 °C and 2,4-dichlorobenzyl chloride (154 mg, 0.788 mmol) was slowly added to the mixture. The reaction mixture was allowed to stir for 24 h at ambient temperature and then neutralized with aqueous HCl (1 M) at 0 °C. The resulting slurry
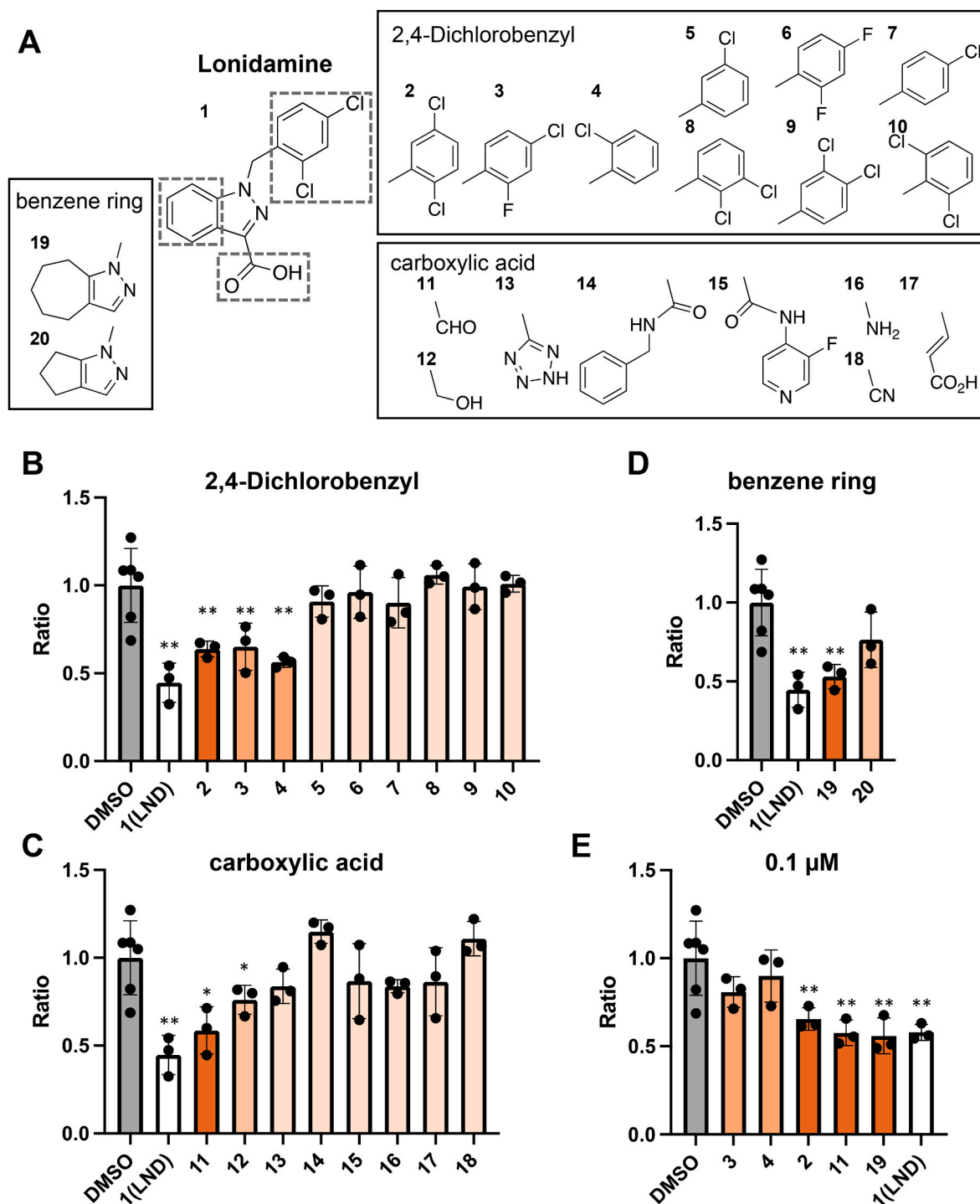
Fig. 1. Evaluation of focus area expansion inhibitory activity by LND derivatives
(A) Structures of the synthesized LND derivatives.
(B–D) Inhibitory activity for focus expansion of each compound was evaluated at 1 μM. E: For compounds that suppressed focus expansion at 1 μM, the inhibitory activity at 0.1 μM was evaluated (mean ± standard deviation (SD)). Mean and SD were obtained from measurements of six wells for the DMSO controls and three wells for the LND derivatives. The y-axis presents the ratio of the value at each concentration to that of the DMSO-treated control (0 μM) (mean ± SD; Mean and SD are obtained from measurements of six wells for DMSO and three wells for the others, *p < 0.05, **p < 0.01; a representative result of two experiments is presented).

was poured into ice-cold water and the resulting precipitate was collected by filtration. Silica gel column chromatography of the precipitate (CH$_2$Cl$_2$: MeOH = 10: 1) afforded compound **19** (22 mg) in 12% yield.

2.6.5. 1-(2,4-dichlorobenzyl)-1,4,5,6-tetrahydrocyclopenta [c]pyrazole-3-carboxylic acid (20)

Compound **20** was obtained from 1,4,5,6-tetrahydrocyclopenta [c] pyrazole-3-carboxylic acid (100 mg, 0.549 mmol) according to the same protocol described above for **19** (73 mg) in 73% yield.
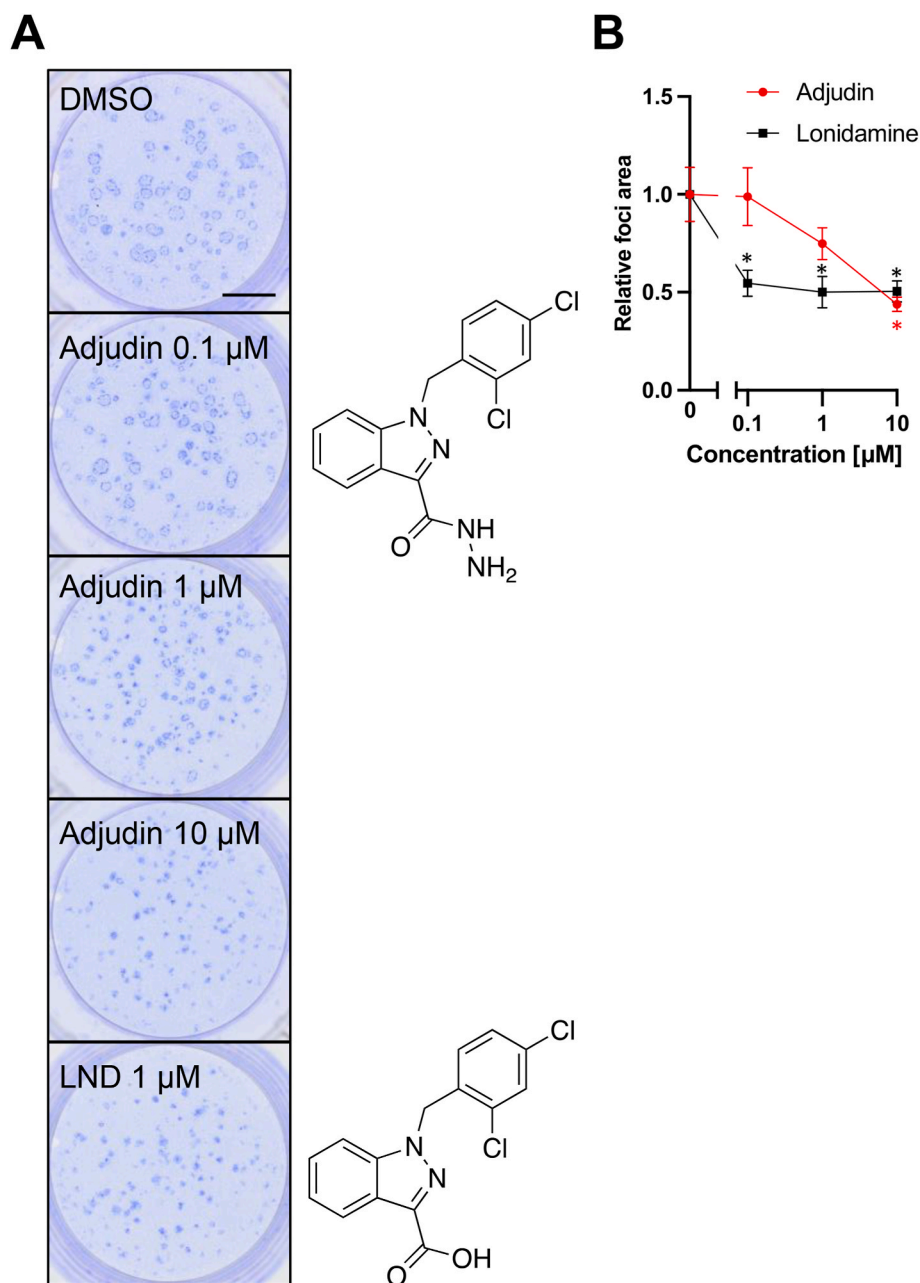
Fig. 2. The inhibitory activity of adjudin is weaker than LND
After 7 days of mixed culture focus formation assay with or without adjudin or LND, oncogenic foci were stained with 0.01% crystal violet. Quantification of the total area (B) of oncogenic foci based on the images of (A) (mean ± SD). Data obtained from measurements of three wells. The y-axis presents the ratio of the value at each concentration to that of the DMSO-treated control (0 μM) (mean ± SD; Mean and SD are obtained from measurements of three wells, $*p < 0.05$; a representative result of two experiments is presented). Scale bar = 5 mm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3. Results

### 3.1. Evaluation of the inhibitory activity of focus expansion of LND or its derivatives

LND is a derivative of 1H-indazole-3 carboxylic acid. Therefore, we first evaluated the inhibitory activity of focus expansion of indazole and other compounds that are substructures of LND using the mixed culture focus formation assay (Supplementary Figs. 1A, 2, 3). While LND suppressed focus expansion of transformed cells, as previously described [9], none of the LND substructures demonstrated any inhibitory activity at 1 μM (Supplementary Fig. 3).

Next, we synthesized 19 analogous compounds to LND (compounds **2–20**, Fig. 1A, physical property data for each compound can be found in the Supplementary Fig. 1B) and focus expansion inhibition of these compounds was evaluated using the mixed culture focus formation assay (Fig. 1B–D, Supplementary Fig. 4 and 5A–C). These 19 compounds were

classified as having activity comparable to, or less than, LND at 1 μM (Fig. 1B–D). Compounds with inhibitory activity at 1 μM (**2, 3, 4, 11** and **19**) were further evaluated at 0.1 μM; however, the inhibitory activity of these compounds were slightly inferior to LND (Fig. 1E, Supplementary Fig. 5D). Furthermore, the inhibition activity of adjudin, a derivative of LND with demonstrated antispermatogenic activity [12], was less than that of LND (Fig. 2).

3.2. Induction of actin stress fiber or change of ZO-1 localization correlates with the inhibitory activity of focus expansion

In our previous study, we reported that LND enhanced the formation of actin stress fibers in NMuMG cells under confluent conditions [9]. Therefore, on the basis of the aforementioned screening results, we selected representative compounds to further study such morphological changes in affected cells. Compounds that demonstrated inhibitory activity for focus expansion (**1** (LND), **2, 11**, and **19**) were selected along
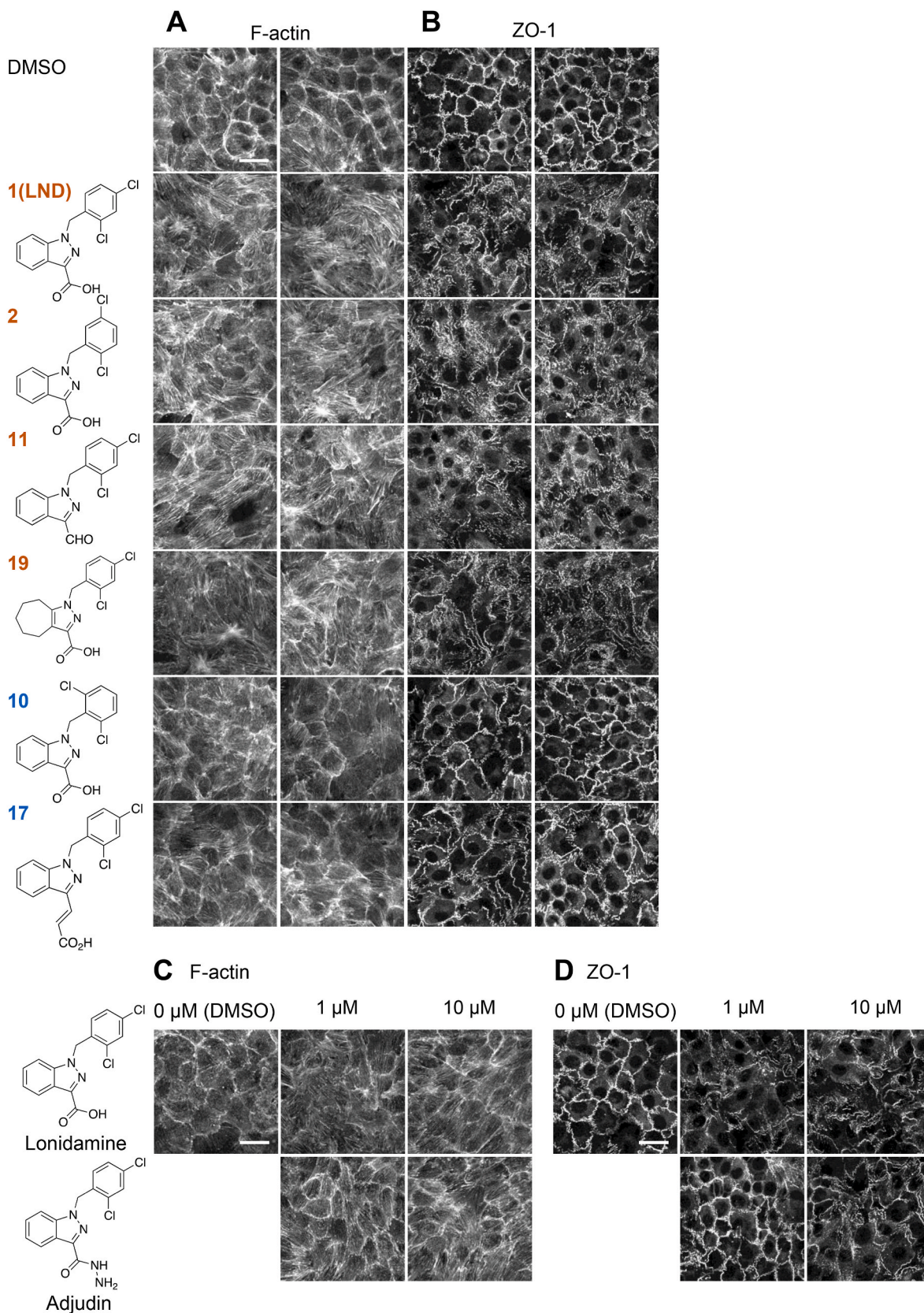
Fig. 3. Inhibition of focus expansion correlates with the ability to induce localization changes of ZO-1
Nontransformed NMuMG cells were treated with each compound (1 μM) or adjudin (1, 10 μM) for 48 h, stained with phalloidin (F-actin, (A), (C)), and immuno-stained for ZO-1 ((B), (D)). The results obtained from one or two images are shown (scale bar = 25 μm). A representative result obtained from two experiments is presented.

with compounds that did not exhibit inhibitory activity for focus expansion (**10** and **17**). More enhanced stress fiber formation was observed when cells were treated with compounds **1** (LND), **2**, **11**, **19**, and adjudin (focus expansion inhibitors) than when treated with compounds **10** and **17** (not focus expansion inhibitors) (Fig. 3A, C [F-actin]). These results suggested that focus expansion inhibition by LND and its derivatives was correlated with induction of actin stress fiber formation.

We next performed immunostaining studies on ZO-1, a protein that interacts with F-actin at tight junctions [15]. ZO-1 is normally localized at the cell–cell adhesion surface, but treatment with LND and other analogous compounds significantly altered the localization of ZO-1 on the NMuMG cell surface (Fig. 3B, D [ZO-1]). Conversely, little change in ZO-1 localization was observed for compounds without inhibitory activity (**10** and **17**). These results suggested that the change in ZO-1 localization is correlated with focus expansion inhibition.

4. Discussion

We evaluated the inhibitory activity of 19 analogous compounds to LND (**1**) through a structure–activity relationship analysis (Fig. 1). Effective inhibitors contained a halogen at the benzene ring C-2 position. The presence of halogens at C-4 or C-5, or absence of a halogen at C-3 to C-6 position maintained the activity (compounds **2**, **3**, and **4**). Compounds **8** and **10** had Cl at the C-2 position but contained a second Cl at C-3 or C-6. The second Cl may have weakened the inhibitory activity. Inhibitory activity was found to have reduced when F was the halogen (compound **6**). These results suggest that the benzene ring interacts with the target proteins and halogenation strongly affects this interaction. Changing the benzene ring to a cycloalkane did not negatively affect the activity (compounds **19** and **20**), although the activity was slightly reduced for compound **20**. Turning to the carboxylic acid moiety, inhibition activity is maintained when the carboxylic acid is converted to an aldehyde or alcohol (compounds **11** and **12**). With noted exceptions (**16**), compounds with a higher octanol/water partition coefficient logP value (calculated by ChemDraw 19 program) than **1** were less active (compounds **13**–**18**). It is possible that the C=O or OH group present in parts of the carboxylic acid is important for the inhibitory activity. These results suggest that the position of Cl atom in the benzene moiety, the carboxylic acid moiety itself, and the overall hydrophobicity of the molecule may be involved in the inhibitory activity of **1**.

Immunostaining studies revealed a correlation of focus expansion inhibition with the strong induction of actin stress fiber formation (Fig. 3A, C), further supporting a previous study that reported LND could suppress focus expansion via promotion of actin stress fiber formation [9]. In addition to actin stress fiber formation, we discovered that changes in ZO-1 location correlated well with focus expansion inhibition (Fig. 3B, D).

LND was first developed as an antispermatogenic agent. Corsi et al. synthesized various lH-indazole-3-carboxylic acid derivatives and evaluated the activity of each compound as an antispermatogenic agent [10]. They found that substitution of the Cl group at benzene ring C-2 position was slightly more active than Cl substation at the C-3 or C-4 position when only one Cl atom was on the ring. When two Cl atoms were present, substitution at C-2 and C-4 positions was necessary for maintaining activity. While we were not able to evaluate all of the compounds studied by Corsi et al., our current work corroborates well and suggests that focus expansion inhibition and antispermatogenic activities are correlated.

The mechanism of action for adjudin (1-(2,4-dichlorobenzyl)-1H-indazole-3-carbohydrazide, AF-2364), a derivative of LND, has been studied as an antispermatogenic drug in detail because it is less cytotoxic than LND [12]. Adjudin alters actin dynamics to affect apical ectoplasmic specialization (apical ES), a testis-specific structure created at the boundary between Sertoli cells and spermatocytes. Consequently, adjudin induces transient infertility by causing the release of spermatocytes in an immature state [16–18]. Adjudin has also been reported

to alter the localization of ZO-1 [19], which is consistent with our observations (Fig. 3D). Adjudin is less effective than LND at inducing the disruption of Sertoli cell–sperm cell adhesion and the strength of the inhibitory effect by LND and adjudin on focus area expansion shown in this study correlate well with a mechanism affecting apical ES (Fig. 2).

Collectively, these findings suggest that the effect of LND and adjudin on the cytoskeletal system may lead not only to antispermatogenic activity but also to anticancer activity. Further study of LND antitumor activity in the context of cancerous cell area expansion inhibition is necessary.

In conclusion, we synthesized 19 LND derivatives and evaluated their inhibitory activity for focus expansion. Monitoring changes in the ZO-1 protein was found to be a useful indicator for studying the inhibitory activity of compounds. The work presented in this study should help to advance the search for novel compounds with higher inhibitory activity for focus expansion.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2023.101480.

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