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Note

Localization and Accumulation Studies of Dacomitinib in Rat Intestines and Skin by Immunohistochemistry

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Dacomitinib, a second-generation tyrosine kinase inhibitor, was irreversible inhibitor forming covalent bonds with the kinase domains of EGFR and other ErbB family receptors. Dacomitinib has been approved for the treatment of locally advanced or metastatic non-small cell lung cancer. In this study, we aimed to develop an immunohistochemistry to detect dacomitinib-ErbB family receptor conjugates. Immunostaining was performed in rat intestine and skin tissues after oral administration of dacomitinib. Following a single oral dose of dacomitinib, strong staining was observed after 24 hr in the ileum and colon, with only slight staining in the duodenum and jejunum. In the skin, strong staining was observed in the epidermis, hair follicles, and sebaceous glands. Moreover, significant amounts of dacomitinib remained for up to 72 hr post-administration in the ileum, colon, and skin. This report is the first to elucidate the localization and accumulation of dacomitinib in the rat intestine and skin and should be valuable during efforts to clarify the mechanism dacomitinib-induced diarrhea or skin toxicities.

Key words: dacomitinib, immunohistochemistry, intestine, skin, localization

I. Introduction

The second-generation oral and irreversible pan-HER tyrosine kinase inhibitors (TKI) dacomitinib and afatinib have very similar chemical structures and selectively bind to the ATP binding pockets of EGFR [2, 8, 9]. These second-generation irreversible TKIs are considered clinically superior to first-generation reversible TKIs because they may block the activity of multiple receptors simultaneously and for a longer duration [4]. On the other hand, the second-generation TKI dacomitinib inhibits normal EGFRs in healthy tissues more strongly than first-generation TKIs, leading to skin rash, acne, and diarrhea [1, 5, 12]. To date, these side effects have not been investigated and the underlying mechanisms remain poorly understood.

Dacomitinib and afatinib form covalent bonds with ErbB family receptors *in vivo* [2, 8]. Therefore, we developed a specific antibody against afatinib and an immunohistochemistry (IHC) system that can detect EGFR-bound afatinib in tissues [7, 13]. Using this IHC system, we revealed the localization of administered afatinib in the intestines and skin for the first time [13]. We also found that afatinib roughly colocalized with EFGRs [13]. However, the localization of dacomitinib in the intestines and skin remains unclear, although it is predicted to be similar to that of afatinib. It is crucial to reveal the precise localization of dacomitinib in the intestines and skin in order to investigate the mechanism underlying dacomitinib-induced diarrhea and skin toxicity.

Afatinib and dacomitinib have very similar chemical structures. The anti-afatinib serum that we previously developed is likely to cross-react with dacomitinib based on its specificity profile [7]. Therefore, in this present study, we tried to develop an IHC system to detect dacomitinib using the anti-afatinib serum to reveal the localization of orally administered dacomitinib in the intestines and skin,

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thereby elucidating the mechanism dacomitinib-induced diarrhea and skin toxicity.

II. Materials and Methods

Chemicals and reagents

Dacomitinib was purchased from Tokyo Chemical Industry (Tokyo, Japan; PubChem ID, 1110813-31-4; Catalog number; D5450). Paraformaldehyde was acquired from Nacalai Tesque (Tokyo, Japan; PubChem ID, 712; catalog number, 26126-25). Histofine Simple Stain MAX-PO (M) was purchased from Nichirei Bioscience (Tokyo, Japan; Catalog number, 414171; RRID; AB_2811178). 3,3'-Diaminobenzidine tetrahydrochloride (DAB) was obtained from Junsei Chemical (Tokyo, Japan; Pubchem ID, 7071; catalog number, 60094-0411). The other reagents and solvents were of the highest grades commercially available.

Enzyme-linked immunosorbent assay (ELISA) for dacomitinib or afatinib

An ELISA for afatinib was performed according to our previous methods [7]. The underlying principle of ELISA is enzyme-labeled and unlabeled drugs competing for a purified immobilized antibody, followed by measuring marker enzyme activity of the resulting immunocomplex bound to the solid phase.

Animals

Specific pathogen-free adult male Wistar rats (180–200 g, 6–8 week old) were obtained from CLEA, Inc. Animals, Tokyo, Japan. The animals were maintained under controlled environmental conditions at 25°C ± 3°C and humidity 60–65% under a 10 hr light/14 hr dark cycle with free access to food and water. Twelve hours prior to experimental procedures, the animals were deprived of water but no food. The principles of laboratory animal care and specific national laws were observed.

Animal groups

Male rats (two rats per group) were administered a single oral dose of dacomitinib at 10 mg/kg. The experiment was conducted 24 and 72 hr later. No abnormalities were observed in these rats after drug administration.

Tissue sample preparation

The rats were orally administered dacomitinib. Thereafter, the rats were anesthetized with pentobarbital and perfused with 2.5% heparin followed by freshly-prepared 4% paraformaldehyde in phosphate buffer (pH 7.4). The intestines and skin were excised and post-fixed in 4% paraformaldehyde in phosphate buffer (pH 7.4) overnight. The tissue was dehydrated with ethanol or xylene and then embedded in paraffin. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Sojo University.

IHC staining procedure

IHC for dacomitinib was performed according to our previous methods [13].

Negative control experiments

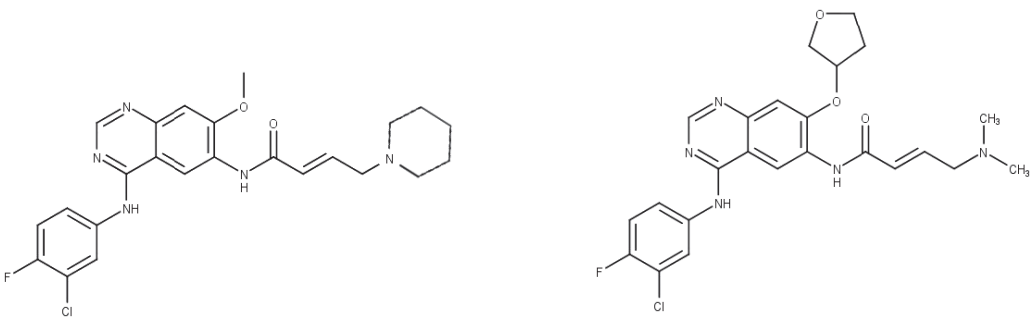
Two different negative control experiments, a conventional control experiment, and an absorption control experiment, were performed in the ileum, colon, and skin 24 hr after the single oral administration of dacomitinib. In the conventional controls, the sections were exposed to normal mouse serum (1:1000) instead of primary anti-afatinib serum. In the absorption controls, diluted primary anti-afatinib serum was preabsorbed with 10 μg/mL dacomitinib before the reactions with the sections.

III. Results and Discussion

We previously generated a specific antibody against afatinib that recognizes a structure other than its 4-(dimethyl-amino)-2-butene moiety [7]. The structure of afatinib is very similar to that of dacomitinib (Fig. 1). The dose-response curves of dacomitinib and afatinib with the anti-afatinib serum are shown in Figure 2. The anti-afatinib serum showed approximately 15% cross-reactivity with dacomitinib. This result suggests that the anti-afatinib serum could be used to analyze dacomitinib. Thus, anti-afatinib serum was used as the anti-dacomitinib serum in IHC for dacomitinib.

As shown in Figure 1, the structures of dacomitinib and afatinib comprise an α,β-unsaturated ketone moiety (4-[dimethylamino]-2-buteneamido moiety) that can act as the acceptor molecule for the Michael reaction. The α,β-unsaturated ketone moiety of these drugs forms covalent bonds with electron-rich sections of proteins (e.g., sulfhydryl or amino groups) [11]. Therefore, these drugs are localized in the tissue by forming covalent bonds with EGFR or other proteins. Both dacomitinib and afatinib are TKIs that act via covalent bonding to ErbB family receptors; therefore, we predict that the localization of dacomitinib and that of afatinib are similar in the intestines and skin. However, the localization of dacomitinib in the intestines and skin has not yet been revealed. Thus, IHC for dacomitinib was developed using afatinib serum, and the localization and accumulation of dacomitinib in the rat intestines and skin was clarified.

Immunostainings were performed with rat intestines (duodenum, jejunum, ileum, and colon) and skin at 24 or 72 hr after single oral administrations of dacomitinib. Following a single oral dose of dacomitinib, strong staining was observed after 24 hr in the ileum and colon, with only slight staining in the duodenum and jejunum (Fig. 3). In the ileum, almost the entire tissue was stained, while in the colon, the epithelial cells and Auerbach's plexus in the muscle layer were specifically stained. To verify the specificity of the immunostaining procedure, two different negative control experiments were performed, the results



Dacomitinib Afatinib

Fig. 1. Chemical structures of dacomitinib and afatinib.

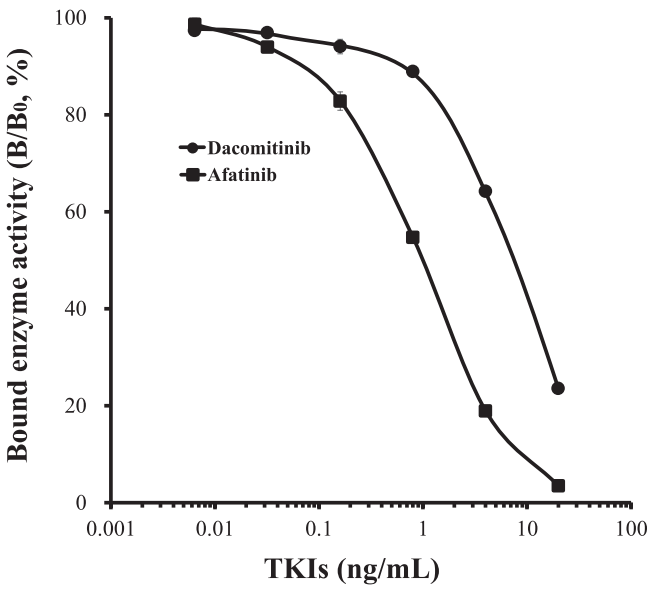


Fig. 2. Dose-response curves for TKIs with the anti-afatinib serum. The curves show the amount (%) of bound enzyme activity for various doses of TKIs (B) as a ratio of that bound using an enzyme-labeled drug alone (B_0). ●, dacomitinib; ■, afatinib. Each point represents the mean ± standard deviation (n = 3).

of which were negative (Fig. 5). These negative control experiments demonstrated that the immunostaining was specific for dacomitinib. These results also indicated that dacomitinib specifically localized in the ileum and colon of the intestines. The expression of ErbB-1, -2 and -4 tends to be higher in the ileum than in other areas of the intestines tract [10]. Qu *et al.* reported that epiregulin expression is confined to epithelial cells in the colon [6]. These findings suggest that the intensity of immunostaining in the intestines is proportional to ErbB family expression in the intestines. In addition, these staining results were nearly identical to those of afatinib in the intestines. Dacomitinib induces severe ileal damage and is accompanied by increased MCP1 expression and gastrointestinal permeability in rats, and that histological changes are most pronounced in the ileum [10]. These findings and our immunostaining results in the ileum strongly suggest that dacomitinib causes damage to the ileum. Moreover, significant amounts of dacomitinib in the ileum and colon remained for up to 72 hr post administration (Fig. 3). This result suggests that the toxicity of dacomitinib in the ileum and colon may last long after discontinuation of the drug.

To elucidate the mechanism of dacomitinib-induced skin toxicities, immunostaining was performed with rat skin sections collected 24 or 72 hr after a single oral administration of dacomitinib. The epidermis, sebaceous glands, and hair follicles were also clearly stained (Fig. 4). Moderate positive staining was observed in fibroblasts in the dermis. In addition, significant amounts of dacomitinib in the skin remained for up to 72 hr post administration (Fig. 4). Two different negative control experiments were performed, both of which gave negative results (Fig. 5). These staining results were nearly identical to those of afatinib in the skin [13]. EGFRs in rat skin have been detected on epithelial cells overlying the basement membranes of the epidermis, sebaceous gland, and regions of hair follicles [3]. The sites that stained strongly corresponded almost perfectly to these sites of EGFR expression. These results strongly suggest that dacomitinib-induced skin toxicities occur through inhibition of EGFRs in the skin by dacomitinib and may last long after discontinuation.

In summary, we successfully developed an IHC protocol to detect dacomitinib using an anti-afatinib serum. IHC for dacomitinib could be a useful tool to enable detection of the localization and accumulation of dacomitinib-ErbB family receptor conjugates. Observation of the localization and accumulation of dacomitinib-ErbB family receptor conjugates is expected to produce important data in studies focusing on the effects and toxicity of dacomitinib. This report is the first to elucidate the localization and accumulation of dacomitinib in the rat intestines and skin and should help to clarify the mechanism dacomitinib-induced diarrhea or skin toxicities.

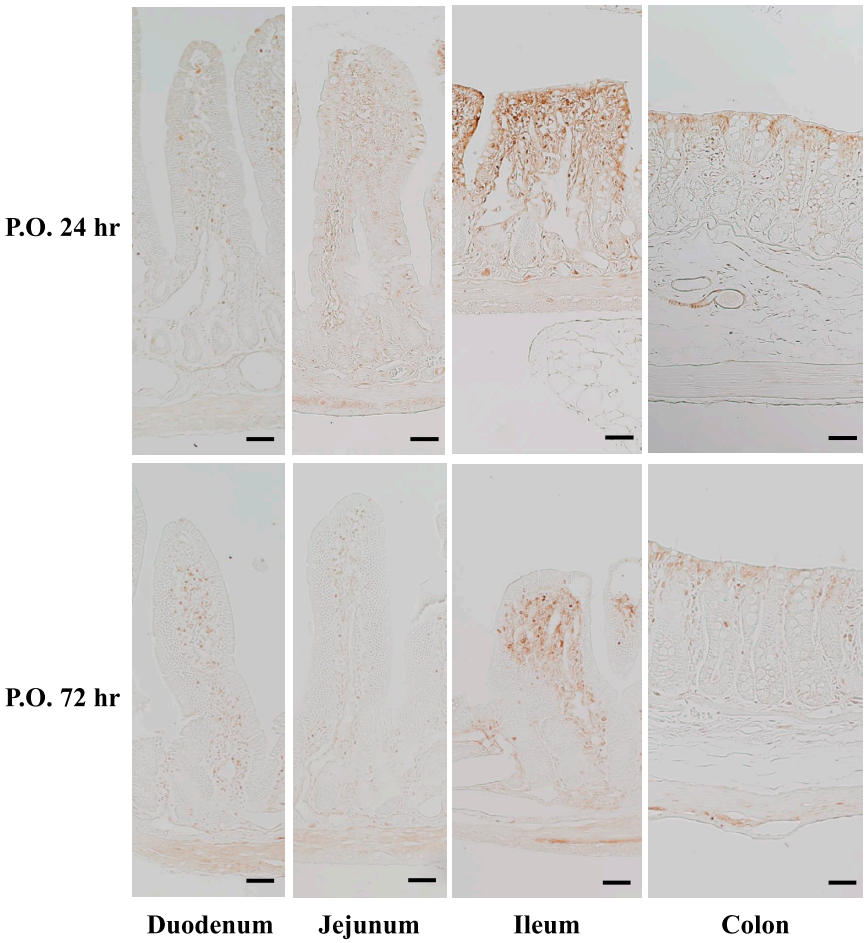


Fig. 3. Localization and accumulation of dacomitinib in the rat intestines. Immunostaining was performed with rat intestinal sections (duodenum, jejunum, ileum, and colon) collected at 24 or 72 hr after a single oral administration of dacomitinib (10 mg/kg). Bar = 50 μm.

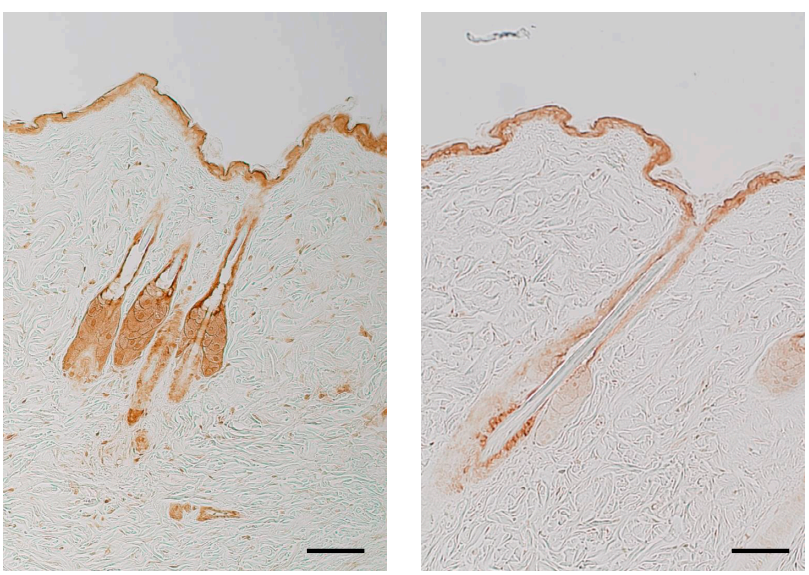


Fig. 4. Localization and accumulation of dacomitinib in rat skin. Immunostaining was performed with rat skin collected at 24 or 72 hr after a single oral dose of dacomitinib (10 mg/kg). Bar = 50 μm.

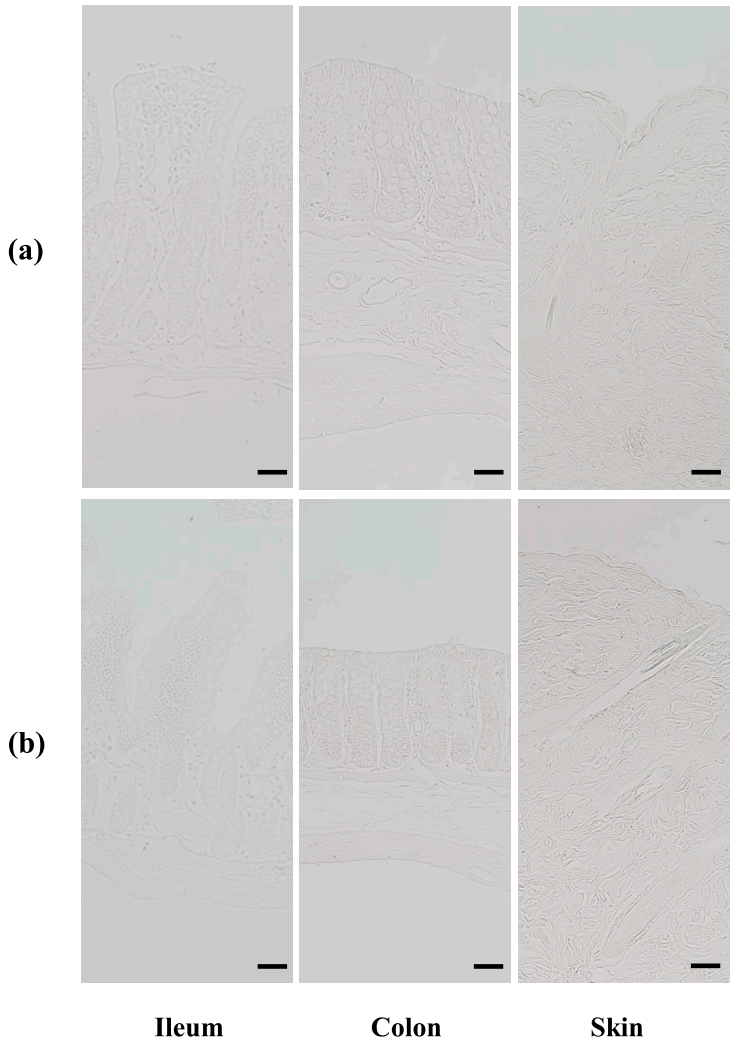


Fig. 5. Two types of negative control experiments in the ileum, colon and skin. Two different negative control experiments (conventional control experiment and absorption control experiment) were performed in the ileum, colon, and skin 24 hr after a single oral administration of dacomitinib. (a) In the conventional controls, the sections were exposed to normal mouse serum (1:1000) instead of primary anti-afatinib serum. (b) In the absorption controls, diluted primary anti-serum was preabsorbed with 10 μg/mL dacomitinib before the reactions with the sections. Bar = 50 μm.

IV. Abbreviation

BSA, bovine serum albumin; DAB, 3,3'-diaminobenzidine tetrahydrochloride; ELISA, enzyme-linked immunosorbent assay; EGFR, epidermal growth factor receptor; IHC, immunohistochemistry; NGS, normal goat serum; PBS, phosphate-buffered saline; TKI, tyrosine kinase inhibitor; TBST, Tris-buffered saline with 0.5% Triton X-100; TBS, Tris-buffered saline.

V. Conflicts of Interest

The authors declare no conflicts of interest.

VI. References

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