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β-eudesmol but not atractylodin exerts an inhibitory effect on CFTR-mediated chloride transport in human intestinal epithelial cells

Phuntila Tharabenjasin [a], Ronaldo P. Ferraris [b], Kiattawee Choowongkomon [c], Pawin Pongkorpsakol [d], Nichakorn Worakajit [e], Sutthipong Sawasvirojwong [f], Noel Pabalan [a], Kesara Na-Bangchang [a,g], Chatchai Muanprasat [e,*]

[a] Chulabhorn International College of Medicine, Thammasat University (Rangsit Campus), Klongnung, Klongluang, Pathum Thani 10120, Thailand
[b] Department of Pharmacology, Physiology and Neuroscience, New Jersey Medical School, Rutgers University, Newark, NJ 07946, USA
[c] Department of Biochemistry, Faculty of Science, Kasetsart University, Ngam Wong Wan Rd, Ladyaow, Chatuchak, Bangkok 10900, Thailand
[d] Faculty of Medicine and Public Health, HRH Princess Chulabhorn College of Medical Science, Chulabhorn Royal Academy, Bangkok 10210, Thailand
[e] Chakri Naruebodindra Medical Institute, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bang Pla, Bang Phli, Samut Prakan 10540, Thailand
[f] Department of Pathology, Faculty of Medicine, Chulalongkorn University, Phayathai Rd, Pathumwan, Bangkok 10330, Thailand
[g] Center of Excellence in Pharmacology and Molecular Biology of Malaria and Cholangiocarcinoma, Chulabhorn International College of Medicine, Rangsit Center, Thammasat University (Rangsit Campus), Klongnung, Klongluang, Pathum Thani 10120, Thailand

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ABSTRACT

Oriental herbal medicine with the two bioactive constituents, β-eudesmol (BE) and atractylodin (AT), has been used as a remedy for gastrointestinal disorders. There was no scientific evidence reporting their antidiarrheal effect and underpinning mechanisms. Therefore, we aimed to investigate the anti-secretory activity of these two compounds *in vitro*. The inhibitory effect of BE and AT on cAMP-induced Cl^- secretion was evaluated by Ussing chamber in human intestinal epithelial (T84) cells. Short-circuit current (I_{SC}) and apical Cl^- current (I_{Cl}-) were measured after adding indirect and direct cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel activator. MTT assay was used to determine cellular cytotoxicity. Protein-ligand interaction was investigated by *in silico* molecular docking analysis. BE, but not AT concentration-dependently (IC_{50} of ~1.05 μM) reduced cAMP-mediated, $CFTR_{inh}$-172 inhibitable Cl^- secretion as determined by transepithelial I_{SC} across a monolayer of T84 cells. Potency of CFTR-mediated I_{Cl}- inhibition by BE did not change with the use of different CFTR activators suggesting a direct blockage of the channel active site(s). Pretreatment with BE completely prevented cAMP-induced I_{Cl}-. Furthermore, BE at concentrations up to 200 μM (24 h) had no effect on T84 cell viability. *In silico* studies indicated that BE could best dock onto dephosphorylated structure of CFTR at ATP-binding pockets in nucleotide-binding domain (NBD) 2 region. These findings provide the first evidence for the anti-secretory effect of BE involving inhibition of CFTR function. BE represents a promising candidate for the therapeutic or prophylactic intervention of diarrhea resulted from intestinal hypersecretion of Cl^-

1. Introduction

Secretory diarrhea which includes cholera is a serious gastrointestinal disorder that disproportionately targets the young [1,2]. About 2 million people worldwide, mostly children under 5 years of age, die from consequences of diarrhea each year [2,3]. Morbidity and mortality are commonly attributed to hypovolemic shock caused by severe dehydration [4]. This disease is most commonly caused by *Vibrio cholera* and *Escherichia coli* [5]. The pathogenesis of this type of diarrheas involves enterotoxin-induced intestinal chloride (Cl^-) secretion, resulting in loss of intestinal fluids [5,6]. After natural infection, cholera toxin produced by *V. cholerae* binds to the intestinal ganglioside receptor before being endocytosed into the enterocyte and stimulating adenylyl cyclases, thereby elevating cyclic adenosine monophosphate (cAMP) levels [7] and inducing cystic fibrosis transmembrane conductance regulator (CFTR)-mediated Cl^- secretion. This in turn provides the

Abbreviations: AT, atractylodin; BE, β-eudesmol; cAMP, cyclic adenosine monophosphate; CFTR, cystic fibrosis transmembrane conductance regulator; I_{Cl}-*apical*, Cl^- current; I_{SC}, short-circuit current; MSD, membrane-spanning domains; NBD, nucleotide-binding domains; NKCC, Na^+-K^+-Cl^- cotransporters.

* Corresponding author.
E-mail address: chatchai.mua@mahidol.ac.th (C. Muanprasat).

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driving force to enhance the secretion of Na^+ and water through paracellular routes into the intestinal lumen [7,8]. Treatment with oral rehydration solutions (ORS) and antibiotic drugs is effective, but indirect [6,7]. Therefore, anti-secretory therapies that directly inhibit intestinal hypersecretion of Cl^- provide ancillary treatment options [7,9].

In principle, Cl^- is transported into intestinal epithelial cells from the serosa via basolateral Na^+-K^+-Cl^- cotransporters (NKCC) and transported across the apical membrane into the intestinal lumen via CFTR chloride channels (Fig. 1A) [10,11]. Intestinal Cl^- secretion also requires two other types of basolateral transport proteins that maintain driving force for Cl^- secretion including Na^+-K^+ ATPases and KCNQ1/KCNE3 K^+ channels [5,12,13]. It is widely accepted that elevated intracellular cAMP level stimulates hypersecretion of Cl^- through CFTR [11]. Therefore, inhibition of CFTR is proposed as a potential highly effective anti-secretory treatment.

CFTR consists of five domains including two membrane-spanning domains (MSDs); MSD1 and MSD2, two nucleotide-binding domains (NBDs); NBD1 and NBD2 and a regulatory (R) domain. Both MSDs form a pore for Cl^- transport [14,15]. The two NBDs of CFTR have been modeled as a heterodimer stabilized by ATP binding at two sites in the NBD interface. The phosphorylation of R domain by cAMP-dependent protein kinase A is prerequisite for CFTR channel gating. Furthermore, CFTR gating is controlled by binding and hydrolysis of ATP at the NBDs [16].

Currently, traditional herbal medicine has been applied to the treatment of various ailments [17]. This is partly because it is more affordable and accessible for local communities and aligns with patient's ideology concerning the adverse effects of chemical (synthetic) medicines [17]. Natural remedies in secretory diarrhea may provide alternative treatment [18]. Oriental plant medicine, the dried rhizome of *Atractylodes lancea* (Thunb.) DC. (*A. lancea*: Khod-Kha-Mao or Cang zhu) [19–22] and the bark of *Magnolia officinalis* (Houpu) [23,24], have been

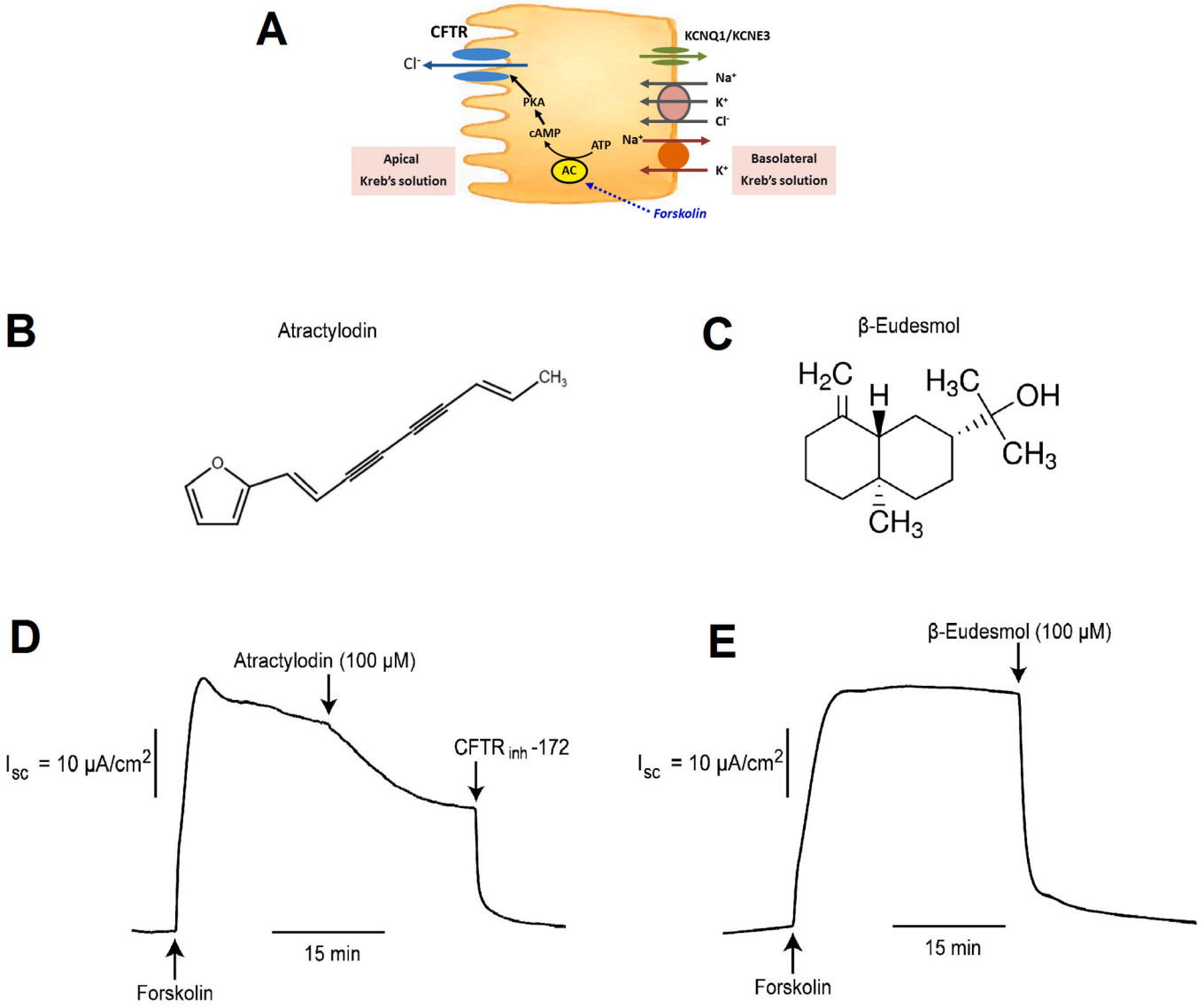

Fig. 1. Effect of BE and AT on cAMP-induced Cl^- secretion in intact T84 cells. (A) Model of transcellular Cl^- secretion in intestinal epithelial cells. AC, adenylate cyclase; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; CFTR, cystic fibrosis transmembrane conductance regulator; KCNQ1/KCNE3, cAMP-activated K^+ channel; Na^+-K^+-Cl^- cotransporters; Na^+-K^+ ATPases; PKA, protein kinase A. (B-C) The structures of AT and BE. T84 cells were mounted in Ussing Chambers. The cells were first incubated with Kreb's solution in both hemichambers. Amiloride (10 μM), an inhibitor of epithelial Na^+ channel (ENaC) was added into the apical solution to eliminate the function of ENaC before adding 20 μM of forskolin into both apical and basolateral sides for 15 min in order to induce cAMP-activated short-circuit current (I_{SC}). AT (D) or BE (E) at concentrations of 100 μM was added into both apical and basolateral solutions. At the end of experiments, 5 μM of $CFTR_{inh}$-172 (specific CFTR channel blocker) was administered into apical side. A representative I_{SC} tracing is shown (n = 5–6).

shown to alleviate various digestive symptoms including flatulence, dyspepsia, nausea and non-infectious diarrheas [25,26]. These plants share common bioactive constituents which is β-eudesmol (BE). Moreover, atractylodin (AT) is one of active ingredients from *A. lancea* (Fig. 1B and C). Although previous studies have shown the biological activities of the BE and AT in therapeutic applications such as anti-angiogenic [27], anticancer [28], antihypertensive [29], anti-inflammatory [22,30], antimicrobial and antipyretic activities [25], and activities on the nervous system and gastrointestinal tract [31–34], the anti-secretory diarrheal efficacy, which are primarily caused by overstimulation of cAMP-induced Cl^- secretion including its underpinning cellular mechanisms has never been elucidated. The objective of this study was therefore to investigate the inhibitory effect of BE and AT on cAMP-induced intestinal Cl^- secretion and its cellular mechanisms in human intestinal epithelial cells using T84 cells as a study model.

2. Methods

2.1. Chemicals and reagents

BE with purity of 98.8% was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and AT with purity of 99.6%, was supplied by Shanghai Run-BioTech CO., Ltd. (Shanghai, China). Fetal bovine serum (FBS), trypsin, penicillin and streptomysin were from Hyclone (Logan, UT, USA). Dulbecco's Modified Eagle's Medium (DMEM-12) was from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Other chemicals were from Calbiochem (San Diego, California, USA) and Sigma-Aldrich (St. Louis, MO, USA). Snapwell inserts were from Corning-Costar Corp (Corning, NY, USA).

2.2. Cell line and cell culture

The T84 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in the mixtures of DMEM and Ham's F-12 medium at a ratio of 1:1. The medium was supplemented with 5% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were maintained at 37 ºC in a humidified incubator under an atmosphere of 95% O_2 and 5% CO_2. For the electrophysiological analysis, 5×10^5 cells/well of T84 cells were grown on the Snapwell inserts in a humidified incubator and culture medium was changed freshly daily for 14 days in order to obtain an electrical resistance of $> 1000\ \Omega\ cm^2$ indicating complete formation of epithelial monolayer [35,36].

2.3. In vitro short-circuit current (I_{SC}) and apical Cl^- current (I_{Cl^-}) measurements

Prior to electrophysiological experiments, transepithelial electrical resistance was measured by EVOM2 volt-ohm meter (World Precision Instruments, Sarasota, Florida, USA). The Snapwell inserts were mounted in the Ussing chamber. I_{SC} and I_{Cl^-} were recorded using a DVC-1000 voltage-clamp (World Instruments, Sarasota, FL, U.S.A.).

For I_{SC} analysis, both apical and basolateral hemichambers were filled with the physiological bathing solution (Kreb's solution) containing (in mM) 120 NaCl, 25 $NaHCO_3$, 3.3 KH_2PO_4, 0.8 K_2HPO_4, 1.2 $MgCl_2$, 1.2 $CaCl_2$ and 10 D-glucose [37].

For apical I_{Cl^-} measurements, different Cl^- concentrations were used to create a basolateral-to-apical Cl^- gradient. Basolateral and apical hemichambers were filled with high and low Cl^- solutions, respectively. The specific compositions of high Cl^- solution (pH 7.4) were 130 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 1.0 $CaCl_2$, 0.5 $MgCl_2$, 10 mM Na-HEPES and 10 mM D-glucose [37,38]. In the apical low Cl^- solution, 65 mM NaCl was replaced with 65 mM Na-gluconate and the $CaCl_2$ concentration was increased to 2 mM in order to balance Ca^{2+} concentration from Ca^{2+} chelating effect of Na-gluconate [38]. Thirty minutes prior to I_{Cl^-} measurement, amphotericin B at a final concentration of 250 μg/mL was added into basolateral solution to induce basolateral membrane permeabilization.

The solution was continuously bubbled with humidified 5% CO_2 in 95% O_2 (for I_{sc}) or 100% O_2 (for I_{Cl^-}), maintained at 37 °C, pH of 7.4 with the osmolality of 289–292 mmol/kg H_2O as determined by a freezing point-based osmometer (model 3320; Advanced Instruments, Norwood, MA, USA).

2.4. Cell viability assays

The T84 cells were grown on 96-well plates at a seeding density of 2×10^4 cells/well overnight. Twenty-four hours after incubation with serum-free culture media containing DMSO (control) or BE at concentrations of 0.25–200 μM, cell viability was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays as previously described [39]. Colorimetry of blue formazan was detected by measuring absorbance at 540 nm using a microplate reader (Victor 2V, PerkinElmer, Waltham, MA, USA). Percentage of cell viability after exposure to BE was calculated by comparing the absorbance of BE-treated group to the control group.

2.5. In silico molecular docking analyses

2.5.1. Computational method

The Crystallographic structure of the phosphorylated, ATP bound CFTR protein (PDB code: 6O2P, resolution: 3.30 Å) and the dephosphorylated, ATP bound CFTR protein (PDB code: 5UAK, resolution: 3.87 Å) were selected in this study. Both structures were downloaded from the Protein data bank (https://www.rcsb.org).

2.5.2. Ligand binding prediction

The Prakweb server (https://prankweb.cz) was used to predict the binding site of CFTR with BE [40]. It was discovered using a machine learning-based method for the protein ligand binding site prediction approaches. For further docking, all predicted sites were investigated and identified as ligand-binding site cavities.

2.5.3. Molecular docking

BE was docked onto the two form of CFTRs (phosphorylated and dephosphorylated- CFTR) using the GOLD CCDC molecular docking program [41]. The hydrogen atoms were added to the protein structure of CFTRs using the set-up window and subsequently, the binding site within 10 Å was identified by their predicted ligand binding sites. Default Genetic Algorithm setting was used for all calculations and a set of 100 solutions were saved for each compound. The one hundred different conformations were generated for each ligand scored using goldscore and were ranked according to their binding energies. LigPlot+ [42] and Pymol (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC.) were used for the post-docking analyzes.

2.6. Statistical analysis

The results are presented as means ± standard error (SE). Multiple comparisons were performed by one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test. The level of significance for all statistical tests was set at p-value < 0.05 (two-tailed). All data were analyzed by GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Effect of BE and AT on cAMP-induced chloride secretion

To evaluate the inhibitory effect of AT and BE on cAMP-induced Cl^- secretion across human intestinal epithelial (T84) cells, I_{SC} was

measured and AT or BE (100 μM) was added into the bathing solutions. Forskolin (20 μM), an activator of adenylate cyclase was used to stimulate CFTR-mediated transepithelial Cl^- secretion as indicated by elevation of I_{SC} (Fig. 1D and E).

BE completely inhibited the cAMP-induced I_{SC} (Fig. 1E), whilst AT inhibited the current by ~40% (Fig. 1D). The remaining I_{SC} was abolished by $CFTR_{inh}$-172, a specific CFTR inhibitor (Fig. 1D), suggesting that the forskolin-stimulated current was primarily CFTR-mediated. This result indicates that BE is more potent than AT in inhibiting cAMP-induced Cl^- secretion in T84 cells. Therefore, the pharmacological properties and mechanisms of action of BE were further examined.

3.2. Concentration-dependence and polarity of BE inhibition of cAMP-induced I_{SC} and apical I_{Cl^-}

We next determined the concentration-dependent inhibition by adding BE into both apical and basolateral compartments. The inhibitory effect of BE on cAMP-induced I_{SC} had an IC_{50} of ~1.05 μM and near maximal inhibition at 20 μM (Fig. 2A and B).

Since cAMP-induced Cl^- secretion is mediated by several transport proteins specifically localized to apical and basolateral membranes [10], we hypothesized that BE inhibition might be side-dependent. I_{SC} was thus evaluated using sequential addition of BE into basolateral and apical hemichambers, respectively. In the intact cell, significant inhibition of cAMP-induced increases in I_{SC} was observed in the presence of BE at apical side compared to basolateral side at a concentration of BE at 2 μM, 20 μM and 50 μM, respectively (Fig. 3). This result indicates that BE inhibits transport protein expressed in apical membrane of T84 cells (i.e. CFTR).

Furthermore, with basolateral membrane permeabilization (Fig. 4A), apical I_{Cl^-} was concentration-dependently attenuated when BE was added into apical side (Fig. 4B). Interestingly, addition of BE into basolateral side produced much less effect compared to that into apical side (Fig. 4C). IC_{50} value of BE added into apical side was ~6.93 μM, whereas BE added into basolateral side was ~76.61 μM (Fig. 4D). These results suggest that BE inhibits cAMP-induced Cl^- secretion predominantly by acting on CFTR in the apical membrane.

3.3. Cellular mechanisms of inhibition of CFTR-mediated Cl^- transport by BE

The inhibitory effect of BE on cAMP-induced Cl^- secretion may not be due to a direct effect on CFTR but rather indirectly to a decrease in cAMP concentrations (Fig. 5A). To examine this hypothesis, T84 cells were pretreated with forskolin alone as a control group (Fig. 5B) or forskolin plus IBMX, an inhibitor of phosphodiesterase/ PDE that catalyzes the

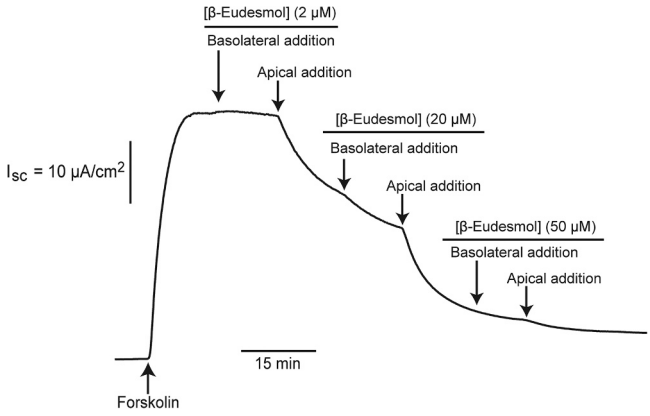

Fig. 3. Polarity of BE inhibition of cAMP-induced I_{SC} in intact T84 cells. BE was sequentially added into basolateral or apical hemichambers at concentrations of 2 μM, 20 μM and 50 μM after stimulation of CFTR by forskolin, respectively. A representative I_{SC} tracing of experiments is shown ($n = 4$).

hydrolysis of cAMP (Fig. 5D) for 15 min before exposure to BE at various concentrations. Results showed that apical I_{Cl^-} was gradually reduced in a concentration-dependent manner in the control with an IC_{50} value of ~1.7 μM (Fig. 5C). The similar concentration-inhibition relationship of BE was also observed in the cells pretreated with IBMX (IC_{50} of ~1.4 μM) (Fig. 5E). Therefore, the potency of BE inhibition of forskolin-induced Cl^- current in the presence of IBMX was not different from that of control. These results suggest that the inhibitory effect of BE on CFTR-mediated Cl^- secretion is not due to a decrease in intracellular cAMP levels by stimulation of PDE activity or does not involve a cAMP degradation process.

3.4. Direct effect of BE on CFTR-mediated chloride transport

Since Cl^- is transported across the apical membrane mainly via CFTR in chloride-secreting epithelia, we further postulated that BE may directly inhibit CFTR function. A natural isoflavone genistein has been reported to stimulate CFTR channel activity by a tyrosine kinase- and protein phosphatase-independent mechanisms [43], we hypothesized BE could suppress apical Cl^- current activated by genistein. Fig. 6 showed that pretreatment with genistein in apical solution enhanced transepithelial Cl^- secretion across T84 cells. BE markedly diminished the genistein-stimulated apical I_{Cl^-} in a concentration-dependent manner with IC_{50} of ~3.23 μM. This finding indicates that suppression of Cl^- secretion by BE may be due to the direct blockage of CFTR channel

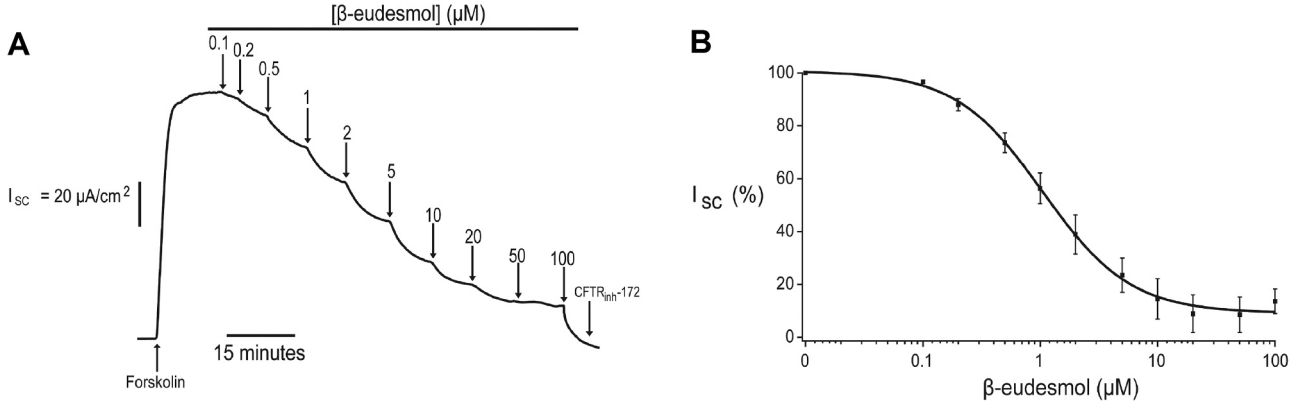

Fig. 2. Concentration-dependence of BE inhibition of cAMP-induced I_{SC} in intact T84 cells. After CFTR activation by forskolin, BE at the indicated concentrations (0.1–100 μM) was sequentially added into apical and basolateral solutions. $CFTR_{inh}$-172 was added to the apical side of the monolayers at the end of experiments. (A) Representative I_{SC} tracing. (B) Relative I_{SC} data are fitted to Hill's equation in order to obtain IC_{50} value expressed as % forskolin-stimulated I_{SC} ± S.E.M. (n = 5–6).

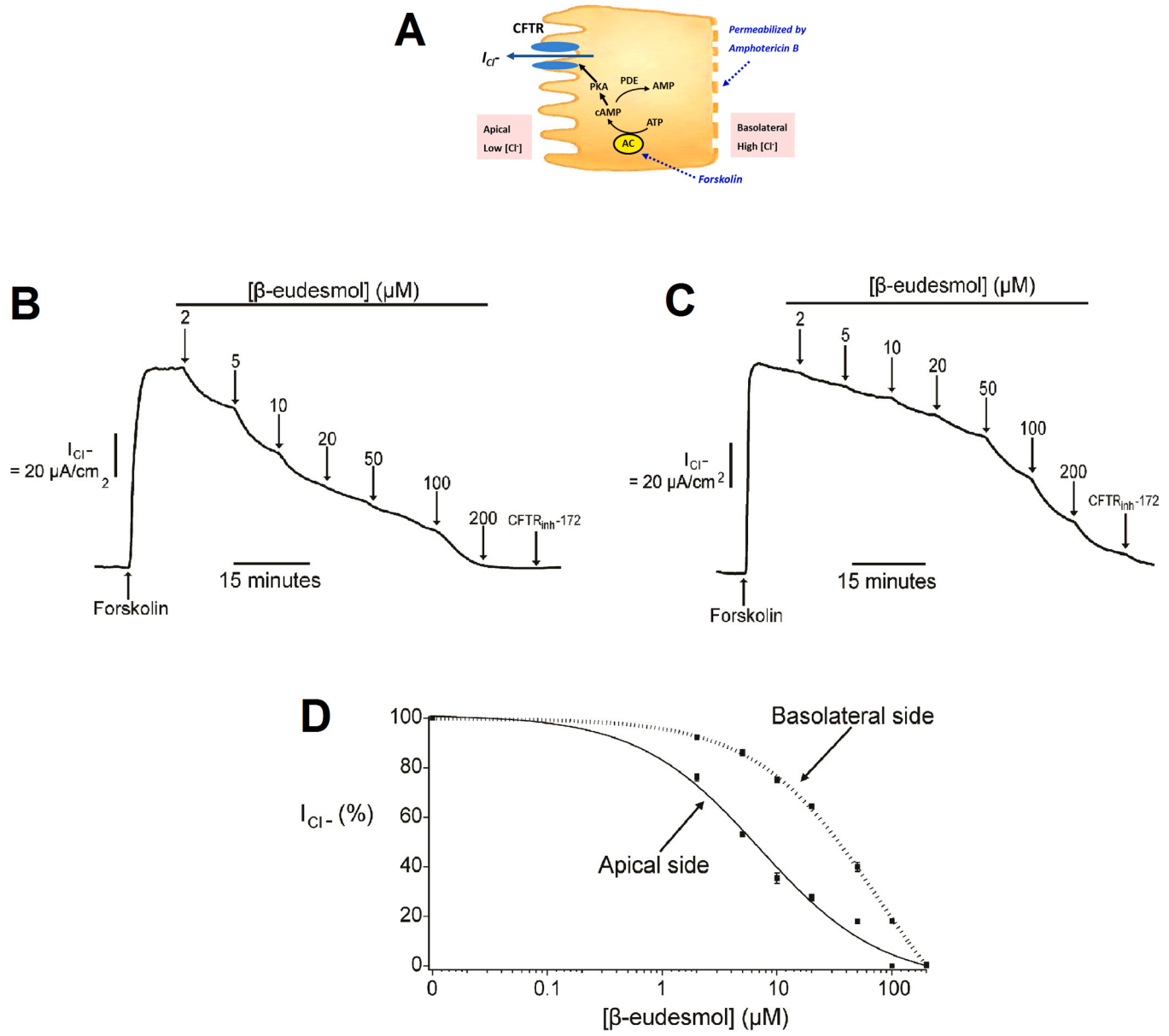

Fig. 4. Concentration-dependence of BE inhibition of cAMP-induced Cl^- secretion in permeabilized T84 cells. CFTR Cl^- channel activity was determined using apical Cl^- current (I_{Cl^-}) analysis. In order to generate Cl^- gradient, basolateral and apical hemichambers were filled with high and low concentration of Cl^- buffers, respectively. After permeabilization of basolateral membrane with amphotericin B (250 μg/mL) for 30 min followed by activation of CFTR by forskolin, BE was sequentially added into apical and basolateral hemichambers in a dose-dependent manner at the indicated concentrations (2–200 μM). At the end of experiments, $CFTR_{inh}$-172 was added to the apical side of the monolayers. (A) Model of experimental protocol. (B) and (C) showed representative tracings of CFTR-medicated apical Cl^- current when BE was added into the apical and basolateral side, respectively. (D) Relative I_{Cl^-} data fitted to Hill's equation are expressed as % forskolin-stimulated I_{Cl^-} ± S.E.M. (n = 5–6).

activity.

3.5. Prevention of CFTR-mediated Cl^- current by BE

To further investigate whether BE could prevent the CFTR-mediated apical I_{Cl^-}, basolaterally permeabilized T84 monolayers were pretreated with 50 μM BE for 15 min before adding forskolin in the apical compartment. As illustrated in Fig. 7, BE could completely prevented the CFTR-mediated Cl^- current. This result indicates that BE is able to prevent the CFTR-mediated Cl^- secretion.

3.6. Cytotoxicity evaluation of BE in T84 cells

MTT assays were performed to determine the effect of BE on cell viability. The T84 cells grown on 96-well plates were treated for 24 h with dimethyl sulfoxide (DMSO) as control or BE at the final concentrations of 0.25–200 μM. BE at concentrations up to 200 μM had no effect ($p > 0.05$) on T84 cell viability (Fig. 8).

3.7. Docking model of BE with the CFTR structure

Since electrophysiological experiments suggested that BE directly inhibited CFTR, molecular docking of protein-ligand (CFTR-BE) interaction would enable us to obtain more insight into the molecular mechanisms underpinning the inhibitory effect of BE on CFTR chloride channels.

In the structure of the phosphorylated complex bound ATP, we discovered four putative ligand binding sites, as shown in Fig. 9A.

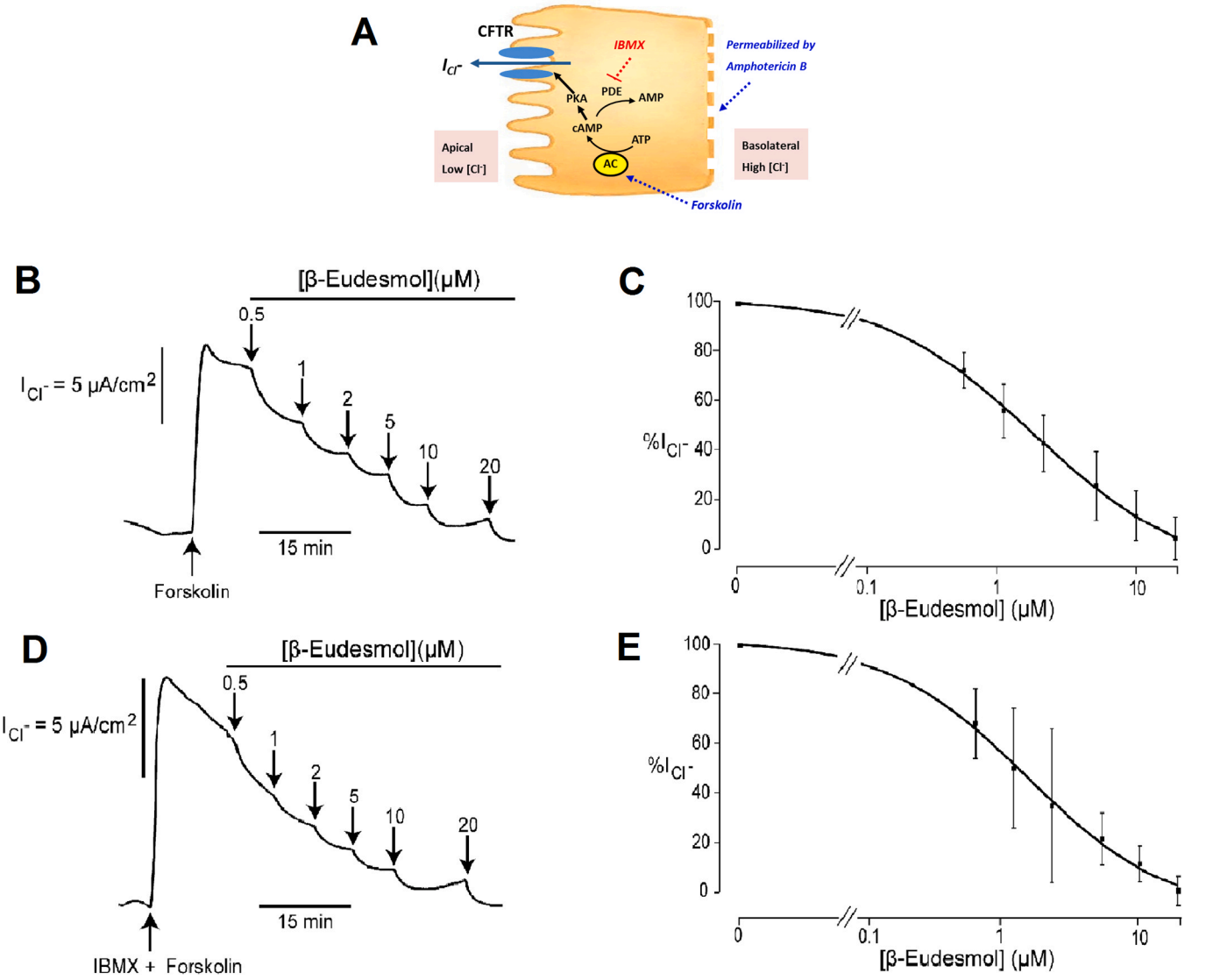

Fig. 5. Inhibition of cAMP-induced Cl^- secretion by BE is not due to alteration in cAMP levels in permeabilized T84 cell. (A) Schematic diagram illustrating the regulatory mechanisms of CFTR activity in T84 cells. AMP, adenosine monophosphate; IBMX, phosphodiesterase inhibitor (3-isobutyl-1-methylxanthine); PDE, phosphodiesterase. Amphotericin B was added into basolateral solutions prior to administration of forskolin alone (as control group) or forskolin plus IBMX (100 μM) (as treated group) for 30 min. BE at the indicated concentrations (0.5–20 μM) was added into both apical and basolateral solutions. Representative I_{Cl^-} tracing and summary of data fitted to Hill's equation are shown for control (B and C) and treated-group (D and E). Data are expressed as means of % agonist-stimulated apical I_{Cl^-} ± S.E.M. (n = 5–6).

Docking analyses showed PSite1–4 has a binding score in the range of 36.67–38.70 kcal/mol (Table 1). There were no significant differences ($p < 0.05$) in binding score on each site. In contrast, the predicted binding sites of the dephosphorylated CFTR structure were presented using Pymol in Fig. 9B. Of note, all sites were chosen as binding sites for docking with BE. The results showed that BE could successfully dock into 5 binding sites, with a binding score ranging from − 132.60 to 39.01 kcal/mol (Table 1). The strongest binding site was represented with the highest energy score. Then, the five binding sites were ranked from the most positive (DSite1) to the least positive docking scores (Dsite5). Next, the Dsite1, which showed the best docking score, was chosen for further investigation of two-dimensional interaction with CFTR by Ligplot+. As stated by molecular structure and amino acid sequence, Dsite1 is the site in an agreement with the NBD2 region of CFTR. The hydrophobic interactions between dephosphorylated CFTR and BE included V1147, L998, I1027, F1026, I1023, I1002, V1001, L1143, and A1146 (Fig. 10A and B). Upon this finding, it could be concluded that Dsites1 is a ligand specific binding site of BE.

4. Discussion

In a secretory diarrhea, an excessive amount of Cl^- secretion caused by enterotoxins leads to massive water and electrolyte loss from the body [7]. Although the benefit of some traditional medicine has been known to alleviate symptom [25,44], no attention has been paid to confirm and determine the underpinning cellular mechanisms of its effects. The present study provides the first scientific evidence showing BE as a phytochemical ingredient exerts a remarkable inhibitory effect on CFTR-mediated Cl^- transport in human enterocytes.

In the intestine, transepithelial Cl^- secretion requires well-synchronized function of several transport proteins. The uptake of chloride anion at the basolateral membrane of enterocytes depends on the Na^+-K^+-Cl^- cotransporter (NKCC) and was subsequently transported into intestinal lumen via cAMP-activated apical Cl^- channels, namely CFTR and IRC (inward rectifying Cl^- channel) [10,11,45]. The function of both channels depends on cAMP concentration. Previous studies have demonstrated CFTR provides the principal route for apical Cl^- secretion,

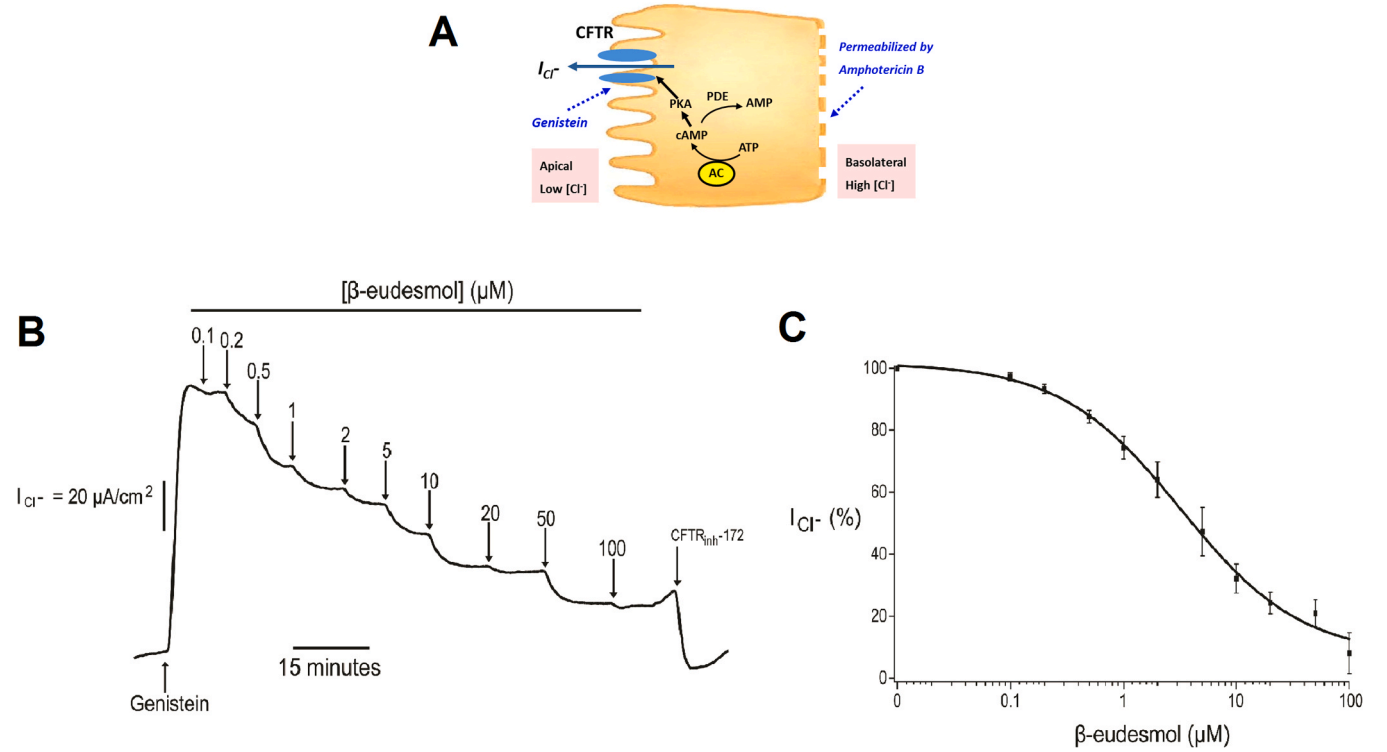

Fig. 6. Effect of BE on genistein-stimulated CFTR Cl^- channel activity in permeabilized T84 cells. (A) Apical side of monolayer was preincubated with genistein (20 μM) as a direct CFTR activator for 15 min, and BE at indicated concentrations (0.1–100 μM) were added into apical hemichamber, followed by $CFTR_{inh}$-172 at the end of the experiment. (B) The representative tracing of CFTR-mediated apical I_{Cl^-} activated by genistein. (C) Summary of the concentration-dependent data, which are expressed as means of % agonist stimulated apical I_{Cl^-} ± S.E.M. (n = 5–6).

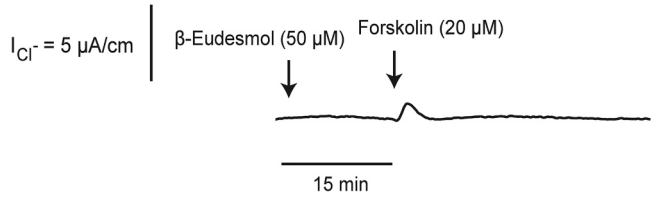

Fig. 7. Effect of preincubation of BE on CFTR-mediated Cl^- secretion in T84 cells. After pretreatment with BE at the concentration of 50 μM, apical Cl^- current stimulated by forskolin was monitored and recorded for 30 min. A representative apical I_{Cl^-} tracing is shown ($n = 3$).

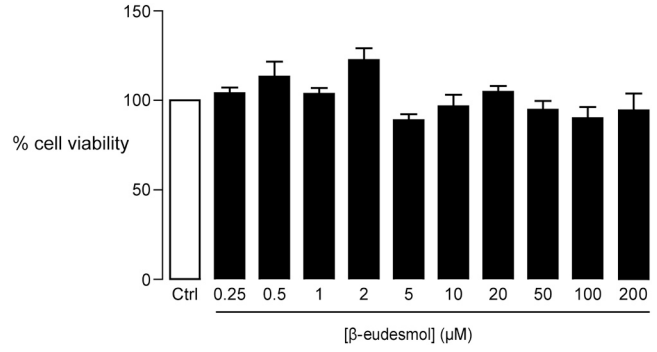

Fig. 8. Effects of BE at various concentrations on T84 cell viability as determined by MTT assays. Cells were exposed to BE at concentration of 0.25–200 μM for 24 h. Data are expressed as % cell viability relative to control (mean ± S.E.M) (n = 6).

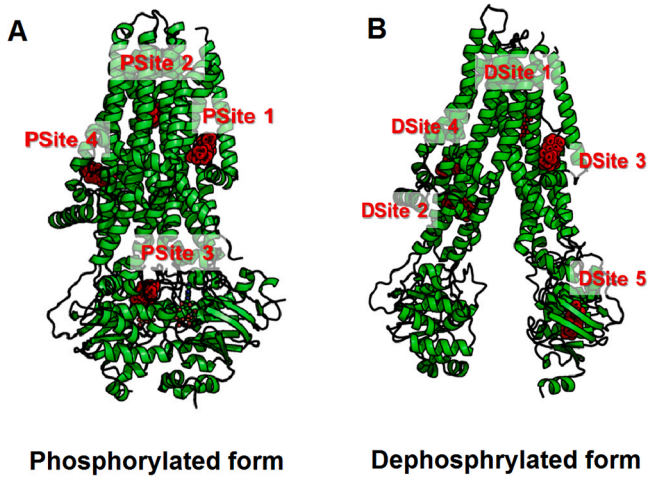

Fig. 9. The ligand-binding site prediction of CFTRs structure. The ligand-binding site prediction of CFTRs structure was identified as shown in red spot. (A) The phosphorylated structure with 4 putative binding sites (PSite1–4). (B) The dephosphorylated structure with five putative binding sites (DSite1–5). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

whereas IRC contributes a significant role only after inhibition of CFTR [45]. Although these two channels are stimulated by cAMP, their sensitivity to channel blockers differs. CFTR is inhibited by $CFTR_{inh}$-172 whereas IRC are sensitive to 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS) [45]. At the basolateral membrane, cAMP-activated basolateral K^+ channels and Na^+-K^+-ATPases are required for recycling K^+ into the cytosol and secretion Na^+ back to the serosa, respectively, which in turn, enables sustained electrochemical driving force for apical

Table 1
The binding score of phosphorylated and dephosphorylated CFTR complexes when bound to BE in each site.

Binding site	Fitness score (kilocalories/mole)	
	Phosphorylated CFTR (P)	Dephosphorylated CFTR (D)
Site 1	38.7	39.01[a]
Site 2	36.67	32.26
Site 3	38.18	-23.69
Site 4	38.16	-8.15
Site 5	–	-132.6

[a] The highest binding score

Cl^- efflux into the intestinal lumen [10]. Cyclic AMP-induced Cl^- secretion proceeds in the presence of these proteins.

Suppression of Cl^- secretion could be the result of functional inhibition of the transport proteins on apical or basolateral sides. We found that addition of BE into apical side produced higher inhibitory effect than that into basolateral side. This results support the conclusion that BE inhibits CFTR, in which BE can get access more readily when it is added into apical side. Because of the limitation of our facilities, additional studies, for example, patch clamp experiment for recording on unitary current flow through single channels could be performed to strengthen our results.

Inhibition of Cl^- secretion across CFTR in the intestine can be direct or indirect [37,39]. The regulation of intracellular cAMP level via the activity of phosphodiesterases (PDE) enzyme is one of possible indirect mechanism, which would affect cAMP degradation and thus CFTR-mediated Cl^- transport. PDE1–5 isoforms are expressed in T84 cells [46–48]. Increased of PDE activity leads to reduction of cAMP [49]. Therefore, the possible mechanism that BE inhibited cAMP-induced Cl^- secretion could be mediated by increased cAMP hydrolysis process upon PDE activity, contributing to suppression of cAMP-induced Cl^- secretion. Since BE inhibition is not altered in the presence of IBMX (a PDE inhibitor that prevents cAMP hydrolysis and thus maintains high intracellular cAMP level) and IC_{50} did not change significantly. Therefore, BE effect was not unquestionably due to stimulation of PDE activity.

The aforementioned findings clearly showed the effect of BE is predominant in apical side. Since BE can absolutely suppress the secretion of Cl^- in the presence of genestein, our results strongly suggest that BE inhibition through a direct effect on CFTR. To substantiate this finding, future investigation is needed to examine the effect of BE on CFTR abundance in plasma membrane. As well, the possible effect of BE on prevention of apical Cl^- secretion was tested in our study. The result showed completely inhibition of cAMP-stimulated I_{Cl^-} after pretreatment with BE, inferring BE could be used as a prophylactic drug. Even so, *in vivo* study should be done to validate this assumption.

Because our results demonstrated the direct effect of BE on CFTR, bioinformatics modeling can provide insight into prediction of the interaction of these two molecules. In regard to the highest protein–compound binding free energies, molecular docking simulation analysis identified the hydrophobic interaction between dephosphorylated structures of CFTR at site 1 (DSite 1) with nearby amino acid residues of V1147, L998, I1027, F1026, I1023, I1002, V1001, L1143, and A1146, which is in agreement with NBD2 domain. Our findings were corresponded to previous study indicating the significant role of NBD2 for regulation of CFTR gating rather than NBD1 [50]. With this particular result, it is feasible BE could be transported across the cell membrane by specific transporter or channel and thus prevent ability of ATP binding and/or hydrolysis at NBD2, which subsequently result in interruption of CFTR Cl^- channel gating. On the other hand, the interaction between BE with phosphorylated CFTR should not be excluded. Even though interpretation of site-specific docking is based on the highest fitness score in dephosphorylated form (39.01 kcal/mol in DSite1), binding score of phosphorylated form (PSite 1) is approximately equal being 38.70 kcal/mol, which could be explained why the effect of BE on inhibition of Cl^- secretion conspicuously seen in our experiment. Further investigation *in vivo* or *in vitro* (e.g. site-directed mutagenesis) should test this specific speculation. Altogether, *in silico* work supports the results associated with functional studies.

Atractylodes lancea (Thunb.) DC. (*A. lancea*) has been widely used in many countries for several pharmacological treatments [22,25]. The two active constituents, AT and BE have been shown to improve gastrointestinal disorders in the animal model [32,33]. Although the ameliorative effects of AT on GI function have been reported [25,44], our study demonstrated virtually no effect of AT on cAMP-induced Cl^- secretion in human intestinal epithelial T84 cells. By contrast, BE robustly exerts inhibitory effect on apical chloride transport through CFTR.

The failure to demonstrate the influence of AT compared to BE may be due to the differences in the solubility and chemical structure. AT and BE belong to two different categories from *A. Lancea* extracts, which are polyacetylenic and sesquiterpenes [19,51], respectively. The higher

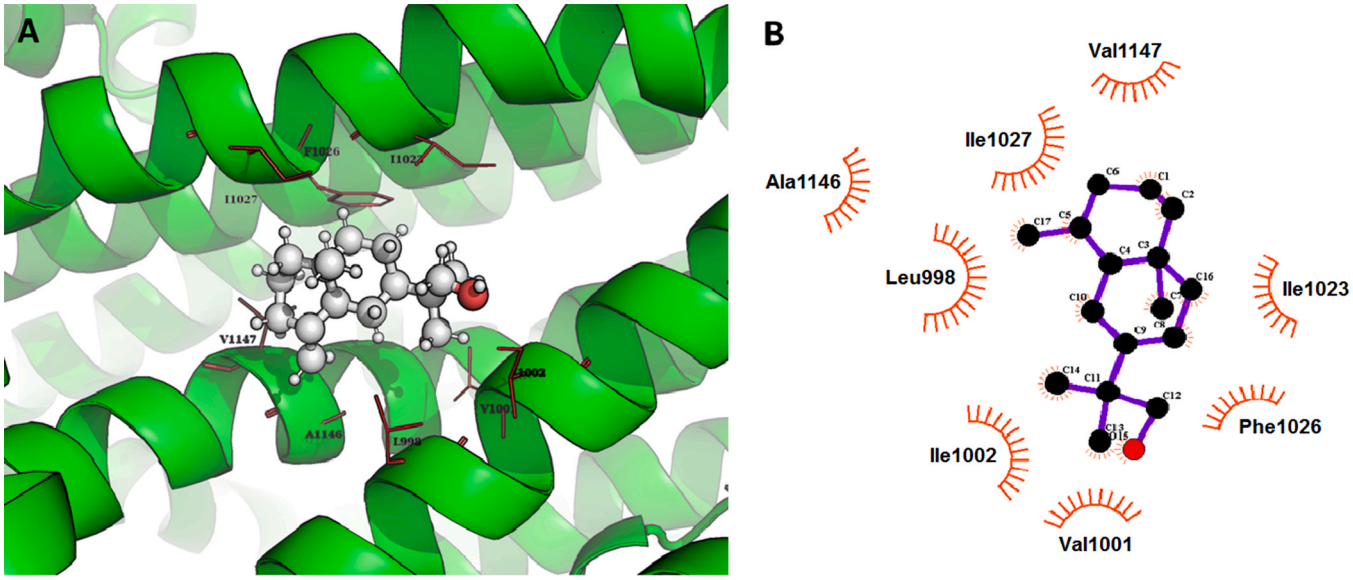

Fig. 10. Interactions of dephosphorylated CFTR with BE. (A) Ribbon diagram of dephosphorylated CFTR bound to the BE (stick model) was performed by Pymol. (B) LigPlot+ showing amino acid residues of dephosphorylated CFTR involved in interaction with BE.

solubility could augment the ability of transport across cell membrane whether active or passive process in BE compared to AT. In addition, one of the possible explanation might be involved molecular structure of BE having more specific interaction to CFTR near the extracellular domain and thus block the channel activity.

The bark of *Magnolia officinalis* has been used in Chinese traditional medicine for more than 2000 years [26]. Various constituents have been isolated and identified from *Magnolia officinalis* including BE as one of essential oil components [24,52]. Even though previous investigations reported therapeutic application in gastrointestinal disorders [23,24,26], the antidiarrheal effect has never been reported. Our finding delineated a new pharmacological activity of BE, implicating *Magnolia officinalis* or other plant species having BE as an active constituent as candidate medicinal herb for diarrheal treatment.

Notably, anti-secretory effect of BE with IC_{50} of ~1.05 μM seems more likely potent than other plant species, for example, black peppers (Piper nigrum L.). In 2015, Pongkorpsakol and colleagues investigated inhibitory effects of piperine as principal alkaloid component in black pepper on intestinal chloride secretion. Even though piperine resulted in completely inhibition of chloride secretion in T84 cell, value of IC_{50} ~9 μM is much higher than our study.

Highlight of our findings rests on the fact that we showed the first scientific evidence to support the use of traditional herb comprising BE for anti-diarrheal drug formulation.

5. Conclusion

The present findings reveal a novel biological activity of a phytochemical compound of oriental herbal medicine, represented by BE as an inhibitor CFTR-mediated Cl^- secretion across human intestinal epithelial (T84) cells. The mechanism underpinning the inhibition of Cl^- secretion by BE involves the direct blockage of CFTR. Molecular docking analysis indicates that BE docks into dephosphorylated structure of CFTR at the NBD2 domain. In addition, we observed prophylactic effect of BE for prevention of Cl^- secretion. Further research and development on BE may provide a drug candidate for the treatment or prevention of secretory diarrheas resulted from the intestinal hypersecretion of Cl^-.

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Disclosure

None.

CRediT authorship contribution statement

P.T., C.M. and K.C. conceived and designed of research; P.T., K.C., P. P. and N.W. performed experiments; P.T., K.C., N.P. and P.P. analyzed data; P.T., C.M., R.F., K.C., P.P. and S.S. interpreted results of experiments; P.T., K.C. and P.P. prepared figures; P.T., C.M. and K.C. drafted a manuscript; P.T., C.M., K.C., R.F., N.P. and K.N. edited and revised manuscript; All authors reviewed and approved final version of manuscript.

Conflict of interest statement

No conflicts of interest, financial or otherwise, are declared by the authors.

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