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Modulation of cellular bioenergetics enhances CFTR-dependent chloride transport in human bronchial epithelium

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Research Article

Keywords: respiratory epithelium, salinomycin, luteolin, CFTR, chloride secretion

Posted Date: November 9th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11469637/v1

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Additional Declarations: No competing interests reported.

Abstract

Airway surface liquid (ASL) homeostasis depends on coordinated epithelial ion transport, with the cystic fibrosis transmembrane conductance regulator (CFTR) playing a central role in chloride secretion. Reduced CFTR activity contributes to ASL dehydration, impaired mucociliary clearance, and chronic airway inflammation. In the present study, we investigated whether modulation of cellular bioenergetics by a mitochondria-targeted conjugate 3a and the flavonoid luteolin enhances CFTR-mediated chloride transport in human bronchial epithelial HBE cells.

CFTR activity was evaluated by Ussing chamber, while mitochondrial respiration, intracellular ATP and Ca^{2+} levels, reactive oxygen species (ROS) production, cell viability, and transepithelial electrical resistance (TEER) were assessed to characterize the cellular responses to treatment. Neither salinomycin nor conjugate 3a affected cell viability or ROS production, whereas luteolin significantly reduced intracellular ROS levels and enhanced epithelial barrier integrity. Conjugate 3a markedly increased mitochondrial oxygen consumption, intracellular ATP levels, cytosolic Ca^{2+} concentration, and CFTR-mediated chloride secretion, while the parent compound salinomycin produced no significant effects. Luteolin also enhanced CFTR activity and increased ATP and intracellular Ca^{2+} levels without stimulating mitochondrial respiration, suggesting a mechanism independent of enhanced oxidative metabolism.

Our findings demonstrate that pharmacological modulation of mitochondrial function may represent a complementary strategy to improve epithelial ion transport and restore airway surface hydration in diseases associated with impaired CFTR function.

Introduction

Ion transport across the airway epithelium is essential for maintaining airway surface liquid (ASL) homeostasis and mucociliary clearance, a critical host defense mechanism of the respiratory tract. Proper hydration of the airway surface depends on coordinated transport of chloride ions, predominantly through cystic fibrosis transmembrane conductance regulator (CFTR), and sodium ions through epithelial sodium channels (ENaC), thereby regulating ASL volume, mucus viscosity, and epithelial barrier function [1, 2] Disruption of finely balanced ion transport leads to ASL dehydration, impaired mucociliary clearance, and chronic mucus accumulation, thereby creating a favorable environment for microbial colonization and persistent inflammation [33, 4].

The CFTR channel is a cAMP-regulated anion channel that plays a central role in maintaining epithelial ion and fluid homeostasis [5, 6]. CFTR activation requires phosphorylation of its regulatory (R) domain, together with ATP binding and hydrolysis at the nucleotide-binding domains (NBDs), which cooperatively control channel gating [7, 88]. Consequently, intracellular ATP availability is an important determinant of CFTR activity and epithelial secretion. Dysfunction of the CFTR channel causes a range of serious diseases, the best-characterized of which is cystic fibrosis (CF). To date, more than 2000 CFTR variants have been identified, with the ΔF508 mutation accounting for the majority of CF cases [9]. Beyond

inherited CF, reduced CFTR activity has also been observed in acquired airway diseases characterized by epithelial dysfunction and chronic inflammation, underscoring the importance of elucidating mechanisms that restore or enhance CFTR-mediated ion transport [10, 11].

Current therapeutic strategies to restore epithelial chloride secretion include direct CFTR modulators and approaches targeting complementary ion transport pathways [12]. Approved CFTR modulators comprise correctors, which improve CFTR protein folding and trafficking, and potentiators, which enhance channel gating. The triple-combination therapy elexacaftor/tezacaftor/ivacaftor (ETI) has shown remarkable clinical efficacy, particularly in individuals carrying the F508del mutation [13, 14, 15, 16]. Nevertheless, therapeutic responses vary considerably among patients, and many rare CFTR variants remain poorly responsive or unresponsive to current modulators. These limitations underscore the need for complementary strategies that enhance CFTR function or restore epithelial ion homeostasis through CFTR-independent mechanisms [9, 17].

Alternative approaches include synthetic ion transporters and ionophores that facilitate transmembrane chloride transport [18. 19]. Several classes of anionophores were shown to partially restore epithelial chloride transport and improve airway surface hydration in cystic fibrosis models [19, 20, 21]. Despite these encouraging findings, the therapeutic translation of many synthetic ionophores has been hindered by several limitations, including limited anion selectivity, excessive lipophilicity, poor pharmacokinetic properties, and dose-dependent cytotoxicity resulting from disruption of intracellular ion homeostasis, mitochondrial dysfunction, and off-target membrane permeabilization [21, 22]. These challenges have prompted the search for next-generation ion transport systems that combine efficient chloride transport with improved biocompatibility and physiological regulation.

Beyond their direct effects on membrane ion transport, ionophores have emerged as modulators of cellular bioenergetics. The polyether ionophore salinomycin, particularly when conjugated to the mitochondria-targeting triphenylphosphonium cation (TPP^+), was shown to enhance mitochondrial respiration and transmembrane electrical currents in epithelial cells [23, 24]. By increasing intracellular ATP production, such derivatives may indirectly enhance ATP-dependent CFTR activity. Naturally occurring flavonoids were also shown to modulate CFTR-mediated chloride secretion through direct effects on channel gating and cAMP-dependent signaling. Compounds including genistein, quercetin, kaempferol, and apigenin have been shown to stimulate CFTR-dependent chloride currents, although with variable efficacy and mechanisms [25, 26]. Among them, luteolin is of particular interest for its antioxidant, anti-inflammatory, and mitochondrial-modulating properties [27]. It was previously demonstrated that luteolin increases basal chloride current in 16HBE14o− cells [28], but its effects on CFTR-dependent chloride secretion and the contribution of cellular bioenergetics remain unclear.

In the present study, we investigated whether modulation of intracellular bioenergetics enhances CFTR-mediated chloride secretion in human bronchial epithelial cells. We hypothesized that increasing intracellular ATP availability would facilitate the CFTR channel gating and stimulate epithelial chloride transport. To test this hypothesis, we examined the effects of luteolin and triphenylphosphonium-

conjugated salinomycin (conjugate 3a) on the 16HBE14σ cell model. The CFTR activity was evaluated by the Ussing chamber technique in differentiated 16HBE14σ cell monolayers.

Materials and methods

Cell culture

The human bronchial epithelial cell line, 16HBE14σ (HBE), was obtained from Sigma-Aldrich (St. Louis, MO, USA) and cultured according to standard laboratory procedures [29]. Cells were maintained in Minimal Essential Medium (MEM; Merck, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 100 U/mL penicillin, 100 µg/mL streptomycin (Merck, Darmstadt, Germany), and incubated for 3 h at 37°C in a humidified 5% CO_2 atmosphere. Cells were grown in T75 flasks (Nunc, Thermo Fisher Scientific, Waltham, MA, USA) and passaged when they reached 70–90% confluence.

Investigated compounds

Salinomycin and its conjugate 3a were synthetized, described [24], and provided by Prof. Huczyński`s laboratory at the Department of Chemistry, Adam Mickiewicz University, Poznań, Poland. Luteolin (98% purity) was purchased from TargetMol Chemicals Inc. (Boston, MA, USA). All compounds used in this study were dissolved in DMSO to prepare 1 mM and 10 mM stock solutions, which were aliquoted and stored at − 20°C until use.

Trypan Blue exclusion assay

Cytotoxicity of the investigated compounds was evaluated using the Trypan Blue exclusion assay. 16HBE14σ cells were exposed for 24h to salinomycin and to conjugate 3a at concentrations of 0.3, 1, and 3 µM, or to luteolin at concentrations of 10, 30, and 50 µM. Following incubation, cells were detached with trypsin, resuspended in cell culture medium, and mixed with Trypan Blue at a 1:1 ratio. Cell viability was determined using a TC20 automated cell counter (Bio-Rad, Hercules, CA, USA) and expressed as a percentage of viable cells relative to the untreated control group.

Measurement of reactive oxygen species (ROS)

Intracellular ROS levels were determined using a 2',7'-dichlorofluorescein diacetate (DCFDA) assay. Briefly, cells were seeded in 96-well plates at a density of 5×10^4 cells per well, cultured until confluence, and then exposed to the compounds under investigation. After 24 h of exposure, cells were washed with phosphate-buffered saline (PBS; VWR) and incubated with 10 µM DCFDA (Sigma-Aldrich, USA) prepared in Hank's Balanced Salt Solution (HBSS; Sigma-Aldrich). Subsequently, 100 µL of the staining solution was added to each well, and the plates were incubated for 3 h at 37°C in a humidified 5% CO_2

atmosphere. DCFDA is a cell-permeable, non-fluorescent probe that is hydrolyzed intracellularly to dichlorodihydrofluorescein (DCFH), which is subsequently oxidized to fluorescent dichlorofluorescein (DCF) by reactive oxidizing species. The assay therefore provides an overall indication of intracellular oxidative activity, with the detected signal being associated, among others, with hydrogen peroxide (H_2O_2)-dependent oxidation and hydroxyl radicals (•OH), rather than representing a specific measurement of a single ROS. Subsequently, fluorescence intensity was measured using a Fluoroskan Ascent microplate reader (Thermo Fisher Scientific, MA, USA) with excitation and emission wavelengths of 485 nm and 520 nm, respectively. Experiments were performed using independent cell batches and repeated twice. Fluorescence values were normalized to the untreated control group.

Transepithelial electrical resistance measurements

Cell monolayer integrity was assessed by measuring transepithelial electrical resistance (TEER), as previously described [28]. 16HBE14σ cells were seeded onto Corning Costar Snapwell inserts (0.45 µm pore size, 1.12 cm² surface area) and maintained for two weeks under liquid–liquid interface (LLI), followed by two weeks of culture under air–liquid interface (ALI) to allow complete epithelial polarization. Prior to treatment, monolayers were rinsed with sterile PBS, and fresh medium supplemented with investigated compounds was applied to the apical compartments at final concentrations of 1 µM for salinomycin and its conjugate 3a and 50 µM for luteolin. TEER values were recorded at 0, 24, 48, and 72 h of exposure using an EVOM2 epithelial voltohmmeter equipped with STX-2 electrodes (World Precision Instruments, Sarasota, FL, USA). Background resistance measured in blank inserts was subtracted from the recorded values, and the resulting resistance was multiplied by membrane surface area to obtain TEER values expressed as Ω·cm². TEER values were normalized to the baseline level measured at 0 h.

Ussing chamber measurements

Short-circuit current (Isc) measurements were performed to assess chloride transport across polarized 16HBE14σ cell monolayers. Cell monolayers treated for 72 hours with the compounds under investigation were mounted in Ussing chambers (Easy Mount Chamber System EM-CSYS-2, Physiologic Instruments, Reno, NV, USA). Transepithelial currents were recorded using an EVC4000 Multi-Channel V/I Clamp (World Precision Instruments, Sarasota, FL, USA) connected to an iWorx118 acquisition system with LabScribe 4.0 software (iWorx, Dover, NH, USA). Both chambers were filled with 5 mL of modified Krebs–Henseleit solution. A transepithelial chloride gradient was established by replacing 145 mM NaCl in the apical solution with an equimolar concentration of sodium gluconate, while the basolateral solution contained 145 mM NaCl, 3.3 mM K_2HPO_4, 10 mM HEPES, 10 mM D-glucose, 1.2 mM $MgCl_2$, and 1.2 mM $CaCl_2$ (pH 7.35). Experiments were performed at 37°C with continuous carbogen (95% O_2/5% CO_2) aeration. Isc was recorded under voltage-clamp conditions. The activity of ion transporting proteins was assessed by several subsequently added pharmacological modulators: the epithelial sodium channel (ENaC) inhibitor amiloride (100 µM; Spectrum Chemical, NJ, USA) to block Na^+ absorption;

forskolin (10 μM; Merck, Darmstadt, Germany), a cAMP agonist, to stimulate CFTR-mediated chloride transport; the CFTR-specific inhibitor CFTRinh-172 (5 μM; Merck, Darmstadt, Germany) and adenosine triphosphate (ATP; 100 μM; Merck, Darmstadt, Germany) to induce purinergic Ca^{2+}-dependent Cl^- secretion. CFTR activity was quantified as the change in short-circuit current following forskolin stimulation and subsequent inhibition with CFTRinh-172 ($\Delta Isc_{Inh-172}$).

High-resolution respirometry

Mitochondrial respiration was analyzed using the Oxygraph-2K system (Oroboros Instruments, Innsbruck, Austria). Cells were harvested, centrifuged, resuspended in cell culture medium at a density of 1×10^6 cells/mL, and transferred to 2 mL respiratory chambers to measure oxygen consumption rate (OCR; pmol $O_2 \cdot s^{-1} \cdot mL^{-1}$). Following stabilization of basal respiration, salinomycin or conjugate 3a was added at final concentrations of 0.1, 0.3, and 1 μM, or luteolin at 10, 30, and 50 μM. At the end of each experiment, 2 μM Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (Merck, Darmstadt, Germany) was added to determine maximal mitochondrial uncoupling. Oxygen consumption rates were normalized to the baseline values.

Measurement of ATP level

Intracellular ATP levels were quantified using the bioluminescent luciferase-based assay (FLAA-1KT, Sigma-Aldrich). Cells were seeded in 6-well plates, grown to confluence, and treated for 24 h with salinomycin (1 μM), conjugate 3a(1 μM), or luteolin (50 μM). Cells were then permeabilized with 0.5% Triton X-100 (Merck, Darmstadt, Germany) for 5 minutes, and cell lysates were mixed 1:1 with ATP assay reagent in 96-well plates. Luminescence was measured immediately using a Fluoroskan Ascent microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). ATP levels were normalized to those of untreated controls.

Calcium flux assay

Intracellular Ca^{2+} levels were assessed using Fura-2-AM ratiometric fluorescent Ca^{2+} indicator (Thermo Fisher Scientific, Waltham, MA, USA) as described previously [30]. Briefly, cells were seeded in 96-well plates and treated for 24 hours with salinomycin (1 μM), conjugate 3a (1 μM), or luteolin (50 μM). Cells were then loaded with 3 μM Fura-2-AM for 45 minutes at 37°C in a humidified atmosphere containing 5% CO_2, washed, and incubated in Ringer solution with or without Ca^{2+}. Cells were maintained in the respective buffer throughout the measurements. Ionomycin (5 μM) (Merck, Darmstadt, Germany) served as a positive control. Fluorescence was measured at excitation wavelengths of 340 and 380 nm, with emission at 538 nm, using a Fluoroskan Ascent microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). Intracellular Ca^{2+} levels were expressed as the F_{340}/F_{380} ratio normalized to untreated control.

Data analysis

Data was analyzed using Microsoft Excel (Office 365, Microsoft Corporation) and GraphPad Prism version 8.0 (Dotmatics, Boston, MA, USA). Statistical analysis was performed using an unpaired Student's t-test. Data are presented as mean ± SEM. Figures were prepared using CorelDRAW 2023 (version 24.3.0.571; Corel Corporation, Ottawa, ON, Canada).

Results

Cell viability and reactive oxygen species production

Cell viability and intracellular reactive oxygen species (ROS) levels were evaluated following treatment with salinomycin, conjugate 3a, and luteolin (Fig. 1B and 1C). The chemical formula of the compounds tested is shown in Fig. 1A. Trypan Blue exclusion assay demonstrated that neither salinomycin nor conjugate 3a significantly affected the viability of HBE cells. Although a slight decrease in cell viability was observed compared with the untreated control, the differences were not statistically significant. At the highest tested concentration (3 μM), cell viability remained at 76.33 ± 12.43% and 76.00 ± 12.48% following treatment with salinomycin and conjugate 3a, respectively (Fig. 1B). Luteolin treatment did not affect the cell viability (Fig. 1B). Treatment with salinomycin or conjugate 3a did not significantly alter intracellular ROS levels. In contrast, luteolin reduced ROS production in a concentration-dependent manner, decreasing the relative luminescence signal to 0.87 ± 0.04, 0.82 ± 0.05, and 0.80 ± 0.06 at 10, 30, and 50 μM, respectively (Fig. 1C).

Transepithelial electrical resistance

Next, the effects of salinomycin, conjugate 3a, and luteolin on epithelial barrier integrity were assessed by measuring transepithelial electrical resistance (TEER) after 24, 48, and 72 h of treatment from both sides of epithelial monolayers (Fig. 2). Treatment with salinomycin or conjugate 3a induced a modest increase in TEER, which was more pronounced following basolateral application; however, these changes did not reach statistical significance (Fig. 2B). In contrast, luteolin significantly increased TEER at all analyzed time points following both apical and basolateral administration. After apical treatment, TEER increased to 1.76 ± 0.24-fold of the control after 24 and 48 h and remained elevated at 1.58 ± 0.15-fold after 72 h (Fig. 2A). Basolateral applications produced a more pronounced response, with TEER increasing to 2.31 ± 0.09-fold after 24 h and 1.89 ± 0.08-fold after 48 h. Although TEER gradually declined over time, it remained significantly higher than the control after 72 h (1.24 ± 0.03-fold; Fig. 2). None of the tested compounds reduced TEER below control values during the experimental period.

Oxygen consumption rate

The effects of salinomycin, conjugate 3a, and luteolin on mitochondrial respiration were evaluated by measuring the oxygen consumption rate (OCR) and normalizing it to basal respiration (Fig. 3B–D). Salinomycin and conjugate 3a were tested at concentrations of 0.1, 0.3, and 1 μM, whereas luteolin was evaluated at 10, 30, and 50 μM. FCCP was added at the end of each experiment to determine maximal

mitochondrial respiratory capacity. Salinomycin and luteolin did not produce significant changes in OCR at any tested concentration (Fig. 3A and Fig. 3C). In contrast, conjugate 3a significantly increased OCR at 1 µM (Fig. 3B). At the highest tested concentrations, the normalized OCR values (mean ± SEM) were 0.92 ± 0.02 for salinomycin, 1.43 ± 0.02 for conjugate 3a, and 1.12 ± 0.08 for luteolin (Fig. 3A–C).

Measurement of intracellular ATP and Ca^{2+} levels.

Intracellular ATP levels and cytosolic Ca^{2+} concentrations were determined following 24 h treatment with salinomycin (1 µM), conjugate 3a (1 µM), or luteolin (50 µM) (Fig. 4). Conjugate 3a and luteolin significantly increased intracellular ATP levels compared with the untreated control, whereas salinomycin produced no significant effect (Fig. 4A). The normalized ATP values (mean ± SEM) were 1.0404 ± 0.0512 for salinomycin, 1.4940 ± 0.0781 for conjugate 3a, and 1.2268 ± 0.0369 for luteolin. Intracellular Ca^{2+} measurements performed under Ca^{2+}-free conditions showed that conjugate 3a and luteolin significantly increased cytosolic Ca^{2+} levels, while salinomycin did not affect intracellular Ca^{2+} concentration (Fig. 4B). The corresponding normalized values (mean ± SEM) were 1.03 ± 0.05 for salinomycin, 1.11 ± 0.04 for conjugate 3a, and 1.25 ± 0.07 for luteolin.

Short-circuit current measurements

The effects of salinomycin, conjugate 3a, and luteolin on CFTR-mediated chloride transport were evaluated by short-circuit current (Isc) measurements following 72 h treatment of polarized HBE cell monolayers (Fig. 5). CFTR activity was quantified as the difference between forskolin-induced channel activation and subsequent inhibition by CFTRinh-172. Following apical treatment, conjugate 3a and luteolin significantly increased CFTR-mediated chloride current when compared with the untreated cell monolayers (Fig. 5). In control monolayers, forskolin increased Isc by 8.44 ± 1.39 µA/cm², and subsequent CFTRinh-172 application reduced the current by − 10.88 ± 1.42 µA/cm². Treatment with 3a resulted in a forskolin-induced current of 6.73 ± 0.42 µA/cm² and a CFTRinh-172-sensitive current of − 17.10 ± 2.49 µA/cm². Luteolin treatment produced a forskolin-induced current of 4.40 ± 1.96 µA/cm² and a CFTRinh-172-sensitive current of − 13.33 ± 1.53 µA/cm². Following basolateral treatment, only 3a significantly increased CFTR-mediated chloride currents (Fig. 6). In these monolayers, forskolin increased Isc by 13.80 ± 1.04 µA/cm², followed by a CFTRinh-172-sensitive current of − 17.87 ± 1.13 µA/cm². Neither salinomycin nor luteolin significantly affected CFTR-mediated chloride currents after basolateral application. Treatment with the investigated compounds altered CFTR-mediated Cl^- transport, as measured by the difference between the forskolin-induced current and the current following CFTR inhibition with CFTRinh-172. This parameter was significantly increased following apical treatment with conjugate 3a and luteolin (Fig. 5C). After basolateral treatment, a significant increase was observed only in cells treated with conjugate 3a (Fig. 6B).

Discussion

The present study demonstrates that mitochondrial targeting substantially modifies the biological activity of salinomycin in human bronchial epithelial (HBE) cells. While neither salinomycin nor its mitochondria-targeted conjugate 3a compromised cell viability or increased intracellular ROS production under the experimental conditions, the conjugate exerted markedly stronger effects on cellular metabolism and epithelial ion transport than the parent compound. In particular, conjugate 3a significantly enhanced mitochondrial oxygen consumption, increased intracellular ATP levels and cytosolic Ca^{2+} concentration, and augmented CFTR-mediated chloride secretion, whereas unmodified salinomycin produced only minimal or non-significant responses. Luteolin, included as a reference compound, displayed a distinct biological profile characterized by pronounced antioxidant activity, improved epithelial barrier integrity, and moderate stimulation of ATP production, intracellular Ca^{2+} signaling, and CFTR function. Collectively, these findings indicate that conjugation of salinomycin with a mitochondria-targeting moiety not only preserves its cellular safety profile but also enhances its ability to modulate mitochondrial function and CFTR-dependent chloride transport in airway epithelial cells.

Safety profile of investigated compounds and their effects on barrier integrity

A prerequisite for interpreting the effects of salinomycin and its derivative on epithelial ion transport is demonstrating that the compounds under investigation do not compromise the viability of non-malignant airway epithelial cells. Neither salinomycin, its mitochondria-targeted conjugate 3a, nor luteolin affected the viability of HBE cells at the concentrations used in subsequent functional assays (1 μM for salinomycin and 3a, 50 μM for luteolin). Likewise, salinomycin and 3a did not increase intracellular ROS levels, whereas luteolin significantly reduced ROS production, consistent with its well-established antioxidant activity [27]. Our results align with previous reports showing that salinomycin and its derivatives exhibit pronounced anticancer activity, while exerting minimal toxicity toward non-malignant cells [24, 31, 32]. Importantly, mitochondria-targeted salinomycin conjugates, including triphenylphosphonium (TPP^+)-modified derivatives such as 3a, have been shown to further potentiate cytotoxic effects on malignant cells by selectively accumulating within mitochondria, thereby enhancing mitochondrial depolarization, ROS generation, and bioenergetic impairment [23, 24]. The absence of cytotoxicity and the increased ROS production observed in our study indicate that the mitochondrial perturbations reported in cancer cells are not reproduced in our non-malignant airway epithelial cell model. This observation is consistent with previous reports demonstrating that salinomycin-associated toxicity in non-malignant cells occurs predominantly at substantially higher concentrations and is highly cell type-dependent [33]. The preservation of cell viability together with the absence of oxidative stress indicates that the functional effects observed in subsequent experiments—including changes in mitochondrial respiration, intracellular ATP and Ca^{2+} levels, and CFTR-mediated chloride transport—are unlikely to result from nonspecific cellular injury. Instead, these findings suggest that mitochondrial targeting of salinomycin modulates epithelial bioenergetics and ion transport through regulated cellular signaling rather than through cytotoxic mechanisms.

Transepithelial electrical resistance and barrier integrity

Measurements of epithelial integrity demonstrated that none of the tested compounds compromised epithelial barrier function. Salinomycin and its mitochondria-targeted conjugate 3a had no effect on TEER following either apical or basolateral application to 16HBE14σ cell monolayers. These findings indicate that neither compound disrupts epithelial barrier integrity under the experimental conditions, suggesting that the functional effects observed in subsequent experiments are unlikely to be secondary to alterations in epithelial permeability or monolayer integrity. Although the effects of salinomycin on canonical tight junction proteins, including claudins, occludin, and ZO-1, have not been directly investigated, several studies have demonstrated that salinomycin modulates epithelial–mesenchymal transition (EMT) by altering the expression of E-cadherin, Snail, and ZEB1 [34, 35]. These observations suggest a potential indirect influence on epithelial junctional organization, although no evidence currently exists that salinomycin or its mitochondria-targeted derivatives directly regulate tight junction proteins or barrier function. To our knowledge, no studies have directly examined the effects of salinomycin on TEER in epithelial cell monolayers. Nevertheless, studies in the intestine have shown that salinomycin can improve intestinal barrier function and preserve epithelial integrity, supporting the notion that this compound does not inherently compromise epithelial barrier properties [36, 37]. In contrast, luteolin significantly increased TEER following both apical and basolateral application. This finding is consistent with previous studies in airway epithelial models [28] as well as intestinal and endothelial systems, where luteolin and related flavonoids enhance barrier integrity by preserving tight junction organization and reducing paracellular permeability [38, 39]. Tight junction integrity is highly sensitive to inflammatory and oxidative stress, conditions that typically reduce TEER by disrupting junctional protein organization [40, 41]. In our study, the preservation of cell viability together with the absence of oxidative stress is consistent with the maintenance of epithelial barrier function. Collectively, these findings indicate that salinomycin, its mitochondria-targeted conjugate 3a, and luteolin do not compromise epithelial barrier integrity in 16HBE14σ monolayers, supporting the conclusion that the functional changes observed in subsequent experiments occur in the absence of epithelial barrier disruption.

Mitochondrial respiration, ATP production, and calcium

Salinomycin was previously shown to disrupt mitochondrial electron transport chain activity in cancer cells, particularly at complexes I and III, leading to metabolic stress and impaired oxidative phosphorylation [42]. However, mitochondria-targeted conjugate 3a increased oxygen consumption and intracellular ATP levels in non-malignant HBE cells, indicating enhanced mitochondrial respiration. Although triphenylphosphonium (TPP^+)-conjugated salinomycin derivatives exert more pronounced mitochondrial effects than the parent compound in cancer cells [23, 24], our findings show that mitochondrial targeting enhances bioenergetic activity in airway epithelial cells without inducing oxidative stress or compromising cell viability, highlighting the cell type-dependent nature of salinomycin responses. The increase in intracellular ATP is particularly relevant for CFTR regulation, as ATP binding and hydrolysis are required for channel gating [7, 8]. In contrast, luteolin increased intracellular ATP without affecting oxygen consumption, suggesting improved metabolic efficiency rather than enhanced respiration. This is consistent with reports showing that luteolin modulates mitochondrial enzymes and

promotes ATP production through enhanced mitochondria–ER coupling and Ca^{2+}-dependent oxidative metabolism [43, 44] Calcium signaling provides another link between mitochondrial function and CFTR regulation. Whereas salinomycin induces Ca^{2+} overload in cancer and neuronal models [45, 46], no changes were observed in HBE cells, further supporting cell type-specific effects. In contrast, conjugate 3a and luteolin increased intracellular Ca^{2+} levels, suggesting modulation of mitochondrial Ca^{2+} handling and epithelial signaling pathways. Together, these findings indicate that salinomycin conjugate 3a and luteolin modulate intracellular ATP and Ca^{2+} homeostasis without inducing oxidative stress or cytotoxicity. Since ATP-dependent channel gating and Ca^{2+}-regulated PKC/PKA signaling converge on CFTR function [47], these coordinated metabolic changes provide a plausible mechanistic basis for the observed alterations in epithelial chloride transport.

Modulation of chloride transport

Several studies have investigated the effects of salinomycin on the electrophysiological properties of epithelial tissues [48, 49]. However, to the best of our knowledge, none have specifically examined its impact on CFTR-mediated chloride transport. In the present study, salinomycin did not affect CFTR-mediated chloride secretion in HBE cells, indicating that the parent compound does not directly modulate CFTR activity. This finding is consistent with the established mechanism of salinomycin as a potassium-selective ionophore that primarily disrupts K^+ gradients and membrane potential rather than directly regulating epithelial anion transport [48]. Accordingly, previous studies have reported that salinomycin does not alter net epithelial chloride or water transport across intact epithelial tissues [50]. Although these measurements reflect integrated transepithelial fluxes rather than CFTR-specific activity, they support the conclusion that disruption of potassium homeostasis alone is insufficient to enhance epithelial chloride secretion. In contrast, the mitochondria-targeted conjugate 3a significantly enhanced CFTR-mediated chloride secretion following both apical and basolateral treatment. This effect was accompanied by increased mitochondrial oxygen consumption, elevated intracellular ATP levels, and enhanced cytosolic Ca^{2+} concentration, indicating improved mitochondrial bioenergetic function. CFTR gating is critically dependent on ATP binding and hydrolysis at its nucleotide-binding domain [7, 8]; therefore, the increased ATP availability observed after treatment with conjugate 3a provides a plausible mechanistic basis for the enhanced chloride transport. The concomitant elevation of intracellular Ca^{2+} may further potentiate CFTR activity through Ca^{2+}-dependent signaling pathways or by facilitating cAMP-dependent regulation of the channel [51]. Luteolin also enhanced CFTR-mediated chloride secretion, although only following apical application. Unlike conjugate 3a, luteolin did not increase mitochondrial respiration despite elevating intracellular ATP and Ca^{2+} levels, suggesting that its effects on CFTR are mediated predominantly through intracellular signaling rather than enhanced oxidative metabolism. This interpretation is consistent with previous reports demonstrating that luteolin modulates epithelial ion transport through kinase-dependent signaling pathways and intracellular Ca^{2+} regulation [25, 28]. Taken together, these findings identify three distinct modes of CFTR regulation in airway epithelial cells. Salinomycin alone does not alter chloride transport, indicating that perturbation of potassium homeostasis is insufficient to activate CFTR. In contrast, mitochondrial targeting of salinomycin markedly enhances CFTR-mediated chloride secretion through metabolic activation, whereas luteolin

stimulates CFTR predominantly through signaling-dependent mechanisms independent of increased mitochondrial respiration.

Study limitations

Several limitations of the present study should be acknowledged. First, all experiments were performed using the 16HBE14o− cell line, which, although widely used as a model of human airway epithelium, does not fully reproduce the structural and functional complexity of primary human bronchial epithelial cells. Second, although conjugate 3a enhanced mitochondrial respiration, intracellular ATP levels, and CFTR-mediated chloride secretion, the study demonstrates an association rather than direct causality between these processes. Future studies employing pharmacological inhibition of oxidative phosphorylation or ATP synthesis will be necessary to determine whether enhanced mitochondrial bioenergetics is required for the observed increase in CFTR activity. Finally, only acute (24–72 h) responses to the investigated compounds were evaluated. The long-term effects of mitochondrial-targeted salinomycin derivatives on epithelial differentiation, barrier function, and ion transport, as well as their efficacy and safety in animal models, remain to be established before considering potential therapeutic applications. Despite these limitations, the present study provides the first evidence that mitochondrial targeting of salinomycin enhances CFTR-mediated chloride transport in airway epithelial cells while preserving epithelial viability and barrier integrity, supporting further investigation of mitochondrial bioenergetics as a modulator of epithelial ion transport.

Conclusions

The present study demonstrates that mitochondrial targeting markedly enhances the biological activity of salinomycin in human bronchial epithelial cells. While the parent compound did not significantly affect mitochondrial function or CFTR-mediated chloride transport under non-cytotoxic conditions, its mitochondria-targeted derivative (3a) increased mitochondrial respiration, intracellular ATP production, cytosolic Ca^{2+} levels, and CFTR-dependent chloride secretion without compromising cell viability, redox homeostasis, or epithelial barrier integrity. In contrast, luteolin enhanced CFTR activity through a mechanism associated with increased intracellular ATP and Ca^{2+} levels but independent of enhanced mitochondrial respiration, while simultaneously exerting antioxidant effects and strengthening epithelial barrier function. Collectively, these findings identify mitochondrial bioenergetics as an important regulator of CFTR function in airway epithelial cells and demonstrate that selective mitochondrial targeting can potentiate epithelial chloride transport. The distinct mechanisms observed for 3a and luteolin further suggest that metabolic and signaling pathways represent complementary approaches for modulating CFTR activity. These results provide a rationale for further investigation of mitochondria-targeted compounds as potential modulators of epithelial ion transport and as adjunctive therapeutic strategies for diseases characterized by impaired CFTR function.

Declarations

Conflict of interest statement

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors contribution for all authors

JH: Investigation, Methodology, Visualization, Writing – original draft; MZ: Formal analysis, Conceptualization, Methodology, Writing – original draft, Writing – review and editing; PB: Conceptualization, Supervision, Writing – review and editing, Funding acquisition, Project administration; MJ: Investigation, Formal analysis, Writing – review and editing; AH: Formal analysis, Writing – review and editing, Funding acquisition.

Disclosure

of using artificial intelligence (AI) in the writing process

Declarations

Ethics, Consent to Participate, and Consent to Publish declarations: not applicable.

This study did not involve human participants or animals. Therefore, ethical approval and informed consent were not applicable.

Funding statement

This work was financed by the OPUS 27 grant (No. 2024/53/B/NZ3/01635) from the National Science Centre (NCN), Poland (to PB) and OPUS 21 grant (No. 2021/41/B/ST4/00088) from NCN (to AH).

Author Contribution

J.H.: Investigation, Methodology, Visualization, Writing – original draft; M.Z.: Formal analysis, Conceptualization, Methodology, Writing – original draft, Writing – review and editing; P.B.: Conceptualization, Supervision, Writing – review and editing, Funding acquisition, Project administration; M.J.: Investigation, Formal analysis, Writing – review and editing; A.H.: Formal analysis, Writing – review and editing, Funding acquisition.

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Figures

Figure 1

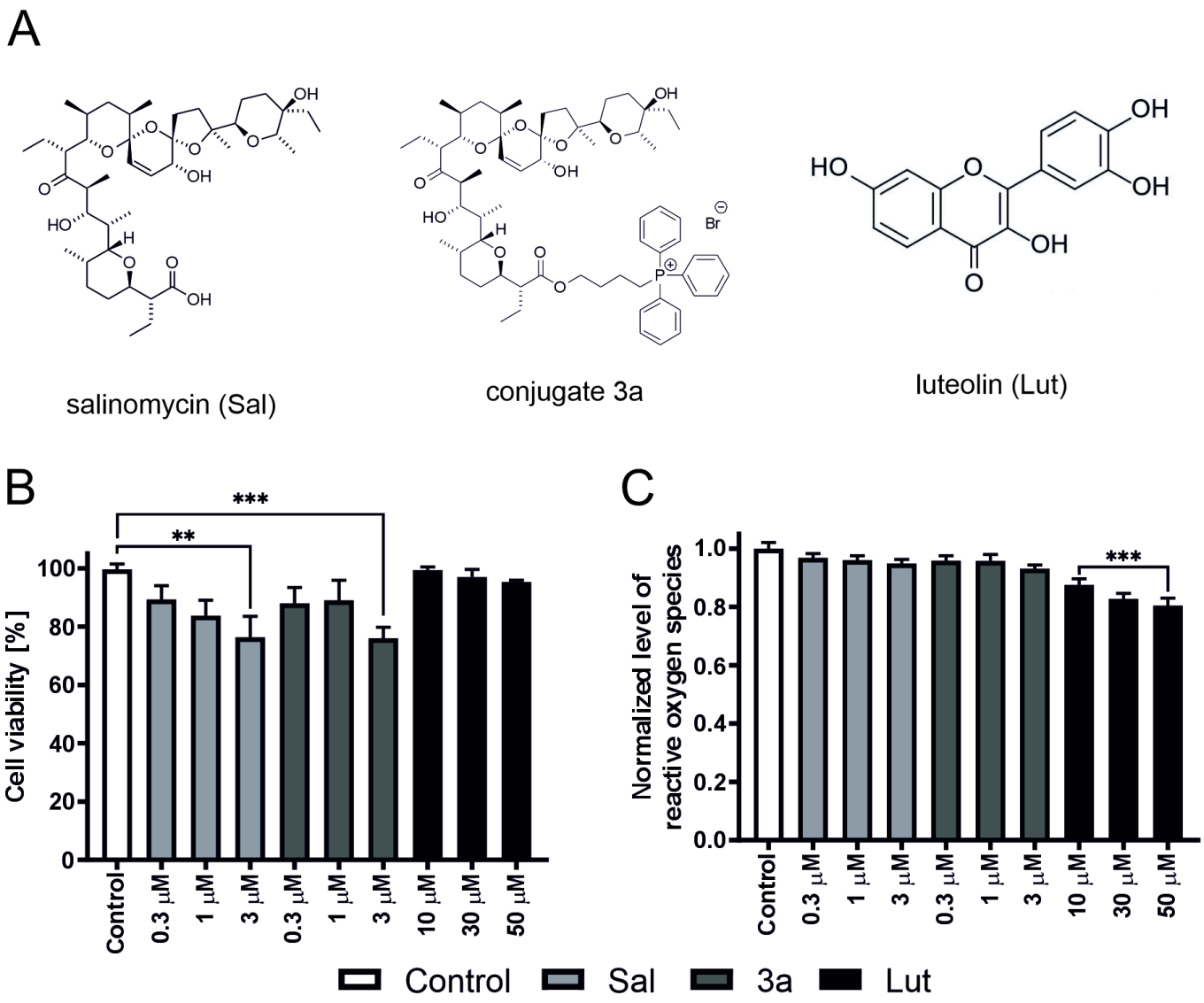


Figure 1

The effect of salinomycin, conjugate 3a, and luteolin on HBE cell viability and ROS production. Cells were treated for 24 h with salinomycin and conjugate 3a at concentrations of 0.3, 1, and 3 μM or with luteolin at concentrations of 10, 30, and 50 μM. **(A)** The chemical formula of the compounds tested. **(B)**Cell viability was assessed by the Trypan Blue exclusion assay. **(C)** ROS levels measured using DCFDA fluorescence assay. Results were normalized to the control and are presented as mean ± SEM (n = 8). Statistical analysis was performed using an unpaired Student t-test (**p < 0.01, ***p < 0.001).

Figure 2

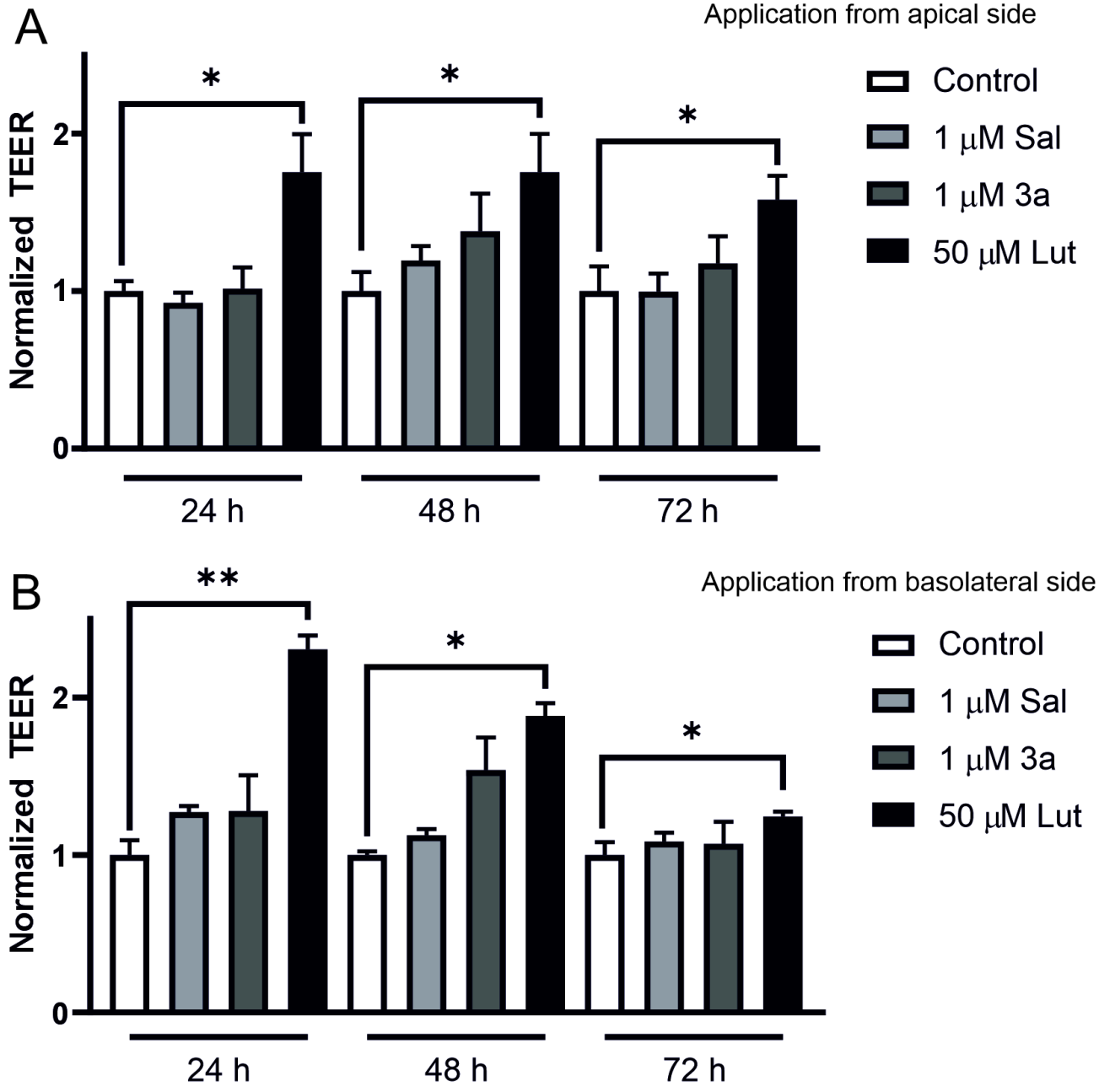


Figure 2

Impact of salinomycin, conjugate 3a, and luteolin on TEER in 16HBE14σ cell monolayers. Cell monolayers were treated apically **(A)** or basolaterally **(B)** with 1 μM salinomycin or conjugate 3a, or 50 μM luteolin. TEER was measured at 24, 48, and 72 h. TEER values were normalized to the baseline level measured at 0 h. Data are presented as mean ± SEM (n = 3). Statistical analysis was performed using an unpaired Student's t-test (*p < 0.05, **p < 0.01).

Figure 3

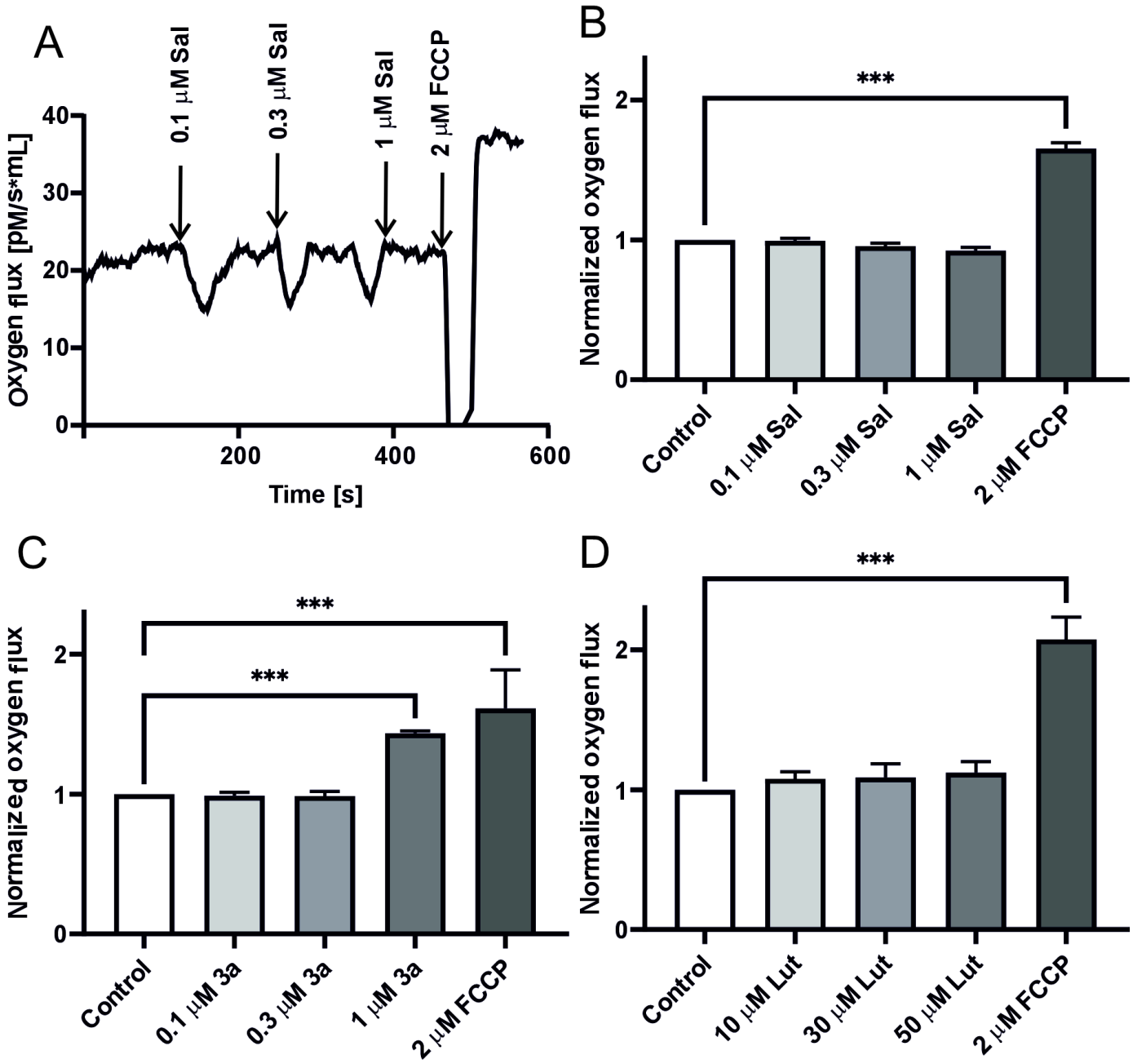


Figure 3

Effects of salinomycin, mitochondria-targeted conjugate 3a, and luteolin on oxygen consumption rate (OCR) in HBE cells. (A) Representative oxygen consumption trace following sequential addition of salinomycin at concentrations of 0.1, 0.3, 1, 3, and 10 μM and 2 μM FCCP **(B)** Salinomycin (0.1, 0.3, and 1 μM), **(C)**conjugate 3a (0.1, 0.3, and 1 μM), and **(D)** luteolin (10, 30, and 50 μM) were directly applied to HBE cells, and OCR was measured using high-resolution respirometry (Oxygraph-2k system). Oxygen consumption rates were normalized to basal respiration (baseline). FCCP (2 μM) was added at the end

of each experiment to determine maximal mitochondrial respiratory capacity and uncoupling. Data are presented as mean ± SEM (n=4). Statistical analysis was performed using an unpaired Student's t-test (***p < 0.001).

Figure 4

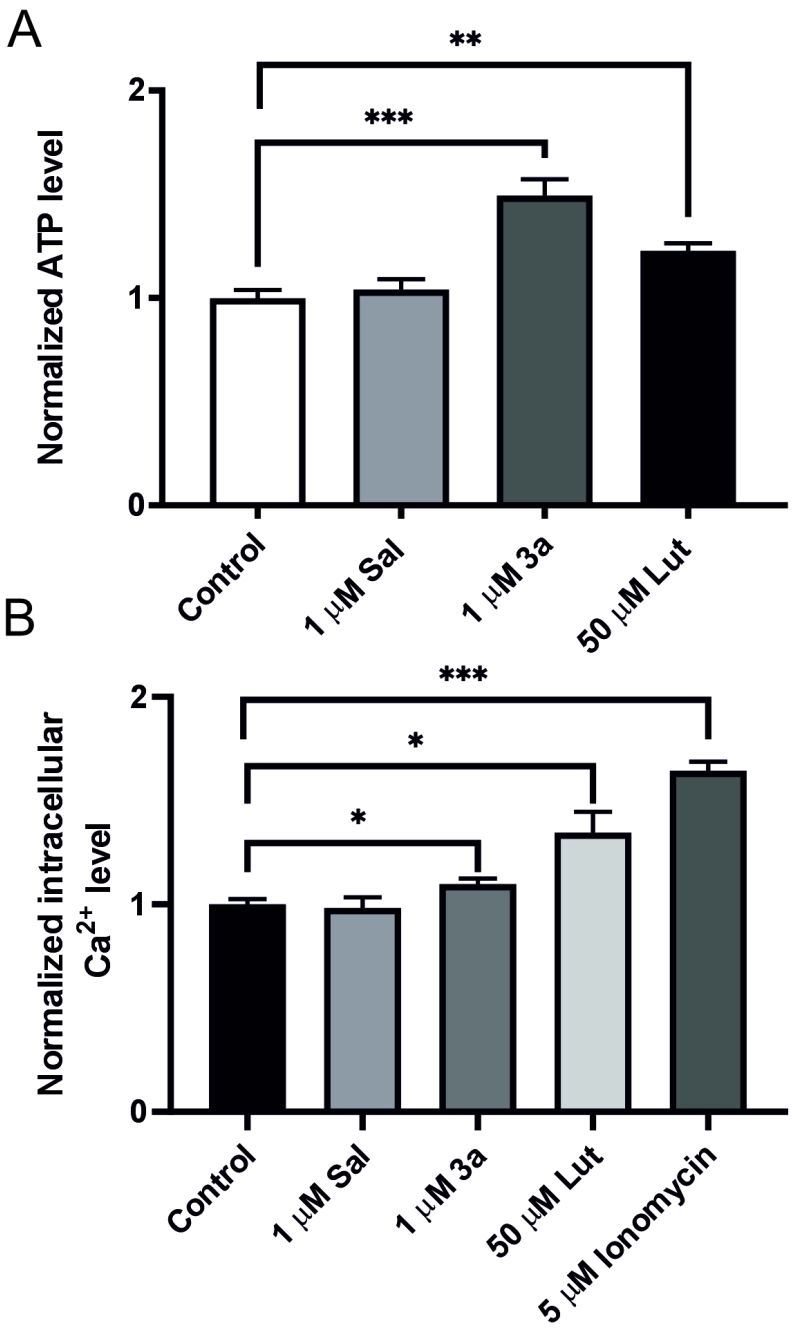


Figure 4

Effects of salinomycin, mitochondria-targeted conjugate 3a, and luteolin on intracellular ATP levels and cytosolic calcium concentration in HBE cells. Cells were incubated for 24 h with salinomycin (1 μM), conjugate 3a (1 μM), or luteolin (50 μM), and all values were normalized to control conditions. **(A)** Intracellular ATP levels were quantified using a luciferase-based bioluminescence assay. **(B)**Intracellular Ca^{2+} levels were measured using the ratiometric fluorescent dye FURA-2 in calcium-free buffer supplemented with EGTA to assess release from intracellular stores (e.g., mitochondria and/or endoplasmic reticulum). Data are presented as mean ± SEM (n=8). Statistical analysis was performed using an unpaired Student's t-test (*$p < 0.05$, **$p < 0.01$, ***$p<0.001$).

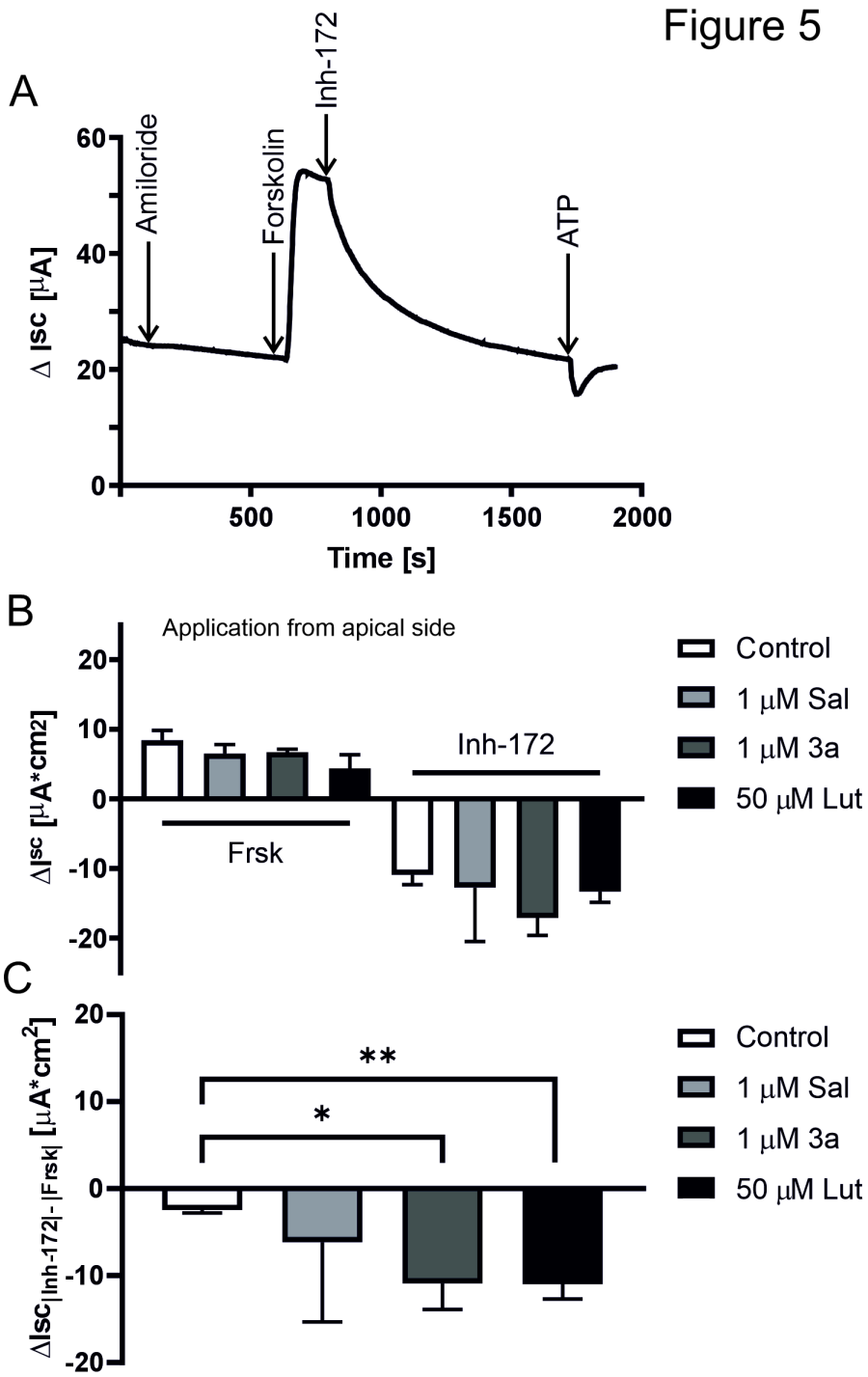


Figure 5

Chloride current changes in 72 h apically treated 16HBE14σ cell monolayers with salinomycin (1 μM), conjugate 3a (1 μM), and luteolin (50 μM). **(A)** Respresentative registration of chloride current flowing across polarized monolayers. **(B)** Effects of sequential addition of forskolin and $CFTR_{inh-172}$ on Isc. **(B)** Quantification of CFTR activity (ΔIsc_{Frsk}- $\Delta Isc_{inh-172}$). Data are presented as mean ± SEM (n=3). Statistical analysis was performed using an unpaired Student's t-test (*p < 0.05, **p < 0.01).

Figure 6

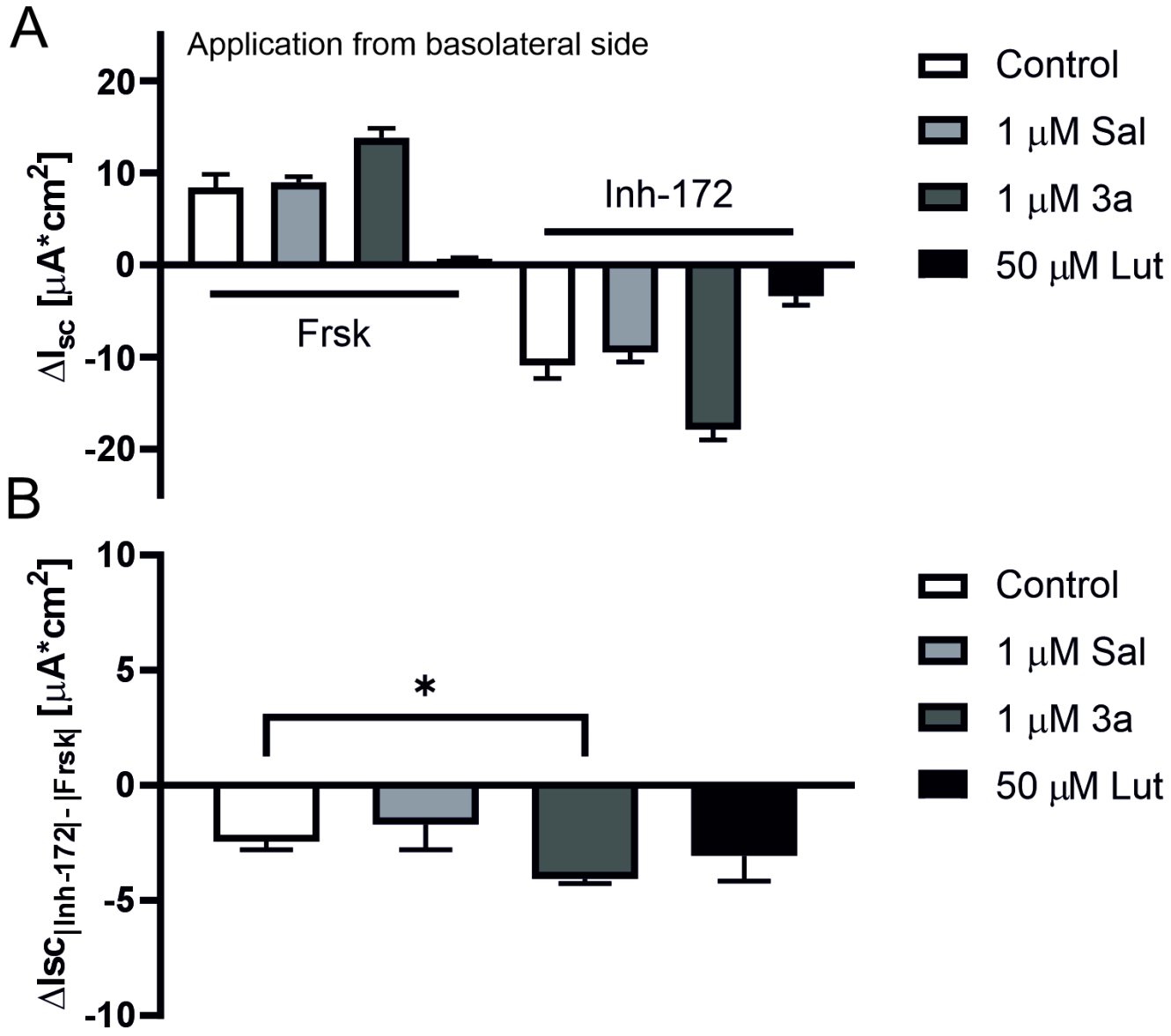


Figure 6

Chloride current changes in 72 h basolaterally treated HBE cell monolayers with salinomycin (1 μM), conjugate 3a (1 μM), and luteolin (50 μM). **(A)** Effects of sequential addition of forskolin and CFTRinh-172 on Isc. **(B)** Quantification of CFTR activity (ΔIsc_{Frsk}- $\Delta Isc_{inh-172}$). Data are presented as mean ± SEM (n=3). Statistical analysis was performed using an unpaired Student's t-test (*$p < 0.05$).