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ATP7B detoxifies silver in ciliated airway epithelial cells

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Abstract

Silver is a centuries old antibiotic agent currently used to treat infected burns. The sensitivity of a wide range of drug-resistant microorganisms to silver killing suggests that it may be useful for treating refractory lung infections. Toward this goal, we previously developed a methylated caffeine silver acetate compound, SCC1, that exhibits broad-spectrum antimicrobial activity against clinical strains of bacteria in vitro and when nebulized to lungs in mouse infection models. Preclinical testing of high concentrations of SCC1 in primary culture mouse tracheal epithelial cells (mTEC) showed selective ciliated cell death. Ciliated cell death was induced by both silver- and copper-containing compounds, but not by the methylated caffeine portion of SCC1. We hypothesized that copper transporting P-type ATPases, ATP7A and ATP7B, play a role in silver detoxification in the airway. In mTEC, ATP7A was expressed in non-ciliated cells, whereas ATP7B was expressed only in ciliated cells. The exposure of mTEC to SCC1 induced the trafficking of ATP7B, but not ATP7A, suggesting the presence of a cell-specific silver uptake and detoxification mechanisms. Indeed, the expression of the copper uptake protein CTR1 was also restricted to ciliated cells. A role of ATP7B in silver detoxification was further substantiated when treatment of SCC1 significantly increased cell death in ATP7B shRNA treated HepG2 cells. Additionally, mTEC from ATP7B^{-/-} mice showed enhanced loss of ciliated cells compared to wild type. These studies are the first to demonstrate a cell-type specific expression of the Ag⁺/Cu⁺ transporters ATP7A, ATP7B and CTR1 in airway epithelial cells, and a role for ATP7B in detoxification of these metals in the lung.

Keywords

silver; copper; antibacterial; ciliated cells; ATP7A; ATP7B; CTR1; airway; mouse

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Conflict of interest statement. The authors AIB and SLB report no conflicts. Both WJY and CLC are officers of Nebusil, Inc., which aims to commercialize SCC1. Neither author received funds from the company.

Introduction

Silver has been used in a variety of ways to control infection since the 18th century and is currently used for the treatment of infected burns and wounds. The efficacy of silver against bacteria, including *Pseudomonas aeruginosa* and *Staphylococcus aureus*, has led to decreased mortality in patients with burns and skin infections (Cason and Lowbury, 1968; Wright *et al.*, 1999; Elechiguerra *et al.*, 2005; Bhattacharyya and Bradley, 2008; Payne and Ambrosio, 2009). Silver has also been shown to kill fungal pathogens, and silver nanoparticles inhibit HIV-1 binding to host cells (Wright *et al.*, 1999; Elechiguerra *et al.*, 2005). In addition, an anticancer activity of silver and silver complexes has been demonstrated recently (Thati *et al.*, 2007; Liu *et al.*, 2008; Medvetz *et al.*, 2008). Thus, silver offers much promise as a therapeutic agent for a broad range of diseases.

Our group has been particularly interested in the efficacy of silver to kill bacteria such as *P. aeruginosa*, since this organism chronically infects the lungs of patients with diseases such as cystic fibrosis and is responsible for an increased mortality (Henry *et al.*, 1992; Aloush *et al.*, 2006). To maximize the treatment effects of silver, reduce systemic toxicity and achieve precise dosing of the active silver cation (Ag^+), we have previously synthesized and characterized a series of silver N-heterocyclic carbene complexes (SCC) (Kascatan-Nebioglu *et al.*, 2006; Hindi *et al.*, 2008). These agents release the active silver cation (Ag^+) when solubilized in aqueous solutions. Almost all carbene compounds evaluated to date, including methylated caffeine silver acetate, designated SCC1, have in vitro antimicrobial activity against numerous respiratory pathogens isolated from the lungs of cystic fibrosis patients, including antibiotic resistant *Pseudomonas* and *Burkholderia* species (Kascatan-Nebioglu *et al.*, 2006; Hindi *et al.*, 2008; Hindi *et al.*, 2009). We have also demonstrated the therapeutic efficacy of nebulized SCC1 against *P. aeruginosa* in mouse infection models (Cannon *et al.*, 2009).

Although the antimicrobial activities of silver have been established, less is known about the mechanisms of prokaryotic and eukaryotic killing or detoxification. Previous studies in bacteria have identified conserved regions of P-type ATPases that are associated with the transport of copper and silver, but not other heavy metals (Solioz and Odermatt, 1995; Stoyanov *et al.*, 2003). In eukaryotic systems, silver has been shown to utilize the copper-transporter, ATP7A to transport intracellular silver in copper-resistant Chinese hamster ovary (CHO-CUR3) cells and human fibroblasts to the plasma membrane for excretion (Petrus *et al.*, 1996; Verheijen *et al.*, 1998). ATP7A and the related protein ATP7B are well characterized as copper transporters. Mutations in the genes that code for these proteins result in two diseases, Menkes and Wilson Disease, respectively (La Fontaine and Mercer, 2007). The major manifestations of these diseases result from an inability to export copper from hepatocytes (Wilson Disease) or neurons (Menkes Disease) through the mutant copper transporters. An additional protein, CTR1 (SLC31A1) has an important role in regulating copper uptake at the cell membrane and can similarly handle silver and other metals (Lee *et al.*, 2002; Petrus *et al.*, 2003; Kim *et al.*, 2009). CTR1, ATP7A and ATP7B are also known to transport platinum and variable expression of these proteins have been shown to affect the cytotoxicity and resistance of platinum-based drugs in cancer cells (Kuo *et al.*, 2007). However, little is known about the expression and function of CTR1, ATP7A or ATP7B in the lung for either silver or copper transport. Because the silver-based antimicrobial SCC1 has shown efficacy for treatment of pulmonary infections when directly nebulized to the lung, the present study was designed to define the roles that the copper transporters CTR1, ATP7A and ATP7B may play in silver transport in respiratory epithelium. Specifically, we sought to test the hypothesis that ATP7A, ATP7B or both may be important for detoxification of silver in airway epithelial cells.

Materials and methods

Chemicals

Synthesis and characterization of methylated caffeine silver acetate (SCC1, MW 375.1294) and methylated caffeine were previously described (Kascatan-Nebioglu *et al.*, 2006; Hindi *et al.*, 2008). Copper (II) chloride dihydrate (CuCl_2 ; Cell culture grade) and silver acetate (CH_3COOAg ; Reagent Plus Grade, 99% pure) were purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture

HepG2 (ATCC, Manassas, VA), HeLa (ATCC, Manassas, VA), and HEK293T (kindly provided by Greg Longmore, Washington University) cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum, penicillin and streptomycin. Preparations of cultured mouse tracheal epithelial cells (mTEC) were derived from wild type C57B6/J (Jackson Laboratories, Bar Harbor, ME), $\text{ATP7B}^{-/-}$ or their wild type littermates mice (kindly provided by S. Lutsenko, Johns Hopkins University; Buiakova *et al.*, 1999), all housed in barrier conditions. Mouse tracheal epithelial cells were cultured using air-liquid interface (ALI) conditions to generate fully differentiated cells composed of ciliated, non-ciliated and secretory cells, as a representative model of the airway (You *et al.*, 2002). Briefly, cells were harvested from tracheas by pronase digestion, seeded on supported polyester semipermeable membranes (12-mm diameter, Transwell, Costar-Corning, Corning, NY) and proliferated in growth-factor-enriched medium until cells were confluent. Subsequently, the air-liquid interface condition was established for induction of differentiation by aspirating apical chamber medium and changing the medium in the lower compartment to a differentiation media supplemented with 2% NuSerum (BD BioSciences, San Diego, CA).

Immunostaining

Cells on supported membranes were fixed and processed for immunostaining as previously described (Ibricevic *et al.*, 2006). Primary antibodies used were: mouse anti- β -tubulin-IV, (1:250, BioGenex, San Ramon, CA), rabbit anti ATP7A (1:1000) and rabbit anti ATP7B (1:2500, both kindly provided by J. D. Gitlin; (Yamaguchi *et al.*, 1996; Hung *et al.*, 1997)) and rabbit anti-CTR1 (1:100, kindly provided by D. J. Thiele; (Lee *et al.*, 2002)). Antibody binding was detected using secondary antibodies donkey anti-rabbit Alexa Fluor 555 or donkey anti-mouse Alexa Fluor 488 (1:700 and 1:500, respectively; Molecular Probes, Carlsbad CA). No detectable staining was observed for species-specific control antibodies. Membranes were mounted on slides with media containing 4', 6 diamidino-2-phenylindole (DAPI) to stain intracellular DNA. Image capture of immunostained cells on the membranes *en face*, and as single cells following disruption from membranes, was performed using a Leica DM5000 microscope (Wetzlar, Germany) with a Retiga 200R charge-coupled device camera (Q-Imaging, Surrey, BC, Canada) interfaced with Q-Capture Pro software (Q-Imaging). Images were globally adjusted for contrast using Photoshop software and composed in Illustrator (both Adobe Systems, San Jose, CA).

Protein blot analysis

Cells were treated with lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate) containing protease inhibitors for immunoblot analysis as previously described (Huang *et al.*, 2003). Tissues were pulverized after freezing in liquid nitrogen prior to incubation in lysis buffer. Lysates containing equal amounts of proteins were separated by 3-8% Tris-Acetate NuPAGE or 4-20% SDS-PAGE. Proteins were transferred to PVDF membranes (Millipore, Bedford, MA) that were blocked with 5% milk and 0.2% Tween-20 (1 h, 25°C or overnight, 4°C), then incubated with primary antibody (1 h, 25°C or

overnight, 4°C). Primary antibodies ATP7A and ATP7B as listed above and mouse anti-GAPDH (1:2000; Sigma-Aldrich) were detected by horseradish peroxidase-labeled secondary antibodies and resolved using enhanced chemiluminescence (ECL, Amersham Pharmacia, Buckinghamshire, England).

ATP7B shRNA

ATP7B mRNA expression in HepG2 cells was inhibited using sequence-specific shRNA delivered by a recombinant lentivirus vector. Bacteria transformed with the pTKO.1 plasmid, containing human ATP7B shRNA sequences and the puromycin resistant cDNA sequence, were purchased from Sigma-Aldrich. Plasmids were amplified and then purified (Qiagen midiprep kit, Hilden Germany). Plasmids containing shRNA sequences were co-transfected with packaging plasmid pHR'8.2ΔR and envelope plasmid pCMV-VSV-G (at a 8:1 ratio, both kindly provided by S. Stewart, Washington University, St. Louis) in HEK293T (human embryonic kidney-derived cells containing SV40 large T antigen) using Fugene 6 (Roche Diagnostics, Indianapolis, IN) as described (Stewart *et al.*, 2003). After 48 h, cell media from the transfected HEK293T cells containing virus was harvested and filtered. HepG2 cells were then infected by incubation overnight with the virus media in the presence of 10 µg/ml polybrene. After an additional 48 h, HepG2 cells that expressed the pTKO-ATP7B shRNA were selected and maintained in media containing 5 µg/ml puromycin for 2 weeks prior to harvest for analysis.

Cell quantification and statistical evaluation

Viable HepG2 cells that were present following incubation with media or ATP7B shRNA and treated with SCC1 (from a 10 mg/ml stock in media) were quantified by hemocytometer using trypan blue. Cells in mTEC cultures were identified by immunostaining and quantified in a minimum of three photomicrographs obtained using the 20x objective for each experiment. Differences in cell numbers in treatment conditions were compared using the Student's t-test or one-way ANOVA using Prism5 (GraphPad Software, Inc., CA). A significant difference between conditions was considered to be $p < 0.05$.

Results

SCC1 dose-dependent loss of ciliated airway epithelial cells

To further characterize SCC1 as a nebulized antimicrobial for the treatment of lung diseases, we sought to determinate if cytotoxicity might occur during treatment. mTEC were assessed for survival after 24 hours by immunostaining with the cell nuclear marker DAPI (Fig. 1A, top row) and the ciliated cell marker β -tubulin-IV (β -tub) (Fig. 1A, bottom row). A marked decrease in ciliated cells was observed at 50 µg/ml, and in all cell types at 500 µg/ml (Fig. 1B). Together, these data suggest not only that the concentration of SCC1 required for bacterial growth inhibition is not toxic, but also that high-concentration SCC1 treatment may induce cell type-specific toxicity in airway epithelial cells.

SCC1-induced loss of ciliated cells is silver and copper dependent

To determine if the SCC1 effect on ciliated cells was specifically related to silver, we compared the relative toxicity of SCC1 to that of silver acetate, as a source of silver cations, and the silver binding component of SCC1, namely, methylated caffeine (Fig. 2). We also assessed the effects of cupric chloride (CuCl_2) since previous studies have shown that the transport of silver and copper ions was shared by P-type ATPases (Petris *et al.*, 1996; Verheijen *et al.*, 1998). Cuprous chloride (CuCl) has a very low solubility in water and could not be tested. Equimolar amounts and an identical range of concentrations that spanned the LD_{50} concentration of SCC1 were used for all compounds (Cannon *et al.*, 2009). The significant dose-related decrease in viable

mTEC, and particularly ciliated cells, was identical for SCC1 and silver acetate. In contrast, there was no observed cell death in mTEC treated with methylated caffeine. Interestingly, cupric chloride was less toxic when compared to SCC1 or silver acetate, resulting in a significant decrease of ciliated cells only at a concentration of greater than 100 $\mu\text{g/ml}$. [Fig. 2A Total cell number: 50 $\mu\text{g/ml}$, $p < 0.01$, for differences between methylated caffeine versus SCC1 and silver acetate; 100 $\mu\text{g/ml}$, $p < 0.0001$ for differences between all but methylated caffeine versus CuCl_2 ; 250 $\mu\text{g/ml}$, $p < 0.0001$ for differences between all but methylated caffeine versus CuCl_2 and SCC1 versus silver acetate; 500 $\mu\text{g/ml}$, $p < 0.0001$ for differences between all but SCC1 versus silver acetate. Fig. 2B Non-ciliated cells: 50 $\mu\text{g/ml}$, $p < 0.01$, for differences between all but methylated caffeine versus CuCl_2 and SCC1 versus silver acetate; 100 $\mu\text{g/ml}$, $p < 0.01$ for differences between SCC1 versus others; 250 $\mu\text{g/ml}$, no differences; 500 $\mu\text{g/ml}$, $p < 0.001$ for differences between all but methylated caffeine versus CuCl_2 and SCC1 versus silver acetate. Fig. 2C Ciliated cells: 50 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$, $p < 0.0001$, for differences between all but methylated caffeine versus CuCl_2 and SCC1 versus silver acetate; 250 $\mu\text{g/ml}$ and 500 $\mu\text{g/ml}$, $p < 0.0001$, for differences between all but SCC1 versus silver acetate.] These findings indicate that both silver and copper exhibit a dose-dependent and ciliated cell-specific cytotoxic effect, suggesting the possibility that shared metal transporters may be utilized for uptake and excretion of both metals.

Cell-type specific expression of ATP7A and ATP7B in airway epithelial cells

Immunoblot analysis was performed to determine if ATP7A and ATP7B P-type ATPases were expressed in mTEC (Fig. 3A). Lysates from HeLa and HepG2 cell lines were used as positive controls for antibodies that detect ATP7A and ATP7B, respectively as previously described (Yamaguchi *et al.*, 1996; Hung *et al.*, 1997). Both ATPases were expressed in mouse lung and mTEC. In contrast to ATP7A, ATP7B expression was not detected in brain tissue samples, as previously shown, likely due to low levels of expression (Kuo *et al.*, 1997). Immunostaining was used to determine if there was cell-specific expression of ATP7A and ATP7B in mTEC. ATP7B was present in ciliated cells that were identified by cilia protein β -tubulin-IV (β -tub), whereas ATP7A was present in non-ciliated cells (Fig. 3B). Merged images of immunostained cells that were distracted from mTEC preparations ("single cells") and overlaid with images obtained using differential interference contrast (DIC) microscopy show the expression of ATP7A and ATP7B in the perinuclear region, consistent with the localization of the trans-Golgi network (TGN) as previously described in other cell types (Guo *et al.*, 2005; Nyasae *et al.*, 2007). Cell-specific expression of ATP7B and evidence that ciliated cells are selectively affected when treated with SCC1 in high dose, suggest that cell-type specific mechanisms for copper and silver transport exist in the airway.

The intracellular localization of ATP7B and the cell-type specific expression of CTR1

Prior reports of the behavior of ATP7A and ATP7B in polarized cells showed that copper induced the relocation of these proteins from the trans-Golgi network to the cell periphery to facilitate the removal of copper. To examine for this translocation effect, mTEC preparations were treated with media only (top row) or with SCC1 (50 $\mu\text{g/ml}$, 24 h, bottom row) (Fig. 4A). In the absence of SCC1, ATP7B remained perinuclear while SCC1 treatment resulted in ATP7B translocation to the sub-apical region of ciliated cells. The application of 10 $\mu\text{g/ml}$ of SCC1 induced a similar effect (data not shown). In contrast, SCC1 did not induce trafficking of ATP7A to either apical or basolateral domains when evaluated by immunostaining (data not shown). SCC1-induced relocation of ATP7B provides evidence that there is a cell-specific, silver-dependent regulation of ATP7B in airway epithelial cells. Because ATP7B responded to extracellular silver, but ATP7A did not, we hypothesized that ciliated cells may express specific copper/silver uptake proteins not present in non-ciliated cells. To address this possibility, the expression of copper importer CTR1 was examined (Lee *et al.*, 2002). As

predicted, expression of CTR1 was restricted to ciliated mTEC cells, suggesting preferential uptake in this cell type (Fig. 4B).

ATP 7B-dependent toxicity of SCC1 in airway epithelial cells

To further analyze the role of ATP7B in detoxifying silver, we reasoned that the absence of ATP7B in cells should increase their sensitivity to silver-induced death. The effect of ATP7B gene silencing on silver-sensitivity was first evaluated in HepG2 cells. We examined the ability of short hairpin sequences designed to target ATP7B mRNA, delivered by a lentivirus vector to silence ATP7B expression in HepG2 cells (Fig. 5A). Among the three target sequences tested (#198, 199, 200), protein blot analysis showed that the most significant decrease in ATP7B protein occurred in the cells treated with shRNA sequence #200. As predicted, SCC1 was significantly more toxic to the cells treated with this shRNA sequence compared to other shRNA-treated cells (Fig. 5B). Similarly, when the cytotoxicity of SCC1-treated mTEC cultures derived from either wild type or ATP7B null mice littermates was compared, loss of total cells and ciliated cells was significantly greater in ATP7B^{-/-} than in wild type mTEC (Fig. 5C). Together, these findings suggest that the expression of the copper transporter ATP7B in ciliated airway cells is required for detoxification of the silver compound SCC1 in the airway epithelium.

Discussion

These studies are the first to describe the cell-type specific expression of known copper transporters ATP7A, ATP7B and CTR1 in airway epithelial cells and demonstrate responses to silver in an airway model. The role of the P-type ATPases and CTR1 in the cellular metabolism of copper and silver has been previously shown in bacteria and cell lines (Solioz and Odermatt, 1995; Petris *et al.*, 1996; Verheijen *et al.*, 1998; Lee *et al.*, 2002; Stoyanov *et al.*, 2003). However, there has been little focus on the handling of silver in mammalian cells. The importance of P-type ATPases for silver metabolism in lung cells is supported by the selective killing of ciliated cells by silver and copper, near exclusive localization of ATP7B in ciliated airway epithelial cells, induction of apical ATP7B trafficking in response to a silver compound, and enhanced silver-induced ciliated cell killing in airway cells from mice genetically deficient in ATP7B. Furthermore, the copper uptake transporter CTR1 is also expressed uniquely in ciliated airway epithelial cells. These findings lead us to propose that uptake of silver through CTR1, followed by detoxification via ATP7B-trafficking and secretion is a key pathway for silver transport in the airway (Fig. 6).

The mTEC system serves as a useful tool for understanding silver and copper metabolism in the lung and as a valuable screen for testing the in vitro cytotoxicity of new silver-containing compounds prior to in vivo testing. Using this preparation, we found that mTEC were more sensitive to killing by silver than copper (Figure 2). This observation may reflect that the copper transport system does not handle the non-essential metal, silver in a fashion identical to that of copper. The mTEC system allowed us to uncover that the ciliated cell-specific responses involved in detoxification of silver were related to the expression of both CTR1 and ATP7B. In our model, CTR1 likely mediates silver uptake while SCC1 induces trafficking of ATP7B to the apical membrane for efflux from the cell. The responses that we observed were similar to that reported to occur in hepatocytes where CTR1 mediates copper uptake and ATP7B traffics to the apical surface to secrete copper into the biliary fluid (Roelofsen *et al.*, 2000; Guo *et al.*, 2005; Bartee and Lutsenko, 2007; Kim *et al.*, 2009). Taken together, we propose that the CTR1-mediated uptake of silver occurs at a greater rate than ATP7B-mediated secretion of this metal at the apical membrane resulting in accumulation of silver in the ciliated epithelial cells.

The function of ATP7A in airway cells has not been fully identified, however, we noted that SCC1 failed to induce the translocation of ATP7A to the plasma membrane of non-ciliated cells at concentrations that induced trafficking of ATP7B in ciliated cells. This lack of translocation is likely due to less efficient uptake of silver by non-ciliated cells that may occur through poorly described CTR1-independent routes (Kim *et al.*, 2009; Larson *et al.*, 2009). In contrast, polarized epithelial cells, such as intestinal or renal cells, in which ATP7A traffics from the TGN towards the basolateral membrane in response to copper, also express CTR1 (Greenough *et al.*, 2004; Nyasae *et al.*, 2007). Interestingly, studies of human fibroblasts and cerebellar tissue have demonstrated that the lack of ATP7A function can be compensated by ATP7B and vice versa (Lockhart *et al.*, 2002; Barnes *et al.*, 2005). However, there was an apparent inability of ATP7A to compensate for ATP7B in the airway epithelium since SCC1-associated ciliated cell death was significantly enhanced in mTEC from ATP7B deficient mice. Thus, cell-type specific expression of ATP7A, ATP7B and CTR1 occurs, although a different combination of transporters may be present in any given tissue.

Recent work has highlighted roles for ATP7A, ATP7B and CTR1 activity in cancer drug resistance (Kuo *et al.*, 2007). Overexpression of ATP7A and ATP7B in human ovarian carcinoma cell lines generated resistance to platinum drugs suggesting cytotoxicity occurs by overloading the P-type ATPase capacity to sequester the drugs (Samimi and Howell, 2006; Dolgova *et al.*, 2009). Furthermore, high levels of ATP7A and ATP7B expression in biopsies of malignant tissues have been associated with more invasive and drug resistant forms of colon and endometrial cancers (Aida *et al.*, 2005; Owatari *et al.*, 2007). Additionally, downregulation of CTR1 has been shown to decrease platinum-based drug cell death in several models (Kuo *et al.*, 2007; Larson *et al.*, 2009). Together, these data suggest that patients with platinum-based drug resistant cancer might have better survival if treated in combination with drugs that would efficiently regulate copper transporters. To date, the ability to target chemotherapy resistance genes has been limited. However, in one study, in vivo gene silencing using ATP7B siRNA in combination with cisplatin resulted in a reduction of platinum resistant ovarian cancer cell growth by 75% compared with controls (Mangala *et al.*, 2009). These studies are directly relevant to the therapeutic use of SCC1, as this and other N-heterocyclic carbene silver complexes have shown activity against ovarian and breast cancer cell lines. More importantly, we have shown SCC1 has therapeutic efficiency in vivo in a mouse model of ovarian cancer (Medvetz *et al.*, 2008).

In summary, our finding that copper/silver transporters are expressed in the airway has a broad relevance in the development of new drugs important in the treatment of resistant lung microorganisms and carcinoma. Our prior in vitro and preclinical in vivo studies indicate that the silver-based SCC1 compound is safe and effective as an antimicrobial and chemotherapeutic agent (Kascatan-Nebioglu *et al.*, 2006; Medvetz *et al.*, 2008; Cannon *et al.*, 2009). Further investigations will evaluate mechanisms for normal copper homeostasis, metal detoxification and roles for these pathways in the potential treatment of pulmonary disease with aerosolized silver compounds either directly in solution or in nanoparticle suspension.

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Abbreviations

SCC1	silver carbene complex 1 (methylated caffeine silver acetate)
mTEC	mouse tracheal epithelial cells
DAPI 4'	6-diamidino-2-phenylindole
β -tub	β -tubulin-IV

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Figure 1. SCC1 dose-dependent loss of ciliated airway epithelial cells

(A) Representative photomicrographs of mTEC treated with the indicated concentration of SCC1 for 24 h then immunostained with ciliated cell marker β -tubulin-IV (β -tub) and DNA marker DAPI. Bar, 100 μ m. (B) Quantification of the effect of SCC1 on cell-type specific survival as in A. Total (DAPI+), non-ciliated (DAPI+, β -tub-) and ciliated (DAPI+, β -tub+) cells are shown as the mean $\pm$ SD from two independent experiments. A significant decrease in cell number between untreated and SCC1-treated samples are indicated for total ($\ddagger$), non-ciliated ($\dagger$), and ciliated (*) cells ($p < 0.01$).

Figure 2. SCC1-induced loss of ciliated cells is silver and copper dependent

mTEC preparations were incubated with media containing the indicated compound for 24 h, then cell types identified and quantified by immunostaining as in Figure 1. Quantification of total (A), non-ciliated (B) and ciliated cells (C) in mTEC preparations following treatment with indicated concentration of SCC1, methylated caffeine (Meth Caff), AgAcetate and CuCl_2 . All compounds were equimolar (e.g., 0.267 mM at the concentration of 100 $\mu\text{g/mL}$ of SCC1). Shown is the mean $\pm$ SD of triplicate samples.

Figure 3. Cell-specific expression of ATP7A and ATP7B in airway epithelial cells
(A) Expression of copper transporters ATP7A and ATP7B in indicated mouse tissues or cells. Equal amounts of protein in each sample were analyzed by western blot. **(B)** Photomicrograph of mTEC preparations immunostained for detection of ATP7A (red; top row), and ATP7B (red; bottom row) and ciliated cells identified by cilia marker β -tubulin-IV (β -tub). En face view shows expression in well-differentiated, confluent mTEC preparations. Single cells of merged immunofluorescent and differential interference contrast (DIC) images show intracellular localization of ATP7A and ATP7B (far right panels). Bar, 10 μ m.

Figure 4. SCC1-induced trafficking of ATP7B and cell-specific expression of CTR1
(A) mTEC preparations were treated with media (top row) or with SCC1 (50 $\mu\text{g/mL}$, 24 h, bottom row), then immunostained for ATP7B (red), and ciliated cell marker β -tubulin-IV (β -tub, green). Arrow indicates apical expression of ATP7B in cell treated with SCC1. **(B)** Expression of CTR1 and β -tubulin-IV (green). DAPI (blue) staining identifies nuclei. Bar, 10 μm .

Figure 5. ATP7B-dependent toxicity of SCC1 in airway epithelial cells

(A) Protein blot analysis of expression of ATP7B and GAPDH in HepG2 cells treated with ATP7B-specific shRNA constructs (#198, 199, 200). (B) Quantification of HepG2 cells survival following treatment of cells (10 µg/ml SCC1, 24 h) with the indicated shRNA construct shown in panel A. Cell survival (percent mean $\pm$ SD) following SCC1 treatment was compared to cells treated with media only for each shRNA construct. A significant difference in cell number between shRNA-uninfected and -infected HepG2 cells is shown (*, $p < 0.01$). A difference between shRNA constructs #198, 199 and 200 is indicated (†). These data are representative of replicate measurements from two independent experiments. (C) Cell survival in mTEC preparations from wild type (WT) and ATP7B^{-/-} mice following incubation for 24 h with media or SCC1 evaluated by immunofluorescence using cell markers as in Fig. 1B. Shown are the mean values $\pm$ SD of 2 independent experiments, and a significant difference in ATP7B^{-/-} compared with WT mTEC (*, $p < 0.01$).

Figure 6. Model of cell-type specific copper and silver transport in the airway epithelium
CTR1 at the membrane imports silver compound SCC1 into the ciliated epithelial cells, inducing trafficking of the P-type ATPase exporter, ATP7B to the apical membrane for metal efflux. Non-ciliated cells express ATP7A, but not CTR1 or ATP7B. Because non-ciliated cells do not express CTR1, intracellular silver concentration likely remains low and ATP7A trafficking is not induced.