Preclinical evaluation of the ENaC inhibitor BI 1265162 for treatment of cystic fibrosis

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Online Supporting Information

Methods

In vitro inhibition of Na^+ transport

The inhibition of Na^+ transport by BI 1265162 was tested in a mouse renal collecting duct cell line (M1) and a human bronchial epithelial cell line (NCI-H441) using a custom-made Ussing chamber.

M1 cells were cultivated in Dulbecco's Modified Eagle Medium, containing 5% foetal calf serum (FCS) and 5 µM dexamethasone for 10–12 days on polyester Transwell® filters. NCI-H441 cells were cultivated in RPMI1640 containing 10% FCS, 1% sodium pyruvate and 5 mL insulin/transferring/selenium on polyester Transwell® filters at 37°C, 5% CO_2 and 95% humidity. After 2 days, the basolateral bath solution was replaced with culture media containing 200 nM dexamethasone. The apical bath solution was removed to create an air–liquid interface. Dexamethasone was used in both cell lines to upregulate ENaC expression and to achieve sufficient short circuit current ($I_{SC;}$ a measure of net ion transport).[1]

Filters containing cell layers were inserted into a Teflon-coated well plate, which fitted into the Ussing chamber. The amount of the transported Na^+ was measured by the application of the I_{SC} that was necessary to bring the measurable voltage difference down to 0 mV.[2] I_{SC} was measured in the voltage clamp mode using a custom-built amplifier (Boehringer Ingelheim, Biberach, Germany), with the software package Lab View used for data acquisition and analysis. Every cell filter was used as its own control. Measurements were only conducted if a minimum transepithelial electrical resistance of 800 Ω or 250 Ω, and an I_{SC} of 1.7 microampere (µA), or between 8 and 17 µA, were reached (dependent on cell type; for M1 and NCI-H441, respectively).

BI 1265162 (Boehringer Ingelheim GmbH & Co.KG, Biberach, Germany) was administered in a stepwise manner at final concentrations of 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} M to the apical solution of each filter. At the end of each experiment, I_{SC} was measured by adding 3×10^{-6} M amiloride, an inhibitor of transepithelial Na^+ transport,[3] to the apical compartment of each filter. Differences in I_{SC} after addition of test compounds were compared with the amiloride effect (maximum inhibition). Results were expressed as inhibition in percent of the amiloride effect, and the inhibition concentration that reduced the effect by 50% (IC_{50}) was calculated by the GraphPad Prism software package (GraphPad Prism for Windows, version 5).

In vitro inhibition of water resorption

ENaC-mediated water transport with and without BI 1265162 was measured using a Transwell® system. M1 cells (5,000 cells/well) were grown in a monolayer on microporous membranes suspended in Lonza PC-1 medium in a Costar 3470 Transwell® plate (E-Figure 1). Experiments were carried out in duplicate.

E-Figure 1: Set-up of the Transwell[®] system
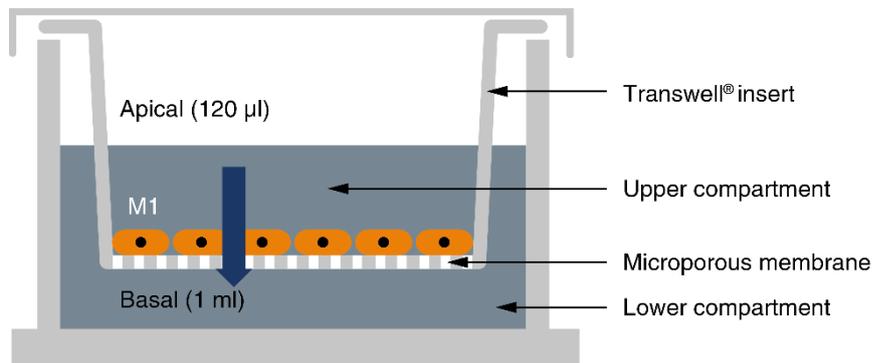
To assure a tight diffusion barrier on Day 10 after seeding, the resistance between the apical and basolateral compartment divided by the cell layer was measured using a voltmeter. Inserts containing cells showing a resistance between 2,200-2,800 Ω were used for measurements.

PC-1 medium (500 µL per well) was added into an assay plate (Costar 3524, basolateral) and equilibrated for 2 hours in a humidified incubator at 37°C, 5% CO_2. The membranes containing M1 cells were put into the assay plate and the apical liquid volume from the M1 cell layers was carefully aspirated. 3 µM BI 1265162 in 120 µL of saline (n=3 replicates for each experiment) or saline alone was added apically. The assay plate was stored for 24 hours in a humidified incubator at 37°C, 5% CO_2.

In order to seal the membrane after the 24-hour incubation, each insert was placed into a Petri dish with silicon paste. Since the volume differences measured in this in vitro assay were very small, a radiometric dilution assay was employed. For determination of volume of water on the apical side, 80 µL of diluted ^{3}H water (Hartmann Analytics, Braunschweig, Germany) was added and mixed with the apical

volume of the Transwell®; 80 µL of this mixture was transferred into a scintillation vial. In parallel, the entire apical volume was transferred directly without mixing into six scintillation vials (measurement of whole activity). 3.5 mL Ultima Gold scintillation solution (Perkin Elmer) was added to each vial, vortexed and the amount of radioactivity (disintegration per minute [dpm]) was measured. Unpaired t-test was carried out and the values $P<0.05$ were nominally considered statistically significant.

In vivo inhibition of liquid resorption

Efficacy of BI 1265162 on inhibition of liquid absorption from the airway epithelium of male Wistar rats was assessed. Animal studies were performed according to the German Animal Welfare Regulations and approved by the Tübingen Regional Administrative Council, licence 12-009.

Rats weighing 260–340 g were randomly assigned to the control and treatment groups (n=8 per group). The animals were anaesthetised with isoflurane prior to intratracheal instillation of test compound or vehicle. For the treatment groups, Ringer Lactate Solution pH 5 (vehicle) alone or with BI 1265162 was instilled in a volume of 5 mL/kg. The following concentrations of BI 1265162 were tested: 10, 3, 0.3 and 0.03 µg/kg of body weight. After this, animals were awake again; three hours after instillation, they were re-anaesthetised once more and blood was withdrawn to obtain serum. Aldosterone was determined via ELISA (Aldosterone ELISA, BIOTREND) from serum. The animals were sacrificed under anaesthesia by cervical dislocation. Lungs were removed, non-pulmonary tissue was dissected, and the weight of the lung was determined. To calculate the inhibition of liquid transport in the current set-up, historical data of lung weights of a negative control group (treated only with 5 mL/kg Ringer Lactate Solution before weighing) and an untreated control

group were used as reference. The variable 'inhibition' used for statistical analysis was calculated from the lung weight using the following equation:

$$y_{inh} = 1 - \left(\frac{1 - \left(\frac{y_i - \bar{y}_u}{\bar{y}_{neg} - \bar{y}_u} \right)}{1/n_p \sum_{j=1}^{n_p} \left(1 - \left(\frac{y_{j,p} - \bar{y}_u}{\bar{y}_{neg} - \bar{y}_u} \right) \right)} \right) * 100$$

where y_{inh} = inhibition [%],

y_i = lung weight of each animal i,

$\bar{y}_u$ = mean lung weight of the untreated (historical) control group,

$\bar{y}_{neg}$ = mean lung weight of the negative (historical) control group,

n_p = number of animals in the placebo (day control) group,

$y_{j,p}$ = lung weight of each animal j in the placebo (day control) group.

The mean lung weight of the historical control groups was measured separately for each experiment. Each treatment group had its own historical control group assigned according to historical body weight. The animals of the historical control groups were chosen by the smallest and highest body weight value of the respective experimental groups. Data from all experiments, carried out on three separate occasions, were combined to estimate the dose-response curve. Effective doses at 50, 70 and 90% of the maximum effect of BI 1265162 were estimated by means of non-linear regression.

Acceleration of MCC in a sheep model

Stimulation of MCC by BI 1265162 (0.1, 1.0, and 10 µg/kg) was tested in a sheep model of whole-lung clearance measuring the retention of a radioactive tracer over 2 hours.

The studies were conducted at Mount Sinai Medical Center (Miami, FL, USA) after approval from the Mount Sinai Medical Center Animal Research Committee.

Adult ewes (25–45 kg, Florida Native; 1–4 years old; Fair Meadows Farm, Ocala, FL, USA) were anaesthetised by local application of lidocaine prior to nasal intubation via bronchoscope, then administered a total of 3 mL (varying compound doses with n=2 in each dose group) using an AirLife™ nebuliser. The animals received the compound or vehicle (deionised water). Aerosolized technetium-labelled sulphur colloid (99mTc-SC; 20 mCi) was administered to measure the effects of the various doses of test compounds or control on MCC by gamma scintigraphy as previously described.[4] Administration of 99mTc-SC occurred at the stated times following administration of drug or control. Serial images were then obtained over a 1-hour period at 5-minute intervals for the first hour and then every 15 minutes for the next hour. Counts from the right lung were corrected for decay and expressed as a percentage of radioactivity cleared relative to the baseline image (% cleared).

Retention of radioactive tracer to determine MCC and plasma potassium levels were expressed as mean ± range. A one-way analysis of variance (ANOVA) was performed on the retention values at 1 hour and 2 hours after radiolabel delivery, versus vehicle control.

Blood was collected and plasma potassium determined. Plasma potassium levels were analysed across time for each dose using a one-way ANOVA.

In vitro effect on water transport and MCC on CF donor cells
The effect of BI 1265162, with or without ivacaftor (IVA; CFTR corrector) and lumacaftor (LUM; CFTR potentiator) and isoproterenol (ISO) (which, as a non-selective β-adrenoreceptor agonist, upregulates CFTR expression) on water

transport, MCC and ciliary beat frequency (CBF) was evaluated using MucilAir™. This is a fully differentiated, pseudostratified 3D model of human epithelium, reconstituted in vitro from either primary human bronchial cells isolated from a donor without CF (MucilAir™) or from a CF donor (ΔF508/homozygous, MucilAir™-CF). MucilAir™/-CF demonstrates the full functionality of the epithelial tissue when cultured on semi-porous membrane. Experiments were carried out on n=5 cultures per treatment.

Proprietary culture medium containing 50 µL BI 1265162 (0.1 and 10 µM) was applied on the apical surface of MucilAir™ and MucilAir™-CF, with 0.1 µM IVA / 3 µM LUM and with or without 100 µM ISO in the basolateral compartment.

Water transport was measured on Days 2 and 5 of the study by weighing the semi-porous membrane inserts containing the cells.

MCC was measured on Day 7 by using a Sony XCD-U100CR camera connected to an Olympus BX51 microscope with a 5x objective. Polystyrene microbeads (30 µM in diameter) were added on the apical surface of MucilAir™ and movements were video-tracked at two frames per second for 30 images at room temperature. Three movies were taken per insert. Average beads movement velocity (µM/sec) was calculated with the ImageProPlus 6.0 software. Data were expressed as mean ± standard error of the mean. Differences, induced by treatments, were tested by a one-way or two-way ANOVA using Prism 6 GraphPad software (La Jolla, CA, USA). Dunnett's multiple comparison post-tests were used to compare every mean to control mean (vehicle) for each series of conditions. The values $P<0.05$ were nominally considered statistically significant.

References

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