Targeting αENaC with an Epithelial RNAi Trigger Delivery Platform for the Treatment of Cystic Fibrosis

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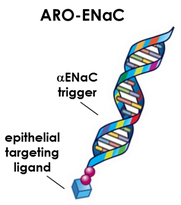
RATIONALE

In cystic fibrosis (CF), mutations in the CFTR chloride channel gene are associated with increased epithelial sodium channel (ENaC) activity which contributes to airway dehydration and reduced airway mucociliary transport[1]. Hypomorphic ENaC alleles increase mucociliary transport[2] and modify CF lung disease to milder phenotypes[3], but the development of inhaled small molecule inhibitors has been limited by their short duration of action and side effects resulting from renal ENaC inhibition[4]. To enable durable, renal-sparing therapeutic ENaC inhibition for CF, we have utilized Arrowhead's Targeted RNAi Molecule (TRiM™) technology to develop ARO-ENaC, an epithelial-targeted conjugate comprised of an optimized RNAi trigger against αENaC paired with an epithelial targeting ligand (EpL) to the integrin αvβ6 receptor[5].

METHODS

- *In vitro* uptake by cultured cells was evaluated by fluorescence microscopy. Tracking conjugates were prepared by conjugating EpL ligands to Cy3-labeled polyacrylate polymer or to Cy3-labeled RNAi triggers
- Integrin αvβ6 receptor-mediated endocytosis of EpL-RNAi conjugates was quantitated by on-cell Western receptor internalization assay (OCW-RIA) in HT29 cells with an antibody to the integrin αvβ6 receptor
- Three versions (v1-3) of EpL-αENaC RNAi trigger conjugate were studied, employing different αvβ6 ligands and configurations, linker chemistries and trigger modifications
- Rats received intratracheal (IT) or oropharyngeal (OP) doses of EpL-RNAi trigger conjugate or RNAi trigger alone; total RNA was isolated from whole lung and kidney homogenates and mRNA expression analyzed by qPCR. Protein expression was evaluated by immunohistochemistry (IHC) with a polyclonal antibody to αENaC
- For nose-only inhalation studies, rats were exposed to EpL-RNAi trigger conjugate aerosolized with an Aeroneb Solo vibrating mesh nebulizer. Deposited pulmonary doses were calculated using the respiratory minute volume, aerosol concentration, exposure time, deposition fraction (estimated at 10%), and body weight

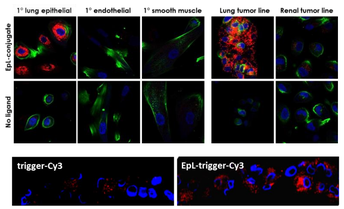
TRiM™ platform



- Rules and algorithms allow selection of optimized RNAi trigger sequences
- Limit cross-reactivity with off-target genes
- Maximize innate stability
- Rational use and placement of modifying chemistries
- Active endosomal escape chemistries not required
- Targeting ligands and linker chemistries improve delivery to target tissues
- Integrin αvβ6 ligands facilitate pulmonary epithelial uptake and endocytosis of triggers

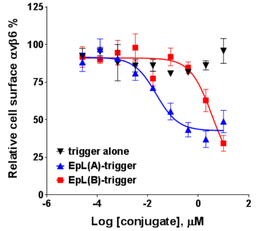
RESULTS

αvβ6 ligands facilitate epithelial uptake of Cy3-labeled tracking conjugates



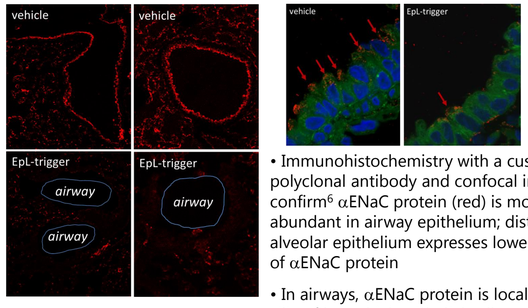
- Cy3-labeled tracking polymer (red) conjugated to αvβ6 (EpL) ligands are selectively endocytosed by αvβ6+ epithelial cells *in vitro* (upper panel)
- EpL ligands enhance uptake of Cy3-labeled RNAi trigger in primary human bronchial epithelial cells maintained in ALI culture (lower panel)

Quantitation of αvβ6 receptor mediated endocytosis of EpL-trigger conjugates by OCW-RIA

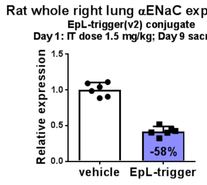


- HT29 cells were exposed to RNAi trigger alone or trigger conjugated to 2 different EpL ligands (A or B)
- Trigger alone does not stimulate αvβ6 receptor internalization monitored by antibody bound to cell surface
- Trigger conjugated to EpL(A) or EpL(B) stimulates αvβ6 receptor internalization with IC_{50} values of 21 nM or 3597 nM

EpL-trigger conjugates eliminate expression of αENaC protein in rat airways

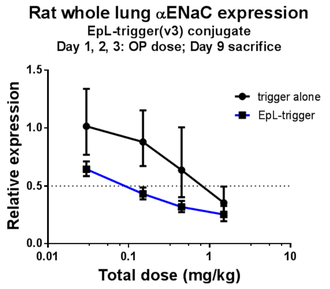


- Immunohistochemistry with a custom polyclonal antibody and confocal imaging confirm[6] αENaC protein (red) is most abundant in airway epithelium; distal alveolar epithelium expresses lower levels of αENaC protein
- In airways, αENaC protein is localized to cilia of bronchial epithelial cells, confirming previous observations[6]



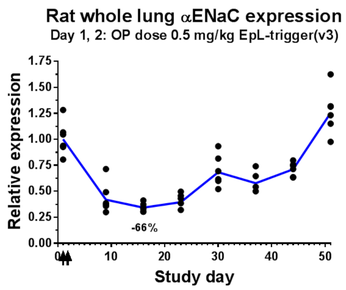
- Single IT doses of EpL-αENaC RNAi trigger that produce ~50% whole lung αENaC mRNA knockdown on Day 9 are associated with significantly reduced airway epithelial αENaC protein expression; remaining αENaC protein predominantly restricted to distal alveolar epithelium

EpL platform increases trigger potency 10x and improves uniformity of whole lung αENaC mRNA knockdown



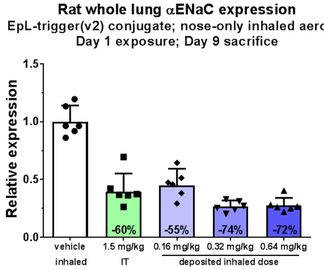
- Rats received three q.d. OP doses of αENaC RNAi trigger alone or EpL-trigger conjugate and sacrificed six days after last dose
- EpL-trigger conjugate reduces lung αENaC mRNA expression >50% with a total dose of 0.15 mg/kg. Equivalent knockdown with trigger alone requires 1.5 mg/kg
- EpL targeting improves uniformity of lung knockdown at low exposures, consistent with improved delivery to airway epithelium

EpL-trigger conjugates mediate durable whole lung αENaC mRNA knockdown



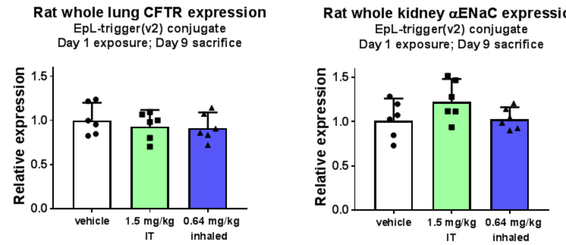
- Rats received two q.d. OP doses of EpL-trigger conjugate; treatment groups were sacrificed weekly
- Maximum reduction in lung αENaC mRNA (nadir) is 2 weeks after dosing; expression fully recovers between 6-7 weeks post-dose
- Durability of knockdown supports an every other week maintenance dose regimen. With other targets, duration of knockdown in primates is typically longer than rodents

Aerosol inhalation of EpL-trigger produces equivalent whole lung αENaC mRNA knockdown at 10x lower exposure



- Rats received a single IT dose or inhaled aerosol exposure of EpL-trigger conjugate and were sacrificed 8 days later
- Nose-only aerosol inhalation improves efficiency of EpL-trigger delivery, producing an equivalent reduction in αENaC mRNA expression at a 10-fold lower exposure than an IT dose

EpL-trigger conjugates mediate lung knockdown with no effect on lung CFTR or kidney αENaC mRNA expression



- At IT and aerosol exposures that reduce lung αENaC mRNA expression by 60% and 72% respectively, no changes are observed in lung CFTR chloride channel or kidney αENaC mRNA expression

CONCLUSIONS

- EpL integrin αvβ6 receptor ligands improve endocytosis of RNAi triggers in cultured epithelial cells
- EpL conjugates employing ligands to the integrin αvβ6 receptor improve functional delivery of an αENaC RNAi trigger to the pulmonary epithelium after inhalation, producing deeper and more consistent reduction of whole lung αENaC mRNA at lower doses
- Loss of airway epithelial αENaC protein expression is observed at exposures that produce ~50% reduction in whole lung αENaC mRNA, with remaining protein expressed in alveolar epithelium
- Reduction of lung αENaC mRNA expression is durable, maintaining >50% knockdown at 3 weeks post-dose and requiring 6-7 weeks for recovery to baseline expression
- Aerosol inhalation improves delivery efficiency of EpL-αENaC RNAi trigger conjugates approximately tenfold over intratracheal administration
- EpL-αENaC RNAi trigger conjugates are well-tolerated with no observed changes in renal αENaC mRNA expression
- ARO-ENaC for cystic fibrosis is Arrowhead's first therapeutic candidate to employ the pulmonary epithelial delivery platform
- The ability of the EpL-RNAi platform to facilitate functional delivery of RNAi triggers to the lung suggests that additional therapeutic targets in the pulmonary epithelium could be considered, particularly those that are currently inaccessible to traditional small molecule or antibody approaches

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