CAT-5571 AS A NOVEL AUTOPHAGY ACTIVATOR THAT ENHANCES THE CLEARANCE OF PSEUDOMONAS AERUGINOSA

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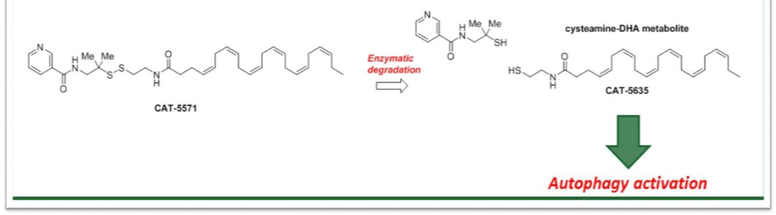
ABSTRACT

In CF patients, the lack of a proper functioning CFTR causes a chronic lung infection that is difficult to treat with conventional antibiotics. *Pseudomonas aeruginosa* is a common and particularly virulent pathogen that can cause a significant level of morbidity and mortality. Intracellular bacterial colonization of *P. aeruginosa* in macrophages, mast cells or epithelial cells is particularly difficult to eradicate with antibiotics, even with those that are cell-permeable. Autophagy is a catabolic process that cells use to degrade various defective proteins/foreign pathogens and convert them into useful cellular building blocks such as amino acids and lipids. In CF, it is known that autophagy is impaired and this can further compromise the patient's ability to clear the chronic lung infection. Autophagy activation enables an alternative mechanism to clear the bacterial infection out of cells; and therefore, could potentially be useful when used in combination with anti-infective agents. The fatty acid cysteamine conjugate CAT-5571 can activate autophagy in cultured primary homozygous F508del human bronchial epithelial (hBE) cells at concentrations as low as 0.3 μM. In an in vitro study involving hBE cells that had been infected with *P. aeruginosa*, a significant reduction in the intracellular bacterial load was observed when cells were pre-treated with CAT-5571. The reduction in the CFU was comparable to that observed when similarly infected hBE cells were treated with Cytochalasin D, a cell-permeable antibiotic. The bacterial clearance was demonstrated first in an in vivo study using female BALB/c mice that were dosed orally with CAT-5571 for 3.5 days prior to infection with a lethal challenge of *P. aeruginosa*. CAT-5571 was then evaluated in a chronic *P. aeruginosa* infection model involving *Cftr* gut corrected mouse B6.129 *Cftr*^{tm1Kth} Tg(FABPCFTR)1Jaw/Cwr (gut corrected F508del) . In this in vivo model of CF lung infection and inflammation, treatment with CAT-5571 resulted in a decrease in bacterial load (82506 ± 36048 without drug to 1657 ± 1406 with drug, n = 7). Although there was no difference in the total white blood cell count in bronchoalveolar lavage fluid, there was a shift away from neutrophils (26.44 ± 10 versus 8 ± 3, n ≥ 6) and increased number of macrophages (71 ± 29 versus 89 ± 9, n ≥ 6) all parameters $p<0.05$, using the analysis of variance between vehicle and CAT-5571 treated animals. CAT-5571 represents a potential new therapeutic to treat the chronic lung infection that is commonly present in CF.

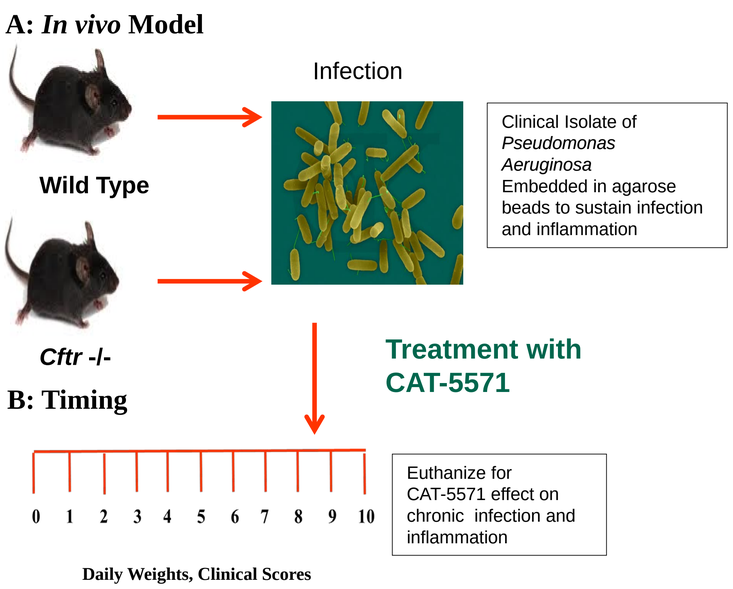
MATERIALS & METHODS

Application: Since patients with CF not only have inflammation, but they are also chronically colonized with bacteria, pre-clinical studies were designed to demonstrate the anti-inflammatory potential of CAT-5571 and the overall impact on *Pseudomonas aeruginosa* colonization. The murine model of CF in which the *Cftr* gene is either knocked out or dysfunctional can provide a consistent and reproducible model in which to measure the differences in the CF host's inflammatory response to pathogens relative to controls with functional *Cftr* providing an ideal window for studying anti-inflammatory drugs in the context of ongoing chronic infection similar to what is seen in patients with CF. As part of the New Anti-Inflammatory Pre-Clinical Modeling Core Center, we explored the potential of CAT-5571 as a new therapeutic for CF, evaluating both it anti-inflammatory potential as well as the impact on pathogen resolution.

CAT-5571 is a novel fatty acid cysteamine conjugate that can synergistically activate autophagy through the simultaneous inhibition of transglutaminase and activation of AMPK



Model Specifics: We used the *Cftr* gut corrected mouse B6.129 *Cftr*^{tm1Kth} Tg(FABPCFTR)1Jaw/Cwr (gut corrected F508del) and controls. For each species (WT and *Cftr* deficient) there were at least two groups: *Pseudomonas areuginosa* infection 10^5 viable-CFUs embedded into agarose beads shown in A. The time line of dosing post infection is shown in B with CAT-5571 BID by gavage. CAT-5571 treatment began 24 hours post infection.



RESULTS SUMMARY

I. In Vitro and In Vivo Development of CAT-5571

CAT-5571 synergistically activated autophagy in human colon and human primary bronchial epithelial cells

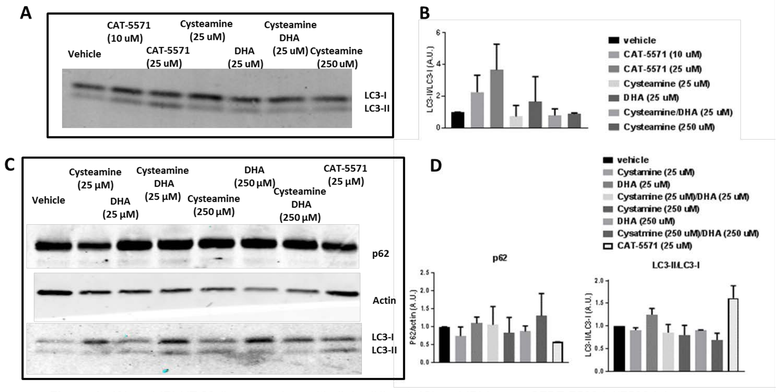


Figure 1. **(A)** and **(B)** HT-29 (human colon epithelial cell lines), **(C)** and **(D)** human CFBE F508del/F508del (KKCFFT004I cells), were treated with CAT-5571, Cysteamine, or DHA (concentrations as indicated) for 24 hrs. Cell lysates were analyzed by immunoblotting with anti-LC3 antibodies (Cell signaling #12741) and p62 antibodies (Cell signaling #8025). **(A)(C)** Representative blots were shown **(B) (D)** Quantification of the immunoblots shown in for the ratio of LC3-II to LC3-I. Error bars represent standard error mean (SEM, n = 3).

Summary: CAT-5571 mediates its anti-inflammatory effects through enhancing autophagy in epithelial cells. Studies to develop CAT-5571 for in vivo administration demonstrate CAT-5571 is effective in vivo at enhancing autophagy in lung tissues

CAT-5571 is orally bioavailable in the mouse, rat and dog. When mice (n = 16) were dosed orally with CAT-5571(100 mg/kg, BID) for 3.5 days, autophagy was activated in lung tissues

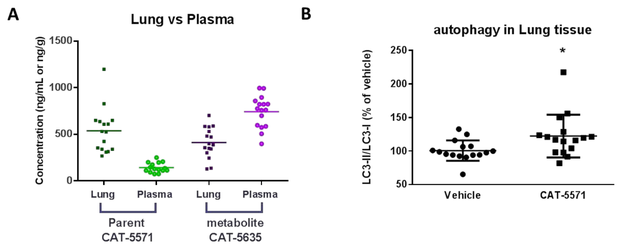


Figure 2. Male C57BL/6 mice (n = 16) were dosed with either vehicle or CAT-5571 (100 mg/kg, p.o. BID for 3.5 days). Plasma and lungs were collected 1 hour after the last dosing. **(A)** Corresponding concentrations of the parent compound (CAT-5571) and the biologically active metabolite CAT-5635 in the plasma and lung tissues. **(B)** Lung tissues were processed and immunoblotting were performed using anti-LC3 antibodies. Quantification of the immunoblots shown in for the ratio of LC3-II to LC3-I. Error bars represent standard error mean (n = 16)

CAT-5571 causes a significant reduction in the intracellular *P. aeruginosa* in F508del CFhBEs

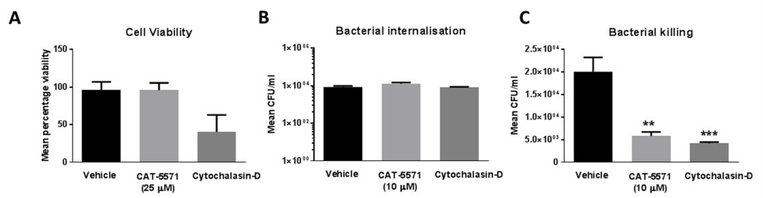


Figure 3. Effect of CAT-5571 on percentage cell viability (A). Human cystic fibrosis F508del/F508del bronchial epithelial cells (Asterand Biosciences, donor ID# 15915) were pre-treated for 24 hours with 500 μl of CAT-5571 (25 μM). As an internal control cells were pre-treated (1 hour) with cytochalasin-D (10 μM). Negative controls consisted of untreated and vehicle only treated control culture and no cells. Data are presented as mean percentage cell viability ± SEM. **Bacterial internalization (B) and Bacterial killing (C).** The same cells were infected with 500 μl of *Pseudomonas aeruginosa* Xen05 at an appropriate MOI (1:50). Prior to bacterial infection, cells were pre-treated for 24 hours with 500 μl of CAT-5571 (10 μM). As an internal control cells were pre-treated (1 hour) with cytochalasin-D (10 μM). Cells were incubated for 2 hours at 37oC with 5% CO2. After incubation the inoculum was removed and all wells were treated with 500 μl of antibiotic mixture (50 U/ml each of penicillin and streptomycin, mixed with 200 μg/ml gentamicin) for 10 minutes (to study bacterial internalization) or 3 hours (to study bacterial killing). Data are presented as mean CFU ± SEM. (**p < 0.01, *p<0.001, one-way ANOVA followed by Dunnet's multiple comparison test).

II. In Vivo Therapeutic Testing of CAT-5571 in the Murine Model of CF Infection and Inflammation

When mice were pre-treated with CAT-5571 for 3.5 days prior to a lethal challenge of *P. aeruginosa*, there was a significant improvement in clinical score and overall survival

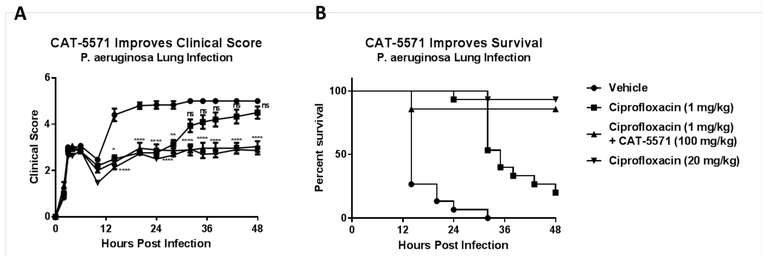


Figure 4. Four groups (n = 15) of female BALB/c mice were used for the study. Animals were treated with CAT-5571 (100 mg/kg BID PO) for 3.5 days prior to the infection and drug treatment was continued until the termination of the study. Control antibiotic were administered as described below: Group 1: Vehicle p.o. (BID from day -3.5) and s.c (BID from 8 hours post infection). Group 2: Ciprofloxacin 1 mg/kg s.c (TBC) (BID from 8 hours post infection), plus vehicle p.o. (BID from day -3.5). Group 3: Ciprofloxacin 1 mg/kg s.c (BID from 8 hours post infection) + CAT-5571 p.o. (BID, 100 mg/kg, from day -3.5). Group 4: Ciprofloxacin 20 mg/kg s.c (BID from 8 hours post infection). **(A)**. Clinical disease scores following infection with 1 X 10^6 CFU *Pseudomonas aeruginosa* Xen 5. Data presented as mean per group (n = 15) ± SEM. Kruskal-Wallis test with Dunn's multiple comparisons (vehicle vs. treatment groups) * < 0.05, ** p < 0.01, **** p < 0.0001. ns = not significant. **(B)**. Survival following infection with 1 X 10^6 CFU Pseudomonas aeruginosa Xen 5. Data presented as percentage survival per group (n = 15) ± SEM.

CAT-5571 lowers bacteria load and reduces pulmonary neutrophilia in *P. aeruginosa* lung infection in a disease relevant mouse model

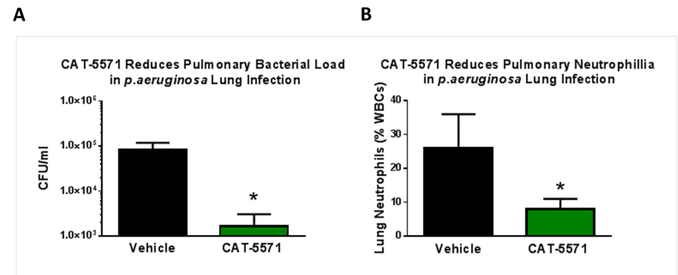


Figure 5. On Day 0, Gut Corrected *Cftr* deficient mice were infected with *P. aeruginosa* embedded in agarose beads to generate a chronic model of infection and inflammation. On Day1, animals were dosed with either CAT-5571 (100 mg/k, BID, PO), or vehicle. Treatment was continued for the next 10 days. On day 3, no significant changes in bronchoalveolar lavage bacterial load, total cell counts or differential of the cell phenotype was observed between the *Cftr* treated animals on CAT-5571 or vehicle. On Day 10, a reduction in bacterial load **(A)** and neutrophil counts **(B)** was observed in CAT-5571 treated animals compared to vehicle controls. All parameters $p<0.05$, utilizing using the analysis of variance between vehicle and CAT-5571 treated animals.

CONCLUSIONS

- CAT-5571 actives autophagy in human colon and human primary bronchial epithelial cells.
- CAT-5571 is bioactive in vivo post-administration into mice, rats and dogs. The end-point of autophagy could be measured in all cases.
- The impact of CAT-5571 appears to be through decreasing intracellular colonization of *Pseudomonas aeruginosa*.
- In the murine model of CF lung infection and inflammation, CAT-5571 decreased bacterial load and lung neutrophils consistent with improved outcomes in the CF murine model.

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