



Original Article

GY971 mitigates inflammation by reducing neutrophil recruitment in cystic fibrosis *Ex Vivo* and *In Vivo* models

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ABSTRACT

Background: There is a prominent need for anti-inflammatory agents for people with Cystic Fibrosis (pwCF), even in the era of CFTR modulators. ETI (Elexacaftor/Tezacaftor/Ivacaftor) reduces but does not eliminate pulmonary inflammation, that chronically damages CF pulmonary tissues and favors recurrent pulmonary exacerbations. Furthermore, although known anti-inflammatory drugs are beneficial to pwCF, their side effects are limiting the clinical use. To address this issue, we developed a new synthetic furocoumarin molecule named GY971, able to reduce the excessive accumulation of neutrophils in the bronchial lumen, by targeting the NF-κB transcription factor (TF).

Methods: To assess its efficacy, GY971 was tested in human primary bronchial and nasal epithelial cells obtained *ex vivo* from different pwCF carrying the *F508del* mutation and infected with *Pseudomonas aeruginosa*. Moreover, GY971 was also administered in a zebrafish model infected with *P. aeruginosa* *in vivo*.

Results: GY971 reduced neutrophil chemotaxis mediators both in CF bronchial epithelial cell lines and in CF primary bronchial and nasal epithelial cells *ex vivo*. The expression of key inflammatory proteins involved in CF lung disease, including IL-8, IL-1β, TNF-α and IL-6, was significantly reduced using nanomolar concentrations of GY971. Importantly, GY971 does not interfere with the ETI-mediated rescue of CFTR protein and showed no cytotoxic effects. Lastly, *in vivo* testing with a zebrafish model confirmed its effectiveness: GY971 decreased neutrophil recruitment in treated larvae across different concentrations, supporting earlier results from murine studies.

Conclusions: GY971 appears to be a promising molecule for the future development of combinatorial anti-inflammatory treatments together with ETI.

1. Introduction

New treatments have significantly improved the prognosis of people with Cystic Fibrosis (pwCF) and today there are more adult patients than children (Supplementary S1).

Despite this progress, CF remains a life-limiting disease, characterized by a progressive reduction of respiratory function conditioned by ongoing respiratory infection and inflammation [1,2].

The CF mucosal epithelium orchestrates chemotactic processes, recruiting neutrophils into the airways, a hallmark of CF lung disease

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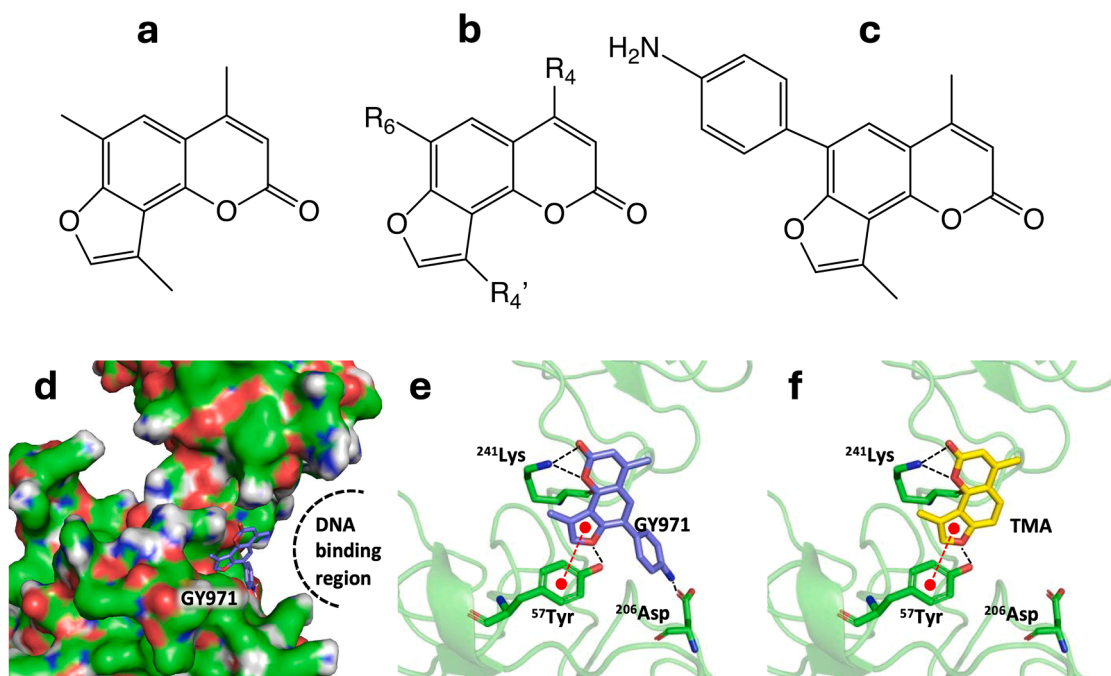


Fig. 1. Chemical structure of TMA (4,6,4'-trimethylangelicin) (a), angular furocoumarin skeleton of TMA derivatives with substitutions in position 4,6,4' (b) and GY971 (6-p-aminophenyl-4,4'-dimethyl-angelicin) (c). Docking simulation (best-ranked pose) between p50 monomer of NF-κB (pdb-id: 1NFK) and GY971 (d, e) and TMA (f), using the Smina software.

[3]. On the contrary, neutrophils accumulate and obstruct the passage of air in multiple lung regions and, once activated by bacterial products and pro-inflammatory cytokines, release DNA filaments, Reactive Oxygen Species and proteases that are ineffective in clearing bacterial infection but produce harmful effects on bronchial walls [2,3].

For all these reasons, there is an urgent need of anti-inflammatory agents for pwCF, acting through novel mechanisms and with a good safety profile. We know from clinical trials that the anti-inflammatory drug ibuprofen significantly prolongs survival and ameliorates the rate of decline of lung function of pwCF, providing a clear proof of concept of the benefits of anti-inflammatory drugs in CF lung disease [4]. Of note, the beneficial effects provided by ibuprofen have been found dependent on the high doses that, possibly by inhibiting NF-κB transcription factor (TF) [5], reduce the recruitment of neutrophils in the conductive airways [4]. Thus, we aimed to reproduce the beneficial anti-inflammatory mechanism of ibuprofen, based on the reduction of recruitment of neutrophils by inhibition of NF-κB, as a promising strategy to ameliorate chronic lung disease of pwCF.

A series of novel synthetic derivatives, including GY971 (6-p-aminophenyl-4,4'-dimethyl-angelicin), was recently designed and synthesized to enhance anti-inflammatory activity while reducing the potential side effects of the lead compound TMA (4,6,4'-trimethylangelicin) (Fig. 1a). These new analogues (Fig. 1b), which can interfere with the formation of the complex between NF-κB TF and the promoter of genes encoding neutrophil chemokines and pro-inflammatory cytokines, were subsequently analyzed to assess their anti-inflammatory effects both *in vitro* and *in vivo* [6,7]. Among all the furocoumarin analogue library tested, GY971 (Fig. 1c) emerged as the most promising candidate, and was designated as an Orphan Drug for CF by the European Medicines Agency in September 2024.

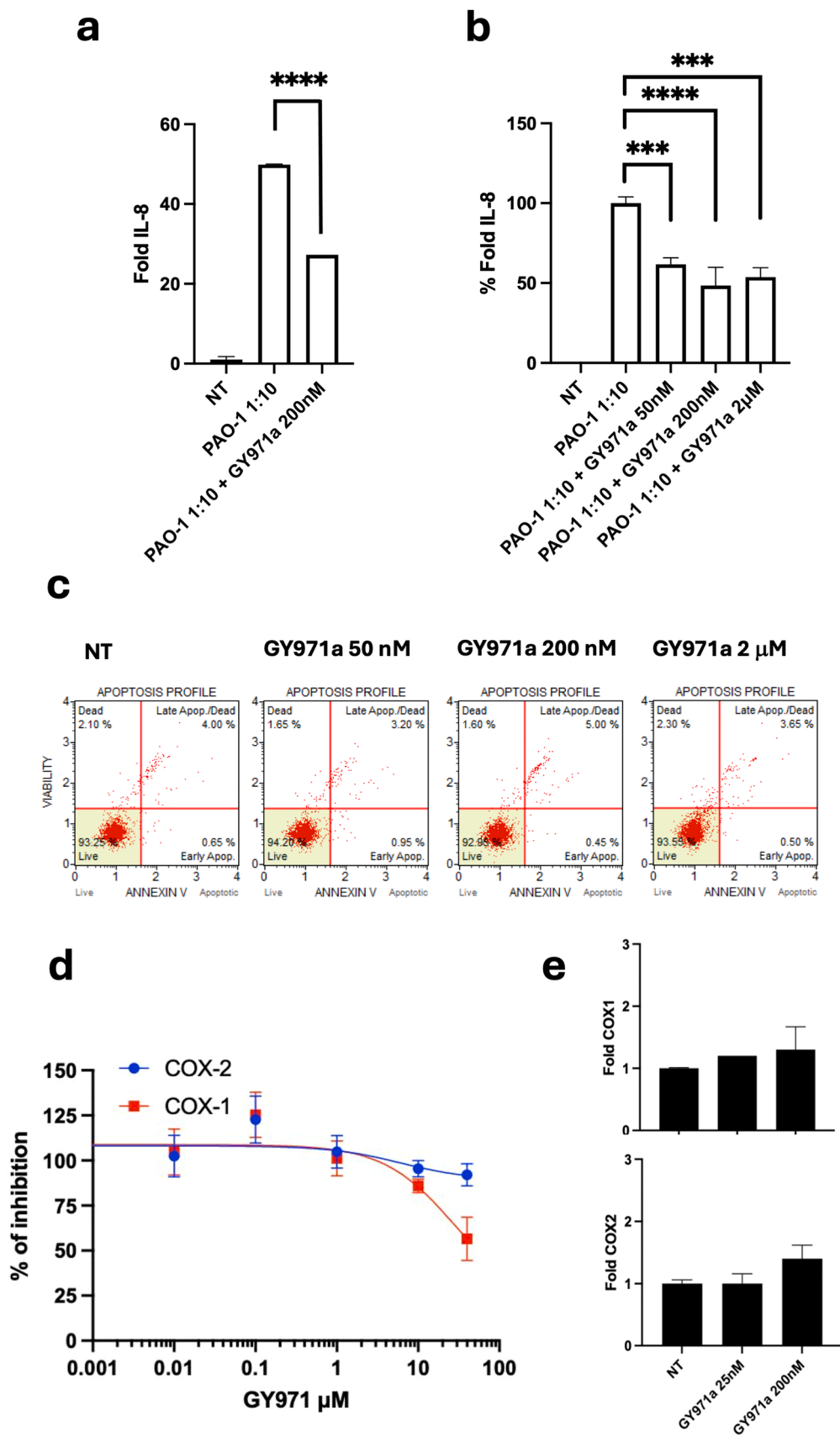
Considering the limitations of the immortalized cell lines model of the previous results [6,7], the first aim of this study is to investigate the anti-inflammatory power of GY971 in primary bronchial and nasal epithelial cells obtained from pwCF *ex vivo*. These cells, that are fully differentiated at the air-liquid interface *in vitro*, mimic as close as possible the potential hindrances of a mucus-producing and tightly polarized surface respiratory layer to the intracellular uptake of the

drugs administered by pulmonary delivery. Thus, GY971 exposed on the apical side of the differentiated layer, could provide preliminary insights on the effectiveness of a pulmonary delivery of this molecule. Since pro-inflammatory mediators have been found to potentiate correction of mutated CFTR protein [8–10], a second aim is to exclude negative interference of the GY971 anti-inflammatory molecule with the rescue of F508del CFTR obtained with Highly Effective Modulator Therapy (HEMT). In addition, recalling the positive results previously obtained in murine lungs infected with *P. aeruginosa in vivo* [7], a third aim is to strengthen the effectiveness of GY971 in a new *in vivo* model of infection-driven induction of neutrophil chemotaxis, such as the zebrafish model. Finally, as GY971 reproduces the mechanism of action on NF-κB, that renders ibuprofen at high dose beneficial to pwCF [4], whereas the clinical use of ibuprofen is abandoned in pwCF due to its severe gastrointestinal side effects dependent on Cyclooxygenase (COX)-1 inhibition, the fourth major aim of this study is to check the effect of GY971 on the enzyme activity of COX-1.

2. Methods

2.1. Synthesis of GY971 and *in silico* evaluation

GY971 (free amine)/GY971a (mesylate salt) has been prepared as previously reported [6]. Molecular docking experiments were conducted using the Smina software. The crystallographic structure of NF-κB (PDB-ID: 1nfk) has been obtained through the Protein Data Bank. The p50 monomer structure was prepared with the “dockprep” tool of Chimera ver 1.17.3 software [11]. GY971 and TMA structures were prepared using Avogadro ver 1.2.0 software. The appropriate “.pdbqt” files were prepared by using MGLTools scripts. The protein was included in a search box large enough to contain the entire structure, *i.e.* a blind docking approach was faced. To ensure a complete evaluation of plausible binding poses, the “exhaustiveness” parameter was set to 100. For both TMA and GY971 the lowest energy pose was selected and analyzed.



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Fig. 2. Preliminary *in vitro* results on human bronchial epithelial CFBE41o- cell line and on whole blood. **a, b:** Fold induction of IL-8 gene expression determined by *Pseudomonas aeruginosa* (PAO-1) infection, and relative reduction after treatment with GY971 mesylate salt (GY971a) 200 nM (**a**). Percent reduction of IL-8 mRNA accumulation induced by GY971a at different concentrations, 50, 200 nM and 2 μ M, on PAO-1-infected CFBE41o- cultures (**b**). **c:** Apoptosis profile, verified through Annexin V assay, of bronchial epithelial CFBE41o- cells treated for 24 h with GY971a at different concentrations, 50, 200 nM and 2 μ M. Statistics were performed by ANOVA, values $\pm$ SD from three independent experiments, using Graph Pad Prism 9.0, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$; ns= not significant. **d:** Concentration-response curves for the inhibition of platelet COX-1 and LPS-induced-COX-2 activity by GY971 (free amine) in human whole blood *in vitro*. Increasing concentrations of GY971 (0.01–40 μ M) were incubated with heparinized whole blood samples, in the presence of LPS (10 μ g/mL) for 24 h; after centrifugation, PGE2 levels were analyzed as an index of LPS-induced-COX-2 activity, by a specific RIA. Furthermore, increasing concentrations of the compound were incubated with human whole; after centrifugation, TXB2 levels were measured as an index of platelet COX-1 activity by a specific immunoassay. Results are depicted as percent inhibition (mean $\pm$ SEM, $n = 6$). **e.** COX-1 and COX-2 gene expression in CF BE41o- bronchial epithelial cells treated with GY971a at different concentrations (25 and 200 nM).

2.2. In vitro studies

CFBE41o- (F508del/ F508del) cell line was utilized in this study [12]. Cultures were infected with *Pseudomonas aeruginosa* (PAO-1) and treated with GY971a at different nanomolar concentrations. After cell growth and apoptosis analysis [7], total RNA and protein were extracted, and RT, SYBR Green RT-qPCR & Western Blot assays were performed, as described in Supplementary S2.

2.3. Ex vivo studies

Two different kinds of human primary airway cells obtained from pwCF were utilized: the bronchial epithelial cells (HBE) [13] and the nasal epithelial (HNE) cells [14,15]. Cultures were infected with *P. aeruginosa* (PAO-1), stimulated with TNF- α , and treated with mesylate GY971 (GY971a). In addition, whole blood was used from different donors to study possible activity on COX-1 and COX-2 inhibition [16, 17]. For further and detailed information, see the Supplementary S2.

2.4. In vivo studies

Zebrafish (*Danio rerio*) were maintained at the University of Milan (Aut. Prot. n. 295/2012-A – December 20, 2012). The zebrafish strains *TgBAC(mpx:EGFP)ⁱ¹¹⁴*, known as *Tg(mpx:EGFP)* [18], was maintained according to international (EU Directive 2010/63/EU) and national guidelines (Italian decree No 26 of the 4th of March 2014). Embryos were collected by natural spawning, staged according to and raised at 28 °C in fish water (Instant Ocean, 0.1 % Methylene Blue) in Petri dishes, according to established techniques [19]. At 24 h post fertilization (hpf), 0.003 % 1-phenyl-2-thiourea (PTU; Sigma-Aldrich) was added to the fish water to prevent pigmentation. Embryos were washed, dechorionated and anaesthetized with 0.016 % tricaine (Ethyl 3-aminobenzoate methanesulfonate salt; Sigma-Aldrich), before observations, microinjection and picture acquisitions. PAO-1 culture was prepared as described [20,21]. Further information on PAO-1 infection and treatments is provided in the Supplementary S2.

2.5. Statistical analyses

These studies are provided in the Supplementary methods S2.

3. Results

To pursue the strategy of reducing neutrophil recruitment by targeting NF-kB, we selected the novel furocoumarin derivative GY971, after a long screening of different TMA analogues. The following section begins by presenting the molecular and computational evidence (Section 3.1) confirming the affinity of GY971 for the NF-kB p50 subunit. We then utilized a CF bronchial epithelial cell line to preliminarily demonstrate the compound's capability to reduce the expression of the key neutrophil chemoattractant, IL-8, following *P. aeruginosa* (PAO-1) infection (Section 3.2).

3.1. Potential mechanism of action at molecular level

Published data suggested that GY971 acts by interfering with NF-kB TF [6,7]. The evidence was further supported by *in silico* docking experiments, that confirmed good affinity of GY971 for the protein pocket of the p50 subunit of the NF-kB (Fig. 1d). GY971 established several hydrogen bonds (dashed black lines) as well as an arene-arene interaction (red dashed line) with residues involved in DNA binding (Fig. 1e). For comparison, we also docked the TMA (the hit compound from which GY971 was developed) against NF-kB. The results showed a binding mode that was almost comparable to GY971 (Fig. 1f), but with less favourable interaction energy (Score_{GY971} = −7.91 Kcal/mol; Score_{TMA} = −5.32 Kcal/mol) due to the lack of interactions with ²⁰⁶Asp, consistent with the biochemical data. Indeed, the concentrations required to disrupt the NF-kB/DNA interactions were 100 μ M and 12.5 μ M for TMA and GY971, respectively.

3.2. Effect of GY971 on IL-8 gene expression on PAO-1-infected CFBE41o- bronchial epithelial cells (F508del/ F508del)

CFBE41o- cells, infected with the *Pseudomonas aeruginosa* laboratory strain PAO-1, represent the cell model used for preliminary experiments utilizing GY971 mesylate salt (GY971a), which confirmed its anti-inflammatory activity [7]. The capability of this derivative in reducing the IL-8 gene expression amplified by PAO-1 is summarized in Fig. 2a and b, without cytotoxic or apoptotic effects (Fig. 2c). Gene expression was significantly reduced by GY971a treatment, showing a 38.3 %, 51.5 %, and 46.3 % decrease at 0.05, 0.2, and 2 μ M (Fig. 2b), respectively (relative to the untreated, infected cells).

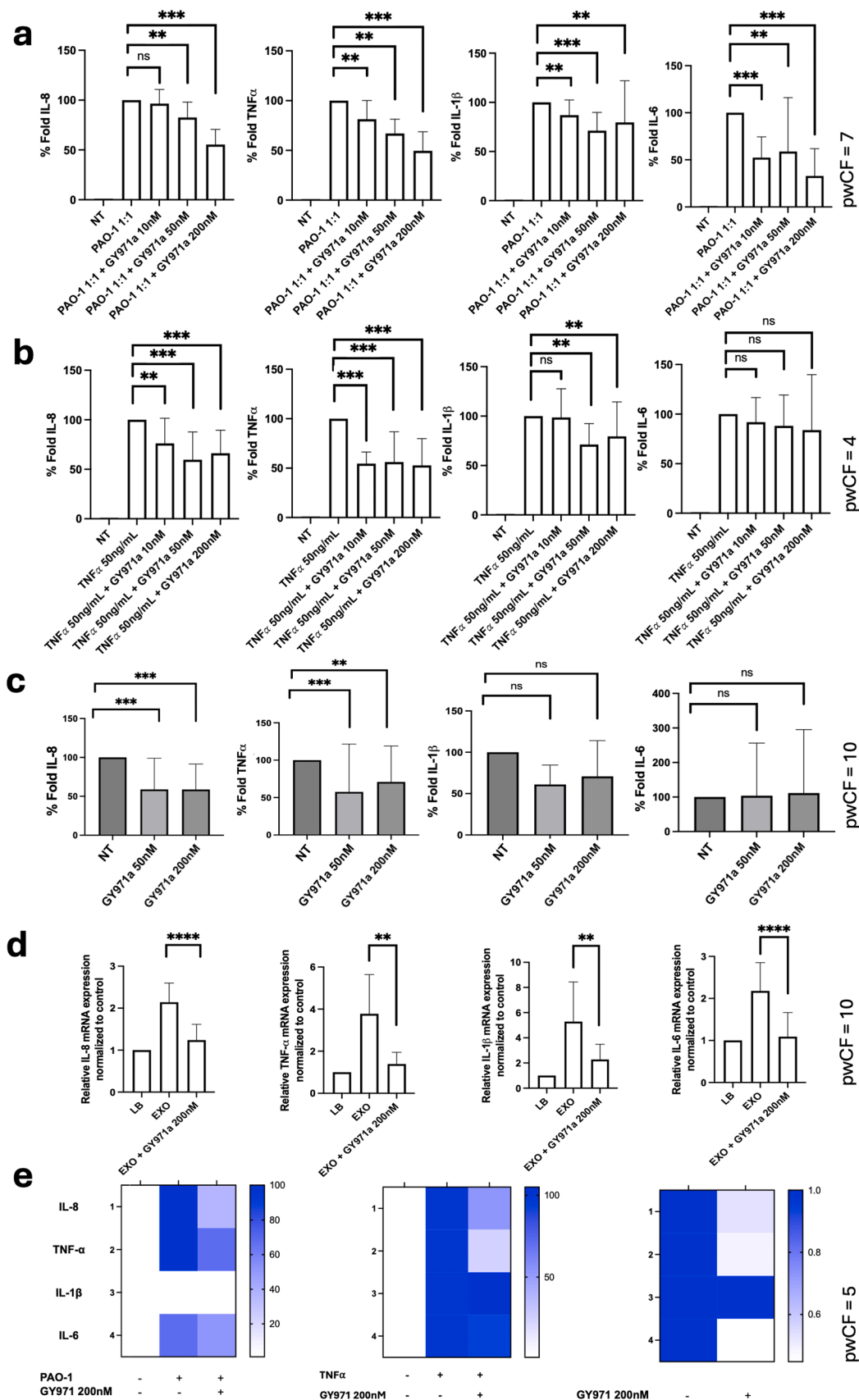
While the preliminary results confirmed that GY971 effectively downregulates PAO-1-induced IL-8 gene expression, a crucial aspect of our design strategy was developing a molecule with a more favorable safety profile than existing anti-inflammatory agents, such as ibuprofen. Therefore, we assessed the potential for gastrointestinal side effects, which are typically caused by strong inhibition of Cyclooxygenase (COX)-1. Section 3.3 presents the evaluation of GY971's inhibitory effects on COX-1 and COX-2 activities.

3.3. Inhibitory effects of the compound on COX-1 and COX-2 activities in human whole blood assays

Despite its clinical effectiveness, the current use of ibuprofen is presently almost abandoned in pwCF due to its severe gastrointestinal side effects dependent on its strong inhibition of COX-1 enzyme [4]. Thus, we wished to investigate the inhibitory effect of GY971 on the enzyme activity of COX in human whole blood assays [16,17], in order to have preliminary insights to exclude a potentially severe adverse effect.

As shown in Fig. 2d, GY971 slightly inhibited platelet COX-1 activity only at the highest concentration (40 μ M) without significantly affecting LPS-induced COX-2 activity in human whole blood. Lastly, GY971 was shown not to induce changes in COX-1 and COX-2 gene expression in CF bronchial epithelial cells (Fig. 2e).

Based on the promising safety profile, we proceeded to validate the



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Fig. 3. IL-8, IL-1 β , TNF- α and IL-6 expression in *P. aeruginosa* diluted 1:1 (PAO-1 strain)-infected (a,e) and TNF- α (50 ng/ml) stimulated (b,e) primary human bronchial epithelial (HBE) cells from different pwCF (F508del/F508del), cultured on Snapwell filters, at the air liquid interface (ALI), and human nasal epithelial (HNE) cells (d) exposed to the exoproducts of *P. aeruginosa* (EXO) obtained from each specific patient after nasal brushing of 10 pwCF bearing F508del-CFTR mutation, grown on transwell filters at the air liquid interface (ALI). Cultures were treated with different nanomolar concentrations (10, 50, 200 nM) of GY971a. The gene expression is indicated as a percentage of fold induction, where PAO-1 (diluted 1:1) is set as 100 % of induction in comparison to 0 % in unstimulated and untreated cells (NT). c: Constitutive IL-8, IL-1 β , TNF- α , and IL-6 gene expression in primary HBE cells from 10 pwCF (F508del/F508del), after treatment with GY971a at different concentrations (50 and 200 nM). e: IL-8, IL-1 β , TNF- α , and IL-6 protein release measured in the basolateral media collected from cell bronchial epithelial cultures derived from 3 pwCF (F508del/F508del). Results are presented as mean $\pm$ SD and statistical significance was assessed by ANOVA, using Graph Pad Prism 9.0, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$; ns= not significant.

therapeutic potential in clinically relevant models. Sections 3.4 and 3.5 detail the comprehensive evaluation of GY971's anti-inflammatory efficacy against key mediators (IL-8, IL-1 β , TNF- α , and IL-6) in primary human bronchial (HBE) and nasal (HNE) epithelial cells obtained *ex vivo* from pwCF carrying the F508del mutation. Moreover, Section 3.6 deepens the compatibility of GY971 with CFTR modulators, such as ETI (Elexacaftor/Tezacaftor/Ivacaftor).

3.4. Effect of GY971 on IL-8, TNF- α , IL-1 β and IL-6 expression in primary human bronchial epithelial (HBE) cells (F508del/ F508del)

The anti-inflammatory efficacy of GY971 was verified in primary CF human bronchial epithelial (HBE) cells derived from pwCF homozygous for the F508del mutation and grown on filters at Air Liquid Interface (ALI). Cells were treated with GY971a at different concentrations (10, 50 and 200 nM), without cytotoxic effects on viability and apoptosis, even at the higher dosage used (Supplementary S3).

IL-8, IL-1 β , TNF- α and IL-6 gene expression and release were evaluated after infecting CF HBE cells with *P. aeruginosa* (PAO-1) or after TNF- α stimulus, and at the basal level. Although variability of the individual immune response should always be considered, the overall and clear effect of GY971 in reducing not only IL-8 expression, but also that of other cytokines is evidenced in the analysis of these samples.

In summary, GY971 reduces the gene expression of IL-8 and affects to some variable extent that of TNF- α , IL-1 β and IL-6 involved in CF lung disease, under PAO-1 infection (Fig. 3a) or using TNF- α stimulation (Fig. 3b). The effect is confirmed also at the constitutive level (unstimulated cells) (Fig. 3c), and in primary human nasal cells (Fig. 3d), as described in the following Section 3.5. Finally, at the protein level (Fig. 3e and Supplementary S4), immunological analysis of the supernatants from primary HBE confirmed data regarding the effect on IL-8, TNF- α and IL-6, while the IL-1 β protein expression wasn't revealed; also, after PAO-1 infection IL-1 β was undetectable. Only after TNF- α stimulation, the IL-1 β concentration was increased.

3.5. Effect of GY971 on IL-8, TNF- α , IL-1 β and IL-6 expression in primary human nasal (HNE) cells (F508del/ F508del)

Recently, we demonstrated that clinical exoproducts (EXO) of *P. aeruginosa* isolated from pwCF showed a variable increase of pro-inflammatory cytokines in clinical strain-specific manner [15]. To consider the individual patient's inflammatory response, we were prompted to investigate the anti-inflammatory effect of GY971 in primary nasal cells exposed to EXO isolated from the corresponding donor. Therefore, we cultured HNE cells in ALI conditions for 16–18 days and then treated for 24 h with 200 nM GY971a at the basolateral side and with clinical EXO at the apical side to mimic the chronic airway infection. Despite the patient-to-patient variability, GY971a significantly reduces the IL-8, TNF- α , IL-1 β and IL-6 mRNA level in infected HNE cultures (Fig. 3d).

3.6. Effect of GY971 on the rescue of F508del CFTR by ETI in CFBE41o-cell line and in primary HBE and HNE cells

Since inflammatory status has been claimed by some reports to increase the rescue of F508del CFTR by CFTR modulators in primary HBE

cells [8–10], we wondered whether the anti-inflammatory effect of GY971a might negatively affect ETI's rescue of F508del CFTR. Therefore, we treated F508del-CFTR CFBE cell line and primary HBE cells with VX-445+VX-661+VX-770. Immunoblotting studies, as shown in Fig. 4a–d, demonstrated that the anti-inflammatory effect of GY971a does not reduce ETI-dependent rescue of F508del CFTR protein processing in both CFBE41o- and primary HBE cells.

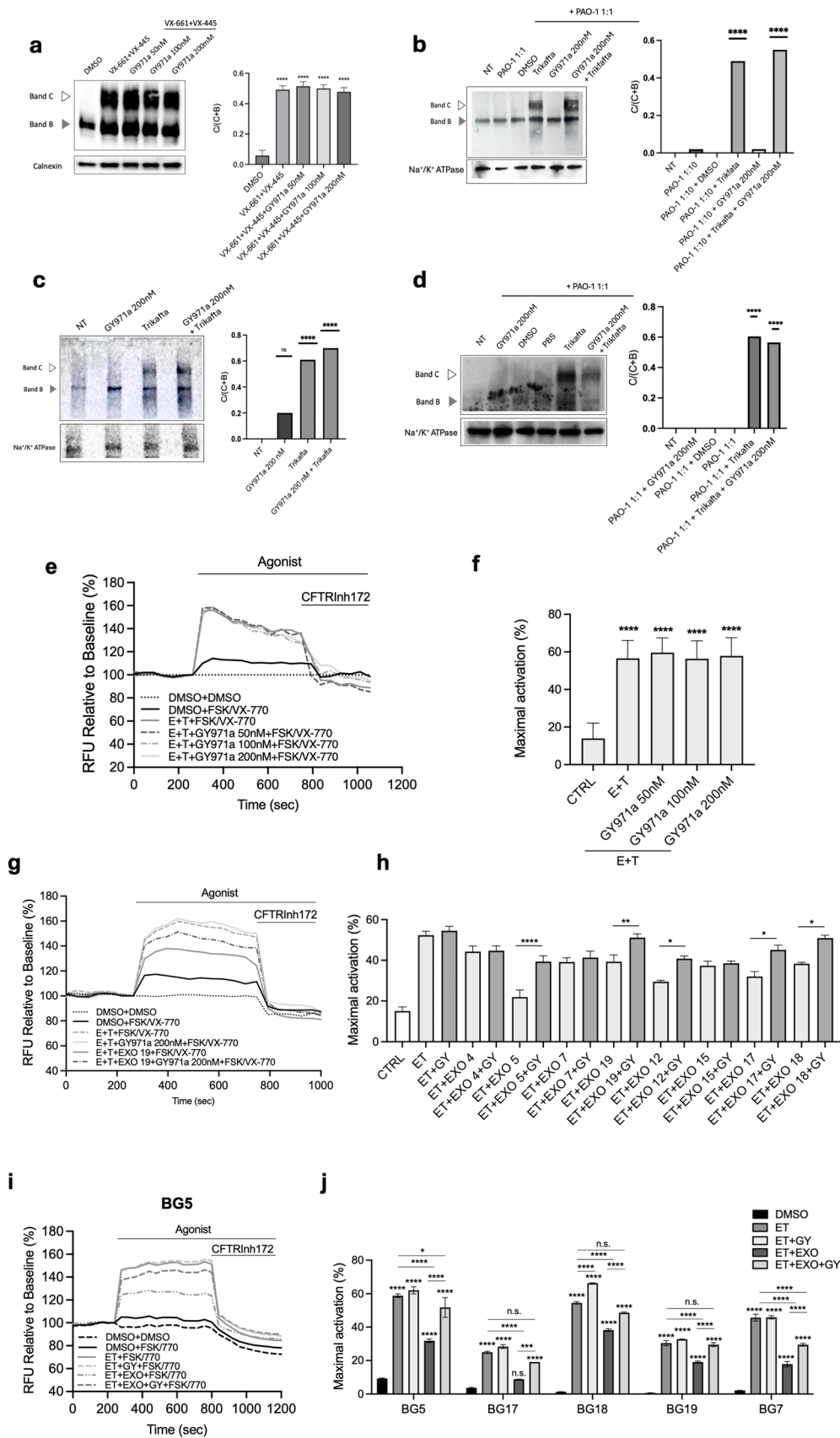
We were then prompted to investigate the effect of GY971a on ETI efficacy in rescuing F508del-CFTR function in airway epithelial cells, both of bronchial and nasal origin. We first employed a fluorescence-based membrane depolarization (FMP) assay to measure F508del-CFTR function in CFBE cells treated with VX-445+VX-661 $\pm$ GY971a at different concentrations (50–200 nM) for 24 h. As shown in Fig. 4e–f, GY971 did not interfere with F508del-CFTR function rescued by ETI in CFBE cells. We then investigated the effect of GY971a on ETI-dependent F508del-CFTR function in the presence or absence of clinical EXO of *P. aeruginosa* from 8 pwCF. As expected [15], ETI-mediated F508del-CFTR function was significantly reduced in the presence of EXO in a strain-specific manner (Fig. 4g–j). Interestingly, GY971a pre-treatment significantly restored ETI-mediated CFTR function in 5 out of 8 clinical EXO (Fig. 4h).

To validate these results in patient-derived tissues, we cultured HNE cells generated from nasal brushing obtained from 5 pwCF heterozygous for F508del mutation. After 16–18 days of ALI differentiation, cells were treated for 24 h with DMSO or ETI $\pm$ GY971a $\pm$ clinical EXO from the corresponding donor (Fig. 4i–j). Interestingly, GY971a, except for one donor where the rescue was increased, did not alter the efficacy of ETI on rescue CFTR function in HNE. However, as expected [15], ETI-mediated CFTR function was reduced by 30–69 % in the presence of EXO, although CFTR function remained significantly higher than DMSO control (except for one donor, BG17). Moreover, pre-treatment with GY971a restored ETI-mediated rescue of F508del-CFTR function in HNE infected with clinical EXO isolated from the corresponding donor.

Having established GY971's robust anti-inflammatory efficacy and its ability to restore ETI function in the presence of bacterial exoproducts, the final stage of our assessment was to validate its therapeutic effect and safety profile within an *in vivo* biological system. We therefore utilized the zebrafish (*Danio rerio*) model infected with *P. aeruginosa*.

3.7. Effect of GY971 administration to zebrafish larvae

To evaluate the safety profile of GY971, zebrafish larvae at 2 or 3 dpf were exposed to increasing concentrations of GY971a, the mesylate salt (1 and 2 μ M), for 24 and 6 h, respectively, in the absence of an inflammatory stimulus (Fig. 5a–e). The doses of GY971a were chosen as 10X concentration based on previous *in vitro* studies, carried out with doses between 50 nM and 200 nM [7]. Treatment times were chosen to evaluate the response to a more or less prolonged exposure to the molecule. No evident alteration in the survival rate between larvae treated with GY971a or the vehicle DMSO were observed in both time exposures (Fig. 5a,c). None of the doses tested showed toxicity or the onset of evident morphological defects (data not shown). Similarly, larvae exposed to a concentration of 2 μ M of GY971a showed comparable total neutrophil count as control larvae in both exposure time settings (Fig. 5b,d,e). Collectively, these findings indicate that GY971a does not induce toxic effects nor modified neutrophil activation in a



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Fig. 4. GY971a (GY) does not reduce the rescue of F508del CFTR by ETI in CF bronchial epithelial cells expressing F508del CFTR protein, and it restores the efficacy of ETI-dependent rescue of F508del-CFTR in airway epithelial cells infected with clinical EXO of *P. aeruginosa*. **a:** Human bronchial CFBE41o- F508delCFTR cells have been pre-incubated for 24 h with CFTR modulators VX-661 and VX-445 in the absence or presence of GY971a (50, 100 or 200 nM). Western blot analysis of CFTR protein showing CFTR bands B and C and densitometry leads graphic representation of the ratio of band C/(C + B); **b:** Human bronchial CFBE41o- F508delCFTR cells have been pre-incubated for 24 h with Trikafta in the absence or presence of PAO-1 and GY971a (200 nM). Western blot analysis of CFTR protein showing CFTR bands B and C and densitometry leads graphic representation of the ratio of band C/(C + B); **c:** HBE primary cells were incubated with Trikafta, and/or with GY971a (200 nM) for 24 h. Western blot analysis of CFTR protein showing CFTR bands B and C and densitometry leads graphic representation of the ratio of band C/(C + B); **d:** HBE primary cells were incubated with Trikafta in the absence or presence of PAO-1 (1:1) and GY971a (200 nM) for 24 h. Western blot analysis of CFTR protein showing CFTR bands B and C and densitometry leads graphic representation of the ratio of band C/(C + B). **e, g:** Representative traces of F508del-CFTR dependent chloride efflux, measured with FMP assay, in F508del-CFTR CFBE cells treated with 0.1 % DMSO, 3 μ M VX-445 (E) + 3 μ M VX-661 (T) $\pm$ GY971 (50–200 nM) $\pm$ EXOs for 24 h at 37 °C. **f, h:** Bar graph shows the maximal activation of F508del-CFTR after stimulation by 1 μ M FSK + 1 μ M VX-770 after 24 h treatment E + T $\pm$ GY971a $\pm$ EXO ($n = 3$); **i:** Representative traces of F508del-CFTR-dependent chloride efflux in HNE cells measured by FMP assay. Cells were treated for 24 h with 0.1 % DMSO, 3 μ M VX-445 (E) + 3 μ M VX-661 (T) $\pm$ clinical exoproducts (EXO) of *P. aeruginosa* isolated from the corresponding patient. **j:** Bar graphs show the mean $\pm$ SEM of maximal activation after 10 μ M FSK + 1 μ M VX-770 after 24 h treatment E + T $\pm$ GY971a $\pm$ EXO. ($n = 3$ technical replicates).

normal *in vivo* condition.

The treatment with GY971a resulted in a significant reduction in neutrophil recruitment to the site of infection, in a dose-dependent manner (x^- PAO-1 ctrl = 32.2; x^- GY971a 1 μ M = 22.6; x^- GY971a 2 μ M = 19.0) (Fig. 5f–j).

To evaluate whether GY971a effect was exerted independently of bacterial viability, neutrophil recruitment was assessed following infection with same amount of heat-inactivated bacterial suspension (HK-PAO-1), to avoid the contribution of bacterial toxins released by living infecting bacteria. Similarly, a significant and dose-dependent reduction in neutrophil migration at the site of inflammation was also observed in comparison with untreated controls. In particular, a significant difference in neutrophil count was found between untreated controls (x^- HK-PAO1 = 24.4) and larvae treated with 2 μ M of GY971a (x^- HK-PAO1+GY971a 2 μ M = 14.8). To note, similar neutrophil recruitment was elicited from both live PAO-1 and HK-PAO-1 (Fig. 5k).

4. Discussion

Although Highly Effective Modulator Therapy (HEMT) has relevantly improved the scenario of treatment of pwCF, HEMT reduces but does not stop a persisting respiratory tissue damage due to chronic inflammation, which also favours recurrent infective exacerbations [22, 23]. A combined daily treatment of anti-inflammatory drugs associated with HEMT is devised as an optimal strategy, with addition of antibacterial drugs during infective pulmonary exacerbations [24]. Among the different anti-inflammatory strategies in the era of HEMT, those targeting neutrophils in the CF bronchial lumen are becoming particularly relevant [25]. Inhibitors of tissue proteases derived from neutrophils are under development, a strategy aimed to block the untoward damage of CF lung bronchial walls. Namely, inhibitors of neutrophil elastase or of neutrophil cathepsin C are undergoing clinical trials in pwCF [26,27]. Both these approaches could obtain an effective inactivation of proteases. However, under this treatment, excessive number of neutrophils in CF conductive airways continue to release both DNA, which worsens the viscosity of airway surface liquid, together with Reactive Oxygen Species, which damage bronchial walls by oxidation. Differently, GY971 wishes to downmodulate the excessive recruitment of neutrophils without increasing the bacterial burden, as shown in a *P. aeruginosa*-infected murine lung model *in vivo* [7] and confirmed here in the zebrafish model (Fig. 5), thus with the potential advantage of mitigating the release of all the three damaging molecules released from neutrophils.

The main mechanism of action of GY971 is based on the inhibition of the activated NF- κ B TF, as demonstrated by previous molecular screenings [6,7], which are consistent with the docking simulation reported here in Fig. 1. This is particularly interesting since the beneficial clinical anti-inflammatory effect of ibuprofen for pwCF has been related to a similar secondary mechanism of action of this drug, that is the inhibition of activation of NF- κ B, which is obtained only at high doses [4, 5]. As the major portion of neutrophil chemokines recruiting neutrophils

in the bronchial lumen is produced by bronchial epithelial cells [3], we believed mandatory to check the effect of GY971 not only in CF immortalized cell lines (Fig. 2 and [7]), but also in mucus-producing polarized CF primary bronchial and nasal cell monolayers grown at the air-liquid interface (Fig. 3). Importantly, in the polarized cellular models, GY971 has been delivered from the apical side *in vitro*, simulating an administration moiety resembling a future pulmonary delivery *in vivo*. Consistently with previous results [7], GY971 reduces the expression of the major neutrophil chemokine IL-8/CXCL8 also in primary bronchial and nasal cells (Fig. 3). Since consensus sequences for the binding of NF- κ B are present in the promoter of different pro-inflammatory genes, it is not surprising to find a parallel reduction of the release of the cytokines TNF- α and IL-6 (Fig. 3). On the contrary, IL-1 β protein levels remained largely undetectable post-infection and were only induced following TNF- α stimulation. On the other hand, we observed increased IL-1 β mRNA levels by RT-qPCR without corresponding secretion in basolateral media, as measured by Bioplex analysis. This discrepancy may be explained by the limited expression or activation of P2 $\times$ 7 receptors in bronchial epithelial cells, where extracellular ATP may not reach levels sufficient to trigger its activation. Moreover, the possible lack of expression of key inflammasome components (e.g., NLRP3, ASC, caspase-1) made the IL-1 β processing inefficient or absent. Additionally, the lack of a secondary signal, like extracellular ATP, could prevent pro-IL-1 β maturation and release [28, 29].

Since soluble inflammatory mediators have been reported to increase the rescue of F508del-CFTR mediated by last-generation CFTR modulators in bronchial epithelial cells [8–10], we wished to check whether the anti-inflammatory molecule GY971 might untowardly reduce the effectiveness of ETI-mediated rescue. A key finding is that GY971 does not interfere with ETI-mediated rescue of F508del-CFTR protein. In CFBE41o- and primary HBE and HNE cells, GY971a did not impair the processing or function of F508del-CFTR restored by ETI. Importantly, in the presence of clinical *P. aeruginosa* EXO, which variably impaired ETI efficacy, GY971a pre-treatment restored ETI-mediated CFTR function in 5 out of 8 EXO samples in CFBE cells. Similar results were observed in patient-derived HNE cells, where GY971a restored CFTR function in the presence of donor-specific EXO. It seems, therefore, reasonable to consider that GY971 could regulate the excessive neutrophil chemotaxis without affecting the beneficial effect of proinflammatory cytokines on CFTR rescue, being GY971 mainly active on the reduction of IL-8 in the different primary respiratory epithelial *in vitro*, with a moderate impact on other cytokines such as TNF- α , IL-6 and IL-1 β .

Based on the pre-clinical results presented here, we speculate that GY971 could mimic the mechanism of reducing neutrophil chemotaxis, which was shown to be the main beneficial action of ibuprofen in clinical trials involving pwCF, possibly through inhibition of NF- κ B activation [4,5]. However, ibuprofen was found to strongly inhibit COX-1, leading to gastrointestinal bleeding and ulcers due to the lack of cytoprotection from PGE2. Here, we observed that GY971 only partially inhibits COX-1 at high concentrations (Fig. 2d), compared to those

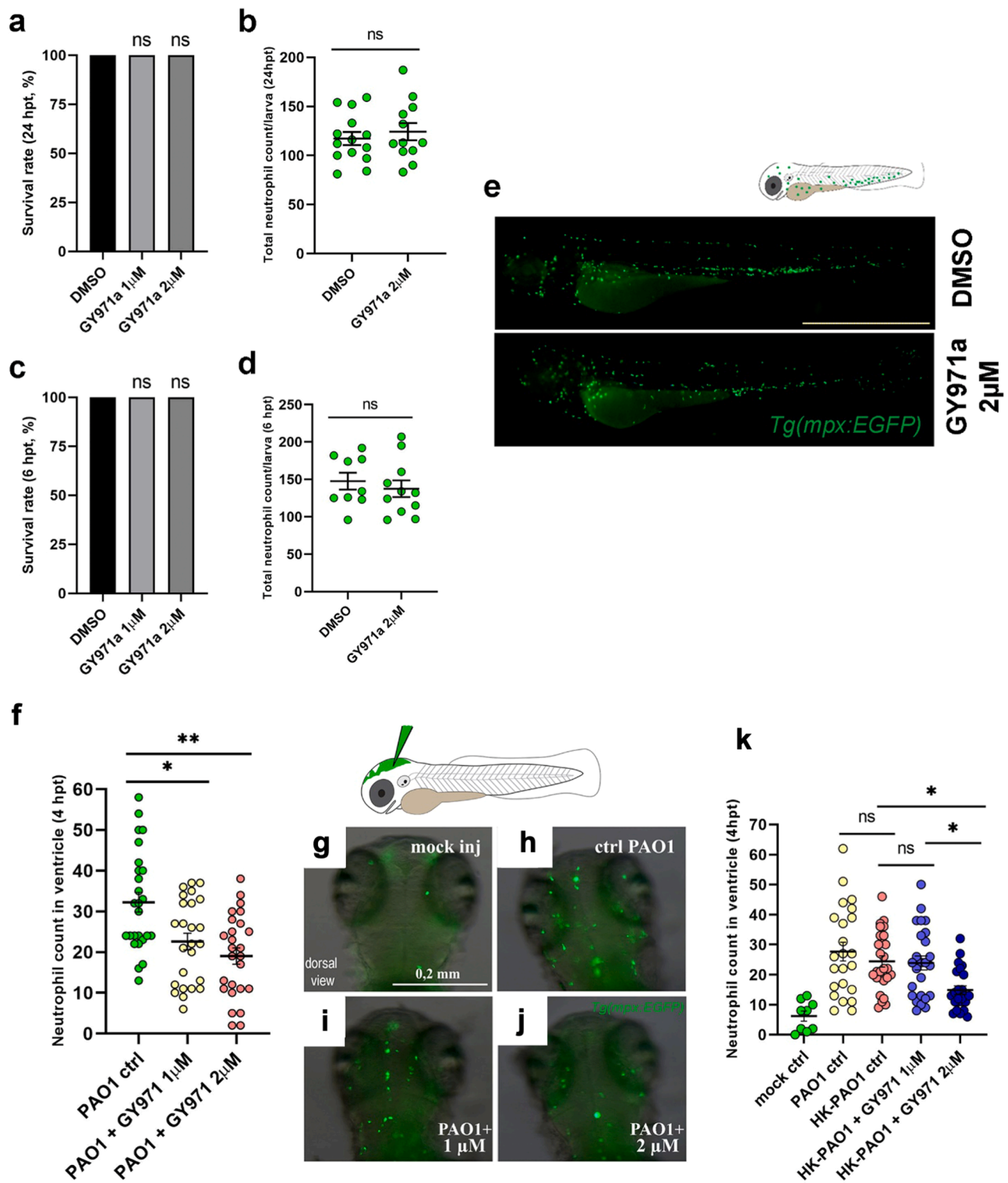


Fig. 5. GY971 does not affect survival rate and total neutrophil activation in zebrafish model (a–e), and it reduces PAO1-induced (f) and HK-PAO1-induced (k) neutrophil chemotaxis in zebrafish model *in vivo*. Different doses of GY971a were tested on *Tg(mpx:EGFP)* embryos in the absence of inflammatory stimuli and the survival rate was evaluated in 2 dpf (a) and 3 dpf (c) at 24 and 6 hpt respectively. 30 larvae for each experimental group were analyzed. Total neutrophils count was assessed in 2 dpf (b) and 3 dpf (d) larvae at 24 and 6 hpt, respectively. e: Representative fluorescent micrograph of 3 dpf larvae treated or not with 2 μM GY971a. Results are presented as mean ± SEM and statistical significance was assessed by unpaired *t*-test with Welch's correction: ns= not significant. Each dot represents a single individual. Quantification of neutrophil recruitment at the brain ventricle of 3 dpf *Tg(mpx:EGFP)* larvae locally infected with 2 nl of PAO-1 suspension (500–1000 cfu/larva) (f) or with 2 nl of same amount of Heat-Killed PAO1 (HK-PAO1) (k) and treated with 1 or 2 μM GY971a or DMSO directly in embryo medium. (f, k) GFP⁺ neutrophils count at 4 h post-treatment (hpt) with GY971a in infected larvae. (g–j) Representative fluorescence micrographs of the different experimental categories (dorsal view of brain ventricle). Results are presented as mean ± SEM and statistical significance was assessed by non-parametric Kruskal-Wallis test in f and by One-way ANOVA followed by Tukey's post hoc test in k: ***p* < 0.01; **p* < 0.05; ns= not significant. Each dot represents a single individual.

needed for optimal anti-inflammatory activity *in vitro*. The toxicity of ibuprofen is linked to an increased risk of upper gastrointestinal bleeding, which is caused by nearly complete inhibition of platelet COX-1 activity [30]. GY971 appears to avoid this risk, as it only partially affects platelet COX-1 activity even at higher-than-therapeutic concentrations. However, the safety of GY971 will need further preclinical and clinical toxicity studies. We believe that GY971, while targeting the same core mechanism as ibuprofen, could provide a significant advantage in terms of side effects, especially if delivered through the lungs.

5. Conclusion

We are aware that the results obtained here present different limitations. The primary bronchial and nasal epithelial cells obtained from pwCF *ex vivo* utilized here, although considered an advanced model of study, cannot completely reproduce the innate immune response we wish to target with the anti-inflammatory effect of GY971, thus more preclinical models *in vivo* are needed. In this respect, the zebrafish model presented here nicely reproduces the mechanism of neutrophil chemotaxis induced by bacterial infection, but cannot represent all the different specificities of a whole mammalian lung model *in vivo*. Thus, future investigation with pre-clinical animal models of chronic lung bacterial infection *in vivo* will more suitably fill the gaps necessary to move forward the pharmaceutical development towards a new anti-inflammatory drug for pwCF.

Being well aware of these limitations, GY971 appears to be a promising derivative for the future development of anti-inflammatory CF treatments. It effectively mitigates inflammation by reducing neutrophil chemotaxis mediators and the expression of key inflammatory proteins, without inducing cytotoxicity or interfering with the therapeutic effects of CFTR modulators. Its ability to restore ETI-mediated CFTR function in the presence of bacterial exoproducts further highlights its potential as a beneficial adjunct therapy.

Contributions

Conceived and designed study, IL, GC; Collected samples and pre-clinical data, IL, CT, AT, NP, VC, AP, MC, OL, CA, PP; Performed research, CT, AT, CA, VC, GM, AC, ST, ADM; Analyzed data, IL, GC, OL, PP, AP. Intellectual contributions, IL, GC, AC, PP. Wrote the paper, IL, GC, AP, OL; All authors read and approved the final version of the manuscript.

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Declaration of competing interest

The authors declare no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.jcf.2025.12.010](https://doi.org/10.1016/j.jcf.2025.12.010).

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