Cystic Fibrosis Mucus Model to Design More Efficient Drug Therapies

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Abstract

Mucus can represent a strong barrier to tackle for oral or pulmonary administered drugs especially in mucus-related disorders. Still little is known about the molecular properties that mediate the interaction of drugs with mucus. This study uses a pathological cystic fibrosis (CF) mucus model to investigate the impact of mucus over the permeability of 45 commercial drugs. An *in vitro* mucosal surface was recreated by coupling the mucus model to 96-well permeable supports pre-coated with structured layers of phospholipids (PAMPA). The mucus model behaved as an interactive filter as different molecular structures reacted differently to mucus. We also found that permeability can be enhanced when calcium salts are formed. This was confirmed also through the use of cystic fibrosis sputum as a rough ex vivo model of CF mucus. Since development of drugs is characterized by a high rate of failure, the mucus platform could aid to reduce at an early stage the number of poor performer drug candidates preventing them to uselessly reach preclinical trials.

1. Introduction

The mucosal surfaces of the human body are constantly exposed to environmental threats. To counteract potential noxious agents, all the wet epithelia are covered with a layer of mucus. Mucus is a dynamic semipermeable network with a heterogeneous composition. It consists of ~95% water and the remaining 5% comprising electrolytes, lipids, DNA fragments and proteins. Maintaining the gel-like properties and keeping together such a huge amount of water requires a strong yet flexible skeleton. The tough job is carried out by mucins. Mucins are long polymeric glycoproteins having high molecular weight, consisting of a peptide backbone to which a huge amount of carbohydrate chains are attached. Such an architecture enables the exchange of nutrients, water, gases and hormones whilst being impermeable to most bacteria and pathogens [1,2].

A delicate balance of mucus production must be achieved as weakening of the mucus barrier makes us more vulnerable to environmental threats. On the contrary, an overproduction or dysfunctional clearance of mucus are hallmarks of the pathogenesis of all the mucus-related pathologies, especially pulmonary diseases[3], such as cystic fibrosis (CF) among others. In these disorders, an overexpression of mucins, accumulation of extracellular DNA as well cellular debris, and the persistent presence of bacteria, confers mucus stasis lending to a vicious cycle of infection and inflammation that can be chronically sustained [4,5]. In CF and bronchiectasis, sputum production is not only a daily reality, but also a crucial marker for physicians during both stable state and exacerbation used to evaluate disease severity and treatment response. Sputum's reliability as an indicator of disease has led it to being proposed as an important clinical prognostic factor in mucus-related diseases. This has been taken a step further by Murray and colleagues, who explored the utility of sputum color in patients with bronchiectasis by developing a quantitative method that predicts bacterial colonization based on the color of sputum [6].

Despite the great advancements in disease management of the last decades, pulmonary failure remains the main cause of morbidity and mortality in patients with CF. The lack of function of the cystic fibrosis transmembrane conductance regulator protein (CFTR) in people with CF, leads to mucus dehydration. As a result, mucus undergoes a reduced clearance, clogging the airways and making it difficult to breath. Physicians have developed advanced clearance techniques (ACTs) based on coughing maneuvers to get rid of the viscous mucus. In addition to the routinely ACTs, CF patients manage their disease by following a regular treatment with medications. The recurrent pulmonary

exacerbations are usually treated with both oral and intravenous antibiotics (*i.e.*, aztreonam, tobramycin, levofloxacin), and anti-inflammatory drugs (*i.e.*, high-doses ibuprofen [7]).

Yet, to enter into the systemic circulation, drugs administered by the oral, pulmonary, nasal or rectal routes, need come in contact and subsequently cross the epithelium in order to reach the capillary circuitry in the lamina propria. Thus, effective drugs administered by these routs should harbor the ability to transverse mucus barriers to be pharmacologically active. In the context of cystic fibrosis, the pathological mucus can strongly limit the absorption of drugs that do not exhibit these difficulties under normal physiological conditions. Also, the pathological CF mucus, is characterized by a reduced mesh size (60-300 nm)[8] with respect to physiological mucus (497-503 nm)[9]. Two main mechanisms are expected to affect drug diffusion through mucus: steric and interactive filtering (Fig. 1). Oligomers of secreted mucins connect with each other creating a complex network that filters molecules bigger than the size of the mesh spacing between mucin fibers [10] (steric filter). Molecules small enough to penetrate the mucin mesh are subjected to the interactive filter which is mainly governed by the structural complexity of mucins. On the highly glycosylated hydrophilic regions, negative charges are exposed due to the presence of sialic acid. On these substrates, hydrogen bonding and electrostatic interactions can be established with polar and hydrophilic molecules. But not the entire peptide core of mucins is glycosylated; cysteine-rich domains are glycans free and usually folds into hydrophobic regions on which lipophilic molecules can attach. And yet, mucin is just one of the components of mucus. Other substances such as lipids, antimicrobial peptides (defensins, histatins, collectins *etc.*,), lytic enzymes (lysozyme) and antibodies (IgA and IgG) are components of mucus as well, and each one of them has the potential to interact with drugs.

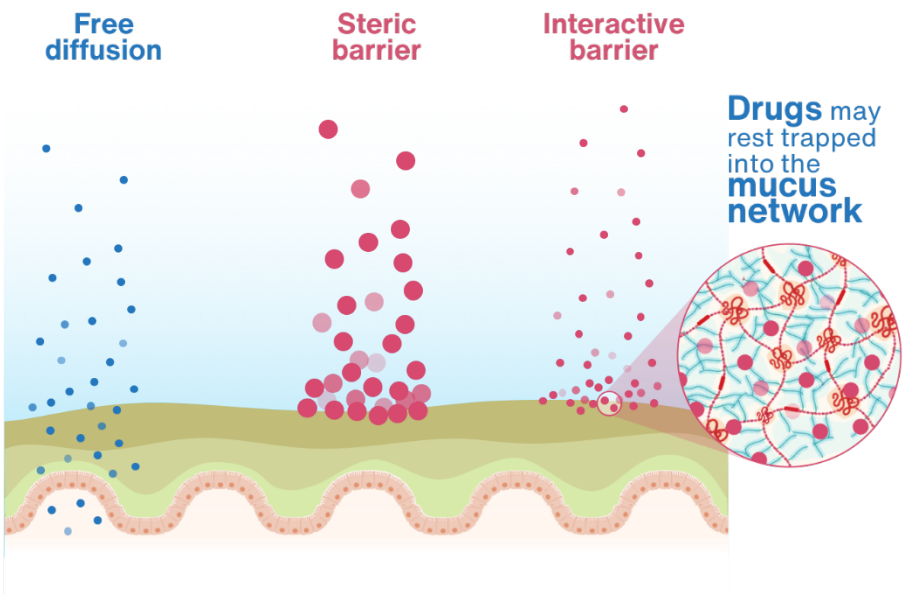

Figure 1. The steric and interactive barriers of mucus. Drugs larger than the mesh spacing between mucin fibers are stacked within mucus because too big to cross the mucus mesh. Similarly, drugs smaller than the mesh but able to interact with mucus components are equally retained by mucus. On the contrary, particles that are small enough and relatively inert to any of the mucus component can freely diffuse through the mucus layer and eventually absorbed.

The importance of developing *in vitro* models able to model both the biological structure and functions of pulmonary mucus is increasingly gaining awareness in modern drug discovery. Today, the most popular *in vitro* models for assessing permeability/absorption of drugs, are those based on artificial membranes, such as parallel artificial membrane permeability assay (PAMPA) [11], systems based on cells, such as Caco-2 [12] and MDCK [13]; and systems based on site-specific tissues [14]. Notably, none of them takes mucus specifically into account. In the last years, a variety of mucus models have been proposed [15]. The proposed models include gastrointestinal mucin-based solutions[16,17], reconstituted oral mucus gels [18], multilayered polyelectrolyte films [19], and *in vitro* cell cultures models incorporating airway mucus or mucus-producing cells [20]. Falavigna *et al*. developed a mucus phospholipid vesicle-based permeation assay that has been used for permeability screening of drugs and formulations [21].

Recently we developed an *in vitro* mucus model which simplifies mucus complexity by mimicking the chemical composition, structural features and viscoelastic properties of pathological CF mucus [22]. The viscoelastic property of CF mucus is achieved by taking advantage of the internal gelation of alginate in the presence of calcium ions. Alginate is an extracellular exopolysaccharide component of mucoid *P. aeruginosa*, a hallmark of CF infection, and has been shown to protect bacteria against certain antibiotics as well

as escape the immune system [23,24]. To reproduce the interactive and steric filters of CF mucus we used commercially available unpurified mucin type III from porcine stomach. One may argue that commercial mucins are different in terms of structure and viscoelastic properties respect to native mucins. Indeed, it has been established that commercial mucins fail to form hydrogels at acidic pH, are only partially purified and are inferior in inhibiting virus infection compared to natively purified mucins obtained in lab [25]. However, the purpose of our mucus model is to have an easy to use and easy to reproduce *in vitro* mucus model suitable for average throughput screening (HTS) applications. Thus, even if in lab extracted mucins are qualitatively superior to commercial mucins, the time consuming and expensive procedures of purification, extraction and concentration at laboratory level are not adapted for HTS purposes.

In this study, we aimed at expanding the applicability and relevance of our *in vitro* cystic fibrosis pathological mucus model. To reach this aim we addressed the following goals: (i) selected 45 commercially available drugs maximizing physicochemical variability; (ii) performed PAMPA measurements in the absence of mucus and assessed the physicochemical determinants of apparent permeability (P_{app}); (iii) we coupled the developed mucus model with PAMPA to mimic *in vitro* cystic fibrosis airway mucosal surface. The permeability in the presence of mucus was compared with the P_{app} obtained in the absence of mucus; (iv) eventually, permeability with cystic fibrosis sputum was measured to support the conclusion drawn from the *in vitro* model herein presented.

Overall, this study highlights the challenging of reproducing *in vitro* the complexity of cystic fibrosis dysfunctional mucus. While PAMPA could be a reasonable model to mimic permeability through cellular membrane, we have shown that it is a too simplistic model to mimic the diffusion across pathological CF mucus. As a first screening tool of poorly permeable molecules, a fully tunable in vitro mucus model, easy to reproduce, and mimicking both the composition and the rheological properties of CF mucus, could be of high usefulness in the early drug discovery.

2. Experimental section

2.1. Computational Part

The drug SMILES codes were retrieved from DrugBank [26]. The csv file of the approved drugs was downloaded from DrugBank (last update on 3 January 2021). The csv file was transformed in xlsx file using Microsoft Excel (v. 16.43). The SMILES of $CFTR_{inh}$-172, which is a non-commercial drug acting as an inhibitor of the CFTR protein, was

retrieved from PubChem[27]. Molecular properties were calculated with DataWarrior (ver. 5.5.0, openmolecules.org) and include physico-chemical properties, druglikeness related properties, various atom and ring counts, molecular shape, flexibility as well as functional groups (Table S2). The molecular charge at pH 7.4 was retrieved from MarvinSketch (Marvin 20.20, 2020, ChemAxon).

The dataset was analyzed with the Principal Component Analysis (PCA) tool implemented in DataWarrior. The correlation matrix of the descriptors was calculated using DataWarrior and represented as a heatmap.

2.2. Materials

Mucin from porcine stomach (PGM Type III, bound sialic acid 0.5-1.5%, partially purified powder), sodium salt of alginic acid, calcium carbonate, D-(+)-gluconic acid δ-lactone ≥99.0% and sodium chloride used to develop the airway mucus model were all purchased from Merck (Germany). Permeability experiments were carried out on Corning® Gentest™ Pre-coated PAMPA, 353015, USA plates. Millipore® grade water (resistivity: 18.2 MΩ cm at 25 °C) was obtained from an in-house Millipore® system. Acetonitrile, ammonium acetate and DMSO were of the highest available grade and purchased from Sigma Aldrich. The drugs used in this study were all commercially available (Fig. S1). Stock solutions were prepared in DMSO and stored at 4 °C.

2.3. Mucus model

The herein used mucus model can be exploited as a platform for drug diffusion either for cystic fibrosis mucus or other mucus-related disorders such as mucus of chronic obstructive pulmonary disease, which unveils its potential for a wide range of applications in drug discovery. The mucus model was prepared as previously described [22]. Briefly, a 21 mg/mL alginate sodium salt solution was dissolved in a 16.3 mg/mL NaCl solution, under slow magnetic agitation. In parallel, a 43.7 mg/mL mucin suspension was prepared in mQ water and left under slow agitation over-night. The alginate and mucin solutions were mixed at a 1:4 proportion using two jointed luer-lock syringes. Then, the alginate and mucin suspension were mixed with a suspension of 7 mg/mL $CaCO_3$ prepared in 16.3 mg/mL NaCl solution. In the last step, a 70 mg/mL GDL solution was freshly prepared in 16.3 mg/mL NaCl and mixed with the previously prepared suspension (alginate, mucin and $CaCO_3$) at a proportion of 1:6. Finally, 40 µL of the mucus model were pipetted directly over the PAMPA membrane in the donor compartment, producing a hydrogel of approximately 500 µm in thickness. The donor plate of the PAMPA was then carefully shacked to uniformly distribute the volume of

mucus over the entire well surface and to get rid of any air bubbles. Afterwards, the mucus within the plate was left to crosslink for 24 h before the addition of drug solutions. Throughout the time course of the permeability experiment, the CF mucus model remained stable with respect to weight and thickness variations. In fact, we previously determined that after 6h of incubation in an aqueous medium, mucus undergoes a thickness variation below 10% which was considered acceptable for our experimental purpose[22].

2.4. PAMPA assay

The apparent permeability (P_{app}) and the effect of mucus on permeability were experimentally determined through PAMPA and mucus-PAMPA assays, respectively. Stock solutions of all drugs were prepared in DMSO at a concentration of 10 mg/mL. Donor solutions of drugs were prepared in PBS (10 mM, pH 7.4, 5% DMSO) at a concentration between 100 to 500 µM, depending on the drugs' specific solubility. Each donor well was filled with 200 µL of drug solution, while the acceptor wells were filled with 300 µL of PBS. The donor plate was then placed on top of the acceptor plate, so the artificial membrane was in contact with the buffer solution below. A lid was placed on top of the donor plate and the whole PAMPA plate was incubated at room temperature for 5 h. At the end of the incubation period, the plates were separated, and the volume of the acceptor wells was collected. Concentrations of drugs in each acceptor well were quantified either by HPLC-UV or HPLC-MS. The apparent permeability coefficient (P_{app}) was expressed using equation 1 derived from Fick's law[28] for steady state conditions:

$$P_{app} = \frac{dQ/dt}{C_0 \times A} \qquad (Eq.\,1)$$

where dQ is the quantity of drug expressed as moles permeated into acceptor compartment at time t (18,000 sec), C_0 is the initial concentration in the donor well and A is the area of the well membrane (0.3 cm^2). The P_{app} was used as an average of all the measures.

The same PAMPA experimental setup was adopted when assessing the effect of individual components of mucus. In particular, we evaluated how the PGM, the NaCl, the alginate hydrogel and calcium impact on permeability. For this purpose, the passive diffusion was measured in the presence of each one of these chemicals. Each of which, were individually added in the donor compartment of the PAMPA. In brief, 40 µL of the alginate gel were deposited over the phospholipid membrane of the donor compartment prior the addition of drug solution. The influence of PGM was assessed by filling the donors with a suspension of drug containing 4.16 mg/mL of PGM (the PGM concentration donor compartment). Similarly, drug donor solutions containing 1.67

mM $CaCl_2$ or 20 mM NaCl were used when investigating the impact of calcium and NaCl over permeability.

2.5. Cystic fibrosis sputum

Sputum collected from cystic fibrosis patients was used as a model of complex biological matrix representative of CF mucus. We measured the permeability of some of the drugs of our dataset using the CF sputum – PAMPA system and compared the results with the permeability obtained in the presence of our mucus model. Sputum samples were a kind concession of Prof. A. Ghigo from the Department of Molecular Biotechnology of University of Turin. Spontaneously expectorated sputum was collected into sterile containers and was processed as described by Oriano *et al.* [29]. Briefly, samples were processed getting first rid of saliva. Then, samples were diluted 8x in PBS, vortexed until sputum dissolution and centrifuged for 15 min at 3000 g. Forty µL of sputum were deposited over the PAMPA membrane in the bottom of the top plate, and eventually 200 µL of drug solution were inserted over the layer of CF sputum, while 300 µL of PBS were inserted into the bottom plate of the PAMPA. The two plates were coupled and incubated for 5h. At the end of the 5h the plates were splitted and the amount of drug diffused into the bottom plate was quantified. Similarly, to test the effect of calcium over the permeability of the drug, we formed drug-calcium complexes by dissolving the drug into PBS containing calcium at the same concentration present in the cystic fibrosis mucus model. The permeability of calcium-drug complexes was measured through the CF sputum PAMPA as previously described.

2.6. Quantification

All compounds, except ebselen, benzoic acid and 3-aminophenol, were analyzed and quantified by HPLC-MS/MS using a Varian HPLC equipped with a 410 autosampler and an Ascentis C18 column (10 cm x 2.1 mm, 3 µm). Gradient mobile phases composed of acetonitrile and water 0.1% formic acid or ammonium acetate 5 mM pH 6.6 as organic and aqueous phase respectively were pumped at a flow rate of 200 µL/min. A flow of 200 µL/min and an injection volume of 10 µL were used. Compounds were detected on a Varian 320 MS TQ Mass Spectrometer equipped with an electrospray ionization (ESI) source operating in positive or negative mode, depending on drugs' method. The detector was used in multiple reaction monitoring (MRM) mode and the transitions of each drug are reported in Table S1.

Ebselen, benzoic acid and 3-aminophenol were quantified on a HPLC Varian ProStar equipped with a 410 autosampler and a PDA 335 LC Detector. The analysis was

conducted on a IAM column (Regis, 10 cm x 4.6 cm 10 μm packing 300 Å pore size) using ammonium acetate and acetonitrile as aqueous and organic mobile phase, respectively. The flow rate was 1 mL/min.

2.7. Statistical analysis

A minimum of 4 replicates were conducted for each compound on each experimental method (with or without the mucus model), some of whom repeated also on different PAMPA plates. Results are expressed as mean ± SD. Student's t-test was applied to detect statistical significance between the permeability recorded with and without mucus. A $p<0.05$ was considered to be a statistically significant difference.

3. Results and discussion

3.1. Dataset selection: assessment of chemical heterogeneity

We included in our dataset a number of anti-inflammatory (ibuprofen, dexamethasone) and antibacterial drugs (tobramycin, ceftazidime, aztreonam, ciprofloxacin, tetracycline) commonly employed in the cystic fibrosis therapy regimes. To properly evaluate the performances of our CF mucus model, we expanded the dataset up to 45 compounds considering it a reasonable number of drugs to be investigated, with good variability in terms of chemical properties (see below).

To assess the distribution of our dataset within the entire drug chemical space, at first, we downloaded the DrugBank database of approved drugs. From DrugBank's database we retrieved the SMILES code for each compound and used them to calculate molecular descriptors (see Methods, Table S2). Using drugs as observations and the selected molecular descriptors as variables, we then computed a principal component analysis (PCA). The drugs within our dataset are small molecules therefore, focus was pointed on compounds of total molecular weight <1,000 Da (yellow dots in Fig. 2A). The variance explained by PC1 and PC2 using 30 molecular descriptors is about 60%. In the score plot defined by PC1 and PC2 is possible to appreciate a good distribution of the tested drugs within the chemical space (red dots in Fig 2A).

To assess chemical variability within our dataset we also evaluated the Lipinski's rule of five (Ro5) molecular descriptors distribution (the number of the hydrogen bond donors (HBD), the number of the hydrogen bond acceptors (HBA), the molecular mass (MW), and the octanol-water partition coefficient (LogP)). Figure 2B shows that all the descriptors categories are well represented by the dataset,

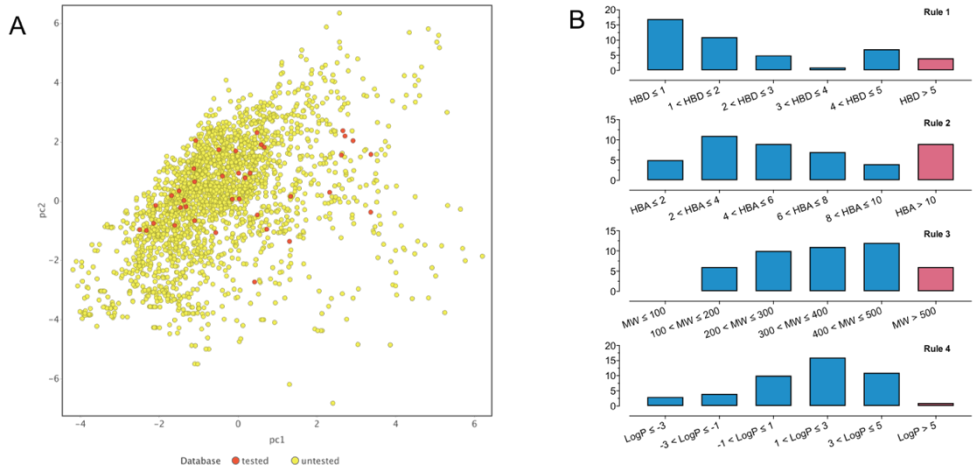

Figure 2. (A) Distribution of the tested drugs (red dots) within the DrugBank database of approved drugs (yellow dots) having total molecular weight ≤1000 Da. (B) Classification and distribution based on Lipinski's rule of five (B). Compounds that violate the Ro5 for each molecular descriptor are represented by the red bars.

3.2. Validation of the permeability setup to measure P_{app}

Before evaluating the effect of mucus on drug permeability, we determined and validated permeability in the absence of mucus through an artificial cellular membrane since our method differs from the standard PAMPA protocol. In fact. in our setup (modified setup), mucus was placed over the PAMPA membrane (a filter plate pre-coated with structured layers of phospholipids). in the top well as is the only way to have a physical support that can sustain mucus. Thus, diffusion takes place from the top to bottom well and drug has been quantified only in the acceptor compartment (bottom well).

Data obtained with our original set-up (Table 1) were firstly validated using a subset of compounds taken from the paper of Chen et al.,[30] (Table S3 and Fig. S2) using the same artificial membrane (r^2=0.610, Fig. S2) . Notably, compounds as propranolol and caffeine known for being high permeable[31,32] could clearly be discriminated from low permeable compounds.

Then we determined which molecular descriptors mostly govern permeability in our system. To do that a correlation matrix between apparent permeability (P_{app}) and a pool of molecular descriptors (Fig. S3 and Methods) were calculated. The highest correlation (r^2 = 0.303) was found to exist with topological polar surface area (TPSA, the surface sum over all polar atoms or molecules, mainly oxygen and nitrogen, also including their attached hydrogen atoms) and in minor extent with hydrogen bond donor and acceptor groups (HBD and HBA). In particular, the higher the TPSA, the lower the P_{app}. This is in line with the literature [33] and again confirms the reliability of our system.

Finally, we verified whether TPSA can distinguish high from low permeable compounds. Although a definitive threshold is missing, in the standard PAMPA setup the P_{app} value for distinguishing low from highly permeable compounds is frequently set at 1.5×10^{-6} cm/s [30]. However, in a modified setup (diffusion from the top to the bottom well) the permeability threshold is higher [34 35]. Here we used 4×10^{-6} cm/s and 1×10^{-6} cm/s to distinguish high, medium and low permeable molecules. Figure 3B shows that TPSA values of 140 Å^2 [36]. and of 75 Å^2 [37,38] are able to predict permeability class of the investigated dataset.

In addition to the discrimination based on the total polar surface area, we plotted the high and low permeable compounds in the chemical space based on their chemical properties. For this purpose, we computed a principal component analysis using as variables the molecular descriptors calculated from DataWarrior. Indeed, we can observe a good separation of the two groups on the first principal component (See Fig. S4 A-C).

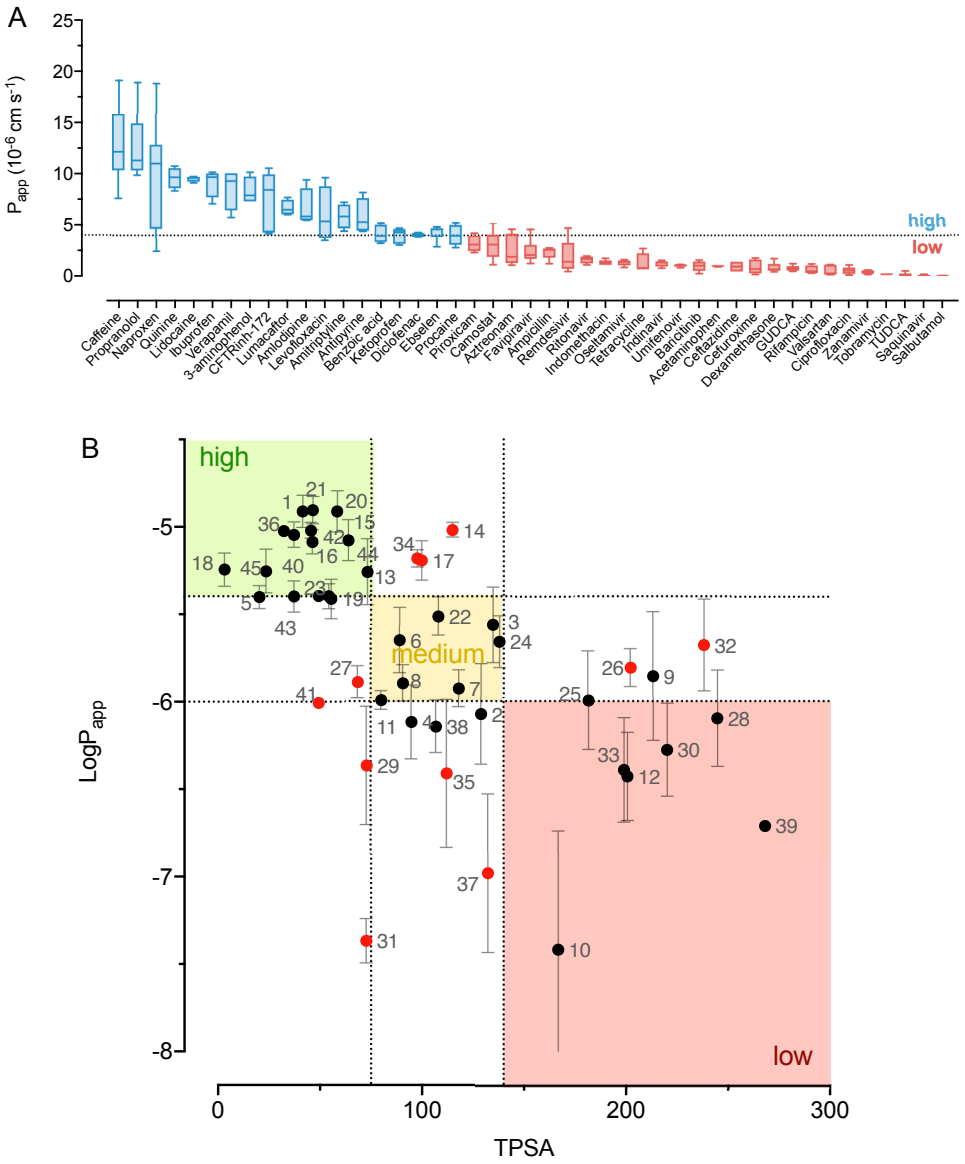

Figure 3. Classification in high and low permeable compounds (A) of the tested drugs based on the determined apparent permeability (P_{app}) Dataset grouping within permeability categories (B): permeability classification: green = high, yellow = medium, red = low. The P_{app} threshold high-medium and medium-low permeable compounds was set at 4 and 1 × 10^{-6}, respectively. TPSA threshold between high-medium and medium-low permeable compounds was set at 75 and 140 Å^2, respectively. The number beneath each dot refers to drug name (see Fig. S1 and Table 2).

Overall, we validated the goodness of the experimentally determined P_{app} in the absence of mucus and confirmed that the P_{app} values can be used as benchmark when assessing the effect of mucus in the mucus-PAMPA system.

3.3. Permeation studies in the presence of a mucus model

The mucus model was adapted to the PAMPA plate by directly pipetting it on top of the phospholipid membrane in the donor compartment. As all the wet epithelia of the human body are covered by mucus, drugs administered by oral or pulmonary routes have to cross both the mucus layer and the cellular membrane to be absorbed, and thus to be effective. With our adapted mucus-PAMPA system we are expecting to be able to mimic *in vitro* the interface at mucosal surfaces. (Fig. 4).

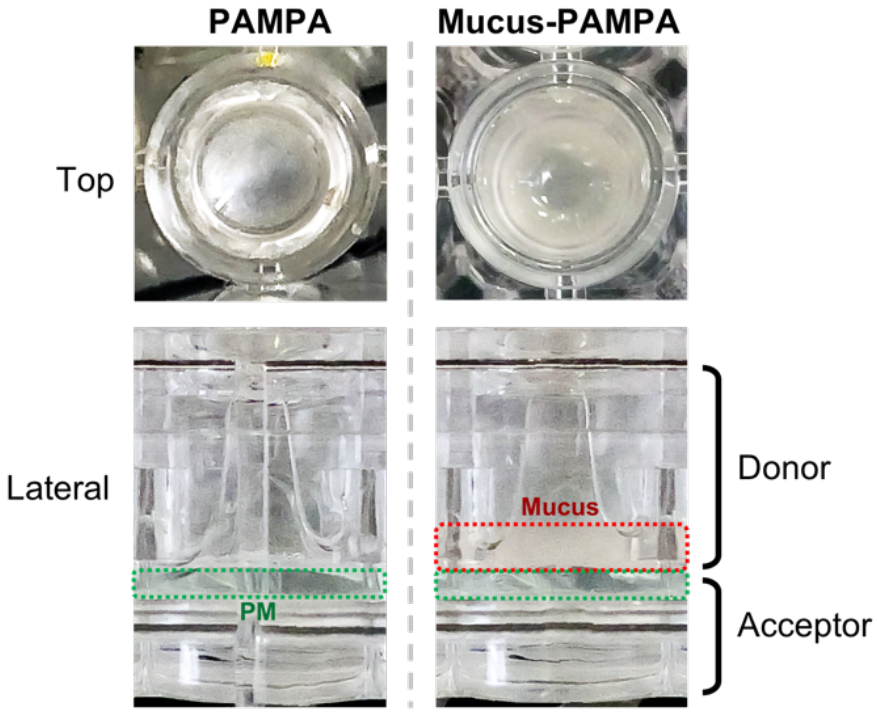

Figure 4. Comparison between PAMPA and mucus-PAMPA system. On top the upper view of the PAMPA wells, on the bottom the lateral view of the donor and acceptor compartments. Dashed lines highlight the mucus layer and the phospholipid membrane (PM).

The effect of mucus was measured in terms of variations of permeability and was considered statistically significant only if the *p*-value between the means of the two groups (PAMPA and mucus-PAMPA) was <0.05. A summary of the P_{app} with and without mucus is reported in Table 1, while the detail of each drug tested is reported in SI (Fig. S6).

Table 1. Summary of the P_{app} recorded on PAMPA and the effect the mucus model played over permeability with the respective P_{app} variations. The terms *increased / decreased* are used only if the difference on permeability is statistically significant. Student's t-test was applied to detect statistical significance between

the permeability recorded with and without mucus. A $p<0.05$ was considered to be a statistically significant difference.

Nr	Compound	MW (g/mol)	Lipophilicity	P_{app} PAMPA (± SD) $\times 10^{-6}$ [cm/s]	P_{app} Mucus-PAMPA (± SD) $\times 10^{-6}$ [cm/s]	Effect of mucus on P_{app}
1	Propranolol	259.3	medium	12.56 (± 2.9)	2.84 (± 1.4)	Decreased (-1)
5	Ebselen	274.2	low	4.01 (± 0.6)	1.82 (± 0.8)	Decreased (-1)
8	Oseltamivir	312.4	medium	1.30 (± 0.3)	0.32 (± 0.1)	Decreased (-1)
11	Umifenovir	477.4	medium	1.03 (± 0.1)	0.18 (± 0.0)	Decreased (-1)
12	Zanamivir	332.3	medium	0.42 (± 0.2)	0.02 (± 0.0)	Decreased (-1)
13	Levofloxacin	361.4	low	5.94 (± 2.7)	0.00 (± 0.0)	Decreased (-1)
14	CFTRinh-172	409.4	medium	7.35 (± 2.9)	3.84 (± 1.2)	Decreased (-1)
15	Verapamil	454.6	low	8.57 (± 2.0)	0.53 (± 0.5)	Decreased (-1)
16	3-aminophenol	109.1	low	8.28 (± 1.4)	0.74 (± 0.6)	Decreased (-1)
17	Amlodipine	408.9	medium	6.60 (± 1.9)	0.00 (± 0.0)	Decreased (-1)
18	Amitriptyline	277.4	high	5.80 (± 1.2)	0.00 (± 0.0)	Decreased (-1)
19	Procaine	236.3	low	4.00 (± 1.0)	0.00 (± 0.0)	Decreased (-1)
20	Caffeine	194.2	low	12.70 (± 3.5)	6.43 (± 4.2)	Decreased (-1)
25	Tetracycline	444.4	high	1.22 (± 1.0)	0.00 (± 0.0)	Decreased (-1)
26	Ritonavir	721.0	high	1.60 (± 0.4)	0.61 (± 0.2)	Decreased (-1)
31	Salbutamol	239.3	low	0.04 (± 0.0)	0.01 (± 0.0)	Decreased (-1)
34	Lumacaftor	452.4	medium	6.64 (± 0.8)	5.40 (± 0.3)	Decreased (-1)
36	Lidocaine	234.3	high	9.47 (± 0.3)	0.05 (± 0.1)	Decreased (-1)
38	GUDCA	449.6	medium	0.76 (± 0.3)	0.22 (± 0.3)	Decreased (-1)
42	Quinine	324.4	low	9.54 (± 1.0)	6.66 (± 0.6)	Decreased (-1)
3	Camostat	398.4	medium	3.04 (± 1.3)	3.28 (± 2.9)	None (0)
4	Dexamethasone	392.5	medium	0.85 (± 0.4)	0.46 (± 0.4)	None (0)
6	Favipiravir	157.1	high	2.44 (± 1.1)	3.17 (± 1.3)	None (0)
7	Indinavir	613.8	low	1.22 (± 0.3)	0.94 (± 0.7)	None (0)
9	Remdesivir	602.6	low	1.86 (± 1.6)	1.63 (± 1.5)	None (0)
10	Saquinavir	670.9	high	0.08 (± 0.1)	0.10 (± 0.1)	None (0)
24	Ampicillin	349.4	high	2.29 (± 0.6)	1.34 (± 0.7)	None (0)
28	Ceftazidime	546.6	low	0.93 (± 0.5)	1.14 (± 1.0)	None (0)
29	Ciprofloxacin	331.3	low	0.54 (± 0.3)	0.31 (± 0.3)	None (0)
30	Rifampicin	823.0	high	0.61 (± 0.4)	0.19 (± 0.1)	None (0)
32	Aztreonam	435.4	low	2.45 (± 1.5)	1.64 (± 0.6)	None (0)
33	Cefuroxime	424.4	low	0.87 (± 0.7)	0.16 (± 0.2)	None (0)
35	Valsartan	435.5	low	0.56 (± 0.5)	0.44 (± 0.5)	None (0)
37	TUDCA	499.7	high	0.17 (± 0.2)	0.04 (± 0.1)	None (0)
39	Tobramycin	467.5	high	0.19 (± 0.0)	0.25 (± 0.1)	None (0)
40	Ibuprofen	206.3	medium	9.09 (± 1.4)	10.33 (± 1.2)	None (0)
45	Antipyrine	188.2	medium	5.75 (± 1.7)	6.19 (± 2.6)	None (0)
2	Baricitinib	371.4	high	0.99 (± 0.5)	2.68 (± 2.1)	Increased (+1)
21	Naproxen	230.3	low	9.57 (± 3.9)	22.02 (± 6.1)	Increased (+1)
22	Piroxicam	331.4	high	3.15 (± 0.8)	6.63 (± 0.1)	Increased (+1)
23	Diclofenac	296.2	medium	4.03 (± 0.2)	5.06 (± 0.1)	Increased (+1)
27	Indomethacin	357.8	medium	1.32 (± 0.3)	2.54 (± 0.9)	Increased (+1)
41	Acetaminophen	151.2	medium	0.99 (± 0.1)	6.22 (± 0.9)	Increased (+1)
43	Benzoic acid	122.1	medium	4.06 (± 0.9)	9.91 (± 0.9)	Increased (+1)
44	Ketoprofen	254.3	low	4.04 (± 0.7)	9.24 (± 1.2)	Increased (+1)

Three kinds of mucus-induced effects were observed (Table 1 and Fig. 5A): a) in the presence of mucus, 44% of drugs showed a decreased permeability, b) 38% had no statistically significant variation, and c) 18% had an increased permeability. Among the compounds which diffusion was reduced by mucus, we cannot outline any dependency on drugs lipophilicity. In fact, mucus reduced the permeability of both hydrophilic and lipophilic drugs which is in agreement with what found by Boegh *et al.*, [18] using a biosimilar mucus on Caco-2 cells, and Falavigna *et al.*, [21] with a mucin-PVPA system.

Data suggests that the CF pathological mucus model did not act as a mere physical barrier. It behaves as an interactive filter as different structures interacted differently with mucus. This can be attributed to low affinity interactions taking place during the diffusion process across the mucus layer and is mostly dependent on the structure of mucins. In fact, due to the complex architecture of mucins, hydrophobic drugs can be retained by the naked domains of the peptide core of mucin, while hydrophilic drugs can entangle with the branched oligosaccharides. In addition to the interactive filter orchestrated by mucins through hydrophobic, electrostatic and hydrogen bonding interactions, mucins can hinder the diffusion of xenobiotics also through a size filtering mechanism dependent on the mucin mesh. To estimate the mesh size of our mucus model we applied the generalized Maxwell model (GMM) as described in our previous work[22]. The estimated mesh size was 54.7 ± 5.35 nm which is in good agreement with what reported in the literature for pathological mucus. Considering that our dataset is composed of only small molecules, thus much smaller than the mesh of our mucus model, we think the steric filter had a minor impact on drug diffusion.

Once we had assessed the effect that mucus plays on the P_{app} (*i.e.*, decreased, no effect, increased) of each compound, we then wanted to quantitatively compute the activity of mucus. Thus, we assigned numerical values to each effect, particularly -1, 0, +1 when the permeability was decreased, unvaried and increased, respectively. The effect of mucus over permeability varies without any apparent relation to any of the molecular descriptors selected, as shown by the correlation matrix (Fig. S3), In fact, the relation between the P_{app} and TPSA registered in the absence of mucus does not hold true anymore. If we previously could correctly predict the permeability of almost 80% of the tested compounds, after the addition of mucus we see that only 53% of the molecules have their permeability correctly predicted (Fig. 5B). For instance, amitriptyline (compound nr. 18) in the absence of mucus belongs to the high permeable group as it has low TPSA and high P_{app}; in contrast, the presence of mucus strongly decreases its permeability. Amitriptyline is a highly lipophilic drug though being positively charged at pH 7.4. Such a reduction of permeability that we observe, may be the result of a combined retention due to interactions with the lipophilic domains and the negatively charged glycans of mucin. As amitriptyline, many other drugs have their permeability decreased (see Table 1 and Fig. 5).

With the drug diffusion studies on the mucus-PAMPA system we show that the pathologic cystic fibrosis mucus model can strongly influence the permeability of drugs. Theoretically, if the observed effects would have been similar for all compounds, one could reasonably state that the rate-limiting factor could be the longer diffusive

pathway when in the presence of mucus. Though, this hypothesis should be discarded as we have found that for some drugs the presence of the pathological mucus model may increase permeability. These results are discussed in the next session.

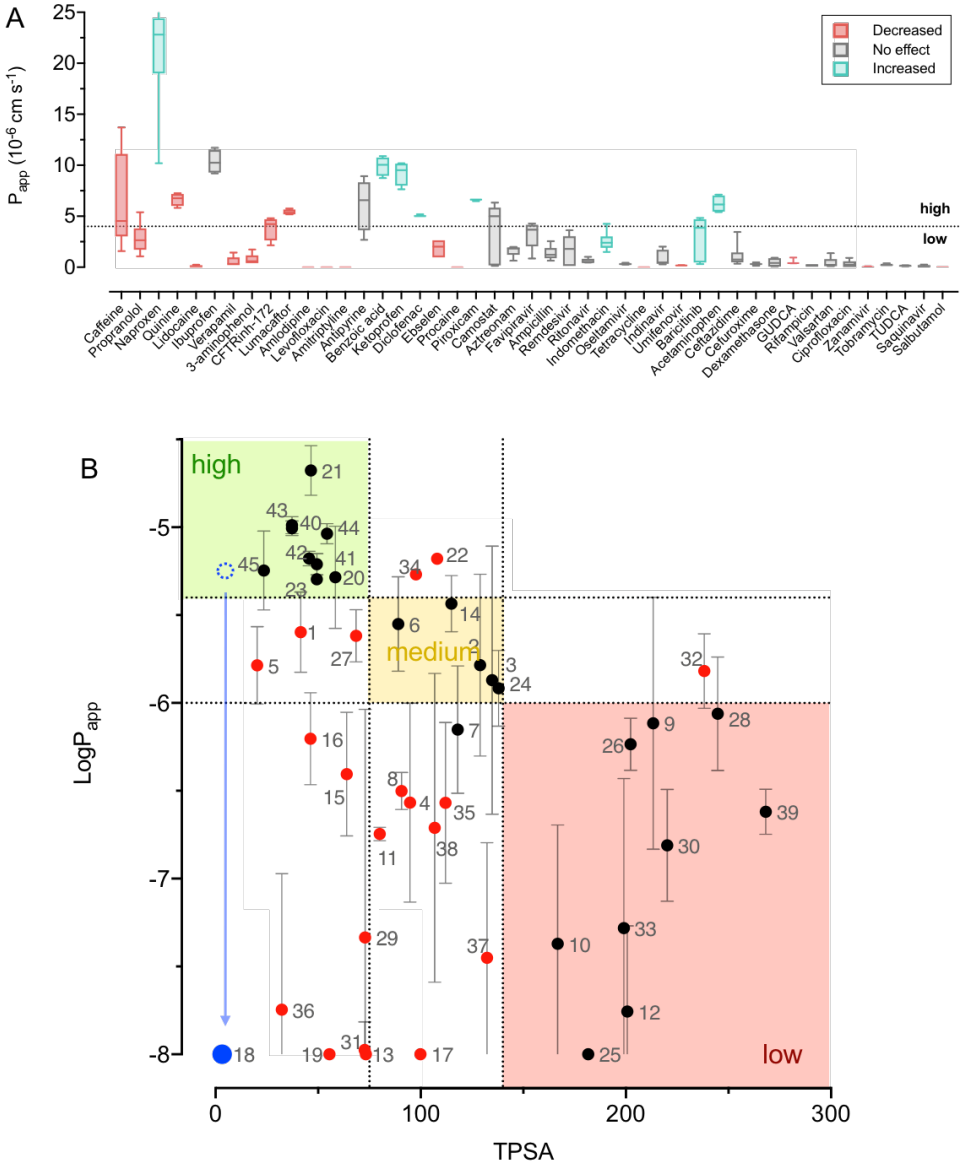

Figure 5. The impact of mucus over permeability. (A) The permeability recorded in the presence of mucus. (B) Grouping within permeability categories of the 45 compounds tested (permeability classification: green = high, yellow = medium, red = low). The P_{app} threshold of high-medium and medium-low is set at 4 and 1 × 10^{-6}, respectively. TPSA threshold of high-medium and medium-low is set at 75 and 140 $Å^2$, respectively. The number beneath each dot refers to drug name (see Fig. S1 and Table 2). As an example, focus is pointed on the variation of permeability of amitriptyline (nr 18, blue dot) in the absence and presence of mucus.

3.4. On the increased permeability in the presence of pathological mucus

We expected the mucus model to reduce or have no effect on the permeability of drugs. Figure 5A clearly reports that some of the compounds we tested (such as naproxen) presented a higher permeability in the presence of mucus than in its absence. Among the tested drugs, 18% (baricitinib, naproxen, piroxicam, diclofenac, indomethacin, acetaminophen, benzoic acid, ketoprofen) had a significant increase of permeability in the presence of mucus. In respect to the entire dataset, the increased-permeability group share some chemical-physical properties; they are relatively small molecules (MW < 380 Da, Total Surface Area < 270 $Å^2$), lipophilic (cLogP > 1), have medium-low polarity (PSA<130 $Å^2$), and 6 out of 8 are negatively charged at pH 7.4.

3.4.1. The role of the mucus components in the enhancement of permeability

To isolate the driving force of the increased diffusion rate observed in the presence of our mucus model, we disassembled the mucus and measured the permeability of naproxen in the presence of each one of the components of mucus. For this purpose, we selected naproxen as a model drug as it is the most remarkable compound for which the permeability increases with mucus (Fig S6). The main components of our mucus model are PGM which is used to mimic the composition of CF mucus; alginate because it is produced by mucoid *P. aeruginosa* infecting the CF mucus; $CaCO_3$ used to crosslink alginate, and NaCl necessary to reproduce the salinity of CF mucus. Thus, we performed a PAMPA assay where naproxen solutions were prepared in PBS buffer containing either PGM, or NaCl or calcium in the same concentrations used in the mucus model. In the case of the alginate gel, it was deposited on the bottom of the top compartment of the PAMPA the day before the experiment to allow alginate to crosslink. (top of Fig. 6). We observed that while the diffusion rate in the presence of PGM or NaCl did not undergo major variations, in the other two systems (*i.e.*, the alginate gel and $CaCl_2$) a net increase of permeability was obtained. It is noteworthy to remind that the alginate gel contains calcium as it is necessary to crosslink the alginate solution hence, to form the hydrogel matrix. The most probable scenario explaining such an activity could rely on ion-pairing as it is known that Ca^{2+} ions have a strong affinity for O, N or F atoms because the metal act as Lewis acid and thus form complexes with many ligands [39]. The binding of calcium ions is highly selective and can form asymmetric complexes that consist of a large radius.

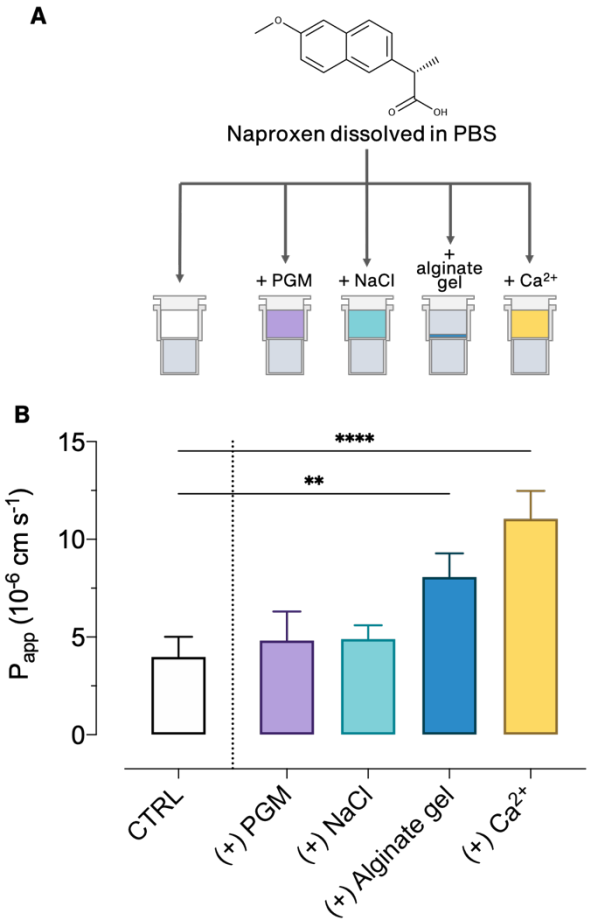

Figure 6. (A) The experimental setup used to isolate the effect of each one of the components of mucus. (B) the permeability of naproxen recorded in the presence of individual components of mucus.

3.4.2. The role of calcium

Once we isolated Ca^{2+} as the reason for the increased permeability, we then wanted to understand if this phenomenon is dependent on the negative charge borne by some of the drugs. Tests were repeated with ketoprofen and indomethacin as anions on which mucus increased their respective permeabilities, as well as $CFTR_{inh}$-172 and cefuroxime as anions with reduced and unvaried permeability (negative controls) in the presence of mucus, respectively. For naproxen, ketoprofen and indomethacin, permeability rises in presence of the alginate hydrogel and gets even higher in presence of the only $CaCl_2$ (Fig. 7A). It has been reported that these three drugs and other non-steroidal anti-inflammatory agents (NSAIDs) can form complexes with calcium[39–41]. Interestingly, Ogiso and colleagues reports that the absorption of indomethacin calcium salt on rat abdominal skin is significantly higher than that from indomethacin alone [41]. When forming complexes with calcium, naproxen, ketoprofen and indomethacin are neutralized; they shift from a negatively charged form to exhibiting no relative charge.

The neutralization implies a decrease of polarity in favor of lipophilicity which we think it actually favors the diffusion through the artificial phospholipid membrane of PAMPA (Fig. 7B). It should be noted, when drug-calcium complexes are formed, the molecular descriptors are completely different from that of the free drugs and cannot be easily calculated. In addition, the higher permeability observed with $CaCl_2$ could be due to larger availability of free Ca^{2+} in solution. In fact, in the system containing alginate, despite having the same Ca^{2+} concentration, part of it is not available because of the ion crosslinking with alginate. On the contrary, we observe that the permeability of $CFTR_{inh}$-172 and cefuroxime is not influenced by the presence of calcium, even though if they are also considered anionic drugs.

Overall, we hypothesized that drug-calcium salts have higher passive diffusion rates through the PAMPA phospholipid artificial membrane with respect to the not-complexed drug. However, the formation of calcium salts is not merely dependent on the negative charge as not all the anionic drugs included in the dataset enhanced permeability.

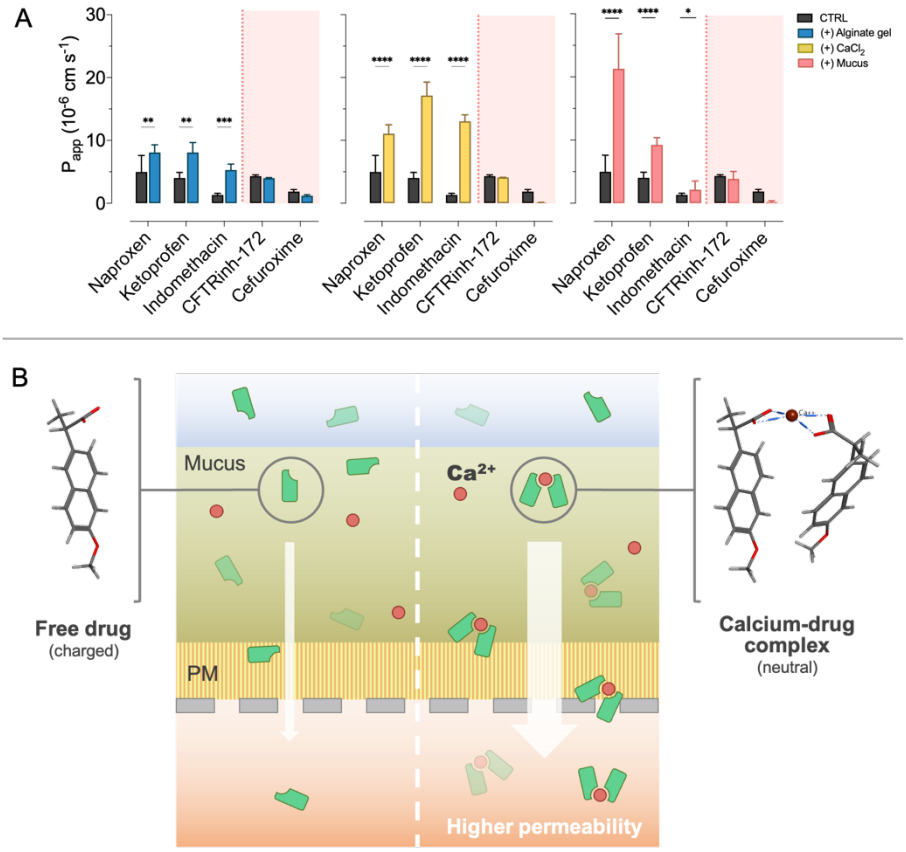

Figure 7. The effect of calcium over the permeability of some anions at pH 7.4. (A) Naproxen, ketoprofen and indomethacin have higher diffusion rates when calcium is present. $CFTR_{inh}$-172 and cefuroxime do not undergo permeability variations in the presence of calcium. Two-way ANOVA comparing each group mean with the mean of each other group was used to compute statistical analysis. (B) Schematic representation of the calcium-naproxen complex and the passive diffusive mechanism through mucus-PAMPA system.

3.4.3. CF patient sputum validation

In the next step we wanted to understand if the results recorded in the presence of our mucus model can be reproduced using a more complex biological matrix to mimic CF sputum. For this purpose, we employed CF sputum as it is often considered a rough *ex vivo* model of CF mucus, and we measured the permeability of naproxen, ketoprofen and indomethacin. In fact, a hallmark of diseases such as CF, COPD and bronchiectasis is the excessive production of sputum. As a consequence of the altered physico-chemical properties, the diseased sputum contains higher concentrations of inflammatory mediators, lytic enzymes (*i.e.*, neutrophil elastase) and bacterial colonization. However, due to the high variability among patients, which depends on the diseases stage, the measurements performed with CF sputum may have a low reproducibility.

Given the high concentration of calcium in our mucus model we wanted to find out if the permeability of some negatively charged drugs results overestimated when using the mucus model developed by us. As expected, in the presence of the CF sputum the permeability of naproxen, ketoprofen and indomethacin was decreased, even though the variation was not statistically significant (FIG. 8A). Such a reduction could be the result of interactions with neutrophil elastase and proteases also present at high concentrations in the CF sputum. Mandel *et al.*[42], reported higher concentrations of calcium (136 ± 33 μg/mL) in submaxillary saliva of CF patients with respect to healthy people (71 ± 19 μg/mL). Based on these values, we can estimate almost a 10x higher concentration of calcium in our model than that of the *in vivo* scenario. Therefore, we can speculate that the permeability of some negatively charged drugs might be overestimated with our CF mucus model.

Despite this, we establish evidence indicating that some negatively charged drugs can form calcium-drug salts which have higher diffusion rates on PAMPA respect to the free drugs. In terms of permeability, these salts might be less affected by the barrier effect of mucus. Thus, we wondered if this mechanism can be exploited to increase the permeability of naproxen, ketoprofen and indomethacin through CF sputum. For this purpose, we repeated the permeability test through CF sputum, this time suspending the drugs into PBS containing calcium. As can be seen from Fig. 8B, the permeability of

all of the three drugs was significantly higher for the samples containing calcium. This is likely a consequence of the formation of calcium complexes which increased more than twice the permeability of the drugs through CF sputum.

Even though the concentration of calcium in the CF mucus is reported to be lower than that of our mucus model, it is still clear the impact that calcium can play on permeability. Drug calcium salts might have better biological activities and should be considered when formulating drugs. For instance, high-dose ibuprofen taken constantly for years has shown to significantly reduce the progression of the lung disease in cystic fibrosis[7]. It would be interesting to investigate if better health outcomes could be reached using a calcium formulation.

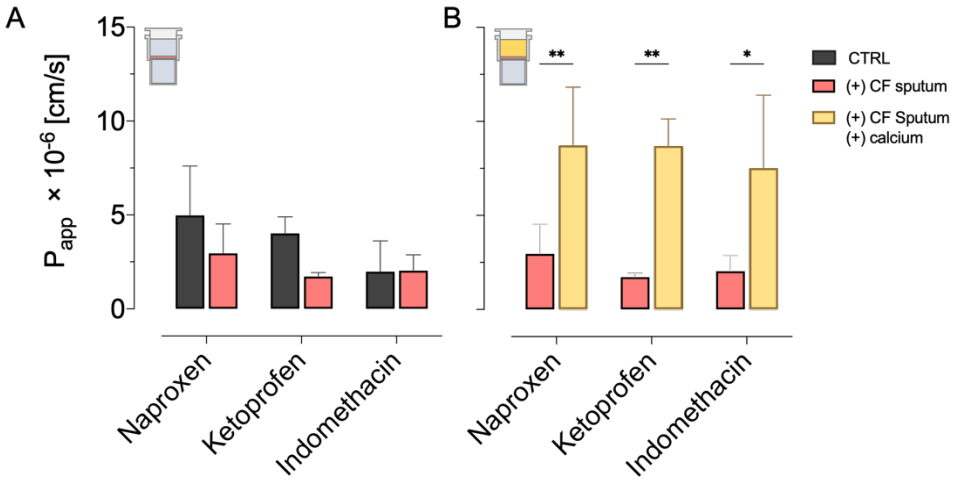

Figure 8. The effect of calcium over the permeability of naproxen, ketoprofen and indomethacin in the presence of cystic fibrosis (CF) sputum. (A) The permeability of the three drugs measured on the CF sputum - PAMPA system and compared with PAMPA (CTRL). (B) The increase of permeability through the CF sputum - PAMPA after the formation of calcium-drug complexes. Two way ANOVA was used to compute statistical analysis.

Conclusions

Here we used an *in vitro* pathological cystic fibrosis mucus model to explore how it can impact over permeability of a dataset of 45 compounds. Overall, our data suggest that the activity of mucus is complex to predict. Determining the specific effects limiting the permeability of drugs through mucus was out of the scope of this work. Yet, it was found that the mucus was not only a physical barrier for the permeability of drugs but also behaved as a dynamic filter as well. The permeability of most of the compounds was reduced, whilst others have not been affected by the barriers of mucus. A poor

correlation of the effect of mucus on permeability was found for all of the selected molecular descriptors. These findings represent an additional evidence of the further need for reliable *in vitro* mucus models to be used for drug screening, especially in mucus related disorders.

We also ascertained that calcium, which is one of the components of our mucus model, enhanced the permeability of a small group of drugs. This was most likely the result of the complexation with the drug. The observed increased-permeability effect induced by calcium was also achieved also through cystic fibrosis sputum, further highlighting the potential to use drugs as calcium salts to pursue higher absorption rates *in vivo*. Additionally, calcium-based formulations could offer new potentialities also for the repositioning of the current therapies.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

CF: cystic fibrosis
COPD: chronic obstructive pulmonary disease
PAMPA: parallel artificial membrane permeability assay.
PBS: phosphate buffer saline
HPLC-MS: high-pressure liquid chromatography – mass spectrometry
DMSO: dimethyl sulfoxide
P_{app}: apparent permeability
GDL: D-(+)-gluconic acid δ-lactone
MRM: multiple reaction monitoring
PCA: principal component analysis
Ro5: Lipinski rule of five
NSAID: non-steroidal anti-inflammatory drug

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