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EDITORIAL

Challenges with drug efficacy prediction of in vitro models of biofilms infecting cystic fibrosis airway

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1. Introduction

Cystic fibrosis (CF) is a genetic disorder caused by a defect in the CF transmembrane conductance regulator gene, characterized by the accumulation of thick, sticky, and acidified sputum in the lungs. This abnormal buildup of sputum, rich in nutrients, and the defective mucociliary clearance render CF patients vulnerable to both acute and chronic bacterial infections of the airways. These infections lead to inflammation, declining lung function, respiratory failure, and premature death [1].

Currently, antibiotics remain the centerpiece of treatment for bacterial infections in CF, but there is a well-known poor correlation between the prolonged and aggressive antibiotic regimens – often utilizing multiple antibiotics simultaneously – and clinical outcomes. This disparity is largely due to the formation of biofilms within CF sputum that grants resident bacteria 10 to 1000-fold greater tolerance to antibiotics compared to their free-living counterparts. This loss of susceptibility is due to the interplay of multiple mechanisms, including decreased antibiotic diffusion through the biofilm exopolysaccharide matrix, reduced growth rate, the phenotypically heterogeneous microenvironment, altered gene expression and the formation of persister cells. Furthermore, the ecological interactions between bacterial species residing in polymicrobial biofilms also contribute to treatment failure [2].

Given the challenges associated with antibiotic therapy, there is an urgent need for new drugs targeting CF-associated infections. While gene replacement therapy remains elusive, significant efforts have been directed toward developing antibiofilm agents. Over the years, an impressive number of research groups and studies have reported antibiofilm drugs; however, only a few have progressed to clinical trials and regulatory approval. This situation brings into question the current predictability of the *in vitro* models regarding antibiofilm drug efficacy.

The process of developing new antibiofilm drugs and bringing them to the market is hampered by the lack of consensus regarding the most appropriate preclinical drug-screening models. Single or polymicrobial-culture experiments using multiwell microtiter plates are the most commonly used *in vitro* model for rapid drug screening and testing [3]. Although these models are inexpensive, reproducible, easy to set up, and allow a high-throughput drug screening ideal to interrogate libraries of novel compounds, they have limited drug predictability because the testing conditions poorly resemble CF biofilms. Preserved lung tissue samples from CF patients have revealed the presence of biofilms suspended in the bronchial sputum, surrounded by numerous polymorphonuclear leukocytes and exhibiting a sponge-like structure [4]. In contrast, biofilms formed in microtiter plates are adhered to an abiotic surface (mostly plastic) and typically present a thick, flat, and lawn-like structure, occasionally displaying a heterogeneous structure with 'holes' or high biomass peaks on the well edges [5]. Distinct biofilm structures integrate different populations that can trigger varied drug responses, including the expression of specific resistance genes that contribute to biofilm persistence [6]. Therefore, *in vitro* microtiter plate-based models are not the most adequate for determining drug efficacy against CF suspended biofilms. Flow-based systems are also used to visualize biofilm formation and ascertain drug effects on biofilms as they offer a real-time and nondestructive approach [7]. However, they share limitations with microtiter-based models, as biofilms are formed on hard plastic or glass that does not replicate the complex microenvironment found in CF lungs. As a result, bacterial tolerance to drugs may be overestimated.

Inhalation stands out as the most attractive drug delivery approach for treating CF-related lung infections. This approach ensures the drug is deposited directly at the site of infection, resulting in higher local drug availability while requiring lower drug dosage for optimal efficacy and avoiding systemic toxicity. To accurately represent inhaled drug delivery, *in vitro* models must recreate the physicochemical environment found in CF lungs, particularly the thick and sticky sputum, because it has been shown to impact the response of biofilms to drug exposure [2,8]. For instance, a transcriptomic analysis of *Burkholderia cenocepacia* grown in CF sputum revealed changes in gene expression associated with efflux pumps, antibiotic degradation (upregulation of ß-lactamases), iron uptake, and host evasion when compared to its growth in minimal salt medium [9]. These changes significantly impact

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antibiofilm drug screening and development. In our laboratory, we identified five natural extracts as potent antibiofilm agents by testing them on single-species biofilms adhered on 96-well microtiter plates and using a standard laboratorial medium. However, only one exhibited antibiofilm activity when tested on suspended biofilms formed within artificial CF sputum (ASM) (data not yet published).

However, adding any of the various available formulations of ASM [10–13] into *in vitro* models is a step forward to a more realistic approach, as it permits the simultaneous growth of planktonic and suspended biofilm-cells as *in vivo*, and ASM-based models must be reshaped due to reported limitations. Transcriptomic profiling of *Pseudomonas aeruginosa* grown in ASM and isolated from CF patients' expectorated sputum samples revealed dissimilarities in quorum sensing, metabolic activity, and antibiotic resistance associated genes. Specifically, the genes involved in amino acid and carbohydrate biosynthesis and TCA cycle were down-regulated, whereas genes associated with efflux systems and DNA damage stress-response were significantly up-regulated in *P. aeruginosa* isolated from CF sputum samples, with respect to the *in vitro* samples [14].

Accordingly, *ex vivo* lung models, such as precision-cut lung slices and isolated and perfused lungs, are considered alternatives to *in vitro* models because they provide an *in vivo* representation fundamental for recapitulating the CF biofilm structure and physiology. *P. aeruginosa* biofilms formed in an *ex vivo* porcine lung model exhibit a lace-like structure with gaps filled with lung fluid more similar to the sponge-like-appearance of *in vivo* biofilms with openings filled with sputum, alginate, or lung fluid [4,15]. However, *ex vivo* biofilms are positioned on the surface of the tissue rather than suspended, as observed *in vivo*. Nevertheless, tolerance of *ex vivo* biofilms to drugs is quite similar to clinical records of CF infection persistence. For instance, *Staphylococcus aureus* biofilms surrounding pig lung tissues exhibited tolerance to flucloxacillin and displayed small colony variant (SCV) phenotype [16]. The main advantages of *ex vivo* models include no purposeful animal sacrifice, as lung tissues are collected, for instance, from post-consumer waste from the meat industry, in opposite to *in vivo* models, and better predictive power when assessing drug efficacy compared to *in vitro* models. Difficulties in maintaining sterility during dissection, a reduced period of time for bacteria to form biofilms in lung tissue, and low reproducibility of results due to tissue heterogeneity and inconsistent tissue cutting are limitations of the *ex vivo* models.

Appropriately characterizing the in-host conditions in a model is fundamental to recapitulate the intricate interactions between the host and the biofilm that can cue a phenotypic switching to drug-tolerant variants, such as SCV [16]. Monoculture of living epithelial cells under static conditions is a classical *in vitro* approach used for decades to represent host conditions, serving numerous purposes, including drug testing. However, cell growth under these conditions lacks certain tissue-specific functions that may be relevant for drug testing, such as the formation of tight junctions and the development of Trans Epithelial Electrical resistance. Therefore, cell coculture models using, for instance, a Transwell support are used to better review key aspects of CF airways, particularly the luminal airflow of the respiratory system. The rigid semi-permeable membrane supports different cell growth both at the apical side (exposed to air) and the basolateral side (nourished by contact with liquid culture medium) in order to establish an air–liquid interface (ALI). This encourages cell differentiation, expression of polarity, and establishment of tight junctions, which are particularly relevant for mimicking inhaled drug deposition onto the epithelial surface. However, due to their expense, time-consuming setup, and lack of high-throughput capability, few tests have been conducted on biofilms.

Efforts have been directed toward engineering living material to include other parameters, such as the extracellular matrix and multiple cell types. Organoids or organ-on-a-chip systems provide 3D platforms or models capable of mimicking structural aspects of the airway tract and, importantly, better integrating analytical tools that significantly contribute to determining and understanding drug effects [17]. For instance, an organ-on-a-chip was developed to model the CF airways and key dysfunctions by integrating primary CF bronchial epithelial cells grown under ALI into a microfluidic chip, recapping sputum accumulation, ciliary beating activity, and the growth of *P. aeruginosa* in sputum. High secretion of, for instance, IL-6, TNF-alfa and GM-CSF was detected, indicating the close resemblance to *in vivo* conditions [18]. However, biofilm formation and antibiofilm drug testing have not been investigated in this model so far.

Recently, a living multicomponent platform, co-assembling bioactive peptides amphiphile with components of ASM into a hydrogel, was able to support the formation of 3D mono and polymicrobial biofilms of the two major CF pathogens, *P. aeruginosa* and *S. aureus*, on the top of lung epithelial cells [19]. This platform provided an *in vitro*-infected epithelial model capable of corroborating the typical biofilm tolerance to ciprofloxacin, but no other antibiotics or CF sputum samples were used to evaluate the predictability of drug efficacy.

2. Expert opinion

Preclinical *in vitro* models are fundamental to the discovery and approval of drugs, and they will definitely continue to play a central role in drug development for CF-associated infections. Recently, O'Toole et al. reviewed the available models for studying polymicrobial infections developed in CF airways and described their strengths and weaknesses [20]. Among the 33 listed models, not all are adequate for drug discovery and development, and they do not specifically address biofilm formation. Therefore, it is imperative to develop or employ specific and physiologically relevant models to advance the discovery of CF antibiofilm drugs and reduce the occurrence of false positives in the drug development pipeline. Increased collaborative efforts among patient organizations, academic centers, networks, and industry are required to develop and use suitable models or platforms for long-term recapitulation of *in vivo* CF biofilm-associated infections. CF biofilms are structurally and

biochemically distinct from the surface adhered-biofilms which complicates their modeling, but there is an urgent need for consensus among the stakeholders on which aspects of CF airway disease must be incorporated into these models or platforms. Key aspects of CF airway disease such as sputum, alternative carbon sources, e.g. fatty acid and aminoacids (released from lung surfactant and by microbial degradation of mucin), iron (released from to tissue damages), low pH, and hypoxia or anaerobiosis have been shown to impact drug testing, both in terms of the delivery and functionality against CF biofilms [21–23]. Therefore, those key factors must be taken into account when designing an *in vitro* model, especially if it is intended to use it for drug testing.

Bioengineered complex *in vitro* models are being developed successfully integrating several features of CF disease to provide conditions reminiscent of those observed *in vivo*. Multi-dimensional cellular models (e.g. 3D and 4D bioprinting of tissues and organs) are emerging realities that capture the complexity of pathological tissues, rendering scientific research faster and more effective. Moreover, it is possible to incorporate omics technologies into these bioengineered *in vitro* models, allowing for the acquisition of more reliable data about *in vivo* drug efficacy.

Through the wide employment of bioengineered systems, there would be conditions to establish thresholds, cutoffs, or breakpoints of parameters long used by clinical community, such as minimal biofilm inhibitory concentration, minimal biofilm-eradication concentration, and biofilm-prevention concentration. These parameters could become the common 'language' for research and clinical laboratories to communicate and exchange knowledge, accelerating and facilitating preclinical therapeutic studies.

The required consensus about *in vitro* models and standard parameters of antibiofilm activity will not be attained only by academic researchers announcing more *in vitro* models or design standards or measuring parameters. Interactions with regulators (e.g. FDA), Health Technologies Assessment bodies and pharmaceutical and biotechnology industry (e.g. Epithelix and MatTek already manufacturing lung models) are also fundamental to provide guidance on technology that can overcome the current limitations in antibiofilm drug discovery and development for CF-associated infections.

The rapid technological advances in drug development and application prompt fast changes in standards, guidelines, recommendations, or regulations regarding adverse effects, safe dosages, and the proven effectiveness of drugs. Researchers must be aware of these changes to guide drug discovery strategies effectively.

Overall, an ideal model would be as simple as possible yet precise enough to reliably predict the drug's performance against CF biofilms in a reproducible and statistical way, regardless of where tests are conducted. It is unlikely that a single *in vitro* model can meet all these requirements. Therefore, the clinical community must select a subset of in vitro models that can predict in vivo drug performance effectively (thus avoiding the arising of false positives in the drug pipeline) while minimizing the need for multiple other models to adequately replicate key aspects of CF disease for drug testing. Having a consensus on the set of *in vitro* CF biofilm models would enable antibiofilm compounds with low performance to be deprioritized, while those possessing the necessary attributes for CF application can advance into *in vivo* testing and the initiation of clinical trials.

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Declaration of interest

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