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Article

Repurposing High-Throughput Screening Reveals Unconventional Drugs with Antimicrobial and Antibiofilm Potential Against Methicillin-Resistant *Staphylococcus aureus* from a Cystic Fibrosis Patient

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Background/Objectives: Antibiotic therapy faces challenges due to rising acquired and biofilm-related antibiotic resistance rates. High resistance levels to commonly used antibiotics have been observed in methicillin-resistant *Staphylococcus aureus* (MRSA) strains among cystic fibrosis (CF) patients, indicating an urgent need for new antibacterial agents. For the first time, this study aimed to identify potential novel therapeutics with antibacterial and antibiofilm activities against an MRSA CF strain by screening a drug repurposing library. **Methods/Results:** Among the 3,386 compounds, high throughput screening identified 2,439 (72%), 654 (19.3%), and 426 (12.6%) drugs active against planktonic cells, biofilm formation, and preformed biofilm, respectively, although at different extents. The most active hits were 193 (5.7%) against planktonic cells, causing a 100% growth inhibition; 5 (0.14%) with excellent activity against biofilm formation (i.e., reduction $\geq 90\%$); and 4 showing high activity (i.e., $60\% \leq$ biofilm reduction $< 90\%$) against preformed biofilms. The potential hits belonged to several primary research areas, with “cancer” as the most prevalent. After performing a literature review to identify other, already published, biological properties that could be relevant to the CF lung environment (i.e., activity against other CF pathogens, anti-inflammatory and anti-virulence potential), the most interesting hits were: 5-(N,N-Hexamethylene)-amiloride (diuretic), Toremifene (anticancer), and Zafirlukast (antiasthmatic), Fenretide (anticancer), and Montelukast (antiasthmatic) against planktonic *S. aureus* cells; Hemin against biofilm formation; and Heparin, Clemastine (antihistaminic), and Bromfenac (nonsteroidal anti-inflammatory) against established biofilms. **Conclusions:** These findings warrant further in vitro and in vivo studies to confirm the potential of repurposing these compounds for managing lung infections caused by *S. aureus* in CF patients.

Keywords: high-throughput screening; *Staphylococcus aureus*; cystic fibrosis; antibacterial; antibiofilm

1. Introduction

Staphylococcus aureus is the most prevalent bacterium isolated from the sputum of cystic fibrosis (CF) patients during their first decade of life. The incidence of methicillin-sensitive *S. aureus* (MSSA) is significantly lower than that of methicillin-resistant *S. aureus* (MRSA) in most CF patients (Craft et al., 2019). Chronic lung infections caused by methicillin-resistant *S. aureus* (MRSA) strains lead to worse clinical outcomes, including a more rapid decline in lung function (measured as forced expiratory volume in 1 second, FEV₁) and increased mortality rates (Dasenbrook et al., 2008; Dasenbrook et al., 2010).

Antibiotic therapy presents challenges due to the rising rates of antibiotic resistance, particularly among biofilm-related infections (Craft et al., 2019). MRSA strains show high resistance to commonly used antibiotics in CF patients, such as ciprofloxacin, clindamycin, erythromycin, and gentamicin. This underscores the pressing need for new antibacterial agents (Xu et al., 2024).

Traditional drug discovery processes are often lengthy, labor-intensive, and expensive. In contrast, drug repurposing offers a more dynamic, cost-effective, and feasible approach to quickly addressing the declining drug discovery pipeline. This strategy involves exploring new applications for already approved pharmaceuticals, thus reducing the time required for drug development, lowering costs, and minimizing the inherent risks of drug innovation (Farha and Brown, 2019).

This study aimed to identify potential novel therapeutics against *S. aureus* by screening a drug repurposing library for antibacterial and antibiofilm activities for the first time.

2. Results

In the present study, we screened the Drug Repurposing Compound Library (MedChem Express) to identify hits with antibacterial and anti-biofilm activity toward *S. aureus*. The library consisted of 3,386 bioactive compounds (2,342 already launched; 1,044 have reached clinical trial stages in the USA: 1 drug in phase I, 606 drugs in phase II, 372 drugs in phase III, and 65 drugs in phase IV) (Figure 1A) used as drugs with several therapeutic indications, including cancer, neurodegenerative, infectious, and cardiovascular diseases (Figure 1B).

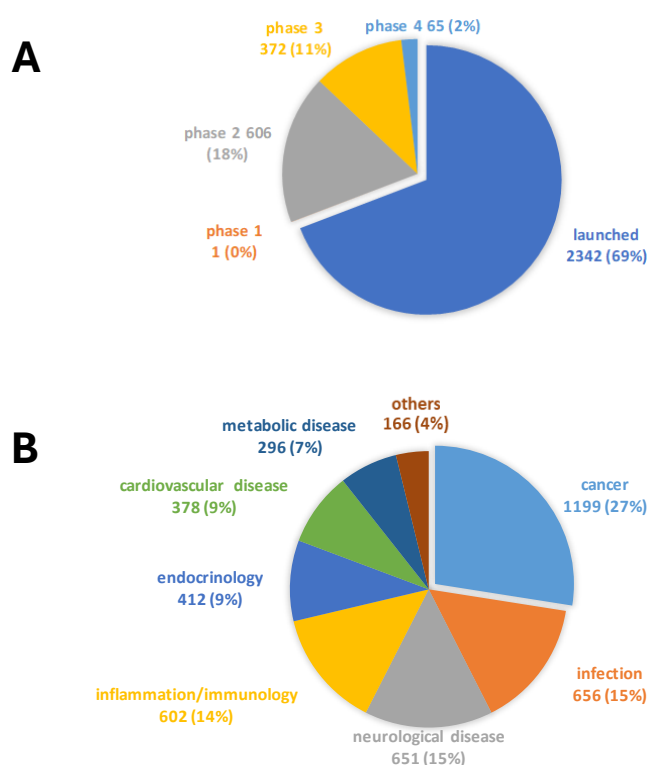


Figure 1. Drug Repurposing Compound Library (MedChem Express) structure: clinical information (a) and research area (b). Reported are the number of drugs and relative percentage. Some drugs belong to more research areas.

2.1. HTS Assay Validation

The screening quality was evaluated using the Z-factor, a standard measure of an HTS's robustness and feasibility. The Z-factor estimates the magnitude of the difference between the positive and negative controls relative to the sum of the respective standard deviations.

The average Z-factor between the negative and positive control in the 96-well test plates was 0.675 (range: 0.500-0.843), well above 0.500 value, indicating the assay could reliably separate positive and negative controls (Figure 2). In addition to the Z-factor, also the coefficient of variation (< 10%) and the signal-to-background ratio (> 10-fold) further supported the feasibility of our anti-*S. aureus* drug screening assay.

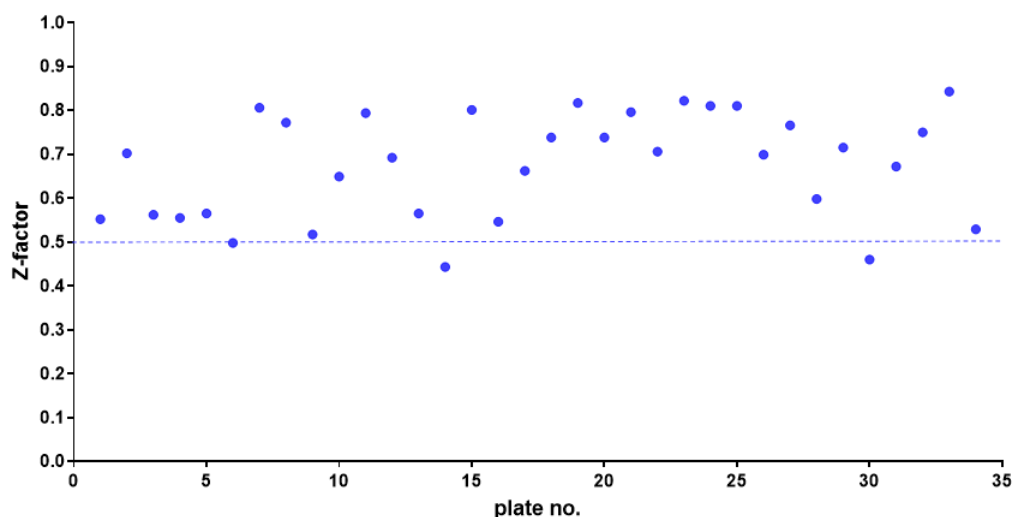


Figure 2. Z-factor plot from high-throughput screening performed in 96-well plates. Solid dots represent the Z' factor from thirty-four 96-well plates. The dashed line indicates the threshold of 0.5.

2.2. Identification of Hits Inhibiting *S. aureus* Growth

We identified compounds active against the methicillin-resistant *S. aureus* Sa2 strain through a 96-well microtiter plate-based high-throughput screening (HTS) of 3,386 compounds belonging to the compound library. All compounds were initially tested at a single concentration of 0.1 mM to pinpoint active hits. The results were expressed as the percentage of inhibition of bacterial growth compared to that in untreated controls, estimated from spectrophotometric readings carried out on supernatant.

This primary screen initially identified 2,439 out of 3,386 (72%) compounds able to affect *S. aureus* Sa2 growth. Among those, 248 hits had “bacterial” as their primary target and were excluded since this work aimed to find new repositionable drugs. Most (n=146) of these excluded compounds showed high (i.e., 60% ≤ growth reduction < 90%) or excellent activity (i.e., growth reduction ≥ 90%), therefore confirming the robustness of HTS.

The remaining 2,191 (64.7%) hits showed a potential for repurposing against *S. aureus*, although at different extents: 617 (18.2%) with low activity (i.e., 10% < growth reduction < 25%); 809 (23.9%) with moderate activity (i.e., 25% ≤ growth reduction < 60%); 350 (10.3%) with high activity (i.e., 60% ≤ growth reduction < 90%); and, more interestingly, 415 (12.2%) compounds with excellent activity (i.e., growth reduction ≥ 90%).

The 415 hits showing excellent activity based on spectrophotometric readings were re-assessed by cell viable count, confirming the efficacy of 364 (10.7%) compounds, listed in Table S1 (in the Supplementary Material) and graphed in Figure 3. Among those, it is worth noting that 193 hits caused a 100% inhibition rate. Based on their known pharmacological profiles, the hits were grouped into classes based on the primary research area (some compounds belonged to multiple areas). Most of the hits belonged to “cancer” (83 out of 193; 43.0%) as a primary research area, followed by “infection” (other than bacterial) (31; 16.1%), “neurological disease” (28; 14.5%), “inflammation” (17; 8.8%), “metabolic disease” and “cardiovascular disease” (12 each; 6.2%), “endocrinology” and “others” (5 each; 2.6%) (Figure 3). Dunnett's multiple comparison tests found no statistically significant differences in antibacterial activity among research areas.

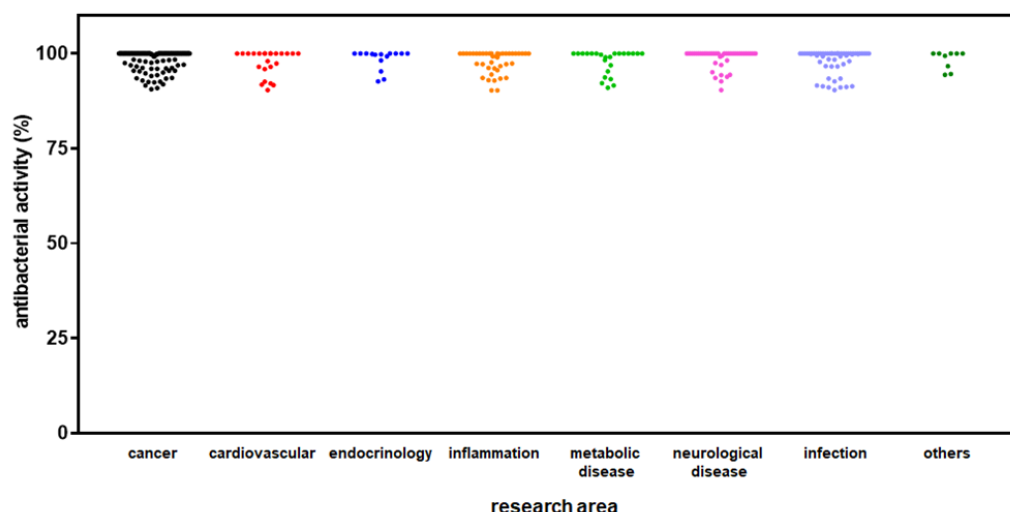


Figure 3. HTS of the compound library to identify active hits against planktonic *S. aureus* Sa2 cells. The antibacterial activity of compounds in several research areas is shown as a percentage referred to as the untreated control.

2.3. Identification of Hits Active Against *S. aureus* Biofilm Formation

The compound library also underwent a 96-well microtiter plate-based HTS to identify hits that could affect biofilm formation by the MRSA *S. aureus* Sa2 strain. The results were expressed as the percentage of inhibition of biofilm biomass formation compared to that in untreated controls, estimated from spectrophotometric readings after a crystal violet assay. The compounds significantly affecting biofilm formation, with no known antibacterial activity and inactive against *S. aureus* Sa2 (i.e., growth inhibition $\leq 10\%$), were considered potential antibiofilm hits.

HTS revealed 654 out of 3,386 (19.3%) compounds can reduce biofilm formation by *S. aureus* Sa2, although at different extents: 128 out of 654 (19.6%) with low activity (i.e., $10\% < \text{biofilm reduction} < 25\%$), 344 (52.6%) with moderate activity (i.e., $25\% \leq \text{biofilm reduction} < 60\%$); 168 (25.7%) with high activity (i.e., $60\% \leq \text{biofilm reduction} < 90\%$); and 14 (2.1%) compounds with excellent activity (i.e., biofilm reduction $\geq 90\%$). Overall, hits belonged to several primary research areas, with “cancer” as the most prevalent, as resumed in Figure 4. Dunnett's multiple comparison tests found no statistically significant differences in activity against biofilm formation among research areas.

The fourteen hits showing excellent activity were retested in a secondary screen - carried out in two independent experiments, each in triplicate - confirming an antibiofilm potential for five compounds (Table S2 in the Supplementary Material). These repositionable hit candidates comprised three anticancer agents (Tipifarnib, Olaparib, and Acefylline), Hemin (“cardiovascular disease” research area), and TMC647055 (Choline salt) (“infection” research area).

2.4. Identification of Hits Active Against Preformed Biofilm by *S. aureus*

Each of the 3,386 compounds was tested at a single concentration of 0.1 mM to pinpoint hits effective towards preformed, 24 h-old biofilm formed by the MRSA *S. aureus* Sa2 strain. The results were expressed as the percentage of biofilm biomass dispersion compared to that in untreated controls, estimated from spectrophotometric readings after a crystal violet assay. The compounds with significant activity against mature biofilms, with no known antibacterial activity and not active against *S. aureus* Sa2, were considered potential antibiofilm hits.

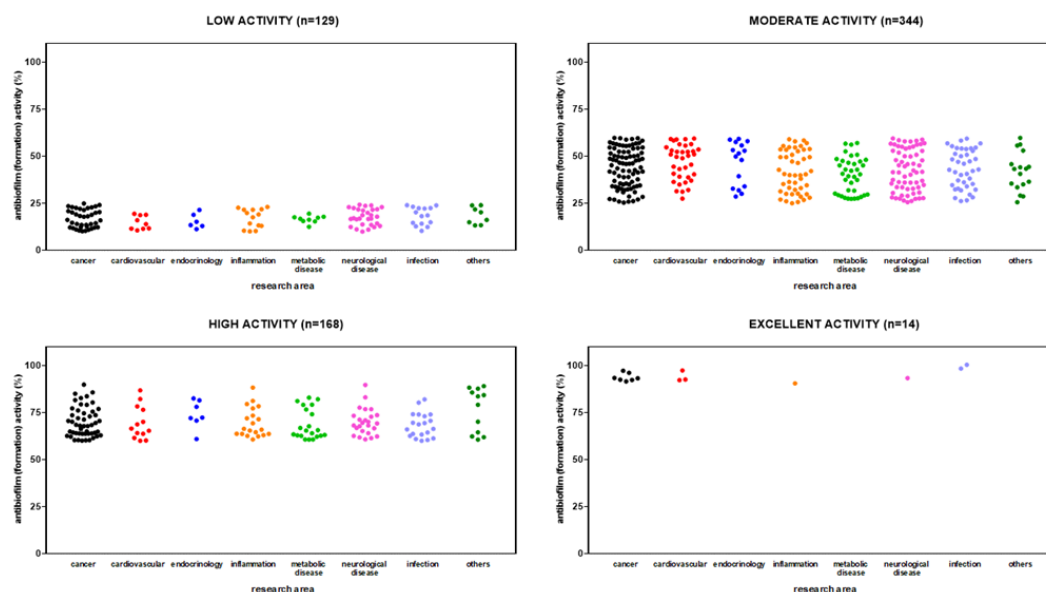


Figure 4. HTS of the compound library to identify active hits against biofilm formation by *S. aureus* Sa2 strain. The anti-biofilm activity of compounds in several research areas is shown as a percentage referred to as the untreated control. The magnitude of the anti-biofilm activity is categorized as follows: low (i.e., 10% < biofilm reduction < 25%), moderate (i.e., 25% ≤ biofilm reduction < 60%), high (i.e., 60% ≤ biofilm reduction < 90%), and excellent (i.e., biofilm reduction ≥ 90%).

The primary screen identified 426 out of 3,386 (12.6%) compounds with the potential for repurposing against mature biofilms by *S. aureus* Sa2, although at different extents. Most of the hits showed low (i.e., 10% < biofilm reduction < 25%) (216 out of 426; 56.1%) or moderate (i.e., 25% ≤ biofilm reduction < 60%) (202, 40.7%) activity, whereas only 8 (3.1%) showed high activity (i.e., 60% ≤ biofilm reduction < 90%). No compound was able to disperse at least 90% of biofilm biomass.

Overall, hits belonged to several primary research areas, although those with low and moderate activity mainly belonged to “neurological disease” and “cancer” areas (Figure 5). Dunnett's multiple comparison tests found no statistically significant differences in activity against mature biofilm among research areas.

The eight hits showing high activity in the primary screening were re-assessed in a secondary screen - carried out in two independent experiments, each in triplicate - confirming 4 repositionable hit candidates, comprising Flumatinib (mesylate) with anticancer activity, the anti-inflammatory Bromfenac (sodium hydrate), Clemastine (fumarate) (“neurological disease”), and Heparin (Lithium salt) (Table S3 in the Supplementary Material).

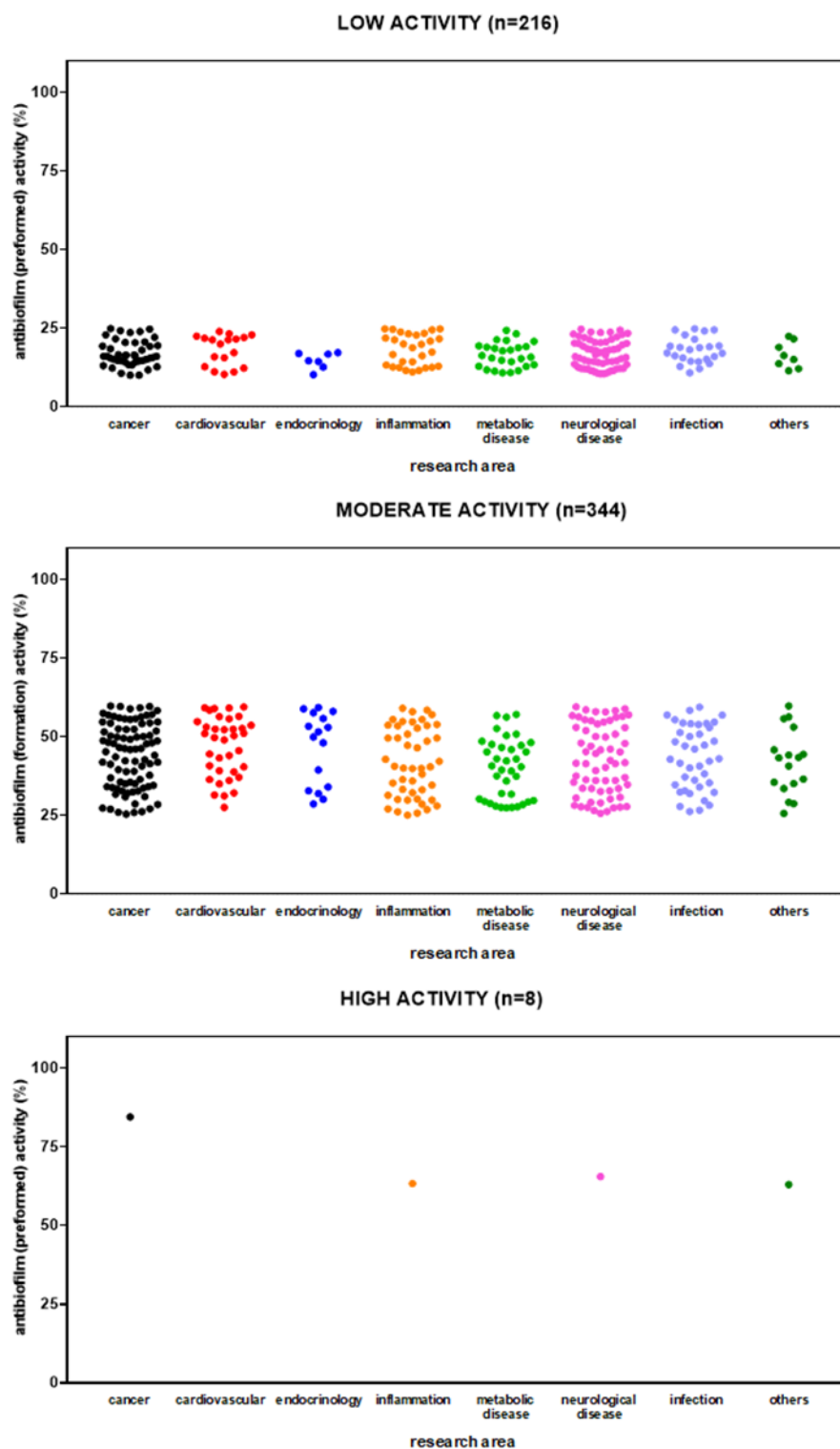


Figure 5. HTS of the compound library to identify active hits against preformed biofilm by *S. aureus* Sa2 strain. The anti-biofilm activity of compounds in several research areas is shown as a percentage referred to as the untreated control. The magnitude of the anti-biofilm activity is categorized as follows: low (i.e., $10\% < \text{biofilm reduction} < 25\%$), moderate (i.e., $25\% \leq \text{biofilm reduction} < 60\%$), high (i.e., $60\% \leq \text{biofilm reduction} < 90\%$), and excellent (i.e., $\text{biofilm reduction} \geq 90\%$).

3. Discussion

In the present study, we screened the Drug Repurposing Compound Library (MedChem Express) to identify hits with relevant potential for antibacterial and antibiofilm activities against an *S. aureus* strain causing a long-term pulmonary infection in a CF patient.

The library we tested in the present study consists of 3,386 bioactive compounds, most already launched and used as drugs with several therapeutic indications, including cancer, neurodegenerative, infectious, and cardiovascular diseases. The library structure could explain the surprisingly high hit rates we observed during the first HTS: 72% (2,440 out of 3,386) for antibacterial activity, 19.3% (654 out of 3,386) for activity against biofilm formation, and 12.6% (426 out of 3,386) for activity against preformed biofilm. Indeed, less than 0.1% hit rates were reported in other HTS studies evaluating large, random chemical libraries of small synthetic molecules that commonly contain numerous non-drug-like molecules (Nilakantan et al., 2002; Langer et al., 2009). The calculation of the Z-factor, the coefficient of variation, and the signal-to-background ratio validated the robustness and feasibility of our anti-*S. aureus* drug HTS assay.

An HTS was initially performed to identify compounds active against planktonic cells of *S. aureus* Sa2 strain. Among the 3,386 compounds screened, 2,191 compounds - with a primary target different from "bacterial" - caused a decrease in Sa2 strain growth, although with different effectiveness. We focused on selected 193 compounds with the highest potential for repurposing since they exhibited maximum activity (i.e., 100% growth reduction), as confirmed by viable cell counts.

A revision of the scientific literature indicated that 135 out of 193 compounds were never reported active against *S. aureus* in previous studies and could be further investigated for repurposing potential. Trying to individuate drugs with a high repurposing potential relevant to the CF lung infection, the selected 135 potential hits underwent a literature review to reveal other biological effects critical to the interplay between the host and pathogens that shape the course of the disease, i.e., activity against other CF pathogens (e.g., *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Haemophilus influenzae*), anti-inflammatory and anti-virulence potential (Bhagirath et al., 2016). In this way, 37 compounds were identified and are listed in Table 1.

Table 1. Compounds (n = 37) with maximum activity against planktonic cells (i.e., 100% growth reduction) of *S. aureus* Sa2 at HTS. Classification, therapeutic category, and mechanism of action are listed, along with other biological properties relevant to cystic fibrosis (CF).

Compound	Classification Therapeutic category and indication(s) Mechanism(s) of action	Anti-bacterial activity	Anti-virulence activity	Anti-inflammatory activity
Perphenazine	- Phenothiazine - Approved for schizophrenia, psychosis, nausea, and vomiting; Investigational for agitation (phase 3), cocaine dependence (phase 2), and depressive disorder (phase 3) - D(2) and D(1A) dopamine receptor antagonist	<i>Neisseria meningitidis</i>, <i>Haemophilus influenzae</i>, <i>Enterobacteriaceae</i>, <i>Streptococcus pneumoniae</i>, group B streptococci, <i>Listeria monocytogenes</i> (Mortensen et al., 1992)		Inhibition of infiltrated mast cells into lesion area (Heo et al., 2020)
Miltefosine	- Hexadecyl monoester of phosphocholine - Approved for trypanosomiasis and cutaneous, mucocutaneous, and visceral leishmaniasis; Investigational for urticaria (phase 2) - Phospholipase A2 inhibitor		- Reduction of <i>S. aureus</i> hemolysis activity in a murine infection model (Koch et al., 2017) - Protective effect <i>vs.</i> murine	T-cell proliferation inhibition (Verhaar et al., 2013)

			pneumonia by inhibition of <i>P. aeruginosa</i> phospholipase C/sphingomyelinase activity (Wargo et al., 2011)	
Diethylstilbestrol	- Synthetic nonsteroidal estrogen - Approved for menopausal and postmenopausal disorders, neoplasm; Investigational for prostate cancer (phase 3) - Estrogen receptor beta agonist		Reduction of <i>S. aureus</i> alpha-toxin, coagulase, deoxyribonuclease, and penicillinase (Yotis, 1977)	Inhibition of phospholipase D activity and degranulation by stimulated human neutrophils (Tou and Urbizo, 2008)
Selexipag	- Pyrazine - Approved for group 1 pulmonary arterial hypertension - Prostacycline receptor agonist			Anti-inflammatory potential (Geerdink et al., 2017)
AZD-9056	- Phenylpropylamine - Investigational for rheumatoid arthritis (phase 2) - P2X purinoceptor 7 antagonist			- In vivo reduction of expression of IL-1β, IL-6, TNF-α, MMP-13, SP and PGE2 (Hu et al., 2016) - Reduction of lipoxin A4 (LXA4), resolvins D1 (RvD1), and 15(S)-hydroxyeicosatetraenoic acid (15(S)-HETE) by alveolar macrophages (Towsend et al., 2023)
Vortioxetine	- N-arylpiperazine - Approved for major depressive disorders 5-hydroxytryptamine receptor 3A and 7 antagonist, Sodium-dependent serotonin transporter inhibitor			Anti-oxidative and immunomodulatory effects by directing macrophages towards the alternative phenotype (Talmon et al., 2018)
Lovastatin	- Fatty acid ester - Approved for hyperlipidemia 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor		Decrease of <i>P. aeruginosa</i> swarming motility (Hennessy et al., 2013)	- Reduction of acute mucosal inflammation via 15-epi-lipoxin A4 (Planagumà et al., 2010) - Macrophage and lymphocyte recruitment reduction in a murine model of the whole lung irradiated Williams et al., 2004) - Decreased proinflammatory

				cytokine levels in lovastatin-treated respiratory syncytial virus-infected cells (Ravi et al., 2013)
Fenretinide	▪ Synthetic retinoid ▪ Investigational for several types of cancer (phases 2-3), lymphoma, and leukemia (phase 1) ▪ Induction of cancer cell apoptosis			▪ Decreased IL-1β and S100A8 expression, improving Cftr-knockout mice's ability to combat <i>P. aeruginosa</i> lung infection (Guilbault et al., 2008) ▪ Arachidonic acid downregulation and increased levels of docosahexaenoic acid in CF patients (Guilbault et al., 2009) ▪ Reduced IL-1β, IL-6, and PGE2 proinflammatory cytokine expression via suppression of JAK-STAT, PI3K-Akt, PKC, and downstream NF-κB signaling pathways in <i>A. actinomycetemcomitans</i>-infected murine monocyte/macrophage (Yu et al., 2013)
Napabucasin	▪ Naphthofuran ▪ Investigational for colorectal and pancreatic carcinoma (phase 3), glioblastoma multiforme (phase 1), and hematopoietic and lymphoid cell neoplasm (phase 1) ▪ Signal transducer and activator of transcription 3 inhibitor	▪ Oral streptococci (Kuang et al., 2021)		▪ Reduced TNF-α and IL-6 levels in neuronal rat cells with post-isolation damage (Wang et al., 2019)
Loratadine	▪ Benzocycloheptapyridine ▪ Approved for symptoms of allergic rhinitis, wheal formation, urticaria, and other allergic dermatologic conditions ▪ Histamine H1 receptor antagonist		▪ Reduced <i>S. aureus</i> biofilm formation, pigmentation, hemolysis (Zheng et al., 2022), and exotoxin production (Balogh et al., 2024)	▪ Inhibition of the release of GM-CSF and IL-8 in A549 human airway epithelial cells (Cheng et al., 2006)
Toremifene	▪ Tertiary amine	▪ <i>Porphyromonas gingivalis</i>, <i>Strepto</i>	▪ <i>S. aureus</i> biofilm	

	- Approved for metastatic breast cancer; Investigational as a preventative agent for prostate cancer (phase 2) - Estrogen receptor modulator	<i>coccus mutans</i> (Geritis et al., 2017a)	formation inhibition in a rat subcutaneous catheter model (De Cremer et al., 2014) Synergy with polymyxin B against resistant <i>P. aeruginosa</i> (Hussein et al., 2017)	
Etifoxine	- Benzoxazine - Approved as an anxiolytic and anticonvulsant - Modulation of GABAergic neurotransmission and neurosteroid synthesis			Reduction in inflammatory mediators and infiltration of leukocytes in the brain (Tian et al., 2022)
5-(N,N-Hexamethylene)-amiloride	- Pyrazine - Approved as diuretic - Na⁺/H⁺ exchangers blocker, apoptosis inducer, antineoplastic agent	<i>Pseudomonas cepacia</i> (Cohn and Rudzienski, 1994)		Downregulation of inflammatory signals and upregulation of anti-inflammatory response by targeting the kappaB-α/NF-kappaB signaling transduction pathway in the alveolar epithelium (Haddad and Land, 2002)
Zafirlukast	- Indole - Approved for asthma; Investigational for breast and ovarian cancer (phase 2) - Leukotriene D4 receptor antagonist	<i>P. gingivalis</i>, <i>S. mutans</i> (Geritis et al., 2017b) <i>Mycobacterium tuberculosis</i> (Pinault et al., 2013)		- In vitro and in vivo inflammatory response suppression of alveolar epithelial cells via reduction of the TLR4/NF-κB/NLRP3 inflammasome pathway (Xue et al., 2024) - Improved clinical score of adult CF patients (Conway et al., 2003)
Mitoquinone (mesylate)	- Organophosphorus compound - Investigational (phase 2) for Parkinson's disease, chronic hepatitis C virus infection, dilated cardiomyopathy, non-alcoholic fatty liver disease, and ulcerative colitis - TPP-based, mitochondrially targeted antioxidant to protect against oxidative damage			Attenuated inflammation, mucus hypersecretion, and oxidative stress induced by cigarette smoke by modulating mitochondrial function and the NF-κB signal pathway (yang et al., 2021)

Crisaborole	▪ Benzoxaborole ▪ Approved for onychomycosis and mild-to-moderate atopic dermatitis ▪ 3',5'-cyclic-AMP phosphodiesterase 4 inhibitor			▪ Inhibition of PDE4 leads to elevated levels of cAMP that inhibit the NF-κB pathway and suppress the release of pro-inflammatory mediators (Fan et al., 2024)
Linsitinib	▪ Quinoline ▪ Investigational for Ewing sarcoma (phase 2), colorectal carcinoma (phase 1), head and neck malignant neoplasia (phase 2), hepatocellular carcinoma (phase 2), and ovarian cancer (phase 1) ▪ Insulin-like growth factor 1 receptor (IGF-1R) and insulin receptor inhibitor	▪ <i>M. tuberculosis</i> (Wang et al., 2022)		▪ Inhibition of the development and progression of thyroid eye disease via infiltration of T lymphocytes and macrophages suppression in a murine model of Graves' disease (Gulbins et al., 2023)
GW 501516 (Cardarine)	▪ Aromatic ether ▪ Investigational for the disorder of lipid metabolism (phase 2) ▪ Peroxisome proliferator-activated receptor delta (PPARδ) agonist			▪ Suppressed inflammatory mediators with protection <i>vs.</i> LPS-induced macrophage inflammation and acute liver failure in mice (Lim and Kwak, 2024)
Darapladib	▪ Substituted pyrimidone ▪ Investigational for acute coronary syndrome (phase 3), atherosclerosis (phase 3), and diabetic retinopathy (phase 2) ▪ Lipoprotein-associated phospholipase-A2 (Lp-PLA2) inhibitor			▪ Reduced expression of nucleotide-binding oligomerization domain-like receptor with pyrin domain 3 (NLRP3) and IL-1β; improved macrophage migration and IL-1β secretion in macrophages by blocking NLRP3 inflammasome activation (Lv et al., 2021)
Bardoxolone	▪ Synthetic triterpenoid ▪ Investigational for lymphoma (phase 1) ▪ Nitric oxide synthase inhibitor			▪ NF-κB signaling suppression in mice with LPS-induced acute lung injury (Pei et al., 2019) ▪ NLRP3 inflammasome and pro-caspase-1 complex assembly inhibition in mice with acute lung injury (he et al., 2024)
Meisoindigo	▪ Second-generation derivative of indirubin			▪ Inhibition of zebrafish

	▪ Approved for chronic myelogenous leukemia; Investigational for active ulcerative colitis (phase 2) ▪ Inhibition of DNA biosynthesis and the assembly of microtubules in cancer cells			leukocyte chemotactic migration (Ye et al., 2017) ▪ Blocked activation of the NLRP3 inflammasome and modulation of the polarization of microglia/macrophages via inhibition of the TLR4/NF-κB signaling pathway (Ye et al., 2019)
Epinastine	▪ Benzazepine ▪ Approved for preventing itching associated with allergic conjunctivitis; Investigational for allergic rhinitis (phase 3) and seasonal allergic rhinitis (phase 3) ▪ Histamine H1 receptor antagonist			▪ Suppression of IL-6 expression in LPS-treated macrophages (Ishikawa et al., 2021)
Salirasib	▪ Salicylic acid derivative ▪ Investigational for non-small-cell lung carcinoma (phase 2) ▪ Serine/threonine-protein kinase mTOR modulator; GTPase NRas antagonist			▪ Inhibition of CD4+ and CD8+ T cell proliferation and IFN-γ, TNF-α, IL-6, and IL-17 release in rheumatoid arthritis (Aizman et al., 2014) ▪ Reduced iNOS expression induced by LPS in murine macrophages (Wu et al., 2019)
Omaveloxolone	▪ Semi-synthetic triterpenoid ▪ Approved for Friedreich ataxia; Investigational for breast cancer (phase 2), liver disease (phase 1), melanoma (phase 1) ▪ Nuclear factor erythroid 2-related factor 2 activator			▪ Improvement of LPS-induced acute murine lung injury by interfering with Bach1-mediated ferroptosis (Wu et al., 2024)
Incyclinide	▪ Chemically modified tetracycline ▪ Investigational for central nervous system neoplasm (phase 1) ▪ 72 kDa type IV collagenase inhibitor			▪ IL-12 and TNF- α inhibition in chronic periodontitis (Alyousef et al., 2017)
Glecaprevir	▪ Aminoisobutyric acid ▪ Approved for chronic hepatitis C virus (HCV) ▪ NS3 protease HCV inhibitor, genome polyprotein HCV inhibitor			▪ Anti-inflammatory potential (Farghaly et al., 2024)
Isradipine	▪ Dihydropyridine			▪ PMN leukocyte function inhibition

	- Approved for hypertension and cardiovascular disease; Investigational for Parkinson's disease (phase 3), bipolar disorder (phase 2), drug dependence (phase 1), and nicotine dependence (phase 1) - T and L type calcium channel inhibitor			by interference with the adenosine system (Capecchi et al., 1993)
Laurocapram	- Caprolactam - Approved as a percutaneous enhancer - Interaction with lipids in the stratum corneum, thus enhancing skin absorption of a hydrophilic chemical		Improved cephalosporin's activity against methicillin-resistant <i>S. aureus</i> (Wang et al., 2024)	
Masitinib	- Benzamide - Approved for amyotrophic lateral sclerosis, mastocytosis, and advanced pancreatic cancer - Proto-oncogene tyrosine-protein kinase Src inhibitor			Reduced survival, migration, and degranulation of mast cells (Ketaforoush et al., 2023)
Mitotane	- Isomer of the insecticide dichlorodiphenyldichloroethane - Approved for adrenal cortex carcinoma and neoplasm; Investigational for carcinoma (phase 3) and prostate cancer (phase 1) - Cytochrome P450 11B1 mitochondrial inducer, Estrogen receptor binder		Synergy with polymyxin B against resistant <i>Acinetobacter baumannii</i>, <i>P. aeruginosa</i>, and <i>Klebsiella pneumoniae</i> (Tran et al., 2018)	
Montelukast	- Quinoline - Approved for asthma, and allergic rhinitis; Investigational for bronchiolitis (phase 2), sepsis (phase 2), leukemia (phase 2), sleep apnea (phase 2), non-small cell lung carcinoma (phase 2), premature birth (phase 2), HIV (phase 1), pain (phase 3) - Cysteinyl leukotriene receptor 1 antagonist			- Decreased eosinophil cationic protein and IL-8 serum and sputum levels, and sputum levels of myeloperoxidase in CF patients (Schmitt-Grohé and Zielen, 2005) - Decreased IL-6, IL-1β, and IL-17 levels in the lung of septic mice (Alnyfach et al., 2022)
Ricolinostat	- Pyrimidinecarboxylic acid - Investigational for breast carcinoma (phase 1), chronic lymphocytic leukemia (phase 1), diabetic neuropathy (phase 2), multiple myeloma (phase 1),			Inhibition of ROS overproduction and TNF-α, IL-1β, and IL-6 expression in LPS-activated RAW264.7 cells (Zhang et al., 2019)

	peripheral nervous system disease (phase 1), hilar cholangiocarcinoma (phase 1), and lymphoma (phase 1) ▪ Histone deacetylase 6 (HDAC6) inhibitor			
Vandetanib	▪ Quinazoline ▪ Approved as an alternative to local therapies for both unresectable and disseminated disease ▪ Vascular endothelial growth factor receptor 2 inhibitor, Proto-oncogene tyrosine-protein kinase receptor Ret inhibitor, Epidermal growth factor receptor inhibitor			▪ Reduced IL-6, IL-10, and TNF-α levels and inflammatory cell infiltrates in the lungs of SARS-CoV2-infected animals (Puhl et al., 2022)
Verteporfin	▪ Benzoporphyrin derivative ▪ Approved as a photosensitizer in photodynamic therapy ▪ Photoabsorption			▪ In vitro and in vivo excellent biocompatibility, safety, and anti-inflammatory responses by suppressing M1 macrophage polarization while inducing M2 polarization in acute lung injury (Cen et al., 2024) ▪ Enhanced PMN-mediated <i>S. aureus</i> killing (Prieto et al., 2020)
Vigabatrin	▪ Gamma-aminobutyric acid analog ▪ Approved for refractory seizures and infantile spasms ▪ 4-aminobutyrate aminotransferase mitochondrial inhibitor			▪ PMN migration inhibition (Raza et al., 2000)
Diroximel (fumarate)	▪ Fumarate ▪ Approved for immune system disease, multiple sclerosis, and relapsing-remitting multiple sclerosis ▪ Neuronal acetylcholine receptor subunit alpha-10 agonist			▪ Reduced iNOS, COX-2, TNF-α, IL-6, and IL-1β expression in the BV-2 microglial cell line (He et al., 2025)
Infigratinib	▪ Phenylurea compound ▪ Approved for advanced or metastatic cholangiocarcinoma, urothelial carcinoma, and achondroplasia ▪ Fibroblast growth factor receptors 1-2-3 inhibitor			▪ Reduced infiltration of lymphocytes and macrophages in a murine model of multiple sclerosis (Rajendran et al., 2023)

Data source: PubChem (<https://pubchem.ncbi.nlm.nih.gov/>). The literature review was performed using PubMed (<https://pubmed.ncbi.nlm.nih.gov/>) using the following keywords: [compound name] and

“antibacterial”, “aureus”, “aeruginosa”, “inflammatory”, “inflammatory lung”, “virulence” and “cystic fibrosis”.

Here, we reported an antibacterial activity for 31 out of 37 selected compounds for the first time. Previous studies referred to the activity of the remaining six compounds against species other than *S. aureus*: the anticancer Toremifene and the antiasthmatic Zafirlukast *vs.* *Porphyromonas gingivalis* (Geritis et al., 2017a; Geritis et al., 2017b), Zafirlukast, the anticancer Napabucasin and the antipsychotic Perphenazine *vs.* oral streptococci (Mortensen et al., 1992; Geritis et al., 2017b; Kuang et al., 2020), Zafirlukast and the anticancer Linsitinib *vs.* *Mycobacterium tuberculosis* (Pinault et al., 2013; Wang et al., 2022), and Perphenazine *vs.* *Neisseria meningitidis*, *Enterobacteriaceae*, and *Listeria monocytogenes* (Mortensen et al., 1992).

Opportunistic polymicrobial bacterial airway infection is a hallmark of CF lung disease and early mortality (VanDevanter et al., 2024). From birth, the airways of individuals with CF are susceptible to infections from microbial opportunists. Over time, more concerning bacterial species often appear in respiratory secretions throughout their shortened lifetimes. The failure of bacterial clearance generates a dominant chronic inflammation, resulting in a toxic pro-inflammatory local microenvironment that damages the lung and the innate immunity, further facilitating infections and thus resulting in the predominant cause of morbidity and mortality in CF patients. In this frame, it is worth noting the previously reported activity of the diuretic 5-(N,N-Hexamethylene)-amiloride and Perphenazine, respectively, against *Pseudomonas cepacia* (Cohn and Rudzienski, 1994) and *Haemophilus influenzae* (Mortensen et al., 1992), since these species have the potential to cause pulmonary exacerbations and lung function decline in people suffering from CF (LiPuma, 2010; Gutiérrez Santana et al., 2024).

Others recent evidence suggest that viridans group streptococci (VGS) may play a crucial role in maintaining a stable microbial ecology within the CF lung, enhancing virulence associated with polymicrobial interactions and directly contributing to the pathology of the lung. *Streptococcus pneumoniae* has recently been associated with an increased severe decline in FEV₁ (Paganin et al., 2015). In addition, VGS may have the ability to aggregate with other important CF bacterial pathogens, such as *S. aureus* and *P. aeruginosa*, promoting the initial attachment and eventual colonization with these pathogens and the development of multispecies biofilms in the CF airways (Rickard et al., 2003). In this regard, the previously reported activity of Perphenazine against *S. pneumoniae* (Mortensen et al., 1992) and Toremifene against *Streptococcus mutans* (Geritis et al., 2017a) adds value to their potential for repurposing, warranting further studies.

The prolonged use of antibiotics has been essential in improving the survival rates of CF patients. However, this approach leads to complications, such as the rise of adaptive antibiotic resistance, adverse effects, and allergic reactions. An attractive alternative recently explored for treating bacterial infections is targeting or reducing the production of virulence factors, such as inhibiting quorum sensing, biofilm formation, iron uptake, and efflux pumps (Carullo et al., 2023; Mudgil et al., 2024). Conversely to antimicrobial therapies, this strategy does not affect bacterial growth and is less likely to lead to resistance, exerting low selective pressure. In this frame, five drugs among the hits we identified in the present study have previously been shown to exhibit anti-virulence potential against *S. aureus* and *P. aeruginosa*, increasing their repurposing potential in CF patients (Table 1). *S. aureus* hemolysis activity has been observed to be significantly affected in over 200 clinical isolates by the antihistamine compound Loratadine (Zheng et al., 2022) and by the antiparasitic Miltefosine in the invertebrate *Galleria mellonella* and a murine model of pneumonia (Koch et al., 2017). The virulence potential of *S. aureus* is also decreased by the synthetic estrogen Diethylstilbestrol - causing reduction of alpha-toxin, coagulase, deoxyribonuclease, and penicillinase production (Yotis et al., 1977) – and Loratadine, affecting biofilm formation, pigmentation (Zheng et al., 2022) and exotoxin production (Balogh et al., 2024). In a rat subcutaneous catheter model, biofilm formation was also inhibited by the anticancer Toremifene (De Cremer et al., 2014). Miltefosine and the anti-hypercholesterolemic agent Lovastatin showed anti-virulence potential in *P. aeruginosa*,

respectively, inhibiting phospholipase C/sphingomyelinase with a protective effect against murine pneumonia (Wargo et al., 2011) and decreasing swarming motility (Hennessy et al., 2013). Other studies revealed synergistic activity of Toremifene and the anticancer Mitotane combined with polymyxin B against multidrug-resistant *P. aeruginosa* (Hussein et al., 2017; Tran et al., 2018) and the percutaneous enhancer Laurocapram combined with cephalosporins against MRSA (Wang et al., 2024).

The main hallmark of CF pathophysiology is excessive inflammation and the inability to resolve lung infections, contributing to morbidity and, eventually, mortality. Therefore, anti-inflammatory properties could be desirable to improve the repurposing potential of the hits found in the present study. Most of the 37 selected hit compounds were reported for anti-inflammatory potential in the literature, although in clinical settings or models not pertinent for infective diseases (e.g., inflammatory bowel disease (Verhaar et al., 2013), allergic dermatitis (Heo et al., 2020), pulmonary arterial hypertension (Geerdink et al., 2017), osteoarthritis (Aizman et al., 2014; Hu et al., 2016), asthma (Towsend et al., 2023), Parkinson's disease (Tian et al., 2022), cigarette smoke (Yang et al., 2021), vascular diseases (fan et al., 2024), thyroid eye disease (Gulbins et al., 2023), and amyotrophic lateral sclerosis (Ketaforoush et al., 2023) (Table 1). Conversely, a few hits have previously been reported for anti-inflammatory potential in models that could be relevant to CF lung infections, thus increasing their potential for repurposing: 5-(N,N-Hexamethylene)-amiloride in lipopolysaccharide (LPS)-exposed alveolar epithelial cells (Haddad and Land, 2002), Zafirlukast in LPS-exposed mice (Xue et al., 2024), GW 501516 (Cardarine) – aromatic ether under investigation for lipid metabolism's disorder (phase 2) – and the anticancer Ricolinostat protect against LPS-activated macrophages inflammation (; Zhang et al., 2019; Lim and Kwak, 2024), Bardoxololone - semisynthetic triterpenoids under investigation for lymphoma - Omaveloxolone - a potent anti-inflammatory in treating Friedreich's ataxia – and Verteporfin – used as a photosensitizer in photodynamic therapy – alleviate LPS-triggered acute lung injury in mice (Pei et al., 2019; Wu et al., 2024; Cen et al., 2024), and Vandertanib – approved for unresectable and disseminated diseases - in SARS-CoV2-infected mice (Puhl et al., 2022) (Table 1). Interestingly, the literature already reported anti-inflammatory properties in CF patients or models for three hits. Specifically, Fenretide increases IL-1 β expression in Cfrt-knockout mice, improving their ability to combat *P. aeruginosa* lung infection (Guilbalt et al., 2008); in addition, it normalizes the fatty acid imbalance by reducing arachidonic acid and increasing docosahexaenoic acid in CF patients (Guilbault et al., 2009). Zafirlukast, a leukotriene receptor antagonist licensed for asthma prevention, significantly improves NIH clinical score observed in a pilot study involving CF patients (Conway et al., 2003), probably due to ameliorated lung tissue pathology and reduced inflammatory cell infiltration reported in LPS-induced lung inflammation, both *in vitro* and in mice (Xue et al., 2024). Finally, the quinoline Montelukast, approved for asthma and allergic rhinitis, decreases eosinophil cationic protein and IL-8 serum and sputum levels and sputum levels of myeloperoxidase in CF patients (Schmitt-Grohé and Zielen, 2005).

Bacterial persistence in CF lungs is influenced by local host defense impairments and the pathogens' ability to adapt to a challenging environment due to selective pressures, such as hyperinflammation, oxidative stress, limited nutrients, anaerobiosis, increased acidity, and antibiotic exposure. Biofilm formation is a key adaptive strategy for bacteria, allowing them to persist even when antibiotics are administered. Nowadays, CF is considered a biofilm-associated disease, and controlling these biofilms is crucial for effective antimicrobial strategies. Several studies reported direct *S. aureus* biofilm visualization in CF patients by scanning electron microscopy and fluorescence electron *in situ* hybridization using a specific peptide nucleic acid and further viability evaluation by confocal laser scanning microscopy (Jean-Pierre et al., 2022). Biofilm formation was a common trait of both MRSA and MSSA strains in CF patients, and high biofilm-formation ability has been associated with fewer pulmonary exacerbations and, conversely, exacerbations negatively impacted biofilm formation (Wieneke et al., 2021).

In this frame, in the second step of the present study, the compound library also underwent a 96-well microtiter plate-based HTS to identify hits that could affect biofilm formation and

preformed-mature - biofilm by the MRSA Sa2 strain. Our findings indicated that 654 compounds affect biofilm formation, although to different extents. Notably, five compounds – i.e., the anticancer Tipifarnib and Olaparib, the bronchodilator Acefylline, Hemin, and the NS5b polymerase inhibitor TMC647055, under investigation for treating hepatitis C – exhibited, for the first time, the highest potential for repurposing due to excellent activity against biofilm formation, which resulted in a reduction of $\geq 90\%$ compared to untreated control (Table 2). A careful revision of the literature aimed at identifying additional biological properties of interest in managing CF patients revealed that Olaparib, Acefylline, and Hemin were previously reported for anti-inflammatory activity in the LPS-induced acute lung injury model (Kapoor et al., 2015; Elzagar et al., 2023; Cheng et al., 2023). Hemin can also affect *S. aureus* virulence by downregulating hemolysins expression (Schmitt et al., 2012). In disagreement with our findings, Tipifarnib and Hemin have previously been shown to inhibit *S. aureus* growth (Ladan et al., 1993; Weber et al., 2021).

Regarding hits active against preformed biofilms, four compounds exhibited the highest activity, i.e., $60\% \leq$ biofilm reduction $< 90\%$ (Table 3). The anti-biofilm activity exhibited by Clemastine (fumarate), a histamine receptor H1 antagonist, is probably due to a decrease in the transcriptional level of the biofilm formation relevant *fnbB*, *icaA*, and *icaB* genes in *S. aureus* (Shang et al., 2022). Conflicting findings come from the literature concerning the antibiofilm potential of Heparin, a highly sulfated glycosaminoglycan with uneven chain length, routinely used in central venous catheters to prevent thrombosis. Our findings disagree with a previous study reporting Heparin augments biofilm formation in *S. aureus* (Shanks et al., 2005; Najarzadeh et al., 2021), probably due to extracellular DNA-binding proteins on the *S. aureus* surface that mediates the incorporation of Heparin into the biofilm matrix (Mishra and Horswill, 2017). A similar pro-biofilm effect was observed for *Escherichia coli* (Wu et al., 2021), *Staphylococcus epidermidis* (Shanks et al., 2005), and in patients with colorectal cancer where Heparin increased the biofilm formed by intestinal flora, thus providing a protective layer in the intestinal tract of patients (Chen et al., 2022). In agreement with our findings, heparinoids, glycosaminoglycans chemically and pharmacologically related to heparin, were found to suppress biofilm formation in *Cutibacterium acnes* by inhibiting the AI-2-mediated QS (Hamada et al., 2024). No evidence of activity against preformed biofilms has been previously published for the anticancer Flumatinib (mesylate) and nonsteroidal anti-inflammatory drug Bromfenac (sodium hydrate). All the five hits here showing high potential for dispersing established biofilm have also been reported to trigger anti-inflammatory activity (Kida et al., 2014; Yi et al., 2015; Chimenti et al., 2017; Motawi et al., 2023), although only Heparin under experimental settings relevant to CF patients, such as human bronchial cells (Yi et al., 2015) and LPS-induced lung injury (Chimenti et al., 2017).

Table 2. Compounds (n = 5) with excellent activity against biofilm formation (i.e., biofilm reduction $\geq 90\%$) by *S. aureus* Sa2 at HTS. Classification, therapeutic category, and mechanism of action are listed, along with other biological properties relevant to cystic fibrosis (CF).

Compound	Classification Therapeutic category and indication(s) Mechanism(s) of action	Anti-bacterial activity	Anti-virulence activity	Anti-inflammatory activity
Tipifarnib	- Nonpeptidomimetic quinolone - Investigational for colorectal cancer, leukemia (myeloid), pancreatic cancer, and solid tumors (phases 1, 2, 3). - Farnesyltransferase inhibitor	<i>Staphylococcus aureus</i>, <i>Staphylococcus epidermidis</i>, <i>Streptococcus pneumoniae</i> (Weber et al., 2021)		Reduced LPS-induced IL1β, IL18, and TNFα secretion in a cellular model of mevalonate kinase deficiency (Marcuzzi et al., 2011)
Olaparib	- N-acylpiperazine - Approved for treating ovarian cancer, breast			Ameliorated LPS-induced acute lung injury by

	cancer, pancreatic cancer, and prostate cancer ▪ Poly (ADP-ribose) polymerase (PARP) inhibitor			downregulation of TNF- α , IL-1 β , and VCAM-1 expression (Kapoor et al., 2015)
Acefylline	▪ Theophylline derivative ▪ Approved for treating asthma, emphysema, acute and chronic bronchitis associated with bronchospasm; Investigational for airway obstruction (phase 4) ▪ Adenosine receptor antagonist	▪ <i>Mycobacterium tuberculosis</i> (Voynikov et al., 2014)		▪ Reduced NO, TNF- α , IL-1 β , and IL-6 levels in LPS-induced RAW 264.7 cells (Elzagar et al., 2023)
Hemin	▪ Iron-containing porphyrin ▪ Approved for acute intermittent porphyria; Investigational for diabetes mellitus, myocardial ischemia, graft failure (phase 2) ▪ 5-aminolevulinic synthase, non-specific, mitochondrial inhibitor	▪ <i>S. aureus</i> (Ladan et al., 1993)	▪ Downregulation of β - and γ -hemolysins expression in <i>S. aureus</i> (Schmitt et al., 2012)	▪ Attenuated LPS-induced lung injury in mice by inhibiting the activation of the nucleotide-binding domain-like receptor protein 3 (NLRP3) inflammasome and oxidative stress (Cheng et al., 2023)
TMC647055 (Choline salt)	▪ Macrolactam ▪ Investigational for treating hepatitis C (phase 1), and chronic hepatitis C (phase 2) ▪ Nonnucleoside Hepatitis C Virus NS5B polymerase inhibitor			

Data source: PubChem (<https://pubchem.ncbi.nlm.nih.gov/>). The literature review was performed using PubMed (<https://pubmed.ncbi.nlm.nih.gov/>) using the following keywords: [compound name] and “antibacterial”, “aureus”, “aeruginosa”, “inflammatory”, “inflammatory lung”, “virulence” and “cystic fibrosis”.

Table 3. Compounds (n=4) with good activity against established biofilm (i.e., 60% $\leq$ biofilm dispersion < 90%) by *S. aureus* Sa2 at HTS. Classification, therapeutic category, and mechanism of action are listed, along with other biological properties relevant to cystic fibrosis (CF).

Compound	Classification Therapeutic category and indication(s) Mechanism(s) of action	Anti-bacterial activity	Anti-virulence activity	Anti-inflammatory activity
Clemastine (fumarate)	▪ Ethanolamine-derivative ▪ Approved for symptomatic relief of allergic rhinitis, and mild, uncomplicated allergic skin manifestations of urticaria and angioedema; Investigational for chronic progressive and relapsing multiple sclerosis (phase 1),		▪ Inhibition of biofilm formation and hemolytic activity in <i>S. aureus</i> (Shang et al., 2022)	▪ Anti-inflammatory and anti-pyroptotic actions via inhibition of the NLRP3 inflammasome in an autoimmune encephalomyelitis rat model (Motawi et al., 2023)

	urticaria and optic neuritis (phase 2), dermatitis (phase 3), and allergic diseases (phase 4)			
	Histamine receptor H1 (HRH1) antagonist			
Heparin	- Sulfated glycosaminoglycan - Approved for preventing blood clots - Antithrombin-III potentiator; coagulation factor X inhibitor	<i>Staphylococcus aureus</i>, <i>Pseudomonas aeruginosa</i>, <i>Escherichia coli</i>, <i>Streptococcus pneumoniae</i>, <i>Haemophilus influenzae</i> (Rosett et al., 1980; Zappala et al., 2007)	- Improved phenol-soluble modulins fibrillation and biofilm formation in <i>S. aureus</i> (Najarzadeh et al., 2021) - Augmented biofilm formation by intestinal microbiota in patients with colorectal cancer (Chen et al., 2022) - Critical for host tissue colonization and invasion of several bacterial pathogens (Patti et al., 1994)	- Decreased COX-2 and CXCL-8 gene expression in human bronchial epithelial cells (Yi et al., 2015) - Reduced IL-6 and TNF-α pulmonary levels in a rat model of LPS-induced acute lung injury (Chimenti et al., 2017)
Flumatinib (mesylate)	- Pyridinylpyrimidine - Investigational for acute lymphoblastic leukemia (phase 3), and chronic myelogenous leukemia (phase 3) - Tyrosine-protein kinase ABL1 inhibitor			
Bromfenac (sodium hydrate)	- Nonsteroidal anti-inflammatory drug - Approved for treating postoperative eye inflammation - Prostaglandin G/H synthase 1 and 2 inhibitor	<i>S. aureus</i>, <i>E. coli</i>, <i>Bacillus subtilis</i> (Yin et al., 2014)		COX-2 inhibition in rabbit retinochoroidal tissues (Kida et al., 2014)

Data source: PubChem (<https://pubchem.ncbi.nlm.nih.gov/>). The literature review was performed using PubMed (<https://pubmed.ncbi.nlm.nih.gov/>) using the following keywords: [compound name] and “antibacterial”, “aureus”, “aeruginosa”, “inflammatory”, “inflammatory lung”, “virulence” and “cystic fibrosis”.

4. Materials and Methods

4.1. Compound Library

The “Drug Repurposing Compound Library” was purchased from MedChem Express (cat. no. HY-L035; Monmouth Junction, NJ, USA). The library was provided in a 96-well plate format with aliquots of 10 mM stocks of drugs in DMSO or water, stored at -80°C.

4.2. Bacterial Strain and Growth Conditions

HTS was performed using *S. aureus* Sa2, an MRSA strain known for causing long-term persistence in a CF patient. Some colonies grew on Tryptone Soya Agar (TSA; Oxoid, Milan, Italy) following overnight incubation at 37°C were suspended in sterile saline 0.9% (Fresenius Kabi Italia, Verona, Italy) to reach an optical density at 550 nm (OD₅₅₀) of 0.3. This suspension was diluted 1:10 in sterile saline to achieve a final 1-2 × 10⁷ CFU/mL concentration. This standardized inoculum was used for all the assays unless differently indicated.

4.3. Antibacterial HTS Assay

The library was screened at a single concentration point to identify antibacterial hit compounds against the *S. aureus* Sa2 strain. Briefly, 5 μL of the standardized inoculum (corresponding to $0.5\text{--}1 \times 10^5$ CFU/well) were added to each well of a 96-well polystyrene microtiter plate containing 100 μL of Tryptone Soya Broth (TSB; Oxoid) and 1 μL of a 10 mM compound stock solution from the MedChem library, achieving a final drug concentration of 0.1 mM. Uninoculated samples with 1% (*v/v*) DMSO (final background in each well) were considered blank. Negative control was also prepared with 50% (*v/v*) DMSO to yield 100% killing. The content of each well was mixed by pipetting, and plates were incubated at 37°C under an aerobic atmosphere. After 24 h-incubation, the survival rate of planktonic cells was assessed spectrophotometrically by measuring the broth culture supernatants OD₅₅₀ using a microplate reader (Tecan Infinite® M PLEX; Tecan Group Ltd Mannedorf, Switzerland). This value was corrected by subtracting the average OD₅₅₀ value of the uninoculated wells (blank). The percentage of growth rate was calculated compared to the inoculated, but not treated, control sample (100% growth). The antibacterial activity of library drugs was classified based on the growth reduction compared to the untreated control sample: i) low efficacy: $10\% < x < 25\%$; ii) moderate efficacy: $25\% \leq x < 60\%$; iii) high efficacy: $60\% \leq x < 90\%$; and iv) excellent efficacy: $90\% \leq x \leq 100\%$. Only drugs causing a $\geq 90\%$ reduction of bacterial burden, subsequently confirmed by cell viable count, were considered potential anti-*S. aureus* hit compounds and underwent further characterization. DMSO at the final concentration of 0.1 mM did not show any activity against *S. aureus* Sa2, as assessed by cell viable count, thus indicating that the antibacterial effects observed were due to the compound only.

4.4. HTS Assay Validation

The results from each HTS microplate were validated by calculating the Z-factor. To validate the degree of separation, the Z-factor and the percent inhibition of the positive and negative controls were determined using the formula:

$$Z - factor = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}$$

where σ_p and σ_n are the standard deviations of the positive and negative controls, respectively, and μ_p and μ_n are the corresponding mean values. A Z-factor between 0.5 and 1.0 indicates an excellent assay and statistically reliable separation between the positive and negative controls.

4.5. Biofilm Inhibition and Dispersion HTS Assays

To evaluate the effect of the drugs in preventing biofilm formation, 5 μL of the standardized inoculum ($1\text{--}2 \times 10^7$ CFU/mL) was added in each well of a TC-treated microplate (Falcon) containing 100 μL of TSB with 1 μL of a 10 mM compound stock solution from the MediChem library, achieving a final drug concentration of 0.1 mM. Control samples were exposed to TSB only. After incubating at 37°C for 24 h, planktonic cells were gently removed by washing twice with 100 μL PBS. After fixing samples (60°C, 1 h), the biofilm biomass was quantified spectrophotometrically. Briefly, 100 μL of 1% (*w/v*) Hucker's crystal violet was added to each well, and after incubation at room temperature for 5 min, each well was washed using tap water. After drying at 37°C, 100 μL 33% (*v/v*) glacial acetic acid was added to dissolve the stained dye for 15 min. The biofilm biomass was determined by measuring the absorbance at 492 nm using a Tecan Infinite® M PLEX microplate reader (Tecan).

To evaluate the efficacy of drugs against preformed biofilms, 24 h-old biofilms were treated with 100 μL of TSB with the selected drug at a final concentration of 0.1 mM, or TSB only (controls). Biofilm samples were incubated at 37°C for another 24 h and then washed with PBS. As previously described, crystal violet staining finally measured the biofilm biomass.

A potential "antibiofilm hit" had to affect biofilm formation or disperse preformed biofilms, whereas it had no antibacterial activity (growth reduction $\leq 10\%$). The magnitude of the hit's activity against biofilm formation or preformed biofilms was categorized as follows: low (i.e., $10\% < \text{biofilm}$

reduction < 25%), moderate (i.e., $25\% \leq$ biofilm reduction < 60%), high (i.e., $60\% \leq$ biofilm reduction < 90%), and excellent (i.e., biofilm reduction $\geq 90\%$).

4.6. Statistical Analysis

All assays were conducted in triplicate and repeated twice (n=6). Statistical analysis was performed using GraphPad Prism 7.0 software (GraphPad Software, San Diego, CA, USA). Data were tested for normal distribution using the Shapiro–Wilk test. Ordinary one-way ANOVA with Dunnett’s multiple comparisons test was applied when assessing differences among three or more groups of unpaired data. The statistical analysis assumed a confidence level of $\geq 95\%$, thus considering p values < 0.05 statistically significant.

5. Conclusions

The present study aimed to identify compounds with high repurposing potential against planktonic and sessile cells of an *S. aureus* strain causing chronic lung infection in a CF patient. In this regard, HTS of a library compound performed in 96-well microtiter plates with a spectrophotometric assessment of the results revealed a helpful platform. We identified 193 hits causing 100% killing of planktonic cells, five causing biofilm reduction of at least 90%, and four effectively dispersing from 60 to 90% of established biofilm. Most hits were anticancer agents, followed by anti-infective (other than antibacterial) and anti-inflammatory drugs. Also considering other, already published, biological properties that could be relevant to the CF lung environment, among the hits active against planktonic *S. aureus* cells, those with the most interesting repurposing potential are 5-(N,N-Hexamethylene)-amiloride (active against other CF pathogens and with anti-inflammatory potential), Toremifene (active against other CF pathogens and with anti-virulence potential), and Zafirlukast, Fenretide, and Montelukast (with anti-inflammatory properties already observed in CF patients). Among hits affecting biofilm formation, Hemin also showed anti-virulence and anti-inflammatory effects. Heparin, Clemastine, and Bromfenac are the most interesting hits that significantly affect established biofilms.

Further *in vitro* and *in vivo* studies are needed to confirm the potential of repurposing these compounds for managing *S. aureus* lung infections in CF patients.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Table S1: Hits (n = 364) showing excellent activity (i.e., growth reduction $\geq 90\%$ vs. unexposed control) against *S. aureus* Sa2 strain, based on spectrophotometric readings and cell viable count; Table S2: Hits (n = 5) showing excellent activity (i.e., biofilm reduction $\geq 90\%$ vs. unexposed control) against biofilm formation by *S. aureus* Sa2 strain, based on spectrophotometric readings; Table S3: Hits (n = 4) showing high activity (i.e., $60\% \leq$ biofilm reduction < 90% vs. unexposed control) against preformed biofilm by *S. aureus* Sa2 strain, based on spectrophotometric readings.

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Abbreviations

The following abbreviations are used in this manuscript:

CF	Cystic Fibrosis
MSSA	Methicillin-Sensitive <i>Staphylococcus aureus</i>
MRSA	Methicillin-Resistant <i>Staphylococcus aureus</i>
FEV ₁	Forced Expiratory Volume in 1 second
HTS	High-Throughput Screening
VGS	Viridans Group Streptococci

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